Edited by
Jean-Marc Cavaillon and
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Inflammation

Inflammation

From Molecular and Cellular Mechanisms to the Clinic

Edited by Jean-Marc Cavaillon and Mervyn Singer

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Preface

Inflammation is older than humanity itself. Indeed, the earliest signs of inflammatory processes can be found on the bones of dinosaurs. Inflammation has always been integral to humans as the key process that protects against sterile or infectious insults. By the end of the eighteenth century, John Hunter was among the first to define inflammation as a salutary function, a concept endorsed 100 years later by Elie Metchnikoff. To limit the side effects of inflammation, the use of herbal anti-inflammatory was introduced in China (2800 BC) and Egypt (1520 BC), well before Hippocrates. Bloodletting was another therapeutic approach widely supported for some 2000 years. While natural products remain an important source of new anti-inflammatory drugs, bloodletting has been recognized to be powerless!

Nowadays, the beneficial effects of inflammation are well recognized when associated with the overlapping innate immune response. In recent years, molecular and cellular players have been well characterized and their precise interactions better understood. New molecular mechanisms have been deciphered such as the inflammasome and epigenetics. However, the word inflammation remains mainly associated with disease. Indeed, many chronic inflammatory disorders have been identified as severe debilitating diseases that may even favor the emergence of certain cancers. Deciphering the molecular and cellular events underlying inflammation has enabled development of new drugs that have revolutionized treatment and outcomes of some of these disorders.

Major achievements have been made in the last few decades allowing new understanding of the cross-talk between immune and nonimmune cells (e.g., cytokines, neuromediators, and eicosanoids) and in the resolution of inflammation (e.g., control by the neuronal system, new lipid mediators). Well-recognized

leaders in the field have contributed their specific expertise to this book, thus making it most comprehensive overview of inflammatory processes and associated diseases.

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1

Inflammation through the Ages: A Historical Perspective

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1.1 Introduction

Inflammation is older than humanity itself and the earliest signs of inflammatory processes can be found on the bones of dinosaurs. Of course, inflammation has always been accompanying humans since they are on Earth as it can be seen on the bones of the first humanoids and of *Homo sapiens*. The first precise diagnoses of inflammatory disorders were made on Egyptian mummies by *Sir Marc Armand Ruffer* (1859–1917). Accompanying the first British Egyptologists, he gave birth to a new science: "paleopathology." He made a pioneer post-mortem diagnosis of arthritis and spondylitis, and by studying the mummy of Ramses II, he diagnosed that the pharaoh had suffered from atherosclerosis. In fact, long before inflammatory processes could be understood or even defined humans had proposed various therapeutic approaches to treat different types of inflammatory diseases.

1.2 The First Treatments

According to Chinese mythology, herbology or the use of plants to cure diseases was introduced by Emperor *Shennong* in 2800 BC, and the first ever book on medicinal plants was published in China in 300–200 BC. Other testimonies of interest on plants to cure diseases or at least to relieve pain and fever were provided by *Edwin Smith* and *Georg Moritz Ebers*, two Egyptologists who obtained fascinating papyruses. These papyruses (around 1520 BC) were copies of even older ones (3400 BC). Not only did they describe case reports of injuries but also listed different plants to be used against various types of injuries (crocodile bites, burns, fractures, bowel diseases, joint pains, etc). For example, infusion of dried myrtle was recommended for rheumatic pain. The interest to use plants to cure inflammatory diseases was perpetuated by the Greeks, and *Hippocrates* (450–370 BC) used extracts from willow bark to relieve pain and

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fever. The study of willow bark ended with the discovery of aspirin in the nineteenth century.

Hippocrates also advocated bloodletting as another therapeutic approach to cure most diseases, including inflammatory disorders. Its use was supported by other erudite Greeks such as *Erasistratus*, *Asclepiades of Bithynia*, or *Galen of Pergamon* and later by the Roman scholar *Aulus Cornelius Celsus*, the Persian medical doctor *Avicenna* (tenth century), or the Spanish Jewish doctor *Moïse Maïmonide* (twelfth century). All physicians of the kings of France were great supporters of bloodletting. *Ambroise Paré* (1509–1590), the physician of Charles IX, explained why he bled a young man 27 times in four days: "I liked to mention this event, so that the young surgeon will not be too shy to draw blood when confronted to large inflammation." *Laurent Joubert* (1529–1583), the physician of Henri III, claimed that it was a way to get rid of the "bad blood" while the best was retained. *Charles Bouvard* (1572–1658) had probably prescribed 47 bloodlettings during the last 10 years of Louis XIII who died of Crohn's disease at the age of 42 years. They used it even when their patients were still young. For instance, *François Vaultier* (1590–1652) bled the young Louis XIV at the age of 9 years when he had smallpox. *Guy Patin* (1601–1672), the dean of the School of Medicine in Paris for a brief period, declared: "There is no remedy in the world that does so many miracles. I have bled my wife twelve times for a pleurisy, twenty times my son for a continuous fever and myself seven times for a cold." [1] Even on the American continent, bloodletting was popular. Thus, on December 14, 1799, Georges Washington, probably suffering from pneumonia, died when 3.7 l of blood was drained out of his body in one single day. The first doctor to question the usefulness of bloodletting was *Pierre Charles Alexandre Louis* (1787–1872) who in 1835 considered this approach to have had very limited advantages. But pitted against him were leading doctors such as *François Broussais* (1772–1838) who was alleged to have had spilled more blood than Napoleon on all battlefields! If the lancet was commonly used to remove blood from patients, then the use of leeches was another method. According to an estimate, 35 million leeches were used in France in 1830 alone. This trend went unabated until *François-Vincent Raspail* (1794–1878) questioned the method in 1845 saying: "But why resort to violent and bloody means? Do you wish to calm fever? You will not succeed by bleeding [. . .] So leave your lancet there, it has made enough troubles since Hippocrates" [2]. In 1856, in Great Britain, *John Hughes Bennet* (1812–1875) also concluded that there was no proven therapeutic advantage of bloodletting.

The other method used to prevent a severe inflammation and infection after a wound was cauterization supported by doctors such as *Giovanni da Vigo* (1450–1525) in Italy and *Paracelsus* (1493–1541) in Switzerland until Ambroise Paré, comparing the relative advantages of cauterization and the use of antiseptics, concluded that the latter were the best. But the word "antiseptic" was coined only in 1750 by *John Pringle* (1707–1782), a Scottish physician, who studied numerous substances able to prevent putrefaction. In 1854, *Florence Nightingale* (1820–1910) advocated hygiene as a means to prevent infection during the Crimean war in order to limit the mortality of wounded soldiers. Of

course, the main advocate of hygiene was *Ignaz Semmelweis* (1818–1865) who succeeded in 1847 to reduce mortality due to puerperal sepsis.

1.3 The Definitions

One of the very firsts to define the parameters of inflammation was *Aulus Cornelius Celsus* (25 BC–50 AD), a Roman encyclopedist to whom we owe the famous statement: "*Notae vero inflammationis sunt quatuor: rubor et tumor cum calore and dolore*" (The signs of inflammation are four: redness, swelling, fever and pain). A fifth element was later added "loss of organ function." Erroneously attributed to Galen of Pergamum, it could have been proposed by either *Thomas Sydenham* (1624–1689) or *Rudolf Virchow* (1821–1902). Of course, inflammation has for a long time been considered a morbid response of the host to any types of insults. However, *John Hunter* (1728–1793), a Scottish surgeon, appropriately defined inflammation in his book published one year after his death: "*Inflammation in itself is not to be considered as a disease, but as a salutary operation, consequent either to some violence or some disease*" [3]. Despite this appropriate definition, one could still read in 1865 in the French dictionary of medicine that "Inflammation is a complex morbid phenomenon, particularly associated with the function of blood circulation." In his lecture on inflammation, *Elie Metchnikoff* (1845–1916) stated in 1891 that phagocytes were the participant of the inflammatory process and that inflammation should no longer be seen as only being deleterious [4]. Since Metchnikoff, many mechanisms accompanying inflammation have been further deciphered and mediators have been characterized.

1.4 Fever

For a while it was believed that fever was consecutive to some obstructions within the blood vessels leading to an accelerated movement within the free vessels. At the beginning of the eighteenth century, a defender of this concept was the Italian physician *Lorenzo Bellini* (1643–1704). *Herman Boerhaave* (1668–1738), a Dutch physician, also thought that increased heartbeats were the source of the accelerated circulation and fever. In 1744, *François Boissier de Sauvages de Lacroix* (1706–1767), while translating in French the book on "haemastatic" written by *Stephen Hales* (1677–1761), added his personal view on fever, confirming the prevailing concept that inflammation was associated with increased blood flow. Thanks to scientists and doctors such as *John Davy* (1790–1868) in the United Kingdom who made the first sets of temperature measurements in different humans and in different environments (1816–1818), *Antoine Becquerel* (1852–1908) in France who invented the pyrometer to measure human temperature (1835), *Thomas Clifford Allbutt* (1836–1925) who invented the clinical thermometer (1866), and *Reinhold August Wunderlich* (1815–1877) of Germany who made more than one million measurements on more than 25 000 patients (1868), normal temperature and fever could be definitively and precisely defined. *William H. Welch* (1850–1934), the first dean of the

Johns Hopkins School of Medicine, offered a wonderful definition of fever in his 1888 Cartwright lecture:

> The real enemy in most fevers is the noxious substance which invades the body, and there is nothing to prevent us from believing that fever is a weapon employed by Nature to combat assaults of this enemy. According to this view, the fever-producing agents light the fire, which consumes them. It is not incompatible with this conception of fever to suppose that the fire may prove injurious also to the patients and may require the controlling hand of the physician [5].

What could be these "noxious substances?" The same year, *Nikolai Fedorovich Gamaleïa* (1859–1949), who worked in Odessa and Moscow, spent some time with Metchnikoff at the Institut Pasteur and reported that an injection of dead bacteria could induce fever in rabbits and sheep. Most fascinatingly, he showed that filtered alcoholic extracts of spleen from pyretic sheep could induce fever within 30 min in rabbits [6]. The following year (1889), Marc *Armand Ruffer*, who joined Elie Metchnikoff and Louis Pasteur shortly after the Institute was set up, published a paper with *Albert Charrin* (1856–1907) who was a professor at the prestigious College de France. They reported that the filtered culture of pyocyanic bacillus (*Pseudomonas aeruginosa*) could induce fever in rabbits, in the absence of alive or dead bacteria [7]. Of note, this report was published three years before the German physician and bacteriologist *Richard Pfeiffer* (1858–1945) coined in 1892 the word "endotoxin" that is nowadays widely used. Most fascinating, the authors wrote, although did not prove, that fever was the consequence of the activation of macrophages. In 1894, *Eugenio Centanni* (1863–1942), an Italian pathologist, recognized the intimate relationship between the pyrogenic and toxic properties of the bacterial poison, which he found to be chemically inseparable. This led him to name his material "pyrotoxina" [8]. In 1890, *Hans Ernst August Buchner* (1850–1902) had shown that this material was also pyogenic [9].

Valy Menkin (1901–1960) was the first to attempt to purify the endogenous mediator that could be responsible of fever. In 1943, he isolated a mediator called "pyrexin" [10]. Unfortunately, further analysis of his work suggested that his factor was contaminated with endotoxin [11]. So, in 1953, *Ivan L. Bennet Jr.* (1922–1990) and *Paul Beeson* (1908–2006) were the first to extract a fever-producing substance from rabbit polymorphonuclear leukocytes [12]. In 1955, *Elisha Atkins* (1921–2005) and *W. Barry Wood Jr.* (1910–1971) isolated a circulating endogenous pyrogen in the blood after the injection of typhoid vaccine [13]. In 1984, Charles Dinarello cloned the human interleukin-1β, known to be the endogenous pyrogen among many other activities [14].

1.5 Phagocytosis

In 1882, *Elie Metchnikoff* (1845–1916), when he was in Messina (Sicilia) with his family-in-law, made his key observation. He stuck rose thorns into starfish larvae

and was surprised to see that many phagocytic cells in the hemolymph of the starfish surrounded the "foreign object." He also observed the process in Daphnia, which he infected with yeast. These cells were able to move, ingest, and destroy the yeast cells within the water fleas. He then understood that this process was an important mechanism of host defense against infectious agents. When he met his Austrian friend Karl Claus in Vienna, he was offered to publish his observation in a Viennese journal and Karl Claus coined the word "phagocyte" (1883) [15]. It is worth mentioning that this discovery that finally led to a Nobel Prize (1908) was not published in a high-profile journal (happy times!). Interestingly, he was not the first one to observe the phenomenon. In 1847, *Alexander Ecker* (1816–1887) in Germany had described erythrocytes inside rabbit spleen cells, an observation confirmed in 1870 by *Nathanael Lieberkühn* (1821–1887) who reported that leukocytes could ingest erythrocytes. In 1871–1873, *Giulio Bizzozero* (1846–1901) provided the first drawings of macrophages that had ingested erythrocytes. He stated that reticular cells could ingest infective particles that were carried by the lymphatic liquid. He made this farsighted statement: ". . . this fact is, perhaps, the cause of the stoppage of some infections to the lymphatic glands which are connected to the part covered by the infection through the lymphatic vessels" [16]. In 1875, *Sir William Osler* (1849–1919) reported that alveolar macrophages of coal miners were full of carbon particles. In 1881, *Alexander Ogston* (1844–1929) published cartoons of groups of cocci, mostly free, but sometimes in or on large nonnucleated masses of protoplasm of leukocytes [17]. While Metchnikoff was in Messina trying to understand the aim of phagocytosis, *Robert Koch* (1843–1910) identified anthrax bacilli within white blood cells. Yet, Koch had interpreted his finding to mean invasion of the host by bacterial pathogens. The discovery of phagocytosis by Metchnikoff was not easily accepted, however. He recalls: ". . . The controversy about the phagocytosis could have killed me, or sooner permanently weakened me. Sometimes, (I remember such attacks of Lubarsch in 1889, and those of Pfeiffer in 1894) I was ready to get rid of life" (Oct. 1913). But some other scientists were fully convinced. For instance, Sir Marc Armand Ruffer was an ardent advocate of phagocytosis in the UK. In 1892, he wrote in the *British Journal of Medicine* [18]: "Should anyone meet a dead lion and find a lamb inside, he, knowing the habits of the lion would not conclude that the lamb had taken refuge in that. True, after a surfeit of lamb, the lion might die of indigestion but the chance of the lamb ever getting out alive would be very small."

Other sources of phagocytes were also discovered. *Nicolas Tchistovitch* (1860–1926) described that what were then considered epithelial cells were in fact alveolar macrophages [19]. *Carl Wilhelm Kupffer* (1829–1902) described stellate cells in the liver. He incorrectly believed that these cells were an integral part of the endothelium of the liver's blood vessels. In 1898, *Tadeusz Browicz* (1847–1928), a Polish pathologist, correctly identified them as macrophages, now known as Kupffer cells. In 1922, *Karl Albert Ludwig Aschoff* (1866–1942) introduced the term "reticulo-endothelial system" that included endothelial cells, fibrocytes, histiocytes, and splenocytes, monocytes and Kupffer cells [20]. However, this concept was of limited help since it mixed cells that undergo pinocytosis and cells that are able to achieve phagocytosis.

In 1903, *Sir Edward Almroth Wright* (1861–1947), a British bacteriologist and immunologist who discovered an effective vaccine for typhoid fever, coined the word "opsonisation" from Greek "οπσονο" meaning "I prepare victuals for . . ." He nicely explained: "The body fluids modify bacteria in a manner which renders them a ready prey to phagocytes" [21]. But the observation had been first reported in 1895 by Joseph Denys (1857–1932), a professor of bacteriology and anatomy at Louvain University. He stated: "In vaccinated rabbit, leukocytes get from sera their power to engulf and destroy *Streptococcus* pyogenes" [22]. In 1904, *Friedrich Neufeld* (1869–1945), a physician and bacteriologist, director of the Robert Koch Institute in Berlin, described the exactly same phenomenon, only based on much more solid experimental works [23]. But the word, "bacteriotropin," he coined did not survive the effects of time, and was not used after 1930.

In 2003, *Aimee M. deCathelineau* and *Peter M. Henson* coined the word "efferocytosis" derived from the Latin prefix *effero*, meaning "to take away, to put away, to carry to the grave, or to bury" [24]. However, Metchnikoff already knew the process as he had observed: "[. . .] many of phagocytes perish and are taken in by other phagocytes, as can be seen in every case a few days after the onset of the inflammation" [25]. But it was described for the first time by Giulio Bizzozero in 1871–1872, studying eye inflammation: "In summary, my observation showed the presence of big cells able to engulf white blood supurative cells or red blood cells in their contractile protoplams. This represents a way through the pus or blood is absorbed from the anterior chamber" [26]. Marc Armand Ruffer, while working with Metchnikoff, described in 1890: "Macrophages are able to swallow microphages (neutrophils) and to destroy and digest them" [27].

1.6 Diapedesis

Galen of Pergamon had considered that the formation of pus was part of the healing process ("*pus bonum et laudabile*," good and commendable pus). Pus was supposed to facilitate the removal of the unhealthy mood of the injured body. Galen's theory prevailed for more than a millennium until *Ugo Borgognoni of Lucca* (1160–1257), a surgeon and the founder of the Faculty of Medicine of Bologna, and his son *Theodorico Borgognoni of Lucca* (1205–1298), clergyman and surgeon, critiqued the doctrine in a four-volume work entitled "Chirurgia" (1267). In 1810, *Alexandre François Ollivier* (1790–1844), a physician accompanying Napoleon's campaign, injected himself with the pus from a wound of a severely injured soldier dying from putrid fever and was the first one to demonstrate its contagiousness [28].

In 1845, two scientists studied pus under the microscope and observed that the cells present in the pus were similar to the cells found in blood. *Alfred Donné* (1801–1878), a French bacteriologist and physician, studied blood from a leukemic patient and thought that it might contain pus cells, although he observed a clear-cut difference between these cells [29]. In contrast, *William Addison* (1802–1881), the physician to the Duchess of Kent, appropriately understood that pus cells were derived from white blood cells (or colorless as they called them): "Colorless blood cells are deposited all over the interior of the vessels;

[. . .] and at length pass into the tissue; [. . .] These facts are all independent of any orifices, rupture or pores in the vessels, allowing the escape of cells" [30]. Yet, the observation of diapedesis was not made. *Augustus Volney Waller* (1816–1870), a British neurophysiologist, was the first to observe natural leukocyte emigration in 1846, using the frog tongue. Hence, the origin of pus cells was not fully demonstrated [31]. In 1858, *Rudolf Virchow* (1821–1902), a medical doctor at Charité Hospital in Berlin, the father of the cell theory, erroneously believed that pus cells were derived from tissue elements following cell division [32]. But his assistant and two of his students made the final demonstration. In 1863, *Friedrich Daniel von Recklinghausen* (1833–1910), a physician and pathologist who left Virchow for a position of professor at the University of Würzburg before joining the University of Strasbourg, induced an experimental keratitis in frogs with silver nitrate. He characterized the pus cells in humor aqueous during acute inflammation and made the seminal observation of the contractility and mobility of colorless cells [33]. In 1867, *Julius Friedrich Cohnheim* (1839–1884) used a combination of colloidal aniline blue injections and microscopy to prove, what Addison had hypothesized, that white blood cells crossed blood vessels to become pus cells [34]. And in 1875, *Julius Arnold* (1835–1915), using an injection of cinnabar to demonstrate the borders of endothelial cells, came to the conclusion that leukocytes moved across blood vessel walls (diapedesis) by passing between endothelial cells at either points of dense staining, "stigmata," or circles of stain, "stomata" [35]. Quite surprisingly, 33 years after Addison's work, *Louis Pasteur* (1822–1895) wrote in 1778: "For us currently, it would be the red blood cells that would be the pus cells from a simple transformation from the first into the second" [36]. This illustrates that great scientists can also make wrong statements!

1.7 Chemotactism

Nowadays, it is well understood that diapedesis of circulating cells toward tissues is under the control of chemoattractant signals. Interestingly, the concept came from an observation made with bacteria. In 1884, *Wilhelm Pfeffer* (1845–1920), a botanist at the University of Tübingen, Germany, observed bacteria swimming toward the vicinity of the tip of a capillary tube filled with nutrient sugar that had been dipped into a bacterial culture broth. He coined the word "chemotaxis" [37]. The *in vivo* phenomenon was first reported in 1889 by *Cornelis Adrianus Pekelharing* (1848–1922), a Dutch physician and professor of physiological chemistry and histology at the University of Utrecht [38]. He put cotton wool soaked with anthrax bacilli in the peritoneal cavity of a frog. Retrieving this cotton wool some time later, he showed that it contained significantly more leukocytes than those that had been soaked with neutral liquid. He came to the conclusion that bacteria produce chemoattractant factors. The following year, in 1890, *Georges Gabritchevsky* (1860–1907), a Moscovite researcher who spent some time with Elie Metchnikoff, drew the same conclusion [39]. He inserted under the skin of frogs, rabbits or axolotl, small capillary tubes filled with alive or dead bacteria. Twenty-four hours later, the capillary tubes were full of leukocytes,

whereas this was not the case if the capillaries had been filled with saline. The same year in Belgium, *Jean Massart* (1865–1925), a doctor in sciences and medicine working with *Charles Bordet*, the brother of the famous Jules Bordet who got the Nobel Prize for his discovery of the complement, was the first to demonstrate that the host can make chemoattractant factors [40]. He injected bovine bile subcutaneously in a frog. Then, he sampled the transudation liquid and transferred it in a capillary tube into the abdominal cavity of another frog. Twenty hours later, the capillary tube was full of leukocytes, whereas this was not observed with a capillary tube filled with normal lymph. Thus, he had demonstrated that chemoattractant factors were produced by the frog. In 1891, the same team performed another elegant demonstration [41]. They injected s.c. bacteria (*Micrococcus prodigiosus*, nowadays known as *Serratia marcescens*) in a rabbit. Thirty-five minutes later, they sampled the blood, prepared the serum, placed it in a capillary tube and finally transferred it into the peritoneal cavity of another rabbit. Eight hours later, the capillary tube was full of leukocytes, whereas this was not the case when the tubes were filled with normal serum. For the first time, they had demonstrated that chemoattractant factors were produced in response to infection. In 1938, *Valy Menkin* (1901–1960) purified a substance from inflammatory exudates that induced an increased capillary permeability followed by a migration of polymorphonuclear leukocytes. He called his factor "leukotaxine" [42]. Unfortunately, like the case for pyrexin, despite all his efforts, he could not succeed and further studies revealed no convincing evidence that "leukotaxine" existed as a distinct chemical entity [43]! The discovery of well-identified chemoattractant factors, later called chemokines (the word was coined in 1992), was made in 1987 and 1988 with the description of interleukin-8 and macrophage inflammatory protein (MIP), renamed CXCL8 and CCL3 according to the revised nomenclature of chemokines [44].

1.8 Infection and Inflammation

The link between microbes, infection, and inflammation was made during the nineteenth century. But some genius had outstanding intuition. This is the case of *Marcus Terentius Varro* (116–27 BC), a Roman scholar who wrote: "There are certain minute creatures which cannot be seen by the eyes, but which flоat in the air and enter the body through the mouth and nose and cause serious diseases." In 1822–1823, *Bernard Gaspard* (1788–1871) and *François Magendie* (1783–1855) showed a link between putrefaction and severe diseases, fever, and death [45,46]. In 1863, *Casimir J. Davaine* (1812–1882) was the first one to convincingly demonstrate the link between bacteria and infectious disease, transmitting anthrax with the blood of a sheep containing the bacteria into rabbits which then died following this injection [47]. The same year, Louis Pasteur established that the first six bacteria identified by *Christian Gottfried Ehrenberg* (1795–1876) in Germany, 30 years earlier, were the ferments of putrefaction [48]. Of note, Ehrenberg had coined the word "bacterium" in 1828. Some infectious diseases were not immediately recognized as such. For example, *Antoine de Jussieu* (1686–1758), a French botanist and physician, claimed that puerperal sepsis

was an inflammation of the uterus. He proposed to use extracts of bark of quassia to reduce fever. Interestingly, this plant was well known to Brazilian Indians who used to relieve pain and fever. *Thomas Denman* (1733–1815) in his book, the first ever to be entirely devoted to puerperal sepsis, mentioned that in addition to an inflammation of the uterus, it could be also an inflammation of the bowel [49]. In 1795, *Alexander Gordon* (1752–1799), a Scottish physician who studied the epidemic puerperal sepsis in Aberdeen, also described inflammation of the abdomen, but he was the very first to claim that it was a contagious disease spread by the hand of the physicians [50]. A similar observation was also made in the United States of America by *Oliver W. Holmes* (1809–1894) in 1843 [51]. Nevertheless, the final demonstration was achieved in 1847 by *Ignaz Semmelweis* (1818–1865), a Hungarian doctor working at the maternity clinic of the Vienna General Hospital. He successfully demonstrated that if medical students cleaned their hands after practicing autopsies before helping women in childbirth, the mortality due to puerperal sepsis was significantly decreased. Yet, doctors were not convinced, and he was asked to leave the maternity clinic. He published his key demonstration 10 years later in Hungarian [52]. However, a short note was published in *The Comptes Rendus* of the French Academy of Sciences on February 21, 1848. Ten years after his demonstration, in Paris, *Paul Dubois* (1795–1871), a professor of obstetrics, claimed that the contamination was due to miasma [53]. Eighteen years after Semmelweis' observation, *Carl Mayrhofer* (1837–1882) was asked by Prof. Carl Braun (1862), the head of the same maternity clinic in Vienna, to demonstrate that airborne organisms were the source of childbed fever. After publishing works in agreement with the ideas of his boss, his following works supported Semmelweis' work, concluding that infection was usually the result of contaminated hands [54]. He was fired! In 1869, *Victor Feltz* (1835–1893) and *Léon Coze* (1819–1896), two Strasbourg physicians, were the first to identify the presence of bacteria in the blood of a lady who died of puerperal sepsis [55]. Ten years later, on March17, 1879, Feltz republished a similar observation [56]. The following day, on 18th March, Louis Pasteur confirmed the observation [57], but challenged the work of Feltz, arguing that his patient had died of anthrax after deliverance.

1.9 Usefulness of Inflammation for Adaptive Immune Response

Gaston Ramon (1886–1963), a veterinary doctor, was hired by the Institut Pasteur to prepare horse immune sera against diphtheria toxin and tetanus toxin. He observed that the antibody titers were quite different from one horse to another. He made a link between these titers and the presence of an inflammatory reaction at the site of injection of the antigen as illustrated by the appearance of an abscess. Then, he purposely created an inflammatory reaction at the site of injection using different substances such as tapioca or lanolin. In 1926, he realized that there was a need to involve an inflammatory reaction at the site of injection of the antigen to enhance immune response, and he had discovered the

concept of adjuvant [58]. Many others used different inflammatory substances, such as lipids. For example, *Jules T. Freund* (1890–1960), an Austria-Hungarian born American immunologist, proposed to create a water oil emulsion to which he added inactivated mycobacterium tuberculosis. In search of the smallest bioactive part that allows the adjuvant properties, the biochemist *Edgar Lederer* (1908–1988) and the immunologist *Louis Chedid* joined forces and discovered the muramyldipeptide [59]. The adjuvants, the so-called "little dirty secrets of the immunologists" led *Charles Janeway* (1943–2004) to redefine the immune response [60]. Since then, what used to be defined as nonspecific immunity was renamed innate immunity. Innate immunity and inflammation are more or less two names for a similar mechanism, namely, the defense of the host against infectious or sterile insults.

1.10 Mediators of Inflammation

If nowadays the complement system is known to contribute to inflammation, particularly through the release of anaphylatoxins, its first identification was associated with its capacity to induce bacteriolysis as shown in 1894 by *Richard Pfeiffer*. In 1898, *Jules Bordet* (1870–1961), who received Nobel Prize in 1919, identified that hemolysis was similarly induced by serum components, but the word "complement" was coined by *Paul Ehrlich* (1854–1915), who shared the 1908 Nobel Prize with Metchnikoff. Surprisingly, when the word anaphylatoxin was employed in a paper reporting a model of anaphylatoxic shock, its existence was denied. Indeed, an unanticipated severe reaction had been observed in 1901 when *Charles Richet* (1850–1935, Nobel Prize 1901) and *Paul Portier* (1866–1962) involuntarily killed dogs after the second injection of a hypnotoxin from physalia. One of the main mediators of anaphylatoxic shock is histamine. The molecule was synthesized for the first time in 1907 by *Adolf O.R. Windaus* (1876–1959, Nobel Prize 1928), and *Henry H. Dale* (1875–1968, Nobel Prize 1936) who discovered its activity on smooth muscle and on endothelium. In 1937, *Anne-Marie Staub* (1914–2012), who worked in the laboratory of *Daniel Bovet* (1907–1992, Nobel Prize 1936) at Institut Pasteur, was the first scientist to save an animal from a shock induced by histamine. However, the employed antihistaminic drug was toxic, and the first antihistaminic to be used in humans (Antergan) was developed by *Bernard Halpern* (1904–1978).

In 1930, *William S. Tillett* (1892–1974) and *Thomas Francis Jr.* (1900–1969), working at the Rockefeller Institute in New York, identified in the serum of a rabbit injected with *Streptococcus pneumoniae* an interaction activity with a carbohydrate extract of the bacteria (called "fraction C"). The C-reactive protein (CRP) was thus discovered and shown to be present in the inflammatory settings independent of any *S. pneumoniae* infection [61]. CRP was purified by *Colin M. MacLeod* (1909–1972) and *Oswald T. Avery* (1877–1955) in 1941 [62] and crystallized in 1947 by *Maclyn McCarty* (1909–1972) [63]. All these investigators were working at the Rockefeller Institute. The pentameric structure was determined in 1977 and its liver origin was demonstrated the following year.

Nowadays, CRP is one of the most common biomarker to confirm the presence of an inflammatory reaction.

In 1935, *Ulf von Euler* (1905–1983) discovered the prostaglandins. This Swedish physiologist and pharmacologist, who also discovered noradrenalin and substance P, was awarded the Nobel Prize in 1970. His father, *Hans von Euler-Chelpin* (1873–1964) was also awarded the Nobel Prize in 1929 for his contribution to the fermentation of sugar and his mother, *Astrid Cleve* (1875–1968), a botanist, geologist, and chemist at Uppsala University, had been the first woman in Sweden to obtain a doctoral degree of science. For their studies on prostaglandins, related biologically active substances, and the discovery that aspirin prevented the production of prostaglandins, the Swedish *Sune K. Bergström* (1916–2004) and *Bengt I. Samuelsson* (1934–) and the British *Sir John R. Vane* (1927–2004) were awarded the 1982 Nobel Prize.

Glucocorticoids was discovered in 1936–1941 by three scientists who were awarded the Nobel Prize in 1950 for their discoveries related to the hormones of the adrenal cortex, their structure, and biological effects. In the United States, *Philip Showalter Hench* (1896–1965) postulated that a natural mediator, which he called substance X, was involved in the improvement of arthritis when patients developed jaundice. He collaborated with *Edward Calvin Kendall* (1886–1972), who purified a large number of substances from adrenals. Among those, was the compound E (dehydrocorticosterone) that was finally considered as substance X [64]. In Switzerland, *Tadeusz Reichstein* (1897–1996), who was born in Poland, also produced numerous substances from adrenals, of which few were bioactive. In 1937, he reported the production of desoxycorticosterone and its first clinical essays [65].

After the identification of the endogenous pyrogen in 1953 as aforementioned, the next cytokine being described was the interferon. In 1954, two Japanese scientists *Yasuichi Nagano* (1906–1998) and *Yasuhiko Kojima* (1928–) noticed that rabbit skin or testis previously inoculated with UV-inactivated virus exhibited inhibited viral growth when reinfected at the same site with live virus. They hypothesized that this was due to some inhibitory factors. They made two major mistakes. The first one was to publish in a French-speaking journal and the second one was not to create a neologism [66]. This explains why the British *Alick Isaacs* (1921–1967) and the Swiss *Jean Lindenmann* (1924–2015), who published three years later in English and coined the word "interferon," have been regularly considered as the discoverers of this cytokine.

In 1966, a new cytokine was discovered by Bloom and Bennett who illustrated the capacity of the supernatants of T cells involved in delayed type hypersensitivity to prevent macrophage migration (macrophage migration inhibitory factor, MIF) [67]. Most fascinatingly, this cytokine was rediscovered later as a product of the pituitary gland. Interestingly, MIF brought Stanley Cohen to create in 1974 his neologism "cytokine" [68]. The words lymphokine and monokine were commonly used, but Cohen had shown that fibroblasts infected with viruses could release MIF. Then, it was obvious that these mediators were not only the products of immune cells but also a universal language of the cells.

Endogenous pyrogen, osteoclast activating factor, hemopoietin-1, catabolin, lymphocyte-activating factor (LAF), epidermal cell-derived thymocyte-activating

factor (ETAF) are various mediators identified between 1953 and 1981, which appeared to be the diverse biological properties of one molecule, namely, interleukin-1. It was later categorized into IL-1α and IL-1β, which are members of a family of 11 molecules, including agonists and antagonists [69]. IL-1 bioactivity was the first to be identified in an inflammatory fluid. In 1982, a French dentist working in Joost Oppenheim's laboratory demonstrated the presence of IL-1 in the gingival fluid of patients with periodontal inflammation [70]. In 1986 in Norway, Waage *et al.* reported for the first time the presence of tumor necrosis factor in the plasma of patients with severe meningococcal sepsis [71]. Later on, all pro- and anti-inflammatory cytokines could be detected in the plasma of patients with severe sepsis or sterile systemic inflammation. These measurements illustrate the cytokine storm observed in many severe inflammatory settings, but those are just the tip of the iceberg [72]. Most fascinating is this statement made by William Osler in 1904: "Except on few occasions, the patient appears to die from the body's response to infection rather than from it."

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Part One

Inducers and Sensors of Inflammation

2

Pathogen-associated Molecular Patterns

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2.1 Definition

For a while, the immune system was defined by its capacity to distinguish between self and nonself, thanks to the specific immunity achieved by B and T lymphocytes with the help of accessory cells. A nonspecific immunity was also recognized but was not considered to be of great interest by the leaders in the field of immunology. Not until in 1989, Charles Janeway (1943–2003) asked: "Why do we need to add adjuvant (noxious substances like mineral oil, mycobacteria, or aluminum hydroxide) in order to get a decent response to a vaccine?" The self–nonself model neither predicted nor explained the need for adjuvant [1]. And he called the adjuvants, the "immunologists' dirty little secret." Then, he proposed that the self–non-self model had reached its end, arguing that the innate immune system was the real gatekeeper. He also argued that the innate immune system used ancient pattern recognition receptors" (PRRs) to recognize a pathogen by its unchanging characteristics. Thereafter, the concept of "pathogen-associated molecular pattern" (PAMPs) was born. PAMPs are compounds present among microorganisms but absent in host cells. They possess rather conserved structures that are required for the survival of pathogens. Often, authors appropriately prefer the term "microorganism (or microbe) associated molecular patterns (MAMPs)." Indeed, the molecular determinants recognized by the "pattern recognition receptors" of the host are not specific to pathogens but are found in any microorganisms being nonpathogenic, pathogenic, or commensal. Indeed, human body is made of 3×10^{13} cells, while it hosts $4 \times 10E^{13}$ commensal bacteria in the throat, nose, gut, or skin. All of these commensal bacteria express similar compounds that can also be found among the pathogenic ones. For example, all Gram-negative bacteria express endotoxin (lipopolysaccharide, LPS) on their surface, and all Gram-positive bacteria display lipoteichoic acid anchored within the peptidoglycan (PGN). Of interest, PAMPs can be expressed on the surface of or inside the pathogens. This means that some PAMPs can be released following the action of the complement system or released within an endosome or the cytoplasm of the phagocytic cells or the

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target cells. Then, these PAMPs will be sensed by intracellular PRRs present either within endosomes or within the cytoplasm.

Of interest is the capacity of the host both to sense these different PAMPs and to specifically adapt its response. This specific recognition and adapted response is not only found in mammals but also exists in insects, which can release antimicrobial peptides adapted to fungal and Gram-negative or Gram-positive bacterial infection. In mammals, the response is different depending on the type of pathogen, and the host can discriminate between viral, bacterial, parasitic, or fungal infections and adapt its response. To illustrate this adapted responsiveness, let us mention the plasticity of dendritic cells, which display different gene expression in response to a bacterium, a virus, or a fungus, while their respective PAMPs, LPS, viral double-stranded RNA, and mannan partially mimic the gene expression induced by the whole pathogen [2]. While Polly Matzinger defined the concept of "damage-associated molecular patterns" (DAMPs) as endogenous alarm signals, which initiate the immune response, it is interesting to note that the concept of exogenous alarm signal was previously proposed by Legrand in his review on the response of the host to endotoxin, undoubtedly the most potent PAMP [3].

2.2 Endotoxin

2.2.1 Brief Historical Background

The basis of contagious diseases was first associated with the presence of miasma in the close environment, before the source of infection was suspected to have been derived from putrid matter. In 1822, *Bernard Gaspard* (1788–1871), a physician in Châlon/Saone, showed that indeed the injection of purulent material could induce death in animals. In 1856, *Peter Ludvig Panum* (1820–1885), a Danish physiologist, showed that the "putrid poison" leads to symptoms and signs similar to those observed in sepsis. Although he initially rejected the possibility that bacteria could play a causative role, in his last publication he hypothesized that "putrid poison" could be a bacterial product. Indeed, his "putrid poison" had striking similarities with endotoxin. In 1888, *Nicolai Gamaleia* (1859–1949), an Ukrainian bacteriologist, after his training with Elie Metchnikoff, reported that heat-killed bacteria injected in rabbits could induce fever. Most fascinatingly, he showed that filtered alcoholic extracts of spleen from pyretic sheeps could induce fever within 30 min when injected into rabbits. In the following year, *Sir Marc Armand Ruffer* (1859–1917), a famous British physician, when working in Paris with *Albert Charrin* (1856–1907), a professor of medicine, showed that the filtered culture of pyocyanic bacillus (*Pseudomonas aeruginosa*) could induce fever in rabbits, in the absence of alive or dead bacteria. Amazingly, the authors proposed, although did not prove, that fever was the consequence of the activation of macrophages. In 1890, *Hans Ernst August Buchner* (1850–1902), a German bacteriologist, showed that bacteria were pyogenic. In 1892, *Richard Pfeiffer* (1858–1945), a German physician and bacteriologist, proposed that toxic properties of bacteria were due to a factor released by lysed bacteria, and coined

the term "endotoxin." In 1894, *Eugenio Centanni* (1863–1942), an Italian pathologist, recognized the intimate relationship between the pyrogenic and the toxic properties of the poison, which he found to be chemically inseparable. This caused him to name his material "pyrotoxina." In 1894, *Giuseppe Sanarelli* (1864–1940), an Italian physician and bacteriologist, observed that animals injected twice with a bacterial endotoxin experience massive disseminated intravascular coagulation with blood clots in the vessels of the kidneys. His observation, called Sanarelli reaction, is also known as the Schwartzman reaction described later in 1928. In 1898, *William B. Coley* (1862–1936), an American surgeon, published that a mixture of dead *Streptococcus pyogenes* and dead *Serratia marcescens* could protect against tumor. On January 24, 1893, the first patient to receive Coley's treatment (known as Coley's toxins) was John Ficken, a 16-year-old boy with a massive abdominal tumor. Every few days, Coley injected his vaccine directly into the tumor mass. By August 1893, the remains of the tumor were barely perceptible. In 1906, *Alexandre Besredka* (1870–1940), an Ukrainian doctor working with Elie Metchnikoff, reported the very first anti-endotoxin antibodies when the German school was claiming that it was impossible [4]. In 1930, *Fritz Kauffmann* (1899–1978) published the first antigenic schema for *Salmonellae*. Based on antibodies against the polysaccharidic region of LPS, 44 serotypes were then listed. In 1935, *Lydia Mesrobeanu* (1908–1978), a scientist at the Cantacuzino Institute in Bucharest, and *André Boivin* (1895–1949), her mentor, reported the first demonstration that endotoxin was made of sugars and lipids. They called it "antigène glucido-lipidique," before it was renamed lipopolysaccharide in the English speaking journals. In 1946, *Paul Beeson* (1908–2006) showed that repeated exposure to typhoid vaccine in rabbits led to a state of tolerance, identified as a reduced fever. The concept was soon called "endotoxin tolerance." In 1952, *Otto Westphal* (1913–2004) described the most popular method to prepare LPS from Gram-negative bacteria by a hot aqueous phenol extraction [5]. In 1956, LPS was shown to be an adjuvant [6] and the same year was demonstrated to be a B-cell mitogen [7]. In 1968, Louis Chedid was the first to propose the concept of a universal antibody, which could neutralize endotoxin during sepsis [8]. Although it was initially shown to be successful [9], this approach was abandoned. In the 1970s, LPS was reported to induce interleukin-1 (IL-1) [10] and tumor necrosis factor (TNF) [11], and TNF was shown to mediate the lethal effects of LPS in 1985 [12]. In the 1980s, the biological characterization of the lipidic moiety (lipid A) and its properties were further deciphered and a synthetic lipid A was produced [13]. In 1990, CD14 was identified as part of the LPS receptor [14], and in 1998 TLR4 was identified to be the other essential moiety of this receptor [15]. In 2009, the US Food and Drug Administration (FDA) approved the monophosphoryl lipid A (MPLA), a subunit of LPS, to be used as an adjuvant in vaccines.

2.2.2 Biochemical Characteristics

Endotoxins are specific of Gram-negative bacteria. The LPS constitutes 75% of the outer membrane of the bacteria and the number of molecule per bacteria is estimated to be around 4 million. They are made of a lipidic moiety called "lipid

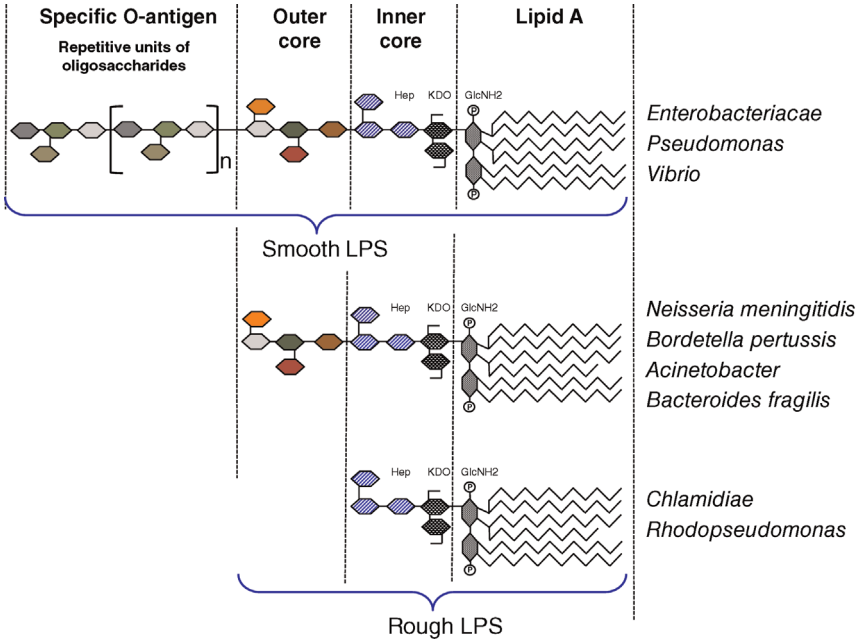


Figure 2.1 Chemical structures of the endotoxins (LPS) of Gram-negative bacteria. Of note, the complete structure (lipid A + core + O-antigen) corresponding to smooth LPS can be shortened in certain rough (R) bacteria. Hep = heptose; KDO: keto-deoxy-octonate; GlcNH2: glucosamine.

A" and a polysaccharidic moiety (Figure 2.1). The lipid A is made of four to seven fatty acids bound to a dimer of phosphorylated D-glucosamine. The length of the fatty acid varies between 10 and 18 carbons. The lipid A is bound to the polysaccharide through one specific eight-carbon acidic sugar (3-deoxy-D-manno-oct-2-ulopyranosonic acid, KDO). The KDO and few heptoses often substituted by phosphate, pyrophosphate, or diphosphoethanolamine are part of the "inner core." The "outer core" region usually consists of neutral or amino-hexoses such as D-glucose, D-galactose, D-glucosamine, D-galactosamine, or *N*-acetyl derivatives. The O-chain structure is made of repeating units (up to 50 ×) consisting of one to eight sugars, and confers the serotype specificity. Enterobacteriacae (e.g., *Escherichia*, *Salmonella*, etc.), *Pseudomonas*, and *Vibrio* display such a complete structure, which is associated with smooth colonies (S LPS). Structures that miss the oligosaccharidic repetitive units are found in mutants of enterobacteriacae and naturally in other bacteria (e.g., *Neisseria*, *Bordetella*, etc.). LPS from some bacteria (*Chlamidiae, Rhodopseudomonas*, etc.) further misses the outer core region. When the repetitive polysaccharidic units are missing, the colonies of the bacteria are rough (R LPS). Because the bacteria are permanently making their LPS, the preparations of LPS are extremely heterogeneous with a mixture of R and S molecules and with intermediate molecular weights reflecting different degrees of polymerization of the repetitive units.

Numerous studies have been carried out to decipher the relationship between structures and activities. Most bioactivities have been regularly linked to the lipid

A moiety. Certain charged structures (i.e., phosphate groups, KDO) enhance the strength of the molecule to activate phagocytes [16] and R LPS are more potent than the purified or synthetic lipid A [17]. The number of fatty acids interferes in the potency of LPS to activate immune cells. Usually, hexa-acylated LPS are the most active ones (e.g., *Neisseiria, Salmonella, Escherichia*, etc.), while lower numbers of fatty acids ($n = 4–5$) are associated with less active LPS (e.g., *Bordetella*). Different degrees of acylation can be found in the same strain of bacteria, depending either on their origin (e.g., laboratory strains versus cystic fibrosis clinical isolates of *P. aeruginosa* [18]) or on their culture conditions (e.g., culture at 37 °C versus 27 °C of *Yersinia pestis* [19]). Similarly, cultures of bacteria in planktonic or biofilm forms affect the exact nature of the LPS [20].

2.2.3 Detection

The hemolymph of horseshoe crab (*Limulus polyphemus*) contains amebocytes of which granules content can be released after contact with endotoxin, resulting in a coagulation cascade. The first available *in vitro* limulus amebocyte lysate (LAL) tests led to a process of jellification within the tube. Nowadays, colorimetric assays are commonly used. Because of the great sensitivity of the assays, they have to be performed with endotoxin-free water and materials. Usually, the calibration curve is performed with *Escherichia coli* LPS and the results are expressed as endotoxin units (EUs). The sensitivity is around 0.1 EU/ml, which corresponds to 10 pg/ml of *E. coli* LPS. Of note, unsurprisingly, because of the great heterogeneity of the LPS structures from bacteria to bacteria, the reactivity within the LAL varies greatly between the LPS of different origins, from 0.14 to 16.7 EU for a similar amount of LPS (1 ng) [21]. Similarly, the capacity of polymyxin B to neutralize LPS activities varies depending on the origin of the LPS [22]. The presence of endotoxin is universal, from drinking water to tobacco leaves and from dust to environmental air. It was reported that a high environmental contamination in farms had been associated with the reduced risk of young children to develop asthma [23]. Interestingly, LPS has also been identified as the main PAMP in filarial nematode as a consequence of the presence of endosymbiotic *Wolbachia* bacteria [24]. Furthermore, LPS contaminants are present in numerous elements employed in *in vitro* culture (fetal calf serum, proteins, monoclonal antibodies, etc.) and have been the source of a large number of published artifacts.

Circulating endotoxin can also be detected in the blood of sepsis patients. The first demonstration was made in 1963, using a rabbit skin test associating subcutaneous injection of epinephrine [25]. The possible presence of endotoxin was then confirmed with the Limulus assay in 1970 [26]. The highest levels were associated with the poorest outcome [27]. In patients with meningococcal infection, plasma levels of LPS were shown to correlate with circulating chemokines [28], and bacterial DNA [29]. Of interest, its presence was also found in patients with Gram-positive bacterial or fungal infection, suggesting that part of its origin can derive from gut translocation [27]. Indeed, circulating endotoxins have been found in many clinical settings as an illustration of endotoxin translocation from the gut (Table 2.1).

Table 2.1 Clinical settings associated with the presence of circulating endotoxin.

Diseases	References
Sepsis	Douglas *et al.* [25] Levin *et al.* [26]
Cirrhosis	Tarao *et al.* [30]
Thermal injury	Jones and Roa [31]
Inflammatory bowel diseases	Palmer *et al.* [32]
Peritonitis	Berger *et al.* [33]
Pancreatitis	Foulis *et al.* [34]
Irradiation	Maxwell *et al.* [35]
Cardiopulmonary bypass surgery	Andersen [36]
Chronic renal failure	Nisbeth *et al.* [37]
Liver transplantation	Miyata *et al.* [38]
Acute respiratory distress syndrome	Parsons *et al.* [39]
Hemorrhagic shock	Roumen [40]
Abdominal aortic surgery	Cabié *et al.* [41]
Trauma	Strecker *et al.* [42]
Chronic heart failure	Niebauer *et al.* [43]
Resuscitated cardiac arrest	Adrie *et al.* [44]
Acute heart failure	Peschel *et al.* [45]
HIV infection	Brenchley *et al.* [46]
Severe malnutrition	Highes *et al.* [47]
Cystic fibrosis	Del Campo *et al.* [48]

2.2.4 Natural Ligands

A great number of natural ligands have been identified in plasma, saliva, and lungs and in granules of neutrophils (Table 2.2). Some have been reported to be acute-phase proteins (LPS-binding protein, LBP; soluble CD14) and further enhance cell activation by LPS [49,50]. LBP has been shown to behave as a shuttle molecule, catalyzing its interaction with CD14. Membrane CD14 is a glycosyl-phosphatidylinositol (GPI)-anchored protein with leucin-rich repeats like those found in the extracellular domain of TLR (Toll-like receptor) molecules. Not only CD14 is a key molecule in the activation of cells by LPS but it is also involved in the interaction of host cells with different PAMPs such as lipoteichoic acid, fimbriae, sugar polymers (e.g., *P. aeruginosa* poly β-1-4-D-mannuronic acid; Streptococcal rhamnose-glucose polymer), bacterial lipoproteins, mycobacterial lipomannan, and trehalose-dimycolate. Apart from being an acute-phase protein, sCD14 can also be released by monocytes/macrophages. Soluble CD14 allows CD14-negative cells, which express TLR4, such as the endothelial cells, to

Table 2.2 Natural LPS ligands.

Plasma	Leukocytes	Saliva	Lungs
LPS-binding protein Soluble CD14 Soluble MD2 Albumin Lipoproteins (HDL and LDL) Antibodies Complement HSP60 Transferrin Hemoglobin Serum amyloid P Lactoferrin	Bactericidal/permeability increasing protein (BPI) Cationic antimicrobial peptides (CAP18 and CAP37) Gangliosides Acyloxylacyl hydrolase	Lysozyme Histatins	Surfactant protein A

respond to LPS. Similarly, LBP binds other PAMPs than only LPS, including glycolipids, lipoteichoic acid, triacylated lipopetide, peptidoglycan, and mycobacterial lipomannan. LBP is a 60 kDa glycoprotein produced by hepatocytes. Its normal concentration in serum is 3 μg/ml and can reach 200 μg/ml in acute-phase serum. LBP can either shuttle the LPS to plasma high-density lipoprotein for its neutralization or to CD14 for initiation of activation [51]. Interestingly, both anti-LBP and anti-CD14 antibodies protect mice against a lethal challenge with LPS, but these antibodies are deleterious when used during a Gram-negative infection [52]. In agreement with the latter observation, LBP-deficient mice are more sensitive to bacterial infection [53].

In contrast, other ligands inhibit LPS activating properties (e.g., cationic antimicrobial peptide CAP 18 and CAP37; bactericidal/permeability increasing protein, BPI). As reflected in the different LPS structures, their inhibitory potency varies from LPS to LPS [54]. BPI share 44% homology with LBP, mainly present in granules of neutrophils, and it can be found in low amounts in human plasma (0.2–2 ng/ml). In abscess fluids, its concentration exceeds that of LBP [55]. BPI is protective against bacterial infection, but its protective efficiency depends on the nature of the bacteria [56]. A recombinant fraction of BPI was shown to significantly reduce the neuroinjury and the need for amputation in patients with severe meningococcal sepsis [57]. Plasma lipoproteins prevent LPS to interact with its receptor, hence limiting its capacity to activate cytokine release [58]. Robert Munford's team has described that the acyloxyacyl hydrolase, which is an enzyme present in macrophages and neutrophils, removes secondary acyl chains from the lipid A, thus detoxifying the endotoxin molecule [59].

2.2.5 Cell Activation

Among the various TLR ligands, LPS is the one, which induces or represses the largest number of genes in macrophage cell line [60]. After its interaction with the MD2 molecule associated with surface TLR4, LPS induces a MyD88-dependent

signaling cascade that leads to the nuclear translocation of the transcription factor NF-κB that acts on the promoters of numerous genes involved in inflammation. In addition, in a CD14-dependent fashion, LPS can be internalized and initiates the TRIF signaling pathway. Of note, human caspase 4 and its murine homolog caspase 11 have also been identified to be the intracellular receptor of LPS [61]. B-lymphocytes, dendritic cells, neutrophils, and monocytes/macrophages are the main LPS responsive leukocytes. Activation of human neutrophils by 100 ng/ml LPS activates or represses 385 genes [62]. A study performed on human monocytes revealed that among the LPS-inducible genes, 25% were identified to encode cytokines and chemokines, followed by proteins related to metabolism (11%), cell surface antigens (9%), nuclear proteins (8%), proteases (6%), proteins related to extracellular transport (4%), and intracellular transducers (4%) [63]. Of interest, in addition to a common core of 415 modulated genes, the gene expression profile in LPS-activated bone marrow-derived macrophages is specific to each mouse strain [64]. *In vivo*, LPS is a potent inducer of cytokines particularly TNF, of which the production that can be detected in the bloodstream is contributed by many tissues, including, heart [65], spleen [66], kidneys [67], brain, and liver [68]. Once again, the plasma levels of TNF depend on the mouse strains [69]. Similarly, in experiments performed with human peripheral blood mononuclear cells, the LPS-induced TNF levels vary from individual to individual [70]. Humans are highly sensitive to endotoxin, and as little as 30 ng/kg induces fever, chills, drop in leukocyte counts, and lower blood pressure, and it also induces interferon expression as shown after a self-injection of LPS [71]. Another self-injection of 15 μg/kg induces a major cytokine storm and a multiple organ failure in humans [72]. In contrast, mice can be injected with 5 g/kg without major deleterious effects. Plasma factors present in murine serum are responsible for the great resistance of mice to LPS [73].

2.2.6 Tolerance

A first activation by LPS up- or downregulates numerous genes, but a second dose soon after does not lead to the same profile of gene modulation. This phenomenon, which is known as endotoxin tolerance, was first claimed not to be specific to endotoxin [74] before being recognized to be common in most TLR ligands, including PAMPs and DAMPs. The terms "leukocyte reprogramming" illustrates this natural event occurring to prevent an overzealous activation that would be deleterious and to initiate mechanisms that aim to dampen the inflammatory reaction. This topic is specifically addressed in Chapter 60.

2.3 Other Bacterial PAMPs

2.3.1 Lipoproteins

In vivo, no cell will ever interact with the purified molecule of endotoxin since its extraction needs a phenol-water extraction that does not occur in any living host. An interesting approach had been undertaken by Shaw Warren's group to identify

how LPS looks like in the host after bacteria have been killed with the complement system. They showed that LPS was associated with a few other PAMPs, including the outer membrane protein A (OmPA), the peptidoglycan-associated lipoprotein (PAL) and the Braun's lipoprotein or murein lipoprotein [75]. OmpA is a small ion channel transmembrane β-barrels protein. OmpA-deficient mutants display markedly decreased virulence [76]. Braun lipoprotein is with LPS the most abundant component of the outer membrane. Together with the PAL molecule, they contribute to maintaining the integrity of the bacterial cell envelope. PAL is highly conserved among enteric Gram-negative bacteria and is a 18–21 kDa lipoprotein of which the protein moiety (152 amino acids) is expressed within the periplasmic domain (i.e., between the outer and the inner membrane). PAL is released into the bloodstream in a mouse cecal ligation and puncture (CLP) model used to induce sepsis and can be lethal in the sensitized D-galactosamine mice[1] [77]. Lipoproteins can be exposed on the bacterial outer membrane and are also expressed by Gram-positive bacteria. For example, PpmA (putative proteinase maturation protein A) and SlrA (streptococcal lipoprotein rotamase A) are lipoproteins belonging to a family of peptidyl isomerases (PPIases) and have been demonstrated to contribute to the virulence of *S. pneumonia* [78]. Synthetic dipalmitoylated lipopeptide (Pam2-Cys-Ser-Lys-Lys-Lys-Lys; Pam2CysSK4) and tripalmitoylated lipopeptide (Pam3-CysSK4) are often used to mimic bacterial lipopeptides and are used as TLR2/TLR6 and TLR2/TLR1 agonists, respectively. Another synthetic derivative of the macrophage-activating lipopeptide (MALP-2) from *Mycoplasma fermentans* is also available as a TLR2/6 agonist.

2.3.2 Lipoteichoic Acid

Lipoteichoic acid (LTA), an ubiquitous cell wall component of Gram-positive bacteria, represents a potent immunostimulatory molecule. LTA is anchored in the bacterial membrane via a diacylglycerol and projects through the peptidoglycan. LTA activates immune cells via TLR-2, requiring CD14 [79]. LTA and its receptor molecules accumulate in lipid rafts and subsequently target the Golgi apparatus after internalization [80]. A complete LTA molecule containing six glycerophosphate units carrying four alanine plus one *N*-acetyl-glucosamine substituents has been synthesized, which displayed the same potency to activate monocytes as native LTA [81]. LTA without any alanine is 100-fold less active than the native molecule [82]. When the molecule is delivered to the lungs in murine models, it induces neutrophil recruitment and inflammatory cytokine expression. Surprisingly, the process does involve not only TLR2 but also TLR4 and the platelet activating factor receptor [83]. On the contrary, when it is delivered to human lungs, LTA elicits neutrophil recruitment but fails to activate them as well as the alveolar macrophages [84]. Indeed, the studies on the capacity of LTA to activate neutrophils led to controversial reports.

1 D-galactosamine is a hepatotoxic agent used to render the mice far more sensitive to endotoxin and other PAMPs.

2.3.3 Flagellin

The bacterial flagellum is a rotary nanomachine that spins at hundreds of revolutions per second. The flagellum is composed of ~30 different proteins. Flagellin, a highly conserved protein of 33–40 000 Da, is the major structural component of the filament of the flagellum. It is recognized in host cells by cell surface receptor TLR5 [85], the cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family members NLRC4 (*NLR family caspase-activating and recruitment domain (CARD) domain-containing protein 4*) [86], and NAIP (*neuronal apoptosis inhibitory protein*) [87]. TLR5 is expressed exclusively on the basolateral surface of intestinal epithelia, thus providing a molecular basis for the polarity of this innate immune response. Therefore, the detection of flagellin by basolateral TLR5 mediates epithelial-driven inflammatory responses to Salmonella [88]. Flagellin not only activates monocytes/macrophages but can also directly stimulate human NK cells, inducing interferon-γ (IFN-γ) production [89].

Intravenous injection of flagellin in mice induces a strong pulmonary inflammation as assessed by leukocyte recruitment and the high levels of cytokines in bronchoalveolar lavages [90]. Chignard's team showed that flagellin was with LPS the main PAMP of *P. aeruginosa* [91]. Of note, some bacteria (e.g., *Helicobacter pylori*, *Campylobacter jejuni*, etc.) display mutated flagellin in 89–96 region that is known to be important for TLR5 recognition, allowing them to escape the innate immunity [92]. Of interest, flagellin could be detected in the serum of sepsis patients and its level correlates with the duration of shock and the lung injury score [90]. Flagellin is also found in the sputum of cystic fibrosis patients [93].

2.3.4 Peptidoglycan

Peptidoglycan is a constituent of both Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, the peptidoglycan area is thin and present between the outer and the inner membranes. In Gram-positive bacteria, the peptidoglycan is thick and constitutes the external structure of the bacteria. PGNs are polymers of β-(1,4) linked *N*-acetylmuramic acid (N-AcMur) and N-acetylglucosamine (N-AcGlc) linked by peptide bonds (Figure 2.2). In Gram-negative bacteria, the peptide bridges are short and contain a very specific amino acid, the diaminopimelate (DAP) or its epimer (mesoDAP). In Gram-positive bacteria, the peptide bonds are longer and contain repeated sequence of glycine. In all cases, alanine is bound to N-AcMur. By attempting to characterize the smallest active moiety of the peptidoglycan displaying adjuvant property, Louis Chedid and Edgar Lederer identified in 1976 the N-acetylmuramyl-L-alanyl-D-isoglutamine (or muramyl dipeptide, MDP) as the smallest active moiety [94]. In 1984, Claude Leclerc showed that intracellular delivery of MDP with the help of antibodies increased 10 000-fold its adjuvanticity [95]. She then postulated that "specific receptors for MDP exist inside the macrophage [96]. To be active, MDP has to be present inside the cells in sufficient concentration" [97]. Seventeen years later, this statement was confirmed with the discovery of NOD1 and NOD2 as the receptor for Gram-negative peptidoglycan-derived minimal structure (N-AcGlc – N-AcMur – L-Ala – D-Glu – mesoDAP), and for both types of bacteria

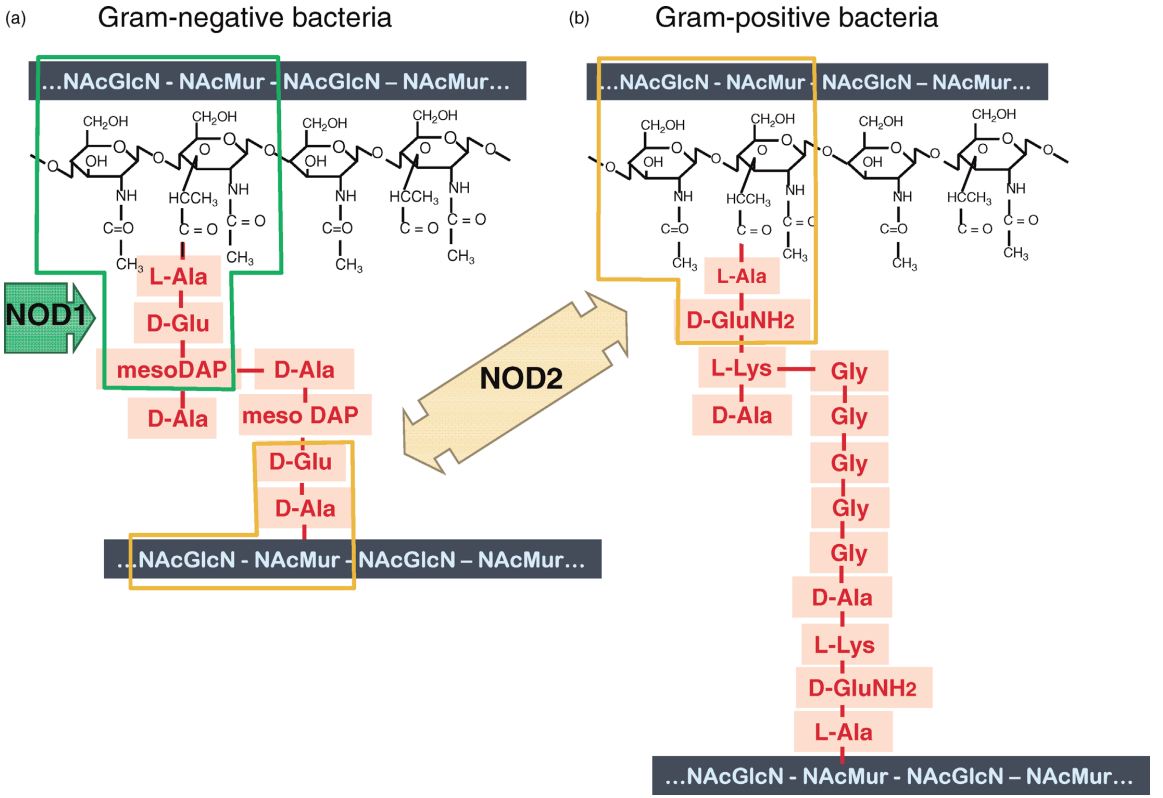


Figure 2.2 Chemical structure of peptidoglycan (PGN) from Gram-negative (a) or Gram-positive (b) bacteria. Of note, a specific amino acid (meso-diaminopimelate) is found mainly in PGN of Gram-negative bacteria, and the peptide bridge is longer in PGN of Gram-positive bacteria. The PGN area recognized by the NOD1, and NOD2 cytoplasmic pattern recognition receptors are shown. NAcGlcN: *N*-acetyl-glucosamine; NAcMur: *N*-acetyl-muramic acid.

(N-AcGlc – N-AcMur – L-Ala – D-Glu) [98,99]. Of note, human and murine NOD1 and NOD2 do not recognize the same minimal structures [100]. If TLR2 can also recognize PGN remains debatable [101,102]. CD14 and LBP also concur to the activation of host cells by PGN [103,104]. Furthermore, peptidoglycan recognition proteins (PGRPs) are PRRs known to interact with PGN. Thirteen PGRP have been described in *Drosophila melanogaster* and four in mammals (PGLYRP-1,2,3,4). The latter are found in different tissues and produced by different cells [105]. Human PGRPs form disulfide-linked homodimers and heterodimers. PGLYRP-1, 3, 3:4, and 4 have antibacterial and bactericidal activities [106] and PGLYRP-2 is an amidase [107].

2.3.5 Fimbriae

Fimbriae are short appendages up to 2 μm long that are exposed on the entire bacterial surface and allow the bacteria to adhere to different types of surfaces. They are made of staking of 500–3000 FimA subunits with additional molecules (FimF, FimG, and FimH) attached on the top. The studies on the interaction of complete fimbriae with epithelial cells, monocytes, dendritic cells, or transfected cells identified TLR2 as the major PRR [108,109]. CD14 and CD11b/CD18 were also shown to interact with fimbriae [109]. The isolated subunit FimA failed to activate epithelial cells [110], but it was shown to weakly activate the macrophage cell line THP-1 via TLR2 [111]. FimH was found to activate macrophages and NK cells via TRL4 [112], and the major fimbrial subunit CsgA of thin curled fimbriae of *Salmonella enterica* serotype Typhimurium is recognized by TLR2 [113].

2.3.6 Glycogolipids

Although the widespread distribution of glycolipids among bacteria is well established, those of mycobacteria have received considerable attention. In particular, mycobacterial dimycolate is a potent macrophage modulator and has been used as an adjuvant. Different receptors have been identified including a C-type lectin named Mincle [114], and the scavenger receptor MARCO, which cooperates with CD14 and TLR2 [115].

Glycosphingolipids (GSLs) are membrane components of every eukaryotic cell. *Sphingomonas* species, *Borrelia burgdorferi*, *S. pneumoniae*, and *H. pylori* have been shown to possess such glycolipid antigens. In contrast, Gram-negative bacteria, if they do, poorly express these molecules [116]. These glycolipids are presented by CD1d molecules of dendritic to the invariant T cell antigen receptor of NKT cells resulting in IFNγ production [117,118].

2.3.7 Heat Shock Proteins

Heat shock proteins (HSPs) are found in both eukaryotic and prokaryotic cells. Of interest, this family of molecules can be classified as PAMPs when derived from pathogens and also as DAMPs when released by damaged host cells. HSPs are intracellular molecular chaperones and play a cytoprotective role when cells are exposed to stressful stimuli. Released into the extracellular milieu, they display

immunoregulatory properties. *Chlamydia pneumoniae* HSP60 activates smooth muscle cells, endothelial cells, monocytes/macrophages, and dendritic cells [119–121]. The specific involvement of TLR2 and TLR4 depends on the cells studied. The *in vivo* role of TLR4 was demonstrated when acute pulmonary inflammation was induced in mice [122]. *H. pylori* HSP60 activates human epithelial cells via both TLR2 and TLR4 [123], and human monocytes via TLR2 [124]. While other studies using *E. coli* HSP (DnaK) and *Mycobacterium tuberculosis* HSP71 suggested that their capacity to enhance antigen processing and presentation was TLR independent [125]. Indeed, the stimulatory properties of *M. tuberculosis* HSP71 were confirmed *in vivo* to be TLR2 and TLR4 independent [126]. However, another *M. tuberculosis* HSP (HSP60) was shown to interact with both TLR2 and TLR4 [127]. Finally, *Francisella tularensis* HSP "DnaK" activates dendritic cells in a TLR4-dependent fashion [128].

2.3.8 Bacterial DNA

Immunostimulating properties of bacterial DNA and oligodeoxynucleotides (ODNs) were first reported when the antitumoral property of *M. bovis* was investigated [129]. Then, the capacity of bacterial DNA and oligodeoxynucleotides to stimulate B-lymphocytes in terms of proliferation and immunoglobulin production was reported [130]. Short ODNs containing an unmethylated cytosine triphosphate deoxynucleotide, phosphodiester bound to a guanine triphosphate deoxynucleotide (CpG), are bioactive. In contrast to eukaryotic DNA, the dinucleotide motif 5′-CpG-3′ is mostly hypomethylated in bacterial DNA. In galactosamine-sensitized mice, bacterial DNA causes lethal shock and induces the production of TNF by macrophage cell line [131]. TLR9 was identified as one of the PRRs for bacterial DNA [132] (Figure 2.3). Bacterial DNAs differ in the percentage of CpG content, and their capacity to activate TLR9 transfected HEK cells is proportional to the CpG content within the bacterial genome. For example, CpG represents 12.2% of the genome in *P. aeroginosa*, whereas it is only 2.2% in *Staphylococcus epidermidis* [133]. In RAW 264.7 macrophage cell line, CpG ODN activated 190 genes at 4 h and 105 genes at 12 h, while Poly (I:C) treatment led to the stimulation of 574 genes at 4 h and 1051 at 12 h [134]. Of interest, some genes that were upregulated neither by CpG nor by Poly (I:C) were significantly upregulated when the combination of both agonists was used. High-mobility group box 1 (HMGB1) is a DAMP that favors DNA delivery to the cells [135]. The cationic antimicrobial peptide LL37/CAP18 allows cells to sense self-DNA [136]. In contrast, some ODN sequences display inhibitory activities [137].

AIM2 (*absent in melanoma 2*) constitutes with ASC (*apoptosis-associated speck-like protein containing CARD*) and procaspase-1 in an inflammasome aiming to sense dsDNA. It is a 343 amino acid protein with an N-terminal pyrin domain and an HIN200 (*hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats*) domain. The inflammasomes are molecular platforms that lead to the activation of caspase-1, the maturation enzyme required for the cleavage of IL-1β and IL-18 precursors. It has been regularly reported that AIM2 binds bacterial dsDNA resulting in the release of IL-1β and IL-18 (Figure 2.4) [138–140].

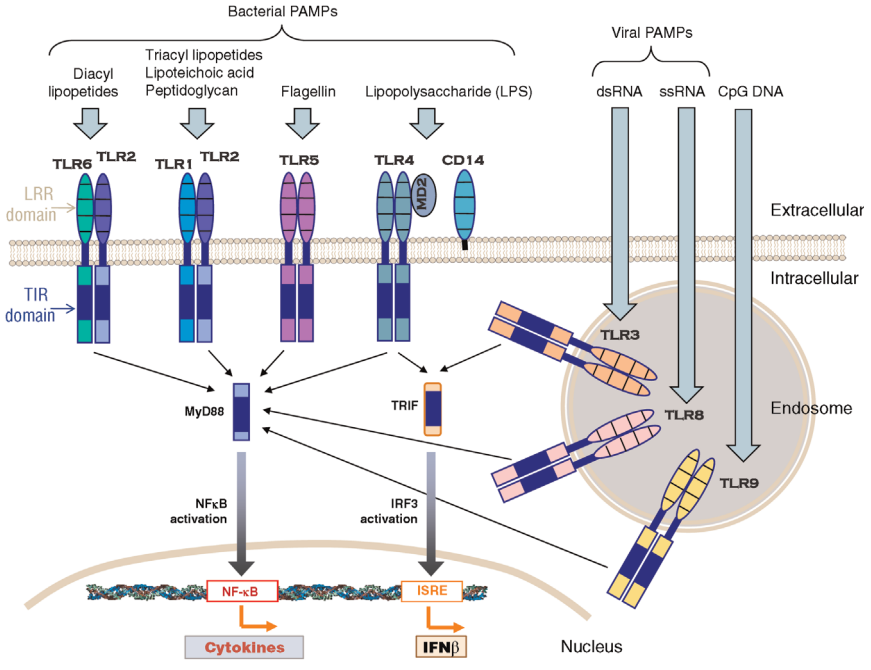


Figure 2.3 Membrane and endosomal Toll-like receptors (TLR) sensing bacterial and viral PAMPs and the two main pathways dependent on adapter molecules MyD88 (myeloid differentiation primary response gene 88) or TRIF (TIR domain-containing adapter-inducing interferon-β). LRR: leucine-rich repeat; TIR: Toll-interleukin-1-receptor; NF-κB: nuclear factor-κB; IRF3: interferon regulatory factor 3; ISRE: interferon-stimulated response element; IFNβ: interferon-β.

2.3.9 Bacterial RNA

Bacterial RNAs are important stimuli of the innate immunity [141] and their sensing can indicate to the host the presence of live bacteria [142]. The immunostimulatory properties of bacterial mRNA was first identified by its capacity to induce the release of IL-12 by human dendritic cells [143] before it was reported that macrophages could also be activated by bacterial RNAs to produce cytokines. Human and murine endosomal TLR7 can sense bacterial RNA and favor the release of type I interferon. In mice, endosomal TLR13, which does not exist in humans, specifically recognizes 23S ribosomal RNA [144]. In humans, endosomal TLR8 was reported to recognize bacterial RNA [145,146]. More precisely, TLR8 recognizes two degradation products of single-strand RNA: uridine and a short oligonucleotide. Uridine binds the site on the dimerization interface and the short oligonucleotide binds a site on the concave surface of the TLR8 horseshoe structure. Both binding sites were essential for activation of TLR8 by ssRNA [147] (Figure 2.4).

Retinoic acid-inducible gene-I (RIG-I) is a cytoplasmic PRR with two CARD domains and a helicase subunit initially recognized to bind 5′-triphosphate viral ssRNA. Different investigations have also reported the capacity of bacterial RNA to activate type I IFN response via RIG-I [148], a process that occurs during

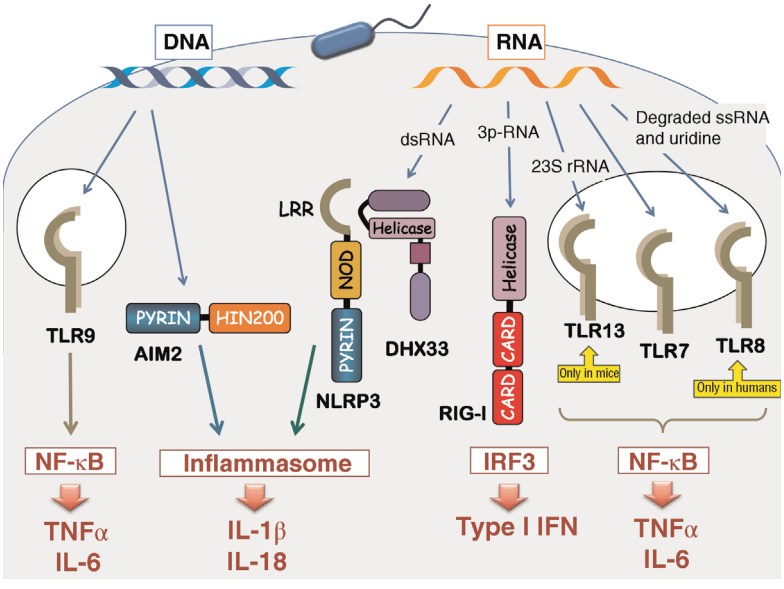


Figure 2.4 Bacterial DNA and RNA can be sensed by endosomal or cytoplasmic receptors, resulting in signaling cascades that end in cytokine production. dsRNA: double-strand RNA; ssRNA: single-strand RNA; NOD: nucleotide-binding oligomerization domain; AIM2: absent in melanoma 2; NLRP: NOD-like receptor family, pyrin domain containing DHX33 (DEAH (Asp-Glu-Ala-His) box polypeptide 33); RIG: retinoic acid-inducible gene; CARD: caspase-activating and recruitment domain; IRF3: interferon regulatory factor 3; NF-κB: nuclear factor-κB.

bacterial infection in immune and nonimmune cells [149]. The specificity of eukaryote mRNA is a 2′O-methyl group at N1. This methylation prevents the RIG-I molecule from being activated by self RNA [150]. Of interest, flaviviruses exploit this tolerance mechanism to escape from the immune system sensing.

The third signaling pathway is consecutive to the capacity of bacterial double-stranded RNA to bind via the helicase domain to DHX33, a nucleolar RNA helicase, allowing the assembly of a DHX33-NLRP3 (*NLR pyrin-domain containing 3*)–ASC–caspase-1 complex that mediates processing and secretion of IL-1β and IL-18 [151].

2.4 Viral PAMPs

Of course, the variety of viral PAMPs is far lower than that of bacteria. Viral PAMPs can be expressed on virus surface structures, be part of the capside or its genomic material and replication products (Figure 2.5). Whole virus such as yellow fever virus used for vaccine activates dendritic cell via TLR2, TLR7, TLR8, and TLR9 to elicit the proinflammatory cytokines [152]. Isolated viral components interact with either TLR2 or TLR4. For example, mouse mammary tumor virus or murine leukemia virus envelope proteins coimmunoprecipitated with TLR4 when expressed in HEK cells [153]. Hepatitis C virus core and

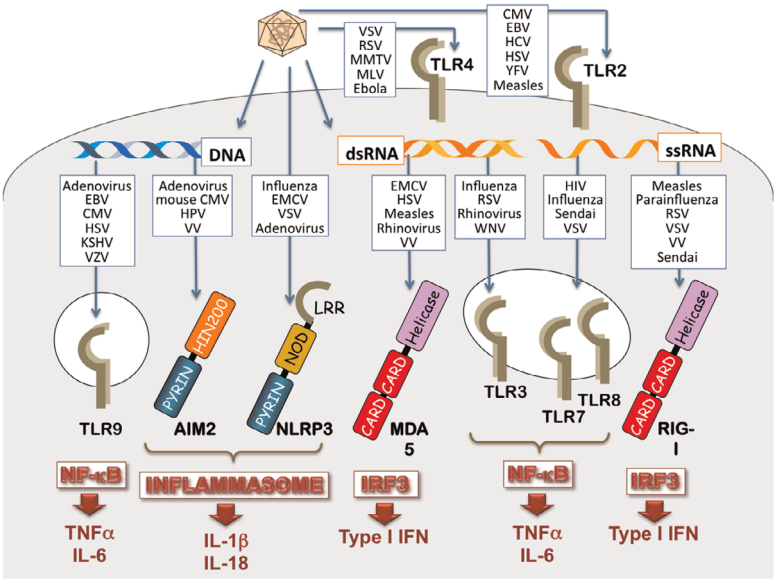


Figure 2.5 Viral DNA and RNA can be sensed by endosomal or cytoplasmic receptors. VSV: vesicular stomatitis virus; RSV: respiratory syncytial virus; MMTV: mouse mammary tumor virus; MLV: murine leukemia viruses; CMV: cytomegalovirus; EBV: Epstein–Barr virus; HCV: hepatitis C virus; HSV: herpes simplex virus; YFV: yellow fever virus; KSHV: Kaposi's sarcoma-associated herpes virus; VZV: Varicella zoster virus; HPV: human papilloma virus; VV: vaccinia virus; EMCV: encephalomyocarditis virus; HIV: human immunodeficiency virus; TLR: Toll-like receptor; AIM2: absent in melanoma 2; NLRP: NOD-like receptor family, pyrin domain containing MDA5 (melanoma differentiation-associated protein 5); RIG: retinoic acid-inducible gene.

nonstructural 3 (NS3) protein activate inflammatory pathways in monocytes via TLR2. TLR2-mediated cell activation was dependent on the conformation of core and NS3 proteins and required sequences in the regions of a.a. 2–122 in core and a.a. 1450–1643 in NS3 [154]. Wild-type measles virus but not vaccine strains activate cells via both human and murine TLR2, and this is a property of the hemagglutinin protein. Its ability to activate cells via TLR2 is abolished by a mutation of a single amino acid, asparagine at position 481 to tyrosine, as found in attenuated strains [155]. Glycoproteins of herpes simplex virus (HSV) and human cytomegalovirus (CMV) are TLR2 ligands, whereas the glycoprotein of vesicular stomatitis virus (VSV) is a TLR4 ligand [156]. Interestingly, Ebola virus leads to a glycoprotein expression on the surface of infected cells, which is shed from the surface by TNF-alpha-converting enzyme (TACE). This glycoprotein is a potent TLR4 agonist that triggers the release of inflammatory cytokines and increases vascular permeability [157].

Genomic ssRNAs of human immunodeficiency virus (HIV), influenza virus, Sendai virus, Coxsackievirus, and VSV are TLR7/8 ligands. 5′-triphosphate ssRNA of flaviviridae, paramyxoviridae, orthomyxoviridae, rhabdoviridae, and filoviridae are recognized by RIG-I. Double-stranded RNA derived from HSV, Epstein–Barr virus (EBV), influenza virus, respiratory syncytial virus (RSV), HIV, and rhinovirus are TLR3 ligands. MDA5 (*melanoma differentiation-associated*

protein 5) is a RIG-I-like receptor of dsRNA generated by the replication of the viruses. MDA5 does not recognize the genomic ssRNA but is the key sensor for the dsRNA replicative intermediate form of positive sense ssRNA viruses [158]. Of note, polyinosinic:polycytidylic acid (poly I:C) is a useful synthetic tool commonly used to mimic dsRNA. Poly I:C is a mismatched double-stranded RNA with one strand being a polymer of inosinic acid and the other being a polymer of cytidylic acid. Finally, viral DNA from HSV, CMV, EBV, Varicella zoster virus, Kaposi sarcoma-associated herpes virus, and Vaccinia virus are TLR9 ligands [156], while others act through AIM2. More surprising was the report that ssRNA of RSV could activate an antiviral innate immune response via NOD2 [159]. Indeed, NOD2-deficient mice were more sensitive to the viral infection and produced a limited amount of IFNβ.

2.5 Parasitic PAMPs

Although the use of different PRR-deficient mice has allowed the identification of some of the innate immune receptors involved in sensing parasitic infections, PAMPs of parasites remain far less characterized than those of bacteria.

In the case of *Plasmodium falciparum*, the malaria parasite digests hemoglobin and converts it into insoluble and chemically inert β-hematin crystals, called hemozoin. This hemozoin favors the recognition of plasmodial DNA by TLR9 [160]. *Plasmodium* glycosylphospatidylinositol (GPI)-anchored glycolipids play an important role in the innate immunity. They are synthesized exclusively at the mid to late trophozoite stages. The parasite's GPI glycolipid consists of four mannoses, one glucosamine, and one inositol. The carbohydrate moieties contain one or two extra phosphoethanolamine. The inositol residue is usually di- or triacylated with C14 to C22 fatty acids, predominantly palmitic and myristic acids. In humans, the GPI glycolipids are mainly recognized by the TLR2/1 heterodimer. In mice, they seem to be recognized by both TLR2/1 and TLR2/6 [161]. CD36, a scavenger receptor, is also involved in the recognition of the GPI glycolipid [162]. The fact that TLR7 was identified as a key sensor of *P. chabaudi* suggests that ssRNA of the parasite also behaves as a PAMP [163]. The observation that *Plasmodium* RNA is a PAMP and is capable of activating a type I IFN response was further demonstrated *in vivo*, and MDA5 was recognized as its cytosolic pattern recognition receptor [164].

Trypanosoma cruzi infections were shown to involve TLR2, TLR4, and TLR9 receptors [165]. GPI-anchored mucin-like glycoproteins distributed at the cell surface membrane of *T. cruzi* trypomastigotes, and glycoinositolphospholipids (GIPLs), which are a subset of GPI anchors from the epimastigote stage of *T. cruzi*, activate cells via TLR2 [166]. The GPI-anchored mucin-like glycoproteins also bind to CD1d, but this interaction with NKT cells does not seem to play a major role in the innate immunity against *T. cruzi* [167]. The *T. cruzi*-released protein, Tc52, has been identified as a potent inducer of TLR2 activation in human and mouse innate immune cells. The *T. cruzi* Tc52-released protein induces human dendritic cell maturation, signals via TLR2 [168]. *T. brucei rhodesiense* and *T. brucei gambiense* express a unique surface coat comprising GPI-anchored variant

surface glycoprotein (VSG) molecules that activates macrophages through scavenger receptor [169]. Other PRRs have been shown to contribute to the innate immune response to *Trypanosoma* (i.e., NOD1, C-type lectin receptor), but the precise nature of the parasitic ligands has not yet been identified. Similarly, the activation of inflammasome by *T. cruzi* has been well established, but the exact nature of the ligand and its receptor remains to be defined [170].

A soluble tachyzoite antigen prepared from *Toxoplasma gondii* induces IL-12 and CCL2 in bone marrow-derived macrophages in a TLR2-dependent fashion [171]. *T. gondii*-derived heat shock protein 70 (*Tg*HSP70) is a tachyzoite-specific virulent molecule expressed before the death of hosts. A vaccine with *Tg*HSP70 was shown to be TLR4-dependent [172]. *T. gondii* activates NLRP1 and NLRP3 inflammasome, but no defined PAMP has yet been identified [173].

Soluble egg antigen (SEA) of *Schistosoma mansoni* is a complex mixture that activates immune cells and others such as endothelial cells. Accordingly, different PRRs have been reported to be involved including TLR2 [174], C-type lectin (i.e., Dectin-2, DC-SIGN, and L-SIGN) [175–177], and the mannose receptor [178]. Glycans and a T2 ribonuclease are part of the SEA. Multifucosylated glycosphingolipids constitute the major egg glycolipids and can behave as other putative PAMPs of *S. mansoni* [179]. In addition, schistosomal lysophospatidylcholine has been reported as a TLR2 agonist [180], a larval preparation has been shown to stimulate macrophages through TLR4 [181], and finally, *Schistosoma* egg-derived double-stranded RNA activate dendritic cells via TLR3 [182].

Lipophosphoglycan is a prominent member of the phosphoglycan-containing surface glycoconjugates of *Leishmania*. When two different lipophosphoglycan biosynthetic genes were knocked out from *L. major*, there was a clear loss of virulence in several steps of the infectious cycle, but no effect on virulence was found with *L. mexicana* [183]. *Leishmania* lipophosphoglycan interacts with TLR2, but contradictory works have been reported on the protective role of this interaction [184–186]. *L. major* specific glycan structure is characterized by a poly-β-galactosyl epitope of which galectin-3 and -9 are the main PRRs [187,188]. Other lectins such as Dectin-1, mannose receptor, and DC-SIGN are other sensors of glycosylated surface antigens [189]. Other PRRs such as NLRP-3 [190] or TLR4 [191] have been identified to sense *Leishmania*, but their corresponding ligands remain to be deciphered. An important PAMP of *Leishmania* is its DNA, which activates dendritic cells via TLR9 [192]. Of interest, the fact that specific activating motifs are present in *Leishmania* and other trypanosomatidae DNA allows human and murine TLR9 to differentiate between parasites and host DNAs [193].

2.6 Fungal PAMPs

The fungal cell wall is the main source of PAMPs. Its composition varies depending on the fungi and their growth stage. Three major cell wall components are found in all medically important fungi. β-glucans, polymers of glucose, are distinct entities [194,195]. For example, the cell wall of *Aspergillus fumigatus* contains β-1,3- and β-1,4-glucan, and α-1,3-glucan, while *Candida albicans*

contains β-1,3- and β-1,6-glucan. Chitin, a β-1,4-linked homopolymer of *N*-acetylglucosamine, provides a protective thin rigid layer on the fungal surface. Finally, mannose-containing structures mannoprotein, mannan (which are chains of several hundred N- or O-linked mannose molecules), and phospholipomannan are expressed mainly on the outer cell wall. Most of these substances are capable of inducing the release of inflammatory cytokines by macrophages. Many PPRs have been reported to recognize these fungal PAMPs. This is the case of C-type lectins that interact with the different forms of mannan (e.g., Galectin-3, Dectin-2, mannose receptor, and DC-SIGN) [195]. Dectin-1 and a scavenger receptor (CD36) have been shown to interact with β-glucan. Glucuronoxylomanann has been demonstrated to interact with TLR4 [196] and phospholipomannan with TLR2/6 [197]. Chitin has been shown to dampen inflammation through its interaction with the mannose receptor and more surprisingly with TLR9 and NOD2 [198]. Finally, as seen for the other pathogens, fungal DNA from *Candida, Cryptococcus*, or *Aspergillus* activates cells via TLR9 [199–201]. Fungal RNA could possibly be a PAMP as suggested in a study on *C. albicans* using TLR7-deficient mice [202]. Of interest, the authors demonstrated a protective role of type I interferon during fungal infection.

2.7 Synergy

One of the key features of the innate immune response to pathogens is the amazing synergy that exists not only between PAMPs but also between PAMPs and host mediators, leading to an increased production of proinflammatory cytokine, nitric oxide, and prostaglandin, dendritic cell maturation, NF-κB activation, and gene expression [203]. These synergies can contribute to enhance probabilities to develop multiple organ failure and lethal shock during severe infections. *In vitro* synergies have been demonstrated between TLRs ligands in terms of cytokines production by macrophages, dendritic cells, or epithelial cells [204–208]. Similarly, this synergy can be observed in human whole blood samples as shown by mixing LPS and PGN [209]. Furthermore, a synergy has been established between TLR agonists and NOD1 or NOD2 ligands [210–212]. Of interest, such a combined effect can be associated with the induction of the expression of certain genes, which are not induced by each PAMP individually [134]. *In vivo*, the injections of two PAMPs such as PGN and LTA synergize to cause a systemic tissue injury (i.e., liver, kidney, heart, pancreas, and lungs) and multiple organ failure [213]. In the galactosamine murine model, another synergy between LPS and bacterial lipoproteins has been shown to increase the incidence of death [214]. This observation was associated with an increased level of circulating TNF. A similar effect was reported between LPS and the *S. aureus* toxic shock syndrome toxin-1 [215]. Interestingly, the increased level of TNF was lowered by anti-IFNγ antibodies, which also protected against lethal shock.

Indeed, once produced, cytokines act in synergy with PAMPs within auto amplificatory loops. IFNγ, initially characterized as a macrophage activating factor, synergizes with LPS [216] as does IL-3 [217]. These results explain the deleterious effects of these cytokines reported in murine models of sepsis [218,219]. The

autocrine loop involving IL-1 and TNF has been nicely demonstrated in the induction of IL-8 in LPS-activated human whole blood samples [220]. The production of IL-12 by dendritic cells requires IFNβ in addition to the activation by different TLR ligands [221]. Similarly, IFNγ and GM-CSF production by NK cells induced by TLR4, TLR2, and TLR9 agonists is amplified by the presence of different cytokines [222]. Anti-inflammatory cytokines can also favor the activation of cells by LPS. For example, IL-4 enhances the IL-1 receptor antagonist (IL-1Ra) production by neutrophils [223] and paradoxically, in the absence of adherence, IL-10 boosts TNF production by LPS-activated human monocytes [224]. Synergy between cytokines has also been reported for NLR ligands as illustrated by the enhanced production of MDP-induced IL-1β and IL-6 by IL-32 [225]. The *in vivo* consequence of such synergies is an increased mortality after LPS injection by IFNγ [226] or TNF [227]. Granulocyte macrophage colony-stimulating factor (GM-CSF) also amplifies the LPS bioactivities *in vivo* [228].

Among the other host mediators produced during the inflammatory process, which act in synergy with LPS, let us mention the anaphylatoxin C5a [229], hemoglobin [230], neurotensin [231], substance P [232], adenosine [233], elastase [234], H_2O_2 [235], or platelet activating factor [236]. Membrane compounds can also amplify the effect of LPS such as triggering receptor expressed on myeloid cells-1 (TREM-1) [237]. Viral infection [238], hypoxia [239,240], ischemia/reperfusion [241], and mechanical ventilation [242] are exogenous signals that enhance the response to LPS and most probably to many other PAMPs.

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3

Damage-associated Molecular Patterns

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3.1 Definition

After Charles Janeway had revisited the current definition of immunology [1], introducing the concept of pathogen-associated molecular patterns (PAMPs) (see Chapter 2), there was still room for further brainstorming in order to conceptualize the aim of the immune system and, more importantly, to define the triggering signals needed to maintain homeostasis and host integrity. In 1994, Polly Matzinger offered a new definition of immunology. For her, the immune system does not discriminate between self and nonself, but recognizes and responds to danger signals. She postulated that any stress undergone by healthy tissues generates danger signals that initiate the activation of the immune response. She proposed the concept of danger as an internal signal, when pathogens are recognized by the tissue damage they generate. She postulated 4 "D"s of her danger model: "Distress, Damage, Destruction, and Death" [2]. While this postulate was fully correct for sterile inflammation, it was only partially true for pathogen-initiated innate immune responses (Figure 3.1). Nevertheless, endogenous danger signals are generated during both sterile and infectious inflammation. These signals – "damage-associated molecular patterns" (DAMPs) – are released by damaged tissues and necrotic cells. They can also be produced and released by activated live cells but not by apoptotic cells. Another term, "alarmins," proposed by Joost Oppenheim [3], was initially restricted to antimicrobial proteins. However, this was later extended to the early warning signals able to activate innate and adaptive immune systems, particularly in recognition of their role in mobilizing the immune system with chemoattractant and immunostimulating properties [4]. In particular, these include enhancing dendritic cell differentiation and maturation, facilitating antigen uptake, processing, and presentation. Evolution has incorporated into the mammalian genome genes encoding receptors aimed at sensing these danger signals. Interestingly, most DAMP receptors are similar to those sensing PAMPs.

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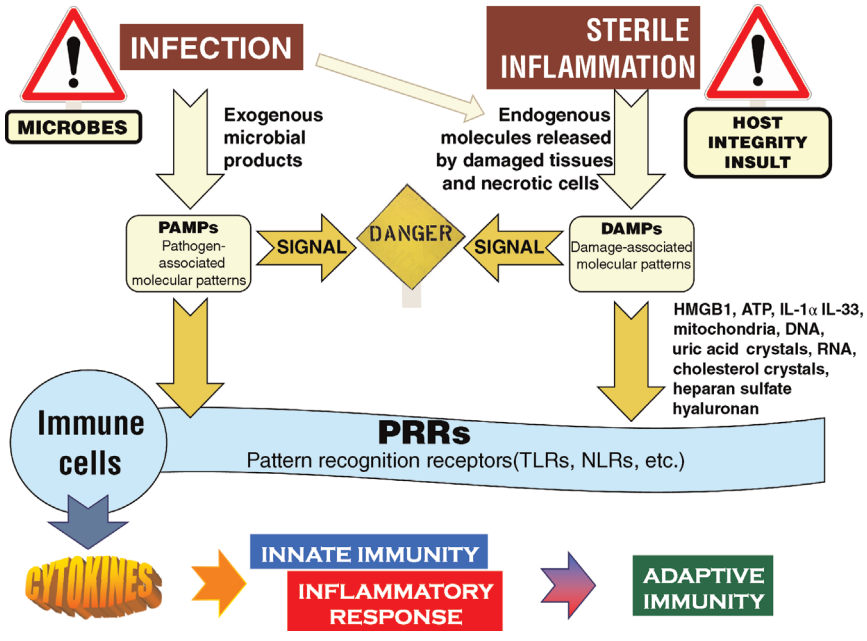


Figure 3.1 Infectious and non-infectious-initiated innate immune responses.

3.2 Necrosis versus Apoptosis

Apoptosis was defined as a silent physiological death aimed at avoiding the triggering of immune cell activation. By contrast, necrosis reflects an unintentional death following insults threatening host integrity. The insult can be of microbial origin or be sterile, for example, burns, trauma, ischemia/reperfusion, hypoxia, radiation, chemicals, and inflammatory particles. The effects of necrotic versus apoptotic cells on macrophages and dendritic cells revealed the capacity of necrotic cells to favor antigen presentation, antitumor activity, NF-κB activation, and cytokine production [5–8]. Of note, a synergy was shown between endotoxin (lipopolysaccharide, LPS) and necrotic cell lysate [9]. In trauma patients, the release of DAMPs is extremely rapid; levels of circulating nuclear DNA, mitochondrial DNA, and HSP70 are increased even at the scene of the trauma [10]. In patients with chronic obstructive pulmonary disease, the extracellular lung fluid levels of several DAMPs (including S100 proteins and high-mobility group box-1) are also increased.

3.3 Receptors of Danger Signals

The nature of the pattern recognition receptors (PRRs) sensing necrotic cells was identified *in vitro* to be Toll-like receptor 2 (TLR2) [11], TLR3 [12], and Nod-like receptor family, pyrin domain containing 3 (NLRP3) [13] (Figure 3.2). Of

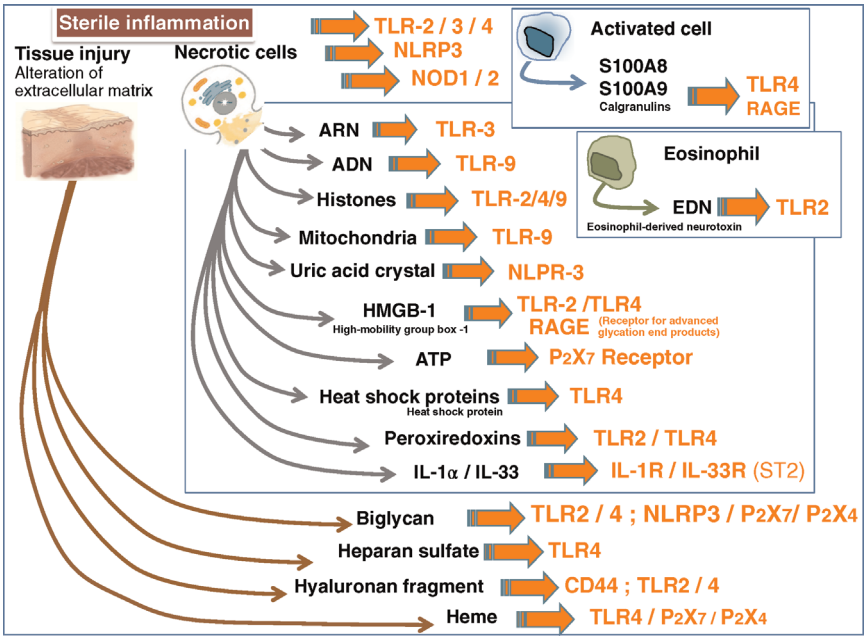


Figure 3.2 Some of the damage associated molecular patterns and their corresponding pattern recognition receptors.

particular interest, the injection of necrotic cells into the peritoneal cavity revealed a minor role for TLR2 and TLR4 but a major role for IL-1α, its IL-1 receptor, and the MyD88-associated pathway [14]. In different *in vivo* models of sterile inflammation, using different types of PRR-deficient mice, liver [15], and kidney [16], myocardial [17] and brain [18] ischemia/reperfusion injuries were found to be TLR4-mediated. Inflammation associated with trauma generated by bone fractures [19], gut injury following trauma-hemorrhagic shock [20], and acute lung injury [21] were also found to be TLR4-dependent. In the latter case, oxidized phospholipid was identified to induce lung injury and cytokine production by lung macrophages via the TLR4-dependent Toll interleukin-1 receptor domain-containing adapter-inducing interferon-β (TRIF) pathway [21]. The same pathway was also shown to be involved in a model of liver ischemia/reperfusion [15]. TLR2-dependent signaling was also identified in a model of myocardial [22] and kidney [23] ischemia/reperfusion. More surprisingly, knowing the initial specificity of the peptidoglycan ligand of nucleotide-binding oligomerization domain (NOD) molecules, NOD1 and NOD2 were demonstrated to be intracellular sensors during kidney ischemia/reperfusion [24]. CD24-Siglec-10 is a receptor that discriminates DAMPs and PAMPs and negatively regulates the stimulatory activity of DAMPs [25]. CD24 associates with HMGB1 and other DAMPs (HSP70 and HSP90), and inhibits NF-κB activity, at least in part through its association with Siglec-10 in humans.

3.4 Main DAMPs

3.4.1 High-mobility Group Box 1

High-mobility group box 1 (HMGB1) (also known as amphoterin) was identified in 1975 as a nuclear protein [26]. Present in all nucleated cells, it binds to DNA, bends it to facilitate the transcription of many genes, and can also interact with nucleosomes. HMGB1 led to the publication of numerous papers in most prestigious journals. The story started in 1999, when Kevin Tracey's group found HMGB1 in the serum of LPS-treated mice, with a late peak at 15 h post-injection [27]. Mice injected with a lethal dose of LPS could be protected by anti-HMGB1 antibodies as HMGB1 acted synergistically with LPS. They also reported HMGB1 in the serum of septic patients, with the highest levels found in nonsurvivors. Similarly, elevated levels of HMGB1 were reported in the sera of patients with hemorrhagic shock [28], trauma [29], and pneumonia [30].

HMGB1 was considered a DAMP when it was reported that necrotic cells release HMGB1, leading to inflammation [31]. However, HMGB1 can also be released by live activated cells in response to inflammatory cytokines [32] or LPS [33]. Other stimuli include polyinosinic-polycytidylic acid (poly I:C) [34], influenza infection [35], or neutrophil NADPH oxidase induced by hemorrhagic shock [36]. During sepsis, a major source of HMGB1 appears to be the spleen [37]. Cardiomyocytes were also found to be a source of HMGB1 after LPS injection [38].

Tracey's group showed that HMGB1 could induce release of inflammatory cytokines by human monocytes [39]. However, it was subsequently shown that highly purified HMGB1 by itself does not induce monocyte activation [40]. Rather, HMGB1 activities are influenced by its redox state: reduced cysteine makes HMGB1 a chemoattractant, whereas a disulfide bond makes it a proinflammatory cytokine, while further cysteine oxidation abrogates both activities [41]. This molecule also has a great capacity to stick to many other microbial agonists such as LPS [42], CpG DNA [43], all immunogenic nucleic acids [44], cytokines, and chemokines such as IL-1β [45] and CXCL12 [46]. Depending on the nature of the complex formed by HMGB1, the nature of the receptor can be TLR2, TLR4, TLR7/8, TLR9, the IL-1 receptor, or CXCR4 [47]. In addition, HMGB1 can be bound by the receptor for advanced glycation end products (RAGE) [48]. HMGB1 promotes recruitment of leukocytes across the endothelial barrier, activates endothelial cells with increased expression of adhesion molecules, disrupts both the endothelial barrier and that of enterocytes, enhances dendritic cell maturation, and acts in synergy with other cytokines to induce production of interferon-gamma by NK cells [49]. It also favors release of prostaglandin E2 by vascular smooth muscle cells [50]. Targeting HMGB1 with specific antibodies protected against acute lung injury [51], experimental models of arthritis [52], remote organ injury following trauma [53], and sepsis [54]. The use of glycyrrhizic acid, which binds and inhibits HMGB1, in a rat model of diffuse axonal injury ameliorated motor and cognitive deficits, reduced neuronal apoptosis, protected the integrity of the blood–brain barrier, reduced axonal injury, and limited the expression of proinflammatory cytokines [55]. siRNA treatment targeting HMGB1 was effective in a cecal ligation and puncture (CLP) model of

sepsis in humanized mice [56]. Human hematopoietic stem cells transferred to immune-deficient nonobese diabetic mice (NOD/SCID/IL2R$\gamma^{-/-}$) contributed to sepsis-induced mortality. Delivery of HMGB1 siRNA targeted at human macrophages reduced the cytokine storm and lymphocyte apoptosis, and rescued humanized mice from CLP-induced mortality. In a mouse model of acute and chronic seizures, antagonists of HMGB1 and TLR4 reduced the clinical symptoms. Together with the observation that increased expression of HMGB1 and TLR4 was found in human epileptogenic tissue, a role for the HMGB1-TLR4 axis in human epilepsy has been suggested [57].

3.4.2 Heat Shock Protein

Heat shock proteins (HSP) are abundant and ubiquitous intracellular proteins. They were initially discovered in 1962 in drosophila exposed to heat. They represent around 5% of total intracellular proteins, but under stressful conditions (high temperature, toxins, oxidative stress, glucose deprivation, etc.), their concentration can rise significantly [58]. Intracellularly, they act as chaperone molecules that are involved in the folding and unfolding of other proteins. They are grouped into families according to their molecular weights (small HSPs, HSP40, HSP47, HSP60, HSP70, HSP90, and HSP100). They also share similarities with their bacterial counterparts. They can activate T-lymphocytes, B-lymphocytes, NK cells, dendritic cells, macrophages, and endothelial cells. A puzzling observation is their capacity to activate cells via TLR2 and TLR4 in a CD14-dependent fashion [59], exactly as endotoxin does. Indeed, numerous investigations came to the conclusion that the macrophage-activating properties of HSP60 [60], HSP70 [61–63], and gp96 [64] were secondary to endotoxin contamination. LPS binds to cell membrane forms of HSP70 and HSP90 [65]. HSPs are released by necrotic but not by apoptotic cells [7], and produced in response to LPS [66,67], to CpG oligonucleotide [68], and in experimental models of hemorrhage [69]. HSP70 levels were increased in the sera of patients with trauma [70], in resuscitated patients after cardiac arrest, and in brain death [71]. In sepsis, HSP70 is enhanced not only in plasma but also in circulating neutrophils [72].

Of great interest are the arguments of van Eden *et al.* [73], who consider that HSPs are not DAMPs but rather "dampers." They state that HSPs exist at homeostasis both at the cell surface and as freely circulating molecules. High concentrations of highly purified HSPs have no stimulatory effects but rather are highly immunogenic and stimulate Treg. Of interest, as noted for HMGB1, they favor the action of PAMPs [74].

HSPs may all not share the same properties. For example, intracellular HSP70 is directly hepatoprotective during liver ischemia–reperfusion injury while extracellular HSP70 does not contribute significantly to the injury response in this model [75]. Indeed, heat stress protects rats and mice against endotoxin shock [76,77]. Similarly, HSP70 was reported to ameliorate experimental acute respiratory distress syndrome [78] and to offer protection in a heart ischemia–reperfusion model [79]. *In vitro*, overexpression of HSP-70 was able to inhibit lipopolysaccharide-induced production of cytokines in human monocyte-derived macrophages. By contrast, HSP90 inhibitors prevented LPS-induced endothelial

barrier dysfunction [80], reduced lung injury, and prolonged survival in a murine model of sepsis [81]. Also in favor of a role for HSPs as DAMPs, a model of myocardial ischemia reperfusion revealed significantly attenuated apoptosis and cytokine expression in wild-type mice treated with anti-HSP60 antibody and in TLR4-deficient mice compared with wild-type controls [82].

3.4.3 RNA and DNA

Hypomethylated CpG sequences among bacterial DNA contrast with the high frequency of methylation in human DNA. This major difference explains why bacterial DNA can activate human TLR9, while human DNA cannot. Similarly, trypanosomatidae DNAs contain 6–13 more agonist sequences than human DNA, as well as fewer inhibitory telomeric motifs [83]. Accordingly, mammalian DNA is, by itself, not a major DAMP. However, when combined with antimicrobial peptide LL37 [84] or HMGB1 [85], self-DNA could activate cells.

Similarly, there are mechanisms that prevent self-RNA to be recognized by the retinoic acid-inducible gene 1 (RIG-I) PRR. A conserved amino acid in the RNA binding pocket prevents recognition of endogenous RNA bearing a N1 - 2′-*O*-methyl group as a marker of "self," flaviviruses exploit this tolerance mechanism to escape the immune system [86]. Double-stranded RNAs are recognized by melanoma differentiation-associated protein 5 (MDA5) and by TLR3. RNA escaping from damaged cells could serve as en endogenous ligand for TLR3 [87]. For example, RNA released from necrotic synovial cells activates rheumatoid arthritis synovial fibroblasts via TLR3 [88]. RNAs are also ligands of TLR7 and TLR8 [89]. As for DNA, the antimicrobial peptide LL37 can bind RNA and the complexes can activate cells through TLR7 and TLR8 [90]. Of note, self-RNA-LL37 complexes are present in psoriatic skin lesions [90].

3.4.4 Mitochondria

Because of the bacterial origin of this organelle, it is not surprising that mitochondria released following the disruption of cells behaves as a DAMP. *In vitro*, they can activate neutrophils while injection of mitochondria causes systemic inflammation and organ injury [91]. As mitochondrial DNA contains hypomethylated CpG DNA repeats similar to bacterial DNA, it is the main activator within the mitochondria via TLR9 [91]. Of note, TNF-induced cell death is associated with release of mitochondria that act as a danger signal [92]. Importantly, mitochondria and mitochondrial DNA can induce cross-tolerance with LPS and, most probably, with any other TLR agonist [93].

3.4.5 Histones

Extracellular histones released in response to inflammatory challenges contribute to endothelial dysfunction, organ failure, and death during endotoxemia and sepsis [94]. This was illustrated by the capacity of antihistone antibody to rescue mice after a lethal injection of LPS or in a peritonitis model. Injection of histones induces massive accumulation of neutrophils in the alveolar vasculature and can

lead to death. During sterile inflammation following liver ischemia/reperfusion, histones activate Kupffer cells via the NLRP3 inflammasome [95]. In trauma patients, histones are found within the bloodstream at concentrations that are toxic for endothelial cells [96]. Histones can trigger cytokine release with the lungs being the predominantly affected organ showing edema, hemorrhage, microvasculature thrombosis, and neutrophil recruitment [96].

3.4.6 Uric Acid Crystals

The cytosol of UV-treated fibroblasts and liver cells contain uric acid. As this is relatively insoluble, high concentrations favor the formation of monosodium urate (MSU) crystals. These activate dendritic cells when released by dying cells [97]. This endogenous adjuvant has been shown to contribute to the adjuvant property of alum [98]. MSU crystals are the etiological agent causing gout. Their capacity to induce IL-1β production reflects their capacity to activate the NLRP3 inflammasome [99]. In this context, it is worth recalling the beneficial effects of IL-1 receptor antagonist treatment in patients with gout [100]. In a bleomycin-induced lung injury model, uric acid released from injured cells activated the NLPR3 inflammasome and contributed to lung inflammation repair and fibrosis [101]. Of note, other crystals, such as cholesterol crystals, act as endogenous danger signals due to their capacity to activate the NLRP3 inflammasome [102].

3.4.7 ATP

Adenosine triphosphate (ATP) is not only an intracellular nucleoside triphosphate utilized by energy-requiring cellular processes but can also serve as a danger signal in inflammatory processes. ATP can be released both by apoptotic cells or autophagic dying cells through pannexin hemichannels and by activated inflammatory cells via connexin hemichannels. ATP also acts as a DAMP in plants [103]. ATP can be released by cells exposed to inflammatory substances such as uric acid crystals, silica, and alum [104]. Anaphylatoxin C3a, which favors IL-1 release [105], also favors ATP release [106]. ATP acts in synergy with LPS to amplify release of IL-1β and IL-18. ATP activates cells via the purinergic P2X7 receptor on macrophages and dendritic cells. High concentrations of ATP are lethal for T-lymphocytes [107]. ATP also contributes to NLRP3 inflammasome activation and the release of IL-1β and IL-18 [108]. Its role, via the P2X7 receptor, has been illustrated in experimental lung inflammation and in patients with idiopathic pulmonary fibrosis [109]. In a murine model of autoimmune encephalomyelitis, alkaline phosphatase, an enzyme that dephosphorylates ATP to ADP thence to AMP and adenosine, significantly reduced neurological signs [110]. Ectonucleoside triphosphate diphosphohydrolase-1 (also known as CD39) is a cell surface molecule expressed on numerous immune cell types; this also contributes to the hydrolysis of ATP to ADP and AMP. In patients with multiple sclerosis, CD39 is increased on microglial cells in white matter lesions [110]. Of note, AMP itself can be converted by CD73 (Ecto-5′-nuclotidase) to adenosine, which possesses anti-inflammatory properties.

3.4.8 S100A8/S100A9

S100 proteins are calcium-binding proteins; more than 20 members of this protein family have been described. S100A8 (also named myeloid-related protein-8, MRP8, or Calgranulin A – 8 kDa) and S100A9 (MRP14/Calgranulin B – 14 kDa) are two abundant cytoplasmic proteins in monocytes and neutrophils comprising 30–60% of total cytosolic protein in neutrophils. They exist as homodimers and, preferentially, as a heterodimer (24 kDa) or a heterotetramer also known as calprotectin (S100A8/A9). Calprotectin contains zinc-binding domains that are involved in its antibacterial activity. Intracellular S100A9 is a critical regulator of TLR3 trafficking, and it acts as a regulator of TLR3 signaling and functions. It colocalizes with TLR3 following poly I:C treatment and facilitates maturation of TLR3 containing early endosome to late endosome [111]. S100A8/A9 is secreted by macrophages and neutrophils activated by TLR agonists, C5a, fMLP, LPS, TNF, or IFNγ. Their increased presence within tissues reflects the inflammatory process as seen in inflamed skin and colon [112,113]. They bind to heparan sulfate proteoglycans, CD36, RAGE, and TLR4. In a murine model, bone marrow cell activation by S100A8, as assessed by TNF production, was absent in cells derived from TLR4-deficient mice [114]. LPS-induced TNF production was lower in S100A9 KO cells, while S100A8 KO mice were more resistant to the lethal effect of LPS + galactosamine [114].

In humans, TLR4 may play a dominant role. Interestingly, the transcriptome analyzed in human monocytes triggered by LPS or S100A8 revealed a common core of 653 modulated genes, while 768 genes specific to S100A8 were modulated [115]. Other experiments identified an association between S100A8 and S100A9 with RAGE in connection with a decrease in cardiomyocyte contractility [116]. S100A8 and S100A9 also contribute to leukocyte migration [117]. Enhanced levels of serum S100A8 and S100A9 were reported in sepsis patients and in human volunteers injected with LPS [118]. Following injection of *Escherichia coli* in mice, liver injury was less severe in S100A9-deficient mice suggesting that S100A9 released during sepsis contributes to its severity and tissue injury [118]. Similarly, during viral infection, S100A9 was released from undamaged virus-infected cells and contributed to the exacerbation of the inflammatory response, cell death, and virus pathogenesis [119]. S100A8/S100A9 has been used as a sensitive biomarker of local (irritative and allergic contact dermatitis) or systemic inflammation (collagen-induced arthritis), with detection even at subclinical activity [120]. Of note, some protective functions of S100A8 and S100A9 molecules have been reported, such as their capacity to enhance bactericidal activity of human neutrophils [121] or to induce IL-10 that initiates a feedback loop that attenuates acute lung injury [122]. Similar to other DAMPs, S100A8/S100A9 can induce cross-tolerance [123].

3.4.9 Interleukin-1α and Interleukin-33

Interleukin-1α is a cytokine that can be normally present within certain cells such as keratinocytes. In addition to its intracellular form, IL-1α also exists as a membrane form. It binds to the same receptor as IL-1β and shares similar

activities. In contrast to IL-1β and IL-33, the precursor form of IL-1α (30 kDa) is biologically active [124]. However, its production and release are independent of the inflammasome, and it possesses intracrine properties, which allows it to act directly within the nucleus. For example, it can induce release of IL-8 within *Chlamydia*-infected cells [125] without being released. In fact, intracellular accumulation of intracellular IL-1 occurs at extremely low concentrations of LPS, far lower than those required for its release [126]. The capacity of necrotic cells (endothelial cells, macrophages, etc.) to stimulate cytokine and chemokine release and an immune response can be counteracted by anti-IL1α and IL-1RA, and was abolished using cell lysates from IL-1α-deficient mice [127,128]. Another study reported that necrotic cells could also stimulate IL-1α release by macrophages [14]. IL-1α released from damaged epithelial cells and during sterile inflammation plays a key role in triggering tissue inflammation, for example, in brain [129], lungs [130,131], skin [132], and heart [133]. Interestingly, IL-1α can stimulate release of other DAMPs such as S100A8/A9 [134].

Similar to IL-1α, IL-33 displays transcriptional regulatory properties that allow it to act as an intracellular nuclear factor in an intracrine fashion [135]. Endothelial cells constitute a major source of IL-33 in chronically inflamed tissues from patients with rheumatoid arthritis and Crohn's disease [135]. Constitutive nuclear expression of IL-33 has also been reported in fibroblasts, epithelial cells, and keratinocytes [136]. IL-33 appears to be an important amplifier of innate immunity, particularly in pulmonary and digestive mucosa [137]. Once released by damaged cells, cathepsin G and elastase released by activated neutrophils contribute to its cleavage and its processing to its active form [138].

3.4.10 Heme

Heme is composed of an atom of iron linked to four ligand groups of porphyrin. Heme is a main component of hemoglobin and is also found in other hemoproteins such as myoglobin and cytochrome catalase. As a free molecule, elevated heme level in blood is a characteristic feature of diseases with increased hemolysis, myolysis, or extensive cell damage. It causes oxidative damage and amplifies the inflammatory response by its capacity to activate macrophages and neutrophils. Heme is scavenged by hemopexin and catabolized by heme oxygenase-1. Heme induces TNF secretion, but neither IL-6 nor CXCL10, by murine peritoneal macrophages in a TLR4-dependent manner [139]. Heme also activates the production of inflammatory proteins by cardiac cells through NF-κB activation [140] and vascular smooth muscle cells via reactive oxygen species [141]. Another study found that heme could activate the NLRP3 inflammasome and IL-1β release via P2X7 and P2X4 receptors [142].

3.4.11 Hyaluronan

Hyaluronan, a major component of extracellular matrix, is a high molecular weight polymer ($>10^6$ Da) glycosaminoglycan composed of the repeating polymeric disaccharides, D-glucuronic acid, and *N*-acetyl glucosamine. After tissue injury and inflammation, it undergoes cleavage, resulting in accumulation of low

molecular weight hyaluronan fragments. Those initiate an inflammatory response in a TLR4- and TLR2-dependent manner [143–145]. TLR4-associated MD2 molecule and CD44 are associated with sensing of hyaluronan fragments [146]. CD44 can promote endocytosis of hyaluronan, which can be further degraded into small saccharide fragments that can trigger the NLRP3 inflammasome and release of IL-1β [147]. Low molecular weight fragments of hyaluronan could trigger the release of β-defensin-2 in keratinocytes [148] and intestinal epithelial cells [149]. Accordingly, hyaluronan fragments contribute to protection of the skin and gut against microorganisms. Furthermore, their presence in human milk, and their capacity to induce production of β-defensin-2 in the gut, contributes toward protective antimicrobial defenses in the newborns [150].

3.4.12 Biglycan

Biglycan is a proteoglycan consisting of a 42-kDa leucine-rich protein core substituted at its N-terminal with two chondroitin/dermatan sulfate side chains. In its matrix-bound form, biglycan cannot activate any PRRs. However, upon tissue stress or damage, biglycan becomes soluble, primarily by partial proteolysis of the extracellular matrix [151]. In this form, biglycan interacts with TLR2 and TLR4, generating a rapid inflammatory response by macrophages [152] in terms of TNF and chemokine production. Biglycan-deficient mice are more resistant to LPS-induced lethality than wild-type mice, illustrating the synergy between PAMPs and DAMPs. Biglycan also induces release of IL-1β following activation of NLRP3 and P2X4 and P2X7 receptors [153]. Biglycan-deficient mice display lower levels of IL-1β in the kidney, lung, and circulation than wild-type mice in response to LPS.

3.4.13 Heparan Sulfate

Heparan sulfate (HS) is a polysaccharide ubiquitously expressed as a proteoglycan in extracellular matrices and on cell surfaces. Heparan sulfate binds to many proteins and contributes to the inflammatory response, particularly by its involvement in the initial adhesion of leukocytes to the inflamed endothelium, and the subsequent chemokine-mediated transmigration through the vessel [154]. HMGB1 is one of its ligands with HS favoring its binding to the RAGE receptor [155]. β-D-endoglucuronidase heparanase is an endoglycosidase that cleaves HS, releasing 4–7 kDa fragments that can activate the release of NF-κB-dependent inflammatory cytokines in a TLR4-, MyD88-dependent manner [156]. Soluble HS is found in the serum of septic shock patients and contributes toward an increased production of mitochondrial reactive oxygen species [157].

3.4.14 Eosinophil-derived Neurotoxin

Eosinophil-derived neurotoxin (EDN) is an eosinophil granule-derived secretory enzyme (RNASE2) belonging to the family of RNase A-type ribonucleases. EDN has broad antiviral activity, targeting RNA viruses. It also displays some chemoattractant activities for dendritic cells, stimulating maturation. It also boosts the production of inflammatory cytokines via TLR2 [158].

3.4.15 Peroxiredoxins

In humans, peroxiredoxin (PRDX) is a family of six antioxidant cytosolic enzymes whose major functions are to regulate hydrogen peroxide signaling through their peroxidase activity and to act as protein chaperones. PRDX are abundant proteins in many cells, particularly erythrocytes. PRDX1 recombinant protein stimulated inflammatory cytokine release in a TLR4-dependent signaling pathway [159]. Their release in ischemic brains contributed to the inflammatory reaction through both TLR2 and TLR4 [160]. In this model, targeting PRDX rather than HMGB1 was more neuroprotective. Of note, LPS and TNF can induce PDRX release by macrophages [161]. Circulating PRDX1 could be measured in cardiogenic shock patients needing extracorporeal membrane oxygenation [162]. Levels correlated with lactate and high levels were predictors of poor outcome. Hypoxia favors release of PRDX1 by human monocytes, while recombinant PRDX1 acted in synergy with hypoxia to induce inflammatory cytokine release of by monocytes [162].

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4

Bacterial Toxins

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4.1. Introduction

Coevolution between host and pathogen is the major determinant of genetics and pathobiology of infections and associated diseases. It is a complex process of reciprocal, adaptive genetic change in two or more species. Changes in gene structure and nuclear organization, driven by selection in one species, generate novel life styles and behaviors that, in turn, select genetic and epigenetic changes in species that share their ecological niche with the first species. Coevolution occurs among any interacting populations: prey and predator, plant and herbivores, competitors or mutualists, but it is expected to be particularly important in host–pathogen systems because of the intimate nature of their association and the strong selective pressures that one species can exert on the other. Pathogen infectivity and host resistance as well as ability of the host to clear an infection versus the strategies of the pathogen to evade or suppress host defenses are examples of reciprocal traits that are potentially involved in the host–pathogen coevolution. In each of these pairings, the effects on the fitness of the host and of the pathogen act in opposite directions: what is advantageous for the pathogen is frequently noxious for the host and vice versa. This paradigm applies to all infectious diseases, even those in which the pathogen, instead of suppressing the host immune response, elicits a strong inflammation as strategy for draining nutrients. In this case, the microorganism usually takes advantage of other mechanisms to escape the host defenses.

The second general mechanism of development of pathogenicity by microorganisms is the one deriving from the generation of proteins that support the growth of the producing species in one environment and that turn out to behave as protein toxins in occasional hosts. This is exemplified by the case of fungi [1].

In this chapter, we will focus on recent advances in the knowledge of the mechanisms evolved by bacteria to face and counteract the inflammatory response; such an action can profoundly impact the survival and spreading of the microorganisms within the host, from the host into other hosts and into the

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environment. More in particular, we will focus on those microbial virulence factors that are released in the host or in the environment and are generally termed bacterial toxins or exotoxins, and will not consider the so-called bacterial effectors that are introduced into target cells by secretion systems.

4.2 Toxins Modulating the Activity of Inflammatory Caspases and Inflammasomes

Caspases are ancient and evolutionarily conserved proteases that play essential roles in development, homeostasis, and immunity. Some caspases are involved in apoptosis, an immunologically silent form of programmed cell death. In contrast, the inflammatory caspase, caspase-1, following its activation initiates a form of lytic cell death termed pyroptosis, which releases inflammatory mediators (cytokines and danger signals), removes the replicative niche of cytosolic bacteria, and exposes intruders to extracellular defenses and neutrophils. In addition, caspase-1 mediates the maturation and secretion of pro-IL-1β and pro-IL-18, two pleiotropic inflammatory cytokines best known for inducing fever and interferon (IFN)-γ secretion, respectively. The canonical inflammasomes, a class of cytosolic pattern recognition receptors (PRR), activate caspase-1 in response to specific signatures of infection. Initially characterized in myeloid cells, inflammasomes are present also in epithelial cells [2].

Recently, a new noncanonical inflammasome pathway has been described that activates another inflammatory caspase, namely, caspase-11 (or the presumed human orthologs caspase-4 and caspase-5) [3]. Caspase-11 lacks the ability to cleave pro-IL-1β and pro-IL-18, but it can promote the release of the mature form of the cytokines by interacting and activating NLRP3 [4]. Similar to caspase-1, it induces lysis of the host cells, but the caspase-11-dependent cell death has features that distinguish it from caspase-1-induced pyroptosis and it was suggested to promote the spreading of intracellular bacterial pathogens [3] rather than contributing to their clearance as in the case of caspase-1-dependent cell death [5]; however, this remains a controversial issue [6]. In addition, it is disputed that caspase-11 rather than caspase-1 may be the critical effector of deleterious inflammatory responses [7].

Listeria monocytogenes is a Gram-positive saprophyte and facultative pathogen that causes self-limited gastroenteritis in immunocompetent individuals. In immunocompromised hosts, *L. monocytogenes* infection can progress to cause sepsis, encephalitis, and death. After internalization into epithelial cells and macrophages by exploiting the two bacterial surface proteins internalin A and B [8], *L. monocytogenes* readily escapes from phagosomes into the cytosol using listeriolysin O (LLO) that assembles transmembrane pores at acidic pH values [9]. LLO is a member of a large group of pore-forming toxins produced by a variety of pathogenic bacteria that includes perfringolysin, streptolysin, pneumolysin, and so on [10]. These toxins bind to cholesterol-containing membranes and form transmembrane pores of large diameter that rapidly quenches ion gradients across membrane causing osmotic imbalance. The pore-forming activity of

purified LLO protein is sufficient to trigger NLRP3 activation, probably via perturbations of the intracellular K^+ levels. Macrophages cultured with *L. monocytogenes* deficient for the toxin LLO do not secrete IL-1β [11].

Streptococcus agalactiae or group B streptococcus (GBS) is a highly pathogenic extracellular Gram-positive bacterium that causes life-threatening infections in neonates, pregnant women, and the elderly [12]. GBS produces two membrane-damaging exotoxins, namely, β-hemolysin/cytolysin and CAMP factor. These bacteria are capable of activating both the signals required for the synthesis and release of IL-1β. Stimulation of pro–IL-1β synthesis by GBS requires an as yet unidentified MyD88-dependent receptor, internalization of whole bacteria, and phagosomal acidification, while the secretion of the mature cytokine is consequent to the activation of the NLRP3 inflammasome by the β-hemolysin [13]. β-hemolysin is an oxygen-stable, nonimmunogenic, pore-forming cytolysin; it has not been purified to homogeneity yet and its precise molecular nature remains unknown [14]. It activates the cytosolic inflammasome platform not only because it permits the efflux of K^+ through the plasma membrane but also because it alters the membrane integrity of phagolysosomes, thus allowing bacterial RNA to escape and to interact with and activate NLRP3 [15]. IL-1β plays a central role in eliciting host defenses against GBS and this action is mediated by the increased production of the neutrophil chemokines CXCL1/CCL3 and the consequent recruitment of neutrophils to infection sites [16]. It was shown that GBS produces a serine protease that cleaves CXCL1 *in vitro* [17]; however, the *in vivo* relevance of such an activity remains to be demonstrated.

Pneumolysin, the thiol-activated cytolysin of Streptococcus pneumoniae, displays a complex action on host defense, not only by inducing necrosis or apoptosis of human neutrophils but also by triggering IL-8 production and stimulating neutrophil transendothelial migration [18]. Pneumolysin also activates the NLRP3 inflammasome and the activation of the inflammatory platform is determinant for the host defense to *S. pneumoniae* [19]. Therefore, pneumolysin can either stimulate or disable host defense depending on expression levels, site, and magnitude of infection and this dichotomy likely applies to all pore-forming toxins [18].

NLRP3 is activated also by the α-hemolysin of *Staphylococcus aureus* but inflammasome activity does not influence the clearance of bacteria during pulmonary infection, while, on the contrary, it plays an important role in pulmonary injury: the latter is independent of IL-β signaling but relies on NLRP3-induced cell death [20]. Similarly, the leukocidin PVL of *S. aureus* triggers the release of IL-β by macrophages upon the activation of the NLRP3 inflammasome [21].

Some extracellular Gram-negative bacteria activate the noncanonical inflammasome pathway and among them *Escherichia coli*, *Vibrio cholerae*, and *Salmonella typhimurium*, a facultative intracellular pathogen. In the case of *V. cholerae*, the toxin responsible for the activation of this pathway is cholera toxin B (the pentameric ganglioside GM1-binding protomer, abbreviated CTB), component of the AB5 holotoxin complex. CTB not only promotes the secretion of IL-1β/IL-18 by activating NLRP3 and caspase-1 but it also induces macrophage cell death in a caspase-11-dependent, NLRP3-independent manner [7].

4.3 Toxins Modulating the Intracellular cAMP Levels

The cAMP signaling pathway, the MAPK pathway, and the cytosolic [Ca^{2+}] are the major signaling systems in mammalian cells, including those of the immune system. Moreover, these three signaling pathways cross-talk among each other at several sites. Therefore, it is not surprising that many bacterial pathogens produce exotoxins that enter into host cells inducing an increase of intracellular cAMP or producing cAMP themselves. This is an effective and powerful strategy of immune evasion as it impacts many different cells of the immune system. The elevation of the cAMP content can be achieved by the activation of the Gsα subunit of heterotrimeric G-proteins coupled to cellular adenylate cyclases, such as in the case of CT and *E. coli* heat-labile enterotoxin (LT). Alternatively, bacteria such as *Bacillus anthracis* or *Bordetella pertussis* release and deliver into target cells an adenylate cyclase toxin: the edema factor (EF) and CyaA, respectively.

CT and LT have been extensively studied because of their strong adjuvant activity and the consequent potential application in human mucosal vaccination protocols [22]. Nevertheless, the molecular mechanisms responsible for their ability to affect the induction of mucosal immune responses have remained ill-defined. Recently, it was shown that the adjuvant activity of CT and of LT is mediated mainly by effects on antigen presenting cells (APCs) and it occurs via the cAMP-dependent protein kinase A and caspase-1/inflammasome-dependent IL-1β signaling [23].

EF produced by *B. anthracis* has a crucial role in disarming the host immune response, an event that occurs early during infection; however, as the activity of EF frequently synergizes with that of the other anthrax toxin (the lethal factor, LF), this topic is dealt with in a dedicated section below.

Interestingly, EF has been reported to enhance the migration of intoxicated macrophages by inducing the activation of cAMP signaling and the transcription factor CREB that, in turn, increases the expression of the proteoglycan syndecan-1 [24], crucial in cell adhesion and migration [25]. The bacterium would take advantage of the modified migratory behavior of macrophages to reach regional lymph nodes wherefrom it enters into the blood circulation, where it rapidly proliferates [24]. The same effect is achieved when edema toxin (ET) is applied to macrophages and it is recapitulated in dendritic cells [26]. On the other hand, ET was also shown to inhibit macrophage migration [27], and it is conceivable that ET may have differential effects on macrophages depending on their phenotypic programming. ET is composed of edema factor and the cell-binding protein, known as protective antigen (PA), which is released as a separate entity. Its name refers to the fact that it is sufficient, by itself, to generate protective immune response to anthrax disease when used as a vaccine [28]. PA binds to two different receptors widely expressed among vertebrate cell types [29,30] and it is processed on the cell surface by removal of a 20 kDa portion. The remaining 67 kDa PA forms a heptamer or octamer that binds either EF or LF [31]. The toxin complex is then internalized via the endocytic route [32] and, at the acidic pH values of late endosomes, PA forms a transmembrane pore with a structure similar to that of α-hemolysin, which mediates the translocation across the membrane into the cytosol of the unfolded EF or LF [31].

The adenylate cyclase toxin CyaA produced by *B. pertussis* is another toxin capable of elevating intracellular cAMP to supraphysiological levels. Upon the translocation of the adenylate cyclase domain across the phagocyte plasma membrane and its activation by binding the cytosolic calmodulin, the enzyme catalyzes the unregulated conversion of ATP to cAMP. Notably, live imaging of cAMP production revealed that EF and CyaA cause cAMP elevation in different compartments of the cell. EF acts mainly in the perinuclear region causing a cAMP gradient of decreasing value from the nucleus to the plasma membrane, while CyaA induces an opposite gradient [33]. CyaA causes a subversion of the bactericidal superoxide and nitric oxide production and neutrophil extracellular trap release (NET) [34–36]. Recently, it was shown that the elevation of intracellular cAMP concentration induced by CyA in macrophages activates the mitochondrial apoptotic pathway in macrophages. This is due to the accumulation of the Bax-activating protein BimEL in the cell cytosol; the stabilization of the proapoptotic protein takes place via its phosphorylation at the Ser^{83} residue by the cAMP-activated protein kinase A [37]. It is also involved in the activity of the tyrosine phosphatase SHP-1 that inhibits ERK1/2 thus preventing phosphorylation of BimL at the Ser^{69} residue that commits to proteosomal degradation [38]. Moreover, the CyA-triggered cAMP signaling upregulates the expression of BimEL through the activation of the transcription factor FOXO3A, leading to a persistent increase in proapoptotic BimEL levels in cell cytosol [39].

4.4 Toxins Modulating MAPK Signaling

Accumulating evidence suggest that p38MAPK plays an essential role in inflammation. In fact, it is involved in the expression of proinflammatory mediators in macrophages such as IL-1β, TNF-α, PGE2, and IL-12 as well as COX-2, IL-8, IL-6, IL-3, IL-2, and IL-1, and for this reason it is considered a good target in the treatment of inflammation-based diseases [40]. However, p38MAPK may also exert an anti-inflammatory role: sublytic concentrations of β-hemolysin of GBS were shown to drive the expression of the anti-inflammatory cytokine IL-10 by triggering the activation of p38MAPK in infected macrophages. This, in turn, results in the inhibition of macrophage killing activity and repression of the expression of IL-12 and NOS2, both critical factors in antibacterial host defense. In accordance, targeted deletion of p38MAPK in macrophages increases resistance to invasive GBS infection *in vivo*. The β-hemolysin capability of activating p38MAPK is shared by two other large pore-forming toxins, streptolysin O and LLO produced by *Streptoccus pyogenes* and *L. monocytogenes*, respectively, and is independent of the osmotic stress induced by membrane permeabilization and of the engagement of a TLR [41].

LF of *B. anthracis* is a zinc-dependent metalloprotease that cleaves the N-terminus of mitogen-activated protein kinase kinases (MAPKKs or MEKs) [42–44]; the impact of interfering with this signaling pathway on the functions of the immune system is tremendous, starting from macrophages that represent the first line of defense irrespective of the port of entry of the bacterial spores [45]. One of the most prominent effects of LT (the binary complex formed

by LF and PA) on macrophages is the induction of apoptosis. At variance from initial studies showing that LT induces apoptosis of activated macrophages via inhibition of the p38MAPKs activation, due to the cleavage of the upstream kinase [46], more recent studies revealed that LT-induced death pathways are more complex than it was initially thought. Indeed, LT also acts at the crossroads of the mitochondria [47], the proteasome and, more importantly, the inflammasome pathways [48,49] to trigger macrophage apoptosis; it is not clear yet whether LT acts by direct physical interactions with components of these pathways or through indirect consequences of MEK cleavage. Mice are killed by LT over days in a manner independent of their macrophage sensitivity to toxin, suggesting that other cell types play a more important role in LT-induced lethality [50,51]. By contrast, rats are killed in a manner directly correlated to the sequence of the NLRP1 inflammasome sensor and thus to the sensitivity of their macrophages to toxin [52]. Notably, the toxin-induced macrophage cell death concerns rodent macrophages and not the human ones that are resistant to LT [49].

LT inhibits the sporicidal and bactericidal activities of macrophages [53–56]. LT also disrupts the production of phospholipase A2 group IIA by alveolar macrophages, which is crucial for their anthracidal activity, by interfering with the MAPK-NF-κB activation pathway [57]. Moreover, while ET enhances spontaneous macrophage migration [24], both LT and ET inhibit macrophage chemotaxis through the respective enzymatic activities [27]. This function is likely to result in suppression of the migratory response of macrophages toward inflammatory chemokines. LT also inhibits the expression of several proinflammatory cytokines in macrophages, both *in vitro* and *in vivo*, thus compromising the recruitment of other immune cells [45,58,59].

4.4.1 Synergy between ET and LT

Beyond the inhibition of macrophage chemotaxis, activity shared by LT and ET, these two toxins act in a concerted way to impair the bacterial clearance by phagocytes and by *B. anthracis*-specific responses. This synergy derives from the intense and multilevel cross-talk that occurs among the cAMP and the MAPK signaling pathways [60]. This permits *B. anthracis* to economize greatly on the amount of protein toxin to be synthesized. This strategy may have evolved under the pressure of amino acid availability inside the phagosomes where *B. anthracis* is located after the first interaction with alveolar or intestinal macrophages. Double and synergic toxin production strategy may have been used by other bacteria: the concomitant production of pertussis toxin and CyaA by *B. pertussis* and of botulinum neurotoxin C1 and toxin C2 by some strains of *Clostridium botulinum* is well known [61,62]; however, the fact that these couples of toxins act in concert remains an ill-defined issue. Although comparatively little has been reported on the effects of LT and ET on the potent antimicrobial defenses provided by PMNs, there is compelling evidence that *B. anthracis* uses both toxins to evade killing by these cells. ET and LT inhibit the oxidative burst elicited by lipopolysaccharide or muramyl dipeptide [63], and ET also inhibits phagocytosis [64]. Moreover, ET impairs neutrophil chemotaxis and when combined with

LT its inhibitory effect is potentiated [65,66]. Collectively, these effects may explain the poor neutrophil response that accompanies early stages of systemic anthrax.

Given the crucial role of DCs in the immune response, it is not surprising that anthrax toxins are capable of crippling DC functions [67,68]. LT and ET impair cytokine secretion by mouse-derived DCs, human monocytes-derived DCs, or primary blood DCs [69,70]. ET also disrupts DC functions [68], alone or in cooperation with LT. Moreover, anthrax toxins may finely tune the balance between signals delivered by DCs, inducing terminal differentiation of T cells toward a Th2 rather than a Th1 immune response. As the Th1 immune response is crucial for host survival, its inhibition by the two toxins may be a major aspect in the pathogenesis of anthrax [71].

4.5 Toxins Affecting Host Cell Motility

The actomyosin cytoskeleton of eukaryotic cells plays a pivotal role in host–pathogen interactions. It is essential for epithelial and endothelial barrier functions, which prevent and limit the invasion and dissemination of pathogens in tissues [72,73]. It also has key roles in different processes carried out by immune cells, including migration and phagocytosis [74], formation of the immune synapse and immune cell signaling [75,76]. Owing to its paramount importance in cell structure, physiology, and movement, the dynamic assembly, disassembly, and orientation of the actomyosin polymers are regulated at various levels by multiple factors among which the Rho GTPases family plays a major part [77]. Therefore, it is not surprising that bacterial toxins targeting the actomyosin cytoskeleton are the second largest family of exotoxins produced by bacterial pathogens, after that of the pore-forming toxins. These bacterial toxins display a number of different biochemical activities and target different GTPases, thus interfering with the corresponding host signaling pathways [78]. A growing number of bacterial protein toxins affects host GTPase activity by catalyzing covalent modifications of the target proteins [78]. The C3 exoenzyme of *C. botulinum* (C3bot) was the first toxin shown to modify Rho-family GTPases [79]. Further, C3 proteins were identified in *Clostridium limosum* (C3lim) [80], *Bacillus cereus* (C3cer) [81], as well as in *S. aureus* (C3stau) [82]. These toxins are small enzymes that transfer the ADP-ribose moiety from cytosolic NAD^+ to different target proteins; interestingly, they consist of only a catalytic domain and do not possess any specific modules for membrane translocation. Accordingly, the cell accessibility of C3-like toxins is often very low except for C3stau (also known as Edin) of *S. aureus* that does not need to be translocated to reach its target, as the bacterium invades the host cell; once within the host cytoplasm, it secretes the toxin that ADP-ribosylates Rho, resulting in the reorganization of the actin cytoskeleton [83]. Monocytes and macrophages take up C3bot and C3lim very efficiently, suggesting that these host cells are preferred targets for the toxin [84]. The same could be true also for C3cer, but no such evidence is yet available. Similarly, the impact of C3stau on phagocytic cells remains unexplored. On the contrary, clostridial C3 enzymes have been extensively studied. These

toxins specifically mono-ADP-ribosylate the isoforms Rho-A, -B, and -C at Asn^{41} rendering them inactive [85]; as a consequence, the subsequent activation of the various cellular Rho-effector molecules is compromised. In monocytes/macrophages, the C3-catalyzed Rho-inhibition prevents migration [86], adhesion [87,88], and phagocytosis [89], suggesting a specific pathogenic role of the C3 protein toxins toward such immune cells [90].

Clostridium difficile, the most common cause of nosocomial antibiotic-associated diarrhea and the etiologic agent of pseudomembranous colitis, produces two large multidomain toxins, TcdA and TcdB, which are capable of entering cells via the endosomal route and membrane translocation into the cytosol similar to the anthrax toxins. Once inside the cytosol, their N-terminal domain displays the glucosyl-transferase activity directed to the Thr^{37} residue of Rho [91]. This covalent modification results in the functional inactivation of the GTP-ase that ultimately culminates in the disruption of the intestinal epithelial barrier [92,93]. In addition to this effect, TcdA and TcdB stimulate the release of inflammatory mediators by immune cells and this relies on two mechanisms, one dependent on the enzymatic activity of the two toxins and the other mediated by the inflammasome [94–96]. There has been considerable debate as to whether TcdA or TcdB is responsible for the inflammatory response and tissue damage associated with the *C. difficile* infection; recent reports suggest that TcdB may synergize with TcdA, enhancing its ability to trigger intestinal inflammation and cause tissue damage [97].

Various cytotoxic necrotizing factors (CNFs) such as CNF1, CNF2, and CNF3 from *E. coli* and CNF from *Yiersinia pseudotuberculosis* persistently activate proteins of the Rho family by deamination of a glutamine residue (Gln^{61} or Gln^{63}) [78] and this is followed by the degradation of the GTPase by proteasomal cleavage [98]. Although the impact of disrupting Rho proteins on the host immune response has been poorly investigated, it may result in a decreased inflammatory response favoring bacterial infection [99].

4.6 Toxins Blocking Protein Synthesis or Protein Folding

Shiga toxins (Stxs) compose a family of structurally and functionally related cytotoxic proteins expressed by enteric pathogens *Shigella dysenteriae* 1 and enterohemorrhagic *E. coli* (Shiga-toxigenic *E. coli*, STEC). Following ingestion of small numbers of Shiga toxin-producing bacteria, humans develop bloody diarrhea and risk the development of life-threatening systemic complications including the hemolytic uremic syndrome and neurological abnormalities [100]. STxs are AB_5 holotoxins, with a structural architecture similar to that of cholera toxin, but capable of inhibiting protein synthesis. These toxins bind the glycolipid globotriaosylceramide at the cell surface and are endocytosed via a retrograde trafficking system from early endosomes to the Golgi stacks and finally to the endoplasmic reticulum (ER). In the ER, toxin A subunits are proteolitically cleaved and a 27 kDa fragment translocates into the cytoplasm via the reverse translocon [101]. In the cytosol, the fragment displays an *N*-glycosidase activity specific for a specific adenine base of the 28 s RNA, thereby blocking protein

synthesis [102]. The treatment of monocytic cells with Shiga toxin type 1 (Stx1, produced by enterohemorrhagic *E. coli*) leads to the activation of the ER stress sensors IRE1, PERK, and ATF6 [103] that are responsible for the initiation of the unfolded protein response (UPR) [104]; with the exception of IRE1 activation that does not require the enzymatic activity of the toxin, the latter is necessary for the optimal activation of PERK and ATF6. ER stress response induced by Stx1 leads to increased cytosolic Ca^{2+} levels, activation of calpain and of caspases-8 and -3 leading to apoptosis [105]. However, it remains to be established whether apoptosis of monocytes occur *in vivo* and whether this is part of a bacterial strategy aimed at evading the host immune response.

Cell life is dependent on the correct folding of proteins taking place in the ER. A central player in this process is the chaperone BiP, which is the target of a toxin produced by Shiga-toxigenic *E. coli* (STEC). The toxin is an AB_5 cytotoxin and it is named subtilase cytotoxin (SubAB); it is extremely cytotoxic *in vitro* for a range of cell types and it is lethal for mice when injected intraperitoneally [106]. SubAB acts by binding via its pentameric B protomer to cell surface glycan receptors terminating in α2–3-linked N-glycolylneuraminic acid [107]. This triggers internalization and retrograde transport to the ER [108], where the A subunit, a highly specific serine protease, cleaves the chaperone BiP [106]. BiP is a conserved ER chaperone essential for the survival of eukaryotic cells. It also maintains the permeability barrier of the ER membrane and plays a crucial role in the UPR. Accordingly, SubAB treatment rapidly triggers the three ER stress-signaling pathways [109]. The toxin also causes a major redistribution of leukocytes and increases leukocyte apoptosis *in vivo* suggesting that it has relevant immunosuppressor effects [110]. Moreover, SubAB was shown to lower the secretion of interleukin-8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1) in human macrophages, colonic epithelial cells, and brain microvascular endothelial cells. The downregulation of secreted chemokines is dependent on the cytotoxic activity of SubAB, as it was not observed in cells treated with an inactive mutant. Thus, SubAB-mediated suppression of secreted chemokines is dependent on the toxin capacity to cleave the ER chaperone BiP [111].

4.7 Conclusions

The study of the impact of bacterial toxins on the innate immune system has revealed important insights into the pathogenesis of several microbial diseases and has highlighted the strategies evolved by bacteria to survive and spread, under the selective pressure exerted by the defense mechanisms of the host. Moreover, deciphering the molecular mechanisms by which the toxins hamper the immune system contributes to implementation of the already large group of toxins that, because of their ability in knocking down or altering a specific cell target and function, represent an excellent tool for unraveling signaling pathways and physiological aspects of the cells, including those belonging to the immune system.

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5

Venoms

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5.1 Introduction

Venom systems are key evolutionary innovations in a broad phylogenetic range of animal lineages, and are used for defense, competitor deterrence, or predation. Venomous animals include the extensively studied and medically important clades (snakes, scorpions, and spiders), and sea anemones, jellyfish, sea snails, cephalopods, centipedes, several insect orders, echinoderms, fishes, lizards, and even some mammals, such as platypus and shrews.

Venoms across the Kingdom Animalia are complex mixtures of substances that include variable combinations of proteins, ranging from multiunit globular enzymes to small peptides, salts, and organic molecules such as polyamines, amino acids, and neurotransmitters [1–6]. Different venom components act synergistically, thus enhancing their deleterious effects [7]. Modes of venom delivery are diverse and include barbs, beaks, fangs or modified teeth, harpoons, neumocysts, pinchers, proboscises, spines, sprays, spurs, and stings.

Targets of venom action include virtually all major physiological pathways and tissues. The targeting of mammalian species by animal venoms has presumably introduced evolutionarily relevant selective pressures to either avoid or minimize the toxic effects of envenomation in a coevolutionary "arms race" [8,9]. In general, animal venoms are potent inducers of tissue damage and inflammation. Understanding the mechanisms involved in venom-induced inflammation has been useful not only to understand the mechanisms of envenomation but also in the more general characterization of inflammatory pathways. The aim of this chapter is thus to describe the present status of the knowledge on the inflammatory response to animal venoms with emphasis on those of medically important animal clades.

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5.2 Inflammation Induced by Snake Venoms

Venomous snakes belong to four main families: Elapidae, Viperidae, Atractaspididae, and Colubridae (*sensu lato*); their venoms contain a vast array of molecules with different biochemical properties and biological activities. The study of snake venom proteomics has unveiled the biochemical complexity of these toxic secretions [10]. Some of these molecules present enzymatic activities, whereas several others are nonenzymatic proteins and polypeptides. The most common enzymes in snake venoms are phospholipases A_2 (PLA_2s), serine proteinases, metalloproteinases, acetylcholinesterases, L-amino acid oxidases, nucleotidases, and hyaluronidases [11,12]. Among these components, PLA_2s [13], metalloproteinases [14–16], and L-amino acid oxidases [17,18] have been described as venom components responsible for triggering inflammatory responses. In addition to enzymatic components, other nonenzymatic venom proteins induce inflammatory events, such as cytotoxins, vasoactive peptides, and C-type lectin-like components [10].

Snakebite envenoming constitutes a serious health problem mainly in the tropical regions of the world that might lead to irreversible sequelae and mortality and affects thousands of individuals every year [19–21]. Snakes of the family Viperidae, which encompasses many different genera, account for the majority of cases of death and morbidity worldwide. In general, viperid snakebite envenomation causes systemic cardiovascular alterations associated with coagulopathy and systemic bleeding, which may evolve into cardiovascular collapse and death. Furthermore, it induces prominent local manifestations, which include myonecrosis, blistering, hemorrhage, and inflammation. Local inflammation is a major characteristic of envenomation by snakes of viperid species [22,23]. Although still fragmentary, a significant body of information has been gathered on the local inflammation induced by venoms of snakes of the genus *Bothrops*, which are responsible for the vast majority of cases in Latin America. Thus, emphasis will be placed in the description of the inflammatory events induced by *Bothrops* sp. venoms (see Figure 5.1).

Bothrops sp. snake venoms induce a set of inflammatory events, including plasma exudation, edema, leukocyte migration, and the synthesis and release of a complex network of mediators that induce multiple actions. These events can progress to resolution, if the amount of injected venom is low, or to an excessive and uncontrolled inflammatory response when a high volume of venom is injected. Prominent local edema is a common clinical finding in viperid snake-bitten victims [22,24] that may cause ischemia and neural compression, leading to permanent tissue loss, disability, and/or amputation [25–27]. In addition, the displacement of vascular fluid to the interstitial space in cases of widespread edema contributes to the pathogenesis of cardiovascular shock. Thus, edema is a relevant clinical manifestation in viperid snakebite envenomation, considering the partial inefficacy of the classical antivenom therapy in the neutralization of viperid venom-induced local manifestations both in humans and in experimental animals [22,28].

It has long been demonstrated that *Bothrops* snake venoms have the ability to induce inflammatory response at the site of their injection in experimental

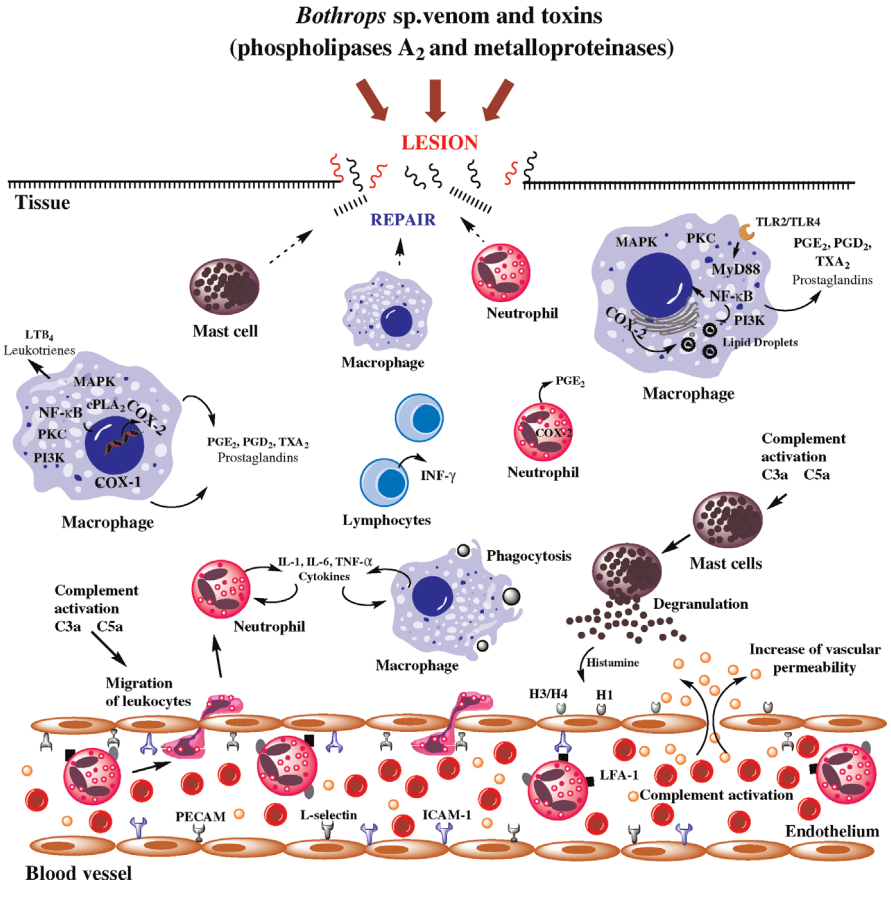


Figure 5.1 Schematic representation of the inflammatory actions of snake venoms. The local inflammatory events caused by *Bothrops* venoms and toxins are summarized.

animals. Many reports have shown that injection of venom of several *Bothrops* species into the mouse and rat foot paw causes edema of rapid onset that can be sustained up to 24 h, depending on the dose of venom. Moreover, it has been shown that edema induced by low dose of venom is not related to the hemorrhagic activity of venom [29–35]. Studies on the dynamics of the local inflammation caused by *Bothrops asper* venom have demonstrated an early plasma extravasation from small venules and adjoining capillary segments after 2 min of exposure to venom, associated with stasis in the microcirculation and dense packing of erythrocytes in the microvasculature [36]. In addition, the ability of this venom to contract collecting lymphatic vessels of mouse mesentery, associated with a halting in the flow of lymph, was demonstrated, thus suggesting that altering of lymphatic system may contribute to the development of edema in envenomed tissues [37].

Regarding the mediators of snake venom-induced edema, despite some variability of data among the available reports, due to the use of different venoms, doses and experimental animal species in the studies, it has been described that

viperid snake venom-induced vascular permeability and edema depend on the local release of well-known synergistic inflammatory mediators, which cause increased vascular permeability and/or vasodilation. Among these mediators are histamine, serotonin, bradykinin [38], platelet activating factor [39], prostaglandins (PGs) [31,39,40], derived from both cyclooxygenase 1 and 2 pathways, mast cell-derived neurokinins, opioid mediators [41], and anaphylatoxins, including the complement components C3 and C5 [42–46]. In addition, participation of both α-1 and -2 adrenergic, but not β-adrenergic mediators, was shown in mouse paw edema induced by *B. asper* venom [29]. In the case of other species, such as *B. lanceolatus*, participation of lipoxygenase rather than cyclooxygenase-derived mediators has been demonstrated [47]. The role of cytokines, such as IL-β, IL-6, and TNF-α, has also been suggested for edema induced experimentally by the venoms of *B. jararaca* [48], *B. asper* [86], *B. jararacussu* [49], *B. bilineata* [50], and *B. atrox* [40], since increased levels of these mediators were detected at the site of venom injection.

Activation of mammalian tissue mast cells contributes to the local edema and inflammation induced by snake venoms, including those from the family Viperidae. Mast cells are tissue-resident effector cells that play key roles in inflammation. Once activated, they rapidly secrete and produce a vast array of inflammatory mediators, which trigger the earliest inflammatory events such as edema [51,52]. The ability of *B. jararaca* and *B. moojeni* venoms to induce degranulation of mast cells was demonstrated by both *in vivo* and *in vitro* experimental models [35,53], and mast cell-derived histamine, acting via H1 and H3/4 receptors, is involved in edema induced by *B. moojeni* snake venom [35]. Among venom components, PLA_2s are potent inductors of mast cell degranulation [54,55]. Apart from the contribution of mast cells in triggering the early events of venom-induced inflammation, some reports have shown that mast cells significantly enhance the resistance of mice to the venom of the snake *Atractaspis engaddensis* (family Atractaspididae), at least in part by releasing carboxypeptidase A and other proteases, which can degrade venom components [56,57]. However, no similar studies are available in the literature with regard to the venoms of species of the family Viperidae.

Besides edema, an important cellular component characterizes the local inflammation induced by the majority of snake venoms. Leukocytes play essential roles in the host defense against offending agents. Upon stimulation by injurious agents, these cells respond with phagocytosis followed by generation and release of microbicide products and mediators. In this regard, several studies have demonstrated that venoms from *Bothrops* sp., as well as from other viperid genera, induce a time-related infiltration of leukocytes into the site of injection. Similar to many other exogenous inflammatory agents, *Bothrops* snake venoms evoke an early neutrophil influx followed by a late mononuclear cell infiltrate, which accumulate at the site of venom injection and adjacent tissues [30,40,58–61]. During the innate immune response, neutrophils are the first cells to migrate into the inflammatory site. This process involves the action of chemotactic mediators and expression of distinct subsets of adhesion molecules by both neutrophils and microvascular endothelium. Studies on the mechanisms involved in *Bothrops* snake venom-induced leukocyte recruitment have provided

contrasting results. Whereas whole *B. jararaca* venom neither triggers oriented cell locomotion nor modifies the intrinsic ability of neutrophils to migrate *in vitro* in response to a chemotactic factor [62], *B. jararacussu* venom has the ability to induce neutrophil chemotaxis *in vitro* by a direct mechanism [49]. This was explained by the presence of a lectin in *B.jararacussu* venom that recognizes glycoligands on the neutrophil surface leading as a consequence to polarization and chemotaxis of neutrophils [63,64]. Further, the ability of *Bothrops* venoms to stimulate *in vivo* the expression of the adhesion molecules L-selectin, LFA-1, ICAM- 1, PECAM-1, and CD-18 by endothelium and leukocytes was described in an experimental model in mice [48,86]. This effect was attributed to a direct action of venom on vessel walls and leukocytes, followed by the release of chemotactic cytokines, chemokines, and leukotrienes [68].

The involvement of cytokines and chemokines in *Bothrops* venom experimentally induced infiltration of leukocytes has been suggested by many authors due to the potent activity of these mediators to induce leukocyte activation, chemotaxis, and expression of adhesion molecules, and to the increased levels of Th1 cytokines IL-1-β, IL-6, IL-8, and TNF-α detected at the site of injection of *Bothrops* venoms in experimental models [39,58,65,66]. This is in agreement with increased levels of IL-6, IL- 8, TNF-α, MIP-1β, and RANTES, also described in the serum of patients bitten by *Bothrops* snakes [67]. In addition, complement system-derived chemotactic fractions have a role in leukocyte infiltration induced by *Bothrops* venoms, and the ability of several venoms to activate the complement system, such as C5a fraction, generating chemotactic fractions, has been demonstrated [69,70]. Furthermore, studies using isolated toxins have demonstrated that PLA_2s and metalloproteinases from *Bothrops* venoms are able to induce leukocyte infiltration into the site of their injection. The effects of PLA_2s were attributed to the action of eicosanoids and cytokines, and those induced by venom metalloproteinases were associated with the release of chemotactic cytokines and complement C5a fraction, as well as with the direct stimulatory action of the metalloproteinase disintegrin-like domain on neutrophils [69,71–73].

Bothrops venoms are also able to activate inflammatory functions of leukocytes as they (1) induce the release of inflammatory mediators that amplify and/or lead to resolution the inflammatory process, (2) induce phagocytosis, and (3) trigger microbicide functions of these cells. Release of cytokines, chemokines, eicosanoids, and nitric oxide by neutrophils and macrophages, as well as neutrophil extracellular traps (NETs) by neutrophils induced by *Bothrops* snake venoms, has been demonstrated in a number of studies [74–77]. Although inflammation induced by *Bothrops* venoms is characterized by a complex network of chemical mediators, the eicosanoids represent major players of this venom-induced response. There is evidence of the involvement of prostaglandins and leukotrienes in edema, hyperalgesia, and leukocyte infiltration induced by the venoms of several species of *Bothrops* [34,39,40,65,68,77,78]. In accordance, *Bothrops* venoms have the ability to induce the expression of COX-2 by peritoneal leukocytes in an *in vivo* experimental model [77] *in vitro* by macrophages and neutrophils in culture, indicating that these cells are relevant sources of eicosanoids under inflammatory conditions triggered by these venoms. Furthermore, venom PLA_2 and metalloproteinases are involved in this effect, since their ability to induce

protein expression of COX-2 enzyme system in macrophages has been described, being the effect of venom PLA_2 dependent on activation of NF-κB and downstream kinase proteins [79–82]. These venom components may thus act synergistically within the context of the whole venom.

In addition, there are studies showing that *B. jararaca* and *B. asper* venoms increase the capacity of phagocytosis by both peritoneal polymorphonuclear and mononuclear leukocytes at the site of their injection. This effect was dependent on the engagement of the phagocytosis complement receptor [83]. Further, it was shown that a PLA_2, named MT-III, from *B. asper* venom increases the phagocytic capacity of macrophages, through mannose and β-glucan receptors [84], whereas a metalloproteinase, named HF3, from *B. jararaca* venom, activates phagocytosis of opsonized-zymosan particles by macrophages via $\alpha M\beta_2$ integrin [85].

With regard to the leukocyte microbicide activities, the ability of *Bothrops* venoms to induce generation of reactive oxygen species (ROS) in macrophages and peritoneal leukocytes has been demonstrated [74,75,83,87]. Moreover, increased production of hydrogen peroxide and nitric oxide by macrophages followed by the generation of the nitric oxide reactive intermediate (RNI) peroxynitrites induced by *Bothrops* venoms [83,86] and the ability of the venom of *Daboia russelli*, another viperid snake of medical importance in Asia, to activate the production of ROS in neutrophils were also described [88,89]. It is known that prolonged or excessive release of leukocyte-derived peroxynitrites and ROS could intensify the inflammatory process and enhance venom-induced tissue damage. However, studies attempting to block the damaging effects of either *B. asper* or *Daboia russelli* snake venoms, by using inhibitors of NO production and/or different antioxidant compounds, showed contrasting results [90,91]. Therefore, the role of leukocytes, mainly neutrophils and their microbicide products, in the host defense against envenomation by snakes remains to be better understood. The beneficial role of neutrophils in the host defense against noxious effects of snake venoms was demonstrated by studies showing that experimentally induced neutrophil depletion in mice reduced skeletal muscle regeneration after myonecrosis induced by *B. asper* snake venom and a myotoxic PLA_2 isolated from this venom. The hindering of arrival of these phagocytic cells to the damaged site impaired their functions related to tissue regeneration, such as the late recruitment of macrophages into the damaged areas and the clearance of necrotic debris [92].

The inflammasome is a large multiprotein complex responsible for the activation of caspase-1, leading to secretion of the proinflammatory cytokines IL-1β and IL-18 and to the onset of a proinflammatory cell death termed pyroptosis. Despite the relevance of inflammasome in the development of inflammation, studies on participation of this multiprotein complex in the inflammatory responses to snake venoms are scarce. In this regard, the ability of *Crotalus atrox* snake venom to activate inflammasome was recently demonstrated [93]. This activation was related to a protective host response to envenomation, since caspase-1-deficient mice were more sensitive to venom-induced pathology. Further studies with other venoms are required to assess the role of the inflammasome in inflammation triggered by snake venoms.

The signaling pathways leading to each specific function of the host defense cells are triggered by pattern recognition receptors (PRRs) that recognize either pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) [94,95]. Toll-like receptors (TLRs) are PRRs expressed in a variety of leukocytes that sense diverse exogenous and endogenous stimuli and activate signaling pathways that result in inflammatory events, such as the synthesis of inflammatory mediators, including cytokines and eicosanoids [96–98]. Adapter proteins, such as myeloid differentiation factor 88 (MyD88), mediate TLR-activated intracellular signaling pathways. Although the impacts of TLRs in triggering inflammation are well appreciated, their participation in snake venom-induced inflammation is less known. Participation of TLRs via MyD88 adapter molecule in inflammation induced by *Bothrops* snake venom-induced inflammation was demonstrated in MyD88 knockout mice, which showed impaired inflammatory response to *B. atrox* snake venom, with reduced infiltration of leukocytes into the site of venom injection, and failure to produce Th1 and Th17 cytokines, CCL-2, PGE_2, and LTB_4 [99]. Similarly, galatrox, a C-type lectin isolated from *B. atrox* snake venom, induced the generation of proinflammatory mediators IL-6, TNF-α, and keratinocyte-derived chemokine from bone marrow-derived macrophages, by a mechanism dependent on activation of the TLR4-MyD88 signaling pathway [100]. In addition, the involvement of TLR2 via engagement of MyD88 adapter molecule in the release of inflammatory mediators (PGE_2, IL-1 β, and IL-10) by macrophages in response to MT-III, a PLA_2 isolated from *B. asper* venom, has been demonstrated [101]. Engagement of TLR2 pathway by this PLA_2 was attributed to its ability to release fatty acids, such as the palmitic and oleic acids, which are TLR2 agonists, from macrophage membranes.

5.3 Inflammation Induced by Scorpion Venoms

Scorpions are arthropods belonging to the class Arachnida. These animals are found everywhere in the world, mainly in subtropical and tropical regions. Envenomation by scorpion stings is considered an important public health issue worldwide, affecting mainly children. Venomous scorpions of medical importance belong to Buthidae family and include the genera *Androctonus, Buthus*, *Mesobuthus*, *Buthotus*, *Parabuthus*, and *Leiurus* in North Africa, Asia, and the Middle East. Species of the genus *Centruroides* are found in North America (United States of America and Mexico) and Central America, and the genus *Tityus* is common both in Central America and South America [102,103].

The venom of scorpions consists of a complex mixture of mucopolysaccharides, hyaluronidase, serotonin, histamine, enzyme inhibitors, and a variety of low molecular mass proteins, most of which are neurotoxic, which target primarily ion channels, mainly voltage-gated Na^+, K^+, or Cl^- channels of excitable cells, such as neurons and muscles. The uncontrolled depolarization of postganglionic nerve terminals by scorpion neurotoxins leads to continuous release of neurotransmitters [104,105], which contribute to the onset of various systemic manifestations of scorpion envenomation. Clinical manifestations of this

envenomation include hyperthermia, psychomotor agitation, local pain, and systemic manifestations resulting from cardiovascular, respiratory, gastrointestinal, genitourinary, hematological, and metabolic alterations [106–109]. The scorpion sting also triggers a strong inflammatory systemic response in the victims, characterized by increased levels of circulating inflammatory mediators such as cytokines, eicosanoids, and platelet-activating factor (PAF), nitric oxide (NO), and kinins, as well as leukocytosis, which are involved in the worsening of clinical symptoms after scorpion stings [110]. The occurrence of multiple organ failure has been documented in severe scorpion envenoming, and this condition is usually accompanied by systemic inflammatory response syndrome (SIRS) [102,106,111–113].

In patients with moderate to severe systemic manifestations, leukocytosis and high plasma levels of the inflammatory cytokines IL-6, TNF-α, IL-8, IL1-β, INF-γ, GM-CSF, and RANTES, as well as the anti-inflammatory cytokines IL-10 and IL-4, are observed [113–117]. A positive correlation between increased levels of cytokines and the severity of scorpion envenomation in humans was described [113]. In this context, studies with experimental models of severe scorpion envenomation have demonstrated that multiple organ dysfunction and death were associated with an increased production of inflammatory cytokines [110,118–123]. The role of IL-6 and TNF-α in hyperglycemia and metabolic deregulation described in severe envenomation by scorpion stings was evidenced after injection of *Androctonus australis hector* venom in mice [124]. Injection of scorpion venom *Tityus serrulatus* causes hyperthermia in rats via release of IL-1 with pyrogenic activity [121]. Moreover, increased brain levels of TNF-α were found in mice injected with tityustoxin (TsTX), a neurotoxin isolated from *T. serrulatus* venom, which were correlated with increased rolling and adhesion of leukocyte in the brain microvasculature [122].

Eicosanoids constitute another group of mediators involved in the systemic inflammatory response and to scorpion venom sting. Participation of eicosanoids derived from the cyclooxygenase (COX) pathway in hyperthermia caused by the scorpion *Leiurus quinquestriatus* sting in children has been shown, since treatment of patients with nonsteroidal anti-inflammatory drugs ameliorated the venom-induced effect [125]. In line with this report, experimental studies have demonstrated that prostaglandins released by the scorpion venom also have a role in the cardiorespiratory, edematogenic, and nociceptive effects induced by the injection of this venom in experimental animals [126–129]. Release of PGE_2 and LTB_4 induced by neurotoxins Ts2 and Ts6 isolated from *T. serrulatus* venom, as well as participation of these mediators in the leukocyte infiltration into the site of injection of these toxins, has been described [123]. With regard to the molecular mechanisms underlying the biosynthesis of eicosanoids, the role of peroxisome proliferator-activated receptor gamma (PPAR-γ) was experimentally shown in macrophages stimulated by *T. serrulatus* venom [130].

In addition, experimental studies have shown that the injection of crude venom or toxin of the scorpions *T. serrulatus*, *Centruroides noxius*, and *Androctonus australis hector* in murine models reproduces the severe symptoms associated with local and systemic inflammatory response and prominent inflammatory cell recruitment similarly to those observed in envenomed humans [114,131–136].

Although leukocytes are recognized as a major source of inflammatory mediators, the stimulatory mechanisms of these cells by the scorpion venom are not completely understood. It is currently accepted that neurotoxins present in the scorpion venom can directly stimulate ion channel receptors on the surface of immune cells, such as macrophages and lymphocytes, thus triggering the production of inflammatory mediators [110,137–142].

Acute pulmonary edema is an important systemic manifestation frequently resulting from scorpion sting, with risk of death due to high propensity of cardiorespiratory arrest [107]. The pulmonary edema induced by scorpion venom is attributed both to an excessive stimulation of the adrenergic system, leading to cardiogenic edema, and to the activation of an inflammatory cascade, which leads to noncardiogenic edema. A number of experimental studies have indicated that lung edema evoked by scorpion venom is due, at least in part, to the release of several inflammatory mediators, with vasodilator and increased vascular permeability activities, such as PAF, nitric oxide, kinins, prostaglandins, and leukotrienes [126,129,131,142–153].

Moreover, stimulation of inflammatory cells, such as mast cells, and neuronal fibers by venom neurotoxins, with release of neuropeptides, including substance P, which activates NK1 receptor, facilitates the release of inflammatory mediators [110,137–141] and contributes to the development of pulmonary edema [145,153–155]. The ability of scorpion venoms to induce degranulation of mast cell and release the vasoactive mediator histamine has also been reported [134,156–161]. In line with these experimental findings, the involvement of the kinin system in the induction of clinical effects caused by *T. serrulatus* envenomation in humans, including pulmonary edema, was demonstrated [161]. Besides edema, a conspicuous leukocyte infiltrate was observed in the lungs of experimental animals injected with this venom (see Figure 5.2). This inflammatory event was dependent on the production of PAF and KC, as well as on the activation of CXCR2 on neutrophils [131].

Studies on the recognition of scorpion venoms by the innate immune cells are scarce. It was experimentally shown that Toll-like receptors 2 and 4 (TLR2 and TLR4) are engaged by *T. serrulatus* scorpion venom and its toxin, toxin 1, in macrophages, playing a role in the synthesis of cytokines IL-6 and TNF-α, which occurs via MyD88-dependent NF-κB phosphorylation [162].

5.4 Inflammation Induced by Spider Venoms

Spiders are arthropods belonging to the class Arachnida. These animals are distributed everywhere in the world, except in the Antarctic region. These arthropods live in a wide variety of ecosystems, including aquatic environments. At present, the World Health Organization considers only four major genera of spiders causing severe envenomation in humans, that is, *Loxosceles*, *Phoneutria*, *Latrodectus*, and *Atrax* [163]. In general, bites of these venomous spiders promote local effects such as edema, erythema, and intense pain associated with acute inflammation [163–165]. Some factors may influence the severity of injuries caused by spider venoms, such as the developmental stage of the spider, the

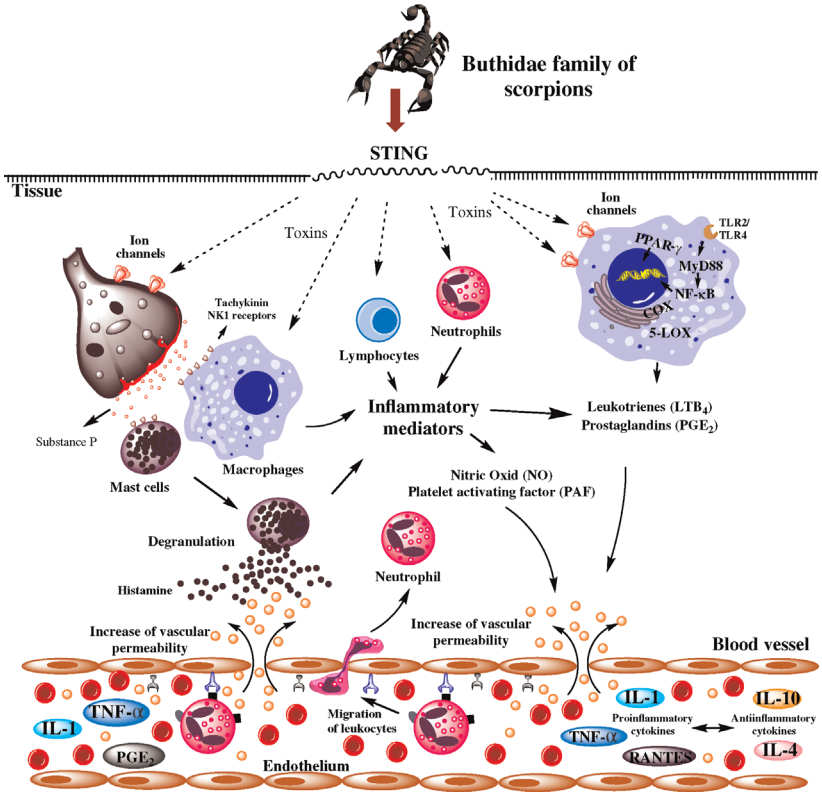


Figure 5.2 Schematic representation of the inflammatory action of scorpion venoms. The local and systemic inflammatory events triggered by venoms of scorpions of the family Buthidae are summarized.

amount and composition of injected venom, and the physiological features of the patient, such as age and physical conditions, among others. In addition, the clinical consequences and the role of inflammatory events are different for each genus of spider of medical importance. Among the medically relevant spiders, the inflammatory effects caused by *Loxosceles* and *Phoneutria* are the most studied and reported in the scientific literature.

Despite being found in four continents, the accidents caused by species of the genus *Loxosceles*, known as brown spiders, are particularly serious in South America [166]. *Loxosceles* ssp. venoms promote classical dermonecrosis at the site of injection, which is a characteristic feature of loxoscelism. This clinical sign is the result of the direct action of venom components, which include proteases, hydrolases, lipases, peptidases, sphingomyelinases, hyaluronidases, metalloproteinases, phospholipases, and collagenases, and is also associated with a prominent inflammatory response in the tissues. Sphingomyelinases are particularly important in the generation of local necrosis. Eventually, in severe cases, systemic symptoms such as intravascular hemolysis and acute kidney injury can lead to death [166–171]. The initial interaction between *Loxosceles* spp. venoms and tissues activates components of the inflammatory process, thus leading to

migration of leukocytes, platelet aggregation, and release of inflammatory mediators, such as lipid mediators, cytokines, chemokines, and growth factors. These elements contribute to pain, disruption of microvasculature integrity, increased vascular permeability, and erythema [166,172]. Following 24 h, the damaged tissue area becomes pale with a white halo, which is caused by ischemia around the affected dermal region. Between 3 and 7 days after *Loxosceles* bites, the lesion may ulcerate and, in some cases, due to failure of healing, surgical removal and grafting in the local damaged skin are necessary [170–173].

Studies have revealed that the intense infiltration of polymorphonuclear leukocytes into the tissue, and the secretion of proteolytic enzymes from the granules of these cells, participate in the pathogenesis of tissue damage caused by *Loxosceles* ssp. envenomation [171]. Some investigations have demonstrated that the upregulation of leukocyte influx induced by *Loxosceles* ssp. spider venoms is a consequence of activation of other cell types, including endothelial cells and fibroblasts. Stimulation of human endothelial cells (HUVEC) in culture with *L. reclusa* and *L. deserta* venoms induces the expression of E-selectin adhesion molecule and the release of leukocyte chemoattractant mediators, such as interleukin-8 (IL-8) and granulocyte colony-stimulating macrophage (GM-CSF), and the monocyte chemoattractant protein-1 (CCL-2) [174,175]. In this context, cultured human fibroblasts stimulated with *L. reclusa* recombinant sphingomyelinase D upregulated gene expression of IL-6, IL-8, CXCL1, CXCL2, and CCL5, which are relevant mediators for chemotaxis of leukocytes. From these observations, it has been postulated that, in addition to activation of endothelial cells of the microvasculature, *Loxosceles* venom acts on fibroblasts present in the dermal region, activating a dysregulated inflammatory leukocyte influx, which in turn contributes to the development of dermonecrosis. The action of *L. gaucho* spider venom on primary cultures of keratinocytes, which are cells that protect skin against external aggressions, was followed by secretion of tumor necrosis factor (TNF-α). The presence of TNF-α, a potent cytotoxic mediator, together with ceramide, which is a lipid generated from the venom sphingomyelinase action on sphingomyelins of endothelial cells, activates mechanisms that culminate in cellular apoptosis or necrosis around the spider bite site [176,177]. The ability of *L. deserta* venom to induce the release of vascular endothelial growth factor (VEGF) from keratinocytes has been demonstrated. Besides inducing angiogenesis, VEGF, by regulating vascular permeability of endothelial cells, can contribute to development of erythema and edema observed after *Loxosceles* ssp. envenomation [178]. Furthermore, the involvement of mast cells and the participation of histamine and serotonin, released from their granules, in the increased vascular permeability and edema induced by *Loxosceles* sp. venom has been demonstrated [179].

Envenomations caused by spiders of the genus *Phoneutria* known as armed-spider, have been described mainly in South America and Costa Rica, in Central America [164]. *P. nigriventer* envenomation causes local symptoms in patients that include intense pain, erythema, and edema. In severe envenomation, systemic effects can be manifested by autonomic effects such as tachycardia, hypertension, diaphoresis, salivation, diarrhea, visual disturbs, tremors, priapism, and lung edema. Occasionally, the evolution of severe cases can lead to death [180,181].

In experimental animals, *P. nigriventer* venom causes paralysis of the upper and lower limbs, convulsion, agitation, neuromuscular blockade, and flaccid paralysis, resulting from the action of neurotoxins present in this venom [182,183]. This spider venom also contains substances that are responsible for edema formation, as demonstrated in rat and rabbit skin [184]. Although the venom contains histamine and serotonin in its composition, these components are not the main culprits of edema formation [184,185]. Edema induced by *P. nigriventer* injection has been related to activation of complex systems linked to the increase in vascular permeability. Early studies demonstrated the participation of bradykinin via B2 receptors in the genesis of edema by this venom [186]. Further studies have evidenced an important role of neurogenic mediators in the inflammatory response to *P. nigriventer* venom. Activation of tachykinin NK1 receptors by vasoactive neuropeptides released from sensory nerve terminals have been shown [187,188]. Moreover, it has been demonstrated that activation of vanilloid receptor and purinergic P2 receptor of ATP and ADP is an important mechanism of the increased vascular permeability induced by *P. nigriventer* venom [189].

Similar pathways, involving activation of peripheral tachykinin NK1 and NK2 receptors in addition to receptors NMDA and AMPA, were described for the nociceptive effect of *P. negriventer* venom [190]. Moreover, 5-HT receptors were demonstrated to be involved in the activation of nociceptive sensory nerve fibers by *P. nigriventer* venom. The exaggerated sense of pain is the most evident manifestation provoked by the venom of this spider. This effect was demonstrated to result from neurotransmitter release in the dorsal horn of the spinal cord, a region of integration of synaptic transfer of sensory inputs from the periphery to the central nervous system. Transmitters such as substance P (SP), neurokinin A, calcitonin gene-related peptide (CGRP), and amine glutamate, contribute to dorsal horn hyperexcitability and pain transmission induced by *P. nigriventer* venom. In addition, nitric oxide (NO) mediates this phenomenon as well as TNF-α, IL-1β, and prostaglandins from the COX-2 pathway. There is evidence that prostaglandins promote an increased release of neurotransmitters in the dorsal horn, including excitatory amino acids, SP, and CGRP. Prostaglandins, in turn, are modulated by glutamate, SP, CGRP, cytokines, and NO, indicating a cooperative link between spinal prostaglandins and neurotransmitters for pain evoked by *P. nigriventer* bite [191]. Interestingly, the neurogenic mechanisms evoked by this spider venom are linked to leukocyte migration, as it has been shown in experimental models that bradykinin is involved in both activation of sensory nerves and leukocyte migration, reinforcing the importance of neurogenic inflammatory mechanisms stimulated by this venom [192].

5.5 Inflammation Induced by Venoms of Bees, Vespids, and Ants

Arthropods from the order Hymenoptera include species of venomous insects capable of promoting accidents in humans. Among them, species of bees and vespids (wasps, yellow jackets, and hornets) belonging to Apidae and Vespidae

families, respectively, are of public health interest. Ants of medical importance, in turn, are included in the Formicidae family [193]. The injuries caused by venomous insects have in common the occurrence of local inflammatory response (erythema, heat, and edema), pain, and hyperalgesia. Rarely, in severe cases, stings may cause more extensive local inflammatory reactions with both erythema and edema signs involving the whole extremity. On the other hand, in some cases, hymenoptera stings can result in the development of an anaphylactic reaction, due to individual hypersensitivity to some components of the venoms, which may be fatal. As local reactions usually disappear within 24 h after being stung, few studies have been conducted to demonstrate the mechanisms governing the inflammatory events promoted by venomous wasps, bees, and ants [194]. Hymenoptera venoms are complex mixtures of pharmacologically active substances that comprise enzymes (phospholipases A_1 and A_2, hyaluronidase), nonenzymatic proteins (antigen 5), peptides (neuropeptides, chemotactic peptides, antimicrobial peptides, apamin, and mast cell degranulating peptides (MCDP), such as melittin and mastoparans), and biogenic amines (histamine, epinephrine, and dopamine) [194–198]. Experimental studies have demonstrated that the components of hymenoptera venoms are responsible for their biological actions, including the inflammatory response, nociception and hyperalgesia. Some of these substances are responsible for massive release of vasoactive amines through degranulation of mast cells. Mast cell degranulating (MCD) peptides such as melittin and mastoparans promote the release of histamine and other vasoactive amines from mast cells. These mediators increase vascular permeability, leading to edema characteristic of the inflammatory response or hypersensitivity reactions caused by these venoms. Honey bee venom contains two polypeptides with MCD activities: (1) melittin, the major component of the venom, and (2) MCDP. Vespid venoms have a type of MCD peptide group named mastoparans, the major components of wasp venom. These peptides exert a detergent action that results in the lysis of cell membranes and the release of biogenic amines from neuronal cells, inflammatory mediators from neutrophils, or vasoactive amines from mast cells [194,199,200]. Beside these peptides, hymenoptera venoms contain pharmacological components, such as histamine, histamine-releasing activity, kinins, and kinin-like components, as described in *Myrmecia genus* ant venom [195,201], in *Apis mellifera* bee venom [202], and in vespid venom [194,203], which may contribute synergistically to local inflammation. The edema induced by venoms of the social wasps *Polybia paulista* and *Polistes lanio lanio* is due to histamine release through a mechanism dependent on the engagement of H1 histamine receptor [204,205]. On the other hand, it has been demonstrated that *P. lanio lanio* wasp venom increases vascular permeability by activating tachykinin NK1 receptors. These receptors may be activated by venom tachykinin-like peptides or by endogenous neuropeptides, such as substance P, released from activation of sensory fibers in response to that venom [205].

It is well known that, besides inflammatory response, melittin also a role in production of pain after bee stings [206]. On the other hand, melittin is not found in wasp venom and the intense pain caused by vespid stings is attributed to

serotonin, kinins, and specific peptides [194,206], as well as to the activation of capsaicin-sensitive afferent fiber and spinal protein kinases pathways [207–211]. Other studies have demonstrated the participation of inflammatory lipid mediators in the genesis of pain induced by melittin. Leukotrienes, lipid mediators derived from lipoxygenases (LOX) pathway, were proved to modulate nociceptive effect induced by injection of melittin and other peptides isolated from social wasp *P. paulista* and *P. sylveirae* venoms in mice [197,203]. This phenomenon was demonstrated to be independent of mediators derived from cyclooxygenases pathway. Further, the involvement of secretory PLA_2 from bee venom in the production of leukotrienes and in hymenoptera venom-induced pain was also demonstrated. Besides mediating the wasp venom nociceptive effects, LTs derived from 5-LOX were proved to mediate polymorphonuclear cell migration into the inflammatory site in tissue injected with *P. paulista* wasp venom [204]. In the case of the jumper ant venom (*Myrmecia pilosula*), there is evidence that, in addition to histamine, eicosanoids are released by factors present in this venom, and potentiate pain and inflammatory response induced by this arthropod sting [212]. In this context, it was shown that a phospholipase-activating protein and melittin from *A. mellifera* bee venom stimulated the release of prostaglandin E_2 (PGE_2) from macrophages [213]. Melittin has been suggested to activate PLA_2. In addition, the ability of mastoparan and PLA_1, from the yellow jacket *Vespula vulgaris* venom, to induce the release of PGE_2 from macrophages in culture was demonstrated [199]. Since PGE_2 is a potent mediator of inflammation and plays an immunomodulatory role, this mediator was associated with the diverse range of biological activities of *V. vulgaris* venom, including enhanced vascular permeability, vasodilation, and nociception [214].

5.6 Conclusions

Inflammation is a general characteristic of envenomation caused by diverse animal species. Complex networks of inflammatory mediators regulating the inflammatory events generated by the actions of animal venoms have been described, but for the majority of venomous animal species the available information is limited or absent; there is, therefore, a need to investigate the inflammatory pathways and mediators involved in the tissue reaction to a variety of animal venoms. Despite the evidence on the ability of some venoms to activate leukocytes, the role of each type of recruited leukocyte in the evolution and resolution of the inflammatory response remains to be clarified. Moreover, the cell receptors sensing venoms that lead to engagement of downstream inflammatory signaling pathways are poorly known, as is the role played by the inflammasome complex in animal venom-induced inflammation. A much better understanding of the mechanisms of inflammatory actions of animal venoms and their toxins, with characterization of the factors and signaling pathways that regulate the inflammatory response to animal venoms could provide important clues to develop novel therapeutic strategies to efficiently treat the dysregulated inflammation commonly resulting from envenomation by many animal species.

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6

Hypoxia as an Inducer of Inflammation

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6.1 Introduction

Despite the proliferation of therapies to treat the critically ill, administration of oxygen is perhaps the most commonly used and cost-effective modality available. Further, at some level, defective oxygen delivery or utilization underlies most severe threats to the well-being of the organism. Therefore, it is not surprising that cell and oxygen deficit constitutes a threat to normal cellular activity. As such, hypoxia constitutes a "danger signal" that is a potent activator of inflammation. This chapter will review the cellular and molecular mechanisms that detect oxygen availability and how these mechanisms activate inflammation.

Cellular hypoxia can be caused by systemic factors and local factors (Table 6.1). On a systemic level, hypoxia is due to (1) decreased fraction of inspired oxygen (i.e. high altitude), (2) ventilation and perfusion mismatch, (3) right to left intracardiac shunting, (4) low cardiac output, (5) anemia, and (6) derangements in the function of the electron transport chain (ETC) (i.e. cyanide poisoning). Locally, hypoxia is due to changes in the vascularity of a tissue bed and the ability of a particular organ to utilize oxygen appropriately (histotoxic hypoxia). The responses to hypoxia include erythropoiesis, increased cardiac output, increased ventilation, and changes in vascular tone.

At its most basic, oxygen utilization is tied to substrate metabolism (Figure 6.1). Oxygen is used by the mitochondrial enzyme complex cytochrome c oxidase (complex IV of the ETC) as the final electron acceptor in oxidative phosphorylation. Thus, oxygen availability is essential to drive the production of ATP and to reestablish, by reduction, sufficient stores of NAD and FAD to support postglycolytic carbohydrate metabolism – the tricarboxylic acid cycle – and utilization of fatty acids.

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Table 6.1 Causes of hypoxia.

Extracellular	Pericellular	Intracellular
Ambient atmosphere	Blood flow	Mitochondrial defect
Pulmonary function	Microcirculation	Exogenous inhibitors (poisons)
Cardiac shunting	Acid-base abnormalities	Electron transport chain defects
Hemoglobinopathy	Extracellular fluid accumulation	
Circulatory failure		

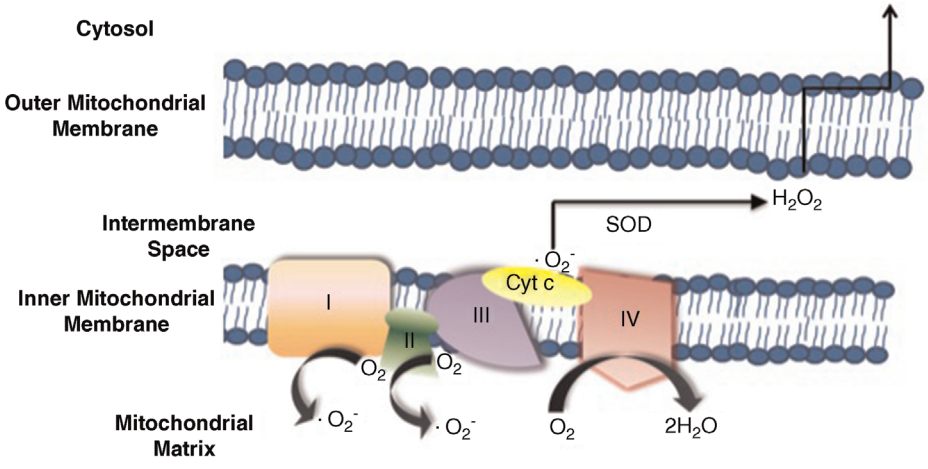


Figure 6.1 Formation of reactive oxygen species by the electron transport chain. Superoxide is formed by mitochondrial complexes I–III of the ETC. At complexes I and II, superoxide (O_2^-) tends to accumulate in the mitochondrial matrix. However, at complex III, O_2^- preferentially diffuses toward the intermembrane space. Superoxide dismutase converts superoxide into hydrogen peroxide (H_2O_2) that diffuses into the cytosol and acts as a trigger for oxygen sensing.

Inadequate oxygen availability/utilization impairs the ETC. Energy production thus comes to rely on glycolysis and not oxidative phosphorylation.

The precise mechanisms by which a transition from oxidative to glycolytic metabolism occurs have been extensively studied over the past 50 years. The link to inflammation has emerged more recently. In this chapter, we will examine oxygen sensing and the molecular response to hypoxia and discuss how hypoxia activates the inflammatory response.

6.2 Oxygen Sensing

It was initially believed that oxygen sensing involved only chemoreceptors in the carotid and neuroepithelial bodies. Hypoxia stimulates these cells to release neurotransmitters that induce cardiovascular and respiratory changes directed

toward restoring oxygen content to steady state levels [1]. However, what constitutes hypoxia varies from tissue to tissue and organ to organ. For example, cells in the lung are most often exposed to oxygen tensions in the 100 mm Hg range. In contrast, renal cortical cells see PO_2 levels around 60 mm Hg, while PO_2 in skeletal and cardiac muscle is much lower at rest and decreases to 10 mm Hg during exertion [2]. A system that regulates oxygen tension in one location only would not be capable of responding to the differing needs of different tissues, let alone cells. As noted by Schumacker, it appears that individual cells have the ability to modulate the PO_2 level at which mechanisms to increase oxygen availability are activated [3]. Researchers have, therefore, sought to identify cellular or subcellular processes that sense perturbations in local oxygen concentration and activate signaling pathways that can restore oxygen tension to a steady state. Most appear to originate in mitochondria.

6.3 Mitochondrial Oxygen Sensing

As oxygen is inhaled, it travels from large airways to smaller airways and ultimately to the millions of alveoli in the lung. It then diffuses from the alveoli into the adjacent capillaries from which it is carried to tissues throughout the body. Via specialized membrane oxygen channels, oxygen diffuses into cells and migrates toward the mitochondria. This process is facilitated by a pressure gradient; the partial pressure of oxygen at the large airways is 150 mmHg and at the mitochondria is 5 mmHg [4]. In addition, oxygen is a highly lipophilic molecule, allowing it to pass through cell membranes. Once inside the mitochondrion, O_2 binds to cytochrome oxidase (complex IV of the ETC) and is reduced to form water [5]. When oxygen tension is adequate this process nets 38 ATP molecules from one molecule of glucose.

Cytochrome oxidase had been touted as the "molecular sensor" signaling hypoxia [6]. However, this enzyme has a very high affinity for oxygen ($K_m < 1\,\mu M$), allowing cellular respiration/oxygen consumption to function at basal rates until the PO_2 falls below 5–7 mmHg [7]. The rate of O_2 consumption by a cell does not fall until a threshold is reached (~1% O_2). The reason that O_2 consumption is maintained is because the redox status in the complexes of the ETC remains constant over a wide range of O_2 concentrations. Even when oxygen tensions are low, cytochrome c, the electron donor for cytochrome oxidase, can be fully reduced right up until the point where the critical threshold – 1% O_2 – is reached. Thus, cytochrome oxidase will continue to consume oxygen at a normal rate even at oxygen tension levels that constitute hypoxia. Oxygen acts to limit the function of cytochrome oxidase only when hypoxia is extreme – where cellular survival is in question [3]. Thus, an alternate mechanism for sensing hypoxia must be operative.

ETC activity, with the transfer of electrons, in the presence of oxygen, can generate the free radical superoxide (O_3^-). Superoxide produced at complex I, complex II, and the ubisemiquinone site of complex III (Figure 6.1) crosses the inner mitochondrial membrane where it can be converted into hydrogen peroxide (H_2O_2) that then exits the mitochondrion [8]. During periods of normal oxygen tension, superoxide formation, and thus H_2O_2 generation, is limited by the immense capacity of cytochrome oxidase to use the majority of mitochondrial oxygen. However, when

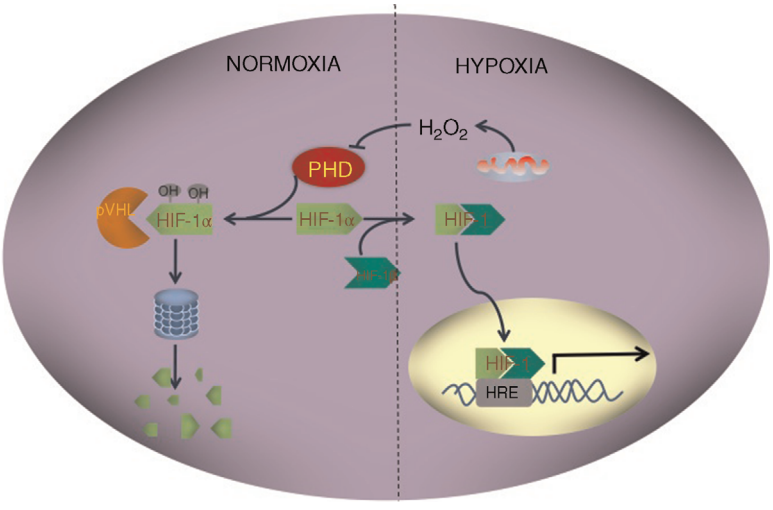


Figure 6.2 Influence of PHD on HIF. Under normoxic conditions, Prolyl hydroxylase dehydrogenase hydroxylates HIF-1α creating a binding site for the Von Hippel–Lindau protein (pVHL). This causes HIF-1α to be degraded by the proteasome. During hypoxia, PHD is inhibited by ROS. This allows HIF-1α and HIF-1β to dimerize, relocate to the nucleus, and lead to the transcription of various genes by binding with the hypoxia response element.

oxygen tensions fall, the ability of cytochrome c oxidase to transfer all the electrons generated by electron transport may become limited. As a result, there is an increase in the transfer of superoxide and other reactive oxygen species (ROS) produced by complexes I–III across the inner mitochondrial membrane onto the matrix, across the outer membrane, and into the cytoplasm. Under normal circumstances, mitochondrial mechanisms (such as mitochondrial superoxide dismutase, mtSOD) can eliminate ROS. However, hypoxia may change this effect [9,10].

ROS are produced during hypoxia and hyperoxia. Studies looking at oxygen tension within the mitochondria demonstrated that, under mildly hypoxic conditions, oxidation in the cytosol and intermembrane space of the mitochondria *increased*, whereas oxidation in the matrix of the mitochondria *decreased*, perhaps reflecting saturation of mtSOD. This effect leads to the development of an ROS gradient between the cytosol and matrix that may drive ROS migration through the intermembrane space and into the cystosol [5]. When coupled with the increase in free electrons in the intermembrane space that occurs as the ability of complex IV to accept electrons is exceeded, free radical transfer into the cytosol can increase substantially in response to relatively small changes in oxygen tension. These free radicals are now believed to activate inflammatory processes, including those that reverse hypoxia [8].

6.4 The Role of Transcription Factors

Cytosolic free radicals alter cellular response via two primary mechanisms. The first involves release of intracellular calcium and the opening of cell membrane

Table 6.2 Transcription factors involved in oxygen sensing.

Hypoxia-inducible Factor
Nuclear factor κ-B
Cyclic AMP response element-binding protein
Activating protein-1
p53
SP-1/SP-3
Early growth response-1
Nuclear factor for interleukin-6
Related transcriptional enhancer factor-1
GATA-2
Signal transducers and activators of transcription
Mammalian Achaete-scute homologous protein-2
Growth arrest and DNA damage-153

calcium channels [6]. The second is the activation of transcription. The myriad transcription factors that have been identified as potential mediators of cellular responses to hypoxia are listed in Table 6.2 [11]. For some of these factors, the pathways involved in oxygen sensing have been intensely studied. Others have been less well investigated.

6.4.1 Hypoxia-Inducible Factor

Hypoxia-inducible factor (HIF) is perhaps the best-studied transcription factor involved in oxygen sensing. The designation *HIF*, in fact, represents a group of molecules that fall into three classes: HIF-1, HIF-2, and HIF-3. HIF-1 is a heterodimer consisting of HIF-1α, a basic helix–loop–helix protein, and HIF-1β, an aryl hydrocarbon receptor nuclear translocator (ARNT) [12]. HIF-1α and HIF-1β have been identified in almost all cells of the human body. HIF-2α and HIF-3α are structurally similar to the HIF-1 subunits but are not as ubiquitous [13,14].

Active HIF-1 can alter the expression of hundreds of genes (Table 6.3) [14]. However, only the HIF-1α/HIF-1β dimer has transcriptional activity. Thus, elimination of one of the two subunits inactivates HIF-1. Studies performed a number of years ago demonstrated that the abundance of the HIF-1α/HIF-1β dimer increases as the FiO_2 decreases and that this effect is reversed when ambient oxygen tension is restored [15]. Additional reports have identified an enhanced, but still incomplete, picture of the mechanism modulating these changes. These findings implicate elimination of the HIF-1α subunit as the key step leading to inactivation of the transcriptional activity of HIF-1. Thus, normoxia stimulates the ubiquitination and proteasomal degradation of HIF-1α. However, under hypoxic conditions, the dimer remains intact and transcription is accelerated [15,16].

Table 6.3 HIF-1 Target genes.

Adenylate kinase 3	α_{1B}-Adrenergic receptor
Adrenomedullin	Aldolase A
Aldolase C	Endothelin-1
Enolase 1	Erythropoietin
Glucose transporter 1	Glucose transporter 3
Glyceraldehyde phosphate dehydrogenase	Heme oxygenase-1
Hexokinase 1	Hexokinase 2
Insulin-like growth factor (IGF)II	IGF binding protein 1
IGF binding protein 3	Lactate dehydrogenase A
Nitric oxide synthase 2	P21
P53	Phosphofructokinase L
Phosphoglycerate kinase 1	Pyruvate kinase M
Transferrin	Transferrin receptor
Vascular endothelial growth factor (VEGF)	VEGF receptor FLT-1

HIF-1α is targeted for ubiquitination and degradation when specific amino acids are hydroxylated, acetylated, or phosphorylated. Hydroxylation involves one of the two enzymes: prolyl hydroxylase dehydrogenase (PHD) and factor-inhibiting HIF (FIH). PHD acts by removing prolyl residues on the α subunit of HIF-1, creating a binding site for the von Hippel–Lindau protein (VHLP) (Figure 6.2). VHLP is a component of the E3 ubiquitin ligase that ultimately causes degradation of HIF-1 [17,18]. The absence of the VHLP tumor suppressor gene is associated with elevated levels of the HIF-1α/HIF-1β dimer in cultured renal carcinoma cells [19]. There are three PHD enzymes: PHD1, PHD2, and PHD3. Of these, PHD2 is predominantly involved in the oxygen-dependent regulation of HIF [20].

FIH hydroxylates asparagyl residues in HIF-1α, decreasing the interaction between the HIF-1 complex and the several essential coactivators. As with PHD, FIH is fully active in normoxic states, but its activity decreases with decreasing oxygen tension. The resultant increase in the interaction of HIF-1 with its cofactors ultimately leads to the transcription of target genes [21].

HIF-1 acetylation at lysine residues is mediated by the enzyme acetyltransferase arrest-defective-1 (ARD1). As with PHD and FIH, ARD-1 provides a site for interaction with VHLP. In contrast to the actions of PHD and FIH, ARD1 activity is not directly affected by changes in oxygen concentration; acetylation may occur even under normoxic conditions. However, the transcription and translation of ARD1 are decreased during hypoxia, providing another mechanism by which the HIF-1α/HIF-1β dimer can be preserved when oxygen availability is low [22].

HIF-1α phosphorylation is mediated by members of the mitogen-activated protein kinase (MAPK) family and involves phosphate binding to both HIF-1α and HIF-2α, increasing transcriptional activity on target genes [23].

PHD activity is oxygen dependent. However, the K_m for oxygen of PHD is higher than that of complex IV. Thus, under hypoxic conditions complex IV will

preferentially consume oxygen, reducing PHD activity and HIF-1α degradation, thus stabilizing the HIF-1α/HIF-1β dimer and enhancing transcription [4]. A number of theories to explain how the activity of PHD, FIH, and ARD-1 are modified by changes in oxygen tension have been proposed. Sabharwal *et al.* demonstrated that mitochondrial scavenging of hydrogen peroxide by peroxiredoxin-5 decreased HIF-1α stabilization in association with a change in PHD activity [24]. It has been suggested that PHD itself senses changes in oxygen tension. Others postulate that PHD is subject to post-translational modification by ROS [25]. An additional hypothesis posits that the activity of PHD is reduced when ROS oxidize cysteine residues on the enzymes [26]. Similar pathways have been proposed to explain the O_2 dependence of FIH, ARD1, and MAPK [17].

6.4.2 Nuclear Factor κB

In addition to effects on HIF-1, PHD also plays a role in the regulation of the nuclear factor kappa B (NF-κB) family of transcription factors. Like HIF-1, NF-κB subspecies (p65, cRel, RelB, p50, p52, etc.) function as homo- and heterodimers whose activity has been linked to inflammation, infection, and cancer. NF-κB species are maintained in an inactive state in the cytoplasm via a complex with IκBα. Phosphorylation by the IκB kinase (IKK) complex targets IκB for ubiquitination and degradation. The released NFκB is free to translocate to the nucleus and stimulate transcription of genes encoding a variety of proinflammatory, anti-inflammatory, apoptotic, and cell cycle enzymes [11,27]. Chandel *et al.* showed that ROS act as a stimulus for the release of NFκB from IκB [28]. NF-κB signals the upregulation of HIF-1α mRNA under both hypoxic and nonhypoxic conditions [29]. Further, the gene for HIF-1α contains an active NF-κB binding site [30,31] and the IKK complex stimulates increased transcriptional activity of HIF-2α [31].

6.5 The Warburg Effect

When oxygen availability decreases, either as a result of global or local tissue hypoxia, ATP production by mitochondria may be impaired. This situation is not uncommon in tumors where rapid growth may exceed oxygen delivery, producing local hypoxia. In 1927, Otto Warburg and colleagues described a phenomenon in tumor cells in which lactate production increased as a result of an enhanced reliance on glycolysis over oxidative phosphorylation [32]. It has been shown that HIF-1 is one of the main regulators of this process, now known as the Warburg effect. The shift from oxidative to glycolytic metabolism preserves ATP production despite hypoxic conditions. While glycolysis produces ATP in a manner that is less efficient than oxidative phosphorylation, an HIF-1-mediated upregulation of glycolytic enzymes via enhanced gene transcription results in an increased rate of glycolysis so that cellular energy provision remains sufficient [33].

Is the Warburg effect a global response to hypoxic conditions or something that is unique to neoplasia? To address this question Cramer *et al.* studied macrophages that were isolated from mice and exposed to a hypoxic environment. These studies

demonstrated that lactate levels were decreased in mice with a knockout of the gene encoding HIF-1α. ATP production in these animals was approximately 15–20% of that found in wild-type mice [34]. These data suggest that HIF-1 has a strong regulatory role over the glycolytic pathway. This conclusion is consistent with the known ability of HIF-1 to enhance the transcription of a number of genes encoding the glycolytic enzymes (Table 6.3) [35]. In addition, the promoters of these genes contain potential HIF-1 binding sites. Further clarification was provided by Zhang *et al*., who exposed both wild-type mouse embryonic fibroblasts and cells with a knockout of the gene encoding HIF-1α to either 1 or 20% oxygen. These studies demonstrated that, even under 1% oxygen, ATP production was preserved in knockout cells, a finding suggesting that the shift from oxidative phosphorylation to glycolysis reflects an HIF-1-mediated response to oxygen content [36]. Further investigations revised our understanding of metabolomics. While hypoxia seems to drive changes in energy production and utilization, these changes appear to reflect the generation of ROS during states of hypoxia. Hypoxia increases ETC production of ROS, which in turn can be toxic to cells and even trigger mitochondrial autophagy. When oxygen concentrations are low, HIF- 1 mediates a metabolic switch to glycolysis, limiting ROS production [37].

6.6 Acute versus Chronic Hypoxia

Different disease states result in varying degrees of hypoxia. For example, an episode of choking may result in hypoxia lasting less than a minute; patients with obstructive sleep apnea (OSA) may have numerous periods of hypoxia, each lasting only a few seconds; those with severe acute respiratory failure may have prolonged hypoxia lasting hours or even days. Although we have thus far described the importance of HIF-1 and NFκB as substantial mediators of gene transcription during hypoxia, studies have shown that they are of lesser importance when hypoxia is not sustained.

Wang *et al*. compared HIF-1 RNA expression in hepatoblastoma cells exposed to 20 or 1% oxygen. Hypoxia was associated with increased levels of HIF-1. Peak expression occurred at 1–2 h with a secondary increase at 16 h. If normoxia was restored, HIF-1 levels returned to baseline within 5 min [12]. These findings suggest that acute, nonrecurrent hypoxia does not result in long-term changes in HIF-1-mediated gene transcription.

To further characterize the activity of the HIF family of transcription factors during intermittent hypoxia, Yuan *et al*. studied rat pheochromocytoma (PC-12) cells. These experiments revealed that intermittent hypoxia increased expression of both HIF-1 and HIF-2α. In addition, lengthening the duration of the hypoxic episode proportionally increased HIF-1 transcriptional activity. During sustained hypoxia, MAPK and phosphoinositol-3 (PI-3) transactivated the activity of the HIF-1 complex. Inhibitors of these enzymes attenuated this response. However, these findings were not replicated during intermittent hypoxia, where gene activation via the HRE continued unfettered [38]. Gene profiling experiments identified two clusters of genes that were activated by intermittent, but not sustained, hypoxia: one was downregulated, the other upregulated [39]. Taken

together, these findings suggest that intermittent and sustained hypoxia both increase HIF-1 activity, but these effects may be mediated via different pathways.

Studies by Ryan *et al*. focused on intermittent hypoxia secondary to OSA. HeLa cells subjected to increasing cycles of hypoxia/reoxygenation were compared to cells cultured in sustained hypoxia. Activation of NFκB, but not HIF-1, was predominantly increased during intermittent hypoxia. Conversely, sustained hypoxia more profoundly increased HIF-1 relative to NFκB [40]. Thus, differences in the activation of the NFκB and HIF-1 pathways may be responsible for the differing responses to intermittent and sustained hypoxia.

6.7 Inflammasome Activation

Mitochondrial and cytoplasmic activities initiated by hypoxia participate in the modulation of inflammation. Most often, hypoxia or other detrimental stimuli stimulate pathways that do not require activation of extracellular mechanisms or the recruitment of others cells, such as those of the immune system. Free radicals are scavenged by endogenous, systems, most notably superoxide dismutase (SOD) molecules in the cytoplasm or mtSOD found in the mitochondrial matrix. In addition, dysfunctional cell components or proteins can be sequestered in the endoplasmic reticulum. In some cases, molecular chaperones such as heat shock proteins (HSPs) facilitate repair. At other times, cellular debris can be confined to *autosomes*, discrete membrane–enclosed "bubbles" that can fuse with similar structures containing digestive enzymes and free radicals – lysosomes – to form an *autophagosome*. Among these contents are senescent or dysfunctional mitochondria [41]. The process by which these contents are deconstructed is *autophagy*. Complete autophagy results in fragments that can be released into the cell and reused or the autophagosome can undergo exocytosis, releasing its contents into the extracellular matrix. Noxious stimuli can increase the generation of ROS and enhance autophagy [41–43]. At times, however, these events can produce ROS and cellular debris in quantities that overwhelm the capacity of the SOD and autophagy systems. In such instances, ROS can accumulate in the cytoplasm, and autosomal contents, including free radicals, may enter into the same space prior to full autophagy (see Figure 6.3). Cellular breakdown products of this type constitute danger-associated molecular patterns (DAMPs) that activate the *inflammasome*. In addition, incomplete autophagy followed by exocytosis of DAMPs into the extracellular space can activate additional components of the inflammatory pathway.

The inflammasome is a primary component of a complex signaling cascade that is activated by intra- or extracellular DAMPs such as molecules that are usually structurally isolated from the cytoplasm (e.g., DNA, RNA, transcription factors, ATP, free radicals, etc.) or microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) such as bacteria or microbial toxins (e.g., LPS). Ultimately, this process activates intracellular defense mechanisms, innate immunity, and inflammation. The inflammasome is the intracellular component of the response system. Its assembly is initiated by ligand binding to a pattern recognition receptor of the NOD-like receptor (NLR), RIG-like receptor (RLR), or Absent

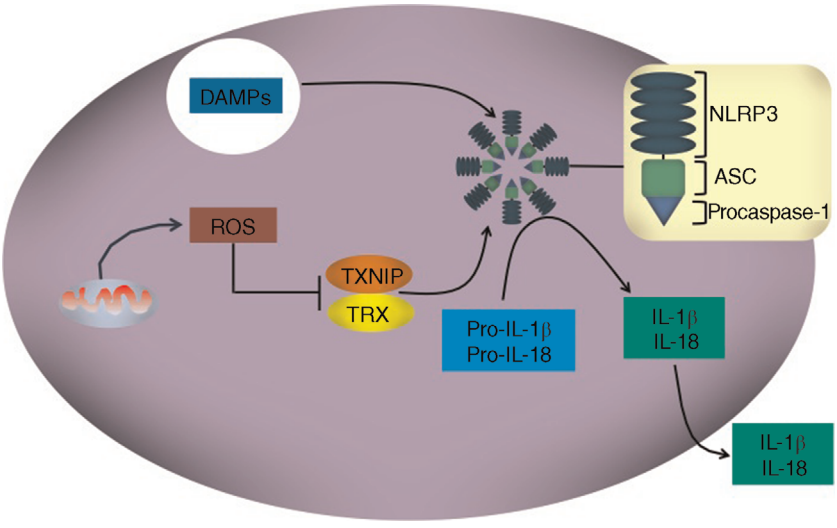


Figure 6.3 Inflammasome activation. The inflammasome is formed when stimulated by intracellular elements containing DAMPs and ROS. Activation of the inflammasome converts procaspase -1 to caspase-1 and then cleaves pro-IL-1b and Pro-IL-18 to their active form for secretion.

in Melanoma-2 (AIM2) types. Upon activation, NLR proteins (NLRPs), RLRPs, or AIM2Ps recruit and assemble a complex that includes an apoptosis-associated speck-like protein (ASC) and procaspase-1. Formation of the complex activates the caspase that, in turn, cleaves pro-IL-1 and pro-IL-18. These cytokines are, in turn, released into the extracellular space and, by interacting with Toll-like receptors (TLRs) on the cell surface, activate additional production of IL-1 and IL-18, as well as other cytokines such as TNFα and IL-6 [41,44–47]. In addition, by a poorly understood mechanism, inflammasomes also initiate a process of cell destruction called *pyroptosis*, in which cells are destroyed by an intense inflammatory reaction [48]. Pyroptosis is characterized by cellular plasma membrane rupture, edema, and ultimately cell lysis accompanied by the release of intracellular components that function as extracellular DAMPs [47].

As discussed above, cellular debris, such as the products of incomplete autophagy that leak into the cytoplasm, activate the inflammasome. In addition, a number of studies indicate that inflammasome activation can be initiated by ROS accumulation in the cytoplasm [47,49]. Zhou *et al*. used inhibitors of ETC complexes I and II in human macrophages to compare wild-type cells and NLRP3 knockout cells. These studies demonstrated that the ROS production induced by ETC inhibition correlated with an enhanced cytoplasmic presence of NLRP3 and increased levels of IL-1β [46]. Bauernfeind *et al.* found that inhibitors of ROS production prevented the activation of NLRP3 [50].

The activation of NLRP3 by ROS is incompletely understood. One proposal involves the action of thioredoxin (TRX), an antioxidant [51]. When in a reduced state, TRX is held inactive by binding to TRX interacting protein (TXNIP) [52]. When electron donation from ROS oxidizes TRX, TXNIP is released, and its binding to NLRP3 activates the inflammasome [53].

6.8 Summary and Conclusions

Based on the material presented, it is possible to construct a model that explains how hypoxia activates inflammation. Decreased O_2 availability limits the transfer of electrons from complex III to IV. These electrons generate ROS that accumulate in the mitochondrial matrix. When the ability of mtSOD to reduce ROS is exceeded, they are transported to the cytoplasm. In the cytoplasm, ROS activate a number of pathways that result in the nuclear translocation of transcription factors, the best studied of which are NF-κB and HIF-1. These in turn stimulate the expression of genes that encode proteins involved in oxygen transport and utilization, as well as those encoding the proteins of the glycolytic pathway. However, ongoing production of free radicals may overwhelm this system as well. Resultant damage to mitochondria can activate autophagy, deconstructing the damaged mitochondria for recycling. Once again, this mechanism may be insufficient. The result will be accumulation of ROS and DAMPs in the cytoplasm. This leads to activation of the inflammasome and ultimately secretion of IL-1 and IL-18. These cytokines stimulate TLRs and, therefore, innate immunity.

Identifying appropriate therapeutic targets is problematic. Our proposed paradigm postulates that initial responses to hypoxia are adaptive, increasing oxygen carrying capacity and redirecting metabolism, via a Warburg-like effect, favoring glycolysis over oxidative phosphorylation. Even stimulation of the inflammasome and, via extracellular mechanisms and TLR on the cell surface, inflammation, results in activation of innate and adaptive immunity. However, activation of the inflammasome can result in pyroptosis and excessive immune activity can be damaging. The only conclusion possible is that additional information about the factors that initiate pyroptosis and the point at which immune activation becomes maladaptive is needed before targeting of the cellular response to hypoxia can be considered as a therapeutic option.

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7

Vaccine Adjuvants

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7.1 Introduction

Vaccines represent one of the greatest successes of modern medicine. Widespread vaccination has dramatically reduced the frequency of infectious diseases among both the susceptible individuals and the general population [1]. In addition to preventing innumerable childhood illnesses, vaccination has led to the eradication of smallpox and freed most of the world from polio. Since not all individuals can be vaccinated and vaccination is not 100% effective, breakthrough infections can and do occur. To prevent such illnesses from spreading, public health officials recognized the importance of establishing "herd immunity." This is achieved by vaccinating such a large fraction of the population that an infected individual is unlikely to come into contact with another susceptible target who could spread the disease further. Herd immunity requires widespread prophylactic vaccination of otherwise healthy individuals (frequently children), a strategy that demands vaccines be extremely safe and well tolerated [1].

Early vaccines were produced from attenuated or heat-killed pathogens. Those preparations primarily induced Th1 biased immune responses that culminated in the production of protective neutralizing Abs. We now know that those early vaccines contained not only pathogen-derived antigenic determinants against which the host developed immunity but also immunostimulatory ligands derived from the pathogen that bolstered the induction of vaccine-specific responses (e.g., endogenous adjuvants) [2]. Today, separately prepared adjuvants are added to vaccines to enhance their immunogenicity. Newer vaccines typically combine purified pathogen-derived Ag with a purified adjuvant designed to boost the immune system. Such formulations have superior safety profiles and can be manufactured with unparalleled consistency [3]. The ability of an adjuvant to enhance vaccine immunogenicity may take a variety of forms, from altering the nature of the response (Th1 versus Th2 versus Th17, cellular versus humoral) to increasing its magnitude and/or

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duration. Adjuvants have two general mechanisms of action. The first involves improving the delivery of the vaccine so as to enhance the uptake, persistence, and/or processing of Ag by professional antigen-presenting cells (APCs) [4–6]. Examples of adjuvants in this category include alum, emulsions, calcium phosphate, liposomes, and "immune stimulating complexes" (ISCOMs). The second category involves agents that directly stimulate immune cells (including elements of the innate and/or adaptive immune systems) to boost their response to the vaccine Ag. Included in this category are the ligands of innate immune receptors such as TLRs, NOD-like receptors (NLRs), C-type lectins, RIG-I-like receptors and cytokines.

Despite the success of licensed vaccines, historically effective strategies have been unsuccessful in confronting the challenges posed by cancer, allergy, and chronic infections such as malaria, TB, and HIV. It is widely believed that vanquishing those diseases will require an enhanced Th1 (or mixed Th1/Th2) response capable of generating large numbers of cytotoxic T cells (CTL). Adjuvants that trigger the innate immune system are of particular interest in this regard as they excel at inducing Th1 biased immunity. This review will provide a general overview of adjuvants in widespread clinical use. It will then describe those in advanced clinical testing, focusing on the development of TLR agonists as vaccine adjuvants.

7.2 Adjuvants Approved for Human Use

Vaccines and the adjuvants they contain have been in widespread use for many decades. Despite this long history and considerable research effort, few adjuvants have been approved for use in humans. These include aluminum-based compounds and monophosphoryl lipid A (MPLA) (licensed for use in the United States and Europe), calcium phosphate, and oil-in-water emulsions (including MF-59, AS03, and AF03) the latter two having been licensed in Europe. Adjuvants currently undergoing clinical testing include a number of TLR ligands, a limited number of cytokines, saponin and its derivative QS-21, ISCOMATRIX, Montanide ISA, and the biopolymer Advax.

7.3 Aluminum Compounds: The Gold Standard

Aluminum compounds including aluminum hydroxide and aluminum phosphate (referred to collectively as alum) are the oldest and most widely used exogenous adjuvants incorporated into human vaccines. Alum is included in vaccines targeting hepatitis A virus (HAV), hepatitis B virus (HBV), diphtheria-tetanus-pertussis (DTP), haemophilus influenza type b (Hib), human papillomavirus (HPV), and pneumococcus. A long history of safety and efficacy combined with low cost make alum the "gold standard" to which other adjuvants are compared [7].

Alum elicits a strong humoral immune response biased toward the production of the Th2-associated IgG1 subclass of Abs [8,9]. This makes alum particularly suitable for use in vaccines that confer protection by stimulating

the production of neutralizing Abs. Conversely, the induction of cellular immune responses by alum is relatively weak, rendering it ineffective for vaccines targeting cancer and chronic infectious diseases [8,9]. Alum does not directly activate cellular elements of the innate immune system, reducing its utility for vaccines composed of poorly immunogenic Ags such as small peptides or recombinant proteins. To compensate for this deficiency, alum can be administered in combination with TLR ligands (reviewed below) to improve the induction of protective immune responses.

Alum's mechanism of action is widely believed to involve acting like a matrix onto which Ag can be adsorbed and stabilized. The slow release of Ag from alum at the inoculation site was postulated to support the induction of a longer lasting immune response. A recent series of studies dispute this interpretation, instead suggesting that alum deposition is not required for adjuvant activity since subsequent removal of the alum did not reduce the induced immune response [10]. Rather, it is proposed that alum stimulates the innate immune system via the inflammasome pathway by interacting with NLRP3, triggering a signaling cascade that proceeds through the recruitment of ASC and caspase-1 to induce inflammasome assembly [11]. The subsequent activation of caspase-1 allows processing of pro-IL-1ß and pro-IL-18 into their active forms. Consistent with such a mechanism, alum-induced innate and adaptive immune responses including alum-dependent Ag-specific IgG1 Ab production were abrogated in NLRP3 and ASC knockout (KO) mice. Unfortunately, other groups have been unable to reproduce these findings such that the overall mechanism(s) of action of alum adjuvants remains uncertain [12].

7.4 AS04

AS04 is a combination adjuvant composed of monophosphoryl lipid A (MPL) and alum. MPL is an immunostimulatory agent that mimics the ability of LPS to trigger cells via TLR4 but with considerably lower toxicity. The activity of MPL is discussed in a subsequent section focusing on TLR4 agonists. AS04 is part of the GSK vaccine against hepatitis B (Fendrix) and is approved in Europe for use in high-risk patients. This adjuvant is also used in GSK's licensed HPV vaccine (Cervarix). In terms of mechanism, the alum component of AS04 is believed to prolong the cytokine responses induced by MPL at the injection site [13,14].

7.5 Calcium Phosphate

Calcium phosphate is being evaluated as an adjuvant for human vaccines against polio, TB, yellow fever, measles, HepB, and in combination with the DTP vaccine in Europe [15,16]. Its mechanism of action is believed to be similar to alum, creating a depot of Ag in particulate form that facilitates and prolongs the uptake of Ag by APCs. When compared to alum, calcium phosphate generally elicits a more potent IgG response but virtually no IgE response in humans [17]. These are important advantages for vaccines that provide protection based on humoral

immunity and for which it is important to avoid an allergic response. As calcium phosphate is a natural constituent for human body, this adjuvant is well tolerated and readily absorbed.

7.6 Emulsions

7.6.1 IFA and CFA

Incomplete Freund's adjuvant (IFA) is the classic example of a water-in-oil emulsion. It is prepared by mixing a water-based solution containing Ag with paraffin oil and mannide monooleate. Extensive animal studies show that IFA induces a predominantly Th2 biased immune response. Although its precise mechanism of action is unknown, IFA creates a depot at the injection site from which Ag is slowly released. This serves to attract and trigger APCs that support the stimulation of Ab secreting plasma cells.

Early clinical trials of IFA involving an influenza vaccine showed that superior protection was achieved due to a higher and more sustained antibody response compared to alum [18–20]. The same improved immune response was observed when IFA was added to a tetanus toxoid vaccine [21]. However, in the latter studies severe side-effects were observed including local swelling, granulomas, and abscesses [21]. Induration was also observed when IFA was used to adjuvant a whole-cell Vibrio cholerae vaccine [22]. Histopathological examination of tissue from the site of these reactions showed that they were caused by a combination of foreign-body reactions and local hypersensitivity. Further development of IFA for clinical use was halted due to the development of granulomas and sterile abscess at the site of injection combined with autoimmune reactions in a small subset of volunteers [23].

Complete Freund's adjuvant (CFA) is made by adding heat-killed *Mycobacterium tuberculosis* to IFA. The mycobacterium expresses ligands for TLRs 2, 4, and 9 and its effect can be replicated by adding these TLR ligands to IFA. The inclusion of mycobacteria boosts activation of the innate immune system, thereby enhancing the development of adaptive immune responses. CFA is highly immunogenic, excels at inducing Th1-dominated immunity, and has been used to elicit strong Ab responses in animals. Unfortunately, the high degree of reactogenicity triggered by CFA makes it unacceptable as an adjuvant in humans. Indeed, even its use in animals has been highly restricted. Side effects include severe pain, swelling, skin ulceration, and necrosis at the injection site [24].

7.6.2 MF59

Extensive efforts to identify safer oil-based emulsions found that the short hydrocarbons present in some oils caused toxicity by disrupting the lipid membrane of tissues at the injection site. Less reactogenic emulsions that were more suitable for human use were subsequently developed. These include mineral oil and the squalene-based adjuvants MF59, AF03, and AS03. All are potent stimulators of both cellular (Th1) and humoral (Th2) immune responses.

Clinical studies showed that MF59 significantly enhanced the potency of an influenza vaccine in young children and the elderly when compared to alum [25–27]. The inclusion of MF59 also led to a broader immune response that protected against heterovariant strains of influenza. Of equal importance, the safety and tolerability profiles of the MF59 adjuvanted flu vaccine were excellent [25–27]. MF59 became the first oil-in-water emulsion licensed for use in Europe, initially as an adjuvant for seasonal flu vaccine and later for pandemic flu vaccines.

MF59 was a more potent adjuvant than alum for the induction of both Ab and CD4 T cell responses [28,29]. MF59, unlike IFA oil-in-water emulsions, did not form a long-lived depot at the injection site [30]. Rather the Ag and emulsion were cleared rapidly but with different kinetics. This might explain the lower toxicity of MF59 when compared to IFA emulsions. MF59 operates through multiple mechanisms, including the induction of a prostimulatory environment at the injection site. MF59 attracts dendritic cells and macrophages to the site of vaccination, thereby facilitating Ag uptake that further supports immune cell recruitment [31,32].

The possibility that oil-in-water emulsions might be combined with other immunostimulatory agents, such as TLR ligands, was investigated in animal models. Adding TLR9 or TLR4 agonists to MF59-adjuvanted vaccines shifted the ensuing response toward Th1 immunity. For example, a tumor vaccine adjuvanted with MF59 plus the TLR9 agonist CpG ODN had superior antitumor activity compared to MF59 alone. The combination inhibited tumor growth and significantly prolonged survival in a melanoma model, whereas the same vaccine lacking CpG ODN promoted melanoma growth [33].

7.6.3 AS03

Adjuvant system 3 (AS03) is composed of α-tocopherol (a bioavailable form of vitamin E) plus squalene in an oil-in-water emulsion. AS03's mechanism of action is likely similar to that of MF59. The inclusion of α-tocopherol is reported to have several benefits: it increases the production of cytokines (particularly IL-6), improves antigen-loading by monocytes, and enhances the recruitment of granulocytes to the draining LNs. This culminates in AS03 inducing an Ab response an order of magnitude higher than nonadjuvanted vaccines [34]. As a result, AS03 was approved for use in Europe and Canada as part of an H1N1 pandemic flu vaccine.

Unfortunately, phase 3 clinical trials suggested that recipients of AS03 suffered from an increased incidence of narcolepsy during the 2009 H1N1 influenza pandemic [35,36]. This was first observed among children and adolescents in Nordic countries [37,38] and subsequently confirmed by findings elsewhere in Europe [39,40]. Clinical results suggested that Pandemrix (an AS03-containing vaccine given to more than 30 million Europeans) could trigger an autoimmune reaction leading to narcolepsy in genetically predisposed individuals. Yet no increase in narcolepsy was observed in Canada or Brazil despite the widespread use an AS03-adjuvanted H1N1 vaccine in those countries [40]. Recent evidence suggests that antibodies to the 2009 A(H1N1)

nucleoprotein antigen cross-reacts with human hypocretin receptor 2, which may provide an possible explanation for the association of this particular influenza vaccine with narcolepsy [41].

7.7 Nano/microparticles Including Liposomes and Virosomes

There is considerable interest in using lipid-based vesicles as vaccine adjuvants. The safety and tolerability of liposome-based delivery systems was established in clinical trials involving the delivery of antifungal and anticancer drugs [42]. In the field of vaccinology, liposomes are commonly produced from a combination of stabilized membrane lipids plus viral fusion proteins to create an antigen delivery system termed virosome [42]. As an example, the hepatitis A vaccine Epaxal is composed of inactivated Hep A viral antigens carried within a virosome composed of highly purified influenza virus surface proteins plus phospholipids and cephalin. These particles retain the receptor binding capacity and specificity conferred by the flu proteins and deliver their Ag payload into the cytosol of target cells [43,44]. In addition to Epaxal, the Inflexal virosome-based influenza vaccine has been approved for use in Europe [45]. Both vaccines show excellent immunogenicity and tolerability profiles. Additional virosome-based vaccines are undergoing clinical trial for use in HIV/AIDS and malaria [46,47]. Preclinical studies suggest that virosomes with a mean diameter of 150 nm may be effective after intranasal or mucosal delivery.

7.8 Immune Stimulating Complexes

ISCOMs are cage-like particles that form spontaneously when a solution of Ag in water is mixed with cholesterol, phospholipids, and Quillaia saponins [48]. The antigen is incorporated into microparticles whose size (typically ≈40 nm) facilitates recognition and uptake by professional APC [48]. ISCOMs promote both humoral and cellular immune responses as characterized by the production of Ab and the activation of cytotoxic T lymphocytes [49]. The saponins in these complexes serve to boost the immunostimulatory activity of the ISCOM. Although ISCOMs were found to improve vaccine immunogenicity in animal studies, there was a problem with toxicity: the saponin component was associated with the development of hemolysis. These and other adverse reactions were observed after human use.

A less toxic derivative of saponin, QS-21, is undergoing clinical evaluation as an adjuvant for several vaccines. These include phase 3 trials of MPL plus QS21 for use in a malaria vaccine and phase 2 and 3 trials in which QS21 is included as a component of AS01, AS02, liposomes, and oil-in-water emulsions for vaccines targeting influenza, HIV, malaria, and cancer. Available results suggest that QS21 supports the immunogenicity of these vaccines without inducing the adverse events associated with saponin [50–52].

7.9 Cytokines

Numerous cytokines influence the nature and strength of vaccine-induced immune responses. Preclinical studies show that depleting cytokines such as IL-2, IL-12, IL-15, and GM-CSF adversely affects vaccine immunogenicity, whereas coadministering such cytokines enhances the induction of Ag-specific immune responses by stimulating T cells and/or APCs. Since recombinant cytokines generally have very short *in vivo* half-lives (on the order of minutes), efforts to use them as adjuvants complicates the method, site, and dose of vaccine delivery. Various strategies have been explored in clinical trials of vaccines designed for use in cancer patients.

7.9.1 IL-12

IL-12 is a major Th1 cytokine. It induces NK, T, and B cells to produce IFNg and plays a key role in supporting the generation of Th1 cells and CTL [53,54]. These abilities support the development of IL-12 as an adjuvant for vaccines targeting chronic infection and cancer. The ability of IL-12 to enhance Th1 immunity was initially demonstrated in a model of *Leishmania* infection in mice [55]. IL-12 was shown to increase IgG2a and decrease IgG1 Ag-specific Ab production compared to Ag alone [56,57], consistent with its induction of a Th1 biased immune response.

In clinical trials, the efficacy of recombinant IL-12 was found to be inconsistent, perhaps being influenced by the route of administration and type of vaccine. Most studies document that it increases Ag-specific CD8 and CD4 T cell responses [58–60]. In terms of safety, when IL-12 was administered systemically at high doses as monotherapy in cancer patients, it was found to be toxic [61]. If IL-12 is to find use as a vaccine adjuvant, it will need to be effective at much lower doses to avoid such toxicity [58–60].

7.9.2 IL-2

IL-2 plays an important role in T-cell proliferation, the induction of regulatory T-cell responses, and the initiation and maintenance of Ag-specific immunity. As such, it can be used to support the generation of CTLs and improve Ab production. In a variety of preclinical models, IL-2 was shown to be effective as a vaccine adjuvant, findings that supported the initiation of clinical trials [62,63]. Moreover, IL-2 was studied and approved as monotherapy for metastatic renal-cell carcinoma and metastatic melanoma in the 1990s [64]. Those early trials helped identify the toxicities associated with IL-2, which include flu-like symptoms, nausea/vomiting, and others.

The first clinical trial evaluating the administration of systemic IL-2 following vaccination reported an improved objective response rate in melanoma patients from 5% in subjects receiving vaccine alone to 42% in recipients of vaccine plus IL-2 [65,66]. Unfortunately, subsequent clinical trials failed to reproduce that outcome. A meta-analysis found that coadministering IL-2 with melanoma vaccine was no more effective than IL-2 alone [67–69]. Given the importance

of IL-2 for the induction and maintenance of T-cell responses, only through studies evaluating the method, dose, and timing of IL-2 delivery may the potential benefits of this cytokine as a vaccine adjuvant be determined.

7.9.3 IL-15

IL-15 is structurally similar to IL-2 and has similar functions [70,71]. In addition to promoting NK and T-cell development/proliferation (especially of CD8 T cells), IL-15 can augment the production of antibodies by B cells [70,71].

The use of a plasmid encoding IL-15 as a vaccine adjuvant was evaluated in a number of infectious disease models [72–74]. Oh *et al.* demonstrated that mice injected with a Vaccinia-based vaccine encoding IL-15 induced long-term CD8 T-cell-mediated immunity, while a vaccine encoding IL-2 was short-lived [75]. These results are consistent with those obtained in rhesus macaques immunized with tetanus and influenza vaccines [76]. Several clinical trials utilizing IL-15 plus chemotherapy or vaccination in cancer patients are ongoing with results expected in the near future [77,78].

7.9.4 GM-CSF

GM-CSF induces the proliferation of macrophages and the differentiation of DC [79,80]. The adjuvant properties of GM-CSF were explored in numerous preclinical tumor models where enhanced Ag-specific Ab and CTL responses were observed [81,82]. Several strategies for administering GM-CSF were examined including coadministration with vaccine [83,84], injection at the vaccination site [85], systemic administration [86,87], and administration as a plasmid/viral vector [88]. Unfortunately, results from clinical trials have been disappointing [83,85,88,89]. As in the case of IL-2, achieving greater activity may depend on improving the dose, route, method, and schedule of GM-CSF administration.

7.10 Adjuvants that Target Toll-like Receptors

7.10.1 Introduction

The innate immune system is responsible for rapidly detecting and responding to viral, microbial, and fungal pathogens. Cells of the innate immune system express pattern-recognition receptors including Toll-like receptors that bind to and are stimulated by molecules expressed by a diverse array of infectious agents. Inducing immunity via TLR triggers the production of proinflammatory cytokines/chemokines as well as type I IFNs that increase the host's ability to eliminate the pathogens [6,90–92]. This innate response supports the subsequent development of adaptive immunity and can be harnessed to accelerate and enhance the induction of vaccine-specific responses [93]. Given the importance of TLR agonists as potential vaccine adjuvants, what follows is a detailed description of the responses they elicit and their safety as determined by clinical trial.

7.10.2 TLR2

7.10.2.1 Overview

TLR2 interacts with a broad and structurally diverse range of ligands, including molecules expressed by microbes and fungi. There is evidence that natural and synthetic lipopeptides, peptidoglycans, various types of polysaccharides, and lipopolysaccharides (LPS) can all act as TLR2 agonists [94,95]. Yet uncertainty exists concerning the mechanisms by which TLR2 could recognize such a wide array of ligands and it seems likely that contamination with lipopeptides (which trigger TLR2 at picomolar levels [94]) may underlie some of the activity attributed to other moieties [96–98]. This section will focus on the use of lipopeptides as vaccine adjuvants, as available data suggest they are certainly agonists for TLR2.

7.10.2.2 TLR2 ligands

Since TLR2 is expressed in many different cell types (including dendritic cells, macrophages, and lymphocytes), the mechanisms by which bacterial lipopeptides manifest their adjuvant properties are diverse. Preclinical testing indicates that lipopeptides coadministered with or physically linked to Ag can (i) induce DC maturation and cause the upregulation of costimulatory signals and Ag-presenting molecules (e.g., MHC class II, CD80, CD83, IFN-γ, and IL-12) [99–101], (ii) stimulate macrophages to release cytokines (e.g., TNF, IL-1, and IL-6) [102,103], (iii) promote the maturation and activation of B cells leading to increased production of Ag-specific IgG and IgM Abs [104,105], and (iv) boost the generation of antigen-specific CD8 T-cell (CTL) responses [106–108]. Animal studies demonstrate that the immunogenicity of peptide-based vaccines can be significantly improved by conjugating them to either bacterial lipopetides or palmitic acid moieties.

7.10.2.3 Clinical Activity

The lipopeptide adjuvant that has undergone the most extensive clinical testing is Pam3Cys. It has been used in a vaccine containing the outer-surface protein A (OspA) of *B. burgdorferi* (the spirochete that causes Lyme disease). This vaccine was tested in over 20 000 volunteers [109,110]. Protective immunity correlated with IgG anti-OspA titers >1400 EIA units/ml. This lipopeptide adjuvanted vaccine (LYMErix™) was licensed by the FDA in 1998 but withdrawn by the manufacturer due to reports of autoimmune side effects [111,112]. Of note, neither the FDA nor the CDC found a connection between the vaccine and the development of autoimmunity.

The same adjuvant was tested in combination with peptide vaccines targeting malaria, HIV, and HBV. The addition of Pam2Cys typically improved or broadened the immunogenicity of these vaccines and boosted humoral responses to peptide-based vaccines in normal healthy volunteers [113,114]. However, their effect on CTL responses was less impressive and they were not effective in postexposure settings. Thus, their utility for the prevention/treatment of chronic infectious diseases (such as HPV or HIV) is uncertain.

7.10.2.4 Safety

Clinical trials indicate that lipopetide vaccines are generally safe with transitory local erythema being the most common adverse event and most systemic reactions being mild [114]. LYMErix™ was well tolerated in clinical trials involving >20 000 individuals followed for up to 6 years. Local adverse events were generally mild or moderate and lasted 2–3 days. Less than 4% of the vaccinees reported systemic complaints, primarily mild myalgia or fever [109,110,115]. In all, 1.4 million doses of LYMErix were administered postlicensure and the Vaccine Adverse Events Reporting System database shows 905 reports of mild self-limiting reactions and 59 reports of arthritis associated with vaccination [116]. This incidence of arthritis did not differ from the background rate in unvaccinated individuals. These findings suggest that lipopeptides interacting with TLR2 can be used safely as vaccine adjuvants.

7.10.3 TLR3

7.10.3.1 Overview

TLR3 is expressed within the endosomal compartment of conventional dendritic cells and macrophage [90]. TLR3 is triggered by double-stranded RNA (dsRNA) generated during the life cycle of most infectious viruses. The interaction of TLR3 with dsRNA initiates a TRIF-dependent signaling cascade that progresses through the activation of NF-kB, MAP kinases, and IRF3 and culminates in the production of inflammatory cytokines and type 1 IFNs [117,118]. Thus, TLR3 is distinct from all other TLRs in not using the MyD88-dependent pathway for signaling.

A potential advantage of TLR3 agonists is that triggering via this signaling cascade facilitates Ag cross-presentation, in which CD8 T cells are primed by exogenous Ag presented in the context of MHC class 1 molecules, improving the generation of cytotoxic T cells [119,120]. Cross-presentation is also enhanced by the type 1 IFNs induced following TLR3 stimulation [121]. Thus, TLR3 ligands are considered excellent candidates as adjuvants for vaccines targeting the induction of a strong cellular immune response [122].

7.10.3.2 TLR3 Ligands and Their Immunogenicity

Poly I:C (polyriboinosinic: polyribocytidylic acid) is a synthetic analog of dsRNA that triggers via TLR3 [123]. In a murine model of influenza virus infection, intranasal administration of poly I:C with an HA-based influenza vaccine induced a strong and protective IgA anti-HA response in the nasal mucosa and IgG response in serum, whereas vaccination without poly I:C was nonprotective [124]. Other studies document that poly I:C enhances the efficacy of peptide-based cancer vaccines by promoting tumor antigen-specific T-cell responses in murine models [125–128].

Despite promising findings in mice, the utility of poly I:C in primates (including man) is limited due to the susceptibility of this adjuvant to degradation by serum nucleases [129,130]. High doses of poly I:C were unsafe, causing shock, renal failure, and coagulopathies in phase 1–2 clinical trials of cancer patients [131]. The FDA has

not approved the use of alternative TLR3 agonists that have shown promise in animal studies but are of uncertain safety and efficacy in humans [132,133].

7.10.3.3 Safety

Initial clinical studies involving poly ICLC showed that high-dose therapy was associated with mild to severe side effects including hypotension, fever, and anemia. Once the dose and frequency of delivery were optimized, this TLR3 agonist was well tolerated in humans [134–141]. Several phase 1–2 clinical trials evaluating the activity and safety of poly ICLC as a cancer vaccine adjuvant are ongoing.

Poly I:C12U was well-tolerated in several long-term phase 1–2 clinical trials involving cancer patients [142]. The reduction in toxicity compared to poly I:C may reflect the accelerated hydrolysis and shorter plasma half-life of polyI:C12U [142,143]. Available data suggest that this agent is the most promising TLR3 ligand under clinical development. Studies of poly I:C12U as monotherapy against viral infection and phase 2 and 3 clinical trials examining its use in HIV and chronic fatigue syndrome are underway [144,145]. Clinical use of this TLR3 ligand as an adjuvant will begin with studies involving a vaccine targeting ovarian cancer [146].

7.10.4 TLR4

7.10.4.1 Overview

TLR4 is expressed by cells of the innate immune system, including conventional dendritic cells and macrophages. It is also expressed by many nonimmune cells including fibroblasts and epithelial cells [90]. Triggering via TLR4 induces a signaling cascade that utilizes both the MyD88- and the TRIF-dependent pathways, leading to NF-kB and IRF3/7 activation, respectively. TLR4 activation generally induces robust IL-12p70 production and strongly enhances Th1 type cellular and humoral immune responses [147,148].

7.10.4.2 TLR4 Ligands

Virtually all clinical trials of TLR4 adjuvants involve derivatives of lipopolysaccharide. LPS is a complex molecule whose lipid A portion mediates interactions with TLR4 [149,150]. Although the immunostimulatory capacity of LPS has been known for decades the intact molecule is highly toxic, preventing its use as a vaccine adjuvant [7]. The monophosphoryl lipid A component is considerably less toxic while maintaining immunostimulatory activity [151–154].

7.10.4.3 Clinical Activity

MPLA can boost vaccine-induced serum Ab titers by 10–20-fold compared to vaccine alone [154]. MPLA preferentially induces the production of IgG2a Abs and supports the generation of Th1 immunity in mice [155,156]. Human vaccine trials indicate that MPLA has a safety profile similar to that of alum [147,156]. Even very high doses of MPLA (up to 100 mg/m^2 delivered i.v.) are safe, inducing neither renal nor hepatic toxicity [157].

As described above, extensive clinical studies were conducted using MPLA absorbed onto aluminum salts [158,159]. Combining the hepatitis B vaccine with MPLA (Fendrix®) improved the speed and magnitude of the resulting anti-HBs Ab response [160]. MPLA was also used as an adjuvant with Cervarix®, a conjugate vaccine that uses virus-like particles to induce immunity against HPV-16 and HPV-18 [158]. Compared to the same vaccine adjuvanted with alum, Cervarix® induced higher anti-HPV16 and HPV18 L1 Ab responses (1.6–3.2-fold, $p < 0.05$) was approved for use in the European Union and the United States [161,162]. MPLA was also tested as an adjuvant for cancer vaccines. A modest enhancement in survival was observed in phase II trials, but further development was not pursued. Instead, a new class of synthetic lipid A mimetics, the aminoalkyl glucosaminide 4-phosphates (AGPs), was developed [148,156]. Clinical trials of the HepB vaccine coadministered with AGP RC-529 demonstrated superior immunogenicity and seroprotection. Based on those findings plus an acceptable safety profile, the RC-529 conjugated HBV vaccine was approved for use in Argentina [163].

7.10.4.4 Safety

In general, adverse events (typically mild to moderate) were more frequent in subjects receiving MPLA or RC-529 adjuvanted vaccines than vaccines adjuvanted with alum. Local reactions were typically mild to moderate and resolved in 1–3 days. The incidence of systemic adverse events was low and did not differ significantly between groups. No serious systemic adverse events related to vaccination were reported.

7.10.5 TLR5

7.10.5.1 Overview

TLR5 recognizes the protein ligand bacterial flagellin [164,165]. TLR5 is expressed on the surface of many types of immune cell, including monocytes, mDCs, Langerhans cells, T cells, and NK cells [166–168]. The adjuvant properties of flagellin have been tested by generating fusion proteins in combination with recombinant Ag. In that form, flagellin induced DC maturation leading to the upregulation of costimulatory signals and Ag-presenting molecules (CD80, CD83, CD86, MHC class II, TNF-α, IL-8, IL-1β, CCL2, and CCL5) [169]. Flagellin also stimulated monocytes to produce IL-10 and TNFa, NK cells to produce IFNγ and α-defensins, T cells to proliferate and produce cytokines/chemokines (e.g., IL-10, IL-8, and IFN-γ), and B cells to increase Ab production by up to 10-fold [165,170,171]. Little is known of the immunogenicity or safety of flagellin-based vaccines in humans.

7.10.6 TLR7/8

7.10.6.1 Overview

TLRs 7 and 8 are phylogenetically and structurally related and will be handled together in this section [172]. Both recognize single-stranded RNA sequences

containing poly-U or GU-rich sequences and are activated by synthetic imidazoquinolines and guanosine analogs [172–175]. TLR 7/8 molecules are localized to the endosomal compartments of human immune cells including DC, monocytes, macrophage, lymphocytes, Langerhans cells, and NK cells [176–178].

The interaction between TLRs 7/8 and their cognate ligands triggers DC to (i) enhance expression of costimulatory molecules (e.g., CD80, CD86, and CD40) [179], (ii) migrate [180], and (iii) produce proinflammatory cytokines including IFN-α, TNF-α, and/or IL-12 (the latter facilitating induction of Th1-type responses). In this context, TLR7-specific activation preferentially triggers IFN-α secretion by pDC, whereas TLR8-specific activation preferentially induces IL-12 production by mDCs [176,181].

TLR 7/8 agonists also stimulate B cells to produce cytokines (e.g., IL-6, TNF-α, etc.) and secrete Ig [182], trigger NK cells to produce IFNγ and T cells to proliferate and produce cytokines including IFNγ, IL-2, and IL-10 [183]. Memory T cells are particularly sensitive to this form of TLR-mediated activation, and TLR7/8 ligands may reduce the immunosuppression mediated by CD4 T regulatory cells [167,184].

7.10.6.2 TLR7/8 Ligands and Their Preclinical Activity

Preclinical studies indicate that imidazoquinolines can improve both the magnitude and the quality of Ag-specific T-cell and Ab responses. In mice and macaques, TLR7/8 agonists enhanced Ag-specific IgG and CTL responses, particularly when the adjuvant was conjugated to Ag [185–187]. TLR7/8 agonists have also been used as adjuvants with cancer vaccines. In a genetically engineered mouse model of breast cancer, a DNA vaccine encoding HER2/neu adjuvanted with imiquimod significantly delayed the development of spontaneous mammary tumors and reduced their incidence by 65% compared to the DNA vaccine alone. These effects were accompanied by a significant increase in Ag-specific Ab production (threefold, $p < 0.05$), a switch from IgG1 to IgG2a Ab isotype, and a 30% increase in CTL activity ($p < 0.05$) [188].

7.10.6.3 Clinical Activity

Imiquimod (Aldara, 3 M) is the only TLR7/8 agonist currently approved for clinical use, having proven efficacious as a topical ointment for the treatment of anogenital warts caused by HPV, for basal cell carcinoma, and for actinic keratosis [189,190].

Topical use of TLR 7/8 in patients with other types of cancer resulted in only modestly enhanced Ag-specific Ab and CD4 responses, although it is unclear whether this was sufficient to impact disease progression.

7.10.6.4 Safety

Topical administration of imiquimod is well tolerated. In contrast, severe side effects were reported after oral and systemic delivery of imidazoquinoline as monotherapy in humans [191,192]. Novel formulations are being developed to overcome this limitation, but their utility awaits the outcome of clinical trials designed to assess their activity and safety.

7.10.7 TLR9

7.10.7.1 Overview

TLR9 is expressed by human B cells and plasmacytoid dendritic cells (pDC). The receptor is localized within endolysosomal compartments and detects the unmethylated CpG motifs present at high frequency in bacterial DNA [193]. Activation of cells expressing TLR9 has broad effects on the immune system, eventually leading to the maturation, differentiation, and/or proliferation of NK cells, T cells, B cells, monocytes, and macrophages [177,193–200]. The resultant immune response is characterized primarily by the production of proinflammatory and Th-1 biased cytokines (including IL-1, IL-6, TNF-α, IFN-γ, and IL-12 [193,194,201–205].

7.10.7.2 TLR9 Ligands

The immunostimulatory activity of bacterial DNA is mimicked by synthetic oligonucleotides (ODNs) expressing CpG motifs [206–208]. While several different "classes" of CpG ODN have been described, "K" type ODN (also referred to as "B" type) are the most widely studied of the lot. They strongly support B-cell activation and induce the maturation of pDCs and monocytes [209–211].

Preclinical studies demonstrate that CpG ODN can accelerate [212], increase the magnitude [212–214], and prolong the duration of vaccine-specific Ab and CTL responses [214–216]. CpG ODNs promote vaccine immunogenicity by improving Ag uptake by professional APC (particularly pDC), triggering the functional maturation of APC, and generating a cytokine/chemokine microenvironment supportive of Ag-specific immunity [217]. These effects are optimized when vaccine and adjuvant are presented to the immune system in close spatial and temporal proximity [213,218,219].

7.10.7.3 Clinical Activity

Clinical trials examined the adjuvant activity of "K" type ODN combined with a number of human vaccines. CpG ODN plus the HBV vaccine boosted anti-HBs Ab titers by 13-fold after primary and 45-fold after secondary immunization [220–222]. When tested in combination with the licensed anthrax vaccine, "K" ODN boosted anthrax-specific Ab titers by six-eight-fold and accelerated the induction of immunity [223]. The effect of adding CpG ODN to the Fluarix influenza vaccine was less impressive. In that trial, CpG ODN had a dose sparing effect, but it did not increase the magnitude of the Ab response [224].

CpG ODNs were tested as adjuvants with a number of cancer vaccines. Improved CTL responses were generally observed, but these had little effect on outcome. Thus, available clinical data indicate that CpG ODNs are most likely to find use as adjuvants for vaccines targeting infectious diseases. This reflects the consistent ability of CpG ODNs to boost Ag-specific humoral immunity in naive subjects. The utility of CpG ODNs as tumor vaccines adjuvants is less clear, as the magnitude of the CTL response needed to clear an established tumor may exceed that which can be induced by the current generation of vaccines.

7.10.7.4 Safety

CpG ODNs have been well tolerated when administered as vaccine adjuvants. However, the frequency and severity of local adverse events (injection site reactions such as pain, swelling, induration, pruritus, and erythema) and systemic symptoms (including flu-like symptoms) were elevated, consistent with the immunostimulatory properties of CpG ODN. Most adverse events were mild to moderate, appeared within 24 h of dosing, and persisted for only a few days. In general, no significant difference in the frequency or severity of AEs was reported for patients receiving vaccine alone versus vaccine plus CpG ODN.

7.11 Adjuvant Combinations

As described in the introduction, vaccine adjuvants generally fall into two broad categories: those that stimulate specific elements of the immune system and those that improve vaccine uptake/persistence/presentation. Not surprisingly, there is considerable interest in combining these two approaches to achieve superior results. It is also hoped that improved safety and lower cost might be achieved if the dose of an immunostimulatory adjuvant could be decreased by pairing it with a delivery system that improved uptake and targeting.

As an example of this approach, TLR agonists such as CpG ODNs have been co-encapsulated with Ag in microparticle delivery systems. Animal studies suggest that this use of microparticles significantly increased the generation of CTL compared to preparations in which Ag, CpG ODN, and microparticles were delivered independently [225–227]. There have been clinical trials in which CpG ODN was paired with alum to adjuvant a novel HBV vaccine "HEPLISAV-B." The addition of the TLR9 agonist was expected to overcome the hyporesponsiveness of some individuals to the commercially available HBV vaccine. Similarly, phase 2 clinical studies indicate that a vaccine composed of the combination adjuvant AS15 (which includes MPL, QS21, CpG ODN, and liposomes) plus the MAGE-3 tumor antigen improves disease-free intervals in a subset of melanoma patients better than the same antigen formulated with AS02 [228].

Also being examined are combination adjuvants designed to modify the Th1: Th2 balance by targeting immunostimulatory ligands to specific receptors. Preclinical studies support the hypothesis that synergy between different TLR agonists can be exploited to improve vaccine efficacy. For example, a vaccine containing a mixture of TLR4 and TLR9 agonists was more efficient than a vaccine containing adjuvants targeting TLR4 or TLR9 alone [229,230]. Similarly, the combinations of TLR7 and TLR9 agonists were more effective against tumor targets than either ligand alone [231].

7.12 Future Directions

There is considerable interest in developing vaccines with improved activity against cancer and chronic infectious diseases. Concepts including combining

immunostimulatory adjuvants with immune checkpoint inhibitors such as anti-CTLA4 or anti-PD-1 are of interest to the oncology community. They hypothesize that checkpoint inhibitors can reduce tumor growth while vaccines enhance adaptive antitumor immunity. Central to any immunologic approach to cancer treatment is the need to overcome the suppressive microenvironment that protects established tumors from immune elimination.

As described in the introduction, novel adjuvants that enhance the generation of the CTL are considered key to the next generation of vaccines that target chronic infectious diseases. Such adjuvants must not only have excellent safety profiles but also induce stronger, longer lasting and/or qualitatively improved immune responses while simultaneously being amenable to consistent manufacture. These multiple requirements highlight the challenges associated with the development of novel vaccine adjuvants.

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8

Pattern Recognition Receptors

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8.1 Introduction

The innate immune system is the first line of defence and is crucial for the initial sensing of microbial infection or tissue injury. Innate immune cells within the body constantly sample the extracellular and intracellular environment through a broad class of proteins known as pattern recognition receptors (PRRs). These germ-line encoded receptors recognize highly conserved motifs known as pathogen-associated molecular patterns (PAMPs) that are associated with microbial pathogens or damage-associated molecular patterns (DAMPs) that are associated with cellular stress. PRRs dictate the initiation of an appropriate and effective innate immune response as well as activation of the adaptive immune response in the face of an infection or sterile inflammation. At present, four major classes of PRRs have been identified, namely, Toll-like receptors (TLRs), C-type lectin-like receptors (CLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and retinoic acid-inducible gene-1-like (RIG-1) receptors (RLRs), as shown in Figure 8.1. In addition, several other classes exist, including DNA-sensing molecules (Figure 8.1). These families of receptors are expressed in a wide variety of cells where they enact functions tailored to the cell and its functional role in the immune system.

Sensing of PAMPs or DAMPs by PRRs triggers specific signaling cascades resulting in transcription of genes involved in the inflammation response. These genes mediate several processes such as release of cytokines, chemokines, antimicrobial peptides, and cell proliferation [1]. Some of these PRRs enable direct phagocytosis of the bound antigen or can enhance the cells' phagocytic ability. Another key mechanism for antimicrobial killing is the generation of reactive oxygen species (ROS), and in the case of neutrophils, neutrophil extracellular trap (NET) formation. Activation of these signaling cascades may also upregulate or downregulate the expression of other PRRs and costimulatory molecules, thus modulating the responsiveness of the cell to stimuli. More recently, there is a growing interest in the contribution of PRRs in mediating the development of adaptive immunity.

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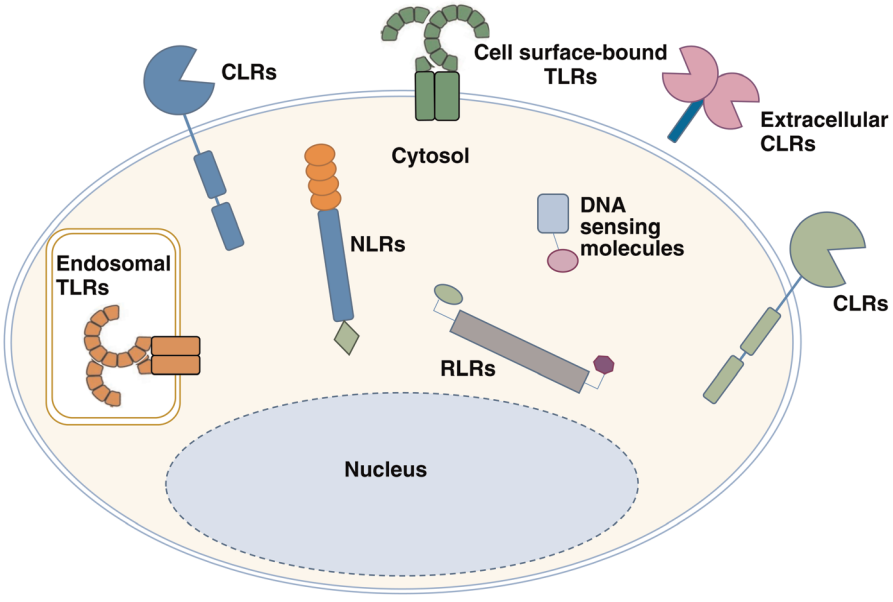


Figure 8.1 Overview of PRR distribution within the cell. TLRs and CLRs can be extracellular or membrane bound that are expressed either at the cell surface or in the endosome. NLRs, RLRs, and DNA sensing molecules are expressed in the cytosol.

In this chapter, we briefly describe the structure and expression of the four major classes of PRRs as well as DNA-sensing molecules and their respective signaling cascades. In addition, we highlight their functional role with selected receptor–ligand examples and their contribution to immunity.

8.2 Toll-like Receptors

The family of Toll-like receptors, initially discovered in *Drosophila*, consists of 10 transmembrane glycoproteins in humans (12 in mice) that recognize a wide range of pathogens. They are highly conserved and share both structural and functional similarities across humans and mice [2]. Structurally, they are characterized by N-terminal leucine-rich repeats (LRRs), a transmembrane region, and a cytoplasmic Toll/IL-1R homology (TIR) domain. Description of the TLR crystal structure revealed that the ligand-binding LRR region gives TLRs their characteristic horseshoe shape. TLRs are monomeric but can form hetero- or homodimers upon ligand binding, thus adapting ligand specificity. They are expressed in a wide variety of cells such as innate immune cells, T and B cells, epithelial cells, fibroblasts, and endothelial cells; however, not all cell types express every TLR [3]. For example, plasmacytoid dendritic cells express a limited repertoire of TLRs, predominantly TLR7 and TLR9, making them particularly responsive to viral and bacterial nucleic acids [4]. Based on their cellular localization, TLRs can be divided into two classes: cell surface-bound and endosomal TLRs.

8.2.1 Cell Surface-bound TLRs

The cell-surface bound TLRs consist of TLR1, TLR2, TLR4, TLR5, and TLR6 and detect lipoproteins with the exception of TLR5. TLR2 recognizes the broadest range of ligands due to its dimerization with either TLR1 or TLR6. TLR2/TLR1 and TLR2/TLR6 heterodimers detect triacyl and diacyl lipoproteins, respectively [5,6]. These lipoproteins can be found on various components of bacteria, fungi, and viruses. TLR2, for example, recognizes lipoarabinomannan from mycobacteria [7–9]. Secreted isoforms of human TLR2 in plasma, saliva, and breast milk may act independent of TLR6 or TLR1 and negatively regulate TLR2 signaling through ligand-binding competition [7,10,11].

TLR5 forms homodimers upon detection of bacterial flagellin and is highly expressed in dendritic cells (DCs) of the lamina propia in the small intestine [12–14]. This ligand specificity and location enable TLR5 to respond to invasive pathogenic flagellated bacteria in the gut [15–17]. In addition, TLR5-deficient mice exhibit uncontrolled microbiota-induced gut inflammation [17]. Due to the increasing importance of the gut microbiota in the development of autoimmune diseases, TLR5 may play an important role in the onset of these diseases [18]. Indeed, TLR5 polymorphisms in humans have already been linked to ulcerative colitis and Crohn's disease [17,19,20]. Sensing of the microbiota by TLR5 may also be important for vaccine responses as the required antibody response cannot be triggered without the initial priming of the innate immune system by either an adjuvant or the microbiota [21].

TLR4 in combination with myeloid differentiation factor 2 (MD2) recognizes lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria and the cause of septic shock [22]. The crystal structure revealed that upon ligand binding a TLR4-MD2-LPS complex assembles and then interacts symmetrically to form a TLR4 homodimer [23]. MD-2 contains a hydrophobic pocket formed by two antiparallel β sheets that directly binds LPS [23]. Polymorphisms in TLR4 have been linked to increased susceptibility to parasitic infections, malaria, leishmaniasis, and typhoid fever [24]. As is expected, TLR4 plays a seminal role in immune responses to bacterial infections; however, there is contradicting evidence when attempting to draw associations between human polymorphisms and bacterial infections [25]. TLR4 is also involved in antiviral innate immunity through the recognition of viral envelope proteins or DAMPs generated during infection [26,27]. For example, infection with H5N1 avian influenza resulted in acute lung injury and the production of endogenous oxidized phospholipids that stimulate TLR4. This recognition is key to avian influenza-induced lethality. Another example, HMGB1, a ubiquitous nuclear protein actively secreted by innate cells or released from damaged cells as a result of sterile tissue injury has been identified as a ligand of TLR4 [28]. It functions as DAMP thereby linking tissue injury and infection. During sepsis, HMGB1 levels are persistently elevated during the later stage of disease [29]. This release of DAMPs can result in a cytokine storm and organ failure, and neutralizing antibodies against HMGB1 was shown to protect against sepsis-induced mortality [30,31].

8.2.2 Endosomal TLRs

This group of TLRs is localized on the endosomal membrane within the cell and is comprised of TLR3, TLR7, TLR8, and TLR9. They predominantly recognize nucleic acids derived from viruses and bacteria but can also recognize self-nucleic acids degraded by DNAses. In order to prevent self-recognition, these TLRs are compartmentalized on the endoplasmic reticulum and translocate to the endolysosome in a regulated manner [32–34]. The multipass membrane protein UNC93B1 loads the TLRs into COPII vesicles that then pass through the Golgi complex to their final destination, the endolysosome [34]. In addition, there is evidence showing that this group of TLRs must be cleaved by various proteases before they are capable of downstream signaling; however, this process is still controversial [35]. These regulatory mechanisms are crucial in preventing autoimmunity.

TLR3 detects viral double-stranded RNA (dsRNA) formed during the replication of positive stranded viral RNA in the cytosol [36]. Double-stranded RNA binds to both N and C terminals of TLR3 and induces dimerization via its cytoplasmic TIR domain [37,38]. A synthetic analog of this ligand, polyinosinic polycytidylic acid (poly I:C) can be used to induce TLR3-driven responses. Inoculation of mice with poly I:C induces the production of Type 1 interferon cytokines essential for viral clearance [39]. Furthermore, TLR3-deficient mice have been shown to be susceptible to a number of viruses including poliovirus and encephalomyocarditis virus [40–43]. TLR7 and TLR8 both recognize viral single-stranded RNA (ssRNA) [44,45]. TLR8 is expressed as a dimer with two ligand-binding sites, situated in a concave face and the other in the dimerization interface [46,47]. TLR7 and TLR9 have both been implicated in the development of lupus through detection of self-nucleic acids [48,49]. TLR9 recognizes bacterial DNA rich in unmethylated CpG motifs [50]. The crystal structure of TLR9 revealed that upon ligand binding, TLR9-CpG-DNA forms a symmetric dimer [51]. TLR9 agonists are increasingly being explored as vaccine adjuvants for several infectious disease vaccines [52].

While the ligand for TLR10 remains unknown, there is evidence that this TLR is indeed functional in humans. Recently, it has been implicated in the recognition of *Helicobacter pylori* by gastric epithelial cells and may act as a heterodimer with TLR2 [53,54]. Further studies are required to elucidate this receptor's biological function.

TLR11 and TLR12 are both present in mice but not in humans. TLR11 is expressed on macrophages, dendritic cells as well as liver, kidney, and bladder epithelial cells. It plays a crucial role during parasitic and urinary tract bacterial infection [55,56]. In addition, TLR11 has been shown to prevent intestinal infection during a murine model of *Salmonella* infection [57]. Similar to TLR11, TLR12 also plays a role in parasitic infection with *Toxoplasma gondii* through recognition of *T. gondii* profilin protein [55,58]. Interestingly, TLR12-deficient mice succumb rapidly to *T. gondii* infection, while TLR11-deficient mice are partially resistant. This difference may be partially attributed to the expression patterns of these two TLRs. TLR12 is expressed on plasmacytoid dendritic cells and, therefore, promotes their function during *T. gondii* infection. In contrast, TLR11 and TLR12 may form heterodimers and are both required for IL-12

production by macrophages and conventional dendritic cells during infection with this pathogen [58].

8.2.3 TLR Signaling

TLR signaling can be roughly divided into two distinct pathways based on the TIR domain-containing adapter molecules recruited to the activated TLR, namely, MyD88-dependent signaling or TIR-containing adapter -inducing IFNβ (TRIF)-dependent signaling. MyD88-dependent signaling leads to activation of the transcription factor NF-κB while TRIF-dependent signaling results in both NF-κB and interferon regulatory factor 3 (IRF3) activation [59,60]. The activation of these transcription factors results in the expression of subsets of genes responsible for proinflammatory immune responses.

8.2.4 MyD88-dependent Signaling Pathway

The MyD88 signaling pathway, summarized in Figure 8.2, is initiated by ligand binding, following which TIRAP (TIR domain-containing adapter protein) is recruited to the TIR domain of the ligand-bound TLR and is activated. In turn, TIRAP activates MyD88, composed of a death domain (DD) and TIR domain. MyD88 is an essential adapter for the downstream signaling of TLRs with the exception of TLR3. This has been demonstrated in both humans and mice; MyD88 knockout (KO) mice exhibit a high mortality rate during bacterial infection, while MyD88-deficient children suffer from recurrent bacterial infections [61]. MyD88 interacts via its N-terminal death domain with IL-1R-associated kinase (IRAK-4) that subsequently recruits other members of the IRAK family and TNFR-associated factor 6 (TRAF6) to form a complex [62]. TRAF6 acts as a ubiquitin ligase and catalyzes the formation of a lysine-63 (K63) ubiquitin chain on itself and an unconjugated ubiquitin chain with the help of ubiquitin ligase enzymes: ubiquitin-conjugating enzyme 13 (UBC13) and ubiquitin-conjugating enzyme E2 variant 1 (UEV1A) [63]. TRAF6 ubiquitination activates the TGF-β-activated kinase 1 (TAK1), TAK1-binding protein (TAB1), TAB2, and TAB3 complex. This complex promotes the phosphorylation of the IκB kinase (IKK) complex, MAP kinase 6, p38, Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). The IKK complex, composed of IKK-α, IKK-β, and NF-κB essential modulator (NEMO), then phosphorylates IKK-β, a NF-κB inhibitory protein. Subsequently, IKK-β is degraded by the ubiquitin-proteasome system releasing NF-κB to translocate to the nucleus and activate transcription of proinflammatory genes. Phosphorylation of MAP kinase 6 results in a MAP kinase cascade and activation of the transcription factor AP-1.

8.2.5 TRIF Signaling Pathway

TLR3, which recognizes dsRNA, induces a MyD88-independent signaling cascade through the recruitment of the adapter molecule TIR domain-containing adapter-inducing IFN-β (TRIF) instead of MyD88. This pathway is summarized in Figure 8.2. Binding to dsRNA induces dimerization of the TIR domain in the TLR

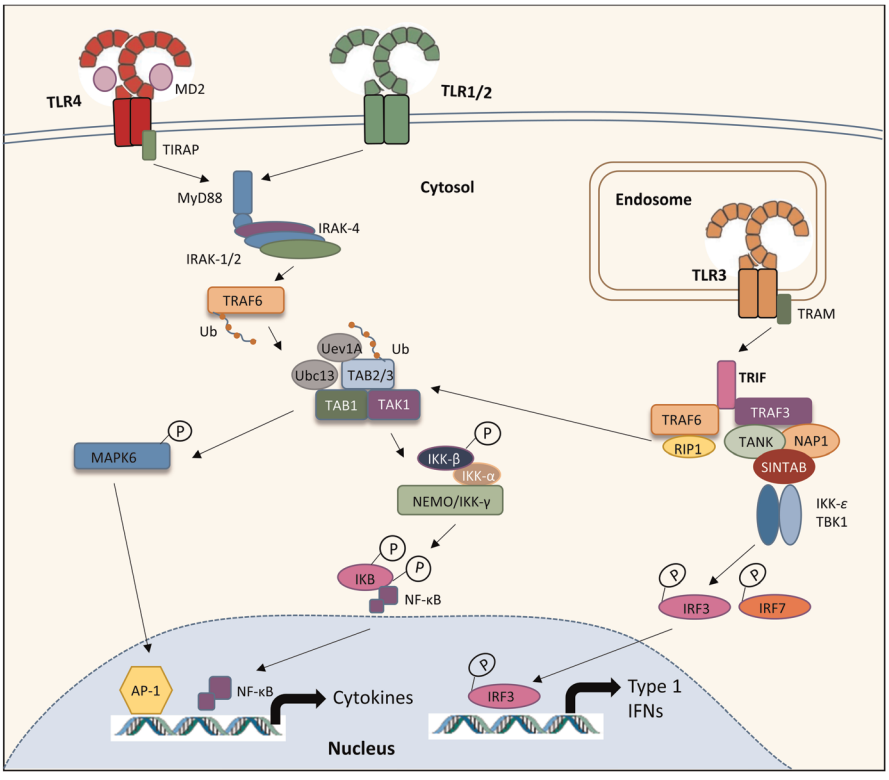


Figure 8.2 TLR signaling pathway. TLR4 and TLR1/6 signal via the MyD88-dependent pathway. MyD88 signaling leads to activation of TAB1-TAB2/3-TAK1 complex, which promotes the phosphorylation and activation of the IKK complex leading to NF-κB activation. The TAB1-TAB2/3-TAK1 complex also activates the MAP kinase pathway leading to activation of the transcription factor AP-1. TLR3 signals via the TRIF-dependent pathway. Following activation of TRIF, TRAF6 and RIP1 activate NF- κB, while TRAF3 activate the transcription factors IRF3 and IRF7 via IKK-ϵ/TBK1 complex.

and interaction with TRIF. TRAF3 and TRAF6 associate with TRIF through TRAF-binding motifs present in its N-terminal portion, while receptor interaction protein (RIP) 1 interacts via its C-terminal RIP homotypic interaction motif (RHIM). TRIF activates NF-κB either by direct interaction with TRAF6 or through RIP-1. TRAF3 is required for activating TANK-binding kinase 1 (TBK1) and IKK-ϵ [64,65]. TBK1 and IKK-ϵ phosphorylate IRF3 and IRF7 that subsequently form homodimers and translocate to the nucleus to initiate transcription of proinflammatory genes. In addition, IKK-ϵ also phosphorylates the transcription factor STAT1 [66]. TLR4 stimulation can trigger both MyD88 and TRIF signaling cascades; however, TLR4 requires TRIF-related adapter molecule (TRAM) to activate TRIF and initiate the cascade [67].

Several proteins modulate the TRIF signaling cascade at various steps. The TNFR-associated death domain protein (TRADD) forms a complex with RIP1 and FAS-associated death domain-containing protein (FADD), thereby mediating RIP1 ubiquitination [68,69]. Activation of TBK1 and IKK-ϵ is modulated by TRAF

family member-associated NF-κB activator (TANK), TBK1 adapter (SINTBAD), and NAK-associated protein 1 (NAP1) [70–72]. In humans, the sterile-alpha and armadillo motif-containing protein (SARM) acts as a negative regulator of TRIF signaling [73].

While TLR-mediated microbial recognition is essential for the host defence, in contrast, exaggerated responses to TLR ligands can induce lethal septic shock syndrome and TLR recognition of self-peptides can result in autoimmunity. Therefore, appropriate and regulated activation of TLRs is crucial for clearance of an invading pathogen and repair of tissue injury with minimal host damage.

8.3 C-type Lectin Receptors

C-type lectin receptors (CLRs) are a large class of proteins characterized by the presence of a C-type lectin-like domain (CTLD) responsible for ligand recognition [74]. CLRs can be found as both soluble and transmembrane proteins that are able to recognize a wide variety of PAMPs and/or DAMPs as well as endogenous ligands. They have traditionally been regarded as carbohydrate-binding receptors; however, they can also bind lipid and protein ligands. Classical carbohydrate-binding CLRs can be divided in two broad groups based on motifs present in the CTLD; those that bind mannose-type carbohydrates via their EPN motif and those that bind galactose-type carbohydrates via their QPD motif [75,76]. For clustering purposes, CLRs have been divided into 17 groups based upon domain organization and phylogeny [76]. Here, we describe a few of the more well-known CLRs, most primarily originating from groups II and V and also from group III, which have established roles in antimicrobial innate immunity and emerging roles in adaptive immunity.

8.3.1 DC-SIGN

One of the most intensively studied CLRs is DC-specific ICAM3-grabbing nonintegrin (DC-SIGN) within group II. As the name suggests, it is expressed in dendritic cells and recognizes fucose and mannose structures via a single extracellular CTLD. Structurally, it is a transmembrane protein with an extracellular stalk of seven complete and one partial tandem repeat with a CTLD and a short cytoplasmic domain with several sorting motifs [77]. Functionally, it exists as a tetramer. DC-SIGN is able to bind a wide variety of pathogens such as HIV-1, *Aspergillus fumigatus*, *Candida albicans*, *Mycobacterium tuberculosis*, and *H. pylori*, for example [78]. It signals through dileucine (LL) and triacidic (EEE) motifs in its cytoplasmic tail. In addition, this receptor can tailor the immune response depending on the pathogen encountered through the formation of the DC-SIGN complex. This complex consists of several proteins including, Raf-1, and scaffold proteins, LSP1, KSR1, and CNK. Raf-1, a serine/threonine MAP kinase, is activated by DC-SIGN and is involved in the acetylation of p65, an NF-κB subunit, which enables prolonged transcription of the IL-10 gene [79]. Recently, triggering of DC-SIGN by fucose and not mannose was shown to activate IKK-ϵ and induce the polarization of T follicular helper cells through the

production of IL-27 [80]. T follicular helper cells play an important role in the induction of the humoral immune response against invading pathogens. Several human polymorphisms in DC-SIGN have been described and, for example, have been shown to increase susceptibility to potentially lethal invasive pulmonary aspergillosis caused by *Aspergillus* infection [81].

8.3.2 Mannose Receptor

The mannose receptor (MR) is a calcium-dependent endocytic receptor within group I that has established roles in homeostasis and immunity. Structurally, it is composed of an extracellular region containing a cysteine-rich (CR) domain, a domain containing fibronectin type two repeats (FNII) and multiple CTLDs, a transmembrane domain, and a short cytoplasmic tail. The cysteine-rich and fribronectin domains recognize endogenous ligands [82]. In addition, it binds and internalizes glycoproteins and immune complexes that contain mannose, fucose, and *N*-acetylglucosamine residues. These residues can be found in glycoproteins on the cell surface of various pathogens including *C. alibicans*, *Leishmania donovani*, *Pneumocystis carinii*, and *Mycobacterium* species [83–88]. Mannose-capped lipoarabinomannan (ManLAM), a major component of the mycobacterial cell wall, is an example of a MR ligand. Recognition of ManLAM by MR results in mediation of phagocytosis and subsequent phagolysosome fusion in macrophages. However, MR-deficient mice exhibit no change in antibacterial activity indicating the redundancy of PRRs in pathogen detection [88–90]. More recently, two studies have demonstrated that MR deficiency results in reduced allergen uptake by mouse and human antigen presenting cells suggesting a possible role in allergy development [91,92]. In addition to the recognition of microbial ligands, MR also detects endogenous self-ligands produced during the inflammatory response such as lysosomal hydrolases [93]. Removal of these potentially detrimental serum glycoproteins enables maintenance of tissue homeostasis and resolution of inflammation.

8.3.3 Surfactant Protein D

Surfactant protein D (SP-D) is a hydrophilic C-type lectin protein present in the lung mucosa. It falls within group III of the CLR superfamily and is characterized by four domains: an N-terminus involved in interchain disulfide bonding, a collagen-like domain, a coiled coil neck domain, and a carbohydrate-recognition CTLD. Each SP-D monomer assembles to form a triple helix at the collagen-like domain that further assembles into a dodecamer. A subpopulation of SP-D can also form an oligomer through interaction of their N-termini. SP-D can bind various ligands present on the cell surface of microbes including lipoproteins, glycoproteins, and phospholipids [94]. Its primary function is interaction and subsequent agglutination of the microbe, thereby preventing dissemination. This interaction also enhances phagocytosis due to opsonization of the microbe with SP-D [94–96]. SP-D has also been shown to play an important role in NET formation. During this process, SP-D binds both the NET and the microbial surface, thus promoting neutrophil killing [97]. In

addition, there is evidence suggesting a direct inhibitory effect on the growth of some microbes [98].

8.4 Natural Killer Gene Complex-associated CLRs

The natural killer gene complex, a genetic locus located on chromosome 12p13.1 in humans and 6F3 in mice, encodes several group II and group V CLRs. In mice, members of two receptor families are encoded by this locus, the NKRP1 and Ly49 families. The ly49 family includes activating receptors such as Ly49D and Ly49YH (Klra8) that recognize epitopes of H2-D and H2-K MHC Class I molecules. Recognition of these epitopes plays an important role in regulating NK cell cytotoxicity. Cells expressing reduced levels of MHC Class 1 molecules are targeted for cell death [99]. The Ly49 family also includes inhibitory receptors such as Ly49Q. These receptors suppress NK cytotoxicity and allow for regulated killing of virus-infected or malignant cells [100–102]. In humans, the equivalent of the Ly49 family is known as killer cell Ig-like receptors (KIRs) and recognizes the human leukocyte antigens, HLA-A, HLA-B, and HLA-C.

Several group V CLRs are also encoded by this locus and can be divided into two distinct clusters: the "Dectin-1" and "Dectin-2" clusters. The Dectin-1 cluster includes receptors such as Dectin-1, myeloid inhibitory C-type lectin-like receptor (MICL or CLEC12A), CLEC1, CLEC2, and CLEC9A, while the Dectin-2 cluster encodes receptors such as Dectin-2, CLECSF8 (CLEC4D or MCL), Mincle (CLEC4E), and DCIR (CLEC4A) [103]. The CLRs encoded by these clusters have been studied primarily in the context of antifungal immunity and also play a role in other pathogen infections, immune homeostasis, and autoimmunity. Several of these receptors are expressed in multiple myeloid lineage cells and natural killer cells but are not restricted to these subsets. Structurally, they consist of an extracellular CTLD, a stalk, a transmembrane region, and a cytoplasmic tail that may contain either an immunoreceptor tyrosine-based activation-like motif (ITAM) or its inhibitory counterpart, immunoreceptor tyrosine-based inhibitory-like motif (ITIM). Functionally, CLRs can be clustered into two groups: activation receptors that signal via their cytoplasmic ITAM or via an association with ITAM-bearing adapter molecules such as FcRγ, DAP10, or DAP12 and inhibitory receptors that signal through their cytoplasmic ITIM. The interplay of these two groups enables the fine balance required for immune homeostasis.

8.4.1 Activation Receptors

Upon ligand binding, the ITAM in the cytoplasmic tail of these receptors or associated ITAM bearing adapter molecules are phosphorylated at tyrosines within the consensus sequences, canonical (YxxI/L (6–12)YxxI/L) and hemITAM (YxxI/L), by Src family kinases. Subsequently, spleen tyrosine kinase (Syk) or zeta chain-associated protein kinase 70 (Zap70) are recruited to the phosphorylated ITAM and activate downstream signaling [104].

Natural killer group 2, member D (NK2GD), is an activating receptor expressed on the surface of natural killer (NK) cells as well as some NKT cells, CD8+

cytotoxic T cells, and γδ T cells [105]. Structurally, it is expressed as a disulfide-linked homodimer on the cell surface as either two isoforms, long or short. NK2GD lacks a signaling motif in its intracellular tail and instead associates with signal-transducing proteins DAP10 or DAP12 via charged residues in its transmembrane domain [100,105,106]. Subsequent activation of these proteins results in Syk, PI3YK, Zap70, and growth factor receptor-bound protein 2 (Grb2) signaling cascades [105]. NK2GD has a remarkable range of ligands due to its ability to recognize distantly related homologues of MHC Class I proteins that include proteins like MHC class-I-chain-related protein A (MICA) and B (MICB) [106]. These ligands are expressed at a low level in all cells but are upregulated during infection or malignant transformation [107]. This recognition of "self-induced" ligands is vital for the NK cytotoxic response to a viral-infected or cancerous cell.

Dectin-1 (CLEC7A) recognizes β-1,3-glucans present in the cell wall of several fungal species as well as undefined ligands on mycobacteria and endogenous ligands [108,109]. It has a typical group V type II tansmembrane receptor structure, as described earlier, with a hemITAM sequence [YxxL] and triacidic [DED] motifs present in its cytoplasmic tail [108,110]. Ligand binding induces signaling through the CARD9-Bcl10-MALT1 complex and results in production of key cytokines necessary for the Th1 and Th17 antifungal response as well as other key cellular effects including actin-mediated phagocytosis, production of reactive oxygen species, endocytosis, and DC maturation [111–113]. Stimulation of dectin-1 with β-1,3-glucans on antigen presenting cells induces the differentiation of Th17 and Th1 Cd4+ T cells through the enhanced production of cytokines IL-6, IL-23, IL-1β, and inhibition of IL-12 [78,114,115]. The importance of dectin-1 is demonstrated by the susceptibility of humans with a dectin-1 deficiency to recurrent candida infections, for example [116]. In addition, dectin-$1^{-/-}$ mice are susceptible to *C. albicans*, *A. fumigates*, and *P. carinii* infection [111,117,118]. Dectin-1 also plays an important regulatory role in NET formation by acting as a microbial size sensor. Through this mechanism, neutrophils are able to clear fungal pathogens while minimizing tissue damage caused by aberrant NETosis [119]. In the gut, dectin-1 has also been shown to be essential for the transcytosis of glycosylated secretory IgAs by intestinal microfold (M) cells, which provide protection for the intestinal epithelium against gut pathogens [120].

In addition to its key proinflammatory role during fungal infections, dectin-1 has also been demonstrated to play a role in homeostasis and autoimmunity. Recently, vimentin, a DAMP secreted during inflammation, was identified as an endogenous ligand of dectin-1. Thiagarajan *et al.* demonstrated colocalization of vimentin and dectin-1 in areas of active inflammation and superoxide anion production in human atherosclerotic plaque sections [121]. However, another study has demonstrated that dectin-1 does not play a role in the development of atherosclerotic plaques [122]. In the context of antitumour innate responses, dectin-1-mediated recognition of tumour cells by macrophages and dendritic cells is vital for the induction of an effective tumourcidal response by NK cells [123]. Interestingly, a human polymorphism in the gene for dectin-1 has been linked to a severe form of ulcerative colitis further highlighting the role of dectin-1 in homeostasis [124].

Dectin-2 (CLEC4N) is also fundamental in antifungal immunity and recognizes α-mannans and glycoproteins containing O-linked mannobiose-rich glycans [125,126]. Moreover, dectin-2 has been shown to play a role in the recognition of various fungi including, *C. albicans*, *A. fumigates*, and *C. neoformans* [127,128]. Dectin-2 can recognize the aeroallergen house dust mite extract, a major allergen during asthma. Activation of this receptor on dendritic cells and alveolar macrophages is crucial for induction of airway inflammation via the Th2 response and the development of severe asthma [129–131]. Mechanistically, triggering of dectin-2 results in the generation of cysteinyl leukotrienes and Th2 cytokines, such as IL-10 and IL-23, which drive Th2 pulmonary inflammation to the major allergen, house dust mite [131]. Signaling occurs through the CARD9-Bcl10-MALT1 complex, similar to dectin-1, but requires an FcRγ signaling adapter bearing an ITAM motif to initiate the cascade [125,132]. Interestingly, several groups have demonstrated that dectin-2 and not-dectin-1 is crucial for the induction of Th17 cell differentiation, important for the defence against fungal infections in humans [128,132].

Mincle (CLEC4E) is predominantly involved in the recognition of mycobacteria but has also been shown to recognize *Candida*, *Malassezia*, and *Fonsecaea* species [133,134]. Several Mincle ligands have been identified including α-mannose, mannitol-linked glyceroglycolipid, mannosyl fatty acids, and trehalose dimycolate (TDM). Furthermore, Mincle acts as a heterotrimeric complex with CLECSF8 (otherwise known as CLEC4D or MCL) and FcRγ to bind mycobacterial cord factor (TDM), for example [135–137]. While Mincle knockout mice do not exhibit an obvious defect during mycobacterial infection, loss of CLECSF8 results in increased inflammation, bacterial burdens, and mortality [138–140]. In humans, a CLECSF8 polymorphism has been identified and is associated with an increased risk of pulmonary tuberculosis [138]. Mincle is also involved in the sensing of damaged cells through recognition of SAP130 providing a homeostatic role for this receptor [141]. Recently, elevated levels of Mincle and SAP130 in the brain and cerebrospinal fluid following traumatic brain injuries were suggested to contribute to inflammation-induced pathology associated with this injury [142].

8.4.2 Inhibitory Receptors

Inhibitory CLRs such as myeloid inhibitory C-type lectin (MICL) and dendritic cell immunoreceptor (DCIR) have been shown to play fundamental roles in autoimmunity and immune homeostasis. These receptors are identified by the presence of ITIMs (I/V/L/SxYxxI/L/V) in their cytoplasmic tails and inhibit cellular activation through a dephosphorylation cascade.

MICL (CLEC12A) is expressed on myeloid cells and recognizes the endogenous ligand, monosodium urate microcrystals (MSU) [143,144]. MSU, the causative agent of gout, is formed when soluble uric acid is released into the extracellular matrix following disruption of the cell membrane. MICL is, therefore, the first inhibitory CLR identified that can bind dead cells [145]. It has been identified as a biomarker in acute myeloid leukaemia but more recently been implicated in rheumatoid arthritis and may control myeloid cell activation during this disease [146,147].

DCIR (CLEC4A) is primarily expressed on antigen presenting cells and is believed to play an immune inhibitory function. Although the ligand for this receptor has not been identified, studies have demonstrated several negative regulatory effects on other receptors including TLR8, TLR9, and B cell receptor [148,149]. In addition, DCIR has been shown to be crucial in the induction of regulatory T cells as well as limiting tissue injuring during Chikungunya virus-induced inflammation, for example [150,151]. Recently, DCIR has been used as a target to improve vaccine responses induced by a HIV Gagp24 protein fused to a DCIR monoclonal antibody. In nonhuman primates, this approach yielded a strong anti-Gagp24 antibody response and may be a promising strategy for HIV vaccines [152]. In the case of autoimmune diseases, DCIR polymorphisms in humans have been associated with both rheumatoid arthritis, systemic lupus erythematosus (SLE), and primary Sjogren's syndrome in the Chinese population [153,154]. Moreover, there is evidence of an immune regulatory role for DCIR in the development of experimental autoimmune encephalomyelitis (EAE), an autoimmune disease model for multiple sclerosis [155]. This receptor may prove to be very useful in the study of the pathology of several autoimmune diseases as well as in future vaccine strategies.

8.4.3 CLR Signaling

C-type lectin receptors in the dectin-1 and dectin-2 cluster signal via Syk-dependent and Syk-independent pathways summarized in Figure 8.3. These pathways lead to the activation of several transcription factors including NF-κB, NFAT, and AP-1 [156]. In the case of activation receptors, phosphorylation of tyrosine residues within the ITAM induces the recruitment of Syk. Following activation, Syk phosphorylates phospholipase C (PLC)γ2 leading to activation of protein kinase C (PKCδ). In turn, PKCδ phosphorylates the heterotrimeric CARD9-Bcl10-MALT1 complex to induce NF-κB translocation to the nucleus. PLCγ2 also promotes calcium release from the endoplasmic reticulum that drives the calcineurin-NFAT pathway.

Syk also causes the noncanonical activation of NF-κB through NK-κB-inducing kinase (NIK) [157,158]. Dectin-1 also mediates a Syk-independent pathway via activation of Raf-1 by Ras (Figure 8.3). Ras interacts with the triacidic motif (DED) in the cytoplasmic tail of dectin-1. Activation of Ras leads to activation of Raf-1 that in turn phosphorylates p65, a subunit of NF- κB. Phosphorylation of p65 results in the formation of p65-RelB dimer that sequesters active RelB and promotes Th1 cytokine gene transcription [79].

In the case of inhibitory receptors, ligand binding induces phosphorylation of tyrosine residues in the consensus sequence of the ITIM by Src kinases. This results in the recruitment of SH2 domain-containing protein tyrosine phosphatises, including SHP-1 and SHP-2, which are able to directly regulate signaling downstream of ITAM motifs through inhibition of Syk [103].

Similar to TLR signaling via MyD88, there is a convergence of downstream signaling of CLRs via the CARD9-Bcl10-MALT1 complex. Humans with a CARD9 deficiency are extremely susceptible to invasive candidiasis and other fungal infections highlighting the central role of CARD9 [159]. In addition,

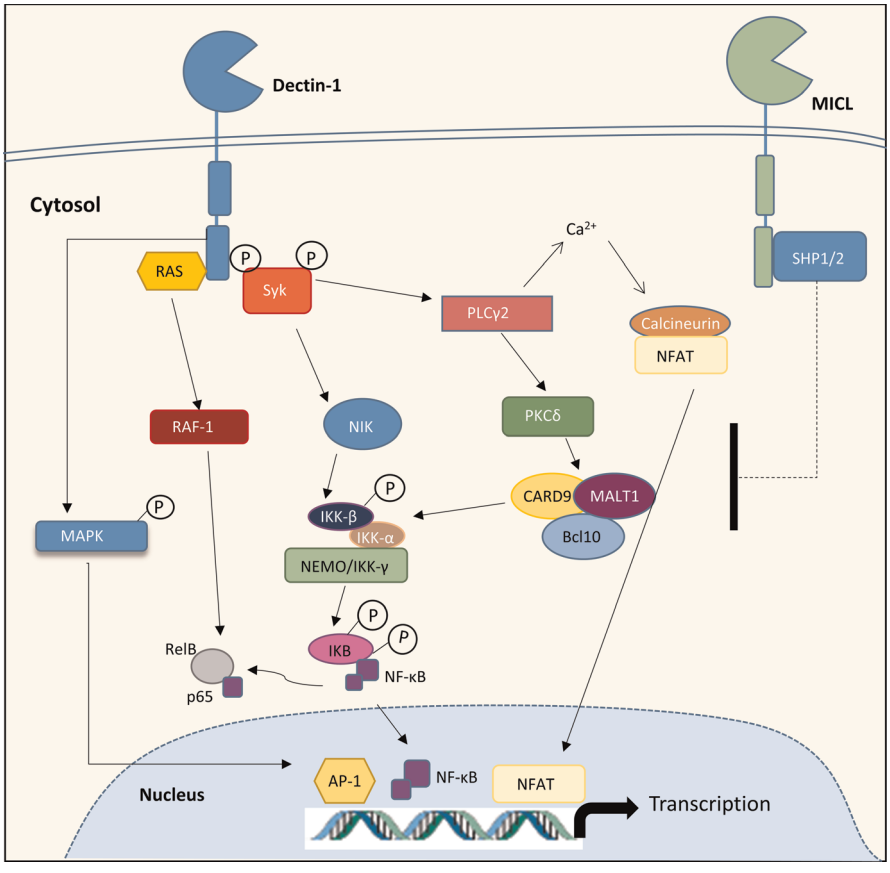


Figure 8.3 CLR signaling pathway. Activation receptors such as dectin-1 signal via Syk. This leads to activation of the CARD9-MALT1-Bcl10 complex via PLCγ2 and PKCδ. CARD9 promotes phosphorylation of the IKK complex resulting in NF-κB activation. Syk can also activate NF-κB via the noncanonical NIK pathway. PLCγ2 promotes calcium release from the endoplasmic reticulum that drives the calcineurin-NFAT pathway. Dectin-1 also signals via the Syk-independent activation of RAF-1. Activation of the RAF-1 pathway results in the formation of a RelB-p65 dimer and modification of gene transcription. Inhibitory receptors such as MICL phosphorylate SHP-1 and SHP-2 enact their downstream inhibitory function.

CARD9-deficient mice exhibit a high mortality during mycobacterial infection due to exacerbated neutrophilic pulmonary inflammation and uncontrolled bacterial replication [160].

8.5 RIG-1-like Receptors

The RIG-1-like receptor family consists of RNA-binding proteins that are expressed in almost all cells and are predominantly responsible for viral recognition in the cytoplasm. Family members include RIG-1, melanoma

differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) [3]. Structurally, RLRs are RNA helicases characterized by two N-terminal caspase recruitment domains (CARDs), which LGP2 lacks, a central DEAD box helicase/ATPase domain and a C-terminal regulatory/repressor domain. They recognize dsRNA as a replication intermediate of ssRNA viruses and genome content of dsRNA viruses [161].

RIG-1 detects short dsRNA that is 5′ triphosphorylated and lacks a 7′ methylguanosine cap [162–164]. In contrast, MDA5 recognizes long dsRNA (more than 2Ykb), and the synthetic analog, poly I:C. [165–168]. Although LGP2 possess the ability for RNA binding, it has not been shown to be crucial for viral RNA recognition and is rather thought to act as a regulator of RIG-1 and MDA5 signaling [169]. RIG-1 and MDA5 signal via activation of IRF3 and NF-κB through the adapter, mitochondrial antiviral signaling protein (MAVS) (also known as IPS-1). Subsequently, transcription of antiviral type 1 interferon genes is upregulated and leads to an antiviral Th1 immune response. RIG-1 and MDA5 have been reported to play a role in immune defense against viruses such as Ebola, measles, dengue, hepatitis C, and many more [170–174].

Recently, a key mutation in the RLR MDA5 rendered this receptor unresponsive to dsRNA but induced lupus-like symptoms in mice [175]. This link between RLR activation and autoimmunity was further confirmed through the discovery of several mutations in humans with three different autoimmune diseases: Aicardi–Goutieres syndrome (AGS), SLE, and Singleton–Merten syndrome (SMS) [176–178]. Interestingly, these mutations all lie within the conserved RNA helicase domain of either MDA5 or RIG-1. A common theme in these diseases and several other autoimmune diseases is the presence of elevated type 1 interferon cytokines, termed the "interferon signature," which drive disease pathogenesis and have since become the target of several therapies [179,180].

8.5.1 RLR Signaling

In uninfected cells, RLRs are maintained in a "closed" conformation with the repressor and CARD domains folded over the PAMP-binding helicase domain as shown in Figure 8.4 [181]. Upon RNA binding, RIG-1 and MDA5 hydrolyze ATP, thereby releasing the CARD domain for oligomerization and enhancing the interaction with MAVS/IPS-1 [182–185]. This oligomerization is further promoted by ubiquitination at N-terminal sites by TRIM25 proteins [186,187]. RIG-1 then translocates from the cytoplasm to the mitochondrial-associated membrane (MAM) where it interacts with IPS-1 [188]. Interaction with IPS-1 leads to activation of TBK1 and IKK-ϵ via TRAF3, TANK, and TRADD. Activated TBK1 and IKK-ϵ subsequently activate the transcription factors IRF3 and IRF7 leading to their translocation to the nucleus [189,190]. Simultaneously, IPS-1 activates NF-κB via RIP1, FADD, and caspase-8/-10. In addition, IPS-1 activates the MAP kinase cascade and subsequent AP-1 activation. As a result, antiviral type 1 interferon genes and proinflammatory cytokines are upregulated and the immune response is initiated.

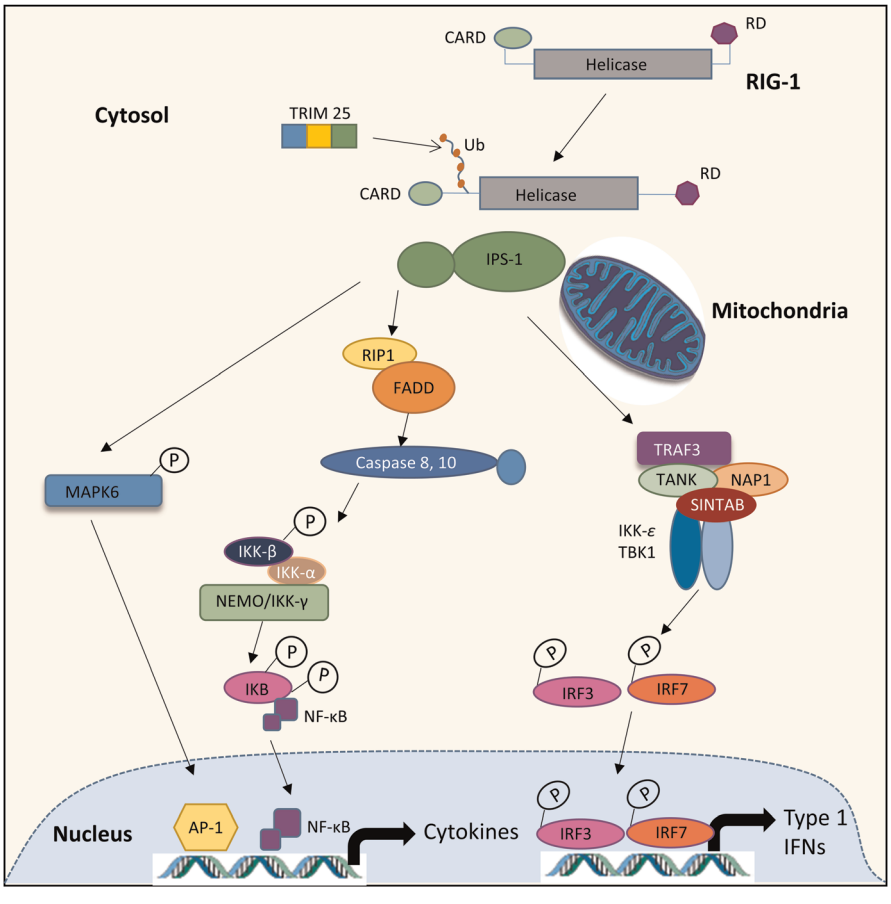


Figure 8.4 RLR signaling pathway. Activation and ubiquitination of RIG-1 induces a conformational change releasing its CARD domain and enhancing interaction with IPS-1. Resultant activation of IPS-1 leads to activation of NF-κB through RIP-1-FADD and caspase-8/-10. IPS-1 simultaneously activates the transcription factors IRF3 and IRF7 via TRAF3.

8.6 NOD-like Receptors

The NLR family consists of 20–30 (34 in mice) cytosolic receptors that have been categorized structurally into five subsets based on their N-terminal effector domain: NLRA, NLRB, NLRC, NLRP, and NLRX. Structurally, NLRs possess a C-terminal leucine-rich repeat domain, a central nucleotide oligomerization domain (NOD), and an N-terminal effector domain used for classification as summarized in Figure 8.5a [191]. NLRA contains an acidic transactivation domain, NLRB, with a baculovirus inhibitor of apoptosis protein repeat, NLRC, with a CARD domain, NLRP, with a pyrin domain and NLRX, has an uncharacterized N-terminal domain (Figure 8.5a) [192]. Traditionally thought to counteract intracellular pathogens by recognition of microbial ligands in the

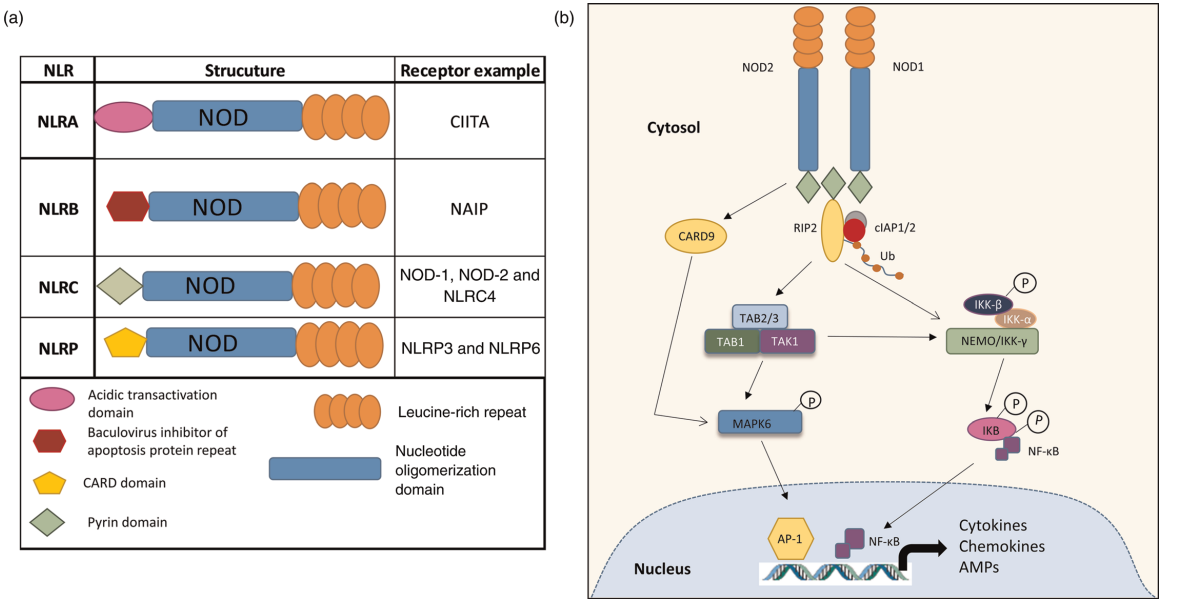


Figure 8.5 NLR classification and signaling pathway. (a) NLR structural classification and receptor examples. (b) NOD1 and NOD2 initiate signaling via activation of RIP2. Ubiquitinated RIP2 activates the IKK complex directly or the TAB1-TAB2/3-TAK1 complex, both of which result in NF-κB activation. Simultaneously, the TAB1-TAB2/3-TAK1 complex activates MAPK6 and initiates the MAPK cascade resulting in AP-1 activation. NOD2 also mediates AP-1 activation via CARD9.

cytosol, NLRs have also been implicated in sensing metabolic changes in the cell, cell death, embryo development, and modulation of inflammation [193–198]. Here, we briefly discuss selected receptor examples from the NLRC and NLRP families.

8.6.1 NOD1 and NOD2

NOD1 and NOD2 within the NLRC family are perhaps the most intensively studied, while NLRPs follow closely behind. NOD1, first identified through sequence homology searches, is expressed in a wide variety of cells including phagocytic cells, dendritic cells, intestinal epithelial cells, lung epithelial cells, and endothelial cells [199–203]. This receptor recognizes mainly Gram-negative and certain Gram-positive bacteria, through peptidoglycan (PGN) fragments from the cell wall containing the *N*-acetlyglucosamine-*N*-acetlymuramic acid tripeptide motif with diaminopimelic acid (DAP) [204–206]. NOD2, closely related to NOD1, contains an extra CARD domain and is also expressed in phagocytic cells, paneth cells, and stem cells within the small intestine and bronchial cells in the lung [207–210]. It detects muramyl dipeptide (MDP), a motif of PGN from both Gram-negative and Gram-positive bacteria [205].

NOD1- and NOD2-deficient mice show enhanced susceptibility to several pathogens [211–214]. Expression of NOD1 and NOD2 in the intestinal epithelium is crucial in the control of enteric bacterial infections caused by *Shigella*, *Salmonella*, *Listeria*, *Yersinia*, pathogenic *Escherichia coli* strains and *Mycobacterium* species [205,212,215–217]. Meanwhile in the lung, NOD1 responds to pathogens such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Chalmydophila pneumoniae*, and *Legionella pneumophilia* [218–222]. In contrast, NOD2 detects *Streptococcus pneumoniae*, *S. aureus*, *E. coli*, *C. pneumoniae*, and *M. tuberculosis* [8,215,223–227]. Ultimately, NOD1 and NOD2 ligation triggers NF-κB activation and subsequent production of proinflammatory cytokines and antimicrobial molecules [228,229]. As such, bacterial pathogens have evolved mechanisms to evade detection and clearance by the NLR-mediated host defense. One of these ways has been described for *L. monocyogenes* and *H. pylori* that evade detection by NOD1 through modification of cell wall PGNs [191,230].

In addition to their role in innate immune response, NOD1 and NOD2 signaling has been implicated in adaptive immune responses. The NOD2 ligand, MDP has been identified as an adjuvant of antigen-specific IgG responses [228,231]. Furthermore, NOD2 and NOD1 stimulation alone induces a predominantly Th2 adaptive immune response in mice [195,232].

Interestingly, human polymorphisms in NOD2 have been linked to Crohn's disease [207,233]. Individuals with genetic variants in NOD2 have a significantly increased risk of developing Crohn's disease, up to 40-fold [234,235]. The mechanisms by which these variants contribute to disease development are controversial; however, it is thought that impaired bacterial recognition and clearance leads to uncontrolled inflammation through NOD2-independent pathways [236].

Although the exact mechanism by which bacterial PGN fragments enter the cytosol is unknown, several routes of entry have been reported. Most recently,

Nakamura *et al.* demonstrated that MDP is transported across the membrane of phagosomes via two peptide transporters, SLC15A3 and SCL15A4 [237]. This is supported by reports demonstrating decreased NOD1-mediated cytokine production in SLC15A3-deficient mice [238]. Other routes of entry include endocytosis, endosomal membrane transporters, bacterial secretion systems, or outer membrane vesicles.

8.6.2 NOD1 and NOD2 Signaling

NOD1 and NOD2 bind cytosolic peptidoglycans and initiate an antibacterial proinflammatory response via the signaling pathways depicted in Figure 8.5b. Following ligand binding, receptor-interacting serine/threonine protein kinase 2 (RIP2) is recruited to the CARD domain of NOD and undergoes ubiquitination by cellular inhibitor of apoptosis protein 1 and 2 (cIAP1 and cIAP2) [199,239,240]. Polyubiquitinated RIPk-2 then interacts with the TAK-1-TAB2/TAB3 complex and the IKK complex [241,242]. TAK-1 is phosphorylated and activates MAPKs p38/JNK and NF-κB pathways. This leads to IKK complex activation and subsequent phosphorylation of IκB that then undergoes polyubiquitination and degradation through the proteasome. Degradation of IκB allows for NF-κB translocation to the nucleus. NOD2 can also induce the noncannonical NF-κB pathway through interaction with NF-κB-inducing kinase (NIK) that in turn activates the p100/p52-dependent NF-κB pathway [243].

8.6.3 NLRC4

NLRC4 is a cytosolic NLR that triggers inflammasome formation in response to bacterial flagellin or components of type 3 bacterial secretion systems (T3SS). It is expressed in myeloid cells as well as intestinal epithelial cells [244]. Similar to the function of TLR5, NLRC4 plays not only an important role in control of the intestinal microflora but also a protective role in the airways. It has been shown to protect mice during pulmonary infection with several bacteria such as *K. pneumoniae* and intestinal infection with *Salmonella typhimurium*, for example [245,246]. Recently, a missense mutation identified in the NLRC4 gene in humans has been linked to a hereditary autoinflammatory disease, familial cold autoinflammatory syndrome (FCAS). This mutation resulted in aberrant NLRC4 inflamasome activation and subsequent IL-1β production leading to uncontrolled inflammation [247]. This study highlights the importance of this receptor not only in bacterial infections but also in the development of autoimmunity.

8.6.4 NLRPs

NLRPs are involved in the formation of the inflammasome that consists of one or two NLR proteins, apoptosis-associated speck-like containing a CARD domain (ASC) adapter molecule and procaspase-1 [191,248]. The NLRP3 inflammasome, expressed in myeloid cells, formed with NLRP3 as its scaffold, mediates the conversion of pro-IL-1β and pro-IL18 to their active forms, IL-1β and IL-18 [249]. These potent proinflammatory cytokines are involved in a mechanism of cell

death termed pyroptosis [249]. NLRP3 is activated by several pathogenic lung microorganisms such as *S. pneumoniae*, *M. tuberculosis*, *A. fumigatus*, influenza virus, and many more [210,250–252]. In addition, there is an emerging role in defense against enteric pathogenic *Clistridium difficile* and *Citrobacter rodentium* [217]. However, there is no evidence for direct ligand binding to NLRP3 in any of these infections. Interestingly, NLRP3 inflammasome production of IL-18 and its subsequent signaling has a protective role against intestinal inflammation during a colitis murine model [253]. The NLRP3 inflammasome is also required for chemotherapy-mediated antitumor response during colorectal cancer. During chemotherapy, dying cells release ATP, thereby activating the NLRP3 inflammasome. This results in IL-1β secretion and subsequent activation of IFNγ producing cytotoxic T lymphocytes that target malignant cells [254].

Aberrant activation of the inflammasome has also been linked to multiple autoimmune diseases. Genetic activation of NLRP3 is associated with multiple autoimmune cryopyrinopathies such as cryopyrin-associated periodic fever syndromes (CAPS), FCAS, MWS (Muckle–Wells syndrome), and NOMID (neonatal onset multisystem inflammatory disorder) [255]. Symptoms of these cryopyrinopathies include periodic fevers, rashes, and arthralgia and can be treated with IL-1β-targeted therapies [256].

NLRP1, a less characterized inflammasome, is activated by the lethal toxin of *Bacillus anthracis* and induces rapid cell death [248,257]. NLRP1 is expressed in myeloid cells, lymphoycytes, and respiratory epithelial cells [258]. As a more recent example, *T. gondii* infection of mice and rats has been shown to activate the NLRP1 inflammasome [259].

NLRP6-deficient mice are resistant to infection with several pathogens such as *L. monocytogenes*, *S. Typhimurium*, and *E. coli* due to enhanced production of proinflammatory cytokines [260]. It is thought that this inflammasome is essential for homeostasis and restriction of commensal bacterial [261]. Despite evidence for these roles, the exact ligand for this receptor remains to be determined.

NLRPs can also negatively control the inflammatory response by reducing NF-κB activation and IFNβ production [262,263]. As an example, NLRP6 inhibits downstream TLR signaling [260,264]. This response may be important in regulating the immune response to gut microflora [264]. NLRP12, another negative regulator of inflammation, expressed in myeloid cells, regulates TLR- and TNFα-induced signaling [265,266]. These roles are crucial for immune and metabolic homeostasis.

8.7 DNA-sensing Molecules

Pathogen DNA or RNA is an important PAMP released during infection with DNA viruses, RNA viruses, or with intracellular bacteria such as *L. monocytogenes* and *L. pneumophila*. Following the discovery of TLR9, there has been a search for alternative foreign nucleic acid receptors. As such, several DNA sensors have been discovered and have been shown to be pivotal in multiple viral and bacterial infections as well as autoimmunity. Although not a formal PRR family, some of these proteins share structural similarity and have since been termed the AIM2-

like receptors (ALRs). These receptors all result in the transcription of type I interferon genes and subsequent proinflammatory phenotype. Here, we briefly mention a few examples of ALRs and others with their respective ligands and signaling mechanisms.

DNA-dependent activation of IFN regulatory factors (DAI) was the first TLR9-independent sensor to be identified [267]. DAI interacts directly with DNA and can signal via the receptor interacting proteins, RIP1 and RIP3, to facilitate NF-κB activation [268,269]. In addition, DAI can also signal via TBK-1 in a STING-dependent manner. The current proposed ligand is AT-rich B form DNA [267].

RNA polymerase III is an indirect sensor of AT-rich DNA. It performs this function by transcribing AT-rich dsDNA into 5′ triphosphate-containing dsRNA, a ligand of RIG-1 [161,270]. Activation of RIG-1 induces signaling via MAVS/IPS-1 and finally the transcription of interferon genes as described above. The RNA polymerase III-dependent pathway has been implicated in sensing of ligands such as Epstein–Barr virus (EBV) RNA as well as HSV-1, adenovirus, and *L. pneumophila* DNA, for example [271,272]. However, it is difficult to fully elucidate the contribution of this enzyme as it is essential for cell survival.

The leucine-rich repeat-containing protein (LRRFIP-1) recognizes AT-rich B-form dsDNA as well as GC-rich Z-form dsDNA. Stimulation of this receptor results in the production of IFN-β through β-catenin rather than NF-κB or IRF3 activation [273]. LRRFIP-1 triggers phosphorylation of β-catenin, which then binds to the C-terminal domain of the transcription activator IRF3 and induces IFN-β expression [273]. This response can be induced through infection of macrophages with vesicular stomatitis virus (VSV) and *L. monocytogenes*, thereby suggesting a role for this receptor during these infections [273].

More recently, a new PRR family has emerged, termed the AIM2-like receptors or more specifically the pyrin and HIN domain (PYHIN) family. This family consists of four members AIM2, MNDA, IFI16, and IFIX. AIM-2 (Absent in Melanoma 2), a cytosolic dsDNA sensor, activation results in the assembly of the inflammasome [274]. Upon activation, AIM2 interacts with ASC, a common adapter of inflammasomes, which leads to cleavage of caspase-1 and production of IL-1β and IL-18 [275]. This receptor plays a central role in the sensing of pathogens including *Francisella tularensis*, vaccinia virus, and mouse cytomegalovirus, for example [275–277]. IFI16 is proposed to sense viral DNA in the nucleus and mediates inflammasome activation of AIM2 as well as IFN-β induction [278]. Following activation, IFI16 recruits stimulator of interferon genes (STING) to activate a TBK1-IRF3-dependent signaling cascade as well as NF-κB activation to intiate an inflammatory response. This response can be induced by DNA oligonucleotides or HSV-1 infection.

8.7.1 Signaling Components of DNA-sensing Molecules

STING is a transmembrane protein present on the endoplasmic reticulum that induces the expression of IFN-β following detection of DNA ligands. It acts as a crucial signaling molecule downstream of DNA sensors such as DAI and upstream of the signaling adapters, TBK1 or IKKβ [279]. STING-dependent signaling has been shown to be crucial for the recognition of viruses such as HIV-

1 and bacteria such as *F. tularensis* [280]. TBK1 directly phosphorylates and activates the transcription factor IRF3, while STING induces the phosphorylation and subsequent degradation of IKKβ resulting in NF-κB activation. IRF3, NF-κB, and IRF7 activation all result in the transcription of type 1 interferon genes crucial for the antiviral or intracellular antibacterial response.

8.7.2 Cross-talk between PRRs

In addition to cross-talk between PRRs within a specific family, there is also cross-talk across PRR families. It is important to note that under physiological conditions there is participation of several PRRs that may act in a collaborative effort to increase the efficiency of the immune response. As microbes can be sensed by multiple PRRs, it is not surprising that in most cases, a loss in one PRR has only modest effects on pathogen clearance *in vivo*. The collaborative effort during bacterial infection has been demonstrated through costimulation experiments of multiple cell subtypes with NOD1/2 and TLR2/4/9 agonists. Costimulation induces a synergistic effect on the expression of proinflammatory cytokines such as TNFα, IL-12p40, and IL-6 [281–283]. As an additional example, NOD1 and TLR4 cooperate to mobilize mature hematopoetic stem cells in the spleen in response to an *E. coli* infection [284].

In the case of viral infection, TLR3 and RLRs can function together leading to enhanced antigen presentation by dendritic cells [285]. RNA viral infection may also induce inflammasome activation through the RLR signaling protein MAVS/IPS-1 [286]. This cross-talk may be particularly important in prevention of viral spread by inflammasome-induced pyroptosis.

During antifungal immunity, dectin-1, a CLR, has been shown to collaborate with TLR4 and TLR2 [287]. In addition, there is evidence to support a functional interaction between dectin-1 and DC-SIGN [288]. Chromoblastomycosis, a chronic skin infection caused by the fungal pathogen *F*onsecaea *pedrosoi*, occurs due to a lack of PRR costimulation. *F. pedrosoi* is recognized only by CLRs and not TLRs, thus resulting in defective proinflammatory cytokine production and chronic infection [289]. Notably, when patients suffering from a chronic chromoblastomycosis infection were treated with imiquimod, a TLR agonist, all four recipients exhibited a marked improvement in their skin lesions [290]. This finding highlights the importance of the collaborative action of CLRs and TLRs in the initiation of an effective innate immune response. In contrast, in order to maintain homeostasis, it is equally likely that there is a negative regulatory effect both within and across the PRR families as mentioned above. However, in many cases, this area is almost completely unstudied.

8.8 Conclusions

Activation of PRRs by PAMPs or DAMPS is essential for the initiation of the innate immune response and triggering of the adaptive immune response. Identifying these innate immune sensors has drastically improved our

understanding of not only how the host defends itself against pathogens but also the development of autoimmunity and immune homeostasis. The implications of this research are not only for the enhancement in the knowledge of basic immunology but also therapeutically with advances in vaccine responses through adjuvant studies and novel drug treatments for infection, autoimmunity, and even cancer. Defining the ligands for these PRRs as well as the dynamic cross-talk of PRRs will continue to aid the clinical aspects of this field of research. In addition, identifying the association of diseases and human PRR polymorphisms and mutations provides valuable insight into the role of these receptors.

Acknowledgments

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Part Two

Inflammatory Cells

9

Monocytes and Macrophages

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9.1 Introduction

Monocytes and macrophages are not only immune cells that serve as the first line of host defense but also key players in organogenesis and development [1]. To perform these distinct roles, monocytes and macrophages show immense functional diversity: triggering inflammation, killing pathogens, tissue remodeling and repair, and immunoregulation [2]. Thus, understanding how these cells perceive different microenvironmental cues and translate them into a "tailored" response as well as how a dysregulation in this process might lead to pathogenic outcomes is of paramount importance. In this chapter, we present a brief account of monocytes and macrophages, focusing on their ontogeny, activation, function, and the molecular mechanism regulating their functional plasticity and diversity.

9.2 Origin and Differentiation

Monocytes differentiate from macrophage/dendritic cell precursor (MDP) in the bone marrow and are released into the circulation from where they are recruited to the tissues under homeostatic or pathological conditions [3]. Based on the differential expression of chemokine receptors CCR2 and CX3CR1, two main subsets of monocytes have been described in mouse: the $Ly6C^{hi/+}CCR2^{+}CX3CR1^{int}$ inflammatory monocytes and the $Ly6C^{lo/-}CCR2^{-}CX3CR1^{hi}$ patrolling monocytes [4]. Corresponding to these murine subsets, inflammatory monocytes in humans correlate to the $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes, whereas patrolling monocytes correlate to the $CD14^{dim}CD16^{+}$ monocytes [5]. Inflammatory monocytes are recruited to the sites of inflammation and infection where they release proinflammatory cytokines to mediate the inflammatory process. In contrast, patrolling monocytes patrol the vasculature, sensing and scavenging cellular

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debris [6,7]. Patrolling monocytes are particularly good at sensing danger signals that stimulate Toll-like receptors (TLRs) 7 and 8 as compared to the inflammatory monocytes that are better at sensing bacteria-related danger signals [5]. Interestingly, a temporal recruitment of respective monocyte subsets has been reported by Auffray *et al.* 2007 [6] during *Listeria* infection, with the patrolling monocytes being recruited first to the sites of inflammation and subsequently inducing the recruitment of inflammatory monocytes (see Section 9.4.4 for further details). More recently, inflammatory and patrolling monocytes have also been shown to have important functions in cancer, with the former contributing to cancer-related inflammation and tumor progression [8–10], while the latter in preventing tumor progression by helping in the natural killer (NK) cell-mediated clearance of metastatic cancer [11]. It was suggested that in the absence of inflammation, mouse inflammatory monocyte subset home back to the bone marrow, where they possibly give rise to the patrolling monocytes [3]. Recent data with fate mapping experiments have indicated $Ly6C^{+}CCR2^{+}CX3CR1^{int}$ monocytes serve as short-lived, obligate precursors for $Ly6C^{-}CCR2^{-}CX3CR1^{hi}$ monocytes under steady state and regulate their life span [12].

For long, it was believed that under steady-state conditions, blood monocytes enter the tissues and differentiate into tissue-resident macrophages. However, fate mapping approach in the last few years has challenged this idea. Except for inflammatory conditions and certain tissue macrophages (e.g., intestinal macrophages), monocytes do not substantially contribute to the differentiation and maintenance of adult tissue macrophages. It is now well accepted that embryonic precursors seed tissues prior to birth and give rise to most adult tissue macrophages, which maintain themselves thereafter by self-renewal [13]. Furthermore, different tissue macrophages have been shown to have different origins, with some derived from yolk sac macrophages (e.g., microglia), fetal monocytes (e.g., alveolar macrophages and Kupffer cells), or from both of these embryonic progenitors (e.g., Langerhans cells), or from adult blood monocytes (e.g., gut) [1]. It is now known that yolk sac erythro-myeloid progenitor (EMP) serve as the embryonic precursor of yolk sac macrophages and fetal monocytes [14,15]. An early wave of c-Myb-independent EMP gives rise to yolk sac macrophages followed by a later wave of $c\text{-}Myb^{+}$ EMPs that gives rise to fetal liver monocytes that following recruitment to the fetal tissues differentiate into the majority of the adult tissue macrophages. However, gut is the only exception that relies exclusively on adult blood monocytes to give rise to intestinal macrophages. In contrast to these steady-state situations, blood monocytes serve as the main precursors of macrophage during infection and inflammation. An exception to this condition is helminth infection as well as adipose tissues, where the macrophages self-renew [16,17].

Colony-stimulating factor-1 (CSF-1) and its receptor (CSF-1R), which is expressed in monocytes and macrophages, is the major regulator of macrophage differentiation. More recently, interleukin-34 (IL-34) was also suggested to contribute to macrophage differentiation, especially in the brain (microglia) and skin (Langerhans cells) [18,19]. Similarly, granulocyte-macrophage colony stimulating factor (GM-CSF) was found to be essential for the differentiation of alveolar macrophages [20], while vascular endothelial growth factor (VEGF)

contributed to the development of osteoclasts [21]. Besides these, the tissue microenvironment also plays an important role in shaping the phenotype of tissue resident macrophages. This is supported by experiments where embryonic-derived macrophages were replaced by adult bone marrow-derived cells upon infection or inflammation, with the latter recapitulating the phenotypic, gene expression, and functional features of the preexisting population [22–24]. As explained above, our understanding of the origin and differentiation of monocytes and macrophages have undergone phenomenal changes in the past few years. However, several issues such as the relationship between monocyte subsets and macrophages differentiated thereof, the identification of specific microenvironmental factors that shape the differentiation and phenotype of different tissue macrophages, and the relative contributions of tissue resident versus monocyte-derived macrophages during pathological conditions await future investigations.

9.3 Activation and Polarization

Monocytes and macrophages are equipped with the ability to respond to their local microenvironmental cues and danger signals by eliciting a functional phenotype that helps them to adapt to or mitigate these conditions. Often, such responses are tailored according to the stimuli these cells encounter. For example, Gram-negative bacterial pathogens (or its product like lipopolysaccharide, LPS) would induce macrophages to produce inflammatory cytokines and antimicrobial products aimed at inducing a full-fledged immune inflammatory response and killing of the pathogen. In contrast, upon encountering tissue microenvironmental stimuli like CSF-1, macrophages trigger an anti-inflammatory phenotype that helps in tissue remodeling and development [2]. Since microenvironmental cues can be immensely diverse and dynamic, these cells can also respond by displaying equally diverse phenotypes as well as switch from one phenotype to the other, paralleling the dynamic changes in their microenvironmental cues. Thus, diversity and plasticity of phenotype and function is a characteristic feature of the monocytes and macrophages [25,26].

Although the functional phenotypes of macrophages can be diverse, early investigators identified two broad activation phenotypes. These include the classical and alternative activation states of macrophages. Classical activation is triggered by inflammatory stimuli such as LPS and interferon gamma (IFNγ) that induces the expression of proinflammatory cytokines (e.g., IL-12, TNF, etc.) and release of reactive nitrogen and oxygen intermediates (RNI and ROI) in macrophages that promotes Th1 effector response and mediates antimicrobial and tumoricidal properties [27,28]. In contrast, alternative activation is triggered by anti-inflammatory cytokines IL-4 or IL-13 wherein macrophages show decreased ability to express proinflammatory cytokines, preferentially metabolize arginine to ornithine via arginase I (Arg1) expression, and show increased scavenger receptor expression and phagocytosis activity [29]. Such alternatively activated macrophages are crucial for the control and clearance of nematode infection [30]. Mills *et al.* [31] noted that macrophages from Th1 mice strains

(C57/B6) and Th2 mice strains (e.g., Balb/c) utilized distinct modes of nitrogen metabolism. Based on this, he proposed that analogous to the Th1 versus Th2 response, macrophages too could display an M1 versus M2 response. Subsequently, this concept was extended by Mantovani *et al.*, who proposed LPS and Th1 cytokines like IFNγ polarized macrophages to the M1 activation state, while those stimulated with anti-inflammatory Th2 cytokines like IL-4, IL-13, or IL-10 polarized them to the M2 activation state, paralleling the Th1 and Th2 nomenclature [32]. Distinct phenotypic and functional characteristics were proposed for M1 and M2 macrophages (see review by Biswas and Mantovani, 2010) [25]. Although different anti-inflammatory stimuli such IL-4, IL-13, IL-10, dexamethasone, or a combination of immune complexes plus LPS can polarize macrophages to a IL-12^{low}IL-10^{high} M2 phenotype, these cells show subtle variations in their phenotypes depending on the particular M2 stimuli used. This led to the proposal of subdivision of M2 phenotype into M2a, b, and c [33] and more recently into M2 (IL-4-polarized macrophages) and M2-like (IL-10-, glucocorticoid or immune complex+LPS-polarized macrophages) phenotypes [25].

The polarized phenotype of macrophages is also evident *in vivo* [34]. For example, M2-like characteristics have been observed for macrophages in the placenta and embryo and macrophages associated with helminth infection, endotoxin tolerance, and cancer [32,35–38]. Under some pathological conditions such as lean versus obesity, allergic skin, myocardial infarction, sepsis progression, or *Listeria* infection, a transition between M1 and M2 phenotypes has also been documented [2,6,39–41]. However, accumulating evidence now reveals further complexity under *in vivo* settings where monocytes and macrophages often fail to show a clear-cut polarized phenotype and even display a mixture of M1 and M2 characteristics [10,42–44]. Recent transcriptome and epigenetic analysis of macrophages support such complexity. For example, human macrophages stimulated with 28 different stimuli demonstrated a transcriptomic profile that suggested these cells to display a spectrum of phenotypes that could not be explained by a simplistic M1–M2 dichotomous model but by a multidimensional model, where macrophage phenotypes are dictated in an input-specific manner [45]. In line with this, a tissue-specific transcriptional and epigenetic regulation was demonstrated in murine tissue macrophages by a recent study [23]. In support of this growing complexity, a stimulus-specific nomenclature system of macrophage activation has been recently suggested. Macrophage phenotypes will be designated by their activating stimuli, for example, M1(LPS), M2(IL-4), and so on so that a common and unambiguous language of defining macrophage activation states can be achieved [46].

9.4 Functions

Monocytes and macrophages play a plethora of functions that relate to host defense, development, and maintenance homeostasis [47], For example, spleen red pulp macrophages help maintain iron homeostasis [48,49], peritoneal cavity macrophages control production of gut immunoglobulins A (IgA) [50], and macrophages in the brain engulf and remodel developing synapses [51]. Some of

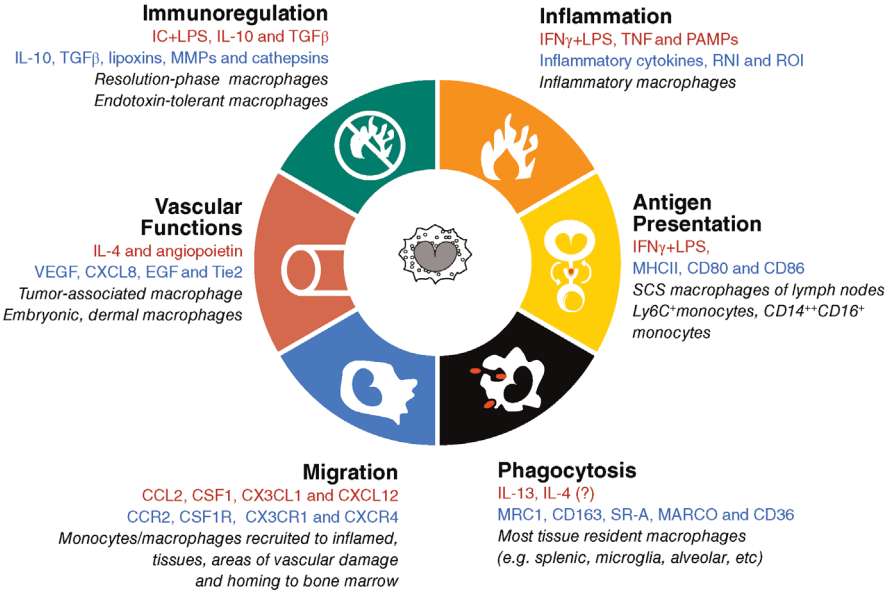


Figure 9.1 Hallmarks of macrophage functional diversity. A schematic representation of the key functions associated with these cells. Red: stimuli inducing the particular functions in macrophages; Blue: the effectors produced by macrophages associated with the particular function; italics: examples of macrophages showing these specific functions. Note: Although IL-4 induces expression of phagocytosis-associated receptors and enhanced endocytosis in macrophages, it impairs phagocytosis of microbes [205]. Tissue repair and remodeling functions of macrophage are integrated into the "immunoregulation" heading.

the hallmark functions of monocytes and macrophages include cytokine production and inflammation, antigen presentation, phagocytosis, migration, vascular functions, and immunoregulation (Figure 9.1). These are further discussed below.

9.4.1 Cytokine Production and Inflammation

Blood monocytes and tissue macrophages act as the first line of host defense against infections. These cells through the expression of pattern recognition receptors (PRRs) on their surface can "sense" pathogens or their products (also referred to as pathogen-associated molecular patterns, PAMPs). For example, TLRs are one of the main classes of PRRs expressed in these cells and can sense a variety of microbial components. Recognition of microbes by TLRs trigger intracellular signaling pathways that activate transcriptional factors such as nuclear factor kappa B (NF-κB) [52] that induce the expression of proinflammatory cytokines such as TNFα, IL-8, IL-6, IL-1, and microbicidal mediators like RNI and ROI. Proinflammatory cytokines and chemokines released by these cells induce the recruitment and activation of other immune cells like T and B cells, promoting a full-fledged immune response. Such an immunoinflammatory response coupled with the release of microbicidal mediators is ultimately instrumental for destruction of the pathogen. However, too much or too long release of

such inflammatory cytokines ("cytokine storm") may lead to serious deleterious effects such as septic shock and organs failure [53]. Indeed, we and other have shown a proinflammatory gene expression profile for monocytes during sepsis [39,47]. In contrast to these acute inflammatory conditions, obesity and cancer are examples of chronic inflammation. Accordingly, obese adipose tissues show increased infiltration of macrophages (ATMs) that are characterized by an M1-like phenotype, expressing high level of TNFα and inducible nitric oxide synthase (iNOS) [54,55]. Similarly, many cancer tissues are characterized by the recruitment of monocytes and macrophages, which by releasing inflammatory cytokines like TNF, IL-1β, and IL-6 contribute to cancer-related inflammation and tumor progression [25,56].

Since an uncontrolled or prolonged release of inflammatory cytokines by monocytes/macrophages can cause serious damage to the surrounding tissues, a tight regulation of this mechanism is necessary. Thus, concomitant with the expression proinflammatory cytokines, these cells also express anti- inflammatory cytokines such as IL-10 and IL-1RA. The tuning down of macrophage inflammatory response is well-documented during resolution of inflammation [57]. This and other immunoregulatory properties of macrophage will be discussed under in Section 9.4.5.

9.4.2 Phagocytosis

Phagocytosis refers to the process by which a cell engulfs a particulate matter (> 0.5 μ) with its plasma membrane to form a vacuole-like structure called the phagosome, within which the target is degraded by acidification and exposure to proteolytic enzymes. Both monocytes and macrophages are "professional" phagocytes. These cells survey their microenvironments for danger, kill potentially harmful microbes, and clear apoptotic cells and tissue debris as part of normal tissue homeostasis and repair [58]. Indeed, tissue-resident macrophages are highly phagocytic. For example, splenic macrophages during erythropoiesis ingest erythrocyte nuclei and contribute to iron recycling. Consequently, PU.1-deficient mice, which lack macrophages, have defective erythropoiesis resulting in lethality [59]. Likewise, resident microglia and recruited monocytes in the brain clear apoptotic neurons [60], thymic macrophages clear apoptotic thymocytes, and Kupffer cells in the liver clear microbial ligands from blood and lymph.

However, not all macrophages are constitutively phagocytic, for example, the $F4/80^{-}CD169^{+}$ sialic acid-binding immunoglobulin (Ig)-like lectin 1 (SIGLEC1)-expressing metallophilic macrophages in the rodent spleen are poorly phagocytic. Similarly, monocytic subsets also differ in their phagocytic ability [61].

The process of phagocytosis is a complex and dynamic cascade of events that initiates with recognition and physiochemical interaction between the particle and its cognate receptors (on the phagocyte), its attachment to the cell surface, its internalization into the phagosomes, and finally its destruction in the phagosome via acidification and proteolytic enzymes [62]. A variety of nonself-entities like microbes or their related molecules as well as self-entities like apoptotic or necrotic cells can be recognized by phagocytic receptors present on the cells surface. Either such receptors can directly recognize these particles (e.g.,

nonopsonizing, microbial ligand receptors) or indirectly recognize via opsonins coated on the surface of these particles (opsonic receptors). Examples of microbial ligand receptors are C-type lectin receptors (CLRs) (e.g., mannose receptor, dectin 1) and scavenger receptors (e.g., SR-A, MARCO, and CD36). Lectin receptors recognize carbohydrate motifs on microbial cell walls or in the side chains of microbial proteins and lipids, as well as those associated with apoptotic cells and extracellular matrix components. In particular, a lot of attention has been focused toward identifying the receptors that recognize and clear dying cells. Receptors like brain-specific angiogenesis inhibitor 1 (BAI1) directly bind to phosphatidyl serine present on apoptotic cells [63]. Similarly, the Tyro3, Axl, and Mer-TK receptors on monocytes and macrophages can recognize Gas 6 and Pros1 (vitamin K-dependent protein S) expressed on the apoptotic cells, thereby initiating a phagocytic cascade [64]. Recently, a C-type lectin receptor CLEC9A was implicated in the clearance of apoptotic and necrotic cells [65]. Foreign materials such as titanium particles and native beads are recognized by scavenger receptors MARCO [66] and SR-A1 [67], respectively, and elicit distinctive gene expression profile in macrophages [68]. However, whether phagocytic receptors can differentially recognize apoptotic versus necrotic cells and how this couples to differential immune response is still not clear.

Opsonin receptors include the immunoglobulin Fc receptors (FcγRI, II, and IIIA) and complement receptors (CR1, CR3, CR4, CRIg, and C1qRp) [58,69]. These receptors recognize opsonins such as immunoglobulins (IgG in the serum, IgA in the mucosal surface, etc.), C-reactive proteins, complement proteins C1q and C3b, mannose-binding lectins, fibronectin, and milk fat globulin (MFG)-E8 (lactadherin) [70].

Besides these phagocytic receptors, several accessory receptors can contribute to the efficiency of phagocytosis by either helping in the tethering of particles to the cell surface or modulating the intracellular signaling pathways. For example, TLRs can cooperate with other phagocytic receptors to regulate phagocytic uptake and signaling. Uptake and processing of particles in the phagosome can trigger various responses that orchestrate actin cytoskeletal remodeling, cell migration, inflammatory response, and microbial killing, many of which are mediated by signaling via TLRs, NOD-like receptors (NLRs), and inflammasome activation [71]. Interestingly, a recent study demonstrated that autophagy can regulate phagocytosis in macrophages as implicated by increase in the expression of both SR-A1 and MARCO receptors in autophagy-related gene 7-deficient ($Atg7^{-/-}$) macrophages [72].

Modulating phagocytosis can have important consequence in diseases. Pathogens and malignant cells exploit different mechanisms to evade removal by innate immune cells. One such mechanism is to display "don't eat me" signals that include CD47 and the signal regulatory protein-alpha (SIRPα) [73]. Blocking CD47-SIRPα axis was shown to increase tumor cell phagocytosis by innate cells including macrophages in a broad range of malignancies [74] as well as regulate response to therapeutic antibodies [65]. Similarly, response to anti-CD20 therapy was shown to involve macrophage-mediated phagocytosis of anti-CD20-bound tumor cells [75]. Besides cancer, phagocytosis is also being actively explored as a possible therapeutic target for other pathological conditions. In diabetes, reduced

phagocytic activity, which may have lead to increased susceptibility of patients to infection, can be improved by insulin treatment [76]. However, in-depth insights into the mechanisms underlying the relationship between phagocytosis, inflammation, and immunosuppression will be necessary before phagocytotic targets can be tested for modulating dysregulated macrophage function in disease.

9.4.3 Antigen Presentation

Macrophages are antigen-presenting cells and, therefore, have the ability to uptake, process, and present antigens to $CD4^+$ and $CD8^+$ T cells to trigger their activation and effector functions [77,78]. However, *in vitro* studies have shown that macrophages are much less efficient in stimulating naïve T cells than dendritic cells (DCs). A possible reason for this is that macrophages rapidly and completely degrade internalized proteins, while DCs do this more slowly and subtly, thereby preserving antigen epitopes for effective T-cell activation [79]. *In vivo* studies did not show such a compelling difference, wherein DCs and bone marrow-derived macrophages upon direct injection into mice showed comparable ability to migrate to the lymph node and activate $CD8^+$ T cell proliferation [80]. However, Moser and Loetscher [81] showed *in vivo* antigen-pulsed macrophages could prime T cells but, compared to DCs, selectively activated Th2 response. Peritoneal macrophages stimulated with CSF-1 have been shown to induce antigen-specific T-cell suppression [82]. Since CSF-1 is more associated with homeostatic functions than inflammatory activation, if using this factor induced such a Th2 or immunosuppressive phenotype is not known. The cytokine milieu definitely dictates the ability of macrophage antigen presentation, as discussed below.

A two-way relationship between macrophage and T-cell subsets has been proposed. For example, IFNγ released by Th1 or cytotoxic T cells can drive macrophages to an M1 phenotype wherein these cells upregulate inflammatory cytokines like IL-12 and TNF, *major histocompatibility complex* class II (MHC class II), and costimulatory molecules, T-cell-specific chemokines (CXCL10), all of which contribute to antigen presentation, activation, and polarization of T cells to a Th1 response [25,83]. In contrast, IL-4 released from Th2 cells or IL-10 from Tregs polarizes these cells into an immunosuppressive M2 phenotype that abundantly express IL-10, promotes an immunosuppression and Treg phenotype [25,84]. In autologous cocultures, antigen-loaded human macrophages when activated with LPS or LPS/IFNγ preferentially induced Th17 or Th1 responses, respectively, whereas those stimulated with IL-4 failed to induce a T-cell response [85,86]. Evidence supporting the role of macrophages in driving polarized T-cell response and vice versa can be found in various *in vivo* settings like bacterial infection, helminth infection, and tumors [25]. Moreover, human lamina propria $CD14^+$ macrophages exhibit antigen-presenting ability, enhancing Th1 response and contributing to Th17 differentiation under Crohn's disease settings [87].

Macrophages can also deliver antigens to B cells. A subset of specialized macrophages located at the subcapsular sinus (SCS) of lymph nodes were shown to be involved in the delivery of antigens to B-cell follicles [88–90]. These were

involved not only in inactivating viral particle-associated antigens [88] but also in delivery of particulate antigens and antigen in the form of immune complexes to B cells [89,90]. Furthermore, macrophages are also known to cooperate with DCs to present dead cell-associated antigens to trigger optimal $CD4^+$ T cell responses.

Human monocytes also show antigen-presenting capacity. The $CD14^+CD16^+$ monocytes have stronger antigen presentation capacity than the classical ($CD14^+CD16^-$) and nonclassical ($CD14^{dim}CD16^+$) monocytes [91]. Elevated expression levels of genes related to MHC class II molecules, antigen processing, and protein turnover in the $CD14^{++}CD16^+$ subset correlated with their stronger antigen presentation and T-cell proliferation capacity. Recently, mouse $Ly6C^+$ monocytes were suggested to carry soluble antigen from tissue to the draining lymph nodes where they presented them to the $CD4^+$ and $CD8^+$ T cells [92]. These monocytes had the intrinsic capacity to uptake dying cells and cross-present cell-associated antigen to $CD8^+$ T cells [93].

9.4.4 Migration

Migration, recruitment, and homing of monocytes between blood, peripheral tissues, and bone marrow is essential for immune surveillance and maintaining homeostasis. For example, blood monocytes are recruited to sites of tissue damage and inflammation, wherein they differentiate into macrophages or DCs, release cytokines and chemokines, and orchestrate an immune response [94,95]. Similarly, sequestering these cells at these sites is critical for eliciting effective immune response as well as avoiding collateral tissue damage. Recruitment and trafficking of monocytes and macrophages between blood, peripheral tissue, and bone marrow is largely guided by the expression of chemokine (or growth factor) receptors in these cells and their cognate ligands in the microenvironment. For example, classical ($CD14^+CD16^-$) monocytes in humans or the inflammatory ($Ly6C^{hi}$) monocytes in mice show high expression of CCR2 but low expression of CX3CR1. Accordingly, these cells respond to the CCR2 ligand CCL2, a key chemoattractant for monocytes [96], which contributes to their migration from blood to tissue and bone marrow to blood. In fact, during a variety of inflammatory conditions including obesity, cancer, and atherosclerosis, CCL2 production by adipocytes, cancer cells, and vessel walls orchestrate the recruitment of inflammatory monocytes into these sites [9,95,97,98]. However, in the absence of inflammation, $Ly6C^{hi}$ monocytes are reported to home to the bone marrow, where they are believed to differentiate into $Ly6C^{lo}$ monocytes [3,99].

Patrolling ($Ly6C^{lo}$) monocytes in mice and $CD16^+$ human monocytes show a $CCR2^{lo}CX3CR1^+$ phenotype [100,101]. These monocytes patrol the vascular endothelium where they help in the scavenging and clearance of cellular debris as well as removal of metastatic tumor cells [7,11,102]. In a recent study, $CX3CR1^+$ patrolling monocytes were suggested to migrate to sites of vascular damage in response to CX3CL1, the ligand for CX3CR1. Upon reaching these sites, these cells express chemokines like CCL3 and CCL5, which recruit NK cells for the removal of blood-borne tumor cells. CX3CR1 is also reported to support the survival of $Ly6C^{lo}$ monocytes [95,103–105]. In a *Listeria* infection model in

mice, patrolling monocytes were shown to arrive rapidly at the site of infection where they released chemokines that subsequently attracted the inflammatory monocyte subset [6]. Similarly, during exercise, $CD16^{+}$ monocytes were reported to increase in number within minutes [106,107], which is conceivable considering their localization to the vascular epithelium that allowed them to rapidly emigrate into the tissue during inflammation.

Recruitment of monocytes and macrophages into inflamed tissues, atherosclerotic lesions, and tumor tissues is also regulated by other receptors such as CX3CR1, CCR5, CSF1R, VEGFR1, and CXCR4 in response to their respective ligands (including hypoxia) in their microenvironment [95,97]. Similarly, adhesion molecules and integrins are important players in the migration of monocytes. For example, inflammatory monocytes use L-selectins, lymphocyte function-associated antigen-1 (LFA-1), macrophage-1 antigen (MAC1), and platelet/endothelial cell adhesion molecule 1 (PECAM1) for migration and adhesion, whereas patrolling monocytes use LFA-1 for their endothelium patrolling functions.

Monocytes and macrophages also undergo trafficking constitutively under homeostatic conditions. This may include homing to bone marrow or seeding to specific tissues. However, how or which chemokines and their receptors play a role in such homeostatic trafficking of these cells still remains poorly understood. Further characterization of tissue macrophages and their ontogeny may shed light on their homeostatic trafficking.

9.4.5 Immunoregulation

In contrast to their inflammatory functions, monocytes and macrophages play an important role in immunoregulation [108,109]. M2-like macrophages have been generally attributed to immunoregulatory activity, although several variations occur within this theme. Some examples of such activity are discussed in this section. A very good example of immunoregulatory function of macrophage is observed during resolution of inflammation. Macrophages during resolution engulf apoptotic cells (e.g., apoptotic neutrophils) as part of clearance of debris and switch to a phenotype characterized by impaired proinflammatory cytokine expression but heightened expression of TGFβ, IL-10, and lipoxins, which relates to proresolving, antifibrotic, and wound repair functions [110–112]. Further study of such cells during resolution of inflammation in mice suggested the existence of $CD11b^{hi}$ and $CD11b^{lo}$ macrophages with phenotypic variations [113]. The $CD11b^{hi}$ macrophages expressed high levels of M2 markers (Arg1), low to intermediate levels of inflammatory markers like NOS2, cyclooxygenase 2 (COX2), matrix metalloproteinase 9 (MMP9), and 12/15-lipoxygenase and were highly phagocytic. These cells resembled the resolution-phase macrophages reported by Stables *et al.* [44]. In contrast, $CD11b^{lo}$ macrophages did not express Arg1, showed lower levels of inflammatory markers than $CD11b^{hi}$ macrophages, high levels of TGFβ, and possessed no phagocytic ability. These cells have been suggested to be involved in antifibrotic and immune regulatory functions [114]. During helminth infection, M2-like macrophages were shown to drastically contribute to rapid resolution of tissue damage [115]. More recently, polarization

of macrophages with TLR ligands in the presence of high-density immune complexes, prostaglandin E2 (PGE2), or adenosine was reported to yield three distinct populations of macrophages with immunoregulatory activity [116]. These cells shared the common characteristics of high expression of IL-10, growth factors, and angiogenic factors, whereas showing reduced levels of inflammatory cytokines (IL-1β, IL-6, and IL- 12). However, the *in vivo* relevance of these populations need further study.

Another variation of immunoregulatory macrophages is the endotoxin-tolerant macrophage. During sepsis, exposure to overt inflammatory cytokines renders monocytes/macrophages to an endotoxin-tolerant phenotype characterized by their inability to respond to further endotoxin challenge [47,117]. Endotoxin-tolerant monocytes/macrophages display downregulated inflammatory cytokines and upregulated anti-inflammatory cytokines along with increased phagocytic activity and tissue remodeling characteristics, suggesting a M2-like phenotype [38,118]. Indeed, monocytes from sepsis patients show such a phenotype [39]. Possibly such an immunoregulatory phenotype is a protective adaptation to the excess inflammation. Tumor-associated macrophages (TAMs) also share immunoregulatory properties since they promote immunosuppression and tissue remodeling processes that are essential for cancer immune evasion, tumor angiogenesis, and metastasis [56,119]. In addition, many of the developmental functions of tissue and embryonic macrophages recapitulate elements of immunoregulation and tissue remodeling [120]. Further discussion on such macrophage functions may be found in Section 9.4.6.

Lastly, it may be emphasized that the monocytes and macrophages show dynamic plasticity switching between inflammatory and immunoregulatory phenotypes. This is not only seen during *Listeria* infections, where patrolling monocytes initially show an inflammatory phenotype and later switch to a tissue resolving M2 phenotype, but also in monocytes during skeletal muscle injury and sepsis [6,39,42]. However, the mechanism of the microenvironmental signals that switch these cells to an immunoregulatory phenotype is still not well understood.

9.4.6 Vascular Functions

Angiogenesis involves the process of new blood vessel formation. Typically, new vessels arise from preexisting ones by sprouting and ultimately fusing (anastomosis) with other vessels. Macrophages play a key role in regulating angiogenesis during development as well as postnatal organ remodeling. By directly secreting proangiogenic factors (VEGF, EGF, CXCL8, and CXCL12) or via secretion of tissue remodeling proteases (e.g., MMPs) that release ECM-bound proangiogenic factors, these cells promote vascular growth as well as contact-dependent anastomosis [121–123]. Indeed, cytokines secreted by activated monocytes and/or macrophages have been long known to induce endothelial cell activation, proliferation, migration, and survival, thereby supporting angiogenesis. At the same time, macrophages are also known to secrete proapoptotic or antiangiogenic factors that induce endothelial cell apoptosis, enabling vascular patterning, and remodeling during development [124–126]. For example, macrophages both support retinal angiogenesis and contribute crucially to hyaloid vessel

regression [122,124–126]. Similarly, in developing neural tissues, fetal macrophages promote as well as restrict the formation of vascular intersections [121–125,127]. It may be pointed out that the vascular modulating role of macrophages ranges from developmental (e.g., retina, brain, etc.) settings to disease settings like wound healing, cancer, retinopathies, and endometriosis [123,128–132]. The role of monocytes and macrophages has been extensively studied in tumor angiogenesis (reviewed in detail elsewhere [133]). Monocytes as well as macrophages by releasing various factors like VEGF, epidermal growth factor (EGF), TNF, and IL-1β can induce tumor angiogenesis [133]. Interestingly, a subset of monocytes/macrophages that express an endothelial marker, Tie2, and localize to perivascular areas in the tumors was shown to possess strong proangiogenic effect as well as regulate tumor response to chemotherapy [134,135]. At a mechanistic levels, macrophage–endothelial cell interaction has been ascribed to interactions involving receptor Tie2 and its ligand angiopoietin 2 (TIE2/ANG2) [136], CXCL12/CXCR4 [137], CSF-1/CSF-1R [138], NOTCH, and delta-like ligand 4 (NOTCH/DLL4) [139] molecules. In addition, embryonic, dermal, and tumor macrophages have also been shown to promote lymphangiogenesis [128,140–143]. Many of these macrophages released VEGFC and VEGFD. Monocytes and macrophages also have an important contribution in arteriogenesis [144,145].

9.5 Molecular Pathways and Mechanisms Regulating Response

In the last decade, our knowledge on macrophage activation, function, and ontogeny has improved immensely. However, information on the molecular mechanisms that connect the impinging microenvironmental stimuli to the diverse gene expression profiles and functions of macrophages is relatively limited. We summarize below the existing information on the molecular mechanisms regulating macrophage activation, polarization, and response, focusing on the signaling, epigenetic, and post-transcriptional mechanisms (Figure 9.2).

9.5.1 Signaling Pathways

Macrophages possess a variety of transmembrane and cytosolic PRRs such as TLRs, CLRs, RIG-I-like receptors (RLRs), and NLRs through which they can sense various danger signals of microbial (e.g., LPS, zymosan, and flagellin) or host origin (e.g., endogenous molecules released from damaged or altered cells) [146]. Upon sensing such a danger signal, the PRRs trigger signaling cascades that lead to a gene expression program that ultimately drives the macrophage functional response to that particular stimulus. In general, macrophage response to Gram-negative bacteria and the classical activating agent, LPS, is mediated through TLR4 [147,148]. Signaling downstream of TLR4 pathway is mediated by two distinct adaptors, Myeloid differentiation primary response 88 (MyD88) and TIR domain-containing adapter-inducing interferon-β (TRIF) [146] (Figure 9.2). The

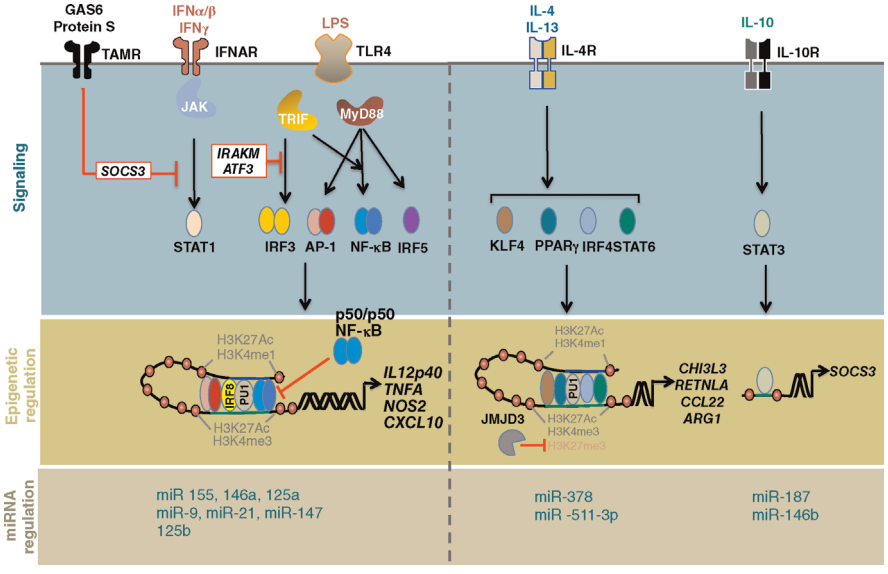


Figure 9.2 Multilevel molecular mechanism regulates the polarized response of macrophages. The figure depicts signaling, epigenetic changes, and miRNA-mediated regulation of macrophage response to polarizing stimuli. Only key pathways are indicated. Not all transcription factors that bind to enhancers and promoters are shown due to space constraints. Note that in basal or unstimulated macrophages, lineage-specific transcription factors such as PU.1 first bind to enhancers making them accessible or "poised" (via histone modifications) for the binding of other stimuli-specific transcription factors such as NF-κB, AP1, IRFs, and so on. Transcription factor binding and epigenetic marks on M2 genes are not well understood, a possible schematic view is presented. Enhancer: blue line; promoter: green line; Gray font: histone modifications. Red "T" sign depicts inhibition.

MyD88-dependent pathway leads to the activation of the master transcription factor NF-κB, which induces the expression of inflammatory cytokine genes such as TNFA, IL1B, IL6, and IL12A. The TRIF-dependent pathway activates the transcription factor interferon regulatory factor 3 (IRF3) that via IFNβ expression activates the transcription factor, signal transducer and activator of transcription 1 (STAT1) [149]. Type I (IFNα/β) and Type II IFNs (IFNγ) through STAT1 activation induces the expression interferon-inducible genes such as NOS2, CCL5, and CXCL10 [150,151]. Thus, M1 polarization of macrophages by IFNγ+LPS induce the activation of various transcription factors such as NF-κB, activator protein 1 (AP-1), IRF5, IRF3, and STAT1 that drives the expression of inflammatory (M1) cytokines like TNF, IL12A, and CXCL10 (Figure 9.2). In particular, IRF5 was demonstrated to be an integral transcription factor in regulating the expression of M1-related inflammatory genes [152].

In contrast to the above, anti-inflammatory stimuli like IL-4 or IL-13 through the IL-4 receptor activates various transcription factors like STAT6, Kruppel-like factor 4 (KLF4), IRF4, c-Myc, and peroxisome proliferator-activated receptor gamma (PPARγ) that induce expression of M2 phenotype-related genes such as ARG1, CCL17, CCL22, and IRF4 (Figure 9.2) [153–155]. The anti-inflammatory

cytokine IL10 via STAT3 activation induces its target genes as well as negative regulators of inflammation such as suppressor of cytokine signaling 3 (SOCS3) in macrophages, promoting immunosuppression. SOCS1 and SOC3 can also be induced via the TAM receptors (e.g., Axl, Tyro3, and Mer), which are known to mediate immunosuppression in sepsis patients [156]. It may be pointed out that many molecules such as p50/p50 NF-κB, hypoxia-inducible factor 1 alpha (HIF1α), negative regulators of TLR signaling (e.g., IRAKM, ATF3, and A20), and pyruvate kinase isoenzyme type M2 (PKM2) that are initially induced by inflammatory stimuli can also switch macrophages to an anti-inflammatory phenotype over time [157–160]. Supporting this concept, IFNβ in some instances can induce IL-4, IL-13, IL-4Rα, and TAM receptors, which facilitates the polarization of inflammatory macrophages into an immunosuppressive, M2-like phenotype [161]. In sepsis, accumulation of p50/50 NF-κB homodimer and HIF1α activation was shown to reprogram macrophages from an inflammatory to an M2-like immunosuppressive phenotype [38,39,162]. However, divergent roles of HIF isoforms, HIF1α, and HIF2α in macrophage polarization have been reported [163–165]. A more detailed account of the various innate signaling pathways and molecular mechanism of macrophage polarization are reviewed elsewhere [25,146]. More recently, another level of complexity to macrophage signaling has emerged with the involvement of signaling molecules and transcription factors such as AMPK, mTOR, PI3 K/AKT, p53, and HIF1α that regulate macrophage response to nutritional and immune–metabolic stimuli [166].

9.5.2 Epigenetic Regulation

Chromatin immunoprecipitation-sequencing (ChIP-Seq), iChIP and RNA-Seq studies in macrophages have advanced our understanding of how the gene expression profile of these cells is regulated at an epigenetic level. Transcription of a gene is often controlled by the participation of multiple transcription factors that assemble at the *cis* regulatory elements like enhancers and promoters for that particular gene (Figure 9.2). However, the accessibility of the chromatin to transcription factors depends on its coiling status that is regulated by nucleosome remodeling and modification of the histone tails associated with the chromatin. Nucleosome remodeling, among many factors, is dictated by cytosine and guanine (C/G) content of the DNA sequence. Thus, areas with extreme C/G content (e.g., "CpG islands") favor nucleosome depletion, thereby giving rapid accessibility to transcription factors [167]. For example, promoters of inflammatory genes coincide with such CpG island and hence have active chromatin that is poised for further stimulus-driven activation [168]. In general, two broad types of transcription factors can be envisaged in macrophages: lineage-specific transcription factors that guide basal gene transcription and stimuli-specific transcription factors that drive gene expression in response to stimuli. In macrophages, lineage-specific transcription factors like PU.1 bind to genomic enhancers and favor nucleosome depletion, thereby making them accessible to other stimuli-specific transcription factors [169,170]. Interferon regulatory factor 8 (IRF8) plays a role in both basal (constitutive) and stimuli-induced (inducible) gene expression in macrophages [171]. In the former case, it cooperates with PU.1

to bind under basal conditions, whereas in the latter case it cooperates with AP1 family members to bind to AP1 consensus sequences.

Histone modification is a key regulatory mechanism that controls the accessibility of chromatin for transcription factors binding. Accordingly, acetylation of histone H3 at lysine-9 and -27 (H3K9ac and H3K27ac) and histone H4 at lysine-5, -8, and -12 (H4K5ac, H4K8ac, and H4K12ac) is associated with active promoters and enhancers. Similarly, methylation of histones is associated with activation or repression depending upon the site of lysine residue. Methylation of histone H3 at lysine-4, -36, and -79 (H3K4, H3K36, and H3K79) associates with active transcription. Promoters exhibit high levels of H3K4me3, whereas enhancers display H3K4me1 (Figure 9.2). Accordingly, PU.1 binding to gene enhancers causes H3K4me1, which facilitates access to other transcription factors like NF-κB, IRF8, IRF5, IRF4, and C/EBPβ in response to activation stimuli (Figure 9.2) [157]. Moreover, some of these transcription factors also cause recruitment of histone acetyltransferase p300 to the enhancer and facilitate transcription [169]. In contrast to the above histone modification, methylation of histone H3 at lysine-9 and -27 and histone H4 at lysine-20 (H3K9, H3K27, and H4K20) promotes transcription repression [172,173]. A good example of gene repression is LPS tolerance. Two classes of genes were identified in a study on LPS tolerance in murine macrophages [174]. These include LPS-responsive nontolerant and LPS-unresponsive tolerant genes. Promoters of both gene classes showed histone methylated, but those of the nontolerant genes displayed histone reacetylation and RNA polymerase II (RNA polyII) recruitment and transcription, when restimulated with LPS. Such differential epigenetic modification has also been proposed to explain "trained immunity" in human monocytes in a recent study [175]. On similar lines, the temporal regulation of macrophage inflammatory gene expression also involves epigenetic regulation. This typically consists of early response genes that have permissive promoters that allow for immediate transcription factor binding. These gene regulatory regions are characterized by nucleosome depletion, CpG-rich islands, relatively high levels of H3K4me3 and acetylated histones, and RNA Pol II recruitment in unstimulated macrophages. Thus, they are poised for rapid transcription upon LPS treatment. In contrast, secondary response genes have nonpermissive promoters that require nucleosome remodeling before transcription factors can bind. In line with these facts, secondary response genes show low-level H3K4me3 [176] and are enriched with H3K27me3 [177].

While most studies have concentrated on the epigenetics of inflammatory genes associated with M1 macrophages, relatively little is known about the regulation of the genes related to the M2 macrophages. In this regard, Jumonji domain containing-3 (Jmjd3), a H3K27 demethylase, was shown to be upregulated in IL-4-polarized macrophages, where it decreased H3K27 methylation at the promoters of M2 genes such as ARG1, YM1 (CHI3L3), and FIZZ1 (RETNLA) to activate their transcription (Figure 9.2) [178]. JMJD3 was also reported to induce the expression of the M2-associated transcription factor IRF4 [179]. Further investigation into the gene regulatory mechanism of M2 macrophages and those involved in homeostatic functions in various tissues is now needed.

9.5.3 Micro-RNAs

Micro-RNAs (miRNAs) or small noncoding RNAs are important regulators of gene expression in immune cells at a post-transcriptional level [180–182]. miRNAs modulate monocyte/macrophage response [182]. Different micro-environmental factors (LPS, IL-4, IL-10, helminth infection, and endotoxin shock) can either induce or downregulate the expression of particular miRNA species in these cells, which in turn act on their target genes to modulate the downstream functional response (Figure 9.2). An excellent review of this topic is presented by Squadrito *et al.* [182].

Inflammation modulates a large number of miRNAs. One of the earliest miRNA studies have shown LPS to rapidly upregulate miR-146a in monocytes [183]. This miRNA acts as a negative regulator of TLR signaling via downregulating the expression of important upstream molecules like TRAF6 and IRAK1 [181,183]. miR-146a also inhibits TNFα at a transcription and translation level during inflammation in monocytes/macrophages [184]. These observations support the role for miR-146a inducing endotoxin tolerance in macrophages [47,185,186]. LPS also upregulates miR-155 in monocytes and macrophages, but the effect is complex. miR-155 inhibits components of TLR4 signaling pathway, but stabilizes TNFA transcripts, thus playing both positive and negative regulatory roles [187,188]. In fact, miR-155 knockin mice show high levels of TNFα [188,189]. Interestingly, LPS differentially regulates the miR-125 family with the upregulation of miR125a and dowregulation of miR-125b in macrophages [190,191]. Both these miRs induced enhanced NF-κB activation [192], while downregulation of miR-125b was required for TNFα production (via stabilization of its gene transcript) [188]. Coordinated regulation of miR-155, miR-146a, and miR-125b has been suggested in pathological settings like endotoxin shock and tolerance [186,188,193]. TLR agonists induce a number of other miRNAs such as miR-9, miR-21, and miR-147, which inhibit proinflammatory response in monocytes/macrophages by downregulation of the NF-κB signaling pathway [194–196]. A recent study showed that miR-10a secreted by endothelial cells could suppress monocyte activation by targeting components of the NF-κB pathway, such as IRAK4 [197].

Anti-inflammatory stimuli may also activate particular miRNAs resulting in the regulation of monocytes/macrophages [182]. Thus, IL-10 alone or with LPS induces miR-187 expression in human monocytes [198]. This miRNA downregulates IL-6 and IL12p40 expression favoring an anti-inflammatory response. IL-10 also induces miR-146b expression in human monocytes that negatively regulates TLR4 signaling and tunes down the inflammatory response [199]. Another anti-inflammatory stimulus, IL-4 induces several miRs in macrophages such as miR-125b and -378 [200]. In this study, miR-378 was shown to target Akt1 signaling serving as negative feedback loop for M2 activation of macrophages. miR- 511-3p is encoded in the fifth intron of the M2 macrophage marker gene, MRC1 (CD206) [201]. miR-511-3p was found to be upregulated *in vitro* and *in vivo* in various M2 macrophages like IL-4-polarized bone marrow-derived macrophages, adipose tissue macrophages, and TAMs. Importantly, this miRNA

was shown to regulate the protumor phenotype of TAMs and hence tumor growth. miR-511-3p serves as a negative feedback loop for M2 macrophages, by targeting ROCK2 that is required for activation of the M2 transcription factor, IRF4 [201].

These examples together with the differential miRNA expression in M1 and M2 macrophages suggest an important role for miRNAs in regulating the M1/M2 macrophage phenotype. This concept is demonstrated *in vivo* in mice with a myeloid-specific gene knockout of Dicer 1 (the key enzyme regulating miRNA processing in mammalian cells) [202]. These authors showed that myeloid-specific Dicer1 deletion in tumor-bearing mice switched the protumor phenotype of the tumor-associated macrophages to a strongly antitumor M1 phenotype that led to robust cytotoxic T-cell response and tumor regression. In fact, Dicer1 deletion led to better outcome of immune checkpoint therapy with anti-PD1 and anti-CD40 in these mice. Surprisingly, Dicer1 deletion specifically affected miRNAs in macrophages, with the Let7-5p family, in particular. It was suggested that under wild-type conditions, Dicer upregulated Let7-5p expression, which targeted the costimulatory molecule CD86, thereby thwarting the activation of an antitumor T-cell response. These results constitute *in vivo* evidence for the role of Dicer (and the miRNA machinery) in driving an immunoregulatory phenotype in tumor macrophages and hence open options for targeting the miRNA machinery for "rewiring" macrophage-mediated antitumor response [203].

9.6 Conclusions

The study of macrophages using fate-mapping approach, transcriptome, ChIP-Seq, and single-cell analysis in last few years has shed unprecedented light on the ontogeny, activation, and functional diversity of these cells. These findings coupled with the characterization of these cells in disease models and patients have established their central role in a large number of pathologies such as obesity, cancer, allergy, liver diseases, and so on. Based on this, strategies modulating monocyte/macrophage lineage such as those targeting CSF-1R are entering into clinical trials [204]. A new area of investigation revolves around the homeostatic roles of these cells and distinguishing between the biology of tissue resident macrophages versus those differentiated from blood monocytes and/or recruited in response to microenvironmental cues. Future multidimensional approaches should provide further insights into these issues [1].

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10

Neutrophils

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10.1 Characteristics

The original report of neutrophils was made in 1865 by Max Johann Sigismund Schultze, who first defined the four different types of blood leukocyte corresponding to what are now documented as the lymphocyte, the monocyte, the eosinophil, and the neutrophil [1]. Paul Ehrlich, on the basis of their characteristic nuclear morphologies, called neutrophil "cells with polymorphous nuclei" [2]. In 1905, Elie Metchnikoff renamed these cells and the term "polymorphonuclear leukocytes" is still used today.

Neutrophils are uniform in dimension with a diameter between 9 and 15 μm. When analyzing neutrophils in a medium, neutrophils have a typical diameter of 8.85 μm [3]. They have copious cytoplasm with a tiny Golgi apparatus, where mitochondria and ribosomes are sparse and the rough endoplasmic reticulum is absent. Two most important morphological characteristics of the neutrophils are the form of their nucleus and their granules. Nucleus is divided into three to five lobules. The evolutionary advantages of having a lobulated nucleus are still unclear. Neutrophils have three varieties of granules: *azurophilic granules* (or "primary granules") that contain myeloperoxidase, bactericidal/permeability-increasing protein (BPI), defensins, and the serine proteases neutrophil elastase and cathepsin G; *specific granules* (or "secondary granules") that contain alkaline phosphatase, lysozyme, NADPH oxidase, collagenase, lactoferrin, and cathelicidin; and *tertiary granules* that contain cathepsin and gelatinase [2]. In normal adult blood, the relative percentage of neutrophils is 60–75% or 4200–5200 mm^{-3}; low neutrophil counts are termed *neutropenia* and it can be hereditary, as in the case of aplastic anemia or some kinds of leukemia, it can also be a side effect of medication or it can be the result of colonization by intracellular neutrophilic parasites [4]. Neutrophils survive 1–4 days once they leave the blood stream. They traverse the connective tissues by ameboid movement and are the most active phagocytes of the three granulocytes. Ameboid movement and, to a lesser degree, phagocytosis is seen moreover in eosinophils and basophils. When

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circulating in the bloodstream and unactivated, neutrophils have a spherical structure. Once activated, they change shape and become more amorphous or ameba-like and can extend pseudopods as they hunt for antigens [5].

10.2 Hematopoiesis

Blood cells are commonly classified into three cell lines: erythrocytes, granulocytes, and megakaryocytes. Hematopoiesis is the process by which blood cells are created.

Hematopoietic stem cells (HSCs) are usually found in the bone marrow, where they self-renew and begin their tour of differentiating into all the singular blood cell types. In the bone marrow, pluripotent stem cells distinguish into either the lymphoid progenitor cells, which are induced to differentiate into B- or T-derived lymphocytes, or the myeloid stem cell (CFU-GEMM) line, which are further induced to become erythrocytes, granulocytes, (neutrophils, eosinophils, or basophils), macrophages, or megakaryocytes (platelets) [6].

Neutrophils are formed within haematopoietic cords interspersed within the venous sinuses of the bone marrow. The bone marrow of a normal adult produces around 100 billion neutrophils daily. The original cells destined to become neutrophils and macrophages that distinguish from the pluripotent stem cells are called granulocyte macrophage progenitor (CFU-GM) cells. The CFU-GM cells are affected by colony-stimulating factors and become either CFU-G or CFU-M cells. The principal regulator of granulopoiesis is granulocyte colony-stimulating factor (G-CSF), whose effects comprise commitment of progenitor cells to the myeloid lineage [7], production of granulocytic precursors, decrease in transit time through the granulocytic compartment [8], and release of mature cells from the bone marrow. G-CSF is made of cells such as monocytes, macrophages, and bone marrow stromal cells, and its action almost entirely concerns with the proliferation of neutrophils. Therefore, CFU-GM cells are encouraged by G-CSF in the bone marrow, pass through the CFU-G stage, and become myeloblasts, which are the greatest primitive neutrophils that can be morphologically distinguished. Myeloblasts continue to divide and differentiate, and they mature into neutrophils, which then lose their ability to divide. Mature neutrophils are not directly released into the blood, but rather are accumulated within the bone marrow. In fact, the neutrophil population in the bone marrow can be subdivided into three pools: the stem cell pool, the mitotic pool, and the postmitotic pool. The stem cell pool contains undifferentiated haematopoietic stem cells (HSCs); however, the mitotic pool refers to committed granulocytic progenitor cells that are undergoing proliferation and differentiation. Finally, fully differentiated complete neutrophils make up the postmitotic pool, which forms the bone marrow reserve, available for release. Neutrophils that have been released into the blood reside in the marginal granulocyte pool or the circulating granulocyte pool, and they later egress into tissues [9]. Studies that have used cells labeled with ^{32}P or ^{2}H have reported that in humans, the transit time through the postmitotic pool is 4–6 days [10,11].

Similarly, the bone marrow reserve of granulocytes in humans has been estimated at 6×10^{11} cells; given this, and the assumption of a 5-day lag between DNA labeling of cells in the marrow and their appearance in blood, the daily turnover of granulocytes through the blood should be 1.7×10^9 cells/kg [7,12,13].

Like M1 versus M2 in macrophages, neutrophils are grouped into either N1 or N2, the former being proinflammatory, whereas the latter being anti-inflammatory. N1 was stimulated under a mild burn injury, whereas N2 was induced under a severe burn damage. N1 was $CD49d^+$ and $CD11b^-$, whereas N2 was $CD49d^-$ and $CD11b^+$.

Burn injury caused systemic infection after subcutaneous infection of a wild-type *P*seudomonas *aeruginosa*. Such mice presented a significant elevation in serum of IL-10 and polarization of neutrophils into an N2 phenotype (IL-10+ IL-12) in the spleen. The surface phenotype of N2 was $Gr1^+$ and $CD11b^+$. Administration of a TLR5 agonist, flagellin, after burn injury, on the other hand, reestablished the neutrophil reply toward a N1 phenotype, resulting in an improved clearance of a wild-type strain of *P. aeruginosa*.

CD177, a GPI-anchored neutrophil antigen, is present in 0–100% of neutrophils, but the portion of CD177-positive neutrophils looks to be constant in all individual [15,16]. CD177 is contained primarily in the membrane of specific granules and in the plasma membrane. Its expression is determined by polymorphisms of the gene.

Olfactomedin 4 (OLFM4), a definite granule protein, is present in roughly 20–25% of neutrophils. Its presence is not regulated at the level of transcription [17]. Both OLFM4-positive and -negative neutrophil subpopulations undergo apoptosis and phagocytose bacteria similarly. Both subpopulations were employed equally to inflammatory positions *in vivo*. OLFM4 was liberated upon NET formation in response to PMA. It was distinguished in only a fraction of the NETs [18].

The relation of these subpopulations with N1 and N2, however, is not known at present. Actually, researchers have not examined cytokine production in these subpopulations [19].

10.3 Adhesion and Migration

10.3.1 Traffic and Margination

Neutrophils are in equilibrium in the blood with a circulating pool, present in large blood vessels, in the axial stream of small vessels, and in a marginating pool. Under physiological conditions, the marginating pool, better called "physiological regional granulocyte pool" [20], comprises granulocytes transiently arrested in narrow capillaries. This retention of neutrophils in capillaries appears to be a mechanical process due to the stiffness of neutrophils compared to the high deformability of erythrocytes and does not involve cell adhesion [21,22].

10.3.2 Adhesion to the Endothelial Wall

On the other hand, during inflammation, neutrophils roll along the endothelial wall of postcapillary venules and integrate inflammatory signals. The recruitment of neutrophils out of blood vessels into injured tissue proceeds in a coordinated series of steps [23]. The traditional three steps of rolling, activation, and firm adhesion have recently been augmented and refined. Slow rolling, adhesion strengthening, intraluminal crawling, and paracellular and transcellular migration are now recognized as separate, additional steps [24]. Capturing is the first contact between neutrophils and the endothelium of postcapillary venules, mediated by selectins and their counterreceptors. During this initial step, neutrophil traffic involves a selectin- and integrin-dependent sequestration in capillaries and postcapillary venules [25]. Intravascular injection of inflammatory mediators results in rapid sequestration that involves a decrease in neutrophil deformability, followed by long-lasting accumulation of neutrophils and by a process involving integrins and L-selectin [21,26]. Reversible interactions of leukocytes with P- and E-selectin on endothelial cells mediate tethering and rolling of leukocytes in inflamed vascular beds, the first action in their recruitment to sites of injury [27]. P-selectin binds with higher affinity to an N-terminal region of P-selectin glycoprotein ligand-1 (PSGL-1) located at the tip of leukocyte microvilli and rolling subsequently involves E-selectin, which appears on endothelial cells 1–2 h after cell stimulation by chemokines or LPS. Furthermore, rolling facilitates binding of neutrophil-expressed chemokine receptors to their chemokines presented on the luminal endothelial surface [28]. Different from P- and E- selectins, L-selectin is constitutively present on leukocytes and directs the migration of naive and central memory T cells to lymph nodes through identification of glycoproteins expressed in high endothelial venules [29]. In addition to its binding to endothelial ligands, PSGL-1 is a counterreceptor for leukocyte L-selectin and there is evidence that neutrophils roll, via L-selectin, on previously adherent neutrophils. This secondary tethering would synergistically enhance neutrophil accumulation on inflamed endothelium [30]. During this tethering step, neutrophils respond to ligands dispatched on the endothelium surface by tissue mediators releasing cytokines that attract free-flowing neutrophils in the bloodstream [31]. These chemokines, tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and (IL)-8 specifically attract neutrophils [32] and their receptors mediate intracellular signaling cascades through activation of two superfamilies of G proteins, the heterotrimeric G proteins and the RAS superfamily of small monomeric GTPases [33]. IL-8 is a major neutrophil chemoattractant, as shown by the complete inhibition of neutrophil recruitment in inflammation sites by anti-IL8 monoclonal antibodies in animal models [34]. While selectins are the key molecules that allow neutrophils' capture and rolling, adhesion mainly depends on molecules of the β_2 integrin family (CD11α, CD11β, and CD11c/CD18) [35]. Ligand binding of β_2 integrins leads to integrin clustering and subsequent outside-in signaling, which contributes to adhesion support and cell motility by controlling cytoskeletal rearrangements [36]. Integrins transmit signals triggered by their clustering and multiple activities with adhesion substrates. Neutrophils integrate these signals and those delivered simultaneously by

inflammatory cytokines or chemoattractants to activate a cascade of intracellular events resulting in cell spreading, locomotion, degranulation, and oxidative burst.

10.3.3 Transendothelial Migration

Neutrophil transmigration happens mainly at the borders of endothelial cells and involves specific neutrophil interactions with endothelial adhesion, through both paracellular and transcellular mode molecules, including P-selectin, ICAM-1, and platelet-endothelial cell adhesion molecule-1 (PECAM-1) [37]. P-selectin has been shown to be concentrated along endothelial borders and may target there neutrophil adhesion. However, extravasation requires modifications of endothelial cell-to-cell adherent junctions. Indeed, disorganization of the junctional components VE-cadherin and β-catenin has been observed in neighborhood regions of mainly adhesion between neutrophils and endothelial cells [38]. PECAM-1, a tyrosine phosphoprotein highly expressed in endothelial cells' junction and on neutrophil surface, modulates neutrophilic transmigration via homophilic PECAM-1/PECAM-1 interaction, but the mechanisms underlying endothelial PECAM-1 function are unknown. Proposed mechanisms consist of (1) formation of a gradient that "guides" neutrophils to the cell–cell border, (2) service as a "passive ligand" for neutrophil PECAM-1, mediating activation of neutrophil β integrins, (3) regulation of endothelial calcium influx, and (4) mediation of SH2 protein association [39]. Two cell adhesion molecules of the Ig superfamily (CAMs) have been shown to be involved in leukocyte transmigration, PECAM-1 and, more recently, the junctional adhesion molecule (JAM) [40]. JAMs are members of an immunoglobulin subfamily, composed of JAM-A, -B, -C, JAM-4, ESAM (EC-selective adhesion molecule), and CAR (coxsackie virus and adenovirus receptor) that localize to cell–cell contacts and are specifically present in tight junctions implicated in leukocyte transendothelial cell migration [41]. JAM-C is unique in terms of its expression and functional profile, and increased luminal expression of JAM-C may promote migration of leukocytes to endothelial cells junctions through increased intravascular crawling, a response that appears to support efficient leukocyte transmigration [42]. Neutrophils move about tissues by haptotaxis rather than through soluble chemoattractants [43]. These chemoattractants, produced by bacteria, dying cells, or epithelial cells of inflamed tissues, are bound to extracellular matrix components because of their negative charge. Cross-talks between chemoattractant receptors and their signaling pathways result in desensitization to one chemoattractant by another, allowing leukocytes recruited by endothelial-derived chemoattractants to migrate source toward their final target [44]. Neutrophils display multiple structurally related receptors for chemoattractants that can generate adhesion, direct cell migration, and promote degranulation and oxidative responses. The link of chemoattractants to such receptors activates phospholipases, via heterodimeric G proteins, resulting in intracellular Ca^{2+} release and activation of proteine kinase C isoforms, tyrosine kinases, and the GTP-binding protein Ras [45]. Chemoattractant receptors, via their coupled G protein heterodimers, activate PI3-kinase, which support the migration of neutrophils; its activation is essential for reorganization of cytoskeleton and membrane organization in response to such agonists, events

resulting in cell polarity and pseudopodia extension [46]. In main inflammatory diseases (gastrointestinal, respiratory, and urinary), neutrophils finally transmigrate across a polarized epithelium to accumulate within a lumen. The initial stage of neutrophil transepithelial migration is characterized by adhesion of the neutrophils to the epithelial membrane. Further, the neutrophils that arrive at the epithelium have a "hystorical previous" in as much as they have just crossed the endothelium and migrated through interstitial tissues before arriving at the base of the epithelium. These accession priorities and migratory events, in conjunction with exposure to the chemoattractants that have stimulated this movement, result in an alteration in the state of activation of the migrating neutrophils [47]. Finally, after traversing the epithelial barrier, neutrophils emerge on the apical surface of the epithelium and adhere to this surface for variables periods of time. During these events, multiple and sequential adhesive interactions must happen in order for the neutrophils to completely traverse the epithelial monolayer. Neutrophil transepithelial migration is mediated by β2 integrin CD11β/CD18 interaction with unknown epithelial ligands, which might include members of the proteoglycan family. Integrin-associated protein CD47 appears to be implicated in neutrophil transepithelial migration as shown by the delayed neutrophil recruitment to inflammation sites in the presence of anti-CD47 blocking antibodies [48]. During transepithelial neutrophil migration, neutrophils seek to migrate in clusters following in the "footsteps" of the leading leukocytes [49]. Migration of great numbers of activated neutrophils results in the formation of large epithelial lacerations due to the mechanical separation of epithelial cells at the site of the interepithelial junction. These wounds represent precursors of the macroscopic areas of denuded epithelium (ulcerated lesions), features of inflammatory mucosal diseases.

10.4 Phagocytosis and Degranulation

10.4.1 Phagocytosis

Phagocytosis is the main microbicidal function of neutrophils, which with macrophages represent the professional phagocytes that are able to engulf and thereby eliminate pathogens and environmental debris [50,51]. Phagocytes are provided with specialized receptors to recognize their targets; this complex machinery mediates internalization and initiates an assortment of degradative mechanisms that culminate in killing of the engulfed particles. Neutrophil phagocytosis can be caused in response to binding of opsonized targets (e.g., immunoglobulin (Ig)- or complement-coated) or by direct bond of target components [52], and it involves two different receptor classes: Fcγ receptors and complement receptors, CR1 (CD35) and CR3 (or CD11β/CD18 integrin). While several different types of receptors are involved in phagocytic uptake of particulate targets [53], a true phagocytic receptor is complicated to define. Among these, the functional phagocytic receptors are FcγRII and CR3, while CR1 and FcγRIIIB appear mostly as coreceptors facilitating the function of the former receptors. Signaling pathways triggered by these two classes of receptors are

different, so are the phagocytic processes. Fcγ receptors are receptors binding IgG-opsonized targets [53]. So, when a small particle is opsonized with IgG, the Fc regions of the IgG molecules may bind to FcR in the neutrophil plasma membrane and trigger a phagocytic reaction. The ingestion of IgG-coated targets is promoted by the aggregation of Fc**γ**RII receptors and the phosphorylation of their cytoplasmic ITAMS (immunoreceptor tyrosine-based activation motifs), through the activation of Src-tyrosine kinases; downstream signaling is propagated by the recruitment of the tyrosine kinase Syk. Syk recruitment is followed by the mobilization of adapter molecules that promote lipid modifications (PLCγ and PI3-kinase), actin remodeling (Cdc42 and Rac1), and Rho protein activation, leading to a localized reorganization of the membrane and the cytoskeleton [54]. This results in an extension of membrane protrusions over the surface of the opsonized particle to form a "phagocytic cup" that engulfs the particle.

CR3-mediated phagocytosis requires an additional signal, resulting in integrin activation, and involves a different process: complement-opsonized targets sink into the cell producing little protrusions [55]. Adhesion of neutrophil CR1 and CR3 to particles exclusively coated with C3b/iC3b is not sufficient to promote phagocytosis, except that neutrophils are activated by formyl-peptides and a contact with fibronectin or laminin [56]. These stimuli result in the phosphorylation of CR1 and trigger the way that activates CR3 binding capacity. Cooperativity between Fcγ and complement receptors occurs when C3b/iC3b-bearing targets are also opsonized by antibodies or display glycosylated CR3 ligands. CR3 can be activated by "inside-out" or "outside-in" mechanisms to generate high-affinity binding sites on the integrin [57].

10.4.2 Degranulation

Neutrophils are specialized killers of invading pathogens to the human organism [58]. Various antimicrobial and cytotoxic proteins are synthesized by neutrophils, packed into different types of granules and released upon cell activation. The phagosome in neutrophils acquires its antimicrobial effects through fusion with secretory vesicles and granules, endomembrane organelles produced from vesicles that bud off the trans-Golgi network and contain a surprisingly heterogeneous and powerful store of microbicidal peptides and proteolytic enzymes that contribute to pathogen elimination [59]. Granule biogenesis follows the granulocyte differentiation pathway, so in different stages of neutrophil growth, azurophil granules are formed first, followed by specific granules containing lactoferrin and collagenase, then it is the turn of gelatinase granules, and finally secretory vesicles appear at the stage of mature neutrophil [60]. Granules are characterized to be readily mobilized upon an inflammatory stimulus at the plasma membrane in reverse order to their formation [61]. Instead, secretory vesicles are endocytic vesicles that represent a reservoir of membrane-associated receptors needed at the earliest phases of the neutrophil-mediated inflammatory response; vesicles are mobilized in response to a wide variety of inflammatory stimuli [62].

The release of granule-derived mediators from neutrophils occurs by tightly controlled receptor-coupled mechanisms, leading to exocytosis. It is known that

exocytosis happens in four discrete steps [63]. The first step is granule recruitment from the cytoplasm to target membrane, which depends on actin cytoskeleton remodeling and microtubule assembly, and it is followed by vesicle tethering and docking, leading to contact of the outer surface of the lipid bilayer membrane surrounding the granule with the inner surface of the target membrane. Translocation and exocytosis of neutrophil granules and secretory vesicles require increases in intracellular Ca^{2+} [64].

10.5 Cytokine Synthesis

Neutrophils are a "factory" of *de novo* synthesized cytokines and also express cytokines at a basal level from preformed stores [61]. Different proinflammatory cytokines and chemokines often exert their proinflammatory functions via an autoregulatory pathway. Neutrophils are favorite targets of proinflammatory cytokines, IL-1 and TNF-α, chemokines such as IL-8, and growth factors such as granulocyte/monocyte colony stimulating factor (G-CSF and GM-CSF). The pattern of cytokines produced by neutrophils also greatly differs depending on the agonist. Also, the production of cytokines by neutrophils can easily be modulated by immunomodulatory cytokines such as IFN-γ, IL-4, IL-10, and IL-13, suggesting that T helper-1 (Th-1) or Th-2 cells may influence neutrophil cytokine production [65].

10.5.1 TNF-α

TNF-α is a pleiotropic cytokine belonging to the superfamily of membrane-anchored and soluble cytokines that are remarkably involved in T cell-mediated immunity. Although it inhibits the growth of tumor cells and it has an enhancing effect on the proliferation of normal cells [66], TNF-α takes part in septic shock, autoimmunity, and inflammatory diseases. Its potent proinflammatory effects mainly results from its capacity to increase expression of endothelial cell adhesion molecules and subsequently support neutrophil adherence to vascular endothelium. Neutrophils are exquisite targets of TNF-α that, under certain conditions, strengthens their expression of adhesion molecules, induces their degranulation and successive release of lysosomal enzymes, causing the production of highly reactive oxygen species (ROS). TNF-α-induced transcellular migration of neutrophils through the production of soluble protein synthesis-dependent chemotactic factors, including IL-8. This testifies cytokine networking involvement in inflammatory cell recruitment and an active role in inflammation [67].

10.5.2 IL-1 and IL-1 Receptor Antagonist

Neutrophils indeed express the mRNA of both IL-1α and IL-1β and release IL-1 proteins [68]. On the other hand, the quantity of IL-1β is greater than IL-1α. Interestingly, it is demonstrated that antineutrophil cytoplasm autoantibodies (ANCA) induce IL-1α mRNA expression in neutrophils, usually released in

concert with TNF-α [69]. The demonstration of a constitutive secretion of IL-1 receptor antagonist (IL-1Ra) by neutrophils using GM-CSF combined with TNF-α as agonists [68] has been followed by the demonstration that neutrophil-derived cytokines have an inhibitory activity on IL-1 production [70].

10.5.3 IL-8 as a Model of Chemokines

IL-8 is a prototype of the C-X-C chemokines family [71]. IL-8 is a potent neutrophil chemoattractant and activator expressed in response to LPS, cytokines, or aggregated immune complexes. Interestingly, IL-8 is the most abundantly secreted cytokine by neutrophils and, on the other hand, neutrophils are the primary cellular target of IL-8 [72].

10.5.4 Modulation of Cytokine Expression and Production by Neutrophils

Proinflammatory cytokines and chemokines upregulate microbicidal activity of neutrophils. All are considered additional immunomodulatory agents to treat serious or refractory infections in humans [73]. Cytokine expression by neutrophils can be easily modulated by the T-cell-derived regulatory cytokines: positively by Th1-type cytokines such as IFNγ and negatively by Th2-type cytokines such as IL-10, IL-4, and IL-13 [74].

IFN-γ exerts a strong enhancing effect on the production of cytokines, upregulating both the production of IL-1Ra and the accumulation of IL-1Ra mRNA. Contrary to IFN-γ, IL-10 exerts an inhibitory effect on neutrophil cytokine production, particularly TNF-α, IL-1-β, and IL-8 production [75]. IL-4 also induces an upregulation of IL-1Ra synthesis in neutrophils and a priming effect on neutrophil production of TNF-α and IL-8 induced by LPS.

One of the important findings is the ability of stimulated neutrophils to release TNF-a, IL-lβ, IL-8, and IL-lRa, and the fact that the type of triggering stimulus determines not only the amplitude but also the intrinsic characteristic of this response [76]. Moreover, recent studies have shown that a wide variety of agonists induce the nuclear accumulation of NF-κB/Rel proteins, the main way of cytokines activation [77]. In contrast, numerous studies have shown that cytokine gene expression can be regulated at the level of mRNA stability and this mechanism explains the upregulation of IL-1β mRNA. The inhibitory effects of IL-10 on neutrophil IL-8 production also result from both an inhibition of IL-8 gene transcription and an enhanced IL-8 mRNA degradation. Finally, a control at the translational level has also been suggested for IL-1Ra under specific experimental conditions [78].

10.6 Neutrophil Apoptosis

Neutrophils live for less than 24 h in the circulation and are characteristically preprogramed to die by constitutive apoptosis. Recent studies suggest that this process is planned by intracellular signaling and changes in gene expression that

define an "apoptosis differentiation program" [79]. Neutrophil apoptosis is a essential, early component of efficacious resolution of inflammation and is likely delayed or defective in nonresolving chronic inflammatory states [80]. This is linked to the loss of expression of adhesion molecules and greatly decreases responsiveness to external stimuli, so that these cells become functionally isolated from their environment. In contrast with necrosis, apoptosis is related with preservation of plasma membrane integrity, so that relieving of harmful neutrophil contents is incomplete, and the inert neutrophils are phagocytosed by local macrophages. Also, phagocytosis of apoptotic neutrophils by human macrophages *in vitro* destroys release of macrophage-derived proinflammatory mediators. Like this, by downregulating neutrophil functions and triggering "silent" clearance by phagocytes, apoptosis provides an instrument for the safe disposal of possibly destructive inflammatory cells [81]. Throughout apoptosis, receptors for proinflammatory signals from the cell membrane are not involved, while phosphatidylserine residues, recognized by phagocytes, are involved. A standard tool for the affinity of apoptotic cells implicates annexin-V binding to phosphatidylserine residues. Interestingly, a putative phagocyte receptor for PS has been found difficult to describe, and three recently identified receptors (Tim4, stabilin2, and BAI1) designate that the complication of this process might have been undervalued. Phagocytosis should be nonphlogistic, suggesting that a professional phagocyte such as the macrophage on recognizing an apoptotic cell is planned to upregulate the production of anti-inflammatory intermediaries such as transforming growth factor (TGF)-β and interleukin (IL)-10 [82]. Developing an understanding of granulocyte behavior has been a study priority and has indicated that a pharmaceutically tractable method to the modulation of granulocyte functional responsiveness might be to influence the longevity of these cells by inducing apoptosis. In addition, research is engendering an increasingly detailed picture of the apoptotic machinery at a molecular level [82,83]. A better appreciation of the processes that drive and delay neutrophil apoptosis will support the development of new drugs appropriate for the management of granulocyte-mediated diseases [84,85].

10.7 Neutrophils in Pathology

The major role of neutrophils is a rapid response to invading microorganisms. On the other hand, neutrophils do not differentiate efficiently between foreign and host antigens without the help of soluble components of the immune system [86]. The host-damaging power of the neutrophils is limited by elimination of the primary event that initiates inflammatory sequences and by mechanisms that inactivate neutrophils such as apoptosis. The blocking of neutrophil influx involves inactivation of mediators and temporal change in the model of chemokines produced. When regulatory mechanisms are impaired or when the acute insult cannot be resolved, neutrophils become the predominant contributor to tissue injury and induce lesions [87]. To exemplify the role of neutrophils in pathology by different diseases, it is appropriate to assess the stimulus or type of mechanism that activates neutrophils.

10.7.1 Bacterial Infection

The ability of neutrophils to sense bacteria and/or their components and migrate across epithelia along a chemotactic gradient is interesting. Neutrophil sequestration is an essential antibacterial defense, but excessive neutrophil infiltration and activation in the infected tissue may partly cause tissue damage. Thus, several life-threatening bacterial lung diseases are caused by excessive neutrophil-mediated inflammation [88]. During pyelonephritis, microscopic examination of infected kidneys shows intensive degranulation of neutrophils containing phagocytosed bacteria, causing severe tubular destruction and occlusion of small capillaries by leukocyte plugs [89]. Indirect evidence suggests that uropathogenic bacterial strains, which induce excessive neutrophil activation, contribute to tissue damage and renal scarring [90]. The bacterial load at the initiation of the inflammatory response and the phenotypic expression of bacteria, determining their ability to cause neutrophil activation and to resist neutrophil phagocytosis, are important factors contributing to neutrophil-induced tissue damage during bacterial infection.

10.7.2 Tissue Injury-induced Inflammation: Ischemia-reperfusion Injury

Tissue injury after ischemia appears to be a consequence not only of tissue hypoxia but also of the process of reperfusion that leads to an inflammatory response. Infiltrating neutrophils are a potential source of reactive oxygen species, proteolytic enzymes, and cytokines, which during reperfusion may play a damaging role; also, capillaries could become clogged by aggregated neutrophils, causing reperfusion of the microcirculation [91]. Several studies have shown a pathogenetic role for neutrophils in ischemic insult to the myocardium [92], liver [93,94], and kidney. Inhibition of leukocyte adhesion with anti-ICAM-1, such as therapeutic approaches on antiadhesion strategies, could be beneficial in ischemia–reperfusion injury.

10.7.3 Crystal-induced Inflammation

Neutrophils appear to be the major effector of certain forms of crystal-induced inflammation, as a consequence of their accumulation in the joint fluid where they actively ingest urate crystals, aggregate, and degranulate. One consequence of this neutrophil action is acute gouty inflammation, due to deposition of monosodium urate crystals in joints [95]. Precipitated urate crystals activate humoral mediator cascades such as complement and contact activation systems; also, urate crystals induce the production of inflammatory mediators by neutrophils (TNF-α and IL-8), amplifying the leukocyte influx [96]. Acute gouty inflammation may be prevented by neutrophil depletion or leukocyte chemotaxis inhibition.

10.7.4 Cytokine-induced Inflammation: Rheumatoid Arthritis

Neutrophils are prominent participants in the joint inflammation of human rheumatoid arthritis (RA) [97]. RA is a predominantly T-cell/macrophage-driven process in the early stage of the disease, but the cell with the greatest capacity to inflict damage within joints is the neutrophil, which is numerous in the synovial

fluid and joint tissues during the early stages of RA and during acute exacerbation of the disease. Neutrophils concentrate at the pannus–cartilage junction, which is the site of early cartilage erosion, contributing directly to the cartilage damage, both through serine and metalloproteases and through the production of ROS and chlorinated oxidants. Neutrophil-mediated cartilage injury is modulated by cytokines such as TNF-α and IL-8 [98]. TNF-α is likely to be an important stimulator of neutrophils within the joints of RA patients, resulting in amplification of the inflammatory response and contributing to the tissue damage caused by neutrophils, while, although IL-8 alone does not exert a direct effect on cartilage, it induces a rapid cartilage destruction [99].

10.7.5 Autoimmunity Against Neutrophil Components: Antineutrophil Cytoplasmic Antibodies and Vasculitis

The neutrophil plays a central role in the pathogenesis of many inflammatory disorders of the vasculature. The presence of neutrophils in vasculitic lesions, including those of Wegener's granulomatosis, leukocytoclastic vasculitis, and others, bears witness to this. Although the effector functions of neutrophils are central to the vascular injury associated with vasculitis, neutrophils must be present at the site in order to contribute to the vasculitic lesion [100]. Neutrophil granule proteins are recognized as target antigens for antineutrophil cytoplasmic antibodies (ANCA) [101]. ANCA can be divided into a group displaying a cytoplasmic staining pattern (C-ANCA) and a second group displaying perinuclear staining (P-ANCA). It appears that the majority of C-ANCA react with PR3 and the correlation between clinical expression of Wegener's granulomatosis and ANCA reactivity has established that PR3 is the target autoantigen, while the major target antigen of P-ANCA is myeloperoxidase; anti-MPO was originally described in patients with microscopic polyangiitis and idiopathic necrotizing crescentic glomerulonephritis [102].

10.7.6 Genetic Disorders of Neutrophil Regulations: Hereditary Periodic Fever Syndromes

The hereditary periodic fever syndromes are a group of disorders characterized by self-limited episodes of fever accompanied by localized inflammation. Of these syndromes, familial Mediterranean fever (FMF) has been the longest recognized [103]. FMF is an autosomal recessive disease characterized by recurrent febrile episodes in association with peritonitis, pleuritis, and arthritis and has ongoing subclinical inflammation during attack-free period [104]. The inflammatory episodes of FMF affect mainly the serosal or synovial membranes, characterized by massive influx of neutrophils into the affected tissues. The FMF gene has been identified by positional cloning and it encodes a transcript expressed predominantly in neutrophils [105]. Other tissues such as mucous membranes that are more exposed to trivial injury are not affected by the inflammatory episodes of FMF, showing that the regulatory dysfunction of neutrophils from these patients is not implicated in all pathways leading to neutrophil stimulation.

Familial Hibernian fever (FHF) is an autosomal dominant disease consisting in recurrent attacks of fever with abdominal pain and severe localized myalgia; the presence of erythematous skin lesions, conjunctivitis, unilateral periorbital edema, and scrotal pain are observed with high frequency, while pleuretic and nonpleuretic chest pain may occur [106]. Mutations that cause this disease are point substitutions within cystein-rich motifs of the extracellular domains of the 55 kDa TNF-α receptor [107].

10.7.7 Cystic Fibrosis: Neutrophil-mediated Tissue Damage and Concomitant Persistence of Infection

Cystic fibrosis (CF) is characterized by chronic progressive bronchiectasis, exocrine pancreatic insufficiency, sterility in males, and abnormally high concentrations of sodium chloride in the sweat. Water reabsorption from the CF airway lumen outweighs limited Cl^- secretion resulting in the airway secretions becoming viscid and clearance of secretions is impaired causing airway obstruction [108]. This is further compounded by colonization of the airway with organisms such as *P. aeruginosa* [109]. The most characteristic feature of inflammation in the CF lung is the persistent infiltration of massive numbers of neutrophils into the airways. Neutrophils are considered responsible for the early onset and the promotion of the inflammatory process in CF, which starts within the first year of a CF patient's life [110]. Proinflammatory chemoattractants have been found to be elevated in bronchoalveolar lavage fluid(BAL) from CF, including IL-8, IL-1, IL-6, and TNF-α and bacterial products, which also act as potent chemoattractants for neutrophils. Also, neutrophil elastase (NE) induces gene activation and secretion of IL-8 and digestion of C3b receptors on neutrophils, which limits the phagocytosis of pathogens [111]; studies in CF patients have shown increased numbers of elastase [112]. With regard to neutrophil functions, myeloperoxidase-dependent activities appear to be significantly higher also in heterozygote parents of CF patients [113], providing some evidence for a genetic component in the altered neutrophil function in CF. Also, there is a decrease in the changing role of L-selectin in stimulated CF neutrophils, which was not observed in either stable or acutely infected non-CF bronchiectasis patients, thus suggesting a disturbed control of the migration process in CF neutrophils [114]. Clinical management of CF presents a paradox because CF involves chronic active inflammation and recurrent infections requiring a therapy combining antibiotherapy and anti-inflammatory drugs [115].

10.8 Conclusions

Polymorphonuclear neutrophils include two-thirds of peripheral blood leukocytes and they are leaders of the innate immune cells that migrate to the place of infection or inflammation for suppression and clearance of infectious elements [58]. Until approximately two decades ago, neutrophils were considered as short-lived cells having a destructive hardware but no software. This notion has

been revised since it became clear that neutrophils were capable to integrate complex arrays of adhesion, chemoattractant, and cytokine driven signs. Neutrophils also were able to synthesize cytokine in reply to inflammatory stimuli. The neutrophil itself acts to be well equipped with an uncommon combination of contrasting molecules: ROS, ubiquitous and short-lived molecules and proteases, and macromolecules with specific long-lasting inactivity. Neutrophil safely keeps them apart through independent intracellular compartmentalization under physiological conditions. These mediator activities regulate pathophysiological processes not exclusively designed at the destruction of invading microorganisms. Finally, neutrophils are activated by anabundant variety of stimuli and are convoluted in different disorders.

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11

Mast Cells: Master Drivers of Immune Responses against Pathogens

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11.1 Introduction

The unique properties of mast cells were first noticed by Paul Ehrlich, who described the distinct morphological appearance and metachromatic staining of these cells in his seminal 1878 doctoral thesis [1]. Now, over 130 years later, we are beginning to gain a full appreciation for the multifaceted capabilities of the mast cell in orchestrating immune responses, particularly in the context of infectious diseases.

Once only thought to serve as part of the defense against parasitic infections and the noxious effectors of allergic disease, it is now becoming apparent that this cell type plays a varied and tunable role in orchestrating immunity to pathogens. As we will describe in this chapter, mast cells have several intrinsic properties that allow them to be such effective drivers and conductors of immune responses. Specifically, mast cells are strategically located at host–environment interfaces, where their expression of a wide range of receptors allows them to respond promptly to extrinsic challenge through degranulation of prestored mediators with both degranulation and secretion of *de novo* synthesized mediators. This leads to potent induction of both innate and adaptive immune responses, which can be exploited in to boost immune responses to vaccines. However, mast cells do not always play protective roles against pathogens. Under certain contexts, mast cell-mediated inflammation may even worsen the disease state. Mast cells also may prematurely trigger anti-inflammatory effects, which can inhibit productive immune responses against pathogens.

11.2 Biology of Mast Cells

Tissue located mast cells originate from bone marrow haematopoetic progenitors that circulate in the blood [2]. Upon entering tissues, these progenitors

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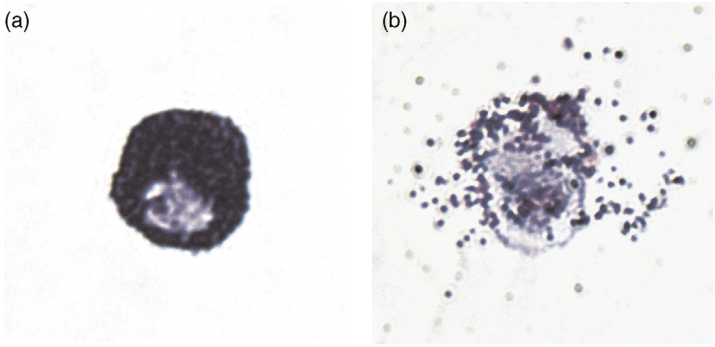


Figure 11.1 Mast cells release prestored granules upon activation. Peritoneal lavage from IgE-sensitized mice injected i.p. with (a) PBS or (b) antigen was stained with Toluidine blue, which stains heparin-containing granules a deep purple. Note the densely packed granules in (a) and the extensive exocytosis of granules in (b).

differentiate into mature mast cells that may live for months [3]. These long-lived cells are capable of several cycles of degranulation and regranulation and are able to proliferate in tissue in response to appropriate signals despite being terminally differentiated [4,5].

The most distinct feature of mast cells is their numerous, electron-dense secretory granules, which occupy a large proportion of the mast cell cytoplasm and upon activation, can be discharged rapidly within seconds to minutes (Figure 11.1). These granules contain high amounts of anionic proteoglycans [6,7] that result in the characteristic metachromatic staining when visualized with cationic dyes such as Toluidine blue. The major constituent of granules are mast cell-specific proteases, which are tightly complexed with sulfated proteoglycans such as heparin and chondroitin sulfate, the specific combinations of which vary with mast cell phenotype. Mast cell granules are also rich sources of biogenic amines, the most notorious of which is histamine, the compound responsible for many of the symptoms of allergic inflammation. The neurotransmitter, serotonin, is also highly expressed in rodent mast cells but less so in human MCs. In addition, as part of their unique function, mast cells also store preformed cytokines and growth factors within granules, such as tumor necrosis factor (TNF) and vascular endothelial growth factor (VEGF) [8].

Notably, mast cells are phenotypically heterogeneous at different anatomical sites, as well as between different animal species. Broadly, rodent mast cells can be categorized into mucosal mast cells, which are present in the epithelia of mucosal tissues such as the gut and lung, and connective tissue mast cells, which are found in the submucosae and muscle layer. Based on their anatomical location, these subtypes of mast cells differ notably in the protein and proteoglycan components of their granules [9]. Connective tissue mast cells express both tryptases and chymases complexed with heparin, while mucosal mast cells express only chymases, complexed with chondroitin sulfate. A similar dichotomy is present in human cells, which are classified into tryptase and chymase-positive (MC_{TC}) and tryptase-positive (MC_T), though there is less stringency in tissue-type specificity [10]. Individual mast cells may also vary with regard to granule

contents, as different environmental stimuli can result in the upregulation of specific granule components. For instance, human mast cells cultured *in vitro* can increase the chymase content of their granules in the presence of IL-4 [11]. In mouse mucosal mast cells, IL-10 can modulate the transcription of the protease mMCP2 [12]. Thus, mast cells can potentially have very heterogeneous responses even to the same stimulus depending on their granule contents.

11.3 Role of Mast Cells in Immune Surveillance

11.3.1 Strategic Peripheral Location

Mast cells are found in most connective tissues throughout the body, but they are most concentrated at interfaces between the host and the environment, such as the skin, gastrointestinal tract, and respiratory tract. Thus, they are ideally situated to be one of the first responders to pathogens and other extrinsic challenges that breach the epithelial barrier of the host [13].

In addition, mast cells are located proximal to blood vessels, lymphatics [14], and nerve fibers [15], suggesting that they are also ideally situated to quickly relay information regarding invading pathogens by releasing mediators in the vicinity of these structures. Interestingly, a recent report suggests that perivascular mast cells may even directly acquire IgE antibodies circulating in the blood by extending cellular processes across blood vessel walls [16]. This ability to sample vascular contents may also apply to detecting pathogens or pathogen-associated molecules in the circulation, and the ability of mast cell products to affect vascular permeability could further facilitate their ability to access microbes and other antigens in the blood.

11.3.2 Direct Recognition of Pathogens

Mast cells express a wide variety of pattern recognition receptors (PRRs) that allow them to directly recognize pathogen-associated molecular patterns (PAMPs). This includes Toll-like receptors (TLRs), C-type lectins (e.g., dectin-1), and other surface molecules such as CD14 [17]. Expression of most TLRs has been described for mast cells at least at the mRNA level, and they have been shown to be either constitutively expressed or are upregulated based on cues from the microenvironment. TLR4 expression in human mast cells derived from cord blood, for example, is inducible by *in vitro* culture in the presence of IFNγ or IL-4, but it is not expressed constitutively [18,19]. Certain pathogen-associated ligands can also activate mast cells by a combination of TLRs and other surface molecules, such as fungal zymosan, which is recognized by a combination of TLR2 and dectin-1 [20], and LPS, which is recognized by TLR4 and CD14 [21].

Differential ligation of different TLRs alone or in combination with other receptors can result in distinct mast cell activation outcomes, such as degranulation or secretion of specific cytokines. TLR2 activation via bacterial peptidoglycan, for instance, results in both mast cell degranulation and cytokine production, while TLR4 ligation via LPS only results in cytokine release [22].

However, different groups have reported conflicting results regarding the ability of various TLRs to stimulate mast cell degranulation or cytokine synthesis, suggesting that the specific responses of mast cells to TLR stimulation may depend on mast cell phenotype. In addition, simultaneous ligation of both TLR and other activating receptors, such as the high-affinity receptor for IgE, FcεRI, can enhance the release of cytokines [23], adding a further dimension to TLR-mediated responses.

Conversely, TLR signaling may influence the phenotype of mast cells, such as by downregulating FcεRI expression, by altering protease composition of granules, and by influencing cytokine secretion [17]. Prolonged interaction of mast cells with pathogens and their associated PAMPs, such as in a chronically inflamed tissue, could therefore further shape mast cell-directed immune responses.

11.3.3 Indirect Recognition of Pathogens

Mast cells express receptors for IgG and IgE on their cell surface, allowing them to become sensitized to antigen previously encountered by the host by binding antigen-specific immunoglobulins. These bound antibodies enable mast cells to specifically recognize and vigorously react to previously encountered pathogens. The high-affinity receptor for IgE, FcεRI, is highly expressed in mast cells and is responsible for protective IgE-mediated responses to metazoan parasites such as roundworms [24]. However, they are also responsible for misdirected IgE-mediated hypersensitivity reactions to harmless antigens. Rarely, IgE can also be produced in response to bacterial antigens [25]. In addition to FcεRI, mast cells also express the FcγR family of receptors for IgG [26], of which FcγRI and FcγRIII are activating and FcγRIIB is inhibitory. Human mast cells express FcγRII constitutively, while FcγRI expression is induced by the presence of IFNγ [27] and FcγRIII is not expressed. In mouse mast cells, FcγRI is not expressed, FcγRIIB is constitutively expressed, and FcγRIII is induced only in the presence of stem cell factor (SCF), such as when bone marrow-derived mast cells (BMMCs) are cocultured with fibroblasts [28]. Cross-linking of activating FcγR by IgG immune complexes or IgE-bound FcεRI by multivalent antigen results in mast cell activation, degranulation, and cytokine secretion. Coligation of FcεRI and the inhibitory FcγRIIB, however, results in the downregulation of FcεRI signaling [26]. Bacterial superantigens such as *Staphylococcus aureus* protein A, which binds certain classes of antibodies independent of their antigen specificity, can also trigger mast cell degranulation through FcR [29].

11.3.4 Activation by Endogenous Danger Signals

During infection many endogenous "danger signals," which are host-derived proinflammatory mediators, are elaborated by stressed or dying cells. These signals, such as complement, antimicrobial peptides, substance P, and endothelin-1, can also activate mast cells [13]. Human mast cells express the G protein-coupled receptor Mrgx2, which can be activated by the antimicrobial peptide LL-37 [30]. This receptor has also been implicated in mast cell-dependent anaphylactoid reactions to ciprofloxin and other irritants [31]. Endothelin-1,

which is produced during sepsis by endothelial cells, can activate mast cells via the receptor ET-1 [32]. Notably, components of the complement system, in particular C5a, can also activate mast cells, inducing their chemotaxis and degranulation and production of cytokines through complement receptors on mast cells [33].

11.3.5 Degranulation and Secretory Responses to Pathogens

The rapid exocytosis of mast cell granules and their associated preformed contents allows for their prompt response to pathogens or other insults within seconds to minutes. This is hugely advantageous, as other immune cells, such as skin-resident DCs or epithelial cells, require considerably more time to mount a response due to the requirement for *de novo* synthesis of mediators. However, mast cells also have a potent second-phase response occurring hours after the initial stimulus, where they *de novo* synthesize and secrete mediators such as cytokines, prostaglandins, and other inflammatory molecules. It is well known that different stimuli, or even intensity of stimuli, can trigger differential production and secretion of these mediators. This two-phase response allows for great plasticity in the duration, nature, and specificity of mast cell-directed responses to pathogens.

During the first phase of mast cell activation, mast cell granules, which are stored as insoluble particles within the cytoplasm, are exocytosed into the extracellular milleau. While some granule-associated mediators, such as histamine, become immediately soluble, most of the membrane-free structure remains insoluble, due to tight electrostatic interactions formed between positively charged proteins (mostly proteases) and negatively charged heparin or chondroitin sulfate. Preformed cytokines such as TNF are entrapped within these particles and are released slowly over time once the particle is exocytosed [34]. In this way, mast cells are able to prolong the half-life of their mediators, as they are extremely susceptible to dilution and degradation in the extracellular medium. In addition, exocytosed granules can drain into the lymphatics and traffic to the draining lymph nodes to modulate cellular infiltration at this distal site [34]. This represents a highly effective way for peripherally placed mast cells to modulate immune responses initiated in the draining lymph nodes.

Within minutes of stimulation, mast cells begin to *de novo* synthesize mediators such as cytokines and eicosanoids. Eicosanoids, which comprise prostaglandins and related compounds such as leukotrienes, can be rapidly enzymatically converted from arachidonic acid to an active form [35]. Once secreted, they function as proinflammatory mediators that enhance vascular permeability and cellular recruitment. Mast cells also produce a wide array of chemokines and cytokines depending on type and strength of stimulus, which have varied effects including cellular recruitment, enhanced bacterial killing, and promotion of antigen presentation. Thus, mast cells can follow up an immediate degranulation response with the synthesis of additional factors that are tailored to the stimulus, allowing for both rapid activation of nearby immune cells and changing the microenvironment to direct immune cells toward appropriate responses to pathogens.

11.4 Mast Cells in Innate Immunity

Mast cells are increasingly being recognized as a pivotal modulator of the innate immune system. Other than directly contributing to degradation of bacteria or other noxious substances, they can also direct the activation and recruitment of appropriate immune cells to the site of infection to facilitate early clearance of the pathogen.

11.4.1 Direct Bactericidal Activity of Mast Cells

Mast cells have been observed to display direct antimicrobial activities, which is in part due to the presence of antimicrobial peptides such as cathelicidins [36] within their granules. In addition, mast cells are able to endocytose enterobacteria, such as *Escherichia coli*, which express type I fimbrae, by binding the fimbral component FimH through the cell surface molecule CD48 [37,38]. Endocytosed bacteria are then killed through oxidative burst and acidification of vacuoles. There is also evidence that mast cells, like neutrophils, are able to trap and kill bacteria via extracellular trap formation (mast cell extracellular traps) [39]. These extracellular traps are composed of nuclear histones, antimicrobial peptides, and tryptases and are released in a reactive oxygen species (ROS)-dependent cell death mechanism. However, due to their low numbers at sites of infection, relative to the large numbers of recruited professional phagocytes and other immune cells, it is unlikely that mast cells contribute significantly to direct bacterial killing *in vivo*.

11.4.2 Resistance to Exo- and Endotoxins

Mast cell granules contain large amounts of active proteases that could potentially cleave and inactivate protein substrates in the microenvironment, including peptide toxins. It has been demonstrated that in a cecal ligation and puncture rodent model of bacterial sepsis, high levels of inflammatory peptides such as neurotensin and endothelin are released, which contribute to mortality of the host. In this model, mast cell-derived carboxypeptidase A3, and possibly other proteases, digested these toxins and increased host survival [32]. Exotoxins present in insect or reptile venoms can cause pathology by mimicking the structure of endogenous inflammatory peptides, and in recent years, mast cells have been hypothesized to play an important role in the defense against these toxins. Mast cell carboxypeptidase has been shown to be protective against sarafotoxin, a component of viper venom that is a homologue of the endogenous inflammatory peptide endothelin [40]. This exotoxin can induce mast cell degranulation through the ET-1 receptor. Interestingly, IgE/antigen-mediated activation of mast cells, even when the antigen was not a component of the venom tested, could enhance immunity to viper venom, perhaps by enhancing immune cell trafficking [41].

11.4.3 Induction of Immune Cell Trafficking to Sites of Infection

Mast cells secrete a wide variety of chemoattractants and cytokines that promote leukocyte trafficking to sites of infection, where they can contribute to pathogen clearance or amplify inflammation by secretion of proinflammatory cytokines. One of the most important cytokines secreted is TNFα, which plays an important role in the trafficking of neutrophils to sites of mast cell activation in order to initiate bacterial clearance [42]. Mast cell granule proteases, released upon degranulation, are also known to have chemotactic activity for leukocytes. Specifically, mouse mast cell protease 6 (mMCP6), but not mMCP7, has been shown to induce neutrophil migration in mice [43,44]. mMCP6 may also contribute to eosinophil recruitment and cytokine production in response to parasitic infection [45]. Mast cells also synthesize leukotrienes (LT), such as LTB4 and LTC4, in response to bacterial challenge, which upregulate P-selectin on endothelial cells, enhancing the rolling phase of the leukocyte adhesion cascade [46]. This activity of mast cell leukotrienes has been demonstrated to facilitate neutrophil recruitment and bacterial clearance in an infectious peritonitis model [47]. Mast cells can also induce cell trafficking in response to viral challenge, presumably after stimulation of TLRs during infection of the cell by virus. Respiratory syncytial virus, while only poorly infective for human mast cells, was able to induce the secretion of multiple chemotactic factors for leukocytes, such as CCL4, CCL5, and CXCL10 [48]. In a mouse model of dengue infection, mast cells upregulate the chemokines CX3CL1, CXCL12, and CCL5, which recruit NK cells to the site of viral infection [49]. This role of mast cells to recruit appropriate pathogen-clearing immune cells to sites of bacterial, viral, and parasitic infections is arguably the most important innate immune activity mediated by these immune surveillance cells.

11.5 Mast Cells in Adaptive Immunity

As previously discussed in Section 11.3.3, mast cells are well known effectors of adaptive immunity by binding immunoglobulin through their Fc receptors. The best known instance of this is in IgE-mediated immunity against helminth parasites. However, mast cells also have critical roles in enhancing and shaping the adaptive immune responses.

11.5.1 Mast Cells as Effectors of Adaptive Immunity

Mast cell and IgE-associated responses are thought to contribute to host defense against helminths and other parasites that are too large to be endocytosed. Mast cell products such as their proteases secreted upon IgE/FcεRI activation and directed at parasite surfaces are believed to contribute to killing and expulsion of parasites [50]. Prior sensitization to insect and reptile venoms can also induce the production of specific IgE. Mast cell-derived carboxypeptidase secreted in response to subsequent challenge with these venoms has been demonstrated to be protective [51].

11.5.2 Recruitment of DCs and Effector T Cells to Sites of Infection

Dendritic cells (DCs) are professional antigen-presenting cells that bridge the innate and adaptive immune systems. DC activation and trafficking to lymph nodes is a hugely important event in the initiation of adaptive immunity. During infection by bacteria such as *E. coli*, mast cells recruit large numbers of DCs into infected tissues, which then go on to migrate into draining lymph nodes [52]. This trafficking was caused by the upregulation on E-selectin on endothelial cells facilitated by mast cell-derived TNF. This mast cell-dependent recruitment of DCs was essential for primary humoral responses against bacteria. In addition, mast cells may also promote the trafficking of effector T cells to sites of infection. In a model of viral peritonitis, mast cell secretion of CXCL10 and CCL4 promoted the recruitment of CD8+ T cells [53].

11.5.3 Recruitment of Dendritic Cells and T Cells to Draining Lymph Nodes

Draining lymph nodes are dynamic lymphoid structures that can entrap large numbers of circulating lymphoctyes during infection, causing them to swell dramatically in size. During infection, T cells recruited to the node interact with antigen-loaded antigen-presenting cells that have trafficked from sites of infection, initiating the adaptive immune response. It is now clear that mast cells can regulate lymph node hypertrophy. Mast cell secretion of TNF recruits T cells and DCs to draining lymph nodes within 24 h after bacterial infection or activation with 48/80, a mast cell activator, a process that involves the upregulation of VCAM-1 on endothelial cells [54]. Interestingly, peripherally located mast cells can also direct distal responses in lymph nodes by the release of stable particles containing inflammatory mediators. These insoluble particles drain via the lymphatics to local lymph nodes, transporting mast cell-derived TNF where it is slowly released [34]. Mast cells may also induce DC migration to the lymph node in a TNF-independent histamine and IL-6-dependent manner when stimulated with bacterial peptidoglycan [55]. Thus, following infection at peripheral sites, mast cells serve to kick-start adaptive immunity by promoting the coordinate trafficking of professional APCs from infection site into the draining lymph nodes and simultaneously sequestering T lymphocytes to facilitate optimal antigen presentation.

11.5.4 Antigen Presentation

While not regarded as professional APCs, mast cells can directly present antigen to T cells and stimulate their response [56]. Constitutively, they express MHC class I, while mast cells isolated from infected tissues have been observed to express MHC class II [57]. They also express costimulatory molecules such as OX40 ligand [58]. Mast cell-mediated MHC class I-dependent antigen presentation to T cells has been reported to promote the activation and proliferation of CD8+ T cells, as well as enhance their production of IL-2 and granzyme B in autoimmunity, suggesting that this modulation of CD8+ T cell function could also possibly occur during infection [59]. However, the contribution of MHC class II-dependent antigen presentation by mast cells *in vivo* is still unclear, as mast cells are comparatively few compared to professional APCs in tissue. Rather, mast

cells are likely to enhance antigen presentation by APCs. Mast cell products can influence DC activation and antigen presentation. For instance, histamine has been suggested to enhance DC uptake of antigen and cross-presentation and the upregulation of costimulatory molecules. Mast cell-derived cytokines can also prime immune responses toward Th2, such as during responses to helminth infection, by the secretion of type 2 cytokines [50].

11.6 Enhancement of Immunity by Boosting Mast Cell Activity

The potent ability of mast cells to drive adaptive immunity, as outlined above, has inspired studies directed at harnessing this activity to improve immune responses to vaccine antigens. One approach is to coadminister small-molecule mast cell activators as adjuvants in vaccine formulations. These mast cell activators, upon administration, would activate local mast cells, greatly enhancing antigen uptake and trafficking of immune cells to the draining lymph node. The second approach is to utilize mast cell granule-inspired synthetic granules loaded with immunomodulatory cytokines such as TNF and incorporate them as adjuvants in vaccine formulations. When injected at a peripheral site, the synthetic granule forumulation, just like natural mast cell granules, would traffic via the lymph to the draining lymph node and release their cargo of immunodulatory cytokines. The resulting cytokine burst should markedly enhance immune cell trafficking into this organ from both the peripheral site of vaccination and the blood, culminating in an enhanced immune response to the vaccine. Both of these approaches are described below.

11.6.1 Mast Cell Activators as Novel Vaccine Adjuvants

The addition of compound 48/80 (C48/80), a mast cell activator, to vaccine formulations has been shown to markedly enhance humoral immunity in an anthrax lethal toxin challenge. Importantly, using this adjuvant resulted in a balanced Th1/Th2/Th17 response without evoking IgE-mediated responses [60]. In addition, intranasal vaccination with C48/80 adjuvant activated mast cells in the nasal-associated lymphoid tissue (NALT) and promoted antigen-specific IgA, a goal considered especially important for evoking effective immunity at mucosal surfaces. Recently, intranasal coadministration of C48/80 with a pneumococcal vaccine has also been demonstrated to be protective against lethal challenge and even nasal pneumococcal colonization compared to a classic mucosal adjuvant, cholera toxin [61]. These reports suggest that it is safe to intentionally activate mast cells in a local fashion in order to promote immunity.

11.6.2 Synthetic Mast Cell Granules

Current vaccine strategies have mostly attempted to enhance immune responses to antigen. However, the nature of the resulting immune response must be carefully tuned to target the specific pathogen. Mast cells evoke predominantly

Th2-type responses, defined by enhanced antibody production and cytokines such as IL-4. These responses are helpful for the production of neutralizing antibodies against pathogens. By contrast, Th1-type responses, characterized by cytotoxic T cell activity and cytokines such as IL-12 and IFNγ, are more effective against intracellular pathogens. Thus, it is important to tailor adjuvants to evoke the type of response that is most effective against a particular pathogen. As discussed previously, mast cell granules contain inflammatory mediators tightly complexed with proteoglycans to form stable particles that can potently induce immune responses at distal sites [34]. Mimicking these particles and including different cytokines to polarize immune responses as needed could, therefore, be a viable strategy in vaccination. Mast cell granule-inspired particles can be readily synthesized by encapsulating cytokines within heparin–chitosan complexes at acidic pH, forming nanoparticles between 200 and 1000 nm in diameter. Indeed, such synthetic mast cell granules containing TNF could protect against lethal flu challenge with efficacy on par with conventional alum adjuvant. Conversely, when particles were loaded with IL-12, a Th1-associated cytokine, this promoted Th1-polarized responses toward an experimental antigen, OVA [62]. Thus, the use of synthetic granules loaded with immunomodulatory cytokines to target lymph nodes in order to enhance and/or polarize immune responses is potentially a powerful new strategy for enhancing immunization. In addition, this approach provides tremendous flexibility in vaccine design by tailoring the specific cytokines encapsulated to the desired immune response.

11.7 Potentially Detrimental Roles of Mast Cell Activation during Immune Responses

While local mast cell activation at mucosal or skin sites is generally beneficial during early infection, these mast cell responses can become harmful if pathogens or pathogen-derived products gain entry into the blood, where they can simultaneously activate mast cells through out the body. Even if the pathogen does not enter the circulation, prolonged activation of mast cells at the site of infection can also lead to significant pathology. Paradoxically, at certain body sites, mast cell activation by microbes can lead to inhibition rather than promotion of immune responses, resulting in impaired clearance of the pathogen.

11.7.1 Overactivation of Mast Cells

Prolonged activation of peripheral mast cells and release of mediators can result in chronic inflammation and tissue remodeling. The best-known examples of this are in allergic diseases such as asthma, where chronic activation of mast cells can result in remodeling of airways [63]. In atopic dermatitis, where most patients are colonized with *S. aureus*, peptidoglycan derived from *S. aureus* can activate mast cells through TLR2, stimulating proinflammatory cytokine production [22]. The *S. aureus* delta-toxin can also potently induce mast cell degranulation directly [64]. Costimulation of FcεRI and TLRs can also enhance cytokine

secretion in mast cells [23], suggesting that viral or bacterial infections have the ability to exacerbate IgE and mast cell-associated disorders. Systemic activation of mast cells can occur either when pathogens bypass the mucosal or skin barrier and reach the bloodstream or when infections are so overwhelming that bacteria or bacterial products enter the circulation, such as during sepsis. In rodent models of sepsis, while local activation of mast cells in the peritoneum can be protective, they can also aggravate inflammation and increase mortality, a complication associated with systemic activation of mast cells and release of IL-6 [65]. In dengue hemorrhagic fever, systemic activation of mast cells and elevated serum protease has been associated with vascular leakage and higher morbidity in human patients [66]. Interestingly, blocking of mast cell degranulation with mast cell-stabilizing drugs such as ketotifen and cromolyn during severe sepsis [65] or dengue hemorrhagic fever [66] has been markedly protective. Mast cells thus seem to be a double-edged sword in immunity, where acute local responses, such as at mucosal surfaces, enhance the clearance of pathogens, while sustained or systemic activation is associated with harmful sequelae.

11.7.2 Negative Modulation of Immunity by Mast Cells

While many mast cell mediators have significant proinflammatory effects, mast cells are also capable of downregulating the immune response. In particular, mast cells secrete IL-10, an anti-inflammatory cytokine. IL-10 can suppress the production of proinflammatory cytokines and chemokines and enhance the ability of DCs to decrease T cell proliferation and cytokine secretion. Mast cell-derived IL-10 has been shown to inhibit proinflammatory responses and limit skin pathology in mouse models of contact hypersensitivity (CHS) and chronic, low-dose exposure to ultraviolet B (UVB) [67]. In these models, tissue swelling and recruitment of leukocytes to the affected skin was reduced in mast cell and IL-10 sufficient mice. In bacterial cystitis, which is an inflammation of the bladder, IL-10 secreted by mast cells limits humoral responses to the pathogen, despite vigorous innate immune responses, resulting in the persistence of bacteria in the bladder, a phenomenon that could contribute to the high rates of recurrence of urinary tract infections. It was found that this limitation was tissue-specific, as bacterial infection of the kidneys (pyelonephritis) did not show the same defect in humoral responses [68]. Other mediators secreted by mast cells are also likely to have negative immunomodulatory effects. One study demonstrated that the ability of UVB to suppress CHS responses to the hapten 2,4,6-trinitrochlorobenzene was dependent on mast cells and histamine [69]. Thus, mast cells have the capability to significantly limit inflammatory responses *in vivo*, potentially hindering the clearance of pathogens.

11.8 Concluding Remarks

Mast cells are one of the first responders to pathogenic insult at host–environment interfaces. The speed of their responses and their ability to evoke both vigorous innate immune responses and long-lasting immunological memory

suggests that they are critical to successfully controlling infections. Therefore, harnessing these properties of mast cells through using adjuvants that stimulate mast cell activity or mimicking the long-distance immunomodulatory effects of their granules are attractive strategies for enhancing productive immune responses to pathogens. However, chronic or systemic activation of mast cells also occurs in some diseases, where they can be severely detrimental to the host. In such cases, inhibiting mast cell activation with specific stabilizers such as ketotifen or cromolyn can have beneficial effects. Thus, understanding the role of mast cells in different contexts is invaluable in order to manipulate their functions to combat infections or reduce inflammation.

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12

Dendritic Cells in Inflammatory Disease

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12.1 What Defines a Dendritic Cell?

Dendritic cells (DCs) are professional antigen-presenting cells that play an essential role in the initiation and the regulation of T-cell responses [1]. By bridging innate and adaptive immunity, they control the balance between tolerance and immunity. They are a relatively newly described cell type, only discovered in 1973 by Nobel laureate Ralph Steinman [2]. From his work, as well as the work of many others, a DC is now described as a cell that has the potential to take up antigen, process it in small peptides for presentation to naïve T cells, and present the antigen in an immunogenic manner, meaning that T-cell costimulatory molecules like CD80 and CD86 and cytokines like IL-12 are made for stimulation of naïve T cells. Another defining characteristic of a dendritic cell (and an important discriminator with macrophages) is the potential of the DC to migrate from the periphery of the body (skin, lung, and intestine) to the T-cell area of draining nodes. Again, there are important exceptions to this conundrum, as some DCs are very sessile, and even called "lymph node resident" DCs, to contrast them with "migratory" DCs. It is also clear now that DCs do more than just stimulate naïve CD4 and CD8 T cells. It was shown that DCs can also stimulate memory T cells, and can transfer unprocessed antigen to B cells [3–5]. In addition, we now realize that DCs can also tolerize and dampen T-cell responses [6]. DCs are considered as promising targets for next-generation immunotherapies aimed at boosting or dampening T-cell responses in inflammatory diseases [7]. This chapter deals with the contribution of DCs in inflammatory disease, beyond the role that these cells have in the initiation of immunity, but focusing mainly on their role in allergic inflammation, the area of our specific expertise.

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12.2 Dendritic Cell Subsets

Initially considered as a single family of cells, distict from macrophages, we now know that there are many subsets of DCs [8,9]. Conventional DC (cDCs) are found in almost all tissues and lymphoid organs and act as sentinels capable of integrating multiple environmental signals and conveying them to CD4 and CD8 T lymphocytes. Plasmacytoid DC produce type I interferons and can also develop into antigen-presenting cells, particularly when stimulated by virus or self DNA [10]. Finally, monocytes can also develop into a cell type called monocyte-derived DC or moDC that not only shares many functional aspects with DCs but also closely resembles macrophages (e.g., they are poorly migratory) [11,12]. moDCs are, however, the form of DCs that has been most exploited for clinical immunotherapy purposes as many cells can be generated *in vitro* from PBMCs or monocytes grown in GM-CSF and IL-4 [13]

The analysis of the various forms of DC is surrounded by complexity. This stems from the fact that DCs are not very abundant in tissues and greatly resemble macrophages and that historically many phenotypes and functions of tissue DCs were described before the development of these cells was properly understood. It has been recently proposed to first classify DCs based on their ontogeny prior to subdividing them based on their microanatomical location or specific functional specialization [14]. This would yield only three subsets of DC: conventional type 1 DC (cDC1), conventional type 2 DC (cDC2), and plasmacytoid DC (pDC). Human and mouse conventional (c)DCs are derived from committed DC precursors (pre-cDC) produced in the bone marrow (BM), distinct from macrophage precursors [8,9]. These pre-cDCs migrate from the BM into the blood and then seed the various tissues where they develop into the two distinct lineages of cDCs. The idea of two distinct lineages is supported by the identification of lineage-defining transcription factors required for development and/or function of cDC1 (IRF8, BATF3, and ID2) and cDC2 (IRF4, ZEB2, Notch2, and KLF4) [14–21]. A separate E2-2-dependent progenitor with prominent pDC potential was recently described [22]. Mature pDCs express Siglec-H, the bone marrow stromal antigen-1, B220, and Ly6C and they can produce large amounts of type I interferons in the presence of viral infection.

With these new molecular insights, it is now clear that cDC belonging to the same lineage are present in various tissues and species and that these cells have been historically characterized by different surface markers (e.g., the marker CD207 has been used to define cDC1 in the mouse skin, CD8α has been used to define cDC1 in the mouse spleen, CD103 in the mouse intestine and lung, and CD141 (BDCA3) in human peripheral blood). In hindsight, it is clear that these markers are not specific to a particular lineage but are imprinted by tissue-specific cues, such as TGF-β or GM-CSF. In addition, monocytes, monocyte-derived cells/moDCs, and macrophages have often contaminated cDC populations in past studies. This is due to the fact that mouse cDCs were typically identified as $CD11c^{+}MHCII^{+}$ cells, while macrophages were identified as $F4/80^{+}$ cells. We now know that many macrophages can express CD11c or MHCII and conversely that cDC2 can express intermediate levels of F4/80 [19,23–27]. The best markers do delineate mouse macrophages from DCs appear to be CD64 and MerTK on

macrophages, whereas CD26 is expressed in cDCs. Distinguishing DCs from macrophages in human tissues has been equally challenging [28–30]. Finally, the lack of conserved markers to identify DCs hampered communication between mouse and human experts within the DC community and was detrimental for fostering translational medicine. The advent of multicolor flow cytometry, allowing unprecedented characterization of these cells through the addition of many novel cellular markers, has even made matters worse by yielding a seemingly ever-growing list of novel DC subsets based on new markers. Therefore, a rational approach simplifying the classification of DC subsets across tissues and species, while still permitting the introduction of new markers to study tissue- and disease-specific activation states, is urgently needed. Generally speaking, within CD64-negative cells (nonmacrophages) expressing CD11c and CD26 ("conventional dendritic cells"), one can use XCR1 (or CADM1) to delineate cDC1s and CD172 (Sirpa) to delineate cDC2 in virtually every tissue and species [31,32].

The different functions of DCs are thought to be performed by different subsets. cDC1s are effective cross-presenters of exogenous antigens to $CD8^+$ T cells and in generating short-lived T effector memory cells [33–35]. The cDC2s excel in presentation to CD4T cells and in generation of T central memory responses (T_{cm}). These DC subgroups with their specific functions are present during the steady state, but during inflammation monocytes migrate to the local tissue to form mo-DCs (inflammatory DCs); this changes the picture as moDCs and cDC2s can also crosspresent antigens quite efficiently uner conditons of inflammation [8,36,37]. Plasmacytoid DCs are mainly proficient in producing type I interferons and can stimulate CD4 and CD8 T-cell responses, particularly to virus [38]. Their antigen-presenting functions are further more boosted by the presence of antigen-specific IgGs [39,40].

12.3 Dendritic Cells and T-cell Polarization

One of the major ways by which DCs influence the inception and maintenance of inflammatory disease is by causing the polarization of naïve CD4 Th0 cells into Th1 cells (making IFNg), Th2 cells (making IL-4, IL-5, and IL-13), Th17 cells (making IL-17 and IL-22), and Treg cells (making IL-10 and TGFβ). The developmental driving force for this has been the tuning of T-cell responses to help clearing infectious threats, but the various extremes of Th-cell polarization are also often associated with allergic (Th2) and autoimmune (Th17) inflammatory diseases. To cause this polarized Th response, DCs express particular costimulatory molecules and produce Th-polarzing cytokines such as IL-1, IL-6, and IL-23 for Th17 development; IL-12 for Th1 development; and TGFβ and IL-10 for Treg development. The precise signals that determine Th2 development are much less clear.

Just like DCs control many aspects of immunity, it is equally important that tolerance mechanisms be installed for ubiquitous environmental antigens such as food antigens or commonly inhaled antigens such as allergens (derived from animal dander, plant pollen, etc.). These are intrinsically inert proteins to which an immune response would be futile and potentially harmfull to the host, causing

inflammation. It has been shown that intestinal DCs are major determinants of oral tolerance mechanisms. First of all, it was shown that intestinal DCs continuously migrate from the lamina propria of the small and large intestine to the mesenteric lymph nodes, where self- and food allergens are presented to naïve T cells that subsequently develop into $Foxp3^+$ iTregs [41–46]. The intestinal CX3CR1+ macrophages of the intestinal villi probe the oral antigens and hand them over to intestinal $CD103^+$ cDCs via gap junctions [47,48]. Intestinal $CD103^+$ cDCs employ various mechanisms to instruct iTreg development in naïve T cells, the most important one being the production of retinoic acid through the enzyme aldehyde dehydrogenase (RALDH), which instructs iTreg development, and a program that ensures that these cells return to the gut mucosa, as well as a differentiation program in B cells that ensures IgA production [49–57]. This program in CD103+ cDCs is driven largely by contact with commensal microbiota [58,59].

In the lung, similar mechanisms of tolerance to commonly inhaled antigens are operative. It was shown that exposure of mice to harmless antigens like ovalbumin is able to subsequently suppress vaccine response to OVA in adjuvant and even prevent the onset of allergic asthma initiated by OVA [60,61]. This induction of tolerance was initially shown to be mediated by DCs, expressing ICOS ligand and producing IL-10 [62]. It was subsequently shown that plasmacytoid DCs are necessary and sufficient to induce inhalation tolerance via induction of Treg cells [63–65]. On the other hand, CD103+ cDCs of the lung that produce RALDH are also able to induce tolerance to inhaled allergens, via induction of Tregs [48,66].

12.4 Dendritic Cells in Allergic Inflammation

12.4.1 Dendritic Cells are Necessary and Sufficient for Induction of Th2 Immunity

The contribution of DCs to the induction of Th2 immune responses has mainly been studied in models of asthma and allergic rhinitis [67], as well as in models employing the proteinase papain [68–70]. Allergic asthma is caused by continuous inhalation of allergens, which leads to chronic eosinophil-rich inflammation, goblet cell hyper/metaplasia, and bronchial hyperreactivity. The end result of these alterations is airway obstruction. Typically, IgE synthesis, eosinophil expansion and activation, mucus overproduction, and hyperreactivity of smooth muscle cells are driven by the cytokines IL-4, IL-5, and IL-13 that are produced by Th2 lymphocytes [71–73]. A crucial contribution for DCs in allergy induction is not so surprising as allergy is dominated by an adaptive Th2 lymphocyte response. When bone marrow DCs or DCs obtained from the spleen were cultured with GM-CSF and pulsed with antigen and then injected into the lungs of mice, they caused sensitization and led to eosinophilic inflammation and asthma features when the mice were challenged with the antigen [74,75]. Some induction of Th17 cells also occurs in this adoptive transfer model, refflecting the mixed Th2/Th17 profile seen in human

asthma [75]. Moreover, repeated injection of DCs into the lungs induces irreversible airway remodeling involving subbasement membrane deposition of components of the extracellular matrix [76]. Likewise, when DCs from the lungs of mice exposed to allergen are transferred to naive recipients, they can induce sensitization and features of asthma [77–79].

The studies described above demonstrate the ability of DCs to induce Th2 immunity in the lung. Recent studies have also demonstrated that induction of Th2 immunity, bronchial hyperreactivity, and goblet cell metaplasia in response to allergens all require lung DCs. These manifestations were abolished when lung DCs were depleted in CD11c-DTR transgenic mice when they were first exposed to the inhaled allergen, house dust mite (HDM) [77]. Studies using sensitization models based on the use of the enzymatic model allergen papain or on helminth allergens indicate that DCs are required for a proper Th2 immune response [68–70,80]. Whether innate ILC2 and DCs interact functionally in asthma has not been studied. In two recent papers, Halim *et al.* showed that in *Rorα*$^{sg/sg}$ mice or ICOS-T mice (lacking ILC2s but not Th2 cells), both the early innate and the late Th2-mediated heightened eosinophilic and IgE responses to inhalation with papain were abolished, pointing to an enforcing role for ILC2s in adaptive Th2 immunity [81,82]. The most striking observation of the study is that ILC2s promote Th2 immunity not through IL-4 (which they hardly produce) but by provision of IL-13. Th2 immunity to papain in *Rorα*$^{sg/sg}$ mice is restored by wild-type ILC2s but not by IL-13-deficient ILC2s. IL-13 stimulates migration of activated DCs to the lung draining nodes, pointing for the first time that ILC2s and DCs cooperate to promote adaptive Th2 immunity [81].

In the context of allergy, it has been repeatedly suggested that basophils and eosinophils are also capable of antigen presentation and that they alone might be able to induce allergic responses [70,83–85]. However, an antibody to the high-affinity IgE receptor FcεRI was used in these studies to deplete basophils, and it is possible that DCs were depleted as well [77]. Direct evidence for the ability of human basophils to present antigen and trigger Th2 immune responses is scanty [86]. More likely, basophils cooperate with DCs to prime for Th2 immunity, possibly by providing IL-4 at an early stage [77,87]. When lung eosinophils were directly compared with DCs, they did not demonstrate a strong antigen presentation function, except maybe for primed Th2 cells [88,89].

12.4.2 Different Subtypes of DCs Induce Th2 Immunity

There has been interest in recent years to know if lung DC subsets induce Th1, Th17, or Th2 responses in different ways. Subsets of CD103$^+$ and CD11b$^+$ cDC sorted *ex vivo* and stimulated with the model allergen OVA induced Th1 and Th2 responses, respectively [90]. This issue was also investigated *in vivo*, but sorting subsets during inflammation is daunting. Monocyte-derived DCs are rapidly recruited to the inflammation site in a CCR2-dependent way. The monocytic marker Ly6C is commonly used to differentiate between CD11b$^+$ Mo-DCs and CD11b$^+$ cDCs. But because Ly6C is downregulated when mo-DCs enter inflamed

tissue, conventional surface marker staining could not distinguish between mo-DC and CD11b cDCs. Better markers are needed, and we and other researchers recently reported that mo-DCs express FcεRI (recognized by MAR-1), CD64, and MerTK, but CD11b cDCs do not [79]. This approach was used to sort DC subsets from the LN of HDM-sensitized mice. Both $CD11b^+$ cDCs and $CD64^+$ moDCs could sensitize acceptor mice after adoptive intratracheal transfer, but $CD103^+$ DCs could not. Mice deficient in Flt3L lacked all cDC subsets and also failed to induce Th2 immunity to low doses of HDM allergen. However, *Flt3l*$^{-/-}$ mice still mounted Th2 immunity when a high dose of HDM was used, thus recruiting moDCs to the lung. Depletion of $CD103^+$ in langerin-DTR mice did not abolish the hallmarks of asthma, confirming the redundancy of the role of CD103+ DCs in the HDM-driven asthma model [79]. The fact that $CD11b^+$ cDCs seem to be necessary for Th2 immunity was also recently confirmed in *CD11c*Cre x *Irf4*$^{fl/fl}$ mice that selectively lack this subset of cDCs. These mice failed to mount Th2 immunity driven by OVA and the Th2 adjuvant alum [91].

Following injection of papain in the skin, basophils collaborate with DCs to induce Th2 immunity [70,84]. Papain is captured by $CD11b^+$ cDCs that also express macrophage galactose-type C-type lectin 2 (CD301b) in the skin and various mucosae [92]. Using the promotor of this gene to drive expression of the diphteria toxin receptor, it was shown that conditional depletion of $CD301b^+$ $CD11b^+$ cDCs abolished the early generation of IL-4 producing Th2 cells to papain as well as to alum and to *Nippostrongylus brasiliensis*, although IgE responses to the parasite were intact [69]. Similarly, *CD11c*Cre x *Irf4*$^{fl/fl}$ mice lack PDL-1^+ $CD301b^+$ cDCs in the skin draining lymph nodes and there was a defect on Th2 response generation to papain [68]. Despite these reports, others have shown that DCs are not necessary to mount Th2 immunity to papain. The most likely explanation is that with very high doses of allergen, there might be other APCs that come into play [84]. For most allergens, including spores of the fungus *Alteraria* or cockroach allergens, there might be a spectrum of innate immune cells that come into play at various time points after allergen exposure. The relative contribution of these innate immune cells to induction of Th2 immunity in the lung depends a.o. on the dose of extract used, the presence of contaminating microbial products in the allergen extracts, and the timing of the experimental obervations. This has created a lot of confusion in the field.

12.4.3 Dendritic Cells are Also Required for Effector Th2 Immune Responses to Allergens

It has been adequately demonstrated that dendritic cells are both necessary and sufficient for inducing Th2 immunity in naive animals, but studies in mice from which DCs can be conditionally depleted have shown that they are also crucial during the allergen challenge period and when primed mice repeatedly encounter inhaled allergens [3,76,93]. In areas of eosinophil-rich type 2 infammation, DCs have an activated phenotype expressing higher levels of costimulatory molecules OX40L, CD80, CD86, PDL-1, and PDL-2 [93]. In a secondary immune response, DCs are found very close to effector T cells and T resident memory (T_{rm}) cells in

the regions of airways and large blood vessels [71–73,94–96]. At these sites, they might secrete chemokines to attract effector T cells or they might restimulate T_{rm} cells by providing costimulatory molecules [79,97]. Allergen-specific IgE and IgG1, by stimulating FcεRI and FcγRIII, respectively, provide strong enhancement because they target inhaled allergens to DCs in primed mice; this further boosts Th2 immunity [98,99].

Though adoptively transferred moDCs can induce Th2 immunity, they more likely play a more important role during the effector phase of the immune response. MoDCs do not express CCR7 and thus do not migrate well to the draining nodes, although a subset of moDCs might upregulate CD209a (DC-SIGN) and migrate efficiently [11]. So, they likely interact with effector Th2 cells that migrate back to the lung or with T_{rm} cells [100]. The observed attraction of effector Th2 cells by chemokines produced by moDCs provides support of this model [79,97,101]. They are also the predominant APC expressing FcεRI and RcγRIII. The exact role of PDL-2 expression on DCs is currently unclear. PDL-2 was identified as a marker for the IRF-4-dependent Th2 inducing DCs of the skin [68].

12.4.4 DCs Get Activated Directly after Exposure to Allergen

Steady-state DCs are immature and must be activated and undergo maturation before they can migrate to the LN (in a CCR7-dependent manner) and initiate an immune response. DCs sense their environment via PRR, such as TLRs, NOD-like receptors, RIG-I-like receptors, and C-type lectin receptors [102,103]. PPRs can detect various molecular patterns on microbes, called pathogen-associated molecular patterns (PAMPs), as well as molecules released by necrotic cells, called damage-associated molecular patterns (DAMPs). DCs also produce different cytokine receptors that can induce activation after they are secreted in an autocrine or paracrine manner. There is evidence that most allergens can activate different classes of PRRs, but the actual Th immune response might depend on the allergen, again creating some confusion in the field [102,103]. For example, glycans in the peanut glycoallergen ara h 1 can mediate DC activation and Th2 induction through DC-SIGN; in contrast, triggering DC-SIGN by the HDM allergen Der p 1 promotes Th1 immunity instead [104,105]. The mannose receptor on DCs mediates the uptake of various allergens through their carbohydrate moieties, leading to Th2 polarization [106]. Triggering of the C-type lectin receptor dectin-2 on DCs by HDM induces the production of cysteinyl-leuktrienes (CysLT), Th2 attractive chemokines, and costimulatory molecules [101,107]. CysLTs then affect DC functioning in an autocrine manner through positive effects on the CysLT(1)R receptor and negative effects through CysLT(2)R. Mice deficient in CysLT(2)R developed a considerably stronger Th2 immune response to HDM, and adoptive transfer of DCs lacking CysLT(2)R had a similar effect. One of the main HDM allergens, Der p 2, is a functional homologue of MD-2, the lipopolysaccharide (LPS)-binding component of the Toll-like receptor (TLR) 4 signaling complex, and so it amplifies TLR4 signaling and DC activation [108].

It is not quite clear how activated DCs, after migrating to the draining nodes, prime for Th2 immune responses because IL-4, which drives Th2 differentiation, is not produced by DCs. The situation is different for cytokines involved in Th1 and Th17 differentiation, namely, TGF-β, IL-6, IL-12, and IL-23. It is possible that Th2 differentiation is the default pathway unless DCs produce Th1 or Th17 polarizing cytokines. Otherwise, ligands interacting with Notch receptors on Th cells might provide a direct polarizing signal for induction of Th2 immune responses [109,110]. Also, the B7 family costimulatory molecule PDL2 was identified as a marker of Th2-inducing DCs, although it remains to be shown if signaling via PD1 on T cells boosts Th2 immunity [68]. It is believed that after migrating to the draining lymph nodes, DCs move to the T-cell zone, where they activate T cells. But based on experiments in a model of *H*eligmosomoides *polygyrus* infection, Leon *et al.* [111] recently challenged the applicability of this proposition to Th2 immunity. They showed that B cells in the draining lymph nodes of infected mice secreted CXCL13 and recruited CXCR5-expressing DCs and T cells, resulting in DC–T interactions outside the T-cell zone. Mice lacking CXCR5 on T cells or on DCs had impaired Th2 immunity against *H. polygyrus* infection, indicating that these interactions are required for optimal Th2 development [111]. It is not known if this model is valid for all Th2-dependent immune responses. Polarizing pro-Th2 cytokines might also be delivered in trans from innate immune cells like basophils, mast cells, or ILC2s (as for IL-13 in the papain response).

12.4.5 Lung DCs Activated Indirectly by Epithelial Cells

Although DCs express PPRs that can directly sense the environment, recent studies have shown that the epithelium is as important for activation of DCs following exposure to allergens [112]. This is certainly true for environmental adjuvants such as diesel particles, fine dust particles, and toxic gases like NO_2, all of which can promote Th2/TH17 sensitization by DCs. Epithelial cells provide more than a physical barrier, because they express TLRs, C-type lectin, and protease-activated PARs [113]. We and others have recently shown that DC activation requires TLR4 triggering on epithelial cells. In radiation-chimera mice lacking TLR4 on radiosensitive hematopoietic cells or radioresistant epithelial cells, the activation and migration of DCs depended on signals emanating from the radioresistant cells following HDM administration. On the other hand, HDM-driven allergic asthma could not be induced in epithelial cells lacking TLR4 [110,114]. Similar effects have been described for other TLR ligands like flagellin acting on TLR5 [115]. The effects of triggering TLR4 seem to depend on the dose, because a high dose of endotoxin can also suppress the development of an allergic response to HDM [116]. Airway epithelial cells produce the cytokines TSLP, IL-33, IL-25, and GM-CSF in a TLR4-dependent manner. These cytokines are essential for DC activation and allergic Th2 sensitization. The relative importance of these cytokines is related to the type of allergen or respiratory adjuvant, which has caused some confusion in the field [117,118]. TSLP is made not only by epithelial cells but also directly by DCs, and it acts on DCs and T cells to promote Th2 immunity [119]. In mice lacking STAT5 (a crucial intermediary

of TSLP signaling) only in DCs, Th2 immune response induction is severely defective. This phenotype resembles that of TSLPR-deficient mice, indicating that TSLP acts mainly through its effects on DCs that require STAT5 to respond to it [120]. IL-33 too affects mainly the activation DCs and ILC2. Production of IL-33 by lung epithelial cells is strictly regulated; under homeostatic conditions, it is suppressed by the recognition and clearance of apoptotic cells [121]. When epithelial cells are exposed to allergen, they also produce trefoil factor 2 (TFF-2), which further increases the production of IL-33 in DCs and epithelial cells [122]. Necrotic cells too can release IL-33, but there is not much in the literature about substantial necrotic cell death in asthma. Moreover, lung macrophages and DCs can also produce IL-33, and allergen-specific IgG1 immune complexes triggering the FcγRIII receptor greatly enhance this process [99].

Besides the well-known role of innate pro-Th2 cytokines, we and others recently demonstrated a central role for IL-1α in the cross-talk between epithelial cells and DCs in HDM allergy. In a manner that was dependent on the NLRP3 inflammasome and caspase-1, HDM triggered epithelial cells to produce IL-1α, and in an autocrine fashion led to the release of IL-33, TSLP, and GM-CSF. Cytokines downstream of IL-1α, for example, GM-CSF and IL-33, also played a role in DC activation. Consequently, mice-deficient in IL-1R were resistant to the asthma caused by HDM [123]. In contrast to this predominant Il-1α role in DC activation, others reported that IL-1β and caspase-1 mediated the adjuvant effects on DC activation and Th2 development in a model of NO_2-enhanced airway inflammation.

There are many studies on how the innate pro-Th2 cytokines are produced in response to various allergens. ECs do produce cytokines, but they may also activate DCs by producing DAMPS in response to allergens. For example, ECs can induce DC activation by producing uric acid (UA), which is released in response to inhaled HDM in both mice and humans and plays a role in increasing cytokine production [124]. UA crystals are classical activators of the NLRP3 inflammasome, but mice deficient in *Nlrp3* or *Asc* had normal HDM and UA-induced Th2 responses in the lung. Another inflammasome believed to negatively regulate other inflammasomes is NLRP10. This inflammasome was found to have a function in the induction of Th2 immune responses by regulating the ability of DCs to migrate to the draining nodes [125].

If epithelial cells are so decisive in controlling the activation of DCs, one can speculate that the threshold for recognition of allergens by epithelial cells is an important check point in the development of Th2 sensitization to inhaled allergens. Many of the receptors expressed by the epithelila cells are TLRs and IL-1R family members, whose signaling cascades are also controlled by negative feedback mechanisms. One such mechanism involves the deubiquitinating molecule TNFAIP3, also known as A20. This enzyme turns off activating lysine-63 (K63) ubiquitin tags from key signaling intermediates in the TLR and IL-1R signaling pathway. Overexpression of A20 and of its binding partner ABIN1 (TNIP1) in the lungs of mice was sufficient to suppress all the salient features of asthma [126,127]. Recently, a genetic polymorphism in TNIP1 and in TNFAIP3 was found to be associated with development of asthma [128,129]. It will be interesting to see how this molecule is regulated and how it affects

epithelial-DC biology. Children growing up on farms are protected from developing allergy. We recently found that farm dust extract induces the expression of TNFAIP3 in lung epithelial cells of mice, and has the potential to suppress HDM allergic asthma in a TNFAIP3-dependent manner. Strikingly, children growing up on farms and still developing asthma had a single nucleotide polymorphism in the TNFAIP3 gene that led to lower levels of the protein, and epithelial cells of asthmatics produced lower levels of TNAIP3 protein [129].

12.5 Inflammatory DCs' Role in Human Allergy

Monocyte-derived DCs have been grown *in vitro* for decades, but they have been hard to trace in human samples. Nevertheless, a recent study identified and characterized inflammatory (Infl)DCs in general terms. Besides expressing CD11c and HLA-DR, (Infl)DCs expressed CD206, CD11b, Sirpα, CD14, CD1a, and FcεR1. Analysis by microarray showed that the inflDCs were enriched for an *in vitro* mo-DC gene signature. This indicates that these inflDCs were derived from monocytes and that they were the *in vivo* counterparts of moDCs. The infDCs produced Th17 polarizing cytokines and preferentially induced Th17 cells. Whether the nature of the pathologic condition affects infDCs T helper lymphocyte polarization is not yet known [37].

Inflammatory FcεR1$^+$ DCs are present not only in allergic lungs but also in the skin of atopic dermatitis patients, in which case they are named inflammatory dendritic epidermal cell [130]. A phenotypically similar cell type has also been observed in the lungs of humans undergoing lung surgery, but we do not know if larger numbers of these moDCs exist in asthma patients [131]. But it is known that circulating and tissue resident DCs of patients with asthma or allergy have higher levels of FcγRI armed with IgE [132,133].

12.6 Dendritic Cells in Autoimmune Inflammatory Disease

Developing autoreactive CD4$^+$ T cells are normally deleted during negative selection in the thymus, yet many tissue antigen-specific autoreactive T cells escape from the negative selection check point, emerging as anergic cells, thymic regulatory T cells (tTreg), or ignorant conventional T cells (Tconv) [134–136]. Dendritic cells have been implicated in controlling negative selection of developing thymocytes, as expression of MHCII exclusively on CD11c+ cells is enough to delete self-reactive cells [137]. Activation of self-reactive CD4 T cells that escape negative selection is a major culprit in organ-directed autoimmune disease, and therefore T cell tolerance to some tissue-restricted self-antigens is shaped entirely by nondeletional processes outside the thymus [138]. The suppression of potentially pathogenic autoreactive CD4$^+$ T helper cells is dependent on peripheral DCs that present tissue-restricted self-antigens derived

mostly from turnover of cells that die by apoptosis [139,140]. In the absence of inflammation, self-antigen presentation by immature DCs results in T cell anergy, the further expansion of natural tTregs or the generation of induced (i) Tregs from Tconv cells, thus promoting peripheral tolerance to the self-antigen [6,141,142]. Not surprisingly, the constitutive lack of steady-state DCs results in a loss of functional Tregs and the subsequent activation of autoreactive Th1/Th17 effector $CD4^+$ T cells, while a constitutive increase in the numbers of immature DCs results in increased iTreg numbers, which prevent autoimmunity [143,144]. It is, however, equally important that DCs presenting self-antigen remain in a quiescent state, devoid of costimulatory molecules or cytokine production that can break peripheral tolerance [145–151]. Removal of inhibitory NF-κB signaling intermediates and inhibitory apoptotic cell receptors on DCs, or artificial extension of DC lifespan, is sufficient to break self-tolerance [152–158]. As only one example, the loss of the deubiquitinating enzyme TNFAIP3 (also known as A20) selectively in CD11c+ dendritic cells leads to spontaneous activation of DCs throughout the body and a failure of DCs to be suppressed by apoptotic cells. Normally, apoptotic cell-derived self-antigen is not presented in an immunogenic maaner to T cells. In DCs lacking A20, apoptotic cell-derived self-antigen was presented to CD4T cells that developed into Th17 producing effector cells. Not surprisingly, mice lacking A20 in DCs developed signs of severe autoimmunity resembling lupus, with development of membranoproliferative glomerulonephritis, thrombocytopenia, and habitual abortion, in conjunction with double-stranded DNA antibodies and antibodies to the phospholipid cardiolipin [154].

Despite these experiments clearly implicating DCs in the regulation of peripheral tolerance and induction of autoimmunity, the precise triggers that can cause activation of DCs and potentially break tolerance to self are incompletely understood [6]. Certain organ-directed infections trigger tissue-specific autoimmunity by promoting the activation of DCs either through direct infection, the triggering of pattern recognition receptors such as Toll-like receptors on DCs, or release of cytokines that causes full DC maturation [159–161]. It has also been proposed that endogenous danger signals like uric acid, ATP, or HMGB1 can lead to activation of DCs in response to sterile tissue injury [162]. These danger signals can be actively secreted from inflammatory cells recruited to the site of sterile injury or directly released from dying cells. It was also shown that exposure of DCs to necrotic cells, but not apoptotic cells, is sufficient to drive DC maturation *in vitro* [163]; however, it is not known if and how sterile inflammation caused by tissue ischemia and necrosis is a driver of tissue restricted autoimmunity *in vivo*.

12.7 Conclusions

It is now clear that there are many subsets of DCs that do much more than just induce primary CD4 and CD8 T-cell immunity. As we review here, DCs are major drivers of autoimmune and allergic inflammation, but it is very likely that they are

at the basis of basically every form of chronic and persistent inflammation. In the future, the challenge will be to selectively target key functions of DCs without undermining the induction of antimicrobial immunity. Alternatively, exploiting the tolerogenic potential of DCs might offer long-term cure for allergic and autoimmune inflammatory disease.

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13

Roles for NK Cells and ILC1 in Inflammation and Infection

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13.1 Introduction

For roughly three decades (1975-2008), NK cells occupied a unique space in the hierarchy of immune defense being the only lymphoid cell subset that retained a close developmental relationship with B and T cells of the adaptive immune system, but could develop independent of the antigen receptor recombinase machinery. This changed recently when several independent reports described peculiar, rare innate lymphocyte populations with "hardwired" effector functions, similar to NK cells [1]. It was subsequently shown that all of these cells comprise a related family of lymphoid lineage cells now referred to as innate lymphoid cells (ILCs) [2,3]. ILCs can be classified into three distinct groups: group 1 ILCs comprise cells that express the transcription factor (TF) T-bet and produce the cytokine interferon gamma (IFNγ). Group 2 ILCs produce type 2 cytokines IL-5, IL-9, and IL-13 and require the TFs GATA-binding protein 3 (GATA3) and retinoic acid orphan receptor alpha (RORα) for their development and function. Finally, group 3 ILCs comprise cells expressing the TF RORγ and producing the cytokines IL-17A and/or IL-22 [4].

All ILCs require cytokines of the γ_c-family (especially IL-2, IL-7, and IL-15) for their development and homeostasis. Nevertheless, NK cells have special features, including granule-mediated cytotoxicity and a capacity to recirculate throughout the body in contrast with other ILCs that appear as tissue-resident [5,6].

NK cells are proposed both to participate in the immune responses to a variety of infectious agents, including viruses, bacteria, and parasites, and to act as modulators of autoimmunity and chronic inflammation [7]. Still, the precise role played by NK cells in these different disease situations remains poorly defined. Over the years, diverse mouse models have been proposed as tools to study the biology of NK cells *in vivo* [8]). Still, many of these models suffer from incomplete NK cell depletion or show effects on other non-NK hematopoietic cell types, thereby clouding interpretations. While more recent studies have allowed for direct targeting of NK cells using "NK cell-specific" gene expression (i.e., NKp46

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expression using the Ncr1 locus), we now know that this gene is also expressed in some γδ T cells, in a subset of intestinal ILC3 and in ILC1 [9,10] that share many properties with "conventional" NK cells. With the identification of ILC1s as a distinct effector subset, it will be important to reassess previous conclusions about NK cell biology. Being able to distinguish between NK cell functions and ILC1 functions in immune responses, however, will be challenging given the phenotypic and functional similarity of NK cells to ILC1s [4,6,10-12].

13.2 Distinguishing Group 1 ILC and NK Cells

Rag-independent innate lymphocytes that are $Tbet^{+}IFN\gamma^{+}$ include previously characterized NK cells and ILC1 in both mice and man. Mouse NK cells are recognized as $CD3^{-}NKp46^{+}$ cells or as $CD3^{-}NK1.1^{+}$ cells (in C57BL/6 or C57BL/10 mice). While the antibody DX5 recognizing CD49b (integrin α2) has also been used to identify NK cells [13], a subset of hepatic NK cells lacks this marker [14]. Liver $DX5^{-}$ NK cells were initially proposed as immature NK cells [14], although recent evidence suggests that they represent a distinct NK subset lacking Eomesodermin (Eomes) [11,12]. A similar $Eomes^{-}$ $NKp46^{+}$ cell subset exists in the mouse intestinal tract and bone marrow [10] with lower cytotoxic activity but stronger cytokine production [10,15]. As such, they resembled thymic $IL\text{-}7R^{+}$ NK cells [16] and were termed "ILC1" to distinguish them from classical NK cells. NK cells and ILC1s express NKp46 (along with NK1.1 in B6 mice) as well as NKG2D, DNAM-1 (CD226), 2B4 (CD244), and NKG2A [6,11,17,18]. In some tissues, mouse NK cells and ILC1s show differential expression of IL-7R, CD49b, and CXCR6 (Figure 13.1).

Human NK cells are $CD3^{-}CD56^{+}$ cells and can be divided into $CD56^{bright}CD16^{-}$ and $CD56^{dim}CD16^{+}$ subsets [19]. $CD56^{dim}$ NK cells predominate in the blood, while $CD56^{bright}$ prevail in lymphoid tissues, especially LN [19]. Atypical $CD3^{-}CD56^{+}$ subsets exist ($NKp44^{+}CD103^{+}$ and $NKp44^{-}CD103^{-}$) in human tonsils that produced IFNγ upon stimulation with IL-12 and IL-15 *in vitro*,

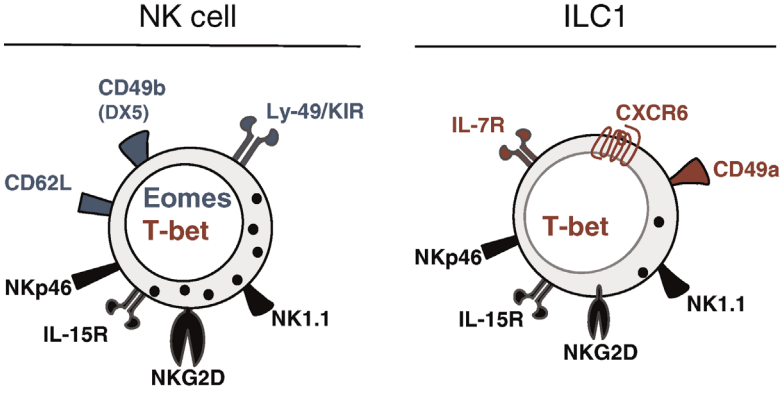


Figure 13.1 Cell surface markers and signature transcription factors expressed by NK cells and ILC1s. Relative expression level is indicated by the size of the corresponding receptor.

but were unresponsive to IL-18 [15]. Based on their expression of CXCR6 and their mucosal location, these cells were termed ILC1s, although they clearly expressed the TF Eomes [15]. These human ILC1 expressed NKG2D, CD244, and CD94, but only $KIR^{-/lo}$, DNAM-1^{lo}, and $NKp44^{+}CD103^{+}$ cells expressed NKp30, distinguishing them from blood NK cells [15]. Unlike other ILC subsets, these atypical $CD3^{-}CD56^{+}$ cells lack IL-7R expression.

More recently, human hepatic $CD56^{bright}$ NK cells have been described expressing CD49b (a2 integrin) and T-bet but lacking Eomes expression. Liver $CD49b^{+}$ NK cells expressed activating receptors (NKp46, NKp30, NKG2D, and DNAM-1) and produced IFNγ, TNF, and GM-CSF upon stimulation [20]. As such, these cells could represent the human counterpart to hepatic ILC1s in the mouse.

Finally, a human $CD161^{+}$ ILC1 subset has been identified that expresses IL-7R but lacks most NK cell markers (CD56, NKp46, NKp44, CD16, and CD94); these cells produce IFNγ but do express neither perforin nor granzyme B, the prototypical cytotoxic molecules expressed by circulating NK cells [21]. Human $CD161^{+}$ ILC1 express T-bet but not Eomes, and *in vitro* culture experiments suggest that they may derive - at least in part - from ILC3s under the influence of the proinflammatory cytokines IL-12 and IL-18 [21].

13.3 NK Cell and ILC1 Activating and Inhibitory Receptors

NK cells express a wide array of germ line-encoded surface receptors that convey either activating or inhibitory signals upon ligand binding. The balance between these opposing pathways "tune" the reactivity of NK cells to have optimal capacity to react to infected, "stressed," "foreign," or transformed cells while remaining tolerant of normal, healthy cells. Engagement of these different activating or inhibitory pathways may occur during development: this process of NK cell "education" has been extensively studied in the context of MHC class I inhibitory receptors (see below) but may also apply to activating receptors.

13.3.1 Natural Cytotoxicity Receptors

Natural cytotoxicity receptors (NCRs) represent a major group of activating receptors expressed by NK cells. Human NK cells express three NCRs, NKp46 encoded by *NCR1*, NKp44 encoded by *NCR2*, and NKp30 encoded by *NCR3* [22]. Of note, NK cells express NKp44 only upon activation [19]. Mouse NK cells express only NKp46. NCRs belong to the immunoglobulin (Ig) superfamily and signal via adapter proteins containing immunoreceptor tyrosine-based activation motifs (ITAMs) [22].

NCRs have been shown to bind viral hemagglutanins (HA) from various viruses, including influenza A (IFA) virus [23-25], vaccinia virus (VV), and ectromelia virus [26], as well as antigens from other viruses, including HCMV [27,28] and West Nile virus (WNV) [29]. Moreover, nonviral NCR ligands can be expressed following infection by *Mycobacteria* [30-32] or *Fusobacterium nucleatum* [33]. NKp46 activation induces cytokine production in NK cells and ILC1 [11].

13.3.2 NKG2D

The majority of NK cells as well as ILC1s express NKG2D homodimers (encoded by Klrk1) [11,12,16,19]. Two mouse NKG2D isoforms exist: $NKG2D_L$ with a long cytoplasmic tail signals via DAP10, while $NKG2D_S$ couples to either DAP10 or DAP12 [34,35]. Human NK cells express a long isoform signaling via DAP10 [36]. NKG2D ligands include stress-induced self-proteins share their structural relation to MHC-I molecules. These include MHC class I chain-related gene (MIC)-A and MIC-B, and the UL16-binding proteins 1-4 in humans, and the retinoic acid early transcript 1 (Rae-1) molecules, murine UL-16 binding-like transcript-1 (MULT-1), and the histocompatibility 60 (H60) family of proteins in mouse [37]. Activation via NKG2D induces cytokine production and cytotoxicity by NK cells [38] and IFNγ production by ILC1 [11].

13.3.3 DNAX Accessory Molecule-1 (DNAM-1, CD226)

The Ig superfamily member DNAX accessory molecule-1 (DNAM-1) is expressed by around 50% of mature NK cells and at higher density on all hepatic ILC1s [17]. DNAM-1 ligands include CD155 (poliovirus receptor, PVR) and CD112 (nectin-2) [39] that trigger NK cell cytotoxicity and cytokine production [39,40]. These ligands are upregulated following cellular stress (e.g., after DNA damage) [41,42] similar to NKG2D ligands and may play a role in antitumor and antiviral immunity [43]. The strong DNAM-1 expression by ILC1 could indicate an important role of this receptor in tissue surveillance.

13.3.4 MHC Class I-specific Receptors and NK Cell "Education"

NK cells express two main classes of MHC class I (MHC-I)-specific receptors that can be either activating or inhibitory depending on the intracellular signaling pathways to which they are coupled. The first set recognize "classical" class I molecules (HLA-A, B, C, and H-2D, K, and L). In mice, these receptors are encoded by distinct genes in the "Ly49 complex" (killer cell lectin-like receptors subfamily A gene family or *Klra*). In humans, they are encoded by killer immunoglobulin-like receptors (KIR). In both species, expression of the activating receptors is restricted to NK cells, while inhibitory receptors can be detected on T cells and myeloid cells [44,45]. Inhibitory receptors bind to different MHC-I haplotypes with variable affinities that depend on peptide occupancy [46,47]. A second set of MHC-I receptors include heterodimers comprised of CD94 and members of the NKG2 family. These receptors bind to nonclassical MHC-I molecules, Qa-1 in mice [48] and HLA-E in man [49]. As cell surface expression of these nonclassical MHC-I requires peptides derived from classical MHC-I, the CD94/NKG2 receptors recognize MHC-I molecules in an indirect fashion. Binding to CD94-NKG2A generates an inhibitory signal, while CD94-NKG2C and CD94-NKG2E engagement results in NK cell activation [50].

NK cells require interactions with class I MHC molecules within the stromal microenvironment in order to gain full functional competence [51-53]. This mechanism is generally referred to as NK cell "education" or "licensing" [54,55]. This poorly understood process is not a simple "on-off" switch, but occurs

progressively; the "rheostat model" proposes an integration of inhibitory and activating signals based on interactions with class I MHC by differentiating NK cell that determines their subsequent cellular responsiveness [56]. Non-MHC-I-based self-receptors also appear to affect this process [57]. Since Ly49 and KIR molecules are expressed in a stochastic manner at the single-cell level [58,59], NK cell education generates a heterogeneous pool of NK cells with different functional capacities. Most NK cells express one-three and up to six class I-specific MHC receptors, although a substantial subset NK cells exists (10-15%) that do not express inhibitory receptors for self-MHC-I receptors and are thus "uneducated" [51,52]. "Uneducated" NK are functionally hyporesponsive and phenotypically indistinguishable (except for the lack of self-MHC-I receptors) from "educated" NK cells [52,55]. Education is not linked to a particular stage during NK cell development but appears to be a perpetual process allowing NK cells to incessantly adapt to their environment. It has been shown that "uneducated" NK cells from MHC-I-deficient mice gain functional competence when placed in a MHC-I-sufficient environment [60,61], while "educated" NK cells become hyporesponsive in a MHC-I-deficient environment [61].

Inflammatory conditions associated with viral infections can promote responsiveness in otherwise "uneducated" NK cells [52,54,55,62,63]. In mouse cytomegalovirus (MCMV) infection, "uneducated" NK cells have superior functional capacities compared to "educated" NK cells in the defense against this virus [62].

13.3.5 Are ILC1s "Educated" Similar to Convention NK Cells?

As indicated above, ILC1 express a variety of activating and inhibitory receptors that could play a role in determining functional competence of this innate effector subset. The vast majority of ILC1 express NCRs, NKG2D, DNAM-1, and CD94-NKG2A heterodimers [12,17,64]. In mouse, ILC1 express Ly49C/I, I, F, and G2 at lower frequencies compared to NK cells [10,12,18,64]. Curiously, Ly49E is predominantly expressed by ILC1s starting in embryonic life [64], although its biological relevance is unclear. In man, ILC1 express a restricted set of KIR molecules [15] with hepatic $CD49a^{+}$ NK cells expressing an oligoclonal KIR repertoire that might indicate selective "antigen-driven" expansion [20].

It is not clear whether the mechanism of "education" that has been described in NK cells also operates in ILC1s. As education seems to enable NK cells with the capacity to distinguish self from nonself (i.e., to become activated when encountering a "nonself" or "self-MHC-I-negative" target and eventually eliminate it). As ILC1s have not been yet implicated in these types of immune responses and lack granule-mediated cytotoxicity, it is not immediately clear why ILC1s would need to be educated or rendered self-tolerant to class I MHC.

13.4 Evidence for Distinct Subsets of NK Cells and ILC1

Current models for NK cell maturation make use of differential expression of surface markers (CD27 (TNFRSF7) and CD11b (Mac-1) to define NK cell subsets with distinct functional properties [65]. $CD27^{+}CD11b^{-}$ NK cells prevail in BM

and LN, while $CD27^{-}CD11b^{+}$ NK cells predominate in blood, spleen, and lungs [66]. $CD27^{+}CD11b^{-}$ NK cells are proposed as the least differentiated cells, while $CD27^{+}CD11b^{+}$ NK cells have the highest cytokine secretion and cytotoxic potential. In contrast, $CD27^{-}CD11b^{+}$ NK cells are terminally differentiated cells with reduced effector functions [65].

ILC1s are present in various organs, including liver, small intestinal lamina propria (siLPL), siIEL, uterus, salivary gland (SVG), and - at lower frequencies - in the spleen, bone marrow, and skin [6,10-12,18]. How ILC1 from these different tissues relate to each other is not known. For example, only hepatic and SVG ILC1s have been shown to express TRAIL [14,67], and ILC1s from all sites express high levels of IL-7Rα except those from the liver [10,11]. Hepatic ILC1s express high levels of CXCR3, while siLPL ILC1s express higher levels of CD69 and CCR9 [18]. It is not clear whether these represent true ILC1 subsets or adaptation to specific tissue microenvironments. ILC1s predominate over conventional NK cells in the gut and uterus [10,12], whereas in lung and skin ILC1s represent less than 5 and 20%, respectively, of $CD3^{-}NKp46^{+}NK1.1^{+}$ cells [12]. In liver, NK and ILC1 coexist at roughly equal frequencies, although in spatially distinct regions with ILC1 restricted to the liver sinusoids [6]. Curiously, all ILC1 express CD27 and lack CD11b [6,10-12] and, therefore, resemble undifferentiated NK cells.

13.5 Effector Functions of NK Cells and ILC1

NK cells possess two principal effector functions: cytotoxicity and cytokine/chemokine secretion [68]. Interferon gamma (IFNγ) is abundantly produced by NK cells and exerts pleiotropic activities on target cells and other hematopoietic (myeloid, macrophage) and nonhematopoietic (epithelial, stromal) cells. NK cell cytotoxicity requires cell-to-cell contact between NK and target cells and can be mediated via granule- or TNF superfamily- (FasL, TRAIL) mediated pathways. Recently, activated liver ILC1s were shown to express granzyme B and perforin, to degranulate CD107a and to lyze YAC-1 tumor cells *in vitro* [6,11,12,18]. As such, granule-mediated killing may be inducible in hepatic ILC1, although it is not clear whether this is involved in antitumor responses *in vivo*.

Mature NK cells carry preformed mRNA encoding IFNγ and RANTES [69] allowing them to rapidly secrete cytokines. Such properties necessitate tight control. As with NK cell "education," effector functions in mature NK cells are regulated by balancing activating and inhibitory signals (Figure 13.2). Under steady-state conditions, normal expression of self MHC-I overrides most NK activation signals. However, during infection, MHC-I may be downregulated in infected cells, unleashing inhibitory "missing self" pathways and allowing for NK cell-mediated killing. In addition, cellular stress can induce NKG2D ligands that override MHC-I inhibition [70]. DNAM-1 ligands might be regulated in a similar manner [42,43], although it is not clear whether they are able to override self MHC-I-driven inhibitory signals.

Importantly, NK cell activation not only relies on cell surface receptor signaling but also on stimulation via cytokines, including IL-2, IL-12, IL-18, IL-21, or IFN I.

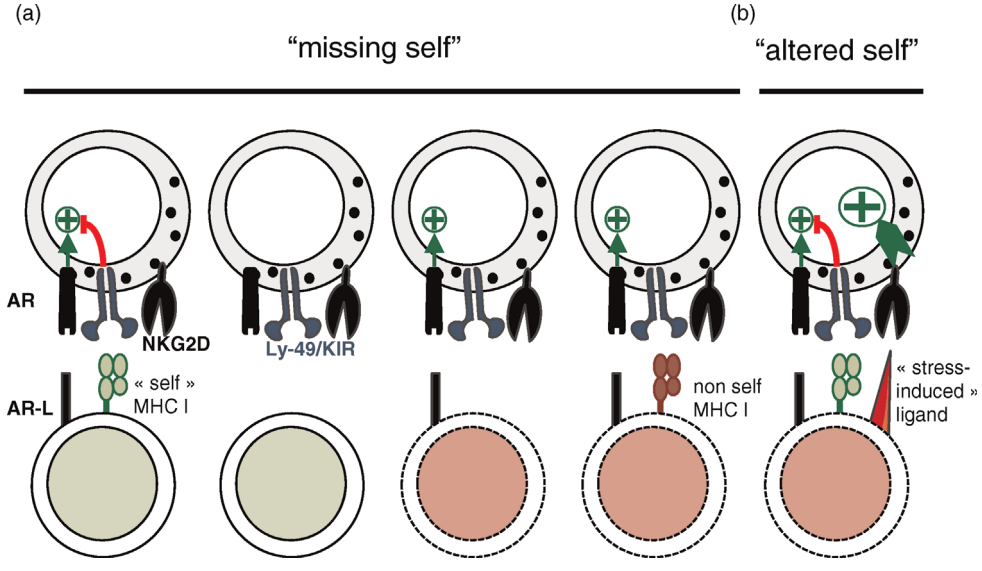


Figure 13.2 Activating and inhibitory signals regulate NK cell activity. "Missing self" (a): NK cells can be stimulated via activating receptors but simultaneously receive inhibitory signals via MHC-I receptors. As a result, NK cells spare target cells (far left). If target cells lack MHC-I expression, NK cell inhibition is released and NK cell target elimination depends on the presence of activating signals that can include target cell nonself MHC I molecules (second from right). "Altered self" (b): cellular stress induces the expression of NKG2D ligands. Activation via NKG2D can override inhibitory signals from MHC I receptors resulting in the elimination of MHC I^+ target cells. AR: activating receptor; AR-L - ligand of an activating receptor.

Combinations of IL-12 with any of the other cytokines are sufficient for NK cell activation in the absence of signals from cell surface receptors.

The full range of activating and inhibitory signals that can regulate effector functions in ILC1 are not known. Much of the published literature has assessed the ability of ILC1 to secrete cytokines (including IFNγ and TNF) in response to soluble factors such as IL-12 and IL-18. Although an exhaustive side-by-side comparison of cytokine production by NK cells and ILC1 from the same tissue has not been performed, it is clear that both are potent producers of IFNγ, while TNF production is enhanced in ILC1 [11,12]. Little is known about the ability of activating receptors (NK1.1, NKG2D, NKp46, and DNAM-1) to stimulate effector functions in ILC1, although cytokine production can be induced in hepatic ILC1 following triggering of NK1.1, NKG2D, or NKp46 [11]. Whether or not ILC1s need to be primed (by inflammatory signals, cytokines, and chemokines) in order to reach full functional competence remains unknown. While some the MHC-I-specific inhibitory receptors are expressed by tissue ILC1, their role in "tuning" cellular responses is unclear. ILC1 express receptors for several soluble factors (IL-10R, TGFβR, and IFNα/βR) that could ultimately modulate their functional properties before, during, or after activation [71].

13.6 NK Cells and Group 1 ILCs in Inflammation and Infection

Inflammation describes the complex process of the body's reaction to tissue damage, often associated with but not restricted to infections with pathogens. NK cells are involved in the immune response to many microbial pathogens; however, in only a few cases have the targets for NK cell recognition been described. In many cases, NK cells are recruited and/or activated via cytokines and chemokines secreted by sentinel cells (epithelial cells, macrophages, and dendritic cells) that initiate immune responses following pathogen detection. NK cell activation and immunity against several different pathogens will be discussed in the next sections.

Sterile inflammation (in the absence of any obvious pathogen) occurs as well, when tissue damage is the consequence of physical, chemical, or metabolic stress [72]. A good example is the case of diet-induced stress that accompanies obesity where systemic inflammation can be observed in visceral adipose tissue (VAT) [73]. An anti-inflammatory state is normally maintained in adipose tissue by a coordinated trio of cells, including alternatively activated (M2) tissue macrophages, ILC2, and eosinophils [74,75]. High-fat diet induces stress in adipocytes leading to the upregulation of NKp46 ligands, resulting in NK cell activation, expansion, and IFNγ production [73]. As a result, adipose tissue macrophages polarize toward a proinflammatory M1 phenotype resulting in the development of a chronic inflammation within the VAT [74,75]. The resultant changes in VAT function lead to insulin resistance and are a risk factor for the development of type 2 diabetes [73].

13.6.1 Mouse Cytomegalovirus

The immune response to infection with cytomegalovirus (CMV) has become a paradigm for studying NK cell functions in mice and man [76]. Cytomegaloviruses are large DNA viruses belonging to the beta-subfamily of Herpesviridae. Several species of cytomegaloviruses have been identified that have a highly restricted host range, including human CMV (also known as human herpesvirus 5 HH5) and MCMV. In immunocompetent individuals, infection with CMV is almost asymptomatic, while it poses a serious threat for immunocompromised individuals [76]. The important role of NK cells in the defense against herpes viruses is evident from patients lacking NK cells or NK cell functions [77]. These patients suffer from severe disease often leading to death [77]. However, even though NK cells are important they are often not sufficient to clear the virus. This requires the coordinated activity of innate and adaptive lymphocytes, especially virus-specific $CD8^+$ T cells (reviewed in [78]).

Strain-dependent genetic differences determine the susceptibility to MCMV. For example, B6 mice are resistant, while BALB/c mice are highly susceptible [78]. The molecular basis for these differences was mapped to the *cmv-1* gene that encodes the activating Ly49H receptor [79]. As indicated above, Ly49 is a member of a family that recognizes MHC class I protein complexes, and it was shown that Ly49H specifically interacts with the MCMV-encoded protein m157 [80,81]. NK cells triggered through Ly49H are amplified (also via cytokines) during the early

phase after infection, with secretion of IFNγ and cytotoxicity that restricts viral spread and allows the adaptive immune response to unfold [82,83].

While mouse and human CMV are rather rapidly cleared from most organs, both can persist for weeks or months in the salivary glands where the virus replicates in the acinar glandular epithelial cells and disseminates via shedding through the saliva. HMCV can be secreted for month following an acute infection in clinically unapparent individuals. Interestingly, NK cells in the SVG are in general hyporesponsive, which translates into much-reduced frequencies of IFNγ-producing NK cells in SVG compared to other organs during acute MCMV infection [84]. This might contribute to MCMV reservoirs within the SVG. Effector $CD4^+$ T cells are important for viral clearance from SVG [85]. However, only lytic virus is cleared from infected individuals, while latent forms persist a lifetime. CMV latency and viral reactivation represents a constant threat for the immune system that can also explain the impact of CMV exposure on global immune signatures [86].

Immune surveillance during MCMV latency is a dynamic situation that requires coordination between innate and adaptive lymphocytes [87]. Effector memory CD8T cells specific for certain viral epitopes gradually increase in CMV latency (a process also termed "memory inflation") [88,89]. This may be driven by limited stochastic viral reactivation [90] or focal transcriptional activities [87] allowing for brief antigen reencounter at barely detectable levels [91]. Depletion of CD8+ T cells or NK cells during viral latency resulted in rapid reactivation of virus within the salivary gland that rapidly reaches the systemic circulation [92]. The same mechanisms leading to CD8T cell memory inflation might also be implicated in the prolonged persistence of "memory" NK (see more below).

The involvement of ILC1s to the anti-MCMV response is not clearly defined. Their lack of Ly49H expression precludes their ability to mount a specific response to m157. ILC1 may possess other receptors that allow for interactions with other CMV viral proteins expressed on productively infected cells. Moreover, given the peculiar ability of hepatic ILC1s to mount recall responses (see more below), they might be able to respond in the context of viral latency. Finally, as tissue-resident cells, ILC1 can rapidly respond to "stress" signals from diverse sentinels enabling them to participate in the early-phase response to MCMV.

13.6.2 Influenza Virus

Influenza A (IFA) is an enveloped RNA virus of the Orthomyxoviridae family. Various serotypes exist based on the type of envelope-encoded hemagglutinins and neuraminidases (NAs), defining HA and NA subtypes (Hynix nomenclature). Direct recognition of IFA-encoded HA by NK cells via NCRs (NKp30 and NKp44 in humans, and NKp46 both in humans and in mice) has been reported [23-25]. HA recognition is essential for the NK cell-mediated viral control in the mouse [93] and mutations in HA allow influenza virus to escape NK cell recognition via NKp46 [94]. Removal of sialic acid residues from viral NA impairs NKp46- [95] and NKp44-mediated [96] recognition.

IFA mainly replicates in the lower airway. In the steady-state, this tissue is rather lymphocyte poor, but interestingly NK cells represent around 10% of

lymphoid cells, which is the highest frequency observed in any tissue [66]. Both beneficial [93] and detrimental [97] roles for NK cells in the anti-IFA response have been reported that are probably due to dosage effects.

13.6.3 NK Cells and Other Viral Infections

NK cells have been shown to become activated during the immune response following infection with Orthopoxviruses (that belong to the Poxviridae family). Two well studied orthopoxviruses are vaccinia virus and ectromelia virus (ECTV, mousepox). B6 mice resist ECTV infection through several mechanisms, including pathways involving CD94 [98]. As such, $CD94^{-/-}$ mice succumb to ECTV infection presumably due to defective CD94-NKG2E function on NK cells [98]. However, this conclusion needs to be revisited in light of studies using $NKG2A^{-/-}$ mice. These mice are highly susceptible to ECTV, which was shown to be independent of NK cell but dependent of $CD8^{+}$ T cell, likely due to a protective role of CD94-NKG2A heterodimers for the function of these adaptive CTL [99].

The implication of NK cells during infections in humans with other viruses including human immune deficiency virus (HIV) (reviewed in [100]), hepatitis C virus (reviewed in [101], hepatitis B virus (reviewed in [102]), dengue virus (reviewed in [103]), and Epstein-Barr virus [104] are well documented. It is out of scope to revisit the literature on human NK cells in these different viral infections, but it is important to underline the fact that defining clear roles for NK cells during the immune response to these infections is quite challenging given the limited access to tissues where the virus is actively replicating and/or achieves latency. In most cases, studies are limited to blood or end-stage diseased tissues. The complex multicellular nature of the early immune responses within draining lymph nodes or acutely inflamed tissues is rarely studied. Another reason for our limited understanding of NK cell responses to these human pathogens is the absence of an accurate small animal model. As these pathogens infect targets with a strict species range (due to species-specific receptors and host restriction mechanisms), normal mouse strains are not permissive. The advent of optimized humanized mouse models holds great promise for future studies of these pathogens [105], especially for studies involving pathogens such HCV and HBV for which compound humanized mice that carry a human immune system and a humanized liver have been recently described [106].

13.6.4 Salmonella

Salmonella spp. are Gram-negative, facultative intracellular, enterobacteria. Infection with *Salmonella* can cause gut-associated, nonsystemic acute inflammation (gastroenteritis), although certain serovars cause more severe typhoid fever [107]. Ingestion of contaminated food represents the dominant cause of infection. Once the bacteria reaches the small intestine, they cross the epithelial barrier and obtain access to the gut-associated lymphoid tissue (GLAT), including Peyer's Patches, from where they disseminate via phagocytes [108]. *Salmonella* spp. are able to survive, divide, and persist inside macrophages that accelerates bacterial spread. Once disseminated via the bloodstream and lymphatics,

endotoxic shock and death rapidly ensue [107]. It has been shown that NK cell-derived IFNγ [109] is critical for the early control of *Salmonella* infection. This cytokine potently polarizes macrophages toward the proinflammatory M1 type and activates their antibacterial activity [110,111]. Accordingly, NK cell depletion results in increased bacteria loads [112]. Another host strategy to restrict pathogen spread involves granuloma formation that assemble a variety of hematopoietic cells (macrophages, neutrophils, NK cells, and T cells). During *Salmonella* infection, granuloma formation and persistence requires TNF [107] that is largely contributed by neutrophils and to a lesser extent by NK cells [113]. The "ex-ILC3" ILC1 are a source of IFNγ in the context of *Salmonella* infection [114] and may also contribute to granuloma formation.

13.6.5 Listeria monocytogenes

Infections with *Listeria monocytogenes* (Gram-positive, facultative intracellular bacteria) result from the ingestion of contaminated food. After crossing the gut epithelial barrier, *L. monocytogenes* reaches the bloodstream and disseminates to the spleen and liver [115]. Innate and adaptive immune cells control *L. monocytogenes* infection [116]. Initial defense involves neutrophils, macrophages, and NK cells [116-118], followed by $CD8^+$ T cells that ensure sterilizing immunity [119]. *L. monocytogenes* are taken up by macrophages but escape destruction via listeriolysin O that destroys the phagosome membrane allowing *L. monocytogenes* to access the cytosol [120]. IFNγ has been shown to be crucial to avoid listeriosis [121] and NK cells secrete this cytokine in an IL-18-dependent manner, thus contributing to the early control of *L. monocytogenes* infections [122]. IFNγ activates the bactericidal activity of macrophages [117] helping to restrict bacterial spread. TNF is crucial for early control of *L. monocytogenes* [123,124], while TRAIL deficiency has been shown to increase the resistance and reduce hepatic pathology [125]. Given that hepatic ILC1s express TRAIL, this might indicate a potential detrimental role for these cells in the defense against *L. monocytogenes.*

13.6.6 Mycobacteria and Other Bacteria

Mycobacterium tuberculosis causes tuberculosis, which frequently involvers the respiratory tract following airborne exposure. Alveolar macrophages phagocytose *M. tuberculosis* leading to the recruitment of neutrophils, NK cells, and T cells. However, the initial immune response is often not successful and *M. tuberculosis* can further disseminate via macrophages and DC to draining lymph nodes and beyond. In most *M. tuberculosis* cases, granulomas are formed that contain the bacteria and generate latent persistent infections. NK cells have been shown to contribute to the anti-*M. tuberculosis* immune response not only by secretion of cytokines (IFNγ and TNF) [126,127] but also through direct bacterial killing via secretion of granulysin [128]. NK cells can lyze *M. tuberculosis*-infected alveolar macrophages and monocytes, a process that appears to involve NK cell recognition of infected cells via NKp46 [31,129] and NKG2D [32]. Taken together, these studies show that NK cells play an active role in restricting *M. tuberculosis*.

Activation of NK cells upon infection with other bacteria, including *Staphyloccocus aureus* [130], *Rickettsia* spp [131]., *Legionella* spp [132]., and *Shigella* spp [133]. has been documented. In these cases, NK cell-derived IFNγ appears to contribute to immune defense against the invading microbe, although some reports suggest that NK cell depletion results in better pathogen clearance (reviewed in [134]).

13.7 NK Cell and ILC1 Memory

While immunological memory is classically associated with adaptive immunity mediated by antigen-specific B and T cells, recent reports have described "adaptive features" of innate immune cells, including those mediated by NK cells (reviewed in [135,136]. As described above, NK cells specifically recognize MCMV in certain mouse strains through the activating Ly49H receptor that specifically interacts with the viral protein m157 [79-81,137,138]. Ly49H activates through an ITAM-based intracellular signaling cascade (similar to antigen receptors), and MCMV was subsequently shown to selectively expand $Ly49H^+$ NK cells [138,139]. Interestingly, virally expanded $Ly49H^+$ NK cells persist for at least 70 days after infection (in contrast with the $Ly49H^-$ subset), suggesting that they represent "memory" NK cells [139]. Moreover, these memory NK cells have enhanced functional capacities compared to "naïve" NK cells ("adaptive features"). Taken together, these results identify a new aspect of innate lymphoid cell "memory" that mirrors the adaptive T-cell response in several important ways. Nevertheless, there are some important differences between MCMV-induced NK-cell and T-cell memory, including the clonal frequency of responding cells (50% for NK cells versus 10^{-5} for T cells), the duration (weeks for NK cells versus months/years for T cells) [140] and the changes in effector functions (about twofold for naïve versus memory NK cells) [139]. Since the molecular signature associated with memory NK cells involves a strong STAT4 response (indicative of IL-12 signaling) and IL-12 blockade abolishes NK cell memory [141], it suggests that inflammatory signals are essential. Whether NK cell memory represents an intrinsic property of NK cells or an environmentally regulated process dependent on inflammatory status remains to be fully explored.

The second type of NK cell memory driven by cytokine stimulation has been described. Exposure of NK cells to IL-12, IL-15, IL-18, and/or IFN results in the generation of long-lived NK cells that produce cytokines more rapidly after restimulation [142,143]. Phenotypically, these NK cells are similar to naïve NK cells and have been termed "cytokine-induced memory-like" (CIML) NK cells. Given their enhanced activities, CIML NK cells might be useful for immunotherapeutic approaches. It remains to be shown if CIML cells are generated *in vivo* and if a similar phenomenon exists for ILC1.

The third type of NK cell "memory" was described in the context of recall responses to haptens in a skin contact hypersensitivity model. Antigens included both classical haptens (DNP) and viral peptides (influenza, VSV) and virus-like particles [6,144,145]. The response could be elicited in $Rag^{-/-}$ mice (excluding a role for adaptive B and T cells), and NK cells were shown to have the capacity to

transfer the contact hypersensitivity phenotype to *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (that are deficient in NK cells and all ILCs). Curiously, only NK cells from the liver were able to perform this type of "memory" responses and this was mapped to subset of hepatic NK cells expressing CXCR6. The specific receptor-mediated hapten-dependent NK cell memory remains undefined. This report suggests that innate lymphocytes can provide "recall" responses in nonlymphoid tissues.

It is interesting to compare and contrast these three situations of NK cell "memory" in light of the dichotomy between NK cells and ILC1. Inflammation is a common theme and suggests that environmental signals, especially elaboration of soluble factors (IL-6, IL-12, IL-18, and IFNs) play an essential role. The necessity for Ly49H in the MCMV model indicates that conventional NK cells and not ILC1 are involved since the latter do not express this receptor [11,12]. Similarly, alloreactivity-mediated through Ly49D would be restricted to NK cells [146]. In contrast, the requirement for CXCR6 in the skin contact hypersensitivity model strongly suggests that ILC1 are involved and not conventional NK cells. Taken together, these observations provide evidence for tissue conditioning of NK and ILC1 phenotypes and functions that may result in "long-term" reactivity for these innate cells. It will be interesting to know if "memory" NK or ILC1 responses play a role in immune responses to other viral, bacterial, or parasitic pathogens or in antitumor immunity.

13.8 Concluding Remarks: NK and ILC1 as Partners in a Type 1 Network

NK cells exert strong effector functions (including cytotoxicity and cytokine secretion) in response to *in vitro* stimulation by tumor cells, viruses, bacteria, and parasites [7,101,147,148] (Figure 13.3). Moreover, some of the signals and pathways that lead to NK cell activation and controlling NK cell effector functions have been identified. Still, NK cells do not act in an isolated manner *in vivo*. Immune reactions are complex and require coordinated activities of multiple diverse cellular actors within tissue-specific microenvironments. Although direct pathogen recognition by NK cells has been described (see above), in many cases of infection and in most cases of inflammation, the specific ligand for NK cell activation is not known. Rather, microbial pathogens are generally detected by macrophages and/or DC, and neutrophil influx occurs subsequent to tissue damage. As such, NK cell-mediated immune responses appear more or less secondary to pathogen-induced alarm signals that involve cytokines such as IL-12, IL-15, and IL-18 that are crucial for the activation of NK cells [149-151] and that also have the capacity to activate ILC1s [6,11,18]. IL-12 is mainly produced by activated DC [152-155], macrophages [156], neutrophils [157], and mast cells subsequent to microbial activation [158]. IL-15 is produced by many cell types, including DC [159-161], macrophages [161], neutrophils [162], and adipocytes [163] and is presented via the IL-15Rα chain "*in trans*" to NK cells [164]. IL-15 is essential for NK cell homeostasis [165-169] and priming [170]. IL-18 is constitutively expressed by barrier cell types [171], including epithelial cells from

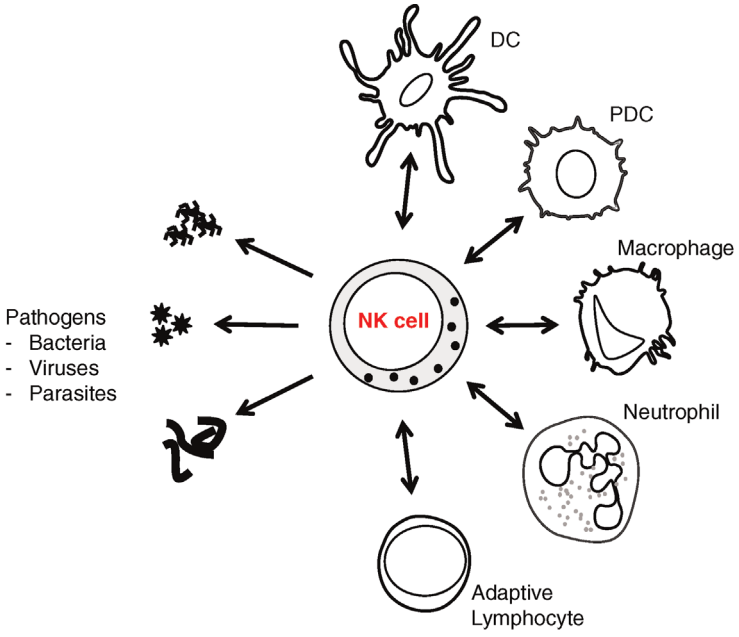


Figure 13.3 NK cells as a "hub" in the type 1 immune network. NK cells interact reciprocally with a diverse set of immune cells. NK cells and ILC1 thereby regulate immune responses to various pathogens, including bacteria, viruses, and parasites.

gut and lung, keratinocytes from skin, and endothelial cells, and also by blood monocytes, macrophages, neutrophils [162,172], and DCs [173]. Its production can be increased upon activation [171] and has also been implicated in the priming of NK cells [174]. As such, NK cells are subject to control by other innate immune cells, including DC, macrophages, and neutrophils, and also by CD4+ T cells that regulate NK cell activity [175].

The interactions of NK cells with innate and adaptive immune cells are far from unidirectional [176-179]. NK cells effector functions impact directly or indirectly on cells within their microenvironment and subsequently influence and regulate them. NK cell-derived IFNγ activates innate and adaptive immune cells [180], induces the maturation, differentiation, and functional capacities of DCs [177,181-183], induces antimicrobial activities of macrophages [110,111] and neutrophils [183], increases the lifespan of neutrophils [184], impacts Ig class switching of B cells [185], and recruits and polarizes T cells toward a Th1-type [187,188]. Activated NK cells have been shown to kill immature DCs [183,189,190], thereby regulating unfolding immune responses and to kill activated $CD4^+$ T cells and consequently indirectly regulating the antiviral $CD8^+$ T cell response and preventing pathology [191]. Similarly, the early restriction of viral load during infection with MCMV prevents pathology as it dampens pDC activation, preserving DC function, and assuring optimal CD8+ T cell control of the virus [82,83,192]. SVG NK cells might help prevent pathology as NK cells reduced $CD4^+$ T cell responses in a TRAIL-dependent manner, while in the

absence of TRAIL MCMV infected mice harbored higher titers of autoantibodies that mimicked Sjogren's syndrome [67].

NK cells and ILC1 have multiple and dynamic connections to the network of innate and adaptive hematopoietic cells that participate in diverse immune reactions. Interactions between these two prominent IFNγ-producing innate lymphocytes with stromal cells, antigen presenting cells, myeloid cells, and adaptive lymphocytes generate a myriad of bidirectional consequences, which focus and intensify immune responses. These intimately intertwined innate lymphoid cells provide the host with an "explosive" mix of defense mechanisms that together provide rapid protection from a large spectrum of potential pathogens. Regulation of innate immune effectors is an important area of future research. Understanding mechanisms of regulation could help improve treatment of autoimmune disease and avoid immune senescence.

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14

Group 2 and 3 Innate Lymphoid Cells: New Actors in Immunity and Inflammation

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14.1 ILCs: A New Family of Innate Effector Cells

Inflammation is associated with many physiological processes, including immune defense, tissue repair, and metabolic regulation [1]. The inflammatory reaction is a complex reaction involving immune cells, soluble factors, and tissue injury in response to infection or trauma; the duration of inflammation can be transient with resolution and restoration of tissue homeostasis or chronic, with tissue dysplasia that can lead to fibrosis or metaplasia. This range of inflammatory outcomes is particularly evident at mucosal sites [2–4]. Recently, a novel family of effector lymphocytes, called innate lymphoid cells (ILCs), has been described that are intimately involved in the inflammatory process [5,6]. ILCs have morphological characteristics of lymphoid cells but lack rearranged antigen receptors, such as the T or B cell receptor, and lack markers associated with myeloid cells and granulocytes. In contrast, ILCs express transcription factors (TF), surface markers, and effector molecules expressed by T helper (T_H) cells or natural killer (NK) cells and can rapidly respond to environment signals with massive production of proinflammatory and immunoregulatory factors [5,6]. As such, ILCs are innate effectors poised to rapidly influence the outcome of immunity following inflammation or infection.

Three major subsets of ILC can be defined by the expression of specific TFs that drive their functional effector programs [6]. Group 1 ILC population (or ILC1) consists of NK cells and others interferon-γ (IFN-γ) producing ILCs require the TFs eomesodermin (EOMES) and/or T-bet (see Chapter 13). Group 2 ILCs (or ILC2) are T_H2-associated cytokines producing ILCs (interleukin (IL) -4, IL-5, and IL-13) that require the TFs GATA-binding protein 3 (GATA-3) and the nuclear hormone receptor retinoic acid (RA) related orphan receptor RORα. Finally, group 3 (or ILC3) includes the cells that express and require the TF RORγt and can produce T_H17-associated cytokines IL-17A and IL-22. Despite these differences, all ILCs share the same lymphoid progenitor and expression of the common γ chain (γ_c; CD132), IL-7 receptor (IL7Rα; CD127), α chain of IL-2 receptor (IL-2Rα: CD25),

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and Thy-1 (CD90) markers [5,6]. Recent studies in human and mouse models have revealed that ILC have a fundamental role in the inflammatory processes. Here, we discuss the development and heterogeneity of ILC2 and ILC3 and their roles in initiation, regulation, and resolution of the inflammatory reaction.

14.2 ILC2 and ILC3 Heterogeneity and Homeostasis

ILC Lineage Commitment

ILC develop in the fetal liver and adult bone marrow following activation of specific transcriptional programs in hematopoietic progenitors [7]. ILCs are generated by the progressive conversion of common lymphoid progenitors (CLPs) into mature ILC subsets via ILC-restricted intermediates [8–11]. Several TFs are involved in CLP generation and prime the lymphoid program including the TFs Ikaros [12], PU.1 [13], and GFI1 (growth factor-independent 1) [14,15]. As a result, CLP have potential to differentiate into all subsets of lymphoid cells, including B and T cells of the adaptive immune system as well as ILCs. Differentiation of adaptive lymphocytes involves genetic modification associated with the generation of antigen-specific receptors. In contrast, ILC develop independent of the recombinase machinery [7,16] suggesting a branching of ILC versus B/T development from CLP at an early stage. The "E" protein family of TF (including E2A) strongly promotes the B/T cell programs; this activity is offset by inhibitors of DNA binding (Id proteins), thereby providing a molecular mechanism to control ILC versus B/T development [17]. Accordingly, the development of all ILCs requires ID2 [18–21]. Interestingly, ablation of other TFs has been shown to strongly impact the development of multiple ILC subsets, including nuclear factor interleukin-3s (NFIL3)-regulated protein [21–25], GATA-binding protein 3 (GATA-3) [26–29], thymocyte selection-associated high-mobility group box protein (TOX) [30,31], and T cell factor 1 (TCF-1) [32]. Taken together, expression of this combination TF "signature" (ID2, GATA-3, NFIL3, TOX, and TCF-1) appears to define the factors that regulate commitment to the ILC lineage and exclusion to the B- and T-cell pathway.

The specific functions and interactions of these TFs in ILC development are not well characterized. Early ILC progenitors (EILPs) are TCF-1^+ but lack IL-7 receptor expression and are largely $ID2^{lo}$ [32]. EILP are proposed to generate committed ILC precursors (ILCP) that are IL-$7R^+$ and $ID2^+$ [10,11,25,33]. How this occurs is not known, but it has been suggested that NFIL3 and IL-7 signaling [25], supported by TOX [31], is required to initiate ID2 expression. ILCP include α-lymphoid precursors (αLP; Lin^- $Id2^+$ $\alpha4\beta7^+$ $CXCR6^+$ cells) [24] and common helper ILCP (CHILP, common helper innate lymphoid progenitor; Lin^- $Id2^+$ $\alpha4\beta7^+$ $CD117^+$ $CD25^-$ cells) [11] that have potential to generate different ILC subsets, including NK cell [11,24]. Nevertheless, the relationship between these different precursors remains unclear. Recent studies suggest that ILCP may progressively lose the ability to generate particular ILC subsets. In this case, ELIP and αLP can generate all ILC lineages (including $EOMES^+$ ILC1 that are conventional NK cells) [7], while CHILPs can give rise to all ILC subsets but lack NK potential [11]. In addition, a subset of CHILPs express the TF

promyelocytic leukemia zinc fingers (PLZF$^+$ ILCP), which have lost the ability to generate CCR6$^+$ ILC3 (including lymphoid tissue inducer cells, LTi cells) but retain potential for ILC1, ILC2, and other ILC3 subsets [10]. Taken together, these observations suggest the following pathway with successive restriction of ILC potental: EILP → αLP → CHILP → PLZF+ ILCP (Figure 14.1)

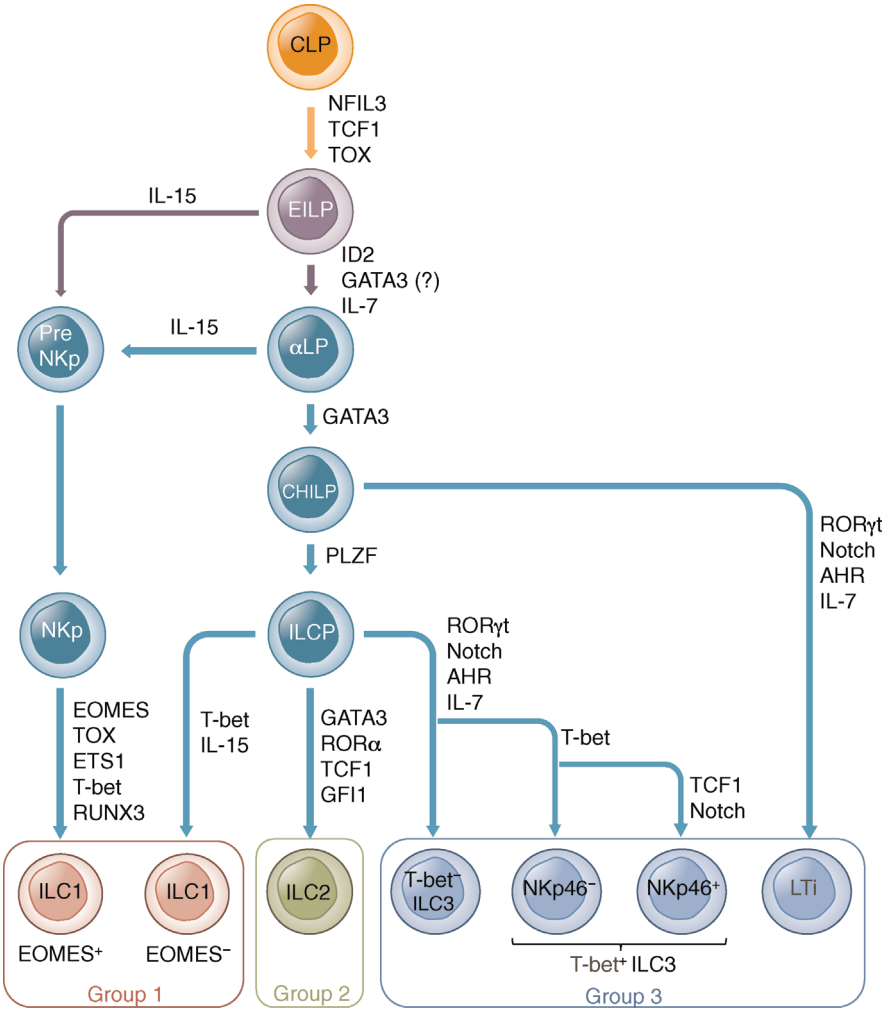


Figure 14.1 A model for ILC development. Innate lymphoid cell differentiate from common lymphoid progenitors and early ILC progenitors in fetal liver or bone marrow via prenatural killer precursors (pre-NKP) and α-lymphoid precursors. These hematopoietic intermediates are regulated by interleukin-7 and the transcription factors Nuclear factor IL-3 induced (NFIL3), inhibitor of DNA binding 2 (ID2), thymocyte selection-associated high-mobility group box protein (TOX), and GATA-binding protein 3 (GATA3). Conventional NK cells (here called EOMES$^+$ ILC1) derive from pre-NKP and NK precursor (NKP) via IL-15 under influence of several TFs including eomosodermin (EOMES), TOX, Runt-related transcription factor 3 (RUNX3), and ETS1. Downstream of the αLP, Id2$^+$ PLZF$^-$ progenitor (or CHILP) and ID2$^+$PLZF$^+$ ILC precursor (or ILCP) direct the differentiation of EOMES$^-$ ILC1, ILC2, or ILC3 subsets based on specific transcriptional programs during fetal and adult life.

Group 2 ILC

ILC2 s were initially denoted "natural helper cells," "nuocytes," or "innate helper 2 cells" [19,34,35] and express IL-7Rα, IL-25R, IL-33R, and TSLP-R and respond by abundant production of type 2 cytokines (including IL-4, IL-5, IL-9, and IL-13) and the EGFR ligand amphiregulin [19,34–36]. Human ILC2 expresses not only IL-7Rα but also the chemoattractant receptor CRTH2 and the natural killer receptor CD161 [37,38]. The TFs GATA-3 [39–42], ROR-α [43,44], GFI-1 [45], and BCL11b [46–48] contribute to ILC2 differentiation and maintenance. In mouse and human, ILC2 are distributed in the blood, spleen, bone marrow, nonlymphoid tissue, and mucosal sites such as liver, adipose tissue, lung, and intestine [19,35,40,41,49–51]. However, ILC2 s seem to be functionally heterogeneous in different tissues and differentially express some receptors including CCR6, CCR9, or KLRG1 likely representing different states of activation or maturation [52,53].

Group 3 ILC

All ILC3 are IL7Rα+ and strongly express the TFs RORγt [9,54–60] and AhR [61–63]. ILC3 s are activated by IL-1β and IL-23, and strongly produce IL-22, IL-17, lymphotoxin-alpha/beta (LTα/β), and/or GM-CSF [55–57,60,64–69]. Several ILC3 subsets have been described, including $CCR6^+$ ILC3 and subsets expressing the natural cytotoxicity receptors NKp46 (in mice and man) and NKp44 (in man) [56–59,70]. Within the $CCR6^+$ subsets are lymphoid tissue inducer cells (LTi cells) that promote formation of secondary lymphoid tissues (lymph nodes, Peyers patches, cryptopatches, and isolated lymphoid follicles) during the fetal life through production of Lt α/β and RANK ligand in response to the microbial signals [71–74] and maternal signals [75]. After birth, LTi cells also contribute to the mucosal defense through production of T_H-17-associated cytokines IL-17A and IL-22 [55,68]. NCR^+ ILC3 s, called "NK22," NCR22," "NK-LTi", or "ILC22" [56–58], and NCR^- ILC3 s [66,76,77] are massive producers of IL-22 or/and IL-17 (for NCR^- ILC3) in response to IL-23 and/or IL-1β stimulation. NCR^+ ILC3 s develop after birth in bone marrow and require the TF T-bet [76,78,79]. T-bet^+ ILC3s are rare in lymphoid tissue but are abundant in mucosal sites such as skin and intestine [56,57,59,66,76,80].

The specific versus redundant roles for $CCR6^+$ and NCR^+ ILC3 in mucosal immunity and intestinal homeostasis are not fully elucidated. Still, all ILC3 s require the presence of commensal bacteria for development notably though the TF AhR [61–63]. Concerning specific signaling pathways, Runx3 [81], Notch [9,78,79], and TGF-β [82,83] are involved in ILC3 differentiation and in the homeostasis of specific ILC3 subsets. In particular, the phenotypic and functional stability of NCR^+ ILC3 is under the control of Notch and TGF-β [82,83].

Maintenance of ILCs in Adult Tissues

Distinct ILC subsets are proposed to contribute to tissue immunosurveillance and homeostasis during the steady state and after aggression (infection, inflammation, etc.). However, the cellular source that maintains ILC pools in the tissue under these conditions remains undefined. Tissue ILCs

homeostasis may involve self-renewal or replenishment from ILC precursors that are stored in "depots" (bone marrow and lymphoid tissue). Recent studies demonstrated a longtime residency of ILC in tissues. Using parabiotic mice to establish a joint bloodstream between congenic mice, it was shown that most ILCs resided in tissues and did not circulate thought the blood. Moreover, ILCs were also locally maintained in tissues during inflammation and after parasite infection. These studies convincingly demonstrate that ILC2 and ILC3 are local tissue resident cells with apparent self-renewal capacity [84].

14.3 ILC2 in Acute and Chronic Inflammation

ILC2 s are critical players in type 2 immune responses having interactions with innate and adaptive immune cells in response to environmental cues relayed through stromal and/or epithelial cells. As such, ILC2 s are an essential part of the network that neutralizes pathogens (including parasites, viruses, etc.). Still, ILC2 responses need to be carefully controlled in order to avoid allergic or atopic inflammatory reactions (Figure 14.2).

ILC2 and Parasitic Infection

Helminth infections are a major cause of chronic human disease in the world causing morbidity or compromising host fitness by anemia and growth retardation [85]. Using mouse models, it has been shown that the cytokine IL-13 is critically involved in parasite expulsion and immune defense against helminths [86]. Recent studies using infection by *Nippostronglus brasiliensis* [19,34,35], *Trichuris muris* [87], or *Strongyloides venezuelensis* [88] have identified ILC2 as one of the most important IL-13 sources. Following infection, epithelial cells release stress mediators (including IL-33, IL-25, and/or TSLP) that activate cytokine production by ILC2 [19,34,35]. Another important ILC2 sentinel involves Tuft cells that constitutively express IL-25 and thereby maintain ILC2 homeostasis and type 2 immune responses [89–91]. ILC2 production of IL13 and amphiregulin can activate several targets, including goblet cells, which produce mucus and limit parasitic infection by production of resistin-like molecule β (RELMβ) [92,93] and the smooth muscle cells to enhance the worm expulsion [19,34,35,94]. Furthermore, the T cell-derived IL-2 [95], epithelial cell activation [19,34,35], IL-9 signaling (by autocrine manner) [96], and TGF-β1 [97] all promote ILC2 expansion, survival, and function. Together, these studies define a complex intercellular "cross-talk" involving ILC2 and nonhematopoietic cells (Figure 14.2).

While ILC2 can directly promote inflammatory reactions, they can also enhance T_H2-associated innate and adaptive immunity. In visceral adipose tissue and in the lung, IL-5-producing ILC2 s promote the accumulation of eosinophils and alternatively activated macrophage (AAM). ILC2 depletion impairs eosinophil accumulation and AAM activation during parasite infection and enhances insulin sensitivity [49]. Interestingly, murine ILC2 express MHC class II molecules [34,43] and can activate T_H cells by antigen presentation [77], similar to

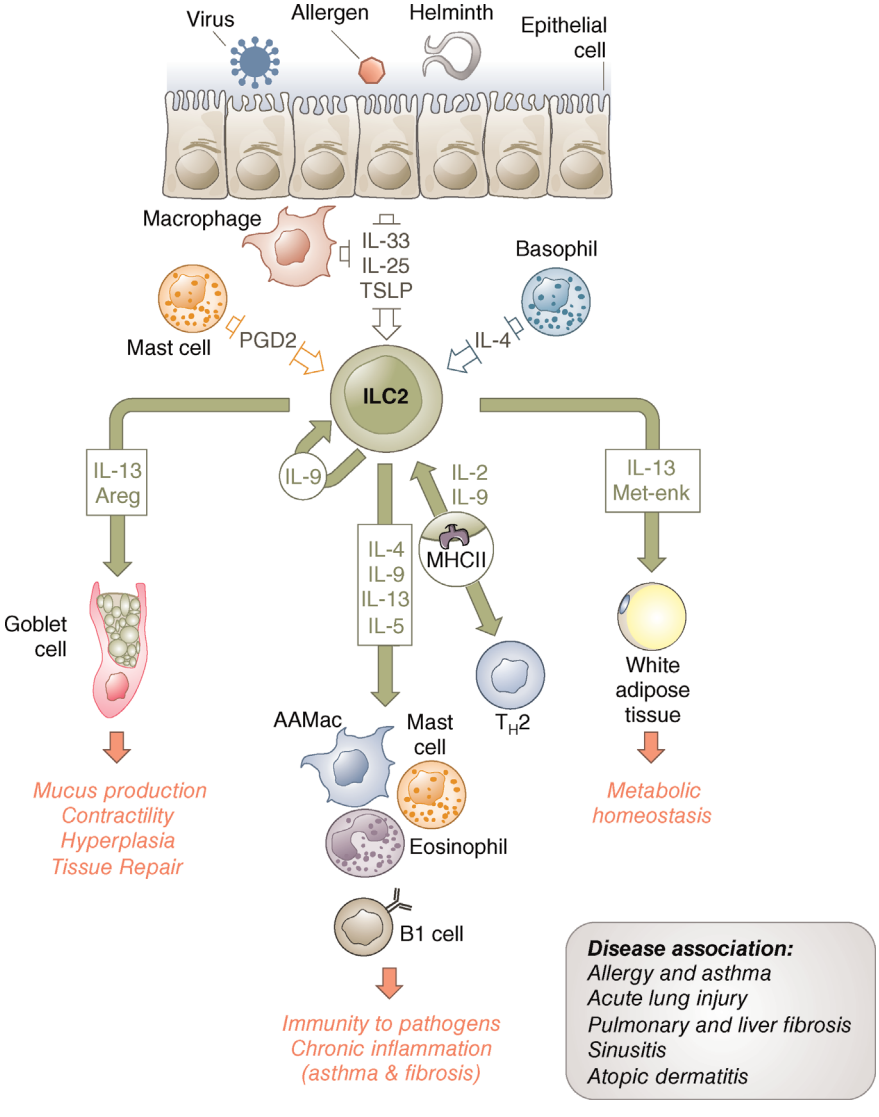


Figure 14.2 ILC2 in infection and chronic inflammation. Epithelial cells, alveolar macrophages, mast cells or/and basophils drive the activation and proliferation of ILC2 via secreted factors including IL-33, IL-25, PGD2, IL-4, and TSLP. Inflammation, allergens, and infection (helminth or viral) activate ILC2 to modulate mucus production and tissue repair through production of type 2 cytokines (IL-4, -5, -9, and -13) and amphiregulin (Areg). ILC2 activation in adipose tissue can promote metabolic homeostasis by stimulation of adipocytes with IL-13 and methionin-enkephalin peptides (Met-enk). Several pathologic conditions associated with ILC2 activation are listed.

mononuclear phagocytes. The absence of ILC2 results in impaired T_H2 responses during worm infection [98,99]. In addition, IL-2 derived from T cells contributes to the ILC2 activation and upregulates MHC expression [98]. Interestingly, ILC2 s seem to respond during dietary stress, notably in vitamin A deficiency, suggesting a link between antiworm immunity and malnutrition [87]. Moreover, recent study shows that the proportion of ILC2 is diminished in young helminth infected children but not older infected patient suggesting a protective preemptive role for ILC2. Interestingly, ILC2 deficiency can be restored after antiparasite treatment. Thus, ILC2 s have essential roles in defense against parasitic infection and blood ILC2 may represent a biomarker for effective resistance to infection [100].

ILC2 and Viral Infection

Viral infection initiates severe inflammation in tissues that can have fatal consequences. In the respiratory tract, influenza viruses replicate locally and induce tissue injury. In addition, the emergence of high pathogenicity influenza strains (such as H5N1) and their potential for pandemic spread represent a significant threat to public health. Taken together, lung-specific immune responses constitute a crucial barrier to contain and eliminate influenza virus [101]. Using a mouse model of influenza infection that acutely induced airway hyperreactivity, ILC2 s were shown to be critical in orchestrating immunity and regulating lung inflammation [36,102]. ILC2 depletion resulted in destruction of epithelial layer and impaired tissue remodeling that strongly impacted on overall lung function. IL-33 production by alveolar macrophages was found to be critical for early immune response and notably for the activation of IL-13 producing ILC2 s [102]. Furthermore, IL-33 enhanced IL-5 production from ILC2 s, which promoted the accumulation of eosinophils during the recovery phase of influenza. ILC2 were shown to be an important source of amphiregulin production that helped restore lung function, barrier integrity, and remodeling of respiratory tissues in the context of acute epithelial injury and asthma [36,94].

ILC2 and Chronic Inflammation

In blood, skin, or lung, deregulation of ILC2 activation has been characterized in multiple chronic inflammatory diseases in human or mice models such as asthma [38,103–106], allergic rhinitis [107,108], chronic rhinosinusitis associated with nasal polyps [37,109], atopic dermatitis [110,111], or pulmonary fibrosis [112]. In this inflammatory context, sentinel cells that initiate ILC2 responses include epithelial cells and various myeloid cell subsets. In a vitamin D analog-induced atopic dermatitis model [113] and in an allergen-induced lung airway inflammation model, IL-4 producing basophils could promote ILC2 proinflammatory responses [114]. In this context, IL5 and IL-13 producing ILC2 s support epithelial cell activation and the recruitment and activation of eosinophils and macrophages to promote chronic inflammation [112,115] and also enhance $CD40^+$-activated dendritic cell (DC) migration into draining lymph to support adaptive T_H2 immunity [116].

ILC2 and Metabolic Disorders

ILC2 s were first characterized in fat-associated lymphoid clusters (or "FALCs") [19]. Since then, ILC2 function within adipose tissue has become a focus of study. Nutrient and metabolic stress impact on systemic homeostasis in the context of obesity or malnutrition [117]. Obesity in human and mice is associated with low frequency of ILC2 s in adipose tissue [51]. In a model of high-fat diet that leads to the build-up of visceral adipose tissue, ILC2 activation by IL-25, IL-33, or vasoactive intestinal peptide (VIP) leads to the production of IL-13 and IL-5 [5,49,118,119]. Thus, ILC2 s appear to control the homeostasis of eosinophils and AAMs that protect the organism in the context of fat-mediated chronic inflammation. ILC2 may also control the accumulation of regulatory T cell (T_{reg}) via direct ICOS–ICOSL interactions [120]. ILC2 regulates adipose function and metabolic homeostasis in white adipose tissue notably through the production of IL-13 and methionine-enkephalin peptides [51,121,122]. This ILC2-dependent activation results in "beiging" of adipocytes that protects against insulin resistance [51,121,122]. Conversely, fat tissue regulates the activation of ILC2 s notably via IFN-γ [120] and through metabolites such as lipoxins or prostaglandins [123] (Figure 14.2).

14.4 ILC3 in Acute and Chronic Inflammation

ILC3 subsets are highly enriched in gut-associated lymphoid tissues (Peyer's patches, mesenteric lymph nodes, cryptopatches, and isolated lymphoid follicles) and are found dispersed throughout the intestinal lamina propria. In this context, ILC3 s are considered as an important contributor to intestinal immune defense to counteract microbial invasion and to maintain equilibrium between the host and the microbial symbionts. As ILC3 s can react rapidly to pathogen or tissue injury signals, they are important actors during bacterial infection or inflammatory bowel disease (IBD) (Figure 14.3).

ILC3 and Commensal or Pathogenic Bacteria

ILC3 populations have been demonstrated to play crucial roles in intestinal homeostasis. ILC3 produce IL-22 that promotes epithelial cell differentiation, glycosylation, and mucus and antimicrobial peptide production [124–126]. In this way, ILC3 s regulate selective containment of commensal bacteria and prevent systemic inflammation [127,128]. In addition, ILC3 s regulate IgA production through specific lymphotoxin α (sLTα3) and β (LTα1β2) control of T cell gut homing and DC functions, respectively [128]. Interestingly, ILC3 s also modulate immune tolerance in the intestine. Expression of MHC class II on specific ILC3 subsets negatively regulates commensal bacteria-specific T_H17 cell responses that limit intestinal inflammation [77] and induce cell death of commensal bacteria-specific $CD4^+$ T cells [129]. Deregulation of ILC3 MHCII expression is correlated with increase in T_H17 cells and commensal bacteria-specific IgG in pediatric Crohn's disease patients [129]. In addition, ILC3 s regulate the homeostasis of mononuclear phagocytes in the gut through production of the granulocyte

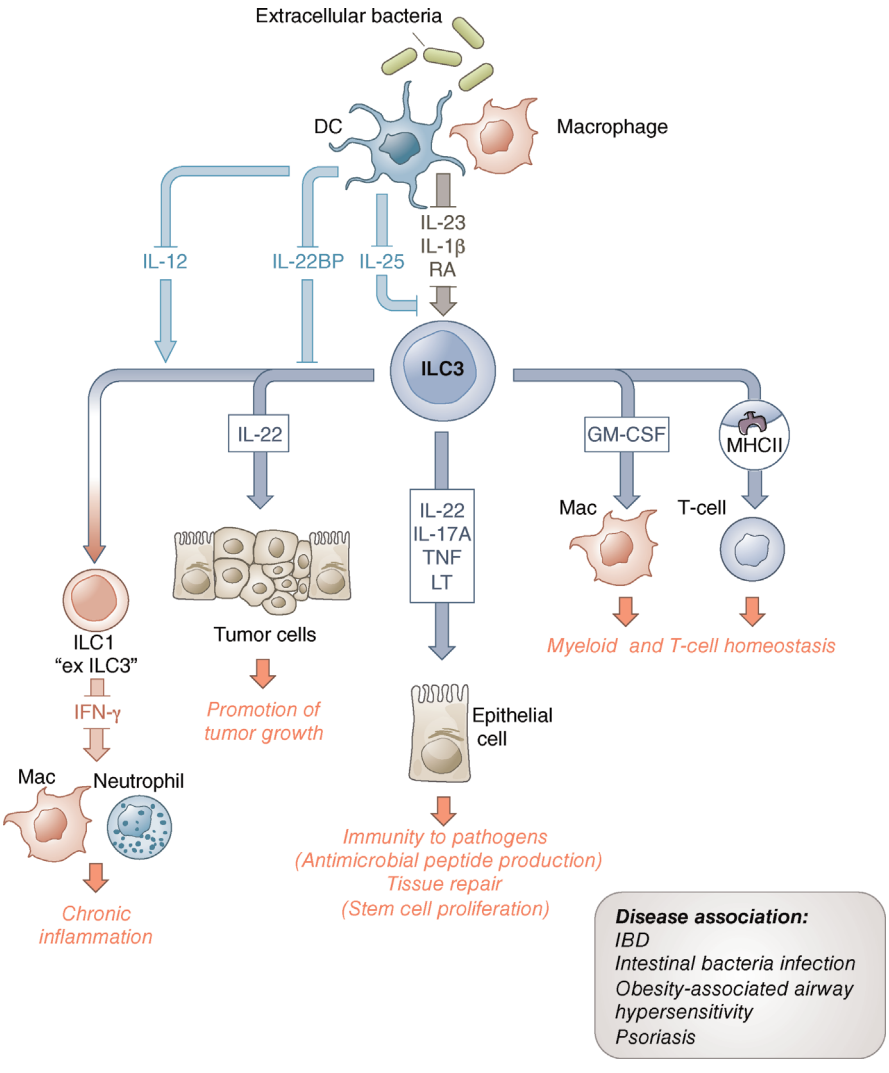


Figure 14.3 ILC3 During gut infection and inflammation. Activation of myeloid cells par microbiota or pathogenic extracellular bacteria mediates ILC3 response via IL-23, IL-1β, or RA. The production of T_H17 cytokines produced by ILC3 increases the antimicrobial activity of epithelial cells mediated by antimicrobial peptides and mucus production. In addition, IL-22 can also contribute to the tumor proliferation and inflammation in mouse colorectal cancer models. IL-25 and IL-22BP secreted by DCs represent negative regulators of this process. ILC3 production of GM-CSF and the surface expression of MHC class II can influence the homeostasis of myeloid cells and pathogenic $CD4^+$ T cells to limit intestinal inflammation.

macrophage colony-stimulating factor (GM-CSF) induced by commensal bacteria [69]. This cross-talk is essential to modulate T_{reg} responses to commensal bacteria and dietary antigens [69]. Altogether, these studies show essential role of ILC3 to maintain intestinal homeostasis that limits the commensal bacteria spread and pathological adaptive immune responses.

ILC3 s promote host protection during *Citrobacter rodentium* (enteric Gram$^-$ pathogen in mouse), *Helicobacter hepaticus* [66], *Salmonella typhimurium* [124], or *Streptococcus pneumoniae* infection [130]. In this context, ILC3 s respond to DC-derived IL-23 and IL-1β [131–133] to produce IL-17 and IL-22 that synergistically promote epithelial cell activation through the STAT3 pathway [126]. Production of chemokines CXCL1 and CXCL9 by ILC3 allows recruitment of neutrophils to the infected tissue [125,134]. Conversely, ILC3 responses can be limited by cell contact with IL-25-activated DCs [135], the presence of IL-22 binding protein (IL-22BP) [136], and through vitamin A metabolites (retinoic acid) [44,75,87,137].

ILC3, Chronic Inflammation, and Cancer

IL-22-producing ILC3 promote tissue repair and are required for protection and recovery from inflammatory colitis [135]. In humans, several inflammatory diseases (including psoriasis, IBD, or autoimmune arthritis) are associated with modulation of IL-17 and IL-22 levels and ILC3 homeostasis appears dysregulated [66,131,138,139]. This may involve changes in phenotype and function of ILC3 subsets ("plasticity") that can occur during inflammation. In mouse models, downregulation of RORγt expression in intestinal ILC3 has been observed, resulting in enhanced T-bet expression and sustained expression of IFN-γ. These RORgt$^-$ T-bet$^+$ ILC3 s have been denoted "ex-ILC3s" [59,76] and appear to represent a ILC3 → ILC1 transition driven by IL-12. Similar findings have been shown in human ILC3 derived from tonsils [140] and have been proposed to occur during inflammatory bowel disease [59,70,140]. ILC3 plasticity suggests the importance of environmental signals in maintaining ILC3 functional diversity in tissues under steady-state and stressed conditions [59,65,76,141]. ILC plasticity is not restricted to ILC3 but has recently been demonstrated in human and mouse ILC2 in the context of chronic inflammation [142–145].

As mentioned previously, ILC3 s promote the epithelial cell proliferation via IL-22 production. Tight regulation of this pathway may be necessary to avoid uncontrolled proliferation that could lead to malignant transformation. Specific roles for ILC3 in tumor development remain unclear. Still, inflammation enhances tumor development and is associated with increased levels of IL-22 [136] and accumulation of ILC3 [146]. IL-22 can directly promote cellular transformation via phosphorylation of STAT3 in colonic epithelial cells. Conversely, this pathway is regulated by DCs that produce a soluble IL-22 binding protein (IL-22BP) capable of downregulating IL-22 activity [136]. Additional cross-regulatory pathways are involved in intestinal homeostasis. IL-23 levels are increased during inflammation [147] resulting in myeloid cell recruitment and blocking of antiapoptotic signals [146] that promote tumor development. In melanoma, CCR7$^+$ LTi and stromal cells have been implicated in tumor progression [148]. CCL21 (CCR7 ligand) expression by tumor cells creates an immunotolerant environment, whereby LTi cells facilitate tumor cell survival and invasion [148]. Together, these observations paint a complex picture of ILC3 regulation of epithelial function that has not been fully explored in the human system. It is likely that ILC plasticity will also play a

key role not only in tumor initiation but also in tumor progression and disease outcome.

14.5 Therapeutically Harnessing ILC2 and ILC3

Because ILCs can rapidly respond to viral or bacterial infection and injury, they represent interesting targets for preventing or enhancing innate immune responses during infection, inflammatory reactions, and in chronic disease settings. Still, ILC-specific targeting approaches that can suppress or enhance their activities have not yet been identified. Still, ILCs share with T cells a plethora of receptors and/or secreted factors, some of which are already targeted in preclinical or clinical trials. It will be important to understand the precise cellular targets for these different therapies (T cell versus ILC) in order to optimize these approaches (Figure 14.4).

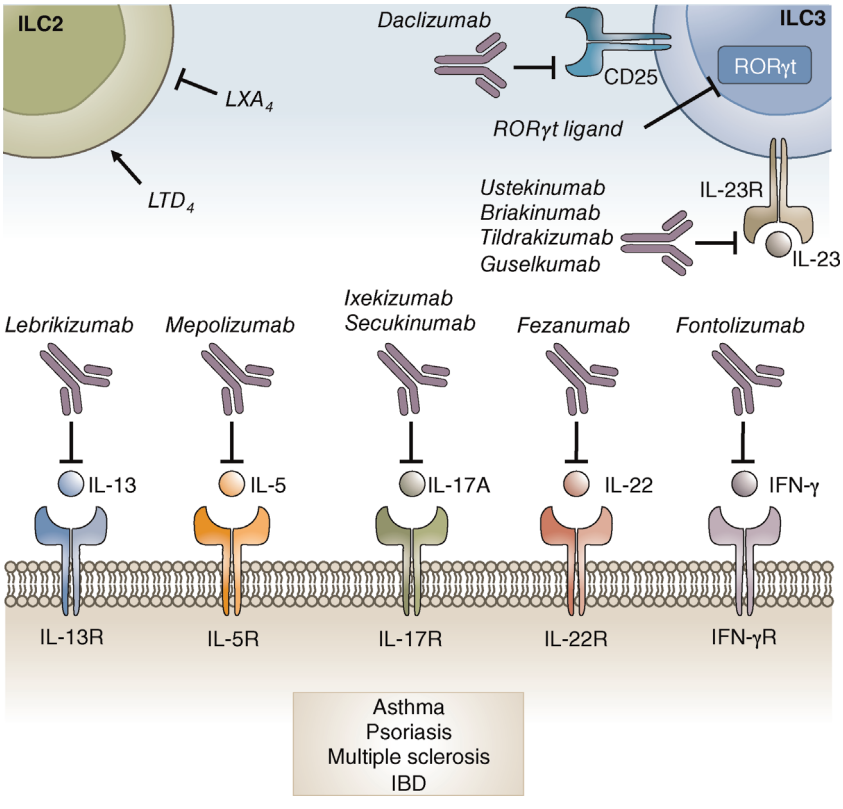


Figure 14.4 Targeting ILC2 and ILC3 in the clinic. ILC2 and ILC3 are associated with inflammatory diseases and ILC activation could be modulated therapeutically using different approaches. Recent preclinical studies and clinical trials show that modulation of ILC2 and ILC3 activity may be developed to control their inflammatory activity using metabolite, ligand, and monoclonal antibodies notably in patients with IBD, asthma, psoriasis, and/or autoimmunity.

ILC2 activity can promote chronic inflammation at barrier sites (e.g., asthma in the respiratory tract). The arachidonic metabolites lipoxin A4 (LXA4) or leukotriene D4 (LTD4) could be used to modulate (negatively or positively) ILC activity in this context [38,123]. Moreover, cytokines IL-5 and IL-13 produced by ILC2 s may be targeted with specific monoclonal antibodies. Along these lines, Mepolizumab (anti-IL-5) and Lebrikizumab (anti-IL-13) are already being used in asthmatic patients [149–151]. In addition, antibodies against IL-25 or IL-33 could be interesting targets in acute allergic diseases [152,153].

During chronic inflammation, activation of potentially pathogenic ILC3 may be involved in local tissue damage. Therapeutic neutralization of IL-23 or IL-1β attenuates ILC-dependent colitis in preclinical mouse models of IBD [66,154,155]. Interestingly, clinical trials with Ustekinumab or Briakinumab (directed against IL-12/23 p40 subunit) or with Tildrakizumab and Guselkumab (against IL-23 p19 subunit) show signs of clinical efficacy in the treatment of psoriasis and Crohn's disease [156–160]. Moreover, the ILC depletion could be an interesting strategy to limit the ILC activity (similar to that used in mouse models with anti-CD90 antibodies) [77,139,154]. Drugs targeting cell ILC subset activation could involve inhibitors of RORγt or cytokine receptors [161–163]. In multiple sclerosis, treatment with anti-IL2Rα (Daclizumab) resulted in a reduced frequency of IL-22 and LTα producing ILC3 s [164]. Finally, selective neutralization of ILC cytokines is other possible strategy. For example, the targeting of IL-17 with Secukinumab, IL-22 with Fezakinumab, or IFN-γ with Fontolizumab presents new opportunities to limit the proinflammatory effects of these cytokines in psoriasis and Crohn's disease [165–167]

14.6 Concluding Remarks

ILCs constitute new actors in innate immunity that have critical roles in maintaining tissue homeostasis, in initiating immune defense against infection, and in regulating inflammatory disorders. The diversity of ILC subsets implies some level of specificity of the innate immune system to protect at individual and distinct mucosal barriers. We have learned much about ILC functions using genetically modified mouse models. In contrast, our understanding of human ILC development, function, and roles in health and disease are less well defined. Recent studies have demonstrated that diverse ILC subsets show functional plasticity, secondary to changes in cytokine and chemokine environments. Because ILCs potently activate and modulate the epithelial cell activity and immune responses, regulation of their activity and plasticity may be important in the context of many inflammatory diseases. Therapeutic modulation of ILCs could be an interesting strategy to bolster immunity against infection, to modulate tissue damage during inflammation, and to prevent cancer. Monoclonal antibodies that can target ILC-derived cytokines might find several applications in the clinic. However, a deeper understanding of ILC biology and responses to environmental and inflammatory signals in humans is necessary before we can fully harness the potential of these novel immune effector cells.

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15

Th9 Cells: From the Bench to the Bedside and Back Again

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15.1 The History of T Helper Cells

Thirty years ago, reports by Mosmann and Coffman provided the first descriptions of T helper cell subsets [1]. Those first papers demonstrated that T-cell clones, upon antigen stimulation, secreted specific and heritable subsets of cytokines. Some clones secreted IFNγ, IL-2, and lymphotoxin and were termed Th1 cells. Other clones secreted IL-4, IL-5, and/or IL-10 and were termed Th2 cells. The ability of T cells to secrete specific sets of cytokines was a striking finding and suggested a separation in the function of these cells as well. Th1 cells could mediate delayed-type hypersensitivity responses *in vivo* and were thought to be responsible for cellular immune responses [2]. Th2 cells demonstrated a greater ability to provide B cell help *in vitro*, and they were thought to be specialized for the development of antibody responses [2].

The concept that T cells could acquire specialized functions was slow to be accepted. Many researchers initially thought these polarized cytokine-secreting patterns were an artifact of long term *in vitro* culture of the T-cell clones. However, the segregated patterns of cytokine production were observed *in vivo* [3,4], and immune responses to particular pathogens appeared to generate immune responses that were consistent with segregated cytokine secretion [4]. Thus, observations of immune responses *in vivo* were validated the previous work on T-cell clones.

The next major advance was in defining the environment that would promote the development of T cells that produced polarized subsets of cytokines. *In vitro* and *in vivo*, IL-4 promoted the generation of Th2 cells [5]. IL-12 stimulated the generation of Th1 cells [6]. The application or blockade of these cytokines allowed responses to pathogens and in disease models to be manipulated, providing the contextual polarization of T cells that impacted the outcome of the inflammatory response.

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The Th1–Th2 paradigm lasted almost 20 years before it became clear that it was not sufficient to explain all immune responses. In the interim, Th1 cells were functionally tied to autoimmunity and resistance to pathogens that evaded host responses by growing within cells [7]. The IFNγ produced by Th1 cells activated macrophages to facilitate phagocytosis and efferocytosis, and induced the production of chemokines that recruited additional cells to sites of inflammation [8,9]. Th2 cells mediated allergic inflammation and promoted immunity to helminthic parasites [10,11]. The IL-4 secreted by Th2 cells was required for class switching to IgE and was required for IgE-dependent mast cell responses [12,13]. Furthermore, Th2 cell-produced IL-5 increased circulating eosinophils that further helped mediate immune responses at mucosal surfaces [14,15].

Central to the ability of IL-4 and IL-12 to promote T helper cell differentiation was the activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway [16–20]. Cytokines that bind to receptors that have JAK tyrosine kinases associated with the cytoplasmic portion of the receptor result in the activation of the tyrosine kinase activity. Phosphorylation of tyrosine residues on the cytoplasmic receptor tail results in recruitment of SH2 domain-containing proteins including the STAT proteins. The STAT proteins are subsequently phosphorylated thereby forming dimers, which are able to move to the nucleus and bind DNA to activate transcription. IL-4 specifically activates STAT6 that is required for Th2 differentiation [13,21–23]. IL-12 activates STAT4 and IFNg activates STAT1, both contributing to Th1 development [17,24–27]. The STAT proteins were the gateway to understanding T-cell differentiation in more detail.

Following STAT protein activation, Th1 and Th2 cells expressed specific transcription factors that activated genes encoding cytokines, cytokine receptors, chemokine receptors, and others that were required for the specialized function of each T helper subset. The T-box transcription factor T-bet was induced by STAT1 and STAT4 and cooperated with a number of other factors including STAT4 itself, the homeobox transcription factor Hlx, and the Runt family transcription factor Runx3 to regulate IFNγ and many other Th1-associated genes [24,28–31]. Th2 cells expressed GATA3 that worked other transcription factors including c-maf and interferon regulatory factor 4 (IRF4) [19,32,33]. Together, these transcription factors not only activated gene transcription but also initiated changes in the chromatin structure that allowed target loci, including the cytokine genes, to be primed for subsequent activation by T-cell receptor signaling.

The paradigms developed in Th1 and Th2 cells served as a blueprint for the rapid characterization of additional T-cell subsets described in the twenty-first century (Figure 15.1). Th17 cells secrete IL-17 and are involved in immunity to bacteria and fungi and promote some types of autoimmune responses [34–37]. T regulatory (Treg) cells limit immune responses, inhibiting the development of autoimmune diseases, and preventing pathogen immunity from causing a chronic inflammatory state [38,39]. Each of these subsets is dealt with in detail in separate chapters in this book. T follicular helper cells are a highly specialized of the T cell that develops in the germinal center and promotes B-cell growth, differentiation, class switching, and affinity maturation [40]. These cells are required for development of high-affinity antibody production, but whether or not they contribute to inflammation is not yet entirely clear. It is thought they might

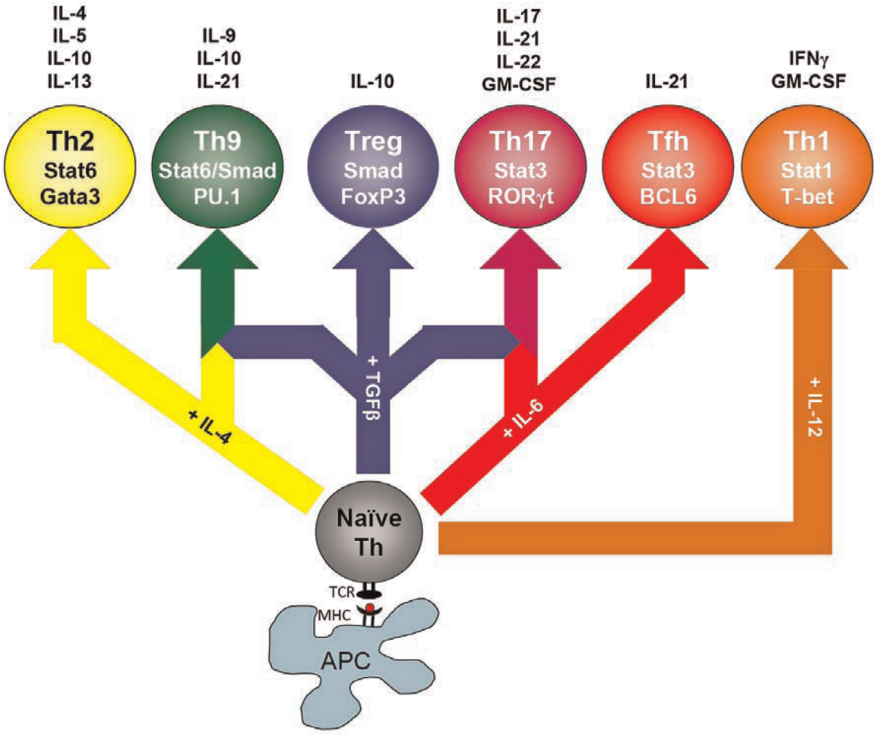


Figure 15.1 Th cell differentiation. Multiple factors are important in determining Th cell fate (e.g., TCR signaling; costimulation); however, research indicates cytokine signaling is the most important factor driving Th cell differentiation. The prototypical cytokines and transcription factors regulating each Th cell pathway are depicted. Predominant cytokines produced by each Th subset are also listed.

be involved in the development of antibody-mediated autoimmune diseases such as systemic lupus erythematosus.

15.2 Introduction to Th9 Cells

15.2.1 Development

The most recently described T helper cell subset is the IL-9-secreting subset of T cells termed Th9 cells. The primary cytokines that promote the differentiation of IL-9-secreting T cells were documented over 20 years and include IL-4, IL-2, and TGFβ [41]. Each of these cytokines activates distinct signaling pathways and increases the expression of transcription factors that are important for IL-9 production and the development of the Th9 phenotype. Because Th9 cells develop from integrated signals activated by IL-4 and TGFβ, Th9 cells share some attributes with Th2 cells (that develop following stimulation with IL-4 alone) and Treg cells (that develop following stimulation with TGFβ alone) [42–44]. Yet,

Th9 cells are a distinct subset that expresses a unique gene signature responsible for the ability of Th9 cells to function in the immune response [43].

Downstream of IL-4, STAT6 activation is critical for the development of Th9 cells and is responsible for the activation of many genes found in the Th9 transcriptional signature [43,45]. Some of these effects may be direct, but STAT6 also activates the expression of a variety of transcription factors important for IL-9 production. Among these are IRF4, basic activating transcription factor-like (BATF), and GATA3 [42,43,46]. These factors activate overlapping sets of genes in the Th9 transcriptional signature, and IRF4 and BATF particularly cooperate in activating Th9 gene expression [43]. STAT6 also represses the expression of transcription factors that impair Th9 development including T-bet, Runx3, and Foxp3, the latter the transcription factor required for Treg development [45].

IL-2 activates STAT5, one of the STAT family members that can contribute to many T helper cell lineages. STAT5 can act directly by binding to the Il9 gene and potentially by interfering with the activity another inhibitory transcription factor, Bcl6 [47,48]. In addition, STAT5 likely promotes Th9 development indirectly. STAT5 induces IRF4 expression, and may activate other Th9 genes as well [47].

TGFβ activates several pathways that are important for Th9 development. One of the common pathways activated by TGFβ and other cytokines in the TGFβ family including activin A (which can replace TGFβ in the induction of Th9 cells [49]) are Smad proteins. The Smad proteins Smad2, Smad3, and Smad4 have each been identified as binding to the Il9 locus and promoting chromatin changes that facilitate Il9 transcription [50,51]. Smad proteins also interact with IRF4, linking the pathways activated by IL-4 and TGFβ [50]. Independent of Smad signaling is the induction of PU.1, an ETS family transcription factor that was one of the first identified with a specific role in Th9 cells [50–53]. PU.1 binds to the Il9 locus and recruits specific histone acetylatransferases that specifically activate IL-9 production [52,54].

15.2.2 Additional Th9-inducing Cytokines

In addition to IL-4, IL-2, and TFGβ, several other cytokines have potent abilities to induce the production of IL-9. Moreover, the function of some of these cytokines is dependent on the ability to induce IL-9. Thymic stromal lymphopoietin (TSLP) has been extensively characterized for its ability to promote allergic inflammation [55]. Much of that function was thought to be dependent upon Th2 cells. However, more recent reports indicate that TSLP function also requires IL-9 and Th9 cells in the generation of allergic airway disease [56]. Mechanistically, TSLP activates STAT5, which as described above, binds directly to the *Il9* locus.

IL-25 is an IL-17 family cytokine member that promotes IL-9 production, and IL-25-induced allergic inflammation requires IL-9 [57]. Interestingly, Th9 cells appear to have the highest IL-25R mRNA expression (*Il17rb*) among the Th subsets [43]. It is not entirely clear how IL-25 promotes IL-9 production, but it could be through activation of NF-κB signaling or by the recently demonstrated ability of IL-25 to activate STAT5 [58].

TL1A is a TNF superfamily cytokine (or costimulatory molecule if surface-bound) that potently activates IL-9 production, parallel to its induction of allergic inflammation [59]. TL1A appears to function by enhancing IL-2 production that feeds back to cells to produce IL-9 in a STAT5-dependent manner.

Finally, IL-1 enhances IL-9 production from Th9 cells through an IRF1-dependent mechanism [60,61]; however, the ability of IL-1 to alter the function of Th9 cells may extend beyond the regulation of IL-9. IL-1-treated Th9 cells have increased both IL-21 production and tumor immunity that is dependent on IL-21, not IL-9 [60]. This altered function will be discussed later in the chapter.

15.2.3 Th9-inducing Cell Surface Ligands

Similar to cytokines that act through cell surface receptors, there are a number of ligands that are cell-associated and also bind to receptors on the developing Th9 cell. A number of these receptors are TNF superfamily members (OX40, 4-1BB, and TL1A) creating a paradigm supporting a general role for TNF superfamily members in the induction of IL-9 [59,62,63]. Although the mechanism of 4-1BB induction has not been investigated, stimulation of OX40 leads to activation of RelB/p52 NF-κB complexes that bind directly to the *Il9* locus [63]. Notch ligands also impact IL-9 production, but the effects can depend on other environmental stimuli [64]. In some contexts, Notch-activated transcription factors can bind directly to the *Il9* gene [64]. However, in other contexts, Notch signaling can induce Th17 or Treg development [65–67], and the precise codependent cues are not clear. PDL2 is unique in being able to inhibit Th9 development following engagement of PD-1 [68]. Most importantly, T-cell activation, through the T-cell receptor and costimulatory molecules such as CD28, induce *Il9* transcription through the activation of NFAT proteins following activation by MHC class II complexes and CD80 or CD86, respectively [69–71].

15.2.4 The Function of Th9 Cells

As predicted by the nomenclature, one of the most important products of Th9 cell activation is IL-9 (see Table 15.1). IL-9 is required for the ability of Th9 cells to mediate allergic inflammation, autoimmune inflammation in colitis models, and tumor immunity, as discussed in more detail in the subsequent section of this chapter. IL-9 has a myriad in the immune system including promoting Th2 cytokine production, enhancing class switching to IgE, and leading to the accumulation of tissue mast cells [72]. IL-9 also impacts structural cells in target organs. It induces chemokine production from airway epithelial cells, airway smooth muscle cells, and tumor stromal cells [72]. IL-9 alters the expression of tight junction proteins in intestinal epithelial cells, decreasing the barrier function of the epithelial layer [73,74]. IL-9 may also have additional functions in particular contexts that have not been explored, and which of these actions are most important *in vivo* is still not entirely clear.

Th9 cells, however, also produce other cytokines (see Table 15.1 and Figure 15.2). IL-3 from Th9 cells impacts the survival of dendritic cells that is important for tumor immunity [75]. In the context of IL-1 stimulation, Th9 cells can also produce

Table 15.1 Soluble mediators and surface markers associated with Th9 cell effector function.

Effector Molecule	Mechanism of Action	References
Soluble mediators:		
BAFF	- promotes B-cell proliferation and differentiation[a)]	[43]
CXCL3	- promotes recruitment of inflammatory cells[a)]	[43]
CCL22	- may promote Th2 cell during Th9-mediated allergic inflammation	[52,53]
IL-1rn	- inhibits IL-1 signaling[a)]	[43]
IL-3	- promotes dendritic cell survival	[75]
IL-9	- antitumor effects - promotes mast cell accumulation and activity - modulates epithelial cell biology (e.g., proliferation) - promotes Th2 cytokine production and ILC2 survival - enhances IgE class switching[a)]	[42,44,72,73,77,78,93]
IL-10	- immune suppression[a)]	[42,44]
IL-21	- antitumor effects - can activate NK and CD8+ T cells - promotes B-cell responses[a)]	[60]
Cell surface mediators:		
CCR3	- ligands include CCL5 and CCL11 - enhances Th9 migration during allergic inflammation	[76]
CCR4	- ligands include CCL17 and CCL22 - associated with homing to skin[a)]	[43]
CCR6	- ligands include CCL20 - enhances Th9 migration during allergic inflammation	[43,76]
CCR8	- ligands include CCL1	[43]
CXCR3	- ligands include CXCL9, CXCL10, and CXCL11 - enhances Th9 migration during autoimmunity	[43,76]
CD103	- localization to mucosal surfaces[a)]	[43]

a) Not investigated with relation to Th9 biology.

IL-21, which is important for tumor immunity as well [60]. Th9 cells were originally identified based on their ability to produce both IL-10 and IL-9 [42,44]. However, the IL-10 producing cells in Th9 cultures largely segregate from the IL-9-producing cells [52], and no specific function has been ascribed to Th9 cell IL-10 production as experiments to assay suppressor activity in colitis studies, Th9 cells activated rather than suppressed disease [42]. Gene profiling studies and subsequent ELISA experiments demonstrated that Th9 cells preferentially secrete IL-1 receptor antagonist (*Il1rn*), BAFF (*Tnfsf13b*), and *Cxcl3* [43]. The Th9-specific functions of these cytokines and chemokines have not yet been defined.

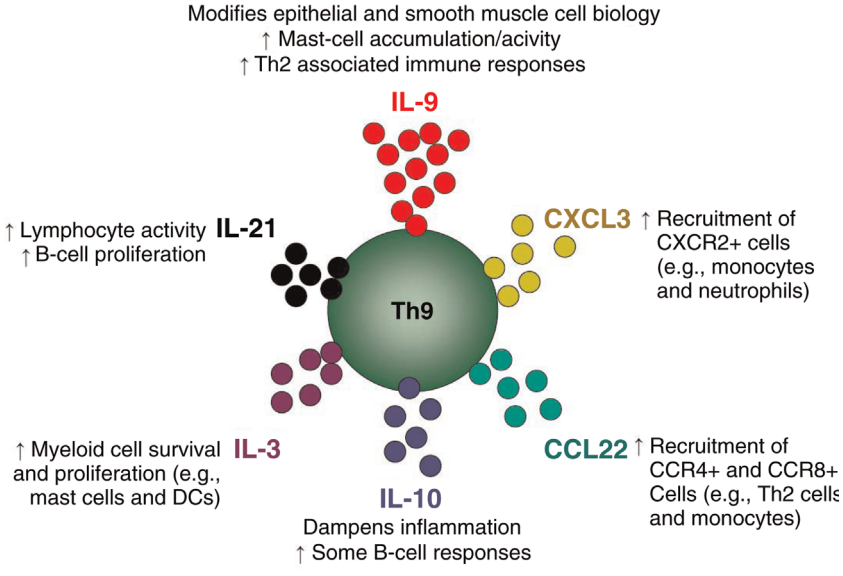


Figure 15.2 Th9-associated cytokines and chemokines. Th9 cells are principally identified by their robust production of the cytokine IL-9; however, cells cultured under Th9 conditions (i.e., IL-4 and TGF-β) are also capable of producing a number of cytokines and chemokines (as depicted above), which may be important in overall Th9-associated pathologies. It is important to note, though, a given cell will not produce all these cytokines. For example, flow cytometry data indicates distinct Th9 cultured cells will produce IL-9 or IL-10 but rarely both.

Th9 cells also preferentially express surface receptors that confer specific functions to these cells (see Table 15.1). Chemokine receptors expressed in Th9 cells include CCR4, CCR8, CCR6, and CXCR3 that are important for recruiting cells to sites of type 2 (allergic) inflammation [43,76]. Th9 cells also express CD103, an integrin that can mediate localization of cells to mucosal interfaces with the environment [43]. Th9 cells also preferentially express cytokine receptors that enhance their function, including IL-25R (*Il17rb*) and TSLPR (*Crlf2*) [56,57]. TSLPR can further be induced by its ligand [56].

15.3 Th9 Cells in Disease: The Helpful and Unhelpful

Here, we explore immune responses and inflammatory disorders that are increasingly recognized as having a Th9 cell component that significantly contributes to the disease process. In the appropriate context, Th9 cells and IL-9 have shown beneficial effects in eradicating both tumor and helminthic infections. In contrast, inappropriate production of IL-9 and Th9 cells has been linked to pathology in colitis and atopic inflammation. In the proceeding segment of this chapter, we will discuss our current understanding of Th9 cells in the clinic supported with background and progress from the bench.

15.3.1 Tumor Immunity

In light of the recent breakthroughs in adoptive immunotherapy for hematological cancers, the role of CD4 effector T cells in the tumor microenvironment of solid tumors is an area of great research interest. Recent studies have demonstrated that Th9-mediated antitumor immunity may prove effective against epithelial tumors such as melanoma and lung adenocarcinoma, two cancers with high rates of incidence and mortality, respectively [77,78]. Adoptive transfer of tumor-specific Th9 cells decreased tumor growth in the mouse model, an effect that was lost with the addition of IL-9 neutralizing antibody. When melanoma cells were coinjected with various T-cell subsets, Th9 cells demonstrated the greatest effect in limiting tumor growth [77,78]. The role of IL-9 in tumor immunity is further supported by a single-nucleotide polymorphism (SNP) in the *IL9* gene that confers an increased risk of malignant melanoma [79].

IL-9 has multiple potential mechanisms of action. Initially, it was found that IL-9 indirectly kills tumors, through the activation of mast cells and granzyme B production [78]. Subsequent studies did not find mast cells in the tumor microenvironment, suggesting that the mast cell-dependent mechanism might be restricted to particular sites or types of tumor progression [77]. In an alternative mechanism, Lu *et al.* found IL-9 acted on epithelial cells in the lung and induced the production of CCL20 in the tumor microenvironment. Increased concentrations of CCL20 act as a chemoattractant for CCR6+CD8α+ DCs to the tumor, thus ensuring greater delivery of tumor-specific antigens to the tumor draining lymph nodes. As a result, greater effector cell activation was found in both the lymph nodes and the tumor microenvironment [77]. More recently, IL-1β was found to enhance Th9 cytokine production of IL-9 and IL-21 resulting in significantly greater Th9-mediated antitumor immunity [60]. Conventional Th9 cells produce large amounts of IL-9 and low amounts of IL-21 with the anticancer effects dependent on the IL-9 production. In the presence of IL-1β, Th9 cells had antitumor activity that was dependent on the IL-21 production. The cytokine IL-21 is known to act in the activation of cytotoxic activity of CD8 T cells and NK cells [60]. Overall, it is unclear at this time if one particular mechanism has a greater contribution to the antitumor activity of Th9 cells or that Th9 cells mediate multiple pathways in the elimination of tumors. Future treatment for melanoma and lung cancer may develop from optimizing strategies to promote Th9 cell production of IL-9 and IL-21 in the tumor microenvironment.

15.3.2 Helminth Immunity

Almost all individuals residing in less developed nations are exposed to intestinal helminth infections. Disease is caused by competition for the host nutrients and inflammation in intestinal mucosa and other organs. Traditionally, helminthic infections have been characterized as a Th2 response, but it has been demonstrated that the Th9 response mediated through IL-9 provides a significant contribution to the host defense as well. For example, patients suffering from filarial lymphedema, a debilitating pathology resulting from parasitic infections, had elevated Th9 and IL-9 production compared to a control population or infected asymptomatic

patients [80]. The degree of filarial lymphedema was directly correlated to the frequency of Ag-induced Th9 cells and IL-9 production in the patient suggesting that Th9 cells are linked to host defense and pathology [80].

There is supportive evidence for Th9 cells and helminthic infections in the widely used experimental murine models of *Trichinella spiralis*, *Trichuris muris*, and *Nippostrongylus brasiliensis. T. spiralis* can be acquired from undercooked meat from infected animals. The larvae hatch in the intestine and if not expelled, they will migrate into skeletal muscle and develop into a nurse cell that is protected from the immune system [81]. In the mouse model, mastocytosis and mouse mast cell protease 1 (mMCP-1) have been identified as important mediators for rapid *T. spiralis* expulsion [82,83]. IL-9 transgenic mice, producing high levels of IL-9 *in vivo*, were resistant to infection with both an increase in mucosal mast cell numbers and mMCP-1 levels. After *T. spiralis* infection, control mice had significant worm burden while IL-9 transgenic mice had no detectable worm burden [84]. When mice were treated with cortisone treatment, thereby inhibiting the adaptive immune response, the effect of IL-9 was reduced [85]. In a second mouse model, *T. muris* is a natural murine parasite that hatches in the small intestine tunneling into the crypts of the mucosal wall [81]. IL-9 blockade, which prevents IL-9 dependent mast cell activation and eosinophil infiltration [86], in resistant mice prevented *T. muris* expulsion [87]. In addition, CD4dnTGFβRII transgenic mice, expressing a dominant negative TGFβ receptor that impairs Th9 differentiation, prevented expulsion of *T. muris* with decreased mast cell numbers in the cecum and low IL-9 levels [44]. Finally, *N. brasiliensis* is a parasite that enters the host through the skin, migrates to the lung, and takes advantage of the host mucus ciliary clearance mechanism to be coughed up and ingested [88]. In *Il9*-deficient mice, there was a decrease in eosinophils, basophils, and mast cells and an increase in worm burden a week postinoculation compared to WT mice [89].

Multiple cell types are known to produce IL-9, including Th9 and type II innate lymphoid cells (ILC2). The major producers of IL-9 in an IL-9 reporter mouse were found to be ILC2 and Th9 cells [89]. In an effort to differentiate the function of ILC2, whose survival is IL-9 dependent [90] in contrast to low IL-9R-expressing Th9 cells [91], an IL-9 receptor mutant (*Il9r*−/−) mouse was utilized in the lung inflammation model. In *Il9r*−/− mice, goblet cell hyperplasia and mast cell accumulation in the lung were not significantly reduced suggesting a role of the Th9 cell in this system. Moreover, Th9 cells when adoptively transferred into IL-9-deficient hosts were sufficient to decrease worm loads [89]. Altogether, it is has been clearly demonstrated that Th9 cells and IL-9 provide a powerful mechanism for protection in the battle between host and parasitic helminthic infection.

15.3.3 Inflammatory Bowel Disease

Inflammatory bowel diseases are chronic relapsing ulcerative disorders that result in life-threatening symptoms of abdominal pain, malnutrition, and diarrhea. The etiology is believed to develop from an inappropriate activation of the mucosal immune system by unknown mediators facilitating defects in barrier function of

the epithelium and the mucosal immune system. Although Th1 and Th2 cells are associated with Crohn's disease (CD) and ulcerative colitis (UC), respectively, Th9 cells may also contribute to inflammation in these diseases.

In human samples, Gerlach *et al.* found that there was an increase in *IL9* mRNA expression in UC patients with active disease compared to both inactive UC and control patients [73]. In mouse models of colitis, IL-9 has been shown to regulate the permeability of the intestinal mucosa through the activation of mast cells and IgE synthesis [92]. Furthermore, it was found that IL-9 downregulated barrier proteins such as claudin 2 in ways that decreased barrier function allowing for the translocation of bacteria across the luminal wall [73]. Interestingly, mice deficient in IL-9 were protected from bacterial translocation and colitis [73]. Finally, IL-9 delays wound healing in intestinal epithelial cell lines [93]. Each of these mechanisms may contribute to the potentially destructive effects of IL-9 in the gut microenvironment.

Although many cells produce IL-9, it is believed that Th9 cells are a major contributor in the intestine in UC. *Spi1* mRNA, encoding the Th9-associated transcription factor PU.1, was upregulated in IL-9 producing cells in patient mucosal biopsies in the inflamed gut [73]. Moreover, specific deletion of PU.1 in T cells diminished autoimmune inflammation in the intestine. In the follow-up study, Nalleweg *et al.* provided convincing evidence that the majority of these cells in patient biopsies producing IL-9 were CD3 T cells. As the disease progressed in severity, the number of cells expressing IL-9 increased in total number and percentage among lamina propria lymphocytes [93]. In addition, human peripheral blood from active UC patients was also found to have a population of CD3+ T cells with IL-9 expression. When these T cells were examined for homing potential, both integrin alpha 4 and beta 7 were present in a proportion of the T cell population leading to the potential for gut homing [93].

15.3.4 Allergic Inflammation

Allergic responses arise from the sensitization of the immune system leading to polarized Th2 and the generation of IgE. These responses are localized to specific organ systems and cause diseases that include asthma, allergic rhinitis, atopic dermatitis, eosinophilic esophagitis, and food allergy. Allergens induce the Th2-associated cytokines IL-5, which acts as a chemoattractant for eosinophils, IL-13, which promotes mucus production and chemokine expression from epithelial cells, and IL-4, which promotes Th2/Th9 differentiation and regulates antibody class switching to IgE. Subsequently, IgE binds to tissue mast cells, and upon reexposure to allergens, IgE mediates activation of mast cells resulting in the release of histamine granules and generation of leukotrienes leading to airway bronchoconstriction, edema, and inflammation. In addition, it has been found that Th9 cells play a necessary and sufficient role in developing allergic inflammation.

Total IgE concentrations are highly correlated in twins concordant for elevated bronchial hyperresponsiveness, a marker of allergic airway inflammation. Further linkage analysis of these siblings found a major gene for atopy on the q arm of chromosome 5 [94]. Investigation in this region identified *IL9* as a candidate gene for asthma risk due to linkage disequilibrium between IgE levels and SNPs within the *IL9* gene [95]. Using contrasting mouse models of airway

hyper- and hyporesponsiveness differences in the *IL9* mRNA were directly correlated with hyperresponsiveness, while there were no differences in the mRNA levels of Th2 cytokines [95]. Additional SNP associations related to the IL-9 pathway in asthma patients were found for *IL9R*, *IL4*, *IL13*, and *STAT6* [96–98].

As a baseline of comparison in healthy human individuals, Th9 cells are found in T-cell subsets tropic to the skin and are regularly absent in the lung and the digestive system [99]. In contrast within atopic children, there is a correlation between IL-9 production from PBMCs and IL-9-positive T cells with serum total IgE [49,52,100]. Furthermore, naïve T cells isolated from the same atopic children and differentiated into Th9 cells expressed a greater level of *IL-9* and *SPI1* mRNA, while no difference was found in Th2 cytokine expression (IL-4, -5, and -13) [100]. In adults, the house dust mite *Dermatophagoides pteronyssinus* was further found to induce IL-9 production, and Th9 cell number providing a marker for asthma and airway hyperresponsiveness correlating to IgE and asthma [49,101]. When IL-9 was increased so were the levels of IL-5 and IL-13 [101].

Adoptive transfer of OVA-specific Th2 and Th9 cells into *Rag1*−/− mice undergoing OVA challenge had enhanced airway inflammatory cell infiltration and mucus production. Blockade of IL-9 greatly decreased airway inflammation and goblet cell metaplasia in mice receiving Th9 cells [46,56]. It is important to note that ILC2 can also produce IL-9 and neutralization of IL-9 might indicate that even the lower concentrations of IL-9 produced by these cells are important for inflammation (46, 56, and 90). Importantly, Th9 cells are required for the ability of TSLP to promote allergic airway inflammation [46,56].

In an IL-9 transgenic airway mouse model, there was increased bronchial hyperresponsiveness when challenged with methacholine. An increased level of IL-9 was found to cause airway inflammation through eosinophil and mast cell infiltration and increased mucus production and muscle cell proliferation [89,102,103]. Interestingly, histamine was not increased [103]. Blockade of IL-9 through autoantibodies prevented IL-9-dependent mast cell activation and eosinophil infiltration [87]. In IL-9-deficient mice, there was a lack of pulmonary goblet cell hyperplasia and mastocytosis, but no difference was found in Th1 or Th2 differentiation or cytokine production [104]. Additional studies where Th9 cell development was prevented also found inhibition of IL-9 production and diminished inflammation [43,105,106]. IL-9 was found to promote airway fibrosis in a mast cell-dependent mechanism [102]. It was suggested that IL-9 effects in lung inflammation and mucus production facilitated the induction of IL-13 production in the lung epithelium, though this is not clear in systems where IL-9 is not overexpressed [107,108].

In allergic disease models, IL-9 clearly promotes mast cell accumulation and inflammation [52,92,104,109]. However, depending on the model, the primary cellular source of IL-9 differs [110]. In a T-cell-independent allergic model, ILC2 s were found to be the significant source of IL-9. In contrast, T-cell-dependent models of allergic inflammation indicate CD4+ T cells are the primary source [110]. Furthermore, mice with a T-cell-specific deletion of PU.1, which impairs Th9 differentiation, restricted allergic inflammation [52]. To further delineate the roles of ILC2 and Th9 cells in the production of IL-9 during allergic

inflammation, mice containing PU.1-deficient T cells were compared with RAG1-deficient mice lacking all T cells that have full functional ILC2. During allergic inflammation, the overall reduction in mast cell numbers was similar between the PU.1-deficient and *Rag1*−/− mice [109], suggesting that Th9 cells are the major T-cell component promoting mast cell accumulation. There was less IL-9 and decreased mast cell numbers and mast cell proteases present in PU.1-deficient mice [52,109]. Altogether, this evidence demonstrates that Th9 T cells are a major producer of IL-9 in models of allergic inflammation.

Further understanding of the role by which Th9 cells promote allergic airway inflammation will be important in preventing associated long-term airway dysfunction. For example, in a chronic allergic mouse model, increased airway resistance and collagen deposition was not resolved 4 weeks after last allergen exposure even though goblet hyperplasia and inflammatory cell infiltrate returned to baseline [102]. Whether these features are dependent on IL-9 production from Th9 cells is not clear. Moreover, whether Th9 cells contribute to other models of inflammation, or more importantly in the pathogenesis of human disease, is still the focus of ongoing research.

15.3.5 Dermatitis

Recent evidence has demonstrated that IL-9 and Th9 cells may have potential roles in various pathologies of the skin. Type I hypersensitivity reactions of the skin in atopic dermatitis have been associated with increased serum levels of IgE compared to normal skin, possibly linked to correlations between IL-9 and serum IgE concentrations [99,100]. Memory helper T cells from healthy donors expressing cutaneous lymphocyte antigen (CLA), a unique skin homing receptor, produced higher IL-9 levels after exposure to *Candida albicans* [99]. In another human study, *Il9* and *Sfpi1* mRNA were found to be significantly elevated in both PBMCs and skin biopsies of atopic dermatitis patients when compared to healthy controls [111]. The percentages of Th9 cells within PBMCs were also elevated in atopic dermatitis patients. In summary, this study indicates that measures of Th9 cell biology correlated with atopic disease severity [111]. However, another report did not identify significant IL-9 CLA+-producing fractions between atopic and normal individuals [112,113] suggesting there may be differences among atopic populations.

Th9 lymphocytes were also found in greater numbers in allergic contact dermatitis skin biopsies compared to normal skin [114]. PBMCs from nickel-allergic patients had increased IL-9 production [114]. In the mouse model of nickel-induced contact dermatitis, there was not a significant difference in disease score between wild-type and IL-9-deficient mice, but the inflammation was skewed to a Th1 phenotype compensating for a decreased Th2 response [114].

What functions IL-9 has in the skin are not yet clear. Certainly, IL-9 might impact recruitment of lymphoid and myeloid cells present in normal or diseased skin. In addition, VEGF, a potent angiogenesis factor, was induced in keratinocytes in cultures with IL-9 [111], suggesting that skin cells themselves might be targets of IL-9. As with other disease models, further work is required to define the relevant IL-9 responding population that results in a pathological response.

15.3.6 Food Allergy

Several reports suggest a link between Th9 cells and food allergy. PBMCs from egg-sensitized infants produce more IL-9 than PBMCs from nonatopic infants when stimulated with anti-CD3 [100]. Microarray analysis of memory Th subsets comparing atopic children with or without peanut allergy found IL-9, IL-5, and IL-13 to be greatly increased in samples from children with peanut allergy [115]. Moreover, IL-9 expression helped discriminate between allergic and clinically tolerant population better than IL-4, IL-5, and IL-13 production [115,116]. In the mouse model, food allergy and antigen sensitization was dependent on IL-9 expression with complete loss of intestinal anaphylaxis in IL-9-deficient mice. In this model, IL-9 increases the permeability of the intestine through mastocytosis and intravascular leakage [92]. The allergy was found to be systemic but limited to oral sensitization through IgE activation [117]. Together, these reports suggest a functional link between IL-9 and food allergy.

15.4 Targeting Th9 Cells Therapeutically

As noted in the preceding section, it is becoming increasingly evident that Th9 cells and IL-9 may promote a variety of human ailments ranging from allergic inflammation to inflammatory bowel disease; however, targeting Th9 cells therapeutically has remained unexplored. In the following sections, we will explore potential approaches to modulate Th9 biology either during differentiation or by neutralizing key effector molecules after differentiation. The key benefits and potential side effects from current and potential therapeutics will be highlighted. Some of the potential therapeutics discussed are in development or have been approved (see Table 15.2).

15.4.1 Th9 Differentiation

15.4.1.1 Cytokines and Costimulators Required for Differentiation

Th9 development is dependent on both IL-4 and TGFβ signaling [41,42,44]; however, the majority of Th9-associated genes are downstream of IL-4 signaling [43,45], indicating inhibition of this pathway would be the most advantageous in impeding Th9 differentiation. Potential therapeutics include Pascolizumab (an IL-4 targeting antibody), Pitrakinra (an IL-4 analog that inhibits IL-4/13 signaling through the IL-4 receptor), and Altrakincept (a decoy IL-4 receptor) [118–120]. IL-4 signaling is also dependent on STAT6 activation by JAK kinases. While no drug specifically targeting STAT6 exists, Teriflunomide, a metabolite of the drug flunomide, can inhibit STAT6 nonspecifically and is currently being used to treat multiple sclerosis [121,122]. Furthermore, specific JAK kinase inhibitors could also inhibit STAT6 signaling. Collectively, these therapeutics would have the additional advantage of impeding Th2 development, which could be beneficial in inhibiting pathologies with a joint Th2 and Th9 immune response such as allergic inflammation.

Table 15.2 Developmental status of therapies potentially beneficial in modulating Th9 responses.

Therapeutic Target	Mechanism of Action	Development Stage	References:
IL-4 signaling: [a]			
Pascolizumab	IL-4 antibody	Phase II	[118]
Pitrakinra	IL-4 analog-binding IL-4 receptor	Phase II	[119]
Altrakincept	Decoy IL-4 receptor	Phase III	[120]
Teriflunomide	Metabolite inhibits STAT6 activity	Approved for treatment of multiple sclerosis	[121,122]
Tofacitinib	Jak1/Jak3 inhibitor	Approved for treatment of rheumatoid arthritis	[172]
TGFβ signaling	Blocking antibodies, peptide inhibitors, and so on.	Numerous in production	[128]
CD28 (Abatacept)	CTLA-4-Ig fusion protein	Approved for treatment of arthritis	[138]
OX40 L		Phase II	
GATA 3 (SB010)	DNA enzyme that inactivates GATA3 mRNA	Phase II	[152]
TSLP (AMG-157)		Phase II	[139]
IL-9 (enokizumab)	IL-9 antibody	Phase II	

a) List is not exhaustive.

A wide range of therapeutics targeting TGFβ signaling are in development or have shown efficacy in animal models including Fresolimumab (a humanized antibody targeting all three TGFβ isoforms), IMC-TR1 (a humanized antibody targeting the TGFb receptor), and Disitertide (a peptide that blocks TGFβ binding to its receptors) [28,123–127]. However, TGFβ signaling is highly complex making this a less than ideal avenue of therapeutic intervention in Th9-mediated pathologies. For one, it was noted that Activin A, a TGFβ superfamily member, could also promote Th9 differentiation [49], potentially necessitating its targeting in any therapeutic regimen. In addition, TGFβ has many functions outside of Th differentiation including effects on cellular proliferation, extracellular matrix deposition, and overall immune suppression [128]. The lethal inflammatory phenotype associated with *Tgfb1*-deficient mice demonstrates the important immune suppressor function of this cytokine [129,130]. It must be noted, however, no complications have been observed in clinical trials targeting TGFβ signaling [128,131]. Furthermore, neutralizing TGFβ signaling in murine studies of autoimmune disease has actually dampened inflammation, likely through inhibiting Th17 development [132,133]. Potentially, TGFβ therapeutics may be beneficial in pathologies involving both Th17 and Th9 responses such as ulcerative colitis and multiple sclerosis and the efficacy might depend on timing of administration and stage of disease.

CD28 and the TNF receptors OX40, 4-1BB (CD137), and T1A have all been linked in stimulating IL-9 production [59,62,63,70,71]. Besides influencing the Th subset fate, these molecules are also associated with promoting survival during activation, enhancing cytokine production, and maintaining T-cell memory independent of TCR stimulation [134–137]. Murine studies have also demonstrated that antagonism of CD28, OX40, 4-1BB, and T1A signaling or genetic deficiencies can impede allergic inflammation among other diseases [59,62,63,70,71,136]. Unfortunately, inhibition of CD28 costimulation with Abatacept, a humanized version of CTLA4Ig, did not alleviate symptoms in people with mild atopic asthma or reduce airway eosinophilia [138]. Furthermore, treatment of patients with OX40 L antagonist also did not alleviate clinical features in atopic patients challenged with allergen extract, although improvement was noted in patient eosinophil numbers and IgE levels [139]. The failure of these trials may be due to overlapping costimulatory function. Thus, greater patient efficacy would be noted with combinational therapy. Furthermore, agonists to these costimulatory molecules may be beneficial in promoting Th9 responses. For example, 4-1BB and OX40 agonists are being explored in promoting antitumor responses [140].

15.4.1.2 Transcription Factors for Differentiation

Each Th subset is defined, in part, by the expression of lineage-determining factors, proteins that not only uniquely drive the fate of a given Th subset but also inhibit the differentiation of most other Th subsets. While the notion of "master" regulators is increasingly becoming anachronistic, there are transcription factors within each Th subset that largely fit this definition: T-BET (Th1), GATA3 (Th2), RORγT (Th17), FOXP3 (Treg), and BCL6 (Tfh) (Figure 15.1). Such proteins would be prominent targets for small molecular inhibitors or other pharmacological interventions for the modulation of a specific Th subset-mediated immune response.

A master transcription regulator of Th9 cells has not been discovered, however. The most specific regulator would be PU.1, as observed in both murine and human studies [45,52–54,141,142]. As mentioned previously, PU.1 is an ETS transcription factor enriched in Th9 cells and regulated by TGFβ signaling [43,45,52], albeit independent of the SMAD pathway [50,51]. In the murine model, PU.1-deficient CD4+ T cells cultured under either Th2 or Th9 conditions have a significantly reduced capacity to produce IL-9 and enhanced production of Th2-associated cytokines [52]. Similar effects were noted in human cells treated with PU.1 siRNA. *In vitro* analysis has revealed that PU.1 interferes with the function of GATA3 and IRF4, resulting in diminished Th2 cytokine production [53,143], and limits Tfh differentiation by inhibiting CD40 L and IL-21 expression [144]. In Th9 cells, PU.1 promotes the expression of IL-9 by recruiting histone acetyltransferases to the *Il9* locus [54]. In summary, PU.1 is the only transcription factor, to date, that induces IL-9 production in other Th cell subsets while also inhibiting an alternative Th fate.

Thus, PU.1 is an attractive target in specifically modifying Th9 development. In murine models of allergic inflammation, PU.1 deficiency did not inhibit Th2 differentiation in the lymphoid tissue [52,109]; however, the impairment of Th9

differentiation did impede the recruitment of Th2 cells to the lung environment potentially due to Th9-mediated production of CCL22 [52] (Figure 15.2), which can promote the recruitment of Th2 cells [145]. It is important to note, though, that PU.1 regulates a small subset of genes outside of IL-9 [43]. Furthermore, several ETS transcription factors are expressed in any given cell type and have overlapping functions [146], which may account for the reduced but still significant numbers of IL-9 producing cells in PU.1-deficient cultures [52]. Thus, targeting PU.1 to inhibit Th9-mediated pathologies may be more efficient in combination with inhibitors of other pertinent ETS factors or Th9-associated transcription factors.

IRF4, downstream of IL-4 signaling [43], is also important in Th9 differentiation [46], as well as Th2 and Th17 cells [33,143,147]. IRF4 promotes *Il9* transcription through binding to the *Il9* promoter and inhibits Th1 cell-associated transcription factors that attenuate Th9 differentiation [43,46]. IRF4 likely cooperates with BATF on the *Il9* promoter to form a transcriptional module [43], a phenomenon also observed in Th17 cells [148,149]. This is highlighted when ectopic expression of both BATF and IRF4 into Th9 cultured cells promotes IL-9 expression greater than expression of either factor alone [43]. Importantly, BATF deficiency in Th9 cultured cells results in a marked defect in the expression of nearly all Th9 cell-associated genes, indicating that BATF is also an important transcription factor outside of the *Il9* gene [43]. Thus, both BATF and IRF4 are attractive therapeutic targets in modulating Th9 activity, although consideration must be given that these factors also regulate other Th subsets.

Although GATA3 is the master transcriptional regulator of Th2 cells, its expression is required for Th9 differentiation as *Gata3*-deficient Th9 cultured cells do not produce IL-9 [42]. Interestingly, ectopic expression of GATA3 in Th9 cells can impede IL-9 production [45]. The differing results in these studies may reflect the amount of GATA3 within the cell, as a gradient of GATA3 expression might be instrumental in driving Th differentiation to one fate or another. Nonetheless, inhibition of GATA3 function could be an attractive candidate in inhibiting both Th2 and Th9 development [42,150,151]. For example, a GATA3-specific DNAzyme has been used to decrease allergen-induced asthmatic responses [152].

15.4.1.3 Factors Required for Promotion of Th9 Biology

Several factors (e.g., cytokines and transcription factors) exist that promote Th9 development and/or effector activity, without being essential for differentiation. Therapeutic interventions in these processes may be of greater importance as by the time of treatment, patients are likely to have established Th9-associated immune responses and ideal therapies would dampen existing immunological disorders.

TSLP, IL-25, and IL-33 are epithelial-derived cytokines that have been linked in the promotion of allergic inflammation and atopic dermatitis [153–159]. Along with their noted activation of ILC2 [155], these factors can also promote Th9 responses. TSLP receptor is expressed on Th9 cells, and TSLP-mediated signaling through STAT5 can substitute for the requirement of IL-2 in promotion of Th9

differentiation [56]. IL-17RB, the receptor for IL-25 signaling, is one of the most highly expressed genes in Th9 cells [43,160]. It has also been demonstrated that IL-25 signaling can promote Th9 responses during allergic inflammation and helminth immunity [57,160]. In the former process, IL-25 promoted the Th9-mediated inflammation independent of Th2 responses and treatment of *T. spiralis*-infected mice with IL-25 neutralizing antibody prevented worm expulsion and decreased IL-9-regulated genes. IL-33 has also been demonstrated to promote IL-9 production in human cultured T cells [161]; however, it is unclear how this cytokine may induce IL-9 expression let alone if there is any impact on Th9-mediated pathology.

AMG-157, a humanized TSLP antibody that prevents receptor interaction, reduced allergen-induced bronchoconstriction in a recent proof-of-concept study [139]. It was also demonstrated that AMG-157 could reduce eosinophil levels in mild asthmatics, and patients trended to have reduced Th2 responses. In addition, an antibody directed against human TSLP receptor has been demonstrated to reduce allergic lung inflammation in a cynomolgus monkey model [162]. While no clinical trials have been reported in therapeutics targeting IL-25 and IL-33, animal models do indicate there is potential in their application. Blockade of IL-25 in a mouse model of rhinovirus-induced allergic exacerbation reduced overall inflammation [163]. Furthermore, administration of an antibody directly against IL-33 or the administration of a decoy receptor to IL-33 reduced allergic inflammation [164]. Careful consideration of patient conditions must be kept in mind with all potential treatments. For example, animal models indicate TSLP, IL-25, and IL-33 may be protective in some diseases including inflammatory bowel disease and arteriosclerosis [165–167].

15.4.2 Th9-associated Effector Molecules

The obvious candidate to neutralize Th9-mediated pathologies would be IL-9 itself; however, clinical trials exploring IL-9 neutralization have been mixed. Initial studies demonstrated that enokizumab (or MEDI-528), a humanized IL-9 neutralizing antibody, had an acceptable safety profile and demonstrated positive effects on patients with mild or exercise-induced asthma [168,169]. These studies, however, had very limited patient numbers, so when studies expanded enokizumab use into a larger patients subset with severe asthma, enokizumab demonstrated no benefit with respect to patients receiving placebo [168]. Thus, IL-9 therapy may be limited only to patients with milder forms of asthma or in conjunction with other therapeutics (e.g., IL-4 and IL-13 antibodies). Furthermore, as increased IL-9 levels are seen early in life in atopic patients and increases are associated with early changes in airway responsiveness [100,170], IL-9 therapy may be more appropriate in high-risk infants to limit atopic disease development. IL-9 therapy may also have greater efficacy in other conditions not tested such as ulcerative colitis.

As noted before, Th9 cells can make a range of effector molecules that could make attractive targets to limit their associated pathologies. Th9 cells can make chemokines including CXCL3 and CCL22 [43,52,53], which can recruit a number of immune cells including Th2 cells and monocytes [145,171]. As noted in the

work investigating Th9 associated tumor immunity, IL-3 and IL-21 are also prominent products [60,75]. In addition, qPCR and ELISA analysis has indicated Th9 cells produce IL-1RN and BAFF [43]. Unlike IL-9, however, no studies have investigated neutralizing these products in an attempt to ameliorate inflammation during Th9-associated pathology. Thus, a number of preliminary studies are required to investigate their potential as therapeutic targets. Furthermore, careful consideration needs to be made on a patient to patient basis to prevent any negative consequences as some effectors (e.g., IL-21) were shown to benefit the host therapeutically [60].

15.5 Summary and Future Directions

Although they are one of the most recently established Th subsets, we learned a tremendous amount about Th9 cells. They participate in immunity responsible for parasite clearance, allergic inflammation, autoimmune diseases, and elimination of tumors. Exactly how and where Th9 cells mediate some of these responses is not entirely clear. Although there are hints that Th9 cells could be involved in bacterial or viral immunity, their contribution is still being defined. Moreover, although we know that IL-9 is a critical mediator of Th9-dependent responses, the extent to which other cytokines produced by Th9 cells contribute to immune responses is only beginning to be explored. The development of Th9 cells requires several transcription factors, although a lineage-defining factor has still not been identified. Similarly, some of the regulatory elements required for *IL9* have been identified, but it is not clear which elements, perhaps yet unidentified, are required for appropriate *IL9* expression in a variety of cell types. As tools for analyzing Th9 cells continue to develop, there should be rapid progress in our understanding of the development and function of IL-9-secreting T cells.

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16

Th17 Cells

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16.1 Introduction

The first line of defense is the immune system with its innate and adaptive components. Its main functions are to recognize and control foreign antigens, to induce an immunologic memory, and to maintain a tolerance to self-antigens. CD4+ T cells play a major role in the initiation and regulation of the immune response, mainly by secreting subset-specific cytokines after being activated and differentiated into distinct effector subsets. The CD4+ population is heterogeneous and different subsets provide effector functions during a large variety of immune reactions. Helper T cells (Th cells) are identified according to their cytokine and transcription factor profiles. The two first described major subsets were Th1 and Th2 cells. Th1 cells provide mainly a response against intracellular bacterial infections, secrete primarily interferon-γ (IFN-γ), and express the transcription factor T-bet. Th2 cells provide mainly a response against parasitic infections and their major cytokine is interleukin-4 (IL-4) and their transcription factor GATA-3 [1]. More recently, a third distinct lineage has been identified, the Th17 subset. Th17 cells produce mainly the proinflammatory cytokine IL-17 and they have a key role in the protection against extracellular pathogens and fungi [1,2]. Furthermore, overactivation of these cells has been associated with autoimmunity and inflammation [1,3]. Despite some common markers with Th1 and Th2 subsets, Th17 cells require specific cytokines and transcription factor for their differentiation [4].

The homeostasis of the immune response is continually controlled by a balance between Th cell activation and suppression of this activation by regulatory T cells (Treg). A disruption of this homeostasis leads to an overactivation of the immune response and chronic inflammation. Th17 cells provide an immediate protective response to foreign pathogens and thus they have an important role in the

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induction of inflammatory process. In contrast, Th17 cells are proinflammatory and pathogenic underlying the development and chronicity of many autoimmune inflammatory diseases. As such, they are an important target for treatment.

16.2 Differentiation of Th17 Cells

IL-17 was identified in 1995 first in the human system as cytokine involved in inflammation and neutrophil activation [5]. The concept of IL-17 production by some but not all T cells was introduced in 1999 using T-cell clones from the joint of patients with arthritis [6]. The identification of the Th17 subset was made in the mouse in 2005 [2], 10 years after the description of the cytokine [7]. The development of Th17 cells shares major similarities in mice and humans, but with some differences (see Figure 16.1).

16.2.1 Th17 Cell Differentiation in Mice

The differentiation of the distinct lineages of Th cells is driven by the influence of different cytokines and transcription factors. Transforming growth factor-β (TGF-β), an immunosuppressive cytokine [8], and IL-6, a proinflammatory cytokine [9] act in concert to induce the highly proinflammatory subset of Th17 cells. These two opposite cytokines appear to be sufficient for the induction of IL-17 expression in naïve T cells [10–12]. In the absence of IL-6, TGF-β induces FoxP3, the transcription factor of Treg cells [13,14]. In the presence of IL-6, FoxP3 induction is inhibited and replaced by the induction of RORγt, the

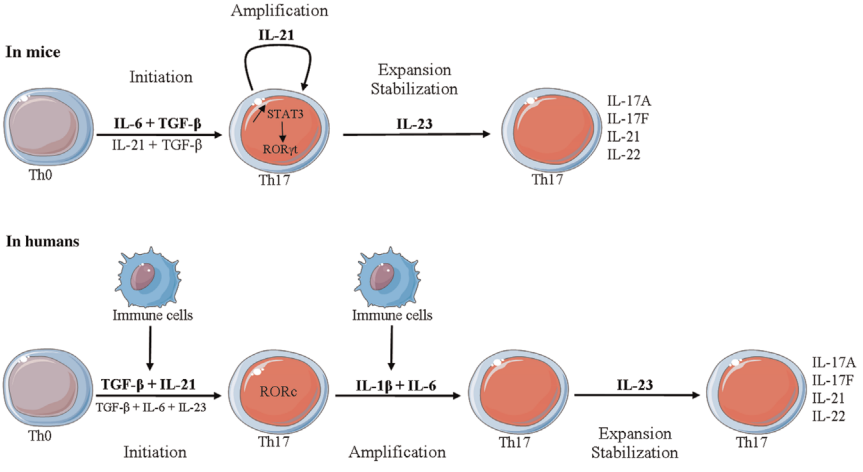


Figure 16.1 Differentiation of Th17 cells in mice and in humans. In mice, TGF-β and IL-6 initiate the differentiation and activate Stat3, which induces RORγt. IL-21 is induced by developing Th17 and in autocrine manner acts on Th17 cell amplification. IL-23 serves to expand and maintain the Th17 cell population. In humans, the differentiation is initiated by TGF-β and IL-21, which induce the transcription factor RORc. IL-1β plus IL-6 are important to enhance the amplification of Th17 cells and IL-23 to maintain the Th17 phenotype. Reproduced with permission from Ref. [111].

transcription factor of Th17 cells. Other proinflammatory cytokines such as tumor necrosis factor-α (TNFα) or IL-1β can enhance TGF-β- and IL-6-mediated Th17 cell differentiation [12].

IL-21, a member of the IL-2 cytokine family, when combined with TGF-β can also induce Th17 differentiation [15–17]. IL-21 is less potent than IL-6 and as it is produced by Th17 cells themselves. It is thus more important in the amplification of Th17 differentiation than in its initiation. IL-21 favors the autocrine loop for Th17 cells. Thus, TGF-β associated with IL-6 initiates Th17 differentiation, while TGF-β associated with IL-21 is a major factor in the autocrine loop of Th17 cells.

After the initiation of differentiation and the expansion of Th17 cells, IL-23, a member of the IL-12 cytokine family, is required for the stabilization of Th17 phenotype [12]. IL-23 alone cannot induce Th17 differentiation as naïve T cells do not express the IL-23 receptor (IL-23R) [18]. IL-23R expression on Th17 cells is induced by IL-6 and IL-21 and also by IL-23 itself [15,19,20] and is thus found only on activated effector or memory T cells. Once activated, T cells become responsive to IL-23 and this leads to the expansion of Th17 cells [21]. IL-23 is not considered as a differentiation factor but as a major player in expansion and stabilization of Th17 phenotype. Accordingly, the differentiation of Th17 cells involves three key steps: the initiation by TGF-β and IL-6, the expansion of Th17 cells by IL-21, and the stabilization of Th17 phenotype by IL-23.

In addition to these specific cytokine patterns, the differentiation of Th17 is driven by a lineage-specific transcription factor, the retinoid-related orphan receptor γt (RORγt) [22]. Cytokines involved in the differentiation of Th17 cells (TGF-β, IL-6, IL-21, and IL-23) induce the expression of RORγt, which depends on Stat3 activation [15,16,23,24]. IL-6-mediated Stat3 activation is crucial for Th17 generation [25,26]. Another transcription factor, RORα, member of the retinoid nuclear receptor family, is also involved in the differentiation of Th17 cells. Its role is similar but not identical to that of RORγt [27]. As RORγt, RORα is strongly induced by IL-6 or IL-21 in association with TGF-β and Stat3 activation increases its expression. This suggests that Th17 cell differentiation could be driven by two lineage-specific transcription factors.

16.2.2 Th17 Cell Differentiation in Humans

The two major cytokines for the differentiation of mouse Th17 cells are TGF-β and IL-6. In humans, the results concerning the role of TGF-β have been confusing. It was demonstrated that TGF-β was dispensable for Th17 differentiation [28,29]. IL-1β in combination with IL-6 [28] or with IL-23 [29] could induce Th17 differentiation. Nevertheless, in these studies, the use of CD45RA+ Th cells purified from peripheral blood as naïve T cells and the presence of serum and platelet contamination, which are important sources of endogenous TGF-β, make the results difficult to interpret.

Other studies, using naïve T cells from cord blood and serum-free medium demonstrated that TGF-β is a key factor, indispensable for the differentiation of Th17 cells. Indeed, the presence of endogenous TGF-β from serum is sufficient to induce Th17 differentiation [30]. Different combinations of TGF-β plus cytokines, TGF-β plus IL-21 and TGF-β plus IL-23 plus IL-6, for instance, can induce

the transcription factor RORc, the human homolog of RORγt, and therefore initiate Th17 differentiation [30–32]. Furthermore, human naïve T cells do not express the IL-1 receptor (IL-1R) and the IL-23 receptor (IL-23R), which are both induced after stimulation by TGF-β and IL-21/IL-6 [33]. Thus in humans, TGF-β has a major role in the initiation of Th17 cell differentiation and it is absolutely required for RORc induction, while IL-6 plus IL-21 is an important combination in the Th17 expansion from central memory cells [30,31]. In conclusion, cytokine requirements and transcriptional program necessary to drive the Th17 cell differentiation in mouse and human do not appear to be so different.

16.2.3 Pathogenic Th17 Cells

The contribution of these various cytokines has an effect on the final pathogenicity of Th17 cells. TGF-β and IL-6 are required for the differentiation of Th17 cells and IL-23 for their expansion and stabilization. Nevertheless, these cytokines also play a role in the regulation of Th17 subtype functions. A continued exposure to TGF-β1 and IL-6 induces not only the production of IL-17 but also the production of IL-10, and these cells produce lower proinflammatory mediators such as IL-22 [34]. Thus, IL-10 production is associated with nonpathogenic Th17 cells. A switch to a pathogenic phenotype can be induced by an IL-23 exposure [34,35]. IL-23 is crucial to confer the Th17 cell ability to induce autoimmunity [35–37]. In the absence of TGF-β signaling, the combination of IL-1β, IL-6, and IL-23 induces pathogenic Th17 cells [38]. Furthermore, IL-23 plays a major role in enhancing the contribution of endogenous TGF-β3 in Th17 development, which has a critical role in inducing pathogenic Th17 cells [39]. Th17 cells induced by TGF-β3 and IL-6 express GM-CSF whose expression is part of the signature of pathogenic Th17 cells [39–41]. Other factors are associated with the Th17 pathogenic signature, including T-bet, IL-23R, and IL-7R [39]. In summary, TGF-β1 and IL-6 induce nonpathogenic Th17 cells, while Th17 cells induced by TGF-β3 and IL-6 are highly pathogenic. IL-23 is also required for Th17 cells to achieve their total pathogenic phenotype, notably by inhibiting the IL-10 production and by promoting TGF-β3 or GM-CSF production.

16.2.4 Circulating versus Tissue Phenotypes

Th17 cells were first identified as a CD4+ T cell population producing IL-17 [42]. Their classic phenotype exhibits also the expression of RORc, IL-23R, and CCR6 [43,44]. Recently, CD161 has been identified as a marker of all circulating IL-17-producing T cells, reinforcing the specificity of the Th17 phenotype [45]. Thus, circulating Th17 cells can be characterized by the phenotype CD4+ RORc+ IL-23R+ CCR6+ CD161+ and IL-17+ (see Figure 16.2). Nevertheless, at this stage, Th17 cells do not produce IL-17, and accumulating evidence indicates that CD4+ T cells are more plastic than previously described. Depending on the environment, Th17 cells can express markers of other Th subsets in addition to IL-17 and can switch their phenotype, notably into Treg cells. However, the double-positive cells are less stable and become single producing cells in inflamed tissue [46–50].

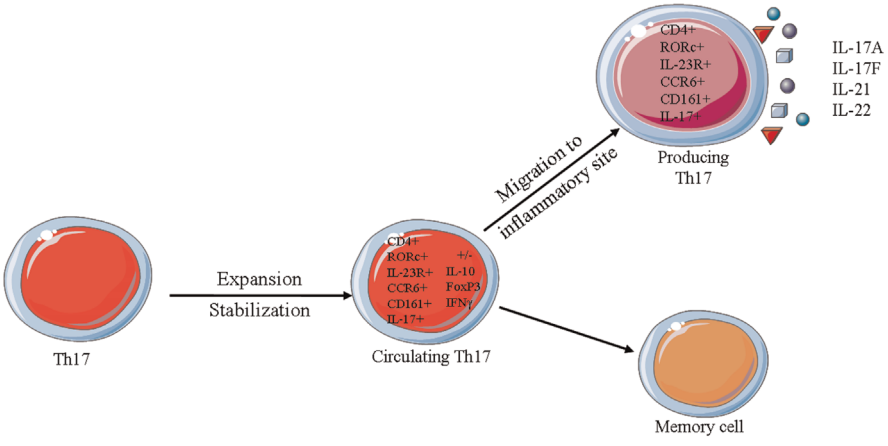


Figure 16.2 Th17 phenotypes. After their expansion and stabilization, circulating Th17 cells are characterized by the phenotype CD4+ RORc+ IL-23R+ CCR6+ CD161+ and IL-17+. At this stage, Th17 cells do not produce IL-17 and can express markers of other Th subsets and can switch their phenotype. The double-positive cells are less stable and become single producing cells in the inflamed tissue, with a plasma cell phenotype.

In most reports, a direct association is made between Th17 cells and IL-17 secretion. The intracellular staining of IL-17, commonly used to identify Th17 cells, is thought to be an equivalent of the actual IL-17 production. Even so the intracellular presence of IL-17 inside Th17 cells does not mean by itself its secretion. *In situ*, Th17 cells interact with local mesenchymal cells in an inflammatory environment and this leads to IL-17 secretion [51]. Furthermore, IL-17+ cells acquire a plasma cell-like morphology under *in vitro* activation and this is correlated with enhanced concentrations of secreted IL-17. In inflamed tissue, IL-17+ cells present the same plasma cell-like morphology [46]. This indicates a major discrepancy between intracellular and secreted IL-17, and it is necessary to distinguish both phenotypes, the plastic circulating Th17 cells and the tissue Th17 cells that secrete IL-17.

16.3 Cytokines and Chemokines Related to Th17 Cells

16.3.1 Major Cytokines and Chemokines Produced by Th17 Cells

Th17 lymphocytes are proinflammatory cells that produce different cytokines and chemokines involved in the pathology of many inflammatory and autoimmune diseases [1,4]. IL-17, IL-21, IL-22, and CCL20 are the most important of these effector molecules [1,4,52].

16.3.1.1 IL-17A (IL-17)/IL-17F

IL-17A and IL-17F have similar biological functions and are genetically linked. IL-17A is the major cytokine produced by Th17 cells and has a stronger effect than IL-17 F. IL-17 signaling goes through a heterodimeric receptor composed of two

chains, IL-17RA and IL-17RC. The distribution of IL-17RA and IL-17RC diverges as IL-17RA is more distributed on immune cells, while IL-17RC is preferentially expressed by nonimmune cells. The binding between IL-17 and IL-17RA induces the recruitment of IL-17RC to form the complex and to transduce the signal. IL-17 plays a central role in the adaptive immune response, mainly against extracellular bacteria and fungi. Excess of IL-17 is involved in a growing list of autoimmune and chronic inflammatory diseases such as rheumatoid arthritis (RA), psoriasis, psoriatic arthritis, multiple sclerosis, or Crohn's disease. The major role of IL-17 is to induce the production of proinflammatory cytokines (TNF-α, IL-6, and IL-1) and chemokines (IL-8 and CCL20), antimicrobial peptides (AMP), and matrix metalloproteinases by many types of cells, such as macrophages, dendritic cells, endothelial cells, or fibroblasts of different origins. This contributes to the recruitment of neutrophils and monocytes at the inflammatory site. IL-17 also plays a role in granulopoiesis by increasing the secretion of G-CSF and GM-CSF. The many proinflammatory functions and the multiple cell targets of IL-17 make it a critical player in autoimmunity and inflammation [1,52–55].

16.3.1.2 IL-21

IL-21 is a cytokine with potent regulatory effects on the immune system and notably on lymphocyte regulation and inflammation [4,52]. IL-21 is also involved in the expansion of activated B cells and in class switching of immunoglobulin isotypes [56]. IL-21 also promotes Th1 responses by enhancing IFN-γ production [57,58] and modulates the functional development of natural killer (NK) cells [59]. Furthermore, it plays a key role in the Th17 pathway, by acting in a positive feedback loop leading to the amplification of Th17 cells and to its own production [15–17]. IL-21 is essential to maintain and amplify the Th17 pool and thus it is an important player in the support of the Th17 response and tissue inflammation [4,52].

16.3.1.3 IL-22

IL-22 is a cytokine produced by terminally differentiated Th17 lymphocytes in response to IL-23. Its receptor IL-22R is widely expressed in different tissue cells such as epithelial and endothelial cells but not in immune cells. Its important local expression is linked to the pleiotropic functions of IL-22 in the biology of epithelia, autoimmunity, and infections [4,52]. For example, by inducing AMP from various cell types such as keratinocytes [60], IL-22 plays a major role in host defense for pathogens and in the immune barrier function of epithelia [60,61]. As a consequence, IL-22 is upregulated in skin and mucosal diseases such as psoriasis, Crohn's disease, and ulcerative colitis [52]. IL-22 is a complex cytokine with both proinflammatory and anti-inflammatory properties, depending on the local conditions, such as during acute liver inflammation [62]. Thus, IL-22 appears as a cytokine used by Th17 cells to interact with tissues but with complex functions that depend on the environment [4,52].

16.3.1.4 CCL20

CCL20 is a chemokine with antimicrobial and chemoattractive activities. It is produced in various tissues by mesenchymal and immune cells [63]. It attracts at

the site of inflammation immature dendritic cells and Th17 cells [64]. Its receptor, CCR6 is expressed by dendritic cells, B lymphocytes, and Th17 cells. Thus, by secreting CCL20, Th17 cells can themselves regulate their recruitment into the inflammatory sites [3].

16.3.2 Major Cytokines Acting on Th17 Cells

Th17 cells contribute to severe tissue inflammation and autoimmunity. Their regulation may be driven in two ways, the persistence of Th17 pool or the inhibition of their differentiation. Two major cytokines IL-23 and IL-27 are involved in this regulation acting in opposite ways.

16.3.2.1 IL-23

IL-23 is a member of IL-12 cytokine family and it has many effects on the immune response. It is mainly produced by innate immune system such as dendritic cells and macrophages and plays a key role in the induction and chronicity of the Th17 response. IL-23 appears as a bridge between the innate immunity and the adaptive immunity [65,66]. After the initiation of Th17 differentiation, T cells start to express the IL-23R and become responsive to IL-23. This leads to the persistence of activated Th17 lymphocytes and to the subsequent production of IL-17. IL-23 is a critical driver for the expansion and the stabilization of Th17 cells and can be the limiting factor to determine if the Th17 response is sustained and pathogenic during immune and inflammatory responses [65–67].

16.3.2.2 IL-27

To counteract the positive effect of IL-23 on Th17 cells, IL-27 acts as a negative regulatory cytokine. IL-27 was first described as an inducer of IFN-γ response [68]. IL-27 and IL-23 belong to the IL-12 cytokine family and are both secreted by antigen-presenting cells, macrophages, and dendritic cells. The anti-inflammatory properties of IL-27 downregulate the immune response during chronic inflammation. One of the mechanisms is the ability to skew T-cell polarization and mainly to inhibit Th17 differentiation. IL-27 can also suppress the production of proinflammatory cytokines, notably IL-17, and thus reduce inflammation [69,70]. In addition to its direct activity on Th17 cells, IL-27 promotes the development of regulatory T cells [71].

16.4 Th17 and Inflammatory Diseases

Th17 cells are highly pathogenic and their deregulation could lead to the development of chronic inflammation and severe autoimmunity. Inflammation is a dynamic process with different stages. At the early stage, neutrophils are recruited first followed by monocytes and finally by lymphocytes. Th17 cells, mainly through the secretion of IL-17, increase the secretion of the proinflammatory cytokines IL-1, TNF, and IL-6 by monocytes and other cell types. Among other secreted molecules, IL-8 is a chemokine for neutrophils and this leads to

their early accumulation at the inflammatory sites. Furthermore, at these sites, Th17 cells interact with local cells and this induces the secretion of metalloproteinases (MMP) involved in matrix destruction [72,73]. Thus Th17 cells contribute largely to inflammation by different mechanisms, which explain their involvement in several inflammatory autoimmune diseases such as psoriasis, arthritic diseases (rheumatoid arthritis, psoriatic arthritis or ankylosing spondylitis), multiple sclerosis, or inflammatory bowel diseases [1,3,4,72,74,75].

16.4.1 Psoriasis

Psoriasis is a chronic immune-mediated inflammatory skin disease characterized by a hyperproliferative epidermis and an immune infiltration, mainly by dendritic cells, macrophages, and T cells in the dermis and neutrophils and T cells in the epidermis. Psoriasis is considered as a Th1/Th17-based disease as both populations are found in psoriatic skin lesions [76]. The infiltrated T cells isolated from the skin lesions present predominantly a Th17 phenotype [77]. Circulating Th17 cells are also increased in psoriatic patients [78]. These increased frequencies of Th17 cells lead to high levels of IL-17, which activates local cells such as keratinocytes to secrete proinflammatory chemokines and cytokines and thus to sustain chronic inflammation. Therefore, Th17 cells contribute largely to the pathogenesis of psoriasis [3,74,75].

16.4.2 Arthritic Diseases

Arthritic diseases combine different pathologies with a common form of chronic immune-mediated inflammatory arthritis. Rheumatoid and psoriatic arthritis are characterized by progressive joint destruction with lack of repair contrasting with the ectopic new bone formation in syndesmophytes of ankylosing spondylitis [79]. Despite this major difference, Th17 cells are involved in rheumatoid and psoriatic arthritis and ankylosing spondylitis pathogenesis. Indeed, Th17 cells are increased in peripheral blood of rheumatoid arthritis [80–82], ankylosing spondylitis, and psoriatic arthritis patients [83–87]. These increased frequencies of Th17 cells exacerbate the inflammatory state of arthritis through the activation of various cell types in the inflamed tissue, such as synoviocytes, dendritic cells, or macrophages. Furthermore, IL-17 produced by Th17 cells in inflammatory site can synergize with other cytokines such as TNF. Interestingly, IL-17 and TNF increase osteoblastogenesis on local isolated cells such as synoviocytes or mesenchymal cells, contrasting with the destructive effect in whole bone [88,89]. The difference between bone loss in rheumatoid arthritis and bone formation in ankylosing spondylitis could be due to the cell composition of the inflammatory environment. In rheumatoid arthritis, osteoclasts are massively activated by IL-17 and TNF and they interact with osteoblasts that leads to bone destruction. Conversely, in ankylosing spondylitis, osteoclasts are not present in tendons and ligaments, and the combination IL-17 and TNF can lead to bone formation as in syndesmophytes. The different effector molecules of Th17 cells synergize to increase the disease activity and this supports the role of Th17 cells in the arthritic pathogenesis by contributing to the chronic inflammatory process [3,74,75].

16.4.3 Multiple Sclerosis

Multiple sclerosis is a chronic inflammatory disease characterized by brain inflammation. Both innate and adaptive immune cells are involved in the inflammation of the central nervous system. As for the previous diseases, the frequencies of Th17 cells and related cytokines are increased in multiple sclerosis patients [90–92]. These cells have the capacity to breach the blood–brain barrier and infiltrate the central nervous system [93]. The role of Th17 cells in multiple sclerosis is reinforced by a lower ratio of Treg/Th17, which correlates with multiple sclerosis severity [91,94]. Furthermore, different treatments used in multiple sclerosis induce a reduction of Th17 cells [74].

16.4.4 Inflammatory Bowel Diseases

The two major forms, Crohn's disease and ulcerative colitis, are characterized by a chronic relapsing inflammatory response to commensal microflora in the gut. Th17-associated cytokines are increased in the inflamed tissues and in the sera and Th17 cells are increased in peripheral blood [95,96]. Furthermore, increased Th17/Treg ratio is associated with disease activity, notably in Crohn's disease patients [97] and activation of the IL-23/Th17 pathway is considered as a biomarker of active disease [98]. As discussed above, IL-17 is also involved in mucosal protection. Although the respective contribution of these opposite mechanisms remains to be elucidated, it is clear that this T-cell subset is important in pathogenesis [3,74].

16.5 Mechanisms in Chronic Inflammation

Inflammation is first a protective response and a reversible mechanism leading to the resolution at inflammatory sites. A dysregulation of the resolution and a sustained inflammatory response lead to irreversible chronic inflammation, which induces various diseases [99,100]. T helper cells, including Th17 cells, are highly present in the inflammatory lesions. As described above, Th17 cells are a pathogenic subset largely involved in the pathogenesis of the chronic inflammatory diseases described above and the number of Th17-associated diseases keeps increasing [75]. Common mechanisms lead to their differentiation and maintenance during chronic inflammation and Th17 cells exhibit similar patterns with increased frequency and elevated Th17 cytokine levels. By activating the innate and adaptive immune cells and local cells in the inflamed site, they contribute to maintain the immune response. Th17 cells act as a bridge between adaptive and innate immunity systems to sustain chronic inflammation.

16.5.1 Differentiation, Recruitment, and Maintenance of Th17 Phenotype during Chronic Inflammation

During the triggering of inflammatory response, immune cells release Th-polarizing cytokines. Among them, TGF-β, IL-6, and IL-1β induce Th17

differentiation. The Th17 transcription factor RORc induces IL-21 production that acts in an autocrine pathway to maintain RORc expression and thus maintain the Th17 phenotype. Once differentiated, Th17 cells express IL-23R and become responsive to this cytokine. IL-23 produced by innate immune cells is a major driver in the maintenance of Th17 phenotype. Furthermore, CCR6 that binds only CCL20 is preferentially expressed by Th17 subset [101] that contributes to the specific recruitment of Th17 cells during inflammation. CCL20, upregulated under inflammatory conditions, is produced by several types of epithelial and immune cells and also by Th17 cells that can themselves regulate their migration [64]. During the inflammatory process, Th17 cells are activated and by secreting proinflammatory cytokines they stimulate local cells that produce, in turn, proinflammatory cytokines and chemokines. These molecules are mainly involved in Th17 differentiation and stabilization. By secreting key cytokines and by interacting with and activating local cells, Th17 cells contribute to their own maintenance. Therefore, the secretion of cytokines and chemokines both by local and immune cells and by Th17 cells contributes largely to the differentiation, the migration, and the maintenance of Th17 cells in the inflammatory site (Figure 16.3). In addition to their own role, another subset of immune cells is crucial to the maintenance of Th17 cells, an inflammatory subset of dendritic cells (inflaDCs) appearing during chronic inflammation. By secreting Th17 cell-polarizing cytokines, inflaDC induce Th17 differentiation from naïve T cells. Furthermore, they can stimulate autologous memory T cells to produce IL-17 [102]. This subset of DC is a major player in the maintenance of Th17 phenotype during chronic inflammation.

16.5.2 Secretion of IL-17 and its Activity

The major effective cytokine of Th17 cells is IL-17 (Chapter 24). During chronic inflammation, the inflammatory environment and the cell interaction lead to activation of cells and release of cytokines that activate intracellular pathway. At molecular level, Stat3 by increasing RORc expression can affect IL-17 secretion. Stat3 can also bind directly the promoter *Il17*. The effective production of IL-17 depends on these two transcription factors, Stat3 and RORc. The secretion of IL-17 is also regulated by other cytokines, such as IL-15, IL-18, and IL-21, which stimulate its production [103,104]. The most efficient cytokine to induce IL-17 secretion is IL-23. Indeed, after IL-23R expression by Th17 cells, IL-23 promotes T cells to produce IL-17 and this requires Stat4 activation for a maximal production [25].

IL-17 exerts various biological functions. Notably, IL-17 stimulates stromal cells to produce proinflammatory cytokines and chemokines, resulting in the maintenance of inflammation [5]. IL-17 can also directly act on the phenotype and survival of cells. Indeed, in combination with TNF, IL-17 increases the survival of rheumatoid arthritis synoviocytes by inducing antiapoptotic molecules, such as synoviolin [105], and contributes to their invasive phenotype [106]. In this way, IL-17 plays a major role in the chronicity of inflammation. Mainly by secreting IL-17, Th17 cells contribute to the irreversible side of diseases induced by inflammation.

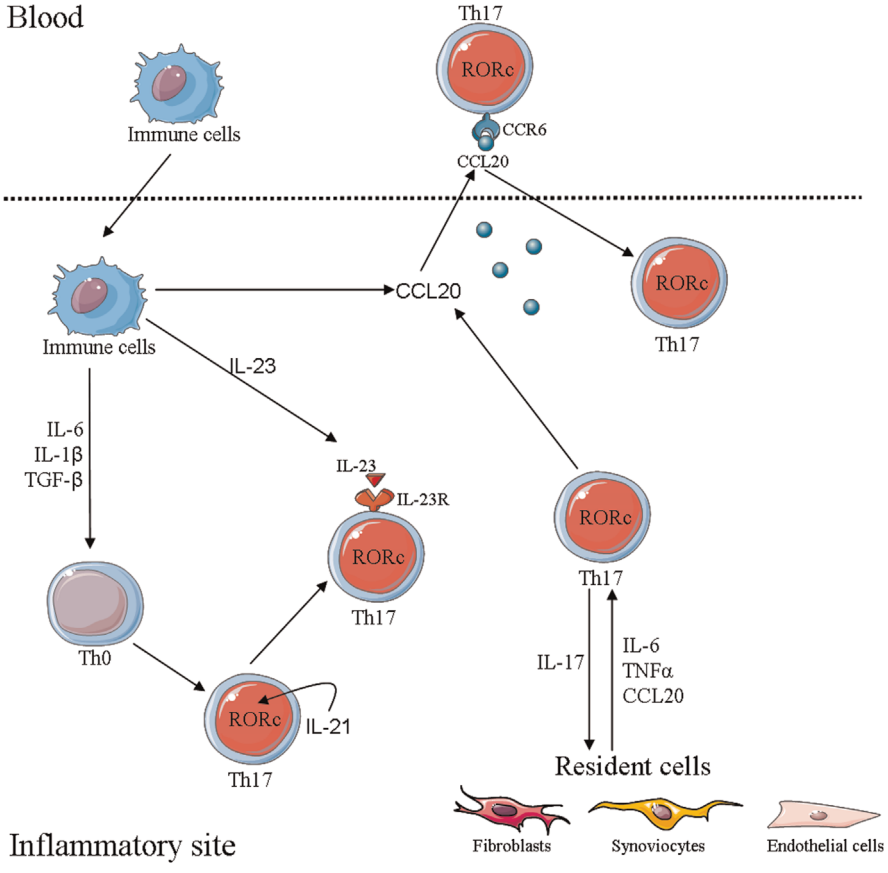


Figure 16.3 Th17 cells during chronic inflammation. During inflammation, immune cells migrate to the inflammatory site and secrete Th-polarizing cytokines. TGF-β, IL-6 and IL-1β induce Th17 differentiation via the induction of the transcription factor RORc. RORc induces IL-21 that acts in autocrine way to maintain the Th17 phenotype. Differentiated Th17 cells express IL-23R and IL-23 drives the maintenance of the Th17 phenotype. Th17 cells express the receptor CCR6, which binds CCL20 and this interaction leads to the recruitment of Th17 cells to the inflammatory site. CCL20 is produced both by mesechymal cells and by Th17 cells themselves. Cytokines produced by Th17 cells, notably IL-17, stimulate local cells such as fibroblasts, synoviocytes, or endothelial cells, to produce proinflammatory cytokines and chemokines involved in Th17 differentiation and stabilization. This way induces the chronicity of the activation of the Th17 cells.

16.6 Plasticity of Th17 Cells

Even if each Th subset requires specific cytokines and transcription factors for their differentiation, Th17 cells keep some degree of plasticity (Figure 16.4) [3,48–50,107]. Thereby, under inflammatory conditions, Th17 cells display plasticity and the produced cytokines can switch from IL-17 to other proinflammatory cytokines, such as IFN-γ. Indeed, in blood, IL-17+ IFN-γ+ double producer cells are easily detected. Different coexpressions during inflammatory

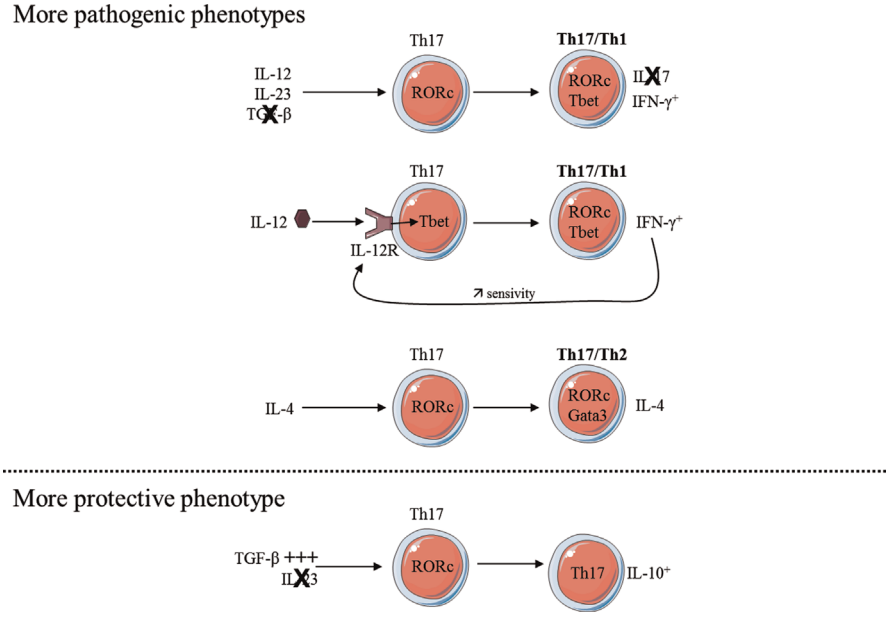


Figure 16.4 Plasticity of Th17 cells. Under inflammatory conditions, the Th17 subset displays plasticity. In the absence of TGF-β, IL-23 and IL-12 suppress IL-17 production and promote T-bet expression that induces IFN-γ expression in Th17 precursors. Exposure of Th17 cells to IL-12, which express IL-12R, induces the switch to Th1 phenotype with the expression of T-bet and the secretion of IFN-γ. In turn, IFN-γ increases the sensitivity of Th17 cells to IL-12. IL-4 can lead to a switch into Th2 phenotype with GATA-3 expression and IL-4 secretion. The Th17/Th1 and Th17/Th2 phenotypes may be more aggressive and pathogenic than unshifted Th17 cells. A high level of TGF-β can induce the secretion of IL-10 by Th17 cells, and this is increased in the absence of IL-23. This phenotype is more protective than the Th17 cells.

responses have been observed, IL-17/IFN-γ, RORc/T-bet, FoxP3/RORc/IL-17, or IL-17/IL-10, and these mixed phenotypes are determined by the inflammatory environment. The prime factors that affect the stability of Th subsets are also those involved in their differentiation. TGF-β plays a major role for maintenance of IL-17 expression. In the absence of TGF-β, IL-23 and IL-12 suppress IL-17 production and promote the emergence of IFN-γ expression in Th17 precursors. This plasticity toward the Th1 profile is dependent on Stat4 and T-bet transcription factors [108]. This coexpression RORc and T-bet leads to a more pathogenic phenotype of Th17 cells. On the contrary, high levels of TGF-β induce the ability to IL-17+ cells to produce also IL-10 and this is reinforced by the absence of IL-23. This phenotype is more protective due to the anti-inflammatory effect of IL-10 [109]. The presence of IL-23 is known to stabilize the Th17 phenotype, but it can also mediate the switch to IFN-γ production during chronic inflammation [110]. Th17 cells express a full IL-12 receptor and thus, in response to exposure to IL-12, they switch to Th1 phenotype by expressing T-bet and secreting IFN-γ. In turn, IFN-γ increases the sensitivity of Th17 cells to IL-12 and contributes to the phenotype Th17/Th1 that coexpressed RORc and T-bet. Furthermore, the interaction with IL-4 can also lead to a switch toward Th2 subset and IL-4 production.

Both phenotypes, Th1/Th17 and Th2/Th17, seem to be more aggressive and more pathogenic than unshifted Th17 cells.

Furthermore, the network between the different transcription factors also affects the stability and the plasticity of Th17 cells, and notably the inhibitory effect of Foxp3 on RORc. The development of Th17 and Treg cells is closely related by TGF-β. Indeed, TGF-β induces both transcription factors, Foxp3 and RORc, but in the absence of inflammation, that is, mainly the absence of IL-6, FoxP3 represses RORc and Treg differentiation is favored. Under inflammatory conditions, RORc is upregulated and induces Th17 differentiation. A transient population made of CD4+ FoxP3+ RORc+ cells can lead to both Th17 and Treg cells according to the local conditions. The plasticity between Th17 and Treg cells is important and plays a major role in the control and outcome of immune responses and inflammation [111]. The plasticity of Th17 cells has important biological implications regarding their ability to switch to a more pathogenic phenotype such as Th17/Th1 or more protective phenotype such as Th17/Treg.

16.7 Clinical Targeting of Th17 Cells

Considering the crucial role of Th17 cells in the pathogenesis of several chronic inflammatory diseases, they have been considered as potential targets for treatment [1,3,72,107]. Various options are available to target the Th17 pathway in inflammatory diseases, cytokine targeting, signaling inhibition, or transcription factor inhibitor.

To act on Th17 differentiation and amplification, inhibitors of IL-6 and IL-1β, largely involved in Th17 differentiation, are currently used in the treatment of inflammatory diseases. Tocilizumab is a humanized anti-IL-6 receptor antibody, anakinra is an IL-1 receptor antagonist, and canakinumab is an inhibitor of IL-1. The first two are currently used in rheumatoid arthritis treatment and the last one for that of autoinflammatory diseases [112]. In contrast, tocilizumab has no clear effect on psoriasis, psoriatic arthritis, and ankylosing spondylitis [113], suggesting different mechanisms by which Th17 cells are involved in these diseases.

Another target is IL-23 as it is critical for the expansion and the stabilization of the Th17 phenotype. IL-23 can be inhibited by two antibodies directed against the p40 chain, a subunit of IL-23, ustekinumab and briakinumab. Ustekinumab is registered for the treatment of psoriasis and psoriatic arthritis. Other indications are in progress [114–118]. All these therapies, by targeting the common p40 chain, affect both the Th17 and the Th1 pathways, and IL-17 and IFNγ, respectively. Targeting the p19 specific chain of IL-23 affects only the Th17 pathways and their cytokines IL-17, IL-21, and IL-22. The anti-IL-23 p19 inhibitors tildrakizumab and guselkumab are now tested in psoriasis and psoriatic arthritis.

Another option is to target the key transcription factor RORc [119], using small molecules. This provides a good opportunity to inhibit Th17 subset but also Th17/Th1 and Th17/Th2 shifted cells, which could be the more pathogenic ones. This will block the entire Th17 program, from Th17 development to

proinflammatory cytokine secretion. Digoxin is an old molecule recently identified as a specific RORγt inhibitor [120]. Other molecules such as ursolic acid and SR1001 bind specifically RORα and RORγt [121]. They inhibit Th17 differentiation and function in murine and human cells and suppress the clinical severity of various models of autoimmune diseases in mice [122]. The use of small molecules to target the transcription factor RORc could provide a simple treatment of chronic inflammatory and autoimmune diseases.

The biologic effects of Th17 cells could also be controlled downstream by targeting directly IL-17. Secukinumab, a fully human anti-IL-17A monoclonal antibody, has been the first inhibitor of IL-17A for the treatment of psoriasis, and registration for psoriatic arthritis and ankylosing spondylitis are in progress [72]. Similar indications have been selected for ixekizumab, a humanized anti-IL-17A [123]. Both antibodies have shown efficacy in rheumatoid arthritis but with a high heterogeneity in the clinical response [124,125]. Another way to inhibit the IL-17 effect is to act downstream on IL-17 receptor complex. Brodalumab is a humanized anti-IL-17RA antibody that has shown efficacy in psoriasis [126,127], in psoriatic arthritis, and ankylosing spondylitis [128] but not in rheumatoid arthritis [129].

16.8 Conclusions

Th17 cells were identified as the third subset of T helper cells, with distinct effector functions from the Th1 and Th2 subsets. Increased knowledge of Th17 cells during the years past has been made in understanding their development and in defining their role in health and diseases. Th17 cells are important effector cells to clear many pathogens. When in excess, this subset becomes highly pathogenic in the induction and the propagation of chronic inflammation. Many diseases are influenced by the activation of the Th17 pathway, such as psoriasis, rheumatoid arthritis, or multiple sclerosis.

Considering their role in inflammatory and autoimmune diseases, the inhibition or the modulation of Th17 cell differentiation and functions could be beneficial under such conditions. Impressive results have already been obtained and more strategies are developed. The final goal is to fine-tune this balance not only to control inflammation but also to induce a cure.

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17

Platelets

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17.1 Introduction

Mammalian platelets are appropriately considered as functioning primarily to support a range of hemostatic mechanisms, both by maintaining the integrity of blood vessels and by contributing to the process of blood coagulation. However, platelets have many other capabilities, although often rudimentary, which appear to be unrelated to their hemostatic function.

Despite approximately 450 million years of evolution [1], mammalian platelets retain many of the functions of amebocytes, the only type of circulating blood cell in the horseshoe crab, *Limulus polyphemus*, which is justifiably considered to be a "living fossil." This phenomenon may have resulted from the retention of multiple functions previously found in a single "all-purpose" circulating cell type, such as the Limulus amebocyte, whose functions include hemostasis. The potential for platelets to be involved in inflammation was suggested by a comparison of reported but historically ignored platelet functions with the multiple functions of the Limulus amebocyte.

The multiple characteristics shared by mammalian platelets and Limulus amebocytes are summarized in Table 17.1. Platelets have rudimentary bactericidal and some phagocytic or phagocytic-like activity [2–6]. However, an ultrastructural study of interactions between human platelets and various-size particles concluded that platelets were not true phagocytes and that the uptake of bacteria involved the channels of the open canalicular system (OCS) [7]. Nevertheless, an exhaustive review of studies that described the interactions of platelets with particulate materials or bacteria presented considerable data, which appeared to support the conclusion that platelets are capable of phagocytosis [8]. Platelets contain endotoxin-binding substances [9] and have been shown to interact with bacteria, endotoxins, viruses, and fungi [10–14]. Murine [15] and human [16] platelets have been shown to express a functional Toll-like receptor-4 (TLR4), the receptor for bacterial

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Table 17.1 Comparison of Limulus amebocytes and mammalian platelets.

Characteristic or Function	Limulus Amebocyte	Mammalian Platelet
Hemostasis	Essential	Essential
Blood coagulation	Essential	Ancillary
Clottable protein	Yes	No
Nucleus	Yes	No
Cellular processes	Yes	Yes
Granules	Yes	Yes
Response to endotoxin	Yes	Yes
Phagocytosis	Yes	Yes (?)
Antibacterial activity	Yes	Yes
Motility	Yes	Yes

endotoxin (LPS) [15]. The extensive role of platelets in antimicrobial host defense has been reviewed [14,17]. Staphylococcus can stimulate human platelets to undergo the release reaction [18].

Pneumococcus (*Streptococcus pneumoniae*) and vaccinia virus can induce the release of serotonin from human platelets [19,20]. Other investigations have demonstrated that two endocarditis-producing strains of *S. viridans* can activate human platelets *in vitro* [21], apparently by direct platelet-bacterial interaction. Fibrinogen in conjunction with other unidentified plasma factors was required for platelet aggregation and secretion [21,22]. The aggregation of human platelets by *S. viridans*, *S. pyogenes*, and *S. sanguis* has been demonstrated [23–25]. *S. mitis* has been shown to possess at least two genomic regions that contribute to platelet binding [26].

Human platelets have been reported to be cytotoxic for parasites by a mechanism involving an IgE receptor on the platelet surface [27,28]. Platelets also have been shown to play a role in the excretion of *Schistosoma mansoni* in the stool of mice [29]. Other studies have demonstrated that platelets enhance the adherence of schistosome eggs to endothelial cells and that interleukin (IL)-6, produced by activated monocytes, markedly increases the cytotoxicity of platelets against schistosomula [30]. Under certain circumstances, platelets demonstrate a chemotactic response and migrate [31–34], as do amebocytes [35]. Platelets have demonstrated gradient-driven migration and have been shown to migrate toward formyl peptides [33]. Overall, it is now clear that bacteria, viruses, fungi, and parasites are capable of interacting with platelets. Depending upon the nature of the infectious particles and other poorly understood variables, this interaction can result in platelet aggregation, release of platelet constituents, phagocytosis of the infectious agent, and ultimately a shortened platelet lifespan. Collectively, these and other observations establish the important roles of platelets in a wide range of inflammatory responses (Figure 17.1 and Table 17.2, modified from Ref. [36].

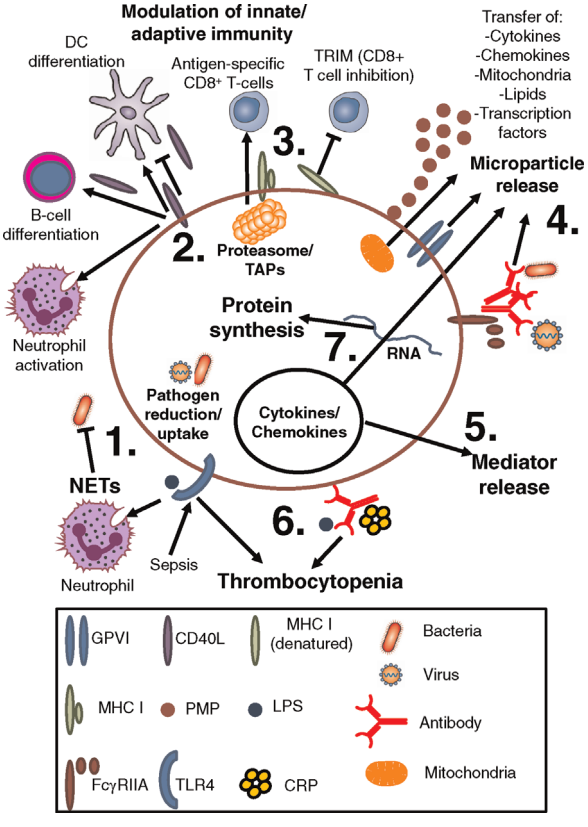


Figure 17.1 The many important roles of platelets in modulating a wide range of inflammatory processes. See also Table 17.2. (Reproduced with the permission from Ref. [36].)

Platelet microparticles (PMPs) are extracellular microvesicles that result from cytoplasmic blebbing. PMPs range in size from 100–1 000 nm, are procoagulant, and have been shown to be present in various inflammatory states [37,38]. PMPs are rich in Il-1 and it has been suggested that they play a role in increasing inflammation [39,40].

Table 17.2 Nonhemostatic components of platelets involved in infection and inflammation.[a)]

MHC Class 1
Cytokines/chemokines
CD40 L (CD154)
TLRs
Transcriptomics
Microparticles (PMPs)
Pathogen reduction

a) Derived from Ref. [36], Table 17.1.

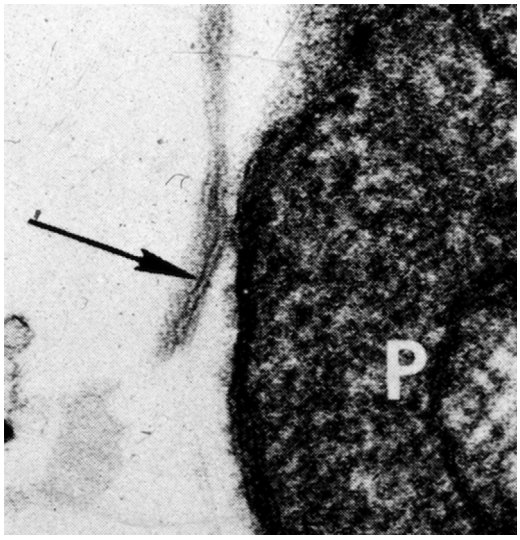


Figure 17.2 Bacterial endotoxin (LPS) adherent to a platelet (P). This also demonstrates the particulate nature of endotoxin, which is an important component of the cell wall of all Gram-negative bacteria.

17.2 Expression of Toll-like Receptors

TLRs are pattern recognition receptors, present on macrophages, neutrophils, dendritic cells, and platelets, which play a role in activation of the immune response to pathogens [41,42]. Importantly, bacterial endotoxin (lipopolysaccharide, LPS) interacts with TLR4 on platelets, perhaps accounting for, at least in part, the ability of bacterial LPS to produce thrombocytopenia. Figure 17.2 demonstrates the adhesion of a piece of the cell wall of a Gram-negative bacterium (containing bacterial LPS) to a platelet. The resultant activation of platelets probably leads to platelet/neutrophil interactions that may lead to bacterial trapping [43]. LPS enhances responses of platelets to immune complexes and aggregates of IgG, demonstrating integration of diverse inflammatory signaling pathways and providing early evidence for a specific receptor for LPS on these cells [44]. Toll-like receptors on both platelets and monocytes, allowing cross-talk, provide another mechanism for the involvement of platelets in immune responses [45,46].

17.3 Interactions between Platelets and Bacteria

Microbicidal proteins, polymorphic protein-1 (PMP-1) and PMP-2, are small cationic peptides released by rabbit platelets, which disrupt the membrane of *Staphylococcus aureus* and cause cell death [47]. It has been demonstrated that staphylococci have a receptor for the Fc fragment of immunoglobulin G (IgG) that provides a mechanism for aggregation of human platelets by the formation of a complex composed of bacteria, IgG, and platelets [48]. Furthermore, it has been shown that platelet binding by *S. aureus* is mediated,

at least in part, by direct binding of clumping factor A (ClfA) to a novel 118-kDa platelet membrane receptor [49]. Certain surface proteins of *S. gordonii* bind to the extracellular portion of the platelet membrane glycoprotein (GP) Ibα [50]. Some streptococcal adhesins bind to platelet glycoprotein GP1b and enhances virulence in endocarditis [51]. In addition, group B *Streptococcus* possess serine-rich repeat (Srr) surface glycoproteins, important adhesins that contain a fibrinogen-binding region [52]. Similarly, *S. agalatiae* (a group B *Streptococcus*) possesses a serine-rich repeat glycoprotein, Srr1, which appears to play a role in invasion of the central nervous system and in the pathogenesis of endocarditis through its role in fibrinogen-mediated platelet binding [53,54]. *S. sanguinis*, a leading cause of infective endocarditis, expresses a serine-rich adhesion, SrpA, similar to platelet-binding adhesins associated with increased virulence in bacterial endocarditis [55]. Oral Streptococci use a Siglec-like domain of SRR adhesins to preferentially target platelet sialogycans; this provides the viridans group of Streptococci a selective advantage in colonizing damaged cardiac vales and producing infective endocarditis [56].

Platelets have been shown to be bactericidal for *Bacillus subtilis* and *Escherichia coli* (but not *S. aureus*), but only in the presence of thrombin and a heat-labile plasma protein fraction >100 kDa [57,58]. The mechanisms of the multiple interactions between platelets and bacteria have been the subject of an exhaustive review [58]. Importantly, it was pointed out that certain infectious agents may utilize various platelet responses to increase virulence [58].

17.4 Interactions between Platelets and White Blood Cells

Platelets which are adherent to endothelium can result in adhesion of polymorphonuclear leukocytes and monocytes [59–62]. In this circumstance, platelets also contribute to leukocyte function and regulated accumulation, including transmigration of polymorphonuclear leukocytes and monocytes on adherent, activated platelets [63]. Overall, it has been clearly established that platelet–leukocyte interactions are an important response of platelets to inflammation, including the formation of neutrophil extracellular traps (NETs) [64–66].

Platelet/leukocyte aggregates have been detected in the blood of patients with a variety of clinical conditions. (Table 17.3) [67]. Platelet/monocyte aggregates may be more stable than platelet/polymorphonuclear leukocyte aggregates [68,69]. A consequence of platelet/leukocyte aggregate formation is increased accumulation of leukocytes at sites of inflammation and vascular injury. Signals produced by activated, adherent platelets trigger activation of β2 integrins on leukocytes, which is required for tight adhesion to vascular surfaces. The effect on the leukocyte of P-selectin on the activated platelet is a key mechanism, together with other signals also delivered by the platelet [63]. The responses of leukocytes to signals delivered by activated, adherent platelets are clearly relevant to inflammatory diseases (Figure 17.1 and Table 17.2).

Table 17.3 Disorders in which platelet–leukocyte aggregates have been detected in the circulation.[a)]

Atherosclerosis	Diabetes
Cystic fibrosis	Sepsis
Ulcerative colitis	SLE[b)]
ARDS[c)]	Stroke
Cirrhosis	Rheumatoid arthritis

a) Derived from Ref. [67].
b) Systemic lupus erythematosus.
c) Acute respiratory distress syndrome.
This list shows only selected disorders.

17.5 Platelet Integrins

Platelets have multiple surface receptors that transmit outside-in signals to biochemical signal transduction pathways. Many of these signaling pathways trigger inflammatory activities of platelets [63]. Stimulation of human platelets by thrombin induces degranulation and release of inflammatory mediators and antibacterial factors and formation of platelet/monocyte aggregates, which triggers additional expression of chemokines, cytokines, and inflammatory enzymes. These contribute to immune functions of activated platelets [63,70,71]. Platelet integrins not only play critical roles in hemostasis but also directly participate in inflammatory signaling in platelets and in interactions of activated platelets with other inflammatory cells. Integrin GPIIb-IIIa, in addition to playing a major role in hemostasis, is involved not only in interactions of platelets with endothelium in inflamed vessels and with leukocytes but also in interactions with some microbial pathogens [59,63,72]. Outside-in signaling resulting from engagement of integrin GPIIb-IIIa by fibrinogen can also trigger inflammatory responses in platelets. Involvement of integrin αIIbβ3 can also induce a variety of other inflammatory activities of human platelets [63]. Synthesis of interleukin-1β by human platelets [73] is stimulated by the interaction between platelets and collagen and this cytokine plays a role in the response to inflammation [74,75]. GPVI, an immunoreceptor, regulates the activity of αIbβ3 and α 2β1 in platelets and induces alpha granule secretion [71,76]. αIIbβ3 has been shown to play a role in the activation of platelets by multiple types of Streptococci; FcγRIIA is involved [77,78]. GPVI also induces platelet microvesicle release, which can then transmit inflammatory signals by transporting IL-1 [79]. Overall, there are multiple pathways by which platelets signal cells within and without the vascular system that have the potential to alter immune events [63,70,80]. Platelets also express Fc receptors that recognize multiple classes of immunoglobulins [71,81–85]. Platelet Fc receptors appear to participate in some immune and infectious syndromes, including heparin-induced thrombocytopenia, bacterial endocarditis, and diabetes [71,84–86].

17.6 Role of Platelets in Immune Responses

Platelets are involved not only in the response to inflammation but also in a wide range of immune responses [87–89]. P-selectin expression in activated platelets can lead to complement activation [90]. Platelets and neutrophils were reported to be associated at sites of acute inflammation [91]. Platelets can affect the immune system by regulating lymphangiogenesis, which then influences lymphocyte trafficking and related cellular events in adaptive immune responses [92]. Activated platelets interact both with lymphocytes and with dendritic cells and have been reported to regulate the functional state of dendritic cells [93–99].

As stated previously, since they also bind to other leukocytes in the circulation, notably monocytes, activated platelets have the potential to markedly influence a wide range of inflammatory responses. Platelet microparticles may be an important mechanism by which platelets affect other circulating blood cells [89,95,100,101].

17.7 Platelets and Inflammatory Disorders

Thrombocytosis or thrombocytopenia is often associated with inflammatory and infectious states, and platelets are often present at infected sites [87]. In some infectious disorders, platelets are especially prominent and thrombocytopenia may be severe [102]. The role of platelets in innate immunity and host defense has been the subject of an entire issue of a journal [103].

Sepsis (systemic inflammatory response syndrome, SIRS) is a serious condition, associated with a high mortality rate [104]. When associated with Gram-negative infections, bacterial endotoxin (LPS) can often be detected in the blood [105]. Both immune and hemostatic systems are pathologically affected. Increased vascular permeability is commonly present [106]. Abnormal concentrations of thrombin, PAF, and cytokines are present [107]. In Gram-negative sepsis, sequential elevations of TNF, IL3, and IL-6 are observed. Platelets are involved [107–109] and moderate to severe thrombocytopenia is almost always present [108,109]. A correlation between thrombocytopenia and multiorgan failure and death has been reported [109]. Disseminated intravascular coagulopathy (DIC) is common, with consumption of both blood coagulation factors and platelets. There is associated vascular injury and thrombosis [110–112]. During an episode of SIRS, platelets activated by LPS via TLR4 have been suggested to trigger formation of neutrophil extracellular traps (NETS) [107]. Fibrin deposition in small vessels is a feature of sepsis; fibrinogen concentrations in plasma are reduced and fibrin degradation products (FDPs) are elevated. Platelets play multiple roles during SIRS [107,109]. It is noteworthy that platelet–white blood cell aggregates have been observed in the circulation in these disorders [67].

17.8 Malaria

Thrombocytopenia is commonly present in *Plasmodium falciparum* and *P. vivax* infections [113,114]. The pathogenesis of malaria-induced thrombocytopenia involves marrow suppression, immune-mediated platelet destruction, adhesion to injured endothelium, and interactions with sequestered parasitized erythrocytes and leukocytes [114,115]. Platelets may play a role in the pathogenesis of severe malaria [63,115,116] and possibly be protective [117–119], and therefore be critical during immune responses to the blood stages of malaria [120]. In contrast, the aggregation of platelets and leukocytes in cerebral microvesicles probably is a cause of mortality in the cerebral malaria that may develop in children infected with *P. falciparum* [115,121]. Platelets also interact directly with red blood cells, causing clumping of the infected erythrocytes and formation of platelet–erythrocyte aggregates. The detection of platelet factor 4 in plasma samples from infected patients indicates that platelet activation had occurred [122]. Other studies indicate that platelets are important immune effector cells in malaria [115,123]. The pathophysiology of malaria provides a clear example of the important interface between inflammation and blood coagulation [123].

17.9 Systemic Lupus Erythematosus

Thrombocytopenia is a feature of systemic lupus erythematosus (SLE). Platelet activation and increased circulating platelet/monocyte complexes have been described in SLE [124,125]. Altered protein expression appears to correlate with cellular activation and vascular abnormalities in SLE [126]. There is excessive production of autoantibodies by B lymphocytes [127], with dendritic cell activation and release of INFα [128]. It has been reported that activated platelets play a role in some of these key pathological events [129]. There is P-selectin expression and formation of platelet/monocyte and platelet/dendritic cell aggregates [129]. Other reports indicated that alterations in the platelet transcriptome in SLE result in increased expression of factors in the type I IFN system [126]. Platelet signaling as a mechanism of renal injury in SLE is also supported by the observation that platelets from patients with SLE can activate mesangial cells, leading to mesangial proliferation and production of profibrotic TGF-β1 [130].

17.10 Conclusions

It has become increasingly clear that platelets, historically believed to be relatively inert fragments of megakaryocyte cytoplasm, are powerful cellular machines with an enormous range of biological activities. Their roles in a wide range of immunological settings and/or when inflammation is present have been increasingly documented [131–132]. It is of particular biological and clinical significance that these insights have provided clear evidence of the important relationships between inflammation, hemostasis, and the control of pathogens [133–138].

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18

Epithelial Cells

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18.1 Introduction

Epithelial cells are the building blocks of epithelial tissues that act as a physical barrier between the body and the external environment. Epithelial cells are tightly arranged into continuous sheets and line the surfaces of organs throughout the body, including the gastrointestinal, urinary, and respiratory tracts, as well as form complex structures such as secretory glands and blood vessels. Under normal conditions, an intact epithelial barrier serves to carefully limit and regulate the bidirectional transport of ions, small molecules, and nutrients, and to prevent unwanted pathogens such as harmful bacteria, viruses, and fungi from entering the host and subsequently the systemic circulation. Beyond providing a physical barrier, epithelial cells also play important roles in coordinating immune responses, secreting signaling molecules to transmit messages between local and distant targets, and initiating mechanisms of self-renewal, regeneration, and repair. These functions are critical to maintain homeostasis locally and throughout the body. However, epithelial cell responses can also contribute to the etiology and pathophysiology of many disease processes, including inflammatory diseases that can induce or result from impaired epithelial cell and/or barrier function. In the intestine, the epithelium is composed of a single continuous layer of simple columnar epithelial cells organized into a series of invaginations and projections into the lumen known as crypts and villi, respectively [1]. These characteristics serve to increase the total surface area and the overall functional capacity of the intestine [2]. In fact, the intestinal epithelium represents the largest surface in the body that is in direct contact with the outside world [3]; therefore, it serves as an archetype for the basis of understanding the structure and function of epithelial cells in health and in the context of inflammatory disease.

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18.2 The Intestinal Epithelium: An Archetypal Epithelial Barrier

Along the surface of the gastrointestinal tract, columnar epithelial cells are tightly packed alongside one another into a single layer, adjacent to the lumen. All epithelial cells are uniformly polarized, with an apical surface facing the lumen and a basolateral surface in contact with the underlying basement membrane. This polarity is required for the specialized bidirectional transport and barrier functions of the epithelium [4]. The apical surface of epithelial cells may exhibit ultrastructural specializations such as microvilli, which compose the brush border and serve to further increase the surface and absorptive area of the small intestine [5]. The composition of terminally differentiated epithelial cells varies across epithelia and within different regions of an organ, but in the small intestine, four principal intestinal epithelial cell subtypes have been identified: absorptive enterocytes, goblet cells, Paneth cells, and enteroendocrine cells [6,7]. Each of these cell subtypes arise from four–five pluripotent stem cells located at the base of intestinal crypts. These cells were first observed with electron microscopy and are referred to as crypt base columnar (CBC) cells, for their location and cell shape [8]. More recently, crypt base columnar cells were found to selectively express the evolutionarily conserved G protein-coupled receptor LGR5 [6], a member of the Wnt signaling pathway characterized by a distinct leucine-rich repeat sequence in the extracellular domain [9]. Undifferentiated *Lgr5*-positive daughter cells begin to migrate from their niche at the base of the intestinal crypt along the crypt–villus axis, forming a pool of highly proliferative transit-amplifying cells (TACs) [10]. These cells differentiate through successive cell divisions and underlie the vast regenerative capacity of the intestinal epithelium.

Intestinal epithelial cells represent one of the most rapidly dividing cell types in the entire body, with epithelial cell turnover occurring within three–five days [11,12]. As TACs migrate from the transit-amplifying zone to the villus, they undergo differentiation and commit to an epithelial cell lineage [13]. Once the differentiated cells reach the tip of the villus, they are rapidly shed into the lumen at a rate of approximately 10 000 cells each day [11,14]. Under normal conditions, intestinal epithelial barrier function is maintained despite the presence of gaps resulting from rapid shedding [15] due to the redistribution of tight junction proteins that mediate cell to cell interactions [16]. Continuous epithelial cell renewal and repair along the crypt–villus axis is closely regulated and critical to maintenance of homeostasis and healthy epithelial barrier function.

18.3 Choosing a Cell Fate: The Absorptive Lineage

Epithelial cells throughout the body are named for their cellular morphology (e.g., squamous, cuboidal, or columnar) and their arrangement is classified as simple (single layer) or stratified (multiple layers). The gastrointestinal, respiratory, and urinary tracts are similar as they are all lined with single layer of columnar epithelial cells, but this is not to imply that the epithelial borders of these organs

are homogeneous. Rather, it is imperative that the proper ratio of terminally differentiated epithelial cell subtypes exists to perform the specialized functions of each organ. In the gastrointestinal tract, the composition of resident epithelial cells is organized to facilitate absorption, secretion, and permit the expansion of the epithelium to accommodate luminal contents. The predominant epithelial cell subtype (~90%) in the lining of the small intestine is the enterocyte [8,17], which can have absorptive or nonabsorptive capabilities based on the spatial distribution and gene expression profile [18]. Absorptive enterocytes have a basal nucleus and a densely packed series of microvilli covering their apical surface [19] that contain negatively charged integral membrane glycoproteins and enzymes to facilitate terminal digestion and absorption of nutrients [20,21]. In addition to their role in digestion and absorption, enterocytes also mediate transepithelial electrolyte and fluid transport. Chloride ion secretion from enterocytes is carefully regulated and can be triggered via an increase in intracellular cyclic nucleotides (cAMP or cGMP) or calcium levels within the cell [22,23]. Increased calcium results in transient chloride secretion from the apical cell surface, while intracellular cyclic nucleotides produce a larger, more sustained response [24]. Anionic chloride (Cl^-) ions in the lumen draw cationic sodium (Na^+) ions in through intracellular spaces, leading to an osmotic gradient that pulls water into the lumen via various specialized transporters, exchangers, and ion channels located at the apical and basolateral membranes of the enterocyte [25]. These include Cl^- channels located at the apical membrane and potassium (K^+) channels and the Na^+-K^+-Cl^- cotransporter at the basolateral membrane [26,27]. In addition, inhibitory signaling mechanisms exist to limit chloride and fluid secretion including carbachol- [28] and epidermal growth factor (EGF)-dependent pathways [29]. The mechanisms for regulating chloride and fluid secretion across epithelial barriers are similar in many organs and are central to both normal physiology and pathophysiology of many diseases, including cystic fibrosis and secretory diarrhea [23,25].

18.4 Choosing a Cell Fate: The Secretory Lineage

18.4.1 Goblet Cells

In addition to enterocytes, three other specialized secretory epithelial cell subtypes differentiate from *Lgr*5-positive stem cells located in the base of intestinal crypts: goblet cells, Paneth cells, and enteroendocrine cells. Goblet cells produce a mucus layer that is critical for maintaining the functional integrity of the mucosal epithelium [30–32]. The mucus layer lowers the coefficient of friction to promote movement of luminal contents along the intestine, protects epithelial cells from physical and chemical injury [30], provides binding sites and nutrient sources for intestinal microbes [33], and limits the host's exposure to harmful antigens [34]. Goblet cells synthesize a variety of large glycoproteins known as mucins that have a protein backbone linked to hydrophilic oligosaccharide side chains [35]. Mucin (MUC) proteins are either bound to the membrane itself (MUC1, MUC3, MUC4, MUC12, MUC13, and MUC17) or stored in large vesicles and secreted from the

apical surface of the intestine (MUC2) [36,37]. These proteins are characterized by large "mucin domains" containing heavily O-glycosylated serine and threonine amino acid residues, which contribute to their distinct structure and the assembly of the mucus layer [34]. Under normal conditions, secretory mucin-containing granules are secreted in small volumes and when combined with water, they expand dramatically to form a viscous gel that protects and lubricates the mucosal epithelium [38], while epithelial membrane-bound mucins create the carbohydrate-rich glycocalyx barrier composed of diverse glycoproteins and lipids along the surface of the brush border [39]. Many transmembrane mucins are expressed by both enterocytes and goblet cells, while secretory mucins such as MUC2 are selectively expressed by goblet cells [40].

The mucus layer is a dynamic component of the epithelial barrier with a distinct microenvironment. MUC2 is the predominant gel-forming mucin protein of the mucus layer [32,41] and is released from the apical surface of the epithelial cell via either constitutive (basal) or stimulated (active) secretion. Constitutive secretion refers to the unregulated, passive release of secretory granules, which occurs via existing electrical or chemical gradients, while stimulated secretion is a calcium-dependent mechanism that results from activation of receptors located on the apical surface of goblet cells. Calcium diffusion neutralizes negatively charged mucins and triggers exocytosis of densely packed granules, which can be initiated by hormones and transmitters acting through intracellular signal transduction systems such as the cAMP/PKA pathway [25]. Exocytosis of the mucin granules leads to rapid expansion of the mucus gel [40], which allows for continuous renewal and replacement as it moves along the intestinal surface toward the distal colon and rectum via waves of peristaltic motor activity [42–44].

In contrast to the small intestine, the large intestine has a mucus layer at the luminal surface containing a loose outer layer and a stratified inner adherent layer [45,46]. The proportion of goblet cells in the epithelium increases distally along the gastrointestinal tract, with the largest number found in the distal colon [47]. Interestingly, the number of bacteria in the gastrointestinal tract also increases in this fashion with over 10^{13} bacteria present in the human colon alone [48]. The outer mucus layer serves as a home for the vast commensal bacteria to colonize, while the densely packed inner layer is devoid of bacteria under normal conditions [49]. This inner mucus layer provides additional protection against bacterial interaction with epithelial cells and bacterial translocation into the host. Furthermore, a clear border exists between the mucus layers, suggesting distinct mechanisms governing the transition between the two layers [48]. Interestingly, the absence of the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- channel involved in transport of Cl^- across the apical surface of epithelial cells, leads to the development of an adherent mucus layer in the small intestine [50]. The commensal bacteria housed in the outer mucus layer produce a variety of metabolites that further confer protection to the host and the underlying epithelium such as the short chain fatty acids acetic acid, propionic acid, and butyric acid [51]. Conversely, intestinal epithelial cells produce antibacterial proteins [52,53] that serve to shape the adjacent bacterial microbiome in a symbiotic fashion.

18.4.2 Paneth Cells

As stated above, the inner adherent mucus layer that separates the bacteria from underlying epithelial cells is absent in the small intestine under normal conditions. There may be multiple reasons for this, including a smaller bacterial load in the small versus large intestine, as well as the increased absorptive capacity of the small intestinal epithelium that an adherent mucus layer may impair. However, additional mechanisms exist in the small intestine to mediate host–microbe interactions and to prevent bacterial translocation and dysbiosis. These include a subtype of secretory epithelial cells called Paneth cells that produce proteins with antibacterial, antiviral, and antifungal actions [53]. These cells are most frequently found at the base of small intestinal crypts and were named after Josef Paneth, one of the two scientists who first described their unique morphology and large granules [54]. In contrast to other terminally differentiated epithelial cell subtypes of *Lgr*5-positive stem cell origin that have a lifespan of roughly three–five days and migrate up the crypt–villus axis, Paneth cells maintain their position in the base of the crypt for approximately 60 days [55]. Similar to goblet cells, Paneth cells rapidly secrete their granules into the lumen via calcium-dependent exocytosis following exposure to bacteria or bacterial antigens [56–59]. Bacteria or bacterial antigens bind to host membrane-bound pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) or nucleotide-binding oligomerization (NOD)-like receptors (NLRs) [60]. Receptor coupling leads to activation of downstream signaling cascades and subsequent release of antimicrobial proteins synthesized and stored in granules at the Paneth cell surface. Paneth cells secrete a variety of antimicrobial proteins, including α- and β-defensin, lysozyme C, angiogenin (ANG) 4, secretory group IIA phospholipase A2 (sPLA2), and regenerating islet-derived protein (REG) 3α [61], thereby limiting luminal bacterial translocation of both commensal and pathogenic bacteria to the small intestine [59,62] and extraintestinal tissues such as mesenteric lymph nodes [59,63] and spleen [59]. Antimicrobial peptides such α-defensins also shape the composition of both pathogenic [64,65] and commensal [66] microbiota of the small intestine, without altering the bacterial load. Furthermore, these changes alter the responsiveness of the immune system [66]. In addition to antimicrobial proteins, Paneth cells also secrete inflammatory cytokines including TNF-α [67], IL-17 [68], and IL-23 [69], supporting an important role for Paneth cells in innate immune function [70].

18.4.3 Enteroendocrine Cells

In addition to goblet cells and Paneth cells, enteroendocrine cells comprise the third terminally differentiated cell type of the secretory lineage derived from *Lgr*5-positive progenitor cells. The gastrointestinal tract is the largest endocrine system in the body and produces more than 20 hormones, peptides, and growth factors that detect and integrate signals from the luminal environment and manifest responses via autocrine and paracrine signaling mechanisms [71,72]. These secretory products are synthesized, stored, and secreted into the lumen by approximately 10 enteroendocrine cell subtypes in the intestine, several of which express multiple functionally related secretory products. The peptide hormones

most frequently coexpressed and secreted by enteroendocrine cells of the intestinal epithelium are cholecystokinin, gastric inhibitory polypeptide, glucagon-like peptide-1, neurotensin, peptide YY (PYY), and secretin [73,74]. The epithelial cells that produce these peptide hormones are derived from a common lineage and are specific to the intestine. In contrast, somatostatin-producing delta (D) cells and serotonin-producing enterochromaffin (EC) cells are found throughout the entire gastrointestinal tract. The spatiotemporal differentiation of enteroendocrine cells in the gastrointestinal tract provides an excellent example of cell fate determination governed by specific networks of transcription factors [75]. Enteroendocrine cells are derived from progenitor cells that express the basic helix–loop–helix (bHLH) transcription factor neurogenin 3 (Neurog3) and downstream transcription factors that contribute to subsequent subspecialization [76]. The fate of intestinal-specific enteroendocrine cells depends on coordination of the transcription factors aristaless-related homeobox (Arx) [76,77] and paired box 4 (Pax4) [76]. In contrast, differentiation of D cells and EC cells results from Pax4 expression alone [76]. Peptide hormones secreted from enteroendocrine cells are capable of acting locally or at distant sites in the body and can coordinate immune responses in the presence of commensal and pathogenic bacteria or their metabolites, nutrients, or other secretory products in the lumen via activation of receptors expressed on their apical surface [70].

18.5 The Epithelium: The Sum is Greater than its Parts

The epithelial cells lining the surface of organs must work together to achieve their unique functions. The epithelium is polarized with specific transporters and receptors at both the apical and basolateral surfaces of individual epithelial cells, while the space between cells is sealed with paracellular/intercellular junctional proteins, including tight junctions, adherens junctions, and desmosomes [78,79]. These junctional proteins form complexes between cells that are critical for the protective functions of the epithelium. Adherens junctions are found in all epithelial linings throughout the body and provide adhesive interactions between epithelial cells as well as contribute to the orientation of the apical–basal polarity axis [4,80]. Their structure is based on epithelial (E)-cadherin-dependent intercellular interactions and the actin–myosin cytoskeleton of neighboring epithelial cells [80]. Adherens junctions are located between adhesive plaque-containing desmosomes facing the basolateral domain of the plasma membrane and the tight junction facing the luminal surface of the epithelium, at the interaction of the apical and basolateral surface between adjacent epithelial cells [81]. Based on its structural orientation and function, the tight junction has been referred to as the "gatekeeper of the paracellular pathway" [78]. In contrast to the adherens junctions, tight junctions are not required for the polarized distribution of membrane proteins [82] or establishment of epithelial apical–basal polarity [4] but are necessary for the regulation of paracellular ion, solute, and water transport across the epithelial surface [83]. Tight junctions are comprised of many individual proteins, with three integral membrane proteins most critical to the regulation of epithelial permeability [78]. Occludins and members of the diverse

family of claudin proteins are key structural elements in a series of parallel tight junction strands linking the plasma membranes of adjacent epithelial cells [84]. A logarithmic relationship exists between the number of these tight junction strands and the permeability of the epithelial barrier [85]. At present, more than 20 isoforms of the claudin family have been identified [86], and these can be classified as pore-forming or barrier-forming, thus playing a critical role in the determination of tight junction permeability [87]. The third integral membrane protein component of the tight junctional complex is the immunoglobulin superfamily molecule junctional adhesion molecule (JAM)-1, thought to contribute to the innate immune function of the epithelium for its role in leukocyte trafficking [88,89].

Transepithelial transport of the epithelial barrier represents the sum of the paracellular transport mechanisms described above as well as transcellular transport via transporters and carriers located on the epithelial cell membrane. Transcellular transport allows for import of molecules from the lumen via specialized transporter proteins expressed on the cell apical surface, while export occurs via transporters on the basolateral membrane. Consistent epithelial cell polarity is critical to transcellular transport, as directed in part by paracellular transport mechanisms. Net ion, small molecule, and nutrient transport across the intestinal epithelium can be studied experimentally using chambers to measure short-circuit current (I_{sc}) as an indicator of transepithelial electrical resistance [90].

18.6 Epithelial Homeostasis: Maintaining the Balance

Epithelial cells are presented with a variety of potential threats to homeostasis on a continuous basis, including bacterial invasion, changes in pH and temperature, mechanical stress, and inflammation due to infection or disease processes. Epithelial homeostasis is the physiological equilibrium that exists between epithelial cell proliferation, migration, and differentiation of pluripotent stem cells and subsequent apoptosis of epithelial cells [91]. As discussed above, terminally differentiated epithelial cells in the intestinal epithelium (with the exception of Paneth cells) are constantly renewed within days [11,12]. Recent advances in the understanding of these processes are owed in part to the identification of *Lgr5* as a specific marker of intestinal stem cells [6] and have revealed the involvement of several key signaling mechanisms. Alterations in these highly conserved pathways also often underlie the pathophysiology of inflammatory diseases involving epithelial cells throughout the body.

18.6.1 Wnt Signaling

Intestinal epithelial cells proliferate in the base of the intestinal crypts via the highly conserved canonical Wnt signaling pathway. β-catenin-dependent Wnt signaling is initiated when an extracellular Wnt ligand binds to one of the frizzled family of receptors [92] and a low-density lipoprotein receptor-related protein (LRP)-5 or LRP-6 coreceptor [93–95], creating a Wnt–receptor complex. The

formation of this Wnt–receptor complex stabilizes and prevents phosphorylation of the key cytoplasmic protein β-catenin, thus allowing it to accumulate in the cell nucleus and subsequently activate downstream target genes [96,97]. In addition, several extracellular cysteine-rich glycoproteins function as antagonists of the Wnt pathway, including the family of secreted Frizzled-related protein (sFRP) [98,99] and Wnt inhibitory factor (WIF)-1 [100]. These secreted proteins inhibit Wnt signaling through competitive binding and interaction with other secreted proteins [101]. Both activators and inhibitors of the Wnt pathway are critical for normal rates of cell proliferation; a reduction in Wnt signaling leads to a decrease in the proliferative capacity of the intestinal epithelium, while sustained Wnt signaling results in hyperproliferation of epithelial progenitors [102]. Furthermore, a series of key experiments have illustrated that when Wnt signaling is inhibited, there is a reduction of terminally differentiated epithelial cells of the secretory lineage (goblet cells, Paneth cells, and enteroendocrine cells), thus disrupting the functional capacity of the intestinal epithelium [103–105].

Recently, key advances in understanding the proliferation, cell fate and differentiation of intestinal stem cells have been made in large part to demonstration of a three-dimensional polarized epithelium grown from individual *Lgr5*-positive stem cells *in vitro* [106]. These technical advances have allowed for experimental interventions and manipulations not previously possible. The first such studies from the laboratory of Hans Clevers showed that intestinal epithelial stem cells cultured with EGF, the bone morphogenetic protein antagonist Noggin, and the Wnt agonist R-spondin-1 could give rise to intestinal organoids displaying the crypt-villus structure *in vitro*, representative of the normal intestinal epithelium [106]. Subsequent studies showed that coculture of this organoid system with differentiated Paneth cells improved organoid formation in culture [107], highlighting the importance of this cell type in the stem cell niche and maintenance and renewal of the intestinal epithelium. As discussed previously, Paneth cells are the only terminally differentiated intestinal epithelial cell to remain in the crypt base, where they lie in close approximation to *Lgr5*-positive stem cells. In addition to secreted antimicrobial proteins, Paneth cells also synthesize and secrete EGF, Wnt ligands, and delta-like ligands of the Notch signaling pathway, which are necessary to support proper functioning of the stem cell microenvironment [107].

18.6.2 Notch Signaling

Commitment to an epithelial cell lineage is another critical element of a properly functioning intestinal epithelium. Appropriate representation of each subtype of terminally differentiated epithelial cells is regulated in part by Notch signaling [108]. The highly conserved Notch signaling pathway underlies broad functions throughout the body and is dependent on cell-to-cell interactions that impact intracellular responses, including inhibiting the differentiation of the secretory cell lineage [109]. Notch is a single-pass transmembrane receptor with a large extracellular domain [110]. The Notch receptor is activated when selective ligands such as mammalian Delta-like and Jagged-1 are secreted from a

neighboring cell, leading to a conformational change and subsequent proteolytic cleavage of the Notch receptor. This results in dissociation of the Notch intracellular domain (NCID), which then translocates to the cell nucleus and modulates downstream target genes and cell processes via binding to the DNA-binding transcription factor RBPj [110]. Math1, a bHLH transcription factor that is a critical regulator of cell fate throughout the body, is required for differentiation of goblet cells, Paneth cells, and enteroendocrine cells from progenitor cells in the intestinal epithelium [111–113]. Notch signaling represses Math1 via activation of the Hes1 transcriptional repressor, limiting differentiation of epithelial cell precursors to the secretory lineage and the associated increase in absorptive enterocytes [109,114]. In instances where Notch receptor expression is low or signaling is inhibited, increased expression of the ligand Delta-1 represses Hes1, thus Math1 levels remain high and cells are able to differentiate into secretory goblet cells, Paneth cells, and enteroendocrine cells.

In addition to an active role in regulating the balance between secretory and absorptive cell differentiation in the intestinal epithelium, the Notch signaling pathway has additional important roles in promoting intestinal epithelial homeostasis. For example, mice with a targeted deletion of the Notch transcription factor RBPj in intestinal epithelial cells show compromised intestinal permeability and develop spontaneous inflammation due to bacterial translocation [115]. This study provides direct evidence for a loss of homeostatic regulation and subsequent dysbiosis resulting from altered Notch signaling. This provides a potential mechanism for immune activation and the development of chronic inflammation due to altered intestinal epithelial cell function and compromised epithelial integrity.

18.6.3 Inflammation-induced Alterations in Epithelial Cells

The signaling mechanisms described above are key to the development and maintenance of epithelial cell homeostasis, yet in situations where the epithelial barrier is impaired and constitutive regulatory mechanisms fail, activation of compensatory responses are required to defend against damage to the host. The etiologies of chronic inflammatory conditions involving the intestinal epithelium such as Crohn's disease and ulcerative colitis, collectively known as Inflammatory Bowel Disease (IBD), are not fully understood but thought to involve an overactive immune response to commensal bacteria mediated in part by a defective epithelium [116]. These responses are associated with subsequent activation of the innate and acquired immune systems, resulting in proinflammatory cytokine release and altered barrier function.

A key feature of the innate immune response during inflammation is the early recruitment of neutrophils [117]. During acute inflammation, migration of neutrophils from the systemic circulation into the inflamed tissue and organs is a protective response against host damage [118]. Recruitment of neutrophils and other immune cells to the site of injury occurs through chemotaxis, which is regulated in large part, by the family of C-X-C motif (CXC) ligand 1 chemokines [119]. Beyond their role in initiating the acute immune response, neutrophils also play a predominant role in the pathophysiology of chronic

inflammatory diseases such as IBD. In Crohn's disease patients, the degree of neutrophil infiltration in the lamina propria of the inflamed intestine is correlated with the severity of disease [120]. Once present at the site of inflammation, neutrophils release proinflammatory cytokines and antimicrobial proteins to shape local innate immune responses. Cathelicidins are a family of antimicrobial proteins released by neutrophils and epithelial cells that are similar in function to defensins [53]. Cathelicidins can be released in response to activation of TLRs by bacteria or bacterial metabolites such as short-chain fatty acids [121] and have been associated with increased mucus release and decreased intestinal epithelial cell apoptosis in mouse studies of experimental colitis [52]. Furthermore, cathelicidins promote intestinal epithelial barrier integrity and wound healing [52,122]. Human cathelicidin LL-37 expression is increased in patients with ulcerative colitis, but not Crohn's disease [123], suggesting that cathelicidins and antimicrobial proteins may underlie distinct and disease-specific host defense mechanisms.

Altered Paneth cell expression has also been noted in patients with intestinal inflammation, with increased expression of the Paneth cell secretory products human alpha-defensin (HD) 5 [124,125] and sPLA2 [126,127] in the colonic epithelium of IBD patients. Under normal conditions, Paneth cells are not found in the colonic epithelium; however, studies in IBD patients illustrate that the degree of Paneth cell metaplasia is directly correlated with the extent of colonic inflammation [126]. Similar to cathelicidins, alpha-defensins are synthesized and released from a variety of cell types in addition to Paneth cells, namely, enterocytes and neutrophils [128]. Alpha-defensins can alter cytokine release from epithelial cells, thus linking defense mechanisms of the innate and acquired immune systems [129,130]. Furthermore, patients with Crohn's disease involving the small intestine have decreased HD5 and HD6 expression as compared to control patients [131] and exogenous HD5 can improve experimental colitis *in vivo* [132]. Interestingly, patients with Crohn's disease of the large intestine have reduced expression of the functionally related beta-family of defensins [133]. Constitutive expression of beta-defensins in colonic epithelial cells is maintained by the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ) [134]. The release of proinflammatory cytokines is regulated in part by the nuclear factor (NF)-kappa B pathway, and beta-defensins can inhibit cytokine expression by preventing phosphorylation of the NF-kappa B subunit p65 [135], consistent with an anti-inflammatory role for these antimicrobial proteins. In addition, beta-defensins increase tight junction protein and mucin expression and decrease intestinal epithelial cell apoptosis during acute inflammation [135].

Several distinct changes reflecting altered epithelial cell differentiation and function have been associated with intestinal inflammation and IBD, reflecting cross-talk between the immune system and the epithelial cells in addition to roles as the first-line defenders against luminal threats. Changes in goblet cells have been noted in the intestinal epithelium of patients with IBD using scanning electron microscopy, including a decrease in MUC2 expression levels [136]. Furthermore, mice with decreased mucus due to a genetic deletion of the *Muc2* gene have increased disease activity and epithelial damage compared to control

mice during early experimental colitis [137], suggesting that a reduction in goblet cell function may contribute to the onset of intestinal inflammation. In addition, recent data suggest a role for goblet cells as luminal sensors for the interface between the innate and the adaptive immune systems, directly sampling antigens from the intestinal lumen and delivering them to dendritic cells in the adjacent lamina propria [138].

18.7 Concluding Remarks

Epithelial cells, regardless of their location in the body, serve similar functions to maintain the integrity of the barrier and promote homeostasis both locally and throughout the body. This requires complex interplay between several subtypes of epithelial cells and maintenance of the connections between adjacent cells. Under normal conditions, tight regulation of epithelial cell proliferation, differentiation, and migration is maintained by several conserved signaling pathways. In the presence of inflammation or disease, epithelial cells must mount rapid responses to the immune system and between individual epithelial cell subtypes to initiate regeneration and repair mechanisms in an effort to minimize damage to the host. Future efforts in the field of epithelial cell signaling and inflammation must focus on identification of new targets to restore normal functioning and homeostasis to limit the development and the consequences of chronic injury or disease.

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19

Inflammation: The Role of Endothelial Cells

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19.1 Introduction

Inflammation is an underlying component of a variety of human diseases, ranging from arthritis, inflammatory bowel disease (IBD), atherosclerosis, and sickle cell disease, to ischemic stroke. A common and critical feature of the inflammatory response in these conditions is endothelial cell dysfunction, which plays an important role in both the initiation and the perpetuation of inflammation. The endothelial cell dysfunction is evidenced in all three segments of the microcirculation and is manifested as an altered capacity of arterioles to dilate, the adhesion of leukocytes and platelets in venules, enhanced coagulation and thrombogenesis, an increased vascular permeability to water and plasma proteins, and increased rates of blood (angiogenesis) and lymphatic (lymphangiogenesis) vessel proliferation (Figure 19.1). The major consequences and benefits of these responses include (1) an enhanced delivery of inflammatory cells and immunoglobulins to the injured/infected tissue, (2) dilution of the injurious/inciting agents in the affected region, (3) isolation of this region from healthy tissue and the systemic circulation, and (4) setting the stage for the regenerative process. Functional changes in the microvasculature are evident during the acute phase of the inflammatory response, that is, within minutes to hours after initiation of the response. However, structural alterations (e.g., increased numbers of blood and lymph vessels) are manifested during the chronic phase of the inflammatory response, that is, days after the initial inflammatory insult [1,2].

Inflammatory reactions and the associated endothelial cell responses are usually short-lived and result in a protective response. However, under certain pathological conditions, excessive and/or prolonged inflammation can lead to extensive tissue damage, organ dysfunction, and mortality. A notable example of such a pathological condition is the IBDs, which include Crohn's disease and ulcerative colitis. These diseases affect millions of individuals worldwide, with the highest incidence and prevalence reported for westernized nations. Furthermore, epidemiological evidence suggests that the global prevalence of IBD will continue

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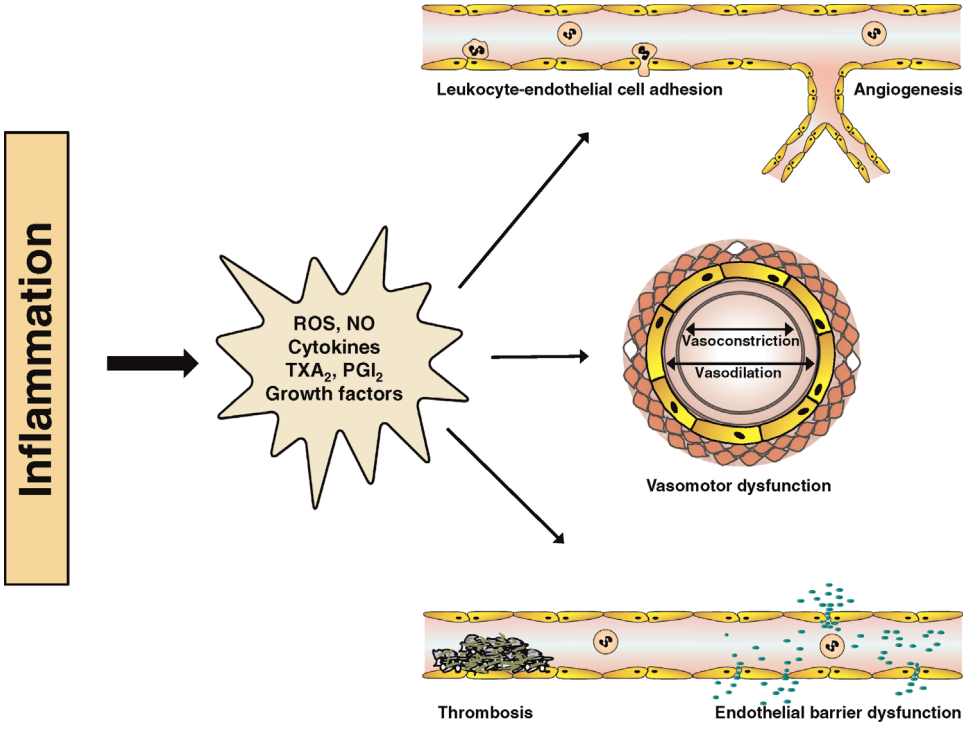


Figure 19.1 Diverse endothelium-dependent responses of the microcirculation to acute and chronic inflammation.

to increase substantially [3]. While it has been proposed that IBD involves a complex interplay among immune, genetic, and environmental factors, the etiology of these inflammatory diseases remains poorly understood. However, there is a large and growing body of evidence from patients with, and animal models of, IBD that implicate a role for the microcirculation and endothelial cell dysfunction in the pathogenesis of these diseases [1,2]. This chapter provides a summary of the endothelium-dependent responses that accompany inflammation, using IBD as a prototype for pathologic conditions that manifest both acute and chronic inflammatory responses.

19.2 Vasomotor Dysfunction

The term vasomotion refers to relaxation or contraction of the smooth muscle cells that surround blood vessels, causing vasodilation or vasoconstriction, respectively. Vasomotion is an important regulator of blood flow to an organ or local tissue, with changes in flow being a necessary response to the requirement for oxygenation, nutrient delivery, and removal of metabolites in tissues. Vasomotor dysfunction refers to an inappropriate regulation of vascular diameter that results from, or contributes to, tissue pathology. In most instances, the

vessels of interest in vasomotor dysfunction are the arterioles, which are called the "resistance vessels" because they are the most influential vessels in controlling the conductance or resistance of tissue perfusion. Flow through a vessel is equal to the pressure gradient (inlet minus outlet) divided by the vascular resistance, with the latter being inversely proportional to the fourth power of diameter. Therefore, even a small change in arteriolar diameter can cause a substantial change in blood flow.

19.2.1 Mechanism of Vascular Smooth Muscle Contraction/relaxation

As with smooth muscle in general, vascular smooth muscle contraction is regulated by the influx of calcium into the cell. Calcium can enter the cytosol through various channels on the outer cell membrane and on the membrane of the intracellular sarcoplasmic reticulum. The entering calcium binds to calmodulin, with the calcium–calmodulin complex activating myosin light chain kinase (MLCK), which can then phosphorylate myosin. The phosphorylation of myosin is critical for binding to actin to cause contraction, which in turn is terminated by dephosphorylation via the enzyme myosin phosphatase. Relaxation of vascular smooth muscle is promoted not only by removal of calcium from the cytosol (by active transport pumps leading back into the sarcoplasmic reticulum or extracellular fluid), but also by inhibition of MLCK. Two inhibitors of MLCK are cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), which is controlled in part by mediators released by the inner layer of the blood vessel, the endothelial cells.

19.2.2 Endothelial Cell-dependent Vasoactivity

The endothelium produces several mediators that contribute to the tone of the adjacent vascular smooth muscle cells. The three main endothelium-dependent vasodilators include nitric oxide (NO), prostacyclin (PGI_2), and endothelium-dependent hyperpolarizing factor (EDHF). Although not a complete list, endothelial cell-derived vasoconstrictors include superoxide, thromboxane A_2 (TxA_2), and endothelin-1 (ET-1).

NO is produced from L-arginine with the aid of NO synthase, which is an enzyme that also can produce the superoxide radical under certain situations (such as substrate or cofactor depletion). Endothelial NO induces vascular dilation by increasing intracellular levels of cGMP in the adjacent smooth muscle. Superoxide can limit NO bioavailability due to the rapid reaction between the two, which results in the formation of peroxynitrite.

Vasomotor tone is often discussed in terms of the balance between NO and superoxide; however, another pair of mediators, TxA_2 and PGI_2, can also create a balance between constriction and dilation. TxA_2 and PGI_2 are both formed during the breakdown of arachidonic acid, which can also yield other prostaglandins. TxA_2 and PGI_2 are often described together in terms of their relative production rates, as their precursor (prostaglandin H_2) can produce either the vasodilator (PGI_2) or the vasoconstrictor (TxA_2), depending in part on the levels of their respective synthases. PGI_2 induces dilation by enhancing smooth muscle

cAMP, whereas TxA_2 causes constriction through an increase in intracellular calcium concentration and enhanced calcium sensitivity.

19.2.3 Endothelium-dependent Vasoactivity in IBD

Vasomotor dysfunction has been documented in patients with IBDs, with the dysfunction involving endothelium-dependent pathways of control. Intestinal blood flow can be altered substantially by chronic gut inflammation. However, the nature and magnitude of the change in intestinal blood flow appear to depend on the phase of progression of the inflammatory response. During the early "exudative" phase of colonic inflammation in patients with either ulcerative colitis (UC) or Crohn's disease (CD), colonic blood flow (especially in the mucosal and submucosal layers) is increased significantly (two–sixfold) [4]. However, in the late "fibrosing" phase of the disease, colonic blood flow is reduced below normal. A reduction in blood flow also has been reported in colonic arterioles of mice in which experimental colitis was induced by either dextran sodium sulfate (DSS) or T-cell transfer into immunodeficient mice [5,6]. However, despite the arteriolar constriction and lower blood flow rates in the individual microvessels, overall blood flow rates to the ileum and colon in both models were only marginally reduced as a result of an approximate twofold increase in vascular density [5–9]. A subset of mice in the T-cell transfer model of colitis does exhibit a significantly reduced overall blood flow, and this is accompanied by mucosal hypoxia and only mild signs of inflammation [10]. In addition, there is evidence that leukocytes recruited into the inflamed bowel may also influence vasoactivity. In a T-cell transfer model of chronic colitis, an association was found between vasodilation of first-order colonic arterioles and venules with the number of circulating leukocytes [11]. However, it should be noted that the dilation was not accompanied by an increase in colonic blood flow, possibly due to the constriction of smaller arterioles mentioned earlier [5].

Studies on colonic arterioles obtained from patients with IBD and from animal models of experimental colitis have revealed an impaired ability of the resistance vessels to respond to endothelium-dependent vasodilators [6,12,13]. The arteriolar dysfunction measured in vessels derived from IBD patients is severe, with acetylcholine-induced dilation reduced to only about 10% of the response measured in normal arterioles. The impaired vasodilatory response appears to result from a reduced ability of arteriolar endothelial cells (ECs) to produce NO, rather than PGI_2. The NO deficiency results from excessive levels of superoxide, which (as mentioned above) rapidly reacts with and inactivates NO, thereby inhibiting smooth muscle relaxation [6,12,13]. The impaired dilation appears to be a consequence of endothelial dysfunction, since vascular smooth muscle function appears to remain unchanged [14]. In addition, recent work has implicated circulating microparticles (which are elevated in plasma of CD patients) in the impaired flow (and NO)-dependent dilation that accompanies IBD [15].

The changes in colonic blood flow in IBD patients also may reflect an altered production of endogenous vasoconstrictors [2,9]. Several reports describe elevated levels of TxA_2 and/or TxA_2 synthase in the colon of IBD patients, with a correlation noted between TxA_2 synthase and disease activity. It remains to be

determined whether TxA_2-induced vasoconstriction contributes to disease progression since TxA_2 synthase inhibitors have shown little benefit in promoting or maintaining remission of IBD. Angiotensin II and ET-1, two other endogenous vasoconstrictors produced by the gut, also are elevated in the colonic mucosa of CD patients [2]. Endothelin-1 has been implicated in the tissue hypoxia that occurs during the ischemic phase of IBD progression [16].

19.2.3.1 Leukocyte– and Platelet–endothelial Cell Adhesion

Leukocyte–endothelial cell adhesion is a long recognized and well-characterized feature of the inflammatory response. This interaction between leukocytes and vascular endothelial cells is observed in postcapillary venules and underlies the recruitment of inflammatory cells to sites of infection and/or tissue injury [17,18]. An important determinant of whether leukocytes adhere to vascular endothelium is the proadhesive force that is generated by adhesion molecules expressed on the surface of both activated endothelial cells and leukocytes. Data derived from both *in vitro* (isolated leukocytes binding to monolayers of cultured endothelial cells) and *in vivo* (intravital microscopic examination of venules) models of leukocyte–endothelial cell adhesion have revealed the relative contributions of different leukocyte and endothelial cell adhesion molecules (CAMs) to the adhesion responses elicited by various inflammatory stimuli [1]. The endothelial cell adhesion glycoproteins that have been implicated in the recruitment of leukocytes in inflamed colonic venules, their ligands (counterreceptors) on leukocytes, and the type of adhesive interaction mediated by the glycoprotein pairs are summarized in Table 19.1. A time-dependent increase in the expression of P- and E-selectin, ICAM-1, VCAM-1, and MAdCAM-1 has been demonstrated in the colonic vasculature of mice with experimental IBD [2,18]. Mucosal biopsy samples derived from patients with active (but not quiescent) ulcerative or Crohn's colitis exhibit similar changes in the expression of endothelial cell adhesion molecules [2,18].

The increased expression of adhesion molecules on colonic endothelial cells is associated with the recruitment of large numbers of rolling, firmly adherent, and

Table 19.1 Endothelial cell adhesion molecules: ligands and functions.

Adhesion Molecule	Leukocyte Receptors	Adhesion Response
P-selectin	L-selectin, PSGL-1	Rolling
E-selectin	L-selectin	Rolling
ICAM-1	CD11/CD18	Adherence, emigration
VCAM-1	VLA_4	Adherence, emigration
MAdCAM-1	Alpha4beta7	Adherence, emigration
PECAM-1	PECAM-1	Emigration

ICAM-1 = intercellular cell adhesion molecule-1; PSGL-1 = P-selectin glycoprotein ligand; VCAM-1 = vascular cell adhesion molecule-1; VLA_4 = very late antigen-4; MAdCAM-1 = mucosal addressin cell adhesion molecule-1; PECAM-1 = platelet endothelial cell adhesion molecule-1.

emigrated (extravasated) leukocytes within (and surrounding) inflamed venules [17,18]. Both leukocyte and endothelial cell adhesion molecules participate in slowing the leukocyte as it exits the capillary and enters the postcapillary venule. The initial low-affinity interaction between leukocytes and venular endothelium is manifested as a rolling behavior. Rolling leukocytes can then become firmly adherent (stationary) to endothelial cells, where the process of transendothelial leukocyte migration can occur when chemotactic signals (e.g., leukotriene B_4) are generated in the perivascular compartment or chemokines presented on the endothelial cell surface. Each of the three stages of leukocyte recruitment, that is, rolling, firm adhesion (adherence), and transendothelial migration, involves the participation of different adhesion molecules, (Table 19.1). The critical importance of adhesion molecules in the genesis of experimental IBD is evidenced by a number of studies demonstrating attenuated inflammatory and tissue injury responses in mice that are genetically deficient in specific leukocyte or endothelial cell adhesion molecules and in animals treated with blocking antibodies directed against these adhesion glycoproteins [1,18].

A number of factors are known to modulate leukocyte–endothelial cell adhesion during inflammation by affecting either the intensity of adhesion molecule expression and/or the ability of these glycoproteins to sustain strong adhesive interactions between the blood cell and the vascular endothelium. Nitric oxide, adenosine, and prostacyclin produced by endothelial cells tend to prevent leukocyte adhesion in the microvasculature, while reactive oxygen species (superoxide, hydrogen peroxide, etc.) produced by activated leukocytes and endothelial cells appear to promote adhesion [17,18]. These endogenous regulators of leukocyte adhesion exert either a direct or an indirect (i.e., altering the production of inflammatory cytokines) effect on the production and expression of endothelial adhesion molecules. Some proadhesive agents, such as histamine, platelet activating factor, and IL-8, rapidly (within 2–3 min) increase the level of activation and/or expression of adhesion molecules on leukocytes (e.g., CD11b/CD18) and/or endothelial cells (e.g., P-selectin), while other agents, such as cytokines, act more slowly to promote leukocyte adhesion by enhancing the transcription-dependent expression of adhesion molecules that prolong the leukocyte rolling (E-selectin) and adherence/emigration (ICAM-1) responses [17,19].

The transcription-dependent expression of endothelial cell adhesion molecules has been linked to specific nuclear transcription factors, most notably nuclear factor kappa-B (NF-κB) and activation protein-1 (AP-1) [17,20]. Both factors represent families of polypeptides with related DNA-binding activity but distinct transactivating potential. NF-κB activation is triggered by the phosphorylation and subsequent conjugation of IκB with ubiquitin, which makes IκB-α (an inhibitory protein that prevents nuclear transport and DNA binding of NF-κB) vulnerable to degradation by the proteasome proteolytic pathway. Point mutations that decrease NF-κB binding to κB response elements result in diminished cytokine-induced E-selectin expression on cultured endothelial cells, suggesting that NF-κB plays an important role in cytokine induction of the E-selectin gene [20]. However, the increased ICAM-1 expression in endothelial cells exposed to hydrogen peroxide [21] appears to be mediated through AP-1 and independent of κB elements [21,22]. Proteasome inhibitors (which prevent the

degradation of IκB and subsequent activation of NF-κB) have been shown to blunt the increased ICAM-1 expression and inflammatory response in animal models of IBD [23].

Nitric oxide, adenosine, and prostacyclin are also potent vasodilators, which raises the possibility these agents also blunt leukocyte–endothelial cell adhesion by altering shear forces generated by the movement of blood through the microvasculature [17]. The number of rolling and adherent leukocytes recruited in inflamed venules is inversely related to wall shear rate, suggesting that it is easier for leukocytes to create strong adhesive bonds with endothelial cells at low shear rates and that high shear rates likely prevent the creation of such bonds. This dependency of leukocyte adhesion on shear rate suggests that the changes in blood flow associated with inflammation may significantly influence the intensity of leukocyte recruitment during IBD [17,24].

Platelets also accumulate in the microvasculature of the inflamed colon [25,26]. The adhesion of platelets to the walls of colonic venules appears to reflect binding to activated endothelial cells rather than adhesion to the collagen-rich subendothelial surface of damaged blood vessels. Intravital microscopic evaluation of platelet binding in inflamed colonic venules has revealed that a relatively small proportion (<10%) of the platelets bind directly to endothelial cells, while most platelets attach to leukocytes that are already bound to the surface of endothelial cells [27]. P-selectin blockade effectively inhibits both the leukocyte-dependent and -independent components of platelet adhesion in colonic venules of colitic mice, suggesting that P-selectin (whether expressed on the platelet or endothelial cell) contributes to both recruitment processes. While platelet binding to endothelial cells is not affected by ICAM-1 or CD18 immunoblockade, the accumulation of leukocyte-bound platelets is dramatically reduced. The findings are consistent with a model wherein leukocytes require P-selectin to roll on venular endothelium and subsequently establish firm adhesion via CD18-ICAM-1 interactions. The adherent leukocytes, which constitutively express PSGL-1, then create a platform onto which platelets can bind using P-selectin [27]. Similar P-selectin (platelet-associated)–PSGL-1 (leukocyte-associated) interactions may also account for the platelet–leukocyte aggregates that are detected in blood of IBD patients. Platelet recruitment into inflamed microvessels may be important because, upon activation, platelets produce/release a variety of proinflammatory mediators (e.g., CD40 L, RANTES, etc.) that can stimulate the production/expression of adhesion molecules on endothelial cells.

19.2.3.2 Enhanced Thrombosis

Another manifestation of the vascular changes and endothelial cell dysfunction that accompanies inflammation is enhanced thrombus development. This response is likely a reflection of the relatively new concept that inflammation and thrombosis/coagulation are interdependent processes that can initiate a vicious cycle wherein each process propagates and intensifies the other [28,29]. Thrombosis and thromboembolism are a significant cause of morbidity and mortality in IBD patients. Clinical studies reveal that the incidence of thrombotic events in IBD patients is ~6.2%, with a 3.6-fold increase in the risk for thrombotic complications compared to the general population [29,30]. A much higher

incidence of systemic thrombotic events (41%) is predicted from postmortem studies. Both the arterial and venous circulations appear to be involved in IBD-associated thrombosis, although venous complications (e.g., mesenteric venous thrombosis) occur more frequently. The thrombotic responses are most commonly manifested as deep vein thrombosis (DVT) or pulmonary embolism (PE), although thromboses have been detected in other regional circulations, including brain, retina, and liver [29].

Endothelial cell dysfunction, platelet abnormalities, and activation of the coagulation cascade have all been implicated in the enhanced thrombosis that accompanies IBD [26]. The altered platelet function that occurs during IBD is manifested as thrombocytosis (resulting from enhanced thrombopoiesis), hyperreactivity to agonist stimulation (e.g., thrombin, ADP, etc.), and hyperaggregability (both homotypic and heterotypic) [26,29]. Intravascular platelet aggregates are detected in mucosal biopsies from patients with ulcerative colitis, and the number of platelet aggregates in mesenteric venous blood is increased in IBD. The enhanced reactivity and aggregability of platelets in IBD are accompanied by evidence of significant systemic activation of the coagulation system. The anticoagulant capacity of endothelial cells is diminished during inflammation, which is manifested as an increased expression of tissue factor and inhibition of the protein C pathway [29–31]. The inactivation of nitric oxide by superoxide has also been implicated in the prothombotic phenotype assumed by endothelial cells during inflammation. Collectively, the functional abnormalities that are manifested by both platelets and endothelial cells during inflammation appear to tip the balance between pro- and anticoagulant mechanisms in favor of thrombus formation.

The hemostatic alterations and endothelial cell dysfunction reported in IBD patients are recapitulated in animal models of these diseases [26,29]. Mice with experimental colitis also exhibit an increased vulnerability to microvascular thrombus formation in vascular beds that are distant from the inflamed bowel. While the enhanced thrombosis in experimental IBD is evidenced in both arterioles and venules, the former exhibit a more consistent and pronounced response [32]. Animal studies have revealed a role for different pro- (e.g., thrombin, tissue factor) and anticoagulants (activated protein C) in the thrombogenic responses to IBD. Tissue factor (TF) immunoblockade experiments in animal models of IBD have revealed an important role for TF in the initiation of thrombus development in DSS colitis, since TF antibody treatment effectively reversed the enhanced onset time (which reflects the initiation phase of thrombogenesis) for thrombus formation in arterioles without altering the time for complete flow cessation (which reflects the propagation/stabilization phases of thrombogenesis). However, transgenic mice that overexpress the endothelial protein C receptor show a significantly attenuated thrombosis response, reflected as a prolonged time to flow cessation, during experimental IBD. Activated protein C (APC) was also shown to protect wild-type colitic mice against thrombosis, while an anti-APC antibody enhanced thrombus formation [33]. Collectively, these findings suggest that TF activation underlies the initiation of thrombus formation and that a blunted production of APC by endothelial cells contributes to the propagation of the thrombogenic response to inflammation.

Cytokines, which are produced and released in high quantities in the inflamed colon, have been implicated as mediators of the distant organ thrombosis response to colonic inflammation. IL-1β, TNF-α, and IL-6 have all been implicated in the accelerated thrombus formation. All three cytokines promote thrombosis, in a dose-dependent manner, when administered at pathophysiologically relevant concentrations. Immunoblockade of either IL-1β or TNF-α, as well as genetic deletion of their receptors, results in a blunted colitis-enhanced thrombosis response. When both cytokines are simultaneously blocked, a more pronounced protective response is achieved, compared to blocking either cytokine alone [26]. Both IL-1β and TNF-α are known to promote the biosynthesis of IL-6, a potent prothrombogenic agent that has been implicated in the coagulopathy of sepsis. Chronic (more than 5 days) administration of IL-6 in otherwise normal mice produces a prothrombotic phenotype that resembles experimental colitis, with an accelerated thrombosis response that is largely evident in arterioles. Furthermore, the colitis-enhanced thrombogenic response is largely prevented in IL-6-deficient mice or following immunoblockade of the IL-6 receptor in wild-type mice in WT mice [34,35].

19.2.3.3 Angiogenesis/vasculogenesis

Angiogenesis, the generation of new blood vessels from existing networks, vasculogenesis, the generation of vascular structures from endothelial progenitor cells, and lymphangiogenesis, the formation of new lymphatic vessels and patterns of vascular remodeling are well-recognized responses of the gut to IBD [36,37]. Although the increased vascular density produced by angiogenesis/vasculogenesis in IBD might be anticipated to improve transport function, angiogenesis in IBD is largely maladaptive and enhances disease severity by activating or intensifying several cellular and molecular inflammatory programs. Consequently, VEGF-depleting antibodies (*bevacizumab* and *ranibizumab*) and reagents that inhibit vascular endothelial growth factor (VEGF) receptor tyrosine kinase signal transduction (*lapatinib*, *sunitinib*, *sorafenib*, *axitinib*, and *pazopanib*) are currently being considered as treatments for human IBD [38,39]. Mesalamine (5-aminosalicylate), a treatment currently used for UC, corrects imbalances in angiogenic and angiostatic regulators in this disease, an effect that at least partly explains its ability to reduce disease activity [40]. Conversely, while angiogenesis in IBD enhances disease activity, lymphatic expansion appears to offer some benefit in some phases of gut inflammation by creating conduits that facilitate the evacuation of accumulated interstitial fluid and immune cells from the inflamed intestine.

Several key cellular events are observed in both angio- and lymphangiogenesis, including proliferation of endothelial cells (in response to the abundance of growth factors and angiogenic stimuli in IBD), extracellular matrix remodeling, cell migration, and vessel maturation. The final events in vessel stabilization appear to be disturbed in IBD such that inflammatory angiogenesis (and lymphangiogenesis) provoke a persistently hyperadhesive, hypermotile, and dedifferentiated endothelial phenotype that is characterized by loss of low-density lipoprotein receptor with mobilization of several endothelial cell adhesion molecules that can bind immune cells and platelets. Vascular remodeling during

angiogenesis also disturbs junctional organization, which contributes to the increased vascular permeability that often accompanies IBD.

At the molecular level, angiogenesis is initiated by binding of VEGF ligands to VEGF receptors (VEGFRs), which are receptor tyrosine kinases expressed on the endothelial surface. Coreceptors for the VEGF/VEGFR complexes include neuropilins-1 and -2 as well as heparin sulfate proteoglycan. In addition, platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) and VE-cadherin and integrins also represent coreceptor components of a mechanotransduction signaling complex with VEGFR-2 that govern responses to fluid shear and coordinate interactions between flow, proliferation, and permeability [41].

The VEGFs potentially active in IBD include VEGF-A, B, C, D, and placental growth factor (PlGF) that interact with three VEGFRs: VEGFR-1 (also Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4). Two additional VEGF isoforms also exist VEGF-E and –F, which are expressed by viruses and snake venoms, respectively. Angiogenesis in IBD is largely mediated by increased levels of VEGF-A, which is derived from both local sources (including intermittently ischemic enterocytes and stromal tissues) and infiltrating peripheral blood monocytes. Interactions between immune cells, platelets and intestinal endothelial cells can intensify angiogenic signaling. It has reported that platelets bound to TNF-α stimulated intestinal endothelium via ICAM-1, fractalkine, and αvβ3 integrin stimulate VEGF-A release and enhance the availability of sCD40 ligand, both of which mediate angiogenic signaling [42].

As the central angiogenic stimulus in IBD, VEGF-A not only activates endothelial cell proliferation but also mobilizes endothelial adhesion molecules, enhances leukocyte adhesion, and increases vascular permeability, which collectively increase disease severity [39]. VEGF-A induces the expression of ICAM-1, VCAM-1, and E-selectin on endothelial cells via NF-kB activation, which enhances immune cell binding (similar to TNF-α, β) synthesis [7,43]. VEGF-A-mediated activation of the adhesive endothelial phenotype seen during active IBD also appears to involve cyclooxygenase-2- and mitogen-activated protein kinase activation [44].

Although VEGF-A_{165} appears to represent the major angiogenic and pathological isoform of VEGF-A, several VEGF-A isoforms exist, and alterations in the ratios of active to inactive VEGF-A isoforms (e.g., VEGF-A_{165b}) may modulate angiogenesis, as well as vascular tone, leakage, remodeling, and leukocyte recruitment in IBD [37]. For example, intestinal recruitment of Mac-2^+ macrophages and neutrophils within colon follicular aggregates increases in experimental UC, during both acute and chronic phases of disease and this recruitment appears to depend on the ratio of active to inactive VEGF-A isoforms, since adenoviral induction of an inhibitory VEGF (VEGF-A_{164b}) reduced immune cell infiltration [37]. However, the available evidence suggests that polymorphisms in VEGF-A structure do not influence IBD induction, progression, or remission.

VEGF receptor signaling is highly complex, with VEGF receptors existing mainly as homodimers, although heterodimers of VEGR-1/2 and VEGFR-2/3 exist. VEGF coreceptors include the neuropilins -1 and -2 and heparin sulfate proteoglycan. Proteolytic modification of VEGFs (-C and -D) also controls ligand receptor preference. VEGF receptors -1 and -2 are found on blood vessel

endothelial cells and govern angiogenesis, while lymphangiogenic responses are mainly mediated by VEGFR-3 and VEGFR-2. VEGF receptors are also expressed in several other cells, including monocytes (VEGFR-1), adventitial and stromal cells (VEGFR-2 and VEGFR-3), and enterocytes (VEGFR-1), suggesting that VEGFs also contribute to extravascular forms of cell signaling regulation. While endothelial VEGFR-1 signaling can modulate immune cell recruitment, VEGFR-2 activity appears to represent the major form of VEGF signaling underlying most physiological blood vascular endothelial responses, as well as the pathological effects of VEGFs in IBD.

VEGF-A binding to VEGFR-2 (and the coreceptor neuropilin-1) activates several NF-kB-dependent gene programs, including endothelial cell adhesion molecules, matrix metalloproteinases (MMPs), and cyclooxygenases that contribute to the vascular disturbances in IBD. In addition to VEGF-A, many angiogenic stimuli and regulators including PlGF, VEGFR1, angiopoietin-2, and the Tie-2 receptor are also significantly elevated in CD [7,45,46]. Similarly, circulating levels of VEGF-A and VEGFR-1 are elevated in active UC, with VEGFR-2 receptor expression significantly increased in both clinical and experimental IBD [39].

In addition to the activity of members of the VEGF family in IBD-associated angiogenesis, hepatocyte growth factor (HGF), transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF-2), and platelet-derived growth factor (PDGF) also regulate angiogenesis in IBD. Like VEGF, elevated circulating HGF levels appear to contribute to the microvascular proliferation and microvascular phenotypic alterations observed in IBD. HGF is released by the inflamed intestinal stroma and is post-translationally activated by HGF activator (HGFA) in response to thrombin generation in inflamed tissues. Unlike VEGF-A activity, however, HGF receptor activation may be beneficial in IBD by aiding in restitution of intestinal epithelia and by opposing thrombin-induced permeability changes.

PDGF is another growth factor that is elevated in IBD. PDGF is stored within and released by platelets, and is also synthesized by macrophages, smooth muscle cells, and endothelium. PDGF participates in attracting adventitial cells (pericytes and smooth muscle) to blood vessels and supports smooth muscle and mesenchymal proliferation, but it also contributes to fibrosis in IBD.

Although TGF-β normally functions as an anti-inflammatory growth factor whose levels are increased in IBD, TGF-β signaling can be compromised in IBD by Smad7, leading to unopposed induction and activation of inflammatory cytokines [47]. VEGF-A appears to regulate TGF-β release by enterocytes, which promotes intestinal homeostasis in an autocrine fashion [48]. Angiopoietins represent another class of growth factors that play important roles in angiogenesis during IBD. Endothelial cell signaling through the TEK/Tie-2 receptor modulates the maturation and stabilization of blood vessels via binding interactions with its ligands angiopoietins 1–4 [36]. While angiopoietins -1 and -4 are agonists of Tie-2-mediated phosphorylation, angiopoietins -2 and -3 antagonize the effects of angiopoietin-1. Ang-2 is stored within and released from endothelial Weibel–Palade bodies in response to PKC activation and acts as an autocrine antagonist of Ang-1. In this sense, Ang-2 plays important roles in releasing both blood and lymphatic vasculatures from their "stable" phenotype and allows them to enter

into angiogenic and inflammatory programs. Ang-1/Ang-2 ratios are low in CD, and Ang-2 gene depletion was found to limit both angio- and lymphangiogenesis, inflammation, and neutrophil infiltration into the gut in experimental ulcerative colitis [36].

Bacterial products and antigens are potent stimuli for intestinal angiogenesis. Ligands for NOD-like (NLR) and Toll-like receptors (TLR) that penetrate beyond the mucosal layer can activate angiogenesis via mitogen-activated protein kinase, nuclear factor-κB, vascular endothelial growth factor receptor 2 (VEGFR-2), and focal adhesion kinase (FAK) signaling [49]. Conversely, other products of bacterial metabolism (e.g., butyric acid) can suppress VEGF-A-mediated angiogenic signaling (proliferation, migration, and capillary genesis) via suppression of cyclooxygenase-2 and reduced interleukin-6 secretion [50].

Disturbances in lymphatic transport function in IBD also seem to underlie not only disease initiation but also progression in both UC and CD. Indeed, the initial descriptions of CD by Crohn implicated lymphangitis, lymphostasis, and lymphatic remodeling as features of the disease, which are also seen in UC and contribute to disease severity. Like blood angiogenesis, lymphatic density increases in both forms of IBD but appears to be largely adaptive. While angiogenic processes are pro-inflammatory in the setting of IBD, lymphangiogenesis appears to relieve tissue edema by transporting accumulated interstitial fluid. While angiogenesis is largely mediated by VEGF-A/VEGFR-2 activity, lymphangiogenesis is governed by VEGFR-3 and its ligands VEGF-C and -D. Experimental blockade of VEGFR-3 intensifies experimental IBD, while administration of VEGR-3 agonists, for example, VEGF-C and -D, has demonstrated protection in IBD models. Paradoxically, lymphatic endothelial exposure to VEGF-C increases expression of the lymphatic marker podoplanin, but simultaneously decreases cadherin-5, which dysregulates lymphatic barrier function [51].

Another modulator of lymphatic valve organization and adventitial structure is the FoxC2 transcription factor. FoxC2 is increased in experimental colitis. Importantly, mice with defects in FoxC2 exhibit no differences in lymphatic density (lymphangiogenesis), but they have significantly more intense disease activity in experimental IBD with significantly lower survival, greater histopathological injury, edema, and increased colonic burden of neutrophil, T-cell, and macrophage penetration [52]. Because this model demonstrates more extensive lymphatic dilation, the failure of lymphatic vessels to fully mature (achieve normal valve organization and adventitital coverage) despite normal levels of lymphangiogenesis argues for the necessity of restituting both vessel integrity and vessel abundance in normal intestinal homeostasis.

19.2.3.4 Junctional and Vascular Permeability

Increased microvascular solute permeability is a hallmark feature of inflammation that observed in both acute and chronic forms of CD and UC. Increased microvascular permeability in IBD delivers albumin and opsonizing antibodies, which help to isolate pathogens, but can severely compromise tissue function and exacerbate the tissue injury response. There are at least two pathways for increased microvascular flux seen during inflammation: (1) the "paracellular" pathway that involves opening of interendothelial tight and adherens junctions

and (2) formation of transendothelial pores (transcytosis) that allow bulk transcellular solute flux [53].

At the molecular level, changes in the paracellular restrictive properties of endothelial monolayers in IBD can represent one or more events that include changes in tight and adherens junctional binding with and organization by the cytoskeleton, transient junctional protein internalization, decreased expression of specific junctional elements, and proteolytic degradation of junction components.

Endothelial cells express tight junctions containing occludin, claudins -1, and their zonula occludens (ZO) linker proteins as well as complexus adherens junctions that contain VE-cadherin (cadherin-5) and the immunoglobulin superfamily molecules PECAM-1 and the junctional adhesion molecules (JAM)-A,-B, and -C. Paracellular leukocyte diapedesis is a complex process involving Ig-CAM family members PECAM-1/CD31, JAM-A, and CD99. While JAM-A expressed at endothelial junctions supports leukocyte diapedesis, JAM-B blocks VEGF/VEGFR-2 signaling and JAM-C binding interaction appears to negatively regulate VE-cadherin junctional integrity [54].

Several classes of inflammatory mediators, including cytokines, oxidants, and bacterial products, can acutely provoke microvascular (and epithelial) solute permeability changes that contribute to the tissue edema and injury seen in IBD [55,56]. Cytokines (e.g., TNF-α, IFN-γ, etc.) depress expression of occludin and cadherin-5 that reduce barrier solute restriction across endothelial monolayers, an effect that can be prevented by pretreatment with interleukin-10 suggesting that the abundance and ratios of Th1/Th2 cytokines in IBD may govern junctional makeup and barrier function [56]. Acute vascular permeability changes in response to TNF-α have been shown to involve protein tyrosine kinase-6 signaling that leads to junctional disintegration [57].

Acute vascular permeability changes in erosive models of UC have been shown to be mediated by Src-kinase tyrosine phosphorylation (Src^{416}) within the colon mucosa early on in the course of acute injury. Src inhibition by PP1 prevented Src^{416} phosphorylation and colon vascular permeability linked with VEGFR-2 activation. Mechanistically, VEGF-A can disturb VE-cadherin junctions via VEGFR-2-mediated VE-cadherin phosphorylation, beta-arrestin-2 binding, and internalization of VE-cadherin [58]. Several other inflammatory mediators including hydrogen peroxide and histamine also transiently increase the internalization of VE-cadherin from the endothelial surface [59]. When not present at the cell surface, the normal level of cell–cell apposition maintained by these junctional proteins is low permitting increased exchange. It is unclear if persistent induction of this type of junction internalization would lead to proteolytic degradation.

Matrix metalloproteinases, a family of zinc-dependent proteases secreted by several cell types as zymogen preforms, can contribute to IBD disease activity by degrading tissue matrices to increase microvascular permeability. Several MMPs are released by neutrophils (MMP-8) and monocytes (MMP-1, -2,-3, -9, -10, -14-17,-19, and -25). Activation of matrix metalloproteinases (e.g., MMP-1, -2, and -9) in IBD by inflammatory cytokines as well as homocysteine can lead to destruction of the subendothelial matrix [60].

Several tight junction (occludin, claudin-5, claudins -1 and -12, and zonula occudens-1) and adherens junction components (VE-cadherin) are also susceptible proteolytic cleavage by MMPs, which can reduce microvascular barrier function [59].

Several cytokines and growth factors including VEGF-A can trigger the production and processing of MMP-1, -3, and -9 to their active isoforms in several cell types of the inflamed intestine. Mechanistically, VEGF-A-mediated increase in vascular permeability involves activation of phospholipase C-γ, protein kinase C, extracellular signal-regulated protein kinase, Akt, Src, focal adhesion kinase, and other pathways [61].

TNF-α can also mobilize endothelial MMPs that modulate barrier function [59]. Elastase released by neutrophils recruited in IBD can also degrade VE-cadherin and beta-catenin. The increased production of reactive oxygen species during active IBD also oxidatively inactivates tissue inhibitors of metalloproteases that would normally suppress the proteolytic activity of MMPs. Although levels of junctional proteins are decreased in the intestine during active IBD, it has been suggested that these changes in junctional protein expression are limited to enterocytes and that levels of endothelial junctional components are preserved even during active IBD [62].

The increased endothelial expression of CD146 (melanoma cell adhesion molecule/MCAM and cell surface glycoprotein/MUC18) within intestinal endothelial junctions has been reported as a specific marker of inflammatory activation in IBD and the release of CD146 has been correlated with increased microvascular permeability [63]. Based on these observations, circulating CD146 levels have been suggested as a potential biomarker in IBD.

19.3 Conclusions

The microcirculation plays an important role in the initiation and perpetuation of an inflammatory response. The diverse responses of the microcirculation to inflammation likely reflect the collective contribution of a variety of cells, including blood cells, endothelial cells, vascular smooth muscle (VSM), and perivascular cells (e.g., macrophages, mast cells, etc.). However, endothelial cells appear to have a unique role in modulating the inflammatory response. The importance of endothelial cells in this process is evidenced by the ability of these cells to regulate the migration of inflammatory cells into tissue, modulate the tone of underlying VSM and consequently blood flow, provide an interface for the activation/deposition of coagulation factors and binding of platelets during thrombus formation, as well as their critical role in the formation of new blood vessels. Each of these responses underlie critical pathological features of an inflammatory response and can contribute to the end-organ damage, morbidity, and mortality that are associated with inflammatory diseases, such as IBD. Consequently, the potential for targeting one or more of these endothelium-dependent inflammatory responses raises hope for the development of effective new drugs for the treatment of inflammatory diseases. Indeed, drugs that target a specific microvascular response to inflammation, such as leukocyte–endothelial

cell adhesion or angiogenesis, have shown promise in both the preclinical and the clinical studies of IBD [37,64]. These promising results justify a continued focus on endothelial cells as a therapeutic target in inflammatory diseases.

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Part Three

Inflammatory Mediators

20

IL-1 Superfamily and Inflammasome

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20.1 Introduction

20.1.1 The IL-1 Family of Cytokines and the Innate Immune System

There are 11 members of the IL-1 family of cytokines and 10 members of the IL-1 family of receptors (Table 20.1). More than any other cytokine family, the interleukin-1 family members are closely linked to damaging inflammation; however, the same members also function to increase nonspecific resistance to infection and the development of an immune response to a foreign antigens (Figure 20.1). These inflammatory and host defense properties are now termed "innate immunity" and augmenting response to antigens is now called "acquired immunity." What characterizes innate immunity is the lack of specificity. Indeed, the numerous biological properties of the IL-1 family are nonspecific. The importance of IL-1 family members to the innate response became evident upon the discovery that the cytoplasmic domain of the IL-1 receptor type I (IL-1R1) is also found in the Toll protein of the fruit fly [1]. The functional domain of the cytosolic component of IL-1R1 [2] is termed the Toll interleukin-1 receptor (TIR) domain. In fact, the TIR domain is highly homologous to the TIR domains of all Toll-like receptors (TLRs). Thus, fundamental inflammatory responses such as the induction of cyclooxygenase type 2, production of multiple cytokines and chemokines, increased expression of adhesion molecules, or synthesis of nitric oxide are indistinguishable responses of both IL-1 and TLR ligands.

Both TLR and IL-1 families nonspecifically augment antigen recognition and activate lymphocyte function. The lymphocyte-activating function of IL-1 was first described in 1979 [3] and is now considered a fundamental property of the acquired immune response. IL-1β is the most studied member of the IL-1 family due to its role in mediating autoinflammatory diseases [4]. Unquestionably, IL-1β evolved to assist host defense against infection [5] and this landmark study established how a low dose of recombinant IL-1β protects mice against lethal bacterial infection in the absence of neutrophils. Although we now accept the concept that cytokines like IL-1β served millions of years of evolution to protect

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Table 20.1 IL-1 family of receptors.

Receptor	Function	Ligand
IL-1R1	Ligand binding	IL-1Ra, IL-1α, and IL-1β
IL-1R2	Ligand binding	IL-1β
IL-1R3	Coreceptor	IL-1α, IL-1β, IL-33, and IL-36α, β, and γ
IL-1R4	Ligand binding	IL-33
IL-1R5	Ligand binding	IL-18
IL-1R6	Ligand binding	IL-36α, β, γ, IL-36Ra, and IL-38
IL-1R7	Coreceptor	IL-18
IL-1R8	Coreceptor	IL-37
IL-1R9	Coreceptor	IL-38
IL-1R10	Coreceptor	?

the host, in the antibiotic and antiviral therapies era of today, we view cytokines also as the cause of disease due to acute or chronic inflammation.

The IL-1 family also includes members that suppress inflammation, specifically within the IL-1 family, such as the IL-1 receptor antagonist (IL-1Ra), IL-36 receptor antagonist (IL-36Ra), and IL-37. In addition, the IL-1 family member IL-38, the last member of the IL-1 family of cytokines to be studied, nonspecifically suppresses inflammation and limits innate immunity.

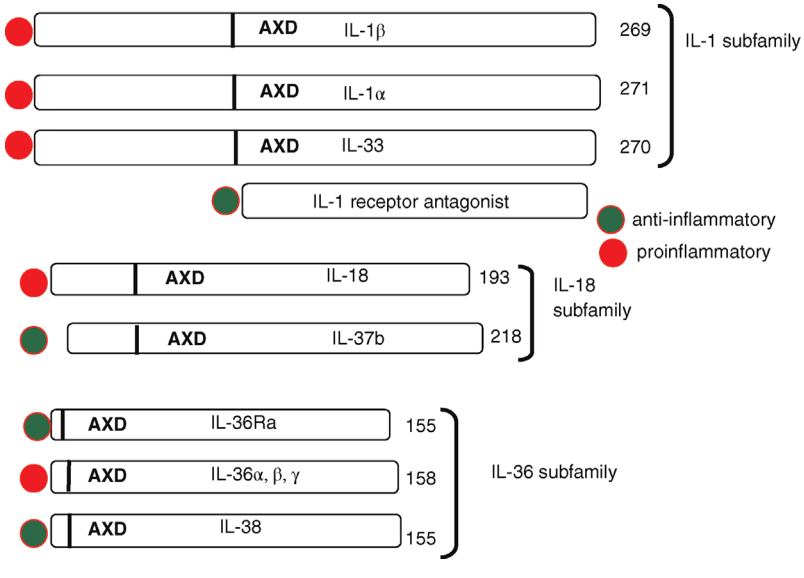


Figure 20.1 Organization of the IL-1 family of ligands into subfamilies. The subfamilies are characterized by similar total number of amino acids of the translated member and the location of the AXD consensus sequence. The vertical bar represents the likely N-terminus based on 9 amino acids forward from the AXD site. The colored circles indicate the primary property of the member, that is, proinflammatory (red) or antiinflammatory (green).

20.1.2 The Family of IL-1 of Receptors

There are 10 members of the IL-1 family of receptors [6]. IL-1R1 binds IL-1α, IL-1β, and IL-1Ra. IL-1R1 binds either IL-1β or IL-1α. IL-1R2 is a decoy receptor for IL-1β. This receptor lacks a cytoplasmic domain and therefore does not signal. IL-1R2 exists not only as an integral membrane protein but also in a soluble form. The term "soluble" is meant to denote the extracellular domain only. The soluble domain of IL-1R2 binds IL-1β in the extracellular space and neutralizes IL-1β. Neutralization of IL-1β by soluble IL-1R2 is greatly enhanced by forming a complex with IL-1R3 (formerly IL-1R1 accessory protein) [7,8]. IL-1R3 is the coreceptor for IL-1α, IL-1β, IL-33, IL-36a, IL-36β, and IL-36γ. IL-1R3 exists as an integral membrane receptor or in a soluble receptor form. In addition, the soluble form of IL-1R3 is produced by the liver and circulates constitutively in health. During inflammation and infection, the liver increases its synthesis and levels of soluble IL-1R3 rise in the circulation.

In that regard, soluble IL-1R2 forms a complex with soluble IL-1R3 or cell bound IL-1R3 and prevents the binding of IL-1β to the IL-1R1. Importantly, the IL-1 family of receptors also contains anti-inflammatory receptors [9]. These are IL-1R8 (formerly SIGIRR, TIR8), IL-1R9 (formerly TIGIRR-2, IL-1RAPL1), and IL-1R10 (formerly TIGIRR-1). In the case of IL-1R8, mice deficient in this receptor develop spontaneous inflammation and when challenged with inflammatory agents, exhibit more severe disease [10]. The IL-18-binding protein (IL-18BP) is not a classic receptor because it is a secreted protein; nevertheless, IL-18BP contains a single IgG domain similar to the extracellular IgG-like domains of the IL-18Rα but no cytoplasmic domain [11]. The IL-18BP functions similarly as do other soluble receptors in the IL-1 receptor family, that is, they bind and neutralize the cognate ligand. In addition to the IL-18BP, there is soluble form of the IL-1R2. As an integral membrane protein, this receptor acts as a decoy receptor [12], but like IL-18BP, soluble IL-1R2 will bind the cognate ligand in the extracellular compartment.

20.1.3 The IL-1 Family Consensus Sequence

With the exception of IL-1Ra, all members of the IL-1 family lack a signal peptide and are not readily secreted. They are found diffusely in the cytoplasm as precursors and each precursor contains a three amino acid conserved consensus sequence A-X-Asp, in which A is any aliphatic amino acid, followed by any amino acid (X) and then aspartic acid [13]. The aspartic acid is not the aspartic acid that indicates a caspase-1 cleavage site. Nine amino acids before the consensus sequence indicate the amino terminal amino acid for optimal folding of the cytokine for receptor binding. For example, truncation of IL-36α, IL-36β and IL-36γ to the ninth amino acid before the A-X-Asp motif results in a 1000-fold increase in bioactivity [13]. In the case of the IL-1β precursor, nine amino acids before the consensus motif (Leu-Arg-Asp) are the caspase-1 cleavage site creating the N-terminus for optimal activity. Other cytokines such as IL-16 and the growth factors, fibroblast growth factor, and vascular endothelial growth factor also lack a signal peptide and are active as precursors. The precursor of IL-1α is also active as a precursor [14]. From cell cultures producing IL-1α, the N-terminus was found at amino acid serine 113 [15]. Serine 113 is not the N-terminus as predicted by the consensus sequence. The processing of human IL-1α from endotoxin-stimulated

macrophages required a calcium-dependent neutral protease [16]. In a mouse macrophage cell line, inhibitors of calpain prevent the processing of the IL-1α precursor [17,18]. Copper is also required for the processing and release of IL-1α via S100A13 in both human macrophages and mouse fibroblasts [19,20].

20.1.4 The IL-1 Family as Therapeutics

Of the 11 members of the IL-1 family, IL-1α, IL-1β, IL-1Ra, and IL-18 have been thoroughly studied *in vitro* in animal models of disease and in humans. In humans, blocking IL-1 activity, particularly IL-1β, is used to treat a broad spectrum of diseases [21]. The IL-1Ra is a specific inhibitor of the activity of both IL-1α and IL-1β and the recombinant form is a pharmaceutically known as anakinra. Anakinra is approved in several countries for treating the signs, symptoms, and joint destruction of rheumatoid arthritis as well as cryopyrin-associated periodic syndrome (CAPS). Over 1000 patients with rheumatoid arthritis have been treated with anakinra, some for over 10 years. Blocking IL-1 activity with anakinra results in an arrest in the progressive joint space narrowing that is one of the hallmarks of rheumatoid arthritis. In a placebo–controlled randomized trial, anakinra appeared to benefit patients with stroke [22]. Anakinra is now the standard therapy for patients with systemic onset juvenile idiopathic arthritis [23], refractory adult Still's disease [24], and other systemic and local inflammatory diseases. These and other diseases are now classified as "autoinflammatory" diseases and are to be distingished from "autoimmune" diseases. The IL-1 Trap, a bivalent construction of the extracellular domains of the IL-1 receptor type I (IL-1R1) and the IL-1R3 [25], has been approved for the treatment of autoinflammatory diseases such as familial cold-induced autoinflammatory syndrome and Muckle–Wells syndrome [26].

For many years, there has been a great interest in the ability of IL-1β to damage the insulin-producing pancreatic beta cell [27]. But recent studies in humans have demonstrated a role for IL-1 in type 2 diabetes. In a placebo–controlled, blinded randomized trial in patients with poorly controlled type 2 diabetes, 13 weeks of anakinra therapy improved glycemic control and the function of the insulin-producing beta cell [28]. In a second study, neutralizing monoclonal antibodies to IL-1β also improved glycemic control and beta-cell function in type 2 diabetic patients [29]. Therefore, destruction of the insulin-producing beta cell appears to be an IL-1β-mediated autoinflammatory disease [30]. Patients with type 2 diabetes at high risk for cardiovascular events such as myocardial infarction and cerebrovascular stroke were treated with statin-type drugs to reduce the atherosclerotic process. Serum C-reactive protein (CRP) levels are used to monitor the risk of a cardiovascular event. When treated with canakinumab (a human antihuman IL-1β) a further decrease in CRP predicts reduced cardiovascular events. To test this hypothesis, a randomized placebo–controlled trial of canakinumab has enrolled over 10 000 patients [31]. This is the largest trial of an anticytokine in treating a specific disease. The trial tests three subcutaneous canakinumab at doses of 5, 15, 50, or 150 mg monthly compared to placebo. In a preliminary study for 4 months, the antibody-treated groups reported statistically significant reductions in circulating CRP, IL-6, and fibrinogen levels compared to the cohort receiving placebo [32].

Another member of the IL-1 family used clinically is the IL-18BP [11], the naturally occurring inhibitor of IL-18 activity. IL-18BP has a high affinity binding to IL-18 and circulates in healthy subjects at 2–3 ng/ml, which is greater than 100-fold molar excess of IL-18 levels [33]. Although IL-18BP is elevated to 5–6 ng/ml in several autoimmune and autoinflammatory diseases, the level of free IL-18 is associated with disease severity [34–38]. IL-18BP has been administered to patients with rheumatoid arthritis and plaque psoriasis [39]. IL-18BP is being tested in patients with macrophage activation syndrome, adult onset Still disease and mutations in NLRC4. In patients with the NLRC4 missence mutation Thr337Ser, there is increased release of IL-1β and IL-18 associated with flares of macrophage activation syndrome [40].

20.2 IL-1α

20.2.1 Historical Background

In 1974, while purifying the endogenous fever-producing protein "leukocytic pyrogen" from the supernatants of human blood monocytes, two molecular weights (36 and 15 kDa) and two distinct isoelectric points (pI 5.1 and 6.8) were observed [41]. The 36 kDA and pI 5.1 leukocytic pyrogen was recognized at IL-1α in 1984; the 15 kDa and pI of 6.8 became IL-1β. Historically, IL-1α was studied for many years in murine immunology as lymphocyte-activating factor (LAF) [42]. LAF activity was defined as an augmentation of mouse thymocyte proliferation to phytohemagglutin (PHA). In general, supernatants from activated macrophages were added to cultures of mouse thymocytes stimulated with PHA and the activity of LAF was an increase in the proliferation of the thymocytes as measured by increased uptake of tritiated thymidine. In other assays, LAF activity was measured by augmented T-cell responses to antigen. In 1984, a mouse macrophage cell line, a cDNA, was isolated and when expressed in *Escherichia coli*, the recombinant LAF increased in the proliferation of the thymocytes [15]. Also in 1984, a cDNA was isolated from human blood monocytes and the recombinant protein increased the proliferation of a mouse T-cell line stimulated with PHA. The two cDNAs were related but not the same. The mouse IL-1 was later named IL-1α and the human IL-1 was named IL-1β. The primary amino acid sequences of IL-1α and IL-1β are only 26% identical.

20.2.2 Systemic Effects of IL-1α in Humans

Following the cDNA cloning of mouse IL-1α in 1984, recombinant IL-1α became available for study in humans with cancer. As a result, clinical studies were performed in cancer patients based on studies in mice that showed an antitumor effect, perhaps related to the immunological properties of cytokine. Humans receiving IL-1α developed fever, generalized systemic inflammation at doses as low as 30 ng/kg; at 100 ng/kg, patients developed hypotension [43,44]. Similar febrile and systemic inflammation developed in cancer patients receiving infusions of IL-1β (reviewed in Ref. [45]. Understandably, IL-1α or IL-1β is not used to treat cancer today; instead, blocking IL-1α or IL-1β has resulted in prolongation and improved quality of life [46,47].

20.2.3 IL-1α is a Dual-function Cytokine

IL-1α is a "dual-function" cytokine. Dual-function cytokines are found in the nucleus where they bind to DNA and function; the same cytokine binds to its cell membrane receptor and initiates signal transduction. In the case of IL-1α, there is a nuclear localization sequence in the precursor region of the cytokine, and one of the functions of IL-1α in the nucleus is as a transcription factor [48]. In that context, nuclear IL-1α functions to increase gene expression, for example, the chemokine IL-8 [48]. Another member of the IL-1 family, IL-33, also is a dual-function cytokine. When the nuclear localization sequence of IL-33 in mice is deleted, cytokine is found only in the cytosol; in these mice, overwhelming unresolved inflammation occurs early after birth, dominated by large numbers of eosinophils [49]. Thus, nuclear localization of IL-33 acts as a sink for its proinflammatory properties. Nuclear translocation of IL-1α can also be a sink for its proinflammatory properties. For example, the IL-1α precursor shuttles between the cytosol and the nucleus within a few nanoseconds [50]. When the cell is exposed to a proapoptotic signal, IL-1α leaves the cytosolic pool and rapidly migrates to the nucleus where it binds tightly to chromatin. Whole lysates of these cells fail to induce inflammation since the chromatin bound IL-1α is not available to bind to IL-1 receptors on adjacent cells. In contrast, when the cell is exposed to a necrotic signal, IL-1α migrates from nucleus to the cytosol and the lysates of these cells are highly inflammatory [50]. There is a clinical correlate of these findings. The epithelial lining cells of the intestine contain large amounts of the IL-1α precursor and these cells routinely undergo apoptosis naturally without producing inflammation. In contrast, when exposed to toxic agents such as dextran sodium sulfate (DSS), cell death occurs by necrosis and the IL-1α precursor is released from the cytosolic pool resulting in lethal inflammation [51].

Following genotoxic stresses such as UV light or hydrogen peroxide, DNA is damaged. Unexpectedly, IL-1α precursor localizes specifically to the damaged foci on the DNA [52]. The localization of IL-1α to the damaged sites is distinct from the nuclear localization of the IL-1α precursor in cells undergoing apoptosis [50]. During genotoxic stress, lysine 82 in the nuclear localization sequence becomes acetylated, which facilitates the binding to the damaged foci. In addition to the nuclear localization of acetylated IL-1α following genotoxic stress, the precursor is released from the cell without loss of membrane integrity [52]. Although the IL-1α precursor is active [14], processing by calpian, granzyme B, mast cell chymase, and neutrophil proteases results in a more active form. However, in the cells undergoing genotoxic stress, the release of the IL-1α precursor takes place without evidence of processing [52].

20.3 Cell Sources, Production, and Secretion

20.3.1 Large Amounts of Constitutive IL-1α in Healthy and Disease Cells

IL-1α is an unusual member of the IL-1 family because IL-1α is found constitutively in epithelial and mesenchymal cell types of healthy subjects, whereas IL-1β is primarily induced under disease conditions. The keratinocytes of the skin contain

large amounts of the IL-1α precursor as are intestinal lining cells, fibroblasts, hepatocytes, brain astrocytes, and renal tubule cells. Endothelial cells contain IL-1α, and cytokine is present in vesicles comprised of the endothelial membrane [53]. These vesicles are termed "apoptotic bodies," but despite the name, they are highly inflammatory [53]. IL-1α is also present in most tumors of mesenchymal origin. As discussed below, tumor-derived IL-1α is released as tumors outgrow their vascular supply, resulting in paraneoplastic syndromes such as fever and loss of appetite. Under conditions of primary cell stimulation *in vitro*, human blood monocytes do not process or readily secrete IL-1α and IL-1α is not commonly found in the circulation during systemic inflammation. However, as cytokine is released from dying cells [50,54], IL-1α is a classic "alarmin" because the IL-1α precursor is active and mediates sterile inflammation [14]. For example, in many ischemic diseases such as myocardial infarction, cell death is by necrosis and IL-1α is released into the extracellular space where it stimulates the production of chemokines resulting in the infiltration of neutrophils first followed by monocytes. This transition from neutrophils to monocytes in ischemic tissues has been demonstrated in cells exposed to hypoxic conditions where the IL-1α precursor is released [55]. Since nearly all tumors contain the IL-1α precursor, upon death by necrosis, the IL-1α precursor triggers the infiltration of monocytes and chemokines.

20.3.2 IL-1α as a Growth Factor

Most cell lines including tumor cell lines contain constitutive levels of IL-1α [48,56,57]. The concept that IL-1α acts as an autocrine growth factor assumes that the intracellular IL-1α precursor regulates normal cellular differentiation. In support of this concept, an antisense oligonucleotide to IL-1α reduces senescence in endothelial cells [58]. Although the concept that IL-1α acts as an autocrine growth factor in fibroblasts or endothelial cells *in vitro*, the data must be interpreted carefully since mice deficient in IL-1α show no demonstrable defects in growth and development, including skin, fur, epithelium, and gastrointestinal function [59]. However, mice deficient in IL-1α still retain the propiece that functions as nuclear factor. The term precursor is used for the entire IL-1α molecule. The term mature is used for the carboxyl end and the term propiece for the N-terminus. In the case of IL-1α, the N-terminal 115 amino acids translocates to the nucleus and these amino acids are often termed the propiece.

Is there a role for intracellular precursor IL-1α in normal cell function? The IL-1α precursor is constitutive in all epithelial cells and large amounts of the intracellular form of IL-1Ra (icIL-1Ra) are also present in these cells [60,61]. This form of IL-1Ra also binds to IL-1RI and prevents signal transduction. In fact, icIL-1Ra is produced in the same cells expressing the IL-1α precursor and thought to compete with the intracellular pool of precursor IL-1α for nuclear-binding sites.

20.3.3 Membrane IL-1α

Precursor IL-1α can be found on the surface of several cells, particularly on monocytes and B lymphocytes, where it is termed membrane IL-1α [62]. Membrane IL-1α is biologically active [63]; its biological activities are neutralized by

antibodies to IL-1α but not against IL-1β. Thus, membrane IL-1α is an integral membrane protein. Membrane IL-1α plays an important role in inflammation as mice deficient in IL-1α exhibit reduced inflammation in models in which cell death and the release of intracellular IL-1α does not take place [64]. In models of immune-mediated killing of tumor cells, membrane IL-1α has a role to augment recognition of neoantigens on cancer cells [65]. Constitutively expressed IL-1α is critical for several IFNγ activities. Using the WISH epithelial cell line, what was considered to be intrinsic IFNγ activities depended largely on constitutively expressed IL-1α. IFNγ activities inhibited antibodies to IL-1α, but not to IL-1β [57].

20.3.4 Studies in IL-1α-deficient Mice

Mice deficient in IL-1α are born healthy and develop normally. Following subcutaneous injection of turpentine, which induces a local inflammatory response, wild-type and IL-1α-deficient mice develop fever and acute-phase proteins, whereas IL-1β-deficient mice do not [59]. In addition, although the induction of glucocorticoids after turpentine injection was suppressed in IL-1β-deficient mice, this suppression was not observed in IL-1α-deficient mice. Expression of IL-1β mRNA in the brain decreased 1.5-fold in IL-1α-deficient mice, whereas expression of IL-1α mRNA decreased more than 30-fold in IL-1β-deficient mice. These data suggest that IL-1β exerts greater control over production of IL-1α than does IL-1α over the production of IL-1β. In caspase-1-deficient mice, IL-1α production is also reduced [66], suggesting that production of IL-1α is under the control of IL-1β.

The effects of IL-1 on atherogenesis by a high-cholesterol diet were evaluated in mice deficient in either IL-1α or IL-1β [64]. Serum amyloid A protein, a marker of inflammation in atherogenesis, was markedly lower in IL-1α-deficient mice compared to wild type or IL-1β-deficient mice. IL-1α-deficient mice had significantly higher levels of nonhigh-density lipoprotein cholesterol. Reduced atherosclerosis and inflammatory cytokines were observed in apolipoprotein-E-deficient mice lacking bone marrow-derived IL-1α [67]. Transplantation of bone marrow cells from IL-1α-deficient mice resulted in a reduction in aortic lesion size twice that observed in mice transplanted with bone marrow from IL-1β-deficient mice. Using deficient mice, there is a signifcant role for IL-1α in the transformation of steatosis to steatohepatitis and liver fibrosis in hypercholesterolemic mice [68]. However, the role of IL-1α in steatohepatitis stems from the hepatocyte, rather than the myeloid compartment.

20.4 IL-1β

20.4.1 Historical Background

In 1977, IL-1β derived from the supernatants of freshly obtained human blood monocytes stimulated with heat-killed *Staphylococcus epidermidis* was purified to homogeneity [69]. At the time, IL-1β was termed leukocytic pyrogen because the

activity was that of the endogenous fever-producing molecule. Purified human leukocytic pyrogen characteristically produced a monophasic fever in rabbits at 10 ng/kg [69]. The specific activity was unexpectedly low compared to other bioactive proteins, but recombinant human IL-1β [70] causes fever in humans at 10 ng/ml, as reviewed in Ref. [71]. After the cDNA cloning of human IL-1β in 1984 [70], recombinant IL-1β was available to study and during the following years, the biological properties of IL-1β expanded greatly. Although the properties of recombinant IL-1β indicate the likely role in inflammation, a causative role for IL-1β in disease was shown in clinical trials using specific neutralization of IL-1β by monoclonal antibodies as reviewed in Ref. [72].

20.5 Systemic Effects of IL-1β in Humans

20.5.1 Transcription, Translation, and Synthesis of IL-1β

Although nearly all microbial products induce IL-1β via TLR ligands, IL-1 induces itself both *in vivo* and in monocytes *in vitro* [73]. Other studies supporting this concept have been reported [74,75]. Following LPS stimulation in human blood monocytes, IL-1β mRNA levels rise rapidly within 15 min, reach peak levels in 4 h but begin to decline 4 h thereafter due to the half-life of the mRNA or the action of micro-RNA. However, using IL-1 itself as a stimulant, IL-1β mRNA levels are sustained for over 24 h compared to microbial stimulants [76]. Raising intracellular cAMP levels with histamine enhances IL-1-induced IL-1 gene expression and protein synthesis [77]. The IL-1β precursor accumulates in the cytosol and processing by caspase-1 is triggered by ATP activating the P2X7 receptor. In freshly cultured human blood monocytes, endogenous levels of ATP rise and are sufficient for triggering the assembly of the inflammasome [78].

Non-TLR ligands such as the complement component C5a, hypoxia, adherence to surfaces or clotting of blood induce the synthesis of large amounts of IL-1β mRNA in monocytic cells without significant translation into the IL-1β protein. In these cells, the IL-1β mRNA assembles into large polyribosomes, but there is no significant elongation of the peptide [79]. This failure to complete the translation into IL-1β protein may be due to the instability element present in the coding region. This instability region is also found in IL-18 and IL-37 and appears to limit the production of these cytokines [80]. However, completion of translation of the mRNA into the respective cytokines can be accomplished by adding low concentrations of TLR ligands or IL-1 itself to the "primed" monocytes [81].

20.5.2 Regulation of IL-1β Production

Unlike most cytokine promoters, IL-1β regulatory regions are distributed over several thousand base pairs upstream from the transcriptional start site. In addition to a cAMP response element, there are NFκB-like and activating protein-1 sites. IL-1β gene regulation has been reviewed in detail [82]. The primary sources of IL-1β are the blood monocyte, tissue macrophages, and

dendritic cells. B lymphocytes and NK cells also produce IL-1β. Fibroblasts and epithelial cells, however, generally do not produce the cytokine. In healthy humans, the circulating blood monocyte or bone marrow cells do not constitutively express mRNA for IL-1β. Several malignant tumors express IL-1β as part of their neoplastic nature, particularly acute myelogenous leukemia, melanoma, multiple myeloma, and juvenile myelogenous leukemia, each of which exhibits constitutive expression of IL-1β.

20.5.3 The NLRP3 Inflammasome-dependent Processing and Secretion of IL-1β

Regardless of the stimulus, in monocytes and macrophages, specific inhibitors of caspase-1 reduce the secretion of mature IL-1β, while precursor IL-1β accumulates inside the cell. The rate-limiting step in the processing and secretion of IL-1β takes place with activation of the inflammasome. Several intracellular proteins form a complex with NLRPe, also termed cryopyrin. NLRP3 was initially discovered in patients with "familial cold autoinflammatory syndrome," a genetic disease characterized by constitutional symptoms, fevers, and elevated acute-phase proteins following exposure to cold [83]. Assembly of the inflammasome components with inactive procaspase-1 takes place following a fall in intracellular potassium. ATP activation of the P2X7 receptor opens the potassium channel, and simultaneously as potassium levels fall, caspase-1 is activated by the inflammasome [84–87].

The cleavage of the IL-1β precursor by active caspase-1 can take place in the specialized secretory lysosomes or in the cytoplasm. However, more than one pathway seems available for processed IL-1β to exit the cell. These include exocytosis of the secretory lysosomes [84,85], shedding of plasma membrane microvesicles, direct release via transporters or multivesicular bodies containing exosomes [88], or a process termed pyroptosis [89]. In general, the release of processed IL-1β takes place before there is significant release of lactate dehydrogenase [90], although *in vitro* cell death eventually takes place. Pyroptosis is a caspase-1-dependent form of cell death and employed by certain bacteria using Ipaf, a member of the NLR family of intracellular receptors [91]. An increase in intracellular calcium is also required for the mature IL-1β to exit the cell and is phopholipase C dependent [85].

The function of caspase-1 inflammasome is primarily to convert inactive procaspase-1 into the active enzyme and this step requires activation of the P2X7 receptor by ATP. However, in freshly obtained human blood mononuclear cells from healthy subjects, the p10 subunit of active caspase-1 is present even in the absence of stimulation [78] and during the subsequent incubation, extracellular levels of ATP increase [78]. Upon differentiation into macrophages, activation of the inflammasome requires exogenous ATP [78]. But in monocytes from patients with single amino acid mutation in cryopyrin, activation of caspase-1 occurs without a requirement for a rapid fall in the level of intracellular potassium [92]. Therefore, mutated cryopyrin allows for the assembly of the complex of interacting proteins in the presence of normal intracellular levels of potassium. Although often studied using LPS-induced synthesis of the IL-1β

precursor [93], it is unlikely that LPS plays a role in autoinflammatory diseases. On the other hand, spontaneous secretion of IL-1β from monocytes of patients is due to endogenous IL-1β stimulation. In patients with NOMID, there is a decrease in steady-state levels of procaspase-1 mRNA with IL-1Ra treatment [74], suggesting that IL-1β stimulates its own production and processing. Thus, any disease process that includes an increase in the steady-state levels of procaspase-1 mRNA, components of the inflammasome, or the IL-1β precursor explains the "autoinflammatory" nature of the disease. Type 2 diabetes appears to be an example of an autoinflammatory disease where glucose induces IL-1β production from the insulin-producing beta cell and IL-1β induces the beta cell to produce its own IL-1β [30].

20.5.4 P2X7 and the Activation of the Inflammasome

Patients with classic autoinflammatory diseases such as FMF or NOMID have nearly identical clinical parameters, secrete more IL-1β, and respond dramatically to IL-1 receptor blockade yet have no mutation in NALP3. It is, therefore, possible that mutations in P2X7 itself or regulation of the other genes controlling potassium channels [23] may account for dysfunction secretion of IL-1β. For example, monocytes from patients with rheumatoid arthritis are more sensitive to release of IL-1β following ATP activation of the P2X7 receptor compared to monocytes from healthy controls [94]. However, monocytes from subjects with a P2X7 Glu496Ala loss-of-function polymorphism secrete significantly less IL-1β [95]. Monocytes from subjects homozygous for this polymorphism also released significantly less IL-18 [96]. Another P2X7 receptor polymorphism is associated with increased mortality in patients undergoing allogenic stem cell transplantation [97]. Bacteremia was documented in 68% of patients with this polymorphism compared to 18% in wild-type control patients [97].

In mice deficient in P2X7 receptors, inflammation, pain, and IL-1β-mediated IL-6 production are markedly reduced [98]. In addition to a fall in intracellular potassium, ATP triggers formation of peroxynitrite, which is required for caspase-1 activation since peroxynitrite scavengers prevent IL-1β secretion [99]. Pannex-1, a mammalian protein that functions as a hemichannel for the uptake of dyes, is required for caspase-1 processing and release of IL-1β via the P2X7 receptor [100]. Pannexin-1 can also function for LPS-induced IL-1β synthesis in the absence of TLR4 [101]. P2X7 receptor activity is also regulated by "regeneration and tolerance factor" [102].

20.5.5 Noncaspase-1 Processing of IL-1β

A caspase-1 site exists for the IL-1β, IL-18, IL-33, and IL-37 precursors. However, for each of these cytokines, noncaspase-1 mechanisms also exist and generate active forms. For example, sterile inflammation induces fever, elevated IL-6, and increased production of hepatic acute-phase proteins. These responses are absent in mice deficient in IL-1β but present in mice deficient in caspase-1 [103]. Sterile inflammation is often associated with neutrophilic infiltration, and neutrophils produce IL-1β. Because neutrophils are short-lived cells dying within hours upon

emigration, release of the IL-1β precursor from intracellular stores is not unexpected. Therefore, processing of the IL-1β precursor extracellularly into an active cytokine has been reported for the common neutrophil protease, proteinase-3 [104]. Proteinase-3 also contributes to the processing of IL-18 [105]. Other proteases such as elastase, matrix metalloprotease 9, and granzyme A process the IL-1β precursor extracellularly. In addition, a mast cell chymase generates active IL-1β.

Mice with a targeted IKKβ deletion in myeloid cells are more susceptible to LPS-induced shock than control mice [75] and markedly elevated levels of IL-1β are found in the circulation associated with a prominent neutrophilia [75]. The elevated levels of IL-1β are lethal since blockade with IL-1Ra protects these mice from death. The source of the IL-1β in these mice is the neutrophil. When incubated with proteinase-3, cleavage of the IL-1β precursor is observed with molecular weights of 25 000 and 15 000 Da [75]. In fact, the products of proteinase-3 cleavage of the IL-1β precursor are biologically active. In other studies, the molecular weight of active IL-1β at 23–25 000 Da has been observed [86]. Therefore, under inflammatory conditions such as urate crystal arthritis, which is characterized by a prominent neutrophilic infiltration, proteinase-3 cleavage of extracellular IL-1β precursor likely takes place. Mice deficient in caspase-1 are not protected against urate-induced inflammation. Although IL-1Ra is effective in treating gout, IL-1Ra would be equally effective in any disease with extracellular processing of the precursor [106]. The importance of extracellular processing of the IL-1β precursor by serine proteases may explain, in part, the anti-inflammatory properties of alpha-1-antitrypsin [107].

20.5.6 Effects in Mice Deficient in IL-1β

After 10 years of continuous breeding, mice deficient in IL-1β exhibit no spontaneous disease. However, upon challenge, IL-1β-deficient mice exhibit specific differences from their wild-type controls. The most dramatic is the response to local inflammation induced by a subcutaneous injection of turpentine. Within the first 24 h, IL-1β-deficient mice injected with turpentine do not manifest an acute-phase response, do not develop anorexia, have no circulating IL-6, and have no fever [108,109]. These findings are consistent with those reported in the same model using anti-IL-1R type I antibodies in wild-type mice [108]. IL-1β-deficient mice also have reduced inflammation due to zymosan-induced peritonitis [110]. In contrast, IL-1β-deficient mice have elevated febrile responses to LPS, IL-1β, or IL-1α compared to wild-type mice [111]. IL-1β-deficient mice injected with LPS have little or no expression of leptin mRNA or protein [112].

20.6 IL-18 and IL-18-binding Protein

IL-18 was first described in 1989 as "IFNγ-inducing factor" isolated in the serum of mice following an intraperitoneal injection of endotoxin. Days before, the mice

had been pretreated with *Proprionibacterium acnes*, which stimulates the reticuloendothelial system, particularly the Kupffer cells of the liver. With purification from mouse livers and molecular cloning of "IFNγ-inducing factor" in 1995 [113], the name was changed to IL-18. Unexpectedly, the new cytokine was related to IL-1β. Similar to IL-1β, IL-18 is first synthesized as an inactive precursor and without a signal peptide remains an intracellular cytokine. The tertiary structure of the IL-18 precursor is closely related to the IL-37 precursor and the intron–exon borders of the IL-18 and IL-37 genes suggest a close association. Since 1995, many studies have used neutralization of endogenous IL-18- or IL-18-deficient mice to demonstrate the role of this cytokine in promoting inflammation and immune responses (reviewed in Refs [114–116]). However, the biology of IL-18 is hardly the recapitulation of IL-1β. There are several unique and specific differences between IL-18 and IL-1β. For example, both in healthy human subjects and in healthy mice, gene expression for IL-1β in blood mononuclear cells and hematopoietic cells is absent and there is no evidence that the IL-1β precursor is constitutively present in epithelial cells [117]. In contrast, the IL-18 precursor is present in blood monocytes from healthy subjects and in the epithelial cells of the entire gastrointestinal tract.

20.6.1 Processing of the IL-18 Precursor by Caspase-1

The IL-18 precursor has a molecular weight of 24 000 Da and is processed by caspase 1, which cleaves the precursor into an active mature molecule of 17 200 Da. As with the processing of IL-1β, inactive procaspase-1 is first converted into active caspase-1 by the nucleotide-binding domain and leucine-rich repeat pyrin containing protein-3 (NLRP3) inflammasome. Following cleavage of the IL-18 precursor by active caspase-1, mature IL-18 is secreted from the monocyte/macrophage, although over 80% of the IL-18 precursor remains unprocessed inside the cell. Importantly, any phenotypic characteristic of capsase-1-deficient mice should be studied to know if the deficiency is due to reduced IL-1β or IL-18 activity. For example, the caspase-1-deficient mouse is resistant to dextran sodium sulfate-induced colitis [118], but the IL-1β-deficient mouse is susceptible in the same disease model [51]. Since neutralizing antibodies to IL-18 are protective in the DSS colitis model, caspase-1 deficiency appears to prevent the processing of IL-18 [118,119] rather than IL-1β.

Similar to IL-1α and IL-33, the IL-18 precursor is constitutively expressed in endothelial cells, keratinocytes, and intestinal epithelial cells throughout the gastrointestinal tract. Macrophages and dendritic cells are the primary sources for the release of active IL-18, whereas the inactive precursor remains in the intracellular compartment of mesenchymal cells. Also, similar to IL-1α and IL-33, the IL-18 precursor is released from dying cells and processed extracellularly, most likely by neutrophil proteases such as proteinase-3 [105].

20.6.2 Signal Transduction by IL-18

IL-18 forms a signaling complex by binding to the IL-18 alpha chain (IL-18Rα, now called IL-1R5), which is the ligand-binding chain for mature IL-18; however,

this binding is of low affinity. In cells that express the coreceptor, termed IL-18 receptor beta chain (IL-18Rβ, now IL-1R7), a high-affinity complex is formed, which then signals. The complex of IL-18 with the IL-1R5 and IL-1R7 chains is similar to that formed by other members of the IL-1 family with the coreceptor IL-1R3. Following the formation of the heterodimer, TIR domains approximate and a cascade of sequential recruitment of MyD88, the four IRAKs, and TRAF-6 takes place. These steps are followed by the degradation of IκB and release of NFκB. Thus, IL-18 signaling is nearly identical to IL-1 signal transduction [120]. However, there are differences between IL-1 and IL-18 signaling. With few exceptions, IL-1α or IL-1β is active in cells in the low nanogram/milliliter range and often in the picogram/milliliter range. In contrast, IL-18 activation of cells expressing the two IL-18 receptor chains requires 10 to 20 ng/ml and sometime higher levels [121,122].

20.6.3 Role of IL-18 in the Production of IFNγ

Together with IL-12, IL-18 participates in the Th1 paradigm. This property of IL-18 is due to its ability to induce IFNγ either with IL-12 or with IL-15. Without IL-12 or IL-15, IL-18 does not induce IFNγ. IL-12 or IL-15 increases the expression of IL-18Rβ, which is essential for IL-18 signal transduction. Importantly, without IL-12 or IL-15, IL-18 plays a role in Th2 diseases [123]. The importance of IL-18 as an immunoregulatory cytokine is derived from its prominent biological property of inducing IFNγ from NK cells. Membrane IL-18 is expressed in 30–40% of M-CSF-primed macrophages, but LPS treatment is necessary for the release of membrane IL-18 [124]. A major immunoregulating role for IL-18 is in the NK cell. Several human autoimmune diseases are associated with elevated production of IFNγ and IL-18. Diseases such as systemic lupus erythematosus, rheumatoid arthritis, type 1 diabetes, Crohn's disease, psoriasis, and graft versus host disease are thought to be mediated, in part, by IL-18.

20.6.4 IL-18, IL-17, and Gamma/delta T-Cell Activation

The role for IL-17 in the pathogenesis of autoimmune diseases has not only been studied in animal models but also validated in humans treated with either neutralizing antibodies to IL-17 or the IL-17 receptor. Is there a role for IL-18 in the production of IL-17? Attention has focused on a role for IL-18 in Th17 responses primarily because both IL-1β and IL-18 are processed into active cytokines via caspase-1. Using a model for multiple sclerosis termed experimental autoimmune encephalomyelitis (EAE) [125], a role for IL-18 was studied. As expected, using the adjuvant of *Mycobacterium tuberculosis* plus the myelin-derived immunogen for EAE, bone marrow-derived mouse dendritic cells released IL-1β and IL-18, which was dependent on caspase-1 [126]. The primed dendritic cells induced IL-17 from T cells, which when transferred to nonimmunized mice resulted in encephalomyelitis. However, the disease did not develop when the dendritic cells were exposed to a caspase-1 inhibitor [126]. Treating mice with either IL-1β or IL-18 restored the ability of the T-cell transfer to induce the disease. Moreover, treating the recipient mice with the caspase-1 inhibitor

both reduced the disease and reduced the production of IL-17 from CD4-positive T cells and from gamma-delta T cells. Gamma-delta T cells produce IL-17 when stimulated with IL-18 plus IL-23, as these T cells express high levels of the IL-18 receptor alpha chain. Thus, similar to caspase-1-dependent IL-1β, IL-18 induces T cells to produce IL-17 and promote autoimmune responses to specific antigens.

20.7 IL-18 and Inflammation

20.7.1 Proinflammatory Properties of IL-18

In the absence of IL-12 or IL-15, IL-18 exhibits characteristics of other proinflammatory cytokines of the IL-1 family, such as increase in cell adhesion molecules, nitric oxide synthesis, and chemokine production. In general, inhibition of IL-18 activity results in a reduction in disease severity. Blocking IL-18 activity reduces metastasis in a mouse model of melanoma; this is due to a reduction in IL-18-induced expression of vascular call adhesion molecule-1 [127]. The induction of fever, a well-studied property of IL-1α and IL-1β as well as acute-phase proteins, TNFα, and IL-6, is not a significant property of IL-18. Injection of IL-18 into mice or rabbits does not produce fever [128,129]. In a clinical study of intravenous IL-18 dosing in patients with cancer, chills and fevers were not common and were grade 1 (low fevers). Unlike IL-1 and TNFα, fever in humans was observed in all patients at doses of 10 ng/kg, whereas IL-18 fevers were observed in 3 of the 21 patients and only at doses of 100 and 200 μg/kg [130].

Unlike IL-1 and TNFα, IL-18 does not induce cyclooxygenase-2 and hence there is no production of prostaglandin E2 [122,131]. IL-18 has been administered to humans for the treatment of cancer in order to increase the activity and expansion of cytotoxic T cells. Not unexpectedly and similar to several cytokines, the therapeutic focus on IL-18 has shifted from its use as an immune stimulant to inhibition of its activity [39,115].

Because IL-18 can increase IFNγ production, blocking IL-18 activity in autoimmune diseases is an attractive therapeutic target since anti-IL-12/23 reduces the severity of Crohn's disease as well as psoriasis. As discussed below, there appears to be a role for blocking IL-18 in Crohn's disease. However, there are several activities of IL-18 that are independent of IFNγ. For example, IL-18 inhibits proteoglycan synthesis in chondrocytes [132] and proteoglycan synthesis is essential for maintaining healthy cartilage. IL-18 also increases VCAM-1 expression in endothelial cells independent of IFNγ. VCAM-1 plays a major role in multiple sclerosis, in other autoimmune diseases, and in the metastatic process [133].

A commonly used mouse model for colitis is dextran sodium sulfate, which is added to the drinking water and which damages the intestinal wall. Thus in DSS-induced colitis, the epithelial barrier defenses against luminal bacterial products are breached. In this model, reducing IL-18 with a neutralizing antibody is protective and linked to a reduction in IFNγ [119]. Blocking IL-18 with the IL-18-binding protein (IL-18B) also reduces colitis induced by antigen sensitization [134]. Since the generation of active IL-18 requires caspase-1, studies have also been performed in mice deficient in caspase-1 and subjected to DSS colitis.

Nevertheless, despite many studies, the role of caspase-1 in DSS colitis remains unclear. The first study showed that mice deficient in caspase-1 were protected [118,135]. In addition, treatment of mice with a specific caspase-1 inhibitor was also effective in protecting against the colitis [136–138]. In both studies, the effect of caspase-1 deficiency was linked to reduced IL-18 activity, whereas reducing IL-1 activity with the IL-1Ra was ineffective [118]. In support of the role of IL-18 in DSS colitis, inhibition of endogenous merprin β to reduce the generation of active IL-18 was protective in DSS colitis [139].

However, a conundrum has developed whether caspase-1-deficiency is protective or detrimental in DSS colitis. DSS colitis is not the optimal model for Crohn's disease as the model is one of the rapid loss of the protective barrier of the intestinal epithelium exposing the lamina propria mononuclear cells to a large amount and variety of bacterial products. Using the same DSS model, mice deficient in the adapter protein inflammasome component ASC experienced increased disease, morbidity, and precancerous lesions compared to wild-type mice exposed to DSS [140]. Similarly, mice deficient in caspase-1 died rapidly from DSS compared to wild-type mice [141], whereas mice deficient in caspase-12, in which caspase-1 is enhanced, were protected [141]. Administration of exogenous IL-18 restored mucosal healing in caspase-1-deficient mice [141]. Also, mice deficient in NLRP3 were more susceptible to either DSS or TNBS-induced colitis and exhibited both decreased IL-1β and decreased beta-defensins [142]. Macrophages from NLRP3-deficient mice failed to respond to MDP [142]. Mice deficient in NLRP6 are also more vulnerable to DSS [54,143] and the susceptibility appears to be due to lack of sufficient IL-18.

20.7.2 IL-18 in Heart Disease

Heart disease includes coronary vessel disease with associated myocardial infarction, postviral myocardopathies, autoimmune heart disease, and chronic heart failure. Increasing numbers of animal and clinical studies indicate a role for IL-18 in heart disease. The myocardium of patients with ischemic heart failure express the alpha chain of the IL-18 receptor and have elevated levels of circulating IL-18 and are associated with death [144]. Daily administration of IL-18 results in ventricular hypertrophy, increased collagen [145], and elevated left ventricular diastolic pressure in mice [146]. In a model of myocardial suppression associated with septic shock, mice injected with LPS and a neutralizing antibody to murine IL-18 were protected [147]. In another study, human atrial muscle strips were obtained from patients undergoing by-pass surgery and the atrial tissue exposed to ischemia. The addition of IL-18BP to the perifusate improved contractile function from 35% of control to 76% with IL-18BP [148]. Serum from patients with heart failure intravenously administered to mice results in myocardial suppression. However, using mice deficient in IL-18 or IL-18R5 did not develop the suppression [149]. Mice pretreated with IL-18BP or caspase-1 inhibition also were protected. Although myocardial suppression in mice is induced by IL-1β, it was not expected that IL-18 was required. Thus, IL-1β induces heart failure in mice by an IL-18-dependent pathway. Since the IL-18 precursor is present in myocardiocytes and the endothelium, caspase-1 is induced by IL-1β, which

then processes the IL-18 precursor into an active cytokine. Another role for IL-18 in heart is a model of isoproterenol-induced cardiomyocyte hypertrophy. Mice transgenic for IL-18BP are protected [150].

20.8 IL-18 as a Protective Cytokine

There are a growing number of studies supporting a protective role of IL-18. Mice deficient in caspase-1 experience increased disease severity when subjected to DSS colitis and administration of exogenous IL-18 restored mucosal healing in these mice [141]. Mice deficient in NLRP3 are more susceptible to DSS colitis, which is thought to be due to decreased IL-18 [142]. Mice deficient in NLRP6 are also more vulnerable to DSS [54,143] and the susceptibility appears to be due to lack of sufficient IL-18. A protective role for IL-18 is not limited to the gastrointestinal track. In the eye, a condition resembling "wet macula degeneration" worsens with antibodies to IL-18 [151]. In a mouse model of "wet" age-related macular degeneration, the disease was worse in mice deficient in NLRP3 but not in IL-1RI-deficient mice [151]. Therefore, IL-18 rather than IL-1α or IL-1β was protective and upon administration of IL-18, the disease severity improved.

Starting at 16 weeks of age, IL-18-deficient mice begin to overeat, become obese, and exhibit lipid abnormalities; there is increased atherosclerosis, insulin resistance, and diabetes mellitus reminiscent of the metabolic syndrome [152]. IL-1R7-deficient mice also develop a similar phenotype. The higher body weight is attributed to enhanced food intake, in which the IL-18-deficient mice begin to diverge from wild-type animals at a relatively early age and to reach values 30–40% higher than that of wild-type mice. Others have observed similar findings [153]. A striking finding was an increase of more than 100% in adipose tissue in the IL-18-deficient animals that was accompanied by fat deposition in the arterial walls. The insulin resistance in these mice is corrected by exogenous recombinant IL-18. The unexpected and unique mechanism responsible for the higher food intake in the IL-18-deficient animals appears to be due a central nervous system loss of appetite control. IL-18-deficient mice eat throughout the day, whereas wild-type mice eat once, nocturnally.

20.9 IL-18-binding Protein

20.9.1 Background on IL-18BP

The discovery of the IL-18BP took place during the search for the soluble receptors for IL-18 [11]. With a clear signal peptide, IL-18BP is a constitutively secreted protein. IL-18BP with an exceptionally high affinity for IL-18 of 400 pM, although recent studies indicate an affinity of 5 pM. IL-18BP is present in the serum of healthy humans at a 20-fold molar excess compared to IL-18 [33]; thus, IL-18BP may contribute to a default mechanism by which a Th1 response to foreign organisms is blunted. Although IL-18BP is readily secreted, it falls into the functional category of being a shed soluble receptor. IL-18BP contains only one

IgG domain, whereas the IL-1 receptor-2 (IL-1R2) contains three domains. In this regard, the single IgG domain of IL-18BP is similar to IL-1R8 (SIGIRR), which also has a single IgG domain and also functions as a decoy receptor. The salient property of IL-18BP in immune responses is in downregulating Th1 responses by binding to IL-18 and thus reducing the induction of IFNγ [123]. Since IL-18 also affects Th2 responses, IL-18BP also has properties controlling a Th2 cytokine response [123].

20.9.2 The Concept of Free IL-18

Because a single IL-18BP molecule binds a single IL-18 molecule, one can calculate bound versus free IL-18 in a mixture of both molecules [33]. If one examines immunologically mediated diseases where IFNγ plays a pathological role such as Wegener's granulomatosis and systemic lupus erythematosus, one must consider the level of free IL-18 compared to IL-18 bound to IL-18BP. In fact, in these diseases both IL-18BP and IL-18 are high [34,35], but the level of IL-18BP is not high enough to neutralize IL-18 and, therefore, the level of free IL-18 is higher than in healthy subjects. In macrophage activation syndrome, where IFNγ plays a pathological role, both IL-18BP and IL-18 are also high, but the clinical and hematological abnormalities correlate with elevated free IL-18 [36].

A unique property of IL-18BP is that the molecule also binds IL-37 [154] and in doing so, enhances the ability of IL-18BP to inhibit the induction of IFNγ by IL-18. IL-37 binds to the IL-18Rα with a very low affinity, but in mice expressing human IL-37, a profound anti-inflammatory effect is observed [155], particularly of LPS-induced cytokines and dendritic cell maturation [155]. Human IL-37-expressing mice are also resistant to colitis [156]. Thus, the anti-inflammatory property of IL-37 can be affected by the concentration of IL-18BP. As the concentration of IL-18BP increases and binds IL-37, there is the possibility that IL-37 becomes less available as an anti-inflammatory cytokine. Indeed, this has been observed in mice injected with IL-18BP. At low dosing of IL-18BP, there is reduced inflammation in a model of rheumatoid arthritis, but as the dosing of IL-18BP increases, the anti-inflammatory properties of IL-18BP are lost [157].

20.9.3 Regulation of IL-18BP

IL-18BP is highly regulated at the level of gene expression and unexpectedly, IFNγ increases gene expression and synthesis of IL-18BP [158,159]. Therefore, IFNγ driving an increase in the natural and potent inhibitor of IL-18 falls into the category of a negative feedback loop. The concept is supported by clinical data showing that patients being treated with IFNα for hepatitis have elevated levels of IL-18BP [37,160]. IL-27, like IFNγ, functions as both a pro- and an anti-inflammatory cytokine and both may accomplish their roles as anti-inflammatory cytokines at the level of increased production of IL-18BP. In the skin, IL-27 also acts through a negative feedback loop for inflammation. IL-27 acts, as does IFNγ, by induction of IL-18BP gene expression and synthesis [161].

20.9.4 Viral IL-18BP

Natural neutralization of human IL-18 by IL-18BP takes place during a common viral infection. In *Molluscum contagiosum* infection, characterized by raised but bland eruptions, there are large numbers of viral particles in the epithelial cells of the skin, but histologically there are few inflammatory or immunologically active cells in or near the lesions. Clearly, the virus fails to elicit an inflammatory or immunological response. Amino acid similarity exists between human IL-18BP and a gene found in various members of the poxviruses; the greatest degree of homology is found to be expressed by *M. contagiosum* gene [162]. The ability of viral IL-18BP to reduce the activity of mammalian IL-18 likely explains the lack of inflammatory and immune cells in the virally infected tissues and provides further evidence for the natural ability of IL-18BP to interfere with IL-18 activity.

20.9.5 Hemophagocytic Lymphohistiocytosis and Macrophage Activation Syndrome

These are life-threatening conditions characterized by a severe hyperinflammatory state. There is a rare genetic form of hemophagocytic lymphohistiocytosis. However, macrophage activation syndrome (MAS) is not a hereditary disease and can be secondary to infections and lymphoma. Development of MAS is associated with several infectious diseases, notably due to Epstein–Barr virus, cytomegalovirus, herpes virus, or intracellular bacteria and parasites and also various lymphomas, especially of T-cell lineage. In addition, patients with rheumatological conditions, particularly systemic onset juvenile arthritis (sJIA), and also systemic lupus erythematosus, Kawasaki disease, or systemic vasculitis can develop MAS [163–166]. One of the most prominent hematologic and metabolic characteristics of MAS is thrombocytopenia and hepatic injury, respectively. Indeed, IFNγ may be responsible for the thrombocytopenia as well as several of the immunological abnormalities of the disorder. Although treatment with anti-IL-1β or anakinra is the treatment of choice for MAS, the mechanism of resolution of the hyperinflammatory state may be the reduction in IL-18. For example, some patients enrolled in anakinra trials for the treatment of septic shock have MAS. In those patients treated with anakinra, all-cause 28-day survival was 65% compared to patients treated with placebo ($p = 0.007$) [167].

20.9.6 IL-18 in the Hemophagocytic Syndromes

In the case of familial hemophagocytic lymphohistiocytosis or MAS, gene expression for IL-18 is upregulated in peripheral mononuclear cells [168,169] and serum IL-18 is unusually elevated [36,170–173]. Although levels of IL-18 in the circulation are below 1 ng/ml in inflammatory diseases such as severe sepsis, in active phase of MAS, serum IL-18 is usually in the range of 5–7 ng/ml, and both in familial hemophagocytic lymphohistiocytosis complicating XIAP gene mutations and in MAS complicating sJIA, levels of circulating IL-18 can be in 20–30 ng/ml range [24,36,174,175]. However, it is necessary to calculate the

level of free IL-18 since IL-18BP is present in the circulation in health and disease [33]. In patients with MAS, free IL-18 but not IL-12 concentrations significantly correlated with clinical status and the biologic markers of MAS such as anemia ($p < 0.001$), hypertriglyceridemia, and hyperferritinemia ($p < 0.01$) and also with markers of Th1 lymphocyte or macrophage activation, such as elevated concentrations of IFNγ and soluble IL-2 and TNFα receptor concentrations [36]. Patients with a mutation in NLRC4 experience a life-threatening hyperinflammation state, mostly manifested by intractable colitis. Free IL-18 is markedly elevated and treatment with IL-18BP provides resolution of the inflammatory state [176].

20.10 IL-33

20.10.1 The Discovery of IL-33

For many years, the IL-1 receptor family member ST2 (proper nomenclature IL-1R4) was studied without knowing its specific ligand. Because IL-1R4 contains the TIR domain, the biology of the orphan ligand (IL-33) was linked to innate immune responses, inflammation, and acquired immunity [177]. For many years, the role of this receptor was established for promoting the Th2 response in diseases such as asthma and parasitic infections. For example, IL-1R4 was required for the production of IL-4, IL-5, and IL-13. Mice deficient in IL-1R4 exhibited reduced allergic responses. Blocking antibodies targeting, this receptor generated similar data, as did the use of the extracellular domain (soluble IL-1R4) and mice deficient in IL-1R4. IL-1R4 was also reported to function in the suppression of inflammation [178]. However, in 2005, using *in silico* research methods, the IL-1 family member IL-33 was identified as the ligand binding to IL-1R4 [179]. Considerable uncertainly surrounded this discovery since other members of the IL-1 family discovered with *in silico* research methods were reported in 2000. Similar to IL-1α and IL-18, IL-33 is present in its precursor form in healthy tissues, particularly in endothelial and epithelial cells and also the brain. In fact, in the brain, the IL-33 precursor is found in microglia, astrocytes, and oligodendroglia.

20.10.2 Processing of IL-33

The IL-33 precursor contains a caspase-1 cleavage site at amino acid aspartic acid 111 and this site was thought to provide a mechanism similar to that of IL-1β and IL-18, that is, processing intracellularly by caspase-1 and secretion of the active cytokine. Indeed, the caspase-1 site was used to produce the first recombinant IL-33 with amino acids 112–270 [179]. However, subsequent studies revealed that caspase-1 and caspase-3 inactivate cytokine [180]. The full-length IL-33 precursor (amino acids 1–270) is active and processing takes place extracellularly by enzymes such as neutrophil elastase and cathepsins [181]. Processing by these and mast cell enzymes results in significantly greater activity of IL-33 [181]. The release of the IL-33 precursor from endothelial cells takes place with damage or

frank necrosis, similar to that of IL-1α [53]. Thus, IL-33 is considered an alarmin; that is, it is released upon cell damage and is active as a precursor. Depending on the neutrophilic infiltration, more active forms of IL-33 are generated. Although the crystal structure of IL-33 with IL-1R4 has been reported, the various N-termini generated extracellularly suggest that signal transduction is triggered differentially depending on the various affinities. The processing of the IL-33 precursor extracellularly generates mature forms, which are more active than the parent precursor [181]. In fact, processing takes place by the same neutrophil and mast cells proteases that process IL-1β [181,182]. There are at least three mature forms of IL-33 resulting from mast cell proteases that are significantly more potent than the precursor and each activates group 2 innate lymphoid cells [182]. It is unclear which N-terminus of IL-33 is present in inflammatory tissues, given the multiple neutrophilic enzymes that cleave the IL-33 precursor.

20.10.3 IL-33 Shares with IL-1 the Coreceptor IL-1R3

IL-33 binds to IL-1R4, which then undergoes a conoirmational change, similar to IL-1β or IL-1α binding to IL-1R1. The affinity of IL-33 for IL-1R4 has a K_d of 74 nM [183]. In the case of IL-33 binding to IL-1R4, there is no signal transduction. However, the conformational changes in IL-1R4 allows for binding to IL-1R3 and the formation of the heterotrimer complex [184], which signals via MyD88, IRAKs, and NFκB. IL-1R3 binds to the side of IL-1R4 [183], again similar to that of IL-1R1 [185]. In this model, there is no contact of IL-1R3 with IL-33.

IL-33 as an anti-inflammatory cytokine. Due to its structure, IL-33 belongs to the IL-1 subfamily with IL-1α and IL-1β. Sharing common coreceptor IL-1R3, IL-33 biological properties are similar to those of IL-1α and IL-1β, that is, proinflammatory. The ST2 tissue and cell distribution determines the Th2 paradigm for IL-33, but IL-1β also falls into the class of Th2, particularly because IL-1 increases antibody production due to its properties as an adjuvant. Although IL-33 is primarily a proinflammatory cytokine in several murine models of human disease, IL-33 has anti-inflammatory properties. For example, IL-33 exerts anti-inflammatory properties in a model of heart disease, obesity, and uveitis. It is unclear if these anti-inflammatory properties of IL-33 are due to a shift to the Th2 paradigm or an independent mechanism. One mechanism for the anti-inflammatory properties of IL-33 appears to be due to the requirement for IL-1R8 [186]. According to a press release, a human trial of anti-IL-33 has shown some efficacy in patients with asthma.

20.10.4 Nuclear Function of IL-33

The nuclear translocation of the N-terminal domain of IL-33 is similar to that of IL-1α. While many researchers focused on recombinant IL-33 and the allergic models, the French scientist Girad and coworkers reported that IL-33 translocated to the nucleus where it bound to chromatin, an observation that had been published earlier in endothelial cells [187]. As described above, the N-terminal domain of IL-1α binds to chromatin via its nuclear localization sequence and acts as a transcription factor [48]; during apoptosis, nuclear IL-1α is sequestered in the

nucleus and not available for binding to its extracellular receptor [50]. A similar mechanism seems to take place with IL-33 as there is suppression of transcription [187]. Further studies on the nuclear function of IL-33 revealed that the full-length IL-33 precursor binds to NFκB specifically via amino acids 66–109 and suppresses activation by IL-1β [188]. Thus, both IL-1α and IL-33 binding to chromatin acts to sequester cytokine and reduce inflammation. A dramatic proof of this mechanism was revealed when the nuclear localization of IL-33 was deleted and the mutated IL-33 expressed in mice. In heterozygous mice expressing IL-33 with the nuclear localization sequence and IL-33 without nuclear localization, overwhelming lethal inflammation was reported [49]. The inflammation consisted of elevated IL-33 plasma levels and massive infiltration of eosinophils in the organs. The inflammation was absent in mice deficient in IL-1R4 [49]. Thus, similar to nuclear IL-1α, chromatin binding of IL-33 acts as a sink to prevent inflammation.

20.10.5 Role of IL-33 in Human Disease

Unlike IL-1α, IL-1β, and IL-18, humans have not received IL-33. Furthermore, unlike IL-1α, IL-1β or IL-18, there are no reports of blocking therapies at the time of this writing that target IL-33, such as neutralizing IL-33 antibodies, blocking antibodies to IL-1R4, blocking antibodies to IL-1R3, or soluble forms of IL-1R4. However, animal studies are numerous and suggest that IL-33 is indeed a target to combat allergic diseases such as asthma. The objective of IL-33 blocking therapies is to reduce Th2 cytokines such IL-4, IL-5, or IL-13. Since IL-33 is upstream of these Th2 cytokines, IL-33 blocking therapies have a rationale basis. In addition to a role for IL-33 in the Th2 paradigm, there are also studies revealing a role for IL-33 in classic Th1 diseases such as inflammatory bowel disease [189] and rheumatoid arthritis [190,191]. IL-33 also increases the production of IFNγ [192].

20.11 IL-36

20.11.1 IL-36 Background

There are four ligands that bind to the IL-36 receptor, IL-36α, IL-36β, IL-36γ, and the IL-36 receptor antagonist, each was reported in 2000–2001 by *in silico* research [193–195]. The IL-36 receptor was previously known as IL-1 receptor-related protein 2 [195]. IL-36 is part of the IL-1 gene cluster located on chromosome 2q13 in a stretch of 360 kb with IL-1 and IL-37 [196].

20.11.2 Role of IL-36 in Disease

A great deal of attention focused on IL-36 when it was reported that a mutation in IL-36Ra resulted in pustular psoriasis [197–199]. Indeed, IL-36 is expressed primarily in the skin.

20.12 IL-37

20.12.1 Background of IL-37

IL-37 was discovered in 2000 using *in silico* research [193]. The earlier name for IL-37 was IL-1 family member 7 (IL-1F7); there are four isoforms due to exon deletions and il37b is the most complete form. IL-37 is unique in the IL-1 family in that unlike other members of the family, IL-37 broadly suppresses not only innate but also acquired immunity. Another distinguishing characterization of IL-37 is that there is no homologue for IL-37 both in mouse and in chimpanzee. IL-37 is often elevated in humans with inflammatory and autoimmune diseases where it likely functions to limit inflammation. But in some diseases, for example, psoriasis, asthma, and calcific aortic valve disease [200], the levels of IL-37 are lower or it is absent, suggesting a relative deficient state. Transgenic mice expressing human IL-37 (*IL37*-tg) exhibit less severe inflammation in models of endotoxin shock, colitis, myocardial infarction, and lung and spinal cord injury, as reviewed in Ref. [201]. *IL37*-tg mice have reduced antigen-specific responses and dendritic cells from these mice exhibit characteristics of tolerogenic dendritic cells [202].

20.12.2 The IL-37 Signaling Complex: Requirement for IL-1R5 and IL-1R8

IL-18 binds to IL-1R5 and recruits IL-1R7. The crystallization of IL-18 allows for binding to IL-1R5 and the conformational changes that allow for the binding of IL-1R7 [203]. The TIR domains of each chain approximate, the adapter molecule myeloid differentiation factor 88 (MyD88) binds to the TIR domains, and a strong proinflammatory signal is initiated. IL-37 also binds to IL-1R5 [154,204,205]; specifically, IL-37 binds to the third domain of IL-1R5 [154,203]. However, IL-1R8 does not recruit IL-1R7, but rather IL-1R8. The TIR domain of IL-1R8 is mutated (termed TIRb) and MyD88 binding to TIRb results in a reduced signal to the nucleus [10,206]. IL-1R8 overexpression decreases the expression of MyD88, IL-1 receptor-associated kinase 4 (IRAK4) and tumor necrosis factor receptor-associated factor 6 (TRAF6) [207]. IL-1R8 functions as a sink for MyD88, depriving MyD88 from binding to native TIR domain, resulting in a weak or no signal. There is a similar sequestration of MyD88 by IL-1R8 for TLR signaling [208]. Regulation of IL-1R8 is relevant for the effects of IL-37. Evidence reveals that expression of IL-1R8 decreases with age in rats, but when rats were subjected to a calorie-restricted diet, expression of IL-1R8 increased [207]. In that study, caloric restriction resulted in decreased NFκB expression.

Although IL-1R5 is expressed on most cells, not all cells are responsive to IL-18. IL-1R5 responsiveness requires IL-1R7, as reviewed in Ref. [209]. IL-1R8 is also expressed by all cells and functions to inhibit innate and acquired immunity [9,210]. Increased expression of IL-1R8 in intestinal epithelial cells is protective in models of colitis [211]. Until recently, no ligand had been identified that binds to surface IL-1R8; nevertheless, the single immunoglobulin

extracellular domain of IL-1R8 is required for its function [208] suggesting an interaction with a ligand. In mice deficient for IL-1R8, there is heightened inflammation in several diverse models, as reviewed in Ref. [10,210,212]. For example, colitis is worse in mice deficient in IL-1R8 [213].

20.12.3 Binding Studies

Early studies indicated that IL-37 binds to IL-1R5 [204,205]. In fact, cells from IL-1R5-deficient mice or WT cells treated with a blocking antibody to IL-1R5 reveal a hyperresponsive state [214]. The anti-inflammatory properties of IL-37 is not observed in cells with a knockdown of IL-1R5 [215] supporting the concept that IL-37 binds to IL-1R5. Specific binding of IL-37 to IL-1R5 has been demonstrated using immunofluorescence, immunoprecipitation, foerster resonance energy transfer (FRET), and proximity ligation assay (PLA) in primary cells expressing constitutive levels of the receptor [215]. In addition to these methods, bioluminescence resonance energy transfer (BRET) and ground-state depletion superresolution microscopy were used to show the formation of the tripartite complex of IL-37–IL-1R5–IL-1R8 [215].

20.12.4 The IL-37, IL-18Rα, and IL-1R8 Complex

Primary macrophages and DCs from IL-1R8-deficient mice do not exhibit anti-inflammatory properties [215,216]. Most impressive are the *in vivo* responses in *IL37*-tg mice deficient in IL-1R8. As summarized above, *IL37*-tg mice are protected from systemic endotoxemia, but in *IL37*-tg mice deficient in IL-1R8, there is a near total loss of this protection [215]. Moreover, cells from *IL37*-tg mice deficient in IL-1R8 lack the suppression of cytokines, transcription factors, and signaling kinases, including suppression of mTOR and the increase in AMPK [215], lending more evidence of the requirement for a complex of IL-37, IL-1R5, and IL-1R8 for anti-inflammatory activity. However, not all changes in IL-37-mediated signaling kinases are IL-1R8 dependent [215]. One explanation for separation of IL-1R8-dependent from -independent functions may be due to the nature of the TIRb domain, which can transmit a "weak" signal, as was shown for another IL-1 receptor chain containing TIRb (IL-1R3b) [217].

In addition to the failure of *IL37*-tg deficient in IL-1R8 to protect against endotoxemia [215], the protection afforded by recombinant IL-37 in inflammatory aspergillosis was also not observed in mice deficient in IL-1R8 [218]. Other studies reveal that the beneficial effects of recombinant IL-37 in WT mice are not observed in mice deficient in IL-1R8. *In vitro*, the reduction in LPS-induced TNFα and IL-6 by recombinant IL-37 in DCs from WT mice was not observed in DCs from IL-1R8-deficient mice [219]. In IL-37-transfected macrophages, an 83% reduction in IL-1β production was observed compared to WT macrophages, but only a 34% reduction was observed when endogenous IL-1R8 was silenced [215]. A similar attenuation of the IL-37 effect was observed in PBMCs by the silencing of IL-18Rα [215]. In summary, IL-1R8 functions as a coreceptor for IL-18Rα and IL-37 is transiently associated with IL-1R8 and IL-18Rα on the cell surface upon stimulation with IL-1β or LPS [215]. This interaction is needed for intracellular

pathways to block inflammation, including not only the anticipated inhibition of MAPKs and NFκB but also the unexpected pseudostarvational effects on the mTOR pathway, inhibition of TAK1 and Fyn, and activation of the anti-inflammatory STAT3, Mer, and PTEN.

20.12.5 Production of IL-37

Using immunohistochemistry, the IL-37 protein is expressed by human blood monocytes, tissue macrophages, synovial cells, tonsillar B cells, plasma cells, T cells, neoplastic cells, and epithelial cells of the skin, kidney, and intestine. IL-37 is not constitutively expressed by blood monocytes from healthy subjects but rather requires stimulation by IL-1β, TLR agonists, or TGF-β [220]; in human keratinocytes, beta-defensin-3 induces IL-37 [221]. In the human lung type II epithelial cell, lipoarabinomannan from *Mycobacterium tuberculosis* induces IL-37 production via upregulating ERK1/2 and p38 [222]. The abundance of IL-37 transcripts is low in human blood monocytes and dendritic cells due to an instability sequence in *IL37*. This instability sequence limits mRNA half-life of IL-37 [80]. Indeed, despite having a CMV promoter, resting mRNA level for IL-37 in *IL37*-tg mice is either absent or low but rapidly increases with inflammation [156,220,223,224]. Even in stimulated human blood monocytes, the level of IL-37 protein is low at 10–20 pg/million cells.

Despite low production, recombinant IL-37 is highly active. Studies have employed picomolar concentrations of recombinant IL-37 to reduce LPS-induced IL-1β, IL-6, and TNF production *in vitro* [219]. IL-37 forms dimers [204], which are biologically inactive. Recombinant forms of IL-37 contain as much as 80% dimer and therefore to trigger a signal, the preparation is diluted in order to reduce the concentration of the dimer, which results in monomeric IL-37. With crystallization of IL-37 [225], two amino acids have been identified that participate in dimer formation: a tyrosine at position 85 and a lysine at position 73.

20.12.6 Processing and Release

Similar to other members of the IL-1 family, the IL-37 precursor contains no classical signal peptide. The precursor can be observed by western blotting of endotoxin-stimulated human blood monocytes [223]. IL-37 in the extracellular space likely reflects an unorthodox mechanism to exit the cell, which is unrelated to cell death. Nevertheless, there is a caspase-1 cleavage site at aspartic acid (D20) in exon 1 and therefore is present in isoforms IL-37b, c, d, and e. In LPS-stimulated human blood monocytes, the addition of exogenous ATP results in a modest release of processed IL-37 [223], but most of the secreted IL-37 is in the precursor form. Also, similar to IL-1α and IL-33, both precursor and processed forms of IL-37 are active [204,219,224].

A form of recombinant IL-37b with the N-terminus at valine 46 [205] was apparently processed naturally during the expression in mammalian cell. A recombinant form of IL-37 from *E. coli* has been generated (V46–218) and is 20–30-fold more active than the precursor form [219]. It remains unclear what enzyme(s) cleaved the N-terminal 45 amino acids, but extracellular proteases

from neutrophils likely generate more than one N-terminus. The high activity of IL-37b (V46–218) is likely due to its proximity to the IL-1 family AXD consensus sequence [13]. In the consensus sequence, A is any aliphatic amino acid, X is any amino acid, and D is aspartic acid. For optimal folding of the beta-fold barrel structure for receptor binding of IL-1 family cytokines, the N-terminus is nine amino acids forward from AXD towards the N-terminus.

20.12.7 Effects of Recombinant IL-37

The administration of 1 μg or less of recombinant IL-37 to WT mice reduces circulating levels of IL-1β, TNF-α, and IL-6 [219], neutrophil infiltration into the lungs [218], myocardial monocyte infiltration following infarction [226], and airway hyperresponsiveness following antigen challenge [227].

Recombinant forms of IL-37 have been shown to be active *in vivo* in WT mice in various models of inflammation. In addition, IL-37-expressing plasmids have been administered to WT mice. Because the N-terminus for endogenous IL-37 may vary, different forms of recombinant IL-37 have been tested in WT mice; the full-length IL-37b precursor (1–218) [218,219] is active *in vivo* and *in vitro*. *In vitro*, recombinant full-length IL-37b (1–218) reduced LPS-induced IL-1β, IL-6, and TNFα in human blood M1 differentiated macrophages associated with a decrease in ERK and p38 MAP kinase [219]. A dose of 1 μg or less of the IL-37 precursor protected mice against acute lung injury following intracellular-tracheal instillation of *Apergillus fumigatus* [218]. The numbers of infiltrating neutrophils were reduced as were gene expression for IL-1β, NLRP3, caspase-1, and several chemokines, whereas IL-10 increased [218]. However, recombinant IL-37b with the N-terminus at valine 46 (46–218) [205] is more active than the IL-37b precursor [219,228]. WT mice subjected to systemic endotoxemia or acute myocardial infarction are similarly protected against inflammation-induced damage [219,226]. In an asthma model of ovalbumin-sensitized mice, a low dose of recombinant IL-37 protected against antigen-challenge airway hyper-responsiveness [227]. Recombinant IL-37a with the N-terminus at lysine 27 is also active [229]. The N-terminus of this form is nine amino acids forward from the IHD consensus sequence.

20.13 Disease Models in *IL37*-tg Mice

20.13.1 IL37-tg Mice

IL37-tg mice were generated using the full-length precursor cDNA of *IL37b* isoform driven by the CMV promoter for constitutive expression [220], as this promoter is commonly used to drive expression in most cells. *IL37*-tg mice breed normally and have no obvious phenotype. However, compared to WT mice, *IL37*-tg mice are protected against LPS challenge, exhibiting significantly less hypothermia, acidosis, hyperkalemia, hepatitis, and dehydration in addition to lower levels of cytokines [220]. *IL37*-tg mice are also protected against spinal cord injury [224] as well as in other models.

20.13.2 Experimental Colitis

In *IL37*-tg mice subjected to dextran sodium sulfate-induced colitis, clinical disease scores and histological indices of colitis were significantly reduced [156]. These reductions were associated with decreased leukocyte recruitment into the colonic lamina propria. Release of IL-1β and TNFα from colonic tissue explants incubated *ex vivo* was decreased 5- and 13-fold, respectively, whereas IL-10 was increased sixfold. Since an IL-10 receptor blocking antibody did not reverse the anti-inflammatory effects on the *IL37*-tg mice, the response in these mice is independent of IL-10. Bone marrow transferred from *IL37*-tg mice into WT mice significantly reduced the severity of the colitis, suggesting that myeloid cells expressing IL-37 provided the protection [156]. Another study showed that mesenchymal stromal cell (MSC) transfected with *IL37* afforded the same level of protection as observed in *IL37*-tg mice subjected to DSS colitis [230]. MSCs without gene expression of IL-37 were not protected [230]. Also, increased myeloid-derived suppressor and T-regulatory cells were observed in mice treated with *IL37* MSC [230], whereas decreased CD4 positive cells expressing IFNγ were present [230].

20.13.3 Ischemic and Damage Models

IL37-tg mice subjected to coronary artery ligation have markedly reduced infarct size and improved left ventricular function compared to similarly challenged WT mice [231]. Reduced accumulation of myocardial mononuclear cells was observed in the *IL-37*-tg mice [231]. Cardiac vascular endothelial cells isolated from *IL37*-tg mice had reduced NFκB activation when stimulated with TLR2 and TLR4 agonists compared to endothelial cells from WT mice [231]. Hydrodynamic injection of *IL-37* in WT mice was used in a mouse model of acute renal ischemia; kidney function was preserved, mononuclear cell infiltration was reduced, and inflammatory cytokine levels were lower compared to mice injected with the control plasmid [232].

20.13.4 Spinal Cord Injury

Traumatic injuries to the spinal cord of humans following accidents leave the subjects with various degrees of lower limb paralysis. In mouse, a localized spinal cord contusion results in reduced motor function and spinal cord tissue damage. *IL-37*-tg mice were protected against the injury and exhibited greater mobility compared to WT subjected to the same injury [224]. The clinical improvement was associated with decreased infiltration of neutrophils and monocytes, fewer numbers of microglia exhibiting activation markers and reduced levels of proinflammatory cytokines [224].

20.13.5 Metabolic Syndrome

Complications of obesity, such as metabolic syndrome and type 2 diabetes, have been studied in the context of IL-18 and IL-1β and, particularly, the role of

caspase-1 [233,234]. *IL37*-tg mice fed a high-fat diet for 16 weeks had reduced numbers of adipose tissue macrophages, less lipid accumulation in the liver, improved response to insulin, and increased glucose tolerance compared to WT mice on the same diet [235]; moreover, the transgenic mice gained less weight than WT animals despite equal food and caloric intake [235]. The adipokine adiponectin is an anti-inflammatory cytokine produced by fat cells. Serum levels of adiponectin in *IL37*-tg mice were markedly higher during the high-fat diet. The anti-inflammatory properties of adiponectin include improved insulin sensitivity, although the mechanism for this property remains unknown.

In 80 human subjects, steady-state IL-37 mRNA levels in subcutaneous adipose tissue were positively associated with appropriate insulin responses, lower adipose tissue leptin, and lower inflammatory status of the adipose tissue [235]. Thus, increased levels of IL-37 in humans seem to buffer the inflammation associated with obesity and raise the possibility of recombinant IL-37 to treat metabolic syndrome in humans. Relevant to a therapeutic use of IL-37, WT mice were subjected to 20 weeks of high-fat diet and then were treated with recombinant IL-37 during the last 2 weeks of the diet. Compared to WT treated with vehicle, insulin sensitivity and glucose disposal increased significantly, secretion of IL-1β from adipose tissue decreased, and plasma levels of IL-1Ra increased by 75% in mice treated with recombinant IL-37 [228].

20.13.6 Aging in IL37-tg Mice

Increased inflammation is a characteristic of aging. The incidence of B-cell cancers such as lymphoma is higher in the elderly. Bone marrow B-cell progenitors in 20-month-old mice exhibited multiple defects in signaling, gene expression, and metabolism compared to 20-month old WT mice. Old *IL37*-tg mice preserved the function of B-cell progenitors and did not develop virus-induced leukemia [236]. The study concluded that chronic inflammatory microenvironments in old age leads to a lower resistance of B-cell bone marrow progenitor populations to oncogenesis [236]. IL-37 seems to provide an anti-inflammatory microenvironment in the aging bone marrow.

20.13.7 Translocation of IL-37 to the Nucleus

In addition to forming cell surface receptor complexes, three members of the IL-1 family, IL-1α, IL-33, and IL-37, translocate to the nucleus where they bind to nuclear DNA and participate in transcription. IL-37 translocates to the nucleus [223,237] and, in this regard, is termed a "dual-function" cytokine, similar to IL-1α [50] and IL-33 [187]. After LPS stimulation of mouse macrophage cell lines, there is a lag phase of 15 h before IL-37 is detected in nuclei [238] and after 18 h, levels of TNFα, IL-1α, IL-6, and chemokines are substantially reduced (72–98%) compared to cells transfected with an empty plasmid [220,237]. The nuclear translocation of IL-37 requires active caspase-1; inhibition of caspase-1 in LPS-stimulated mouse macrophages not only prevents nuclear translocation but also prevents IL-37 from suppressing LPS-induced IL-6 [237]. The caspase-1 cleavage site at aspartic acid 20 (D20) in exon 1 of IL-37b was mutated to alanine

and thereby prevents the cleavage of IL-37 by caspase-1 but without inhibition of caspase-1 itself in the cell. Cells expressing the mutant IL-37D20A not only failed to translocate in the nucleus [223] but also failed to suppress LPS-induced IL-6 [223]. Together, these data reveal an unexpected role of caspase-1 in enabling the nuclear translocation of IL-37 and subsequently suppression of inflammation. As of this writing, it remains unknown how much of the suppression of inflammation or acquired immunity in *IL37*-tg mice is due to nuclear IL-37 or extracellular IL-37.

20.13.8 Requirement for Smad3

Smad3 is a kinase downstream of the TGF-β receptor; Smad3 is associated with the immunosuppressive and anti-inflammatory properties of TGF-β. A proteomics-based search for intracellular proteins that interact with Smad3 identified IL-37 [239]. IL-37 immunoprecipitates with phosphorylated Smad3 and the IL-37–Smad3 complex has been observed in the perinuclear space [220]. Also, a specific inhibitor of Smad3 reversed IL-37-mediated inhibition of IL-6 induced by IL-1β [220]. Depletion of Smad3 by lentiviral shRNA or siRNA also prevented the ability of IL-37 to suppress inflammation both *in vitro* and *in vivo* [220]. As intracellular levels of the IL-37 precursor increase, LPS activation of caspase-1 cleaves IL-37 at D20. One working hypothesis is that the carboxyl domain of IL-37 binds to Smad3 and becomes phosphorylated. It is proposed that this complex then migrates to the nucleus and suppresses gene expression. Unlike IL-1α and IL-33, IL-37 does not contain a classic nuclear localization sequence and, therefore, it is likely that Smad3 transports IL-37 to the nucleus.

The IL-37 precursor exits in the cell by the loss of membrane integrity, by frank cell death, or by an unknown mechanism. Although the precursor is active *in vivo* and *in vitro*, whether there is subsequent processing by extracellular enzymes to a "mature" form is unknown. Nevertheless, from studies with recombinant IL-37, we know that the most active form of IL-37b *in vivo* and *in vitro* is a processed form (46–218) [205,219,224]. Binding to the IL-18Rα has been reported [215,219]. The complex of IL-18Rα and IL-1R8 then signals the cell to initiate a program to reduce inflammation.

20.13.9 A Role for IL-37 in Human Disease

Increased levels of IL-37 have been reported in several human disease conditions by measuring mRNA levels in cells derived from subjects affected with a specific disease as well as the concentrations of IL-37 in the circulation. In addition, immunohistochemistry of tissues and production from cells *in vitro* are also reported. For example, IL-37 is present in colonic tissues from patients with inflammatory bowel disease, but it is absent in biopsies from healthy subjects [240,241]. IL-37 is elevated in patients with rheumatoid arthritis [242–245], but patients with a coding region mutation in IL-37 had lower joint disease scores and less pain compared to patients without the mutation, suggesting a gain of function mutation for the anti-inflammatory properties of IL-37 [246]. In PBMC from HIV-1-infected subjects, steady-state mRNA levels are fourfold higher

compared to noninfected subjects and correlate with the levels of soluble CD14 [247]. These and similar studies support the concept that infection or inflammation increases IL-37 expression, which is an appropriate response to limit disease severity. Anti-inflammatory mediators such as IL-1 receptor antagonist, soluble TNFα receptors, and the IL-18-binding protein are also elevated in several of the diseases.

In contrast, in some diseases, the opposite is observed, that is, there is a lower level of IL-37 compared to the level in healthy subjects. A low level of IL-37 likely contributes to increased disease severity. Patients with a wide range of inflammatory diseases and low levels of IL-37 may reflect a failure to have sufficient levels to combat inflammation. In obesity, increased levels of IL-37 in human adipose tissue are associated with less insulin resistance [235] and therefore are consistent with a functional role of IL-37 in limiting inflammation. In HIV-1, the elevated levels of IL-37 can be interpreted in two ways: the elevation is an appropriate response to limit the inflammation of a chronic infection or the elevation contributes to the immunosuppression of HIV-1 [247].

Missense coding mutations in cytokines such as IL-1β, TNFα, and IL-6 are rare compared to coding mutations in receptors, intracellular proteins, and kinases. IL-37 may be an exception since there are missense sssociated with disease. For example, SNP rs3811047 (A–G in exon 2) results in threonine to alanine change. This SNP is associated with less severe disease in patients with rheumatoid arthritis [246,248]. In the case of humans with IL-37 haplotyope2, decreased levels of IL-37 protein may contribute to more pronounced inflammation [249]. Africans have the highest incidence of haplotype2 (17%), whereas, globally, the incidence is 8% [249].

20.14 IL-38

20.14.1 The Functional Role of IL-1 Family Member 10, Recently Renamed IL-38

IL-38 also was discovered by *in silico* research from the large data bases of the human genome. IL-38 was an orphan cytokine in the IL-1 family until the discovery that recombinant human IL-38 bound to the IL-36 receptor [250]. Interest in IL-38 is derived from the finding that in a large genome-wide association analysis study in over 66 000 subjects with elevated C-reactive protein looking for a missense mutations reported IL-38 was one of the top risk factors [251]. IL-38 belongs to the IL-36 subfamily. Using killed *Candida albicans* yeast cells, recombinant human IL-38-stimulated PBMC released IL-17 and IL-22. The reduction in IL-22 and IL-17 caused by IL-38 was similar to that caused by the naturally occurringIL-36Ra in the same peripheral blood mononuclear cells cultures [250]. When human blood monocyte-derived dendritic cells were used, both IL-38 and IL-36Ra increased LPS-induced IL-6 by twofold. IL-38 bound only to the IL-36 receptor, as did IL-36Ra. The dose-response suppression of IL-38 and that of IL-36Ra of Candida-induced IL-22 and IL-17 was not that of the classic IL-1 receptor antagonist (anakinra), because low concentrations were optimal for

inhibiting IL-22 production, whereas higher concentrations modestly increased IL-22. These data provide evidence that IL-38 binds to the IL-36R, as does IL-36Ra, and that IL-38 and IL-36Ra have similar biological effects on immune

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21

TNF Superfamily

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21.1 Introduction

Tumor necrosis factor superfamily (TNFSF) is a group of cytokines composed of 19 ligands and 29 receptors [1]. This family plays a pivotal role in immunity, in inflammation, and in controlling cell cycle (proliferation, differentiation, and apoptosis). TNFSF receptors can be divided into two different groups depending on the presence or absence of the intracellular death domain (DD). Signaling via the death domain demands the involvement of adapter proteins FADD (Fas-associated death domain) and TRADD (TNF receptor-associated death domain) and leads to activation of caspases that results in apoptotic death of a cell [2]. The second group of TNFSF receptor signals act only via adapter proteins termed TRAFs (tumor necrosis factor receptor-associated proteins), although DD containing receptors can also use this pathway. TRAFs bind either to TRADD or directly to the cytoplasmic part of the receptor and initiate signal transduction pathways that also lead to the activation of several transcription factors, such as AP-1 and NF-*k*B that are responsible for the activation of prosurvival genes, even though they are implicated in proapoptotic signaling [2]. In this context, functional activity of TNFSF receptors depends on the cellular context and the balance between pro- and antiapoptotic factors inside the cell and in the environment. Mostly, the TNFSF members are revealed on cells of the immune system and play a notable function in maintaining the equilibrium of T-cell-mediated immune responses by arranging direct signals required for the full activation of effector and regulatory T cells, regulation of their expansion, contraction of the T-cell effector pool, and survival of memory T cells [3]; for this reason, the TNFSF members are necessary in the development of pathogenesis of many T-cell-mediated autoimmune diseases, in particular asthma, diabetes, and arthritis [4].

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21.2 TNFα

Tumor necrosis factor alpha, also called TNFα or TNFSF2, is classified as homotrimeric transmembrane protein with a preminent role in systemic inflammation. TNFα is expressed as a membrane-bound precursor (tmTNFα), which is later cleaved between Ala^{76} –Val^{77} by a metalloproteinase known as TNFα-converting enzyme (TACE), and called soluble TNFα (sTNFα). Several cell types are able to produce TNFα, including activated macrophages, dendritic cells, monocytes, NK cells, $CD4^+$ T cells, $CD8^+$ T cells, microglia, and astrocytes.

Macrophages/monocytes are able to produce TNFα in the acute phase of inflammation and this cytokine drives a wide range of signaling events within cells, leading to necrosis or apoptosis. TNFα drills its effects by binding, as a trimer, to either a 55 kDa cell membrane receptor defined as TNFR-1 or a 75 kDa cell membrane receptor known as TNFR-2. The TNF superfamily incorporates RANK, CD40, CD27, and FAS. The defining trait of these receptors is represented by an extracellular domain made of two–six repeats of cysteine-rich motifs. Moreover, a number of structurally related "decoy receptors" subsist that act to sequester TNF molecules, thereby rescuing cells from apoptosis. For the first time in 1975, this protein was discoverd in the circulation of animals subsequent to stimulation of their reticuloendothelial system and lipopolysaccharide challenge [5]. This protein has been found to provoke a rapid necrotic regression of certain forms of tumors. This discriminatory TNFα-mediated cytotoxicity may be correlated to the tumor cell's ability to undergo nitrotyrosinylated tubulin-mediated microtubule dysfunction [6]. TNFα and TNFβ structures were defined and these proteins were found as a "pear"/"cone" shaped trimers, TNF receptors, TNFR-1, and TNFR-2; moreover, they were cloned and expressed as 55 and 75 kDa proteins.

21.3 TNFα: Biological Roles

Several biological functons are ascribed to the TNFα and for this reason the mechanism of action is somewhat complex (Figure 21.1). Because this proteine confers resistance to certain types of infections and in parallel cause pathological complications, it carries out contradictory roles. This may be connected to the varied signaling pathways that are activated [7].

TNFα modulates several therapeutic roles within the body, such as immunostimulation, resistance to infection agents, resistance to tumors, sleep regulation, and embryonic development [8]. On the other hand, parasitic, bacterial, and viral infections become more pathogenic or fatal due to TNF circulation [7]. The major role of TNF is explicated as mediator in resistance against infections; moreover, it was postulated that TNF plays a pathological role in a number of autoimmune diseases such as graft versus host rejection or rheumatoid arthritis [9]. In addition, TNF exibits antimalignant cell cytotoxicity in particular in association with interferon [7]. Nevertheless, high concentrations of TNFα are toxic to the host (in fact, the maximum tolerated dose in humans is 200 mg/m^2), and it is necessary to increase the therapeutic index either by decreasing toxicity (e.g., pretreatment with IL-1) or by increasing effectiveness. This may be possible

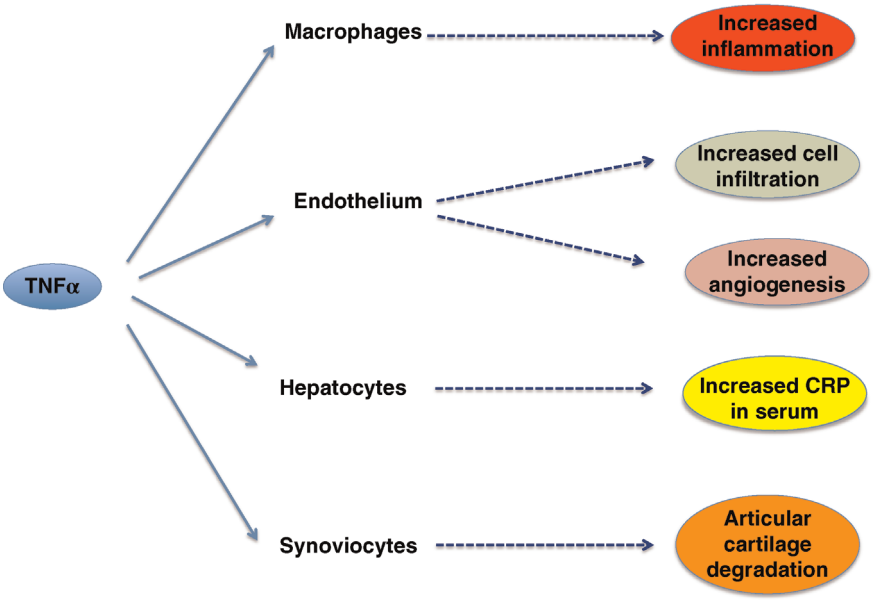


Figure 21.1 Actions of TNF superfamily.

through mutations that reduce systematic cytotoxicity and increase TNF's effectiveness in selectively eliminating tumor cells. TNFα is also implicated in physiologic sleep regulation. Administration of exogenous TNFα is able to induce increased sleep, however, contrary to its inhibition reduces spontaneous sleep. TNFα mRNA is generated during diurnal rhythm in brain with the highest levels occurring during peak sleep periods. The absence of the TNFR-1 receptors resulted in reduced sleep in animal models. Similar effects were observed with the cytokine IL-1. TNF-mediated apoptosis also seems a part of normal embryonic development [8]. TNF can also induce necrotic or apoptotic cell death. Several actions are correlated with the TNFα pathway; for example, TNF related proteins such as RANKL are required for osteoclast differentiation, necessary for bone resorption [10].

21.4 Mechanisms of Action

The TNFα effects are exerted by binding and clustering high-affinity receptors present in great numbers in most cell membranes [11]. The ligand/receptor complex is easily internalized via clathrin-coated pits and ends up in secondary lysosymes where it is degraded. Interestingly, the binding of TNFα to the 75 kDa TNFR-2 is not sufficient to reach cytotoxicity, but rather binding to the 55 kDa TNFR-1 is sufficient to reach TNFα-mediated cell killing. TNFα exerts its effects by activating several secondary proteins that provoke a variety of responses within the cell such as activation of gene transcription and/or production of reactive oxygen or nitrogen radicals (e.g., NO).

Activated proteins include GProtein, transcription factors such as NF-κB and AP-1, protein kinases such as CK II, erk-1, erk-2, and MAP2, phospholipases such as PLA2, PLC, and PLD, and sphingomyelinase mitochondrial proteins such as manganese superoxide dismutase, and serine and cysteine proteases, known as caspases. Many members of the TNF receptor superfamily have intracellular "death domains," which represent protein interaction domains each consisting of 65–80 amino acids; these proteins participate in TNFα-mediated apoptosis process.

21.5 Characterization of the TNFα Gene and Molecule

The gene of TNFα is a single-copy gene, preceded by the TNFβ on the short arm of human chromosome 6 and murine chromosome 17 [12]. TNFα is always 39 to the TNFβ gene and is around 3 Kb long. This gene is composed of four exons, arranged approximately 3 kb of DNA, interrupted by three introns. More than 80% of the mature TNF sequence is encoded in the fourth exon, while exons I and II contain almost entirely leader peptide sequence. The TNFα mRNA seems to be transcribed in a wide variety of cells. The 59 flanking region of the TNFα gene contains several kB homologous sequence enhancer elements and a MHC class II like "Y box." Probably, these sequences seem to be involved in transcriptional activation. The gene also seems to be regulated at the translational level through the UA-rich sequence in the 39 untranslated region of the human TNFα mRNA. The mRNA translates into a 157 amino acid protein in human and 156 in murine cells [12]. Two different forms of TNFα are available: a membrane-bound and a soluble form; each form possibly carries out a distinct physiological role [13]. Human TNFα is expressed as a precursor molecule of a 157 amino acid sequence, preceded by a 76 amino acid presequence (79 in murine). This presequence is highly conserved and seems to serve to anchor the precursor polypeptide in the membrane. Hence, unlike TNFβ, which is secreted, TNFα is produced as a type II membrane protein. TNFα exists in a more diverse range of cells than TNFβ. TNFα is produced in a wide variety of hematopoietic and nonhematopoietic cells, both normal and malignant. Among these macrophages, for instance, there are CD41 and CD81 T-lymphocytes, B-lymphocytes, neutrophils, NK cells, LAK cells, astrocytes, smooth muscle cells, endothelial cells, and a nonhematopoietic tumor cell lines. Actually, the classical cell line for testing TNF cytotoxicity is represented by murine fibrosarcoma cell line L929, although others, for example, WEHI164 clone, display heightened sensitivity. Some cells can be stimulated to produce the two different forms of TNF.

21.6 TNF Receptors and the TNF Receptor Superfamily

TNFα links to two cognate receptors of molecular weight 55 and 75 kDa [14]. A total 434 amino acids form human TNFR-1 and 439 amino acids TNFR-2. Both receptors share very restricted homology in the extracellular region. The superfamily of TNF receptor is determined by the presence of repeating units of

cysteine clusters. No homology is observed in the intracellular regions of the proteins. All known cell types showed a TNF receptors, such as unstimulated T lymphocytes and erythrocytes. Its density ranges is from 200–10 000 per cell. No correlation is reported between the number of receptors present on a cell and the magnitude or direction of the TNF-induced response [15]. In the serum of cancer patients and in human urine, soluble forms of TNFR-1 and TNFR-2 were detected. It was postulated that both forms regulate TNFs activity by decoying the ligand, thereby downregulating the generated signals. Members of the TNF receptor family belong to the type I transmembrane glycoproteins. They share a common cysteine-rich motif repeated two–six times in the extracellular N-terminal domain [9]. The average homology in these repeats is around 25% between the various members. The receptors differ in the possession of a cytoplasmic "death domain," which is missing in a several components of the TNFR family. Both TNFR-1 and TNFR-2 are N-glycosylated, only TNFR-2 is O-glycosylated. All other membrane receptors are glycosylated. The primary sequence of TNFR-1 suggests the presence of a number of potential phosphorylation consensus sites for PKC or CK II. TNFR-1 showed ubiquitous distribution on cells such as epithelial cells and fibroblasts, while TNFR-2 is more restricted, primarily to the cells of hematopoietic origin. CD40 is revealed as a 50 kDa glycoprotein on a variety of cells such as B cells, monocytes, thymic epithelium, dendritic cells, and at a low level of T cells. Fas receptor is expressed in activated T cells, monocytes, B cells, and neutrophils. Fas mRNA is detected in several organs in rodents. CD30 receptor was identified in Hodgkin's cells, but it is expressed in a number of non-Hodgkin's lymphoma cells as well as virally mutated B- and T-cell lines [16]. CD27 is showed on the majority of medullary thymocytes and peripheral blood T cells. It is also present on some B and NK cells. The receptor 4-1BB is manifested on activated CD81 and CD41 T cells and human lung epithelial cells and intestinal intraepithelial T lymphocytes [16].

21.6.1 TL1A

TL1A, also called TNF-like molecule 1A or TNFSF15, was discovered in 2002 and is classified as a member of the TNF superfamily [17]. The human species is caractherized by three isoforms of the protein produced from TNFSF15 gene as a result of alternative splicing, in particular VEGI-174, VEGI-192, and TL1A (VEGI-252), although VEGI-174 is most probably a cloning artifact [17]. VEGI-192 inhibits angiogenesis, whereas TL1A represents a proinflammatory factor embroiled in the pathogenesis of autoimmune diseases such as allergic lung inflammation, arthritis, autoimmune encephalomyelitis, and inflammatory bowel disease (IBD) [18]. Dendritic cells, macrophages, and CD4(+) and CD8(+) T cells express TL1A, whereas very little TL1A was discovered in nonactivated immune cells [18]. TNF, IL-1, Fc fragments of IgG1 antibodies, and certain parasite- or bacteria-related Toll-like receptor ligands, such as synthetic bacterial lipoprotein Pam3CSK4, LPS, polyinosinicpolycytidylic acid, lipopeptide FSL, single-stranded RNA, tachyzoite antigen, and unmethylated DNA sequences, are considered inducers of TL1A expression. DR3 is the main receptor for TL1A with a similar structure of TNFR1. This receptor is upregulated in activated monocytes, NKT

cells, NK cells, B cells, and CD8(+) T cytotoxic cells and CD4(+) T helper. TL1A is considered a proinflammatory molecule able to boost proliferation and effector functions of CD8(+) cytotoxic T cells, as well as Th1, Th2, and Th17 cells, in the presence of TCR stimulation; under physiological conditions, TL1A is not required for the differentiation of these lymphocytes [19]. TL1A stimulates maturation of dendritic cells and production of IL-8, TNF, and monocyte chemotactic protein-1 by macrophages [20]. Moreover, TL1A/DR3 interaction triggers proliferation of regulatory T cells (Tregs) [21]. *In vitro* studies suggested that TL1A inhibited differentiation of Tregs from their precursor cells [22]. TL1A seems to be a promising therapeutic target for patients with T cell-mediated autoimmune diseases, such as IBD, although to this day none of TL1A blocking agents has yet been studied in clinical trials.

21.6.2 FasL

Fas ligands, namely, FasL, CD95 L, or TNFSF6 and its receptors Fas, CD95, and TNFRSF6 are members of the TNF superfamily involved in the pathogenesis of IBD. Fas ligands, namely, FasL, CD95L, or TNFSF6 and its receptors Fas, CD95, and TNFRSF6 are members of the TNF superfamily embroiled in the pathogenesis of IBD. Structurally, FasL is known a transmembrane molecule, although it can be enzymatically derived from cells [23].

Cytotoxic T cells and natural killer cells take FasL to kill tumor cells and virus-infected cells expressing Fas. FasL is also implicated in keeping immune homeostasis and preventing autoimmunity via activation-induced cell death that depends on killing activated T cells following their expansion and differentiation in a FasL/Fas-dependent manner, thus preventing hyperactivation of T-cell mediated immunity [24].

FasL expression is finely regulated and restricted to activated CD4(+) and CD8(+) T cells, NK cells, and monocytes [25]. Phisiologically, Paneth cells represent the only cells of the intestinal epithelium that express FasL [26]. Their expression was also discovered in tissues and organs lacking resident or infiltrating lymphocytes and in neurons and astrocytes as well as in tumors where it may cause the suppression of immune responses via induction of T cell apoptosis [25]. Fas and FasL, while playing an important role in the regulation of apoptosis, have also nonapoptotic functions. Fas holds the death domain and, contrary to TNFR1, has been thought to be implicated only in proapoptotic but not prosurvival signaling [27].

Strong Fas stimulation blocked activation of human CD4(+) helper T cells, while weak Fas stimulation together with TCR signaling augmented their proliferation via activation of MAP kinases, transcription factors, and cell cycle activators [28].

21.6.3 LIGHT

LIGHT, also called lymphotoxin-like inducible protein that compete with glycoprotein D for binding herpes virus entry mediator on T cell, TNFSF14, ligand for the lymphotoxin beta receptor (or LTβR or TNFRSF3), and the herpes virus entry mediator (HVEM or TNFRSF14) is primarily expressed in activated T cells, although it is also found in monocytes, granulocytes, and immature dendritic

cells [29]. Both LTβR and HVEM receptors do not encompass the death domain; therefore, they are considered to be involved in prosurvival signaling [21]. As a matter of fact, interaction between LIGHT and HVEM was found to enhance proliferation and effector functions of CD8(+) cytotoxic T cells [30], boost expansion of CD4(+) helper T cells, and facilitate their differentiation intoTh1 cells [31]. It was reported that LIGHT contributes to the development of intestinal inflammation; for example, transgenic mice with elevated expression of LIGHT naturally develop colitis [32].

21.6.4 DcR3

DcR3 represents a soluble receptor for TL1A, LIGHT, and FasL and is a member of the TNF receptor superfamily that does not possess the transmembrane domain [33]. It is able to inhibit the interaction between its ligands and their membrane-bound receptors, thus repressing their biological activity. A high-level expression of DcR3 was shown in inflamed mucosa and serum of CD and UC patients [34].

21.6.5 TRAIL

TRAIL,TNF-related apoptosis-inducing ligand or TNFSF10, is another member of TNF superfamily expressed in a large variety of tissues including intestines may be implicated in the pathogenesis of IBD [35]. Similar to TNF, TRAIL induces apoptosis and can also stimulate the prosurvival transcription factor NF-κB. Five receptors of TRAIL have been identified: TRAILR1 (TNFRSF10A or DR4) and TRAIL-R2 (TNFRSF-10B or DR5) that possess the death domain in their cytoplasmic fragments and are entailed in functional TRAIL signaling; TRAIL-R3 (TNFRSF10C or DcR1) without death domain and TRAIL-R4 (TNFRSF10D or DcR2) with defective death domain and soluble osteoprotegerin (OPG or TNFRSF11B) are regarded to be decoy receptors [36]. Expression of TRAIL was found to be downregulated in intestinal epithelial cells of IBD patients [180]; however, it was significantly elevated in mononuclear cells of the resected inflamed mucosa in both CD and UC patients with highly active, steroid-refractory disease [37].

21.6.6 TWEAK

TWEAK, also named TNF-like weak inducer of apoptosis or TNFSF12, operates through its receptor Fn14 (TNFRSF12 or TWEAK-R) and has several biological activities, incorporating induction of proinflammatory cytokines and stimulation of cell growth, and, in certain experimental settings, induction of apoptosis. TWEAK protein is expressed in particular in immune cells such as T cells, macrophages, or dendritic cells, although it was discovered in nonhematopoietic cell types such as astrocytes or endothelial cells [38]. Expression of TWEAK receptor, Fn14, was discovered in many types of cells including intestinal mucosa cells. Fn14 does not contain the death domain and its stimulation with TWEAK results in activation of the transcription factor NF-κB [39]. Deficiency or

reduction of TWEAK and its biological activity by anti-TWEAK monoclonal antibodies diminished expression of proinflammatory cytokine, neutrophil, and macrophage infiltration decreasing severity of trinitrobenzenesulfonic (TNBS) acid-induced colitis in rodents [40]. Thanks to *in vitro* studies on rhabdomyosarcoma cell line Kym-1, it was demonstrated that TWEAK upregulated expression of transmembrane TNF that in turn provoked cell apoptosis via TNFR1 [41]. Moreover, TWEAK stimulation enhanced TNFR1-mediated apoptotic cell death of Kym-1 cells [41] that can be clarified by the fact that TWEAK promotes translocation of antiapoptotic adapter protein TRAF2 from the TNFR1 signaling complex, increasing proapoptotic signaling of this receptor [42].

21.6.7 BAFF-R–BAFF

The B-cell-activating factor of the tumor necrosis factor family (BAFF, also called TNFSF13B or CD257 or B lymphocyte stimulator "BLys") was for the first time described as a molecule secreted by T cells and dendritic cells that ensure maturation and survival of peripheral B cells. Its expression was also reported in macrophages, dendritic cells, and neutrophils [43]. BAFF transgenic mice have wired numbers of B and effector T cells and display symptoms similar to that observed in B-cell-mediated autoimmune diseases [44]. For example, immature B cells in the bone marrow manifest BAFF receptor (also BAFF-R) and this expression is upregulated in the development from transitional B-cell stage to mature B cell in the secondary lymphoid organs [45]. BAFF is able to interact with transmembrane activator calcium modulator and cyclophilin ligand interactor (also TACI) and B-cell maturation factor (BCMA) [46]. TACI is expressed in marginal-zone B cells; moreover, BCMA is expressed in germinal center B cells, plasma cells, and memory B cells. BAFF, TACI, and BCMA interact with APRIL, another TNFSF member.

21.6.8 OX40–OX40L

The OX40 or ACT35 or CD134 or TNFRSF4 represents a type I glycoprotein of ~50 kD, expressed in activated T cells, such as Th1, Th2, Th17, Foxp3+ regulatory CD4+ T cell (Treg), and CD8+ T cells [47]. Its ligand, namely, OX40 ligand or OX40 L or gp34, CD252, and TNFSF4, is a glycoprotein expressed in APCs, such as dendritic cells, B cells, macrophages, and endothelial cells. The interaction of OX40–OX40 L gives a costimulatory signal to T cells, which leads to their activation and also cytokine secretion [48]. Blocking OX40–OX40 L interactions protects from several inflammatory autoimmune diseases such as EAE. OX40–OX40 L signaling in CD4+ T cells stimulates IL-12Rβ2 [49] expression and inhibits CTLA-4, Foxp3, and IL-10 expression. Upregulation of IL-12Rβ2 drives Th1 differentiation, whereas loss of Foxp3, CTLA-4, and IL-10 inhibits the Tregs suppressive function. Loss of balance between pathogenic and regulatory cells could relieve autoimmune pathology. OX40–OX40 L interactions regulate CD4+ T-cell subsets' differentiation, and deficiency of OX40 weakens Treg development [50]. Moreover, OX40L–OX40 signaling inhibits Foxp3 expression and plays as a differentiating factor for Th9 in airway inflammation [51].

21.6.9 GITR–GITRL

Glucocorticoid-induced TNFR family-related gene known as CD357 or TNFRSF18 is revealed at the low levels in resting mouse and human T cells, and is upregulated in activated $CD4^+$ and $CD8^+$ T cells. It is constitutively expressed in Tregs [52]. Interaction with GITRL is able to prevent the suppressive function of $CD4^+$ $CD25^+$ Tregs, and blocking these links breaks the peripheral immune tolerance. B and endotelian cells, bone marrow-derived dendritic cells, and macrophages express GITRL [53]. Moreover, signaling through GITR promotes cytokines production such as IFN-γ, IL-4, IL-2, and IL-10 in $CD4^+$ T cells [54].

21.7 TNF Family: Role on Inflammatory Bowel Diseases

IBD patients showed elevated expression of TNF. TNF mRNA was overexpressed in involved colonic tissue of patients affected from CD (Crohn's disease) as well as in both involved and uninvolved colonic tissue of UC (ulcerative colitis) patients compared to healthy subjects [55]. High-level expression of TNF protein was detected in blood serum of CD patients, while other groups had elevated levels of TNF protein in serum and in the intestinal lamina propria of both CD and UC patients as well as the intestinal submucosa of CD patients [56]. The production of TNF in the colon mucosa of UC patients was localized to lamina propria macrophages. Pathogenesis of IBD is correlated with modified expression of TNF receptors since both CD and UC patients revealed elevated expression of TNFR2 on colonic epithelial cells [57]. A positive correlation was ascetained between UC and CD activity and serum concentration of both soluble forms of TNFR1 and TNFR2 [55]. Moreover, upregulated expression of TNFR2 was established in intestinal lamina propria CD4+ cells and in peripheral blood T cells of CD-affected patients [58]. Many reports showed that TNF leads to the disruption of intestinal epithelial barrier that allows for intestinal penetration of luminal antigens and facilitates intestinal inflammation [59]. The integrity of intestinal epithelium is arranged by the presence of TJ (tight junctions) situated in the apical region of intestinal epithelial cells. *In vitro* studies by Ma *et al.* [59] revealed that stimulation of colonic epithelial Caco-2 cells with TNF is able to downregulate expression of TJ-associated zonula occludens-1 proteins and modified their junctional localization in an NF-κB-dependent manner [59]. Moreover, IBD patients showed elevated mucosal expression of another TNF sheddase, namely, metalloprotease ADAM19 that is localized mainly in epithelial cells [60], whereas a mouse study demonstrated that shedding of TNF can also be modulated by matrix metalloproteinase 13 [61]. *In vitro* studies demonstrated that TNF upregulated expression of MLCK in Caco-2 cells pretreated with IFN-γ that increased expression of TNF receptors in the cell surface [62]. A recent study on TNFR1 or TNFR2-deficient mice showed that upregulation of MLCK and the loss of intestinal epithelial barrier in CD4(+) CD45RB (high) T cell transfer model of intestinal inflammation were dependent on TNFR2 expressed in intestinal epithelium but not TNFR1 [63]. TNF signaling upregulated expression of inducible nitric oxide synthase led to increased expression of a proapoptotic

protein p53 [64]. Moreover, TNF takes part in transactivation of epidermal growth factor receptor that upregulates the expression of cyclooxygenase-2 (COX-2) [65]. High-level expression of COX-2 has been linked to enhanced cell resistance to inflammation, apoptosis, and promotion of tumor progression; it is relevant that this aspect of TNF activity might have importance to development of IBD-associated cancers of the GI tract [66]. All components of the TNF superfamily lead to the pathogenesis of IBD and in particular (1) TNF acts to destroy the integrity of intestinal epithelium by mutating the arrangement of adhesion proteins in enterocytes; TNF, FasL, TRAIL, and TWEAK act to elicit apoptotic death of enterocytes and (2) TNF, TL1A, LIGHT, TWEAK, and perhaps FasL encourage the proinflammatory activity of mucosa infiltrating mononuclear cells and influence the activity of regulatory T cells and regulatory macrophages. Recently, TNF superfamily members have drawn a lot of attention as potential therapeutic targets in IBD management. At present, the only TNFSF component employed in clinical treatment of IBD is represented by TNF. Another hopeful target, while still not assessed in clinical trials, is TL1A that looks to be one of the key factors adjusting the inflammatory pathways in IBD. FasL, TRAIL, LIGHT, and TWEAK still require more in-depth studies to better define their function in intestinal inflammation.

21.8 Role in Neuronal Inflammation

The CNS represents as an immune-privileged site and it is caractherized by a network of CNS microvessels. These are constituted by a highly specialized endothelial lining sustained by astrocytes, pericytes, microglial cells, and neurons, which form the blood–brain barrier (BBB). The BBB confines entry of immune cells into the CNS, thereby maintaining a homeostasis. Nevertheless, in inflammatory states, the BBB gets disaggregated and immune cells move into the CNS parenchyma. A lesioned BBB is one of the hallmarks of autoimmune demyelinating diseases such as EAE and MS [67]. A good body of evidence demonstrated that TNF–TNFR interactions are implicated in the pathogenesis of CNS disorders such as EAE and MS. These interactions are able to monitor the disease outcome by fine-tuning peripheral immune response as well as interactions between CNS-resident cells and effector immune cells in the CNS. TNFRSF21 and TNFRSF19 are tuned in the acquisition and development of barrier property in BBB [68,69]. Both these TNFRSF members are downstream objective of the Wnt/beta-catenin signaling pathway in the BBB endothelial cells. Dysregulation of TNFRSF21/TNFRSF19 signaling guides to the rupture of endothelial BBB. Since Wnt/beta-catenin signaling is necessary for CNS angiogenesis but not for peripheral vasculature, an understanding of the molecular mechanism of this signaling would aid in designing novel therapeutics or biologics that target TNFSF–TNFRSF interactions, to control CNS autoimmunity [69].

TNFα operates as a proinflammatory cytokine in the early phase of EAE and indicates immunosuppressive properties during the later phase. The administration of a TNFR-1–IgG fusion protein or neutralization of anti-TNF antibody has been demonstrated to manage the development of EAE [70]. This regimen also

modulated the activation of $CD4^+$ T cell and microglia cells and avoided demyelination in CNS [71]. It was demonstrated that a deficiency of TNFα defers the disease progression and also resulted in disease of comparable severity; moreover, other authors have reported that TNFα-deficient mice expanded a more severe myelin oligodendrocyte glycoprotein (MOG) peptide-induced EAE compared to wild-type littermate controls [72]. TNFα expression, in particular in neurons in transgenic mice, leads to the progression of spontaneous inflammatory demyelination [73]. Deficiency of TNFα, LT-α and TNFR-1 or neutralization of TNF-α and LTα with monoclonal antibodies diminished the severity of EAE [74]. TNFR-2-deficient mice evolved more severe inflammation and demyelination in MOG35–55 -induced EAE [75]. In patients suffering from MS, TNFα was detected to be expressed at high levels in the CNS lesions and cerebrospinal fluids and not in serum [71], suggesting that TNFα was locally made in the inflamed CNS. This indicates that TNFα might be a target for therapy against MS. TNFα inhibitors indicated protection in the mouse model of EAE, but adverse effects were reported in clinical trials in MS patients [76]. Likewise, a clinical trial with TNFR-1 fusion protein in MS patients revealed disease aggravation and the trial was stopped [77]. This incremented demyelination with TNFα antagonist might be due to the some possible reasons such as (a) downregulation of anti-inflammatory cytokine IL-10 and overexpression of inflammatory cytokine IL-12 and IFN-γ; (b) downregulation of TNFR-2, known to modulate proliferation of oligodendrocytes and damage repair; (c) possibility of reduced or nonpermeability of the BBBs to TNFα blockers that would avoid their action through these molecules could still enhance the disease by increasing autoreactive T cells in the peripheral tissues, which can migrate into the CNS and damage the tissues; (d) unmasking of latent infection, which is crucial for inducing the autoimmune demyelination reaction.

The interaction of Fas with FasL on infiltrating cells in the CNS or activated CNS-resident cells such as microglia, astrocytes, and neurons also leads to the induction of apoptosis. This interaction plays an important role in immune cell homeostasis; moreover, its dysregulation leads to various autoimmune diseases. Mice with autosomal recessive mutations in Fas and FasL genes generate a spontaneous autoimmune syndrome accompained by production of autoantibodies and accumulation of CD4–CD8 T cells in the secondary lymphoid tissues, resulting in the progressive development of lymphadenopathy and splenomegaly [78]. Moreover, Fas- and FasL-deficient mice are also resistant to MOG-induced EAE compared to wild-type mice, and intrathecal injection of recombinant FasL protein or neutralizing with anti-FasL antibody suppresses acute EAE [79]. Mice that have genetic deficiency of BAFF-R showed hypersusceptibility to MOG35–55 peptide-induced EAE [80], indicating that this receptor is involved in a regulatory function in EAE. Moreover, BAFF can stimulate the autoreactive Th17 response, leading to increased progression of EAE [81]. Interaction of OX40–OX40 L in CD28-deficient mice brings the identification of OX40–OX40 L signaling as being costimulatory in EAE, and it also blocks interaction-protected mice from EAE [82]. In the rat EAE model, pathogenic OX40+ CD4+ T cells were identified in CNS lesions and neutralization of OX40 by anti-OX40 antibody improved the EAE [83]. Brain endothelial cells are the important component of the BBB and are known to express OX40 L,

which recruit OX40+ autoreactive T cells in the CNS. OX40 treatment of human umbilical cord endothelial cells leads to the upregulation of CCL5, prompting that this signaling modulates endothelial cells to engage a selective population of immune cells at the BBB [84]. Blocking OX40 L by anti-OX40 L antibody diminished the infiltration of OX40+ myelin-specific T cells in the spinal cord without modulating the priming of the myelin-specific CD4+ T cells in the draining lymphnodes in EAE [85]. Therefore, OX40–OX40 L not only controls the activation and differentiation of CD4+ T cells but also potentiates their migration into the CNS. These results indicate that OX40–OX40 L sustains the development of neuroinflammation and CNS autoimmunity.

It was demonstrated that transgenic mice that constitutively express GITRL in B cells (GITRL+ B cells) displayed a higher number of peripheral Tregs, indicating that GITRL+ B cells might play a crucial role in the homeostasis of Tregs [86,87]. Animals receiving B cells pretreated with antimouse GITRL antibody showed a significantly decreased Tregs induction and severe EAE, suggesting that GITRL+ B cells sustain both proliferation and homeostasis of Tregs [87]. Administration of a monoclonal antibody that depletes B cells, including Bregs, takes to severe exacerbation of human ulcerative colitis, suggesting that Bregs play a crucial role in controlling CNS autoimmunity [88].

Several TNF superfamily members have the capacity to modulate neuroinflammatory processess in different ways. Comprehension of their profile, kinetics of expression, and interactions between TNF ligands and their TNFRs on different CNS-resident and infiltrating immune cells at diverse stages of the disease would aid to better design strategies to control neuroinflammation and CNS autoimmunity.

21.9 Conclusions

Since their discovery in 1984 until today, studies conducted during the past two decades have demonstrated the existence of a superfamily of TNF proteins consisting of 19 members that signal through 29 receptors. These ligands are able to regulate

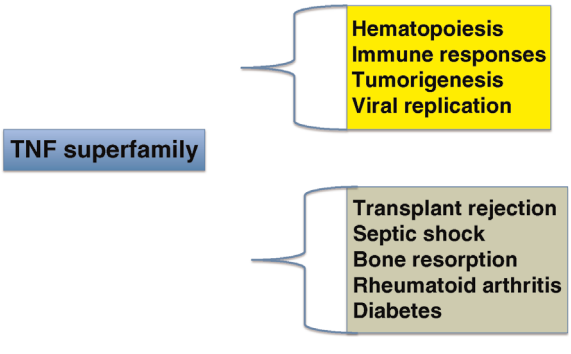


Figure 21.2 TNF superfamily members are fundamental for hematopoiesis, viral replication, immune responses, and tumorigenesis (indicated in yellow); moreover, its dysregulation drives to several diseases (indicated in green).

physiological functions, such as hematopoiesis, morphogenesis, and immune responses, and have also been involved in transplant rejection, septic shock, tumorigenesis, bone resorption, viral replication, rheumatoid arthritis, and diabetes, so pointing to their role as "double-edged swords" (Figure 21.2). Moreover, these cytokines stimulate cellular proliferation, survival, differentiation, and apoptosis. Blockers of TNF have been acknowledged for human use in treating TNF-linked autoimmune and inflammatory diseases. Pathways downstream of receptor ligation supply critical points for interjection for planning new therapeutic strategies.

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22

Interleukin-17 A-E

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22.1 Introduction

The interleukin (IL)-17 cytokine family is composed of IL-17A and five other members (IL-17B, IL-17C, IL-17D, IL-17E, also referred to as IL-25, and IL-17 F), which share a similar protein structure characterized by four highly conserved cysteine residues [1]. IL-17-related cytokines play key roles in defense against extracellular pathogens (e.g., bacteria, fungi, etc.) and autoimmunity, and accumulating evidence indicates that some of these molecules are involved in the amplification and perpetuation of pathological processes in many inflammatory diseases, such as inflammatory bowel diseases (IBDs), psoriasis, rheumatoid arthritis, multiple sclerosis, and allergy [1]. However, the same cytokines can exert anti-inflammatory effects in specific settings and play key role in the control of immune homeostasis [1]. These progresses have paved the way for the development of various compounds targeting IL-17 family members, which have been tested in patients with immune-mediated diseases [2]. In this chapter, we will summarize the biology of IL-17 cytokines and the more recent novelties about the therapeutic armamentarium directed against this group of cytokines.

22.2 Cell Sources of IL-17 Cytokines

IL-17A, a glycoprotein of 155 amino acids originally identified as a product of activated CD4+ T cells, is the signature cytokine of a subset of CD4+ T helper (Th) cells, termed Th17 cells [3,4]. Th17 cells arise from naïve CD4+ T cells under the influence of the polarizing and stabilizing cytokines, transforming growth factor (TGF)-β1, IL-6, IL-21, and IL-1β, and the activity of the master regulator retinoid acid-related orphan receptor (ROR)-γt. Additional transcriptional factors that

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contribute to the optimal induction of cytokines in Th17 cells include Stat3, IRF4, BATF, Runx1, and c-Maf [4–8]. IL-23 receptor (IL-23R) is not expressed in naïve murine T cells and, therefore, this cytokine cannot induce Th17 cell differentiation. However, IL-23 targets IL-23R-expressing Th17 cells, thereby facilitating their maintenance/expansion through a Stat3-dependent mechanism [9]. Human CD4+ T cells and mouse T cells bearing the γδ TCRs, which are prominent in mucosal tissues, express IL-23R and respond functionally to IL-23 with enhanced production of IL-17 [10]. In addition to IL-17A, Th17 cells produce IL-17 F and variable amounts of IL-21, IL-22, and IL-26 [6]. IL-17 cytokines can also be produced by cytotoxic CD8+ T cells, γδ T cells, natural killer (NK) T cells, type 3 innate lymphoid cells, neutrophils, and eosinophils [6]. B cells are a major source of rapid formation of IL-17A *in vivo* in response to infection with *Trypanosoma cruzi* [11]. IL-17A and IL-17 F production is not restricted to immune cells, since these cytokines can be synthesized by intestinal Paneth cells and IL-17A is made by mesenchymal stem cells [12,13]. Both IL-17A and IL-17 F are secreted as disulfide-linked homodimers, even though human and mouse T cells can produce IL-17A/F heterodimers [3,14].

IL-25 is synthesized by Th2 cells, bone marrow-derived mast cells upon IgE cross-linking, activated macrophages, eosinophils and basophils, and nonimmune cells, including lung cells, intestinal epithelial cells, and brain capillary endothelial cells [15]. For a more detailed analysis of factors/mechanisms involved in the control of IL-17 cytokine production, the reader is directed toward other chapters of this book.

22.3 IL-17 Receptor: Structure and Biology

The activity of IL-17 cytokines is mediated by a group of five receptors belonging to the IL-17 receptor (IL-17R) family. IL-17RA, the leading component of this family, can heterodimerize with all the other members to form receptors for all the IL-17-related cytokines except IL-17B [16]. IL-17RA interacts with IL-17RC to form heterodimers and binds both IL-17A and IL-17 F [17,18]. IL-17RA can also associate with IL-17RB to form the functional receptor for IL-25 [19]. Much less is known about IL-17RD and IL-17RE biology and ligands. IL-17 receptors are all single transmembrane-containing domain receptors with an extracellular fibronectin III-like domain and a cytoplasmic SEF-IL-17R (SEFIR) motif, adjacent to a SEFIR extension (SEFEX) domain [20]. After the ligand–receptor interaction, the adapter protein ACT1 is recruited to the SEFIR domain and mediates most of the intracellular events downstream to IL-17 receptors [21]. In particular, ACT1 recruits TNFR-associated factor (TRAF) 6, mediates K63-linked ubiquitination, and leads to activation of mitogen-activated protein kinase (MAPK) and nuclear factor (NF) kB, thus controlling various survival pathways and transcription of innate proinflammatory cytokines [22–24]. Another important group of transcription factors activated by IL-17R-transduced signal is the CCAAT/enhancer-binding protein (C/EBP), which together with NF-kB contributes to the transcription of proinflammatory target genes (e.g., IL-6) in an ACT1-independent manner [25].

22.4 Production and Role of IL-17 Cytokines in Normal and Inflamed Intestine

IL-17-type cytokines are constitutively produced in the gut of normal mice and humans, where they contribute to combat pathogenic bacteria and fungi [1]. Indeed, these cytokines have the ability to promote neutrophilia, tissue remodeling/repair, and production of antimicrobial peptides, such as defensins [26]. Production of IL-17 cytokines, particularly by Th17 cells and epithelial cells, is dependent on luminal bacteria. Indeed, IL-17-producing cells are absent in Germ-free mice but accumulate rapidly in the gut of mice colonized with the gut commensal, segmented filamentous bacterium (SFB) [27]. Moreover, mice overexpressing a human α-defensin in intestinal epithelial Paneth cells are resistant to the colonization of SFB and exhibit decreased number of IL-17-producing cells in the intestinal lamina propria [28]. Luminal bacteria-derived ATP is a major driver for the differentiation and/or accumulation of IL-17-producing cells, even though SFB-mediated Th17 cell intestinal accumulation is independent of ATP [29]. There is also evidence that bacteria can expand Th17 cell responses trough DNA-driven Toll like receptor-mediated mechanisms [30]. Studies in mice indicate that intestinal lamina propria Th17 cells are in part generated in mesenteric lymph nodes and Peyer's patches [31].

IL-17A-producing cells are abundant in inflamed intestine of patients with Crohn's disease (CD) and patients with ulcerative colitis (UC), the major forms of IBD in human beings. In inflamed mucosal areas of IBD patients, there is also marked synthesis of IL-17 F, IL-22, and IL-26 [32–34]. Some of the IL-17A-producing cells coexpress IFN-γ and/or Foxp3, the marker of regulatory T cells [35,36]. The latter cell type shows phenotypic characteristic of both conventional Th17 cells (e.g., expression of ROR-γt, CCR6, and CD161) and regulatory T cells (e.g., high CD101 and low CD127 expression) and has suppressor activity *in vitro* [35]. Analysis of the TCR repertoire revealed a similar TCR-β chain variable region usage by Foxp3-expressing IL-17A-producing T cells and Foxp3-expressing IL-17A-negative T cells, raising the possibility that Foxp3-expressing IL-17A-producing T cells can originate from classical regulatory T cells exposed to specific inflammatory stimuli present in IBD tissue [35]. On the other hand, there is evidence that cells coexpressing IL-17A and IFN-γ are not stable and, under various influences, can undergo transformation into typical Th1 cells. This occurs, for example, if these cells are stimulated with IL-12 [37]. Since TGF-β1 signaling is needed to maintain IL-17A expression by *in vitro* generated Th17 cells, in a milieu lacking TGF-β1, cells initially producing IL-17A can become cells producing other cytokines [38,39]. CD-related inflammation is marked by defective TGF-β1 activity due to high levels of Smad7, an intracellular inhibitor of TGF-β1 signaling [40]. Therefore, it is plausible that, in CD, deviation from IFN-γ-producing Th17 cells to typical Th1 cells can be facilitated by high Smad7. Support to this hypothesis comes from recent work by Weaver's group showing that Th1 cells are generated from IL-17F-expressing Th17 cells in the late stages of colitis development in the absence of TGF-β1 and in the presence of IL-23 [38]. The functional role of Th17-related cytokines in human IBD is not

fully understood, but it is conceivable that these molecules can contribute to amplify some inflammatory pathways that sustain the pathological process. Indeed, IL-17A and IL-17 F can increase the synthesis of inflammatory cytokines, chemokines, and adhesion molecules [41,42]. IL-17A also stimulates the synthesis of extracellular matrix-degrading proteases by stromal cells [43]. In this context, it is, however, noteworthy that evidence generated in mouse models of colitis suggests that IL-17 cytokines are not essential in the development of intestinal inflammation and, in some circumstances, could exert protective rather than proinflammatory effects. Oral administration of dextran sulfate sodium (DSS) in mice causes disruption of the epithelial layer and an acute inflammatory response mediated by luminal bacteria [44]. Excessive production of IL-17 occurs in the colon of DSS-treated mice and administration of a blocking IL-17A monoclonal antibody to wild-type mice exacerbates colitis [45]. Consistently, IL-17A-deficient mice are more susceptible to DSS-colitis compared to wild-type mice [46], thus supporting the counterregulatory role of IL-17A in this acute model of intestinal inflammation. These findings are not surprising as IL-17A stimulates intestinal epithelial cells to produce mucous and defensins, thereby limiting translocation of luminal bacteria to lamina propria [47].

Mice lacking the chemokine receptor CX3CR1 display increased translocation of commensal bacteria to mesenteric lymph nodes, produce high levels of IL-17A, and are more susceptible to DSS colitis as compared to wild-type mice. CX3CR1-deficient mice given a neutralizing IL-17A antibody develop a less severe DSS colitis, indicating that, at least in this specific setting, IL-17A is pathogenic [48]. In line with this latter finding is the demonstration that IL-17RA-null mice or wild-type mice given a neutralizing IL-17RA IgG fusion protein develop less severe colitis following intrarectal administration of trinitrobenzene sulfonic acid (TNBS) [49]. As pointed out above, IL-17RA mediates the effects of both IL-17A and IL-17 F [50]; therefore, it remains unclear whether the pathogenic effect of IL-17RA signaling in TNBS colitis is mediated by either IL-17A or IL-17 F. The possibility that IL-17 F is more inflammatory than IL-17A in the gut is supported by the demonstration that IL-17F-deficient mice are protected against DSS-induced colitis [46].

Even more complex appears the role of IL-17 cytokines in murine models of chronic intestinal inflammation. Administration of an anti-IL-17A antibody ameliorates the spontaneous colitis developed in mice bearing a conditional deletion of Stat3 in Foxp3-positive regulatory T cells [51]. Th17 cells are also pathogenic in the colitis models induced by either transfer of the cecal bacterial antigen-specific C3 H/HeJBir (C3Bir) CD4 (+) T-cell line to C3 H/HeSnJ SCID mice or colonization of mice with enterotoxigenic *Bacteroides fragilis*, a human colonic bacterium [52]. To evaluate the role that Th17 cytokines play in the initiation and progression of colitis, Leppkes *et al.* transferred IL-17A-, IL-17F-, or IL-22-deficient T lymphocytes into RAG1-null mice and showed that the lack of single cytokines did not prevent the development of colitis [53]. Similarly, Noguchi *et al.* showed that colitis induced in RAG mice by transfer of IL-17A-deficient CD4+ T cells does not differ in terms of severity from that induced by transfer of wild-type cells [54]. Leppkes also reported that colitis induced by transfer of IL-17F-deficient T cells was suppressed by a neutralizing anti-IL-17A

antibody, indicating redundant biological effects of IL-17A and IL-17 F. Consistently, RORγ-deficient T cells, which do not produce all Th17 cytokines, cannot transfer colitis to RAG-deficient mice [53]. A different scenario emerges from studies published by the Flavell' group showing that adoptive transfer of IL-17A-deficient naïve CD4+ T cells into recipient immunodeficient mice resulted in severe colitis and wasting disease, with significantly increased colon IFN-γ production [55]. Transfer of IL-17R-deficient T cells induced the same aggressive disease indicating that IL-17 exerts its effects directly on T cells. Furthermore, it was shown that IL-17A blocks differentiation of IL-17RA-expressing CD4+T cells in Th1 cells, raising the possibility that the IL-17A-mediated negative regulation of colitis relies on suppression of pathogenic Th1 cell responses [55].

22.5 IL-17 Blockers are Not Therapeutic in CD

Although IL-17A is overproduced in inflamed intestine of CD patients and this cytokine can trigger inflammatory signals in mucosal cells, a recent clinical trial has shown that neutralization of IL-17A with the blocking antibody Secukinumab was not effective in patients with CD [56]. In another clinical trial, brodalumab, a fully human anti-IL-17RA monoclonal antibody, determined significant CD activity worsening compared to placebo [57]. The reason why IL-17 blockers failed in CD remains unknown even though there are some explanations. A possibility relies on the fact that IL-17A is anti-inflammatory, rather than proinflammatory, in the gut due to its ability to enhance the intestinal barrier integrity [47]. The tissue-damaging immune response in CD is characterized by simultaneous activation of multiple and disconnected inflammatory pathways and some of them (e.g., Th1-mediated signals) are negatively regulated by Th17 cytokines [55]. Therefore, treatment of CD with IL-17 blockers could suppress IL-17A-driven inflammatory pathways but at the same time promote amplification of Th1-driven inflammation.

22.6 IL-17 Cytokines in Psoriasis

Psoriasis is a common, chronic, and immune-mediated skin disorder characterized by distinctive histopathological features (i.e., epidermal acanthosis, hyperkeratosis, and parakeratosis) combined with a rich inflammatory infiltrate of innate and adaptive immune cells [58]. Genome-wide association studies have identified genes encoding proteins involved in the IL-17 pathway associated with psoriasis and psoriatic arthritis. For instance, a single nucleotide substitution in exon 9 of the IL-23R gene is protective against psoriasis [59]. A polymorphism at TRAF3IP2 (the gene encoding Act1) confers increased risk of both psoriasis and psoriatic arthritis, presumably secondary to dysregulation of downstream signaling from the IL-17 receptor [60]. Many descriptive and experimental data have focused on the role of Th17 cytokines, and in particular IL-17 and IL-22, in the complex cross-talk between immune cells and resident epithelial cells. Numbers

of IL-17A-positive cells are increased in lesional skin, of which the vast majority are T cells, both CD4+ and CD8+, mast cells, and neutrophils [61–63]. IL-17A, IL-17C, and IL-17 F RNA transcripts are highly produced in lesional versus nonlesional skin, whereas IL-17B and IL-17D RNA levels are decreased [64], and there is correlation between cutaneous IL-17A RNA expression and disease activity [65]. The relevance of IL-17A in the pathogenesis of psoriasis is supported by the demonstration that clinical response to various therapies is associated with a marked downregulation of IL-17 production in psoriatic skin and functional studies showing the ability of this cytokine to induce or amplify inflammatory signals leading to epidermal pathology [66]. In this context, it is known that IL-17A enhances expression of antimicrobial peptides (i.e., β-defensins and S100A family members) and stimulates keratinocytes to produce chemokines, such as CCL20 (which mediates recruitment of Th17 and dendritic cells to skin) and CXCL1, 3, 5, 6, and 8 (which drive neutrophil recruitment) [67–69]. IL-17A decreases expression of cell adhesion factors, leading to disruption of the skin barrier [70]. IL-17A-driven pathogenic effects can be amplified by other cytokines overproduced in psoriatic lesions, such as TNF [67]. In primary keratinocytes IL-17A and TNF upregulate a multitude of genes, which correlate with the psoriasis gene signature (e.g., β-defensin-2, S100A7, CCL20, CXCL1, CXCL8, and IL-23 p19 subunit) [67]. Studies in murine models of psoriasis-like pathology confirmed the pathogenic role of IL-17A, as no epidermal hyperplasia was seen following IL-23 injection in mice devoid of IL-17 [71] and induction of skin damage in response to IL-21 injection or cutaneous application of TLR7 agonist was paralleled by upregulation of IL-17 cytokines [72,73].

22.7 Anti-IL-17 Therapy in Psoriasis

Various IL-17 inhibitors have been used with success in patients with moderate to severe psoriasis. Initial studies with secukinumab showed that at 3 mg/kg intravenously (i.v.), the drug reduced mean psoriasis area and severity index (PASI) scores compared to placebo at week 4. Secukinumab reduced cutaneous expression of multiple chemokines, cytokines, and proteins involved in the tissue-damaging immune response, including IL-17A, IL-17 F, IL-21, IL-22, IL-12p40, CCL20, β-defensin-4, and keratin 16, a marker of activated keratinocytes [74].

Randomized, placebo–controlled phase 2 trials showed that subcutaneous administration of 75 or 150 mg secukinumab at baseline and weeks 4 and 8 was superior to placebo in increasing PASI 75 response at week 12 primary end point [75]. Better results were seen following subcutaneous administration of secukinumab 150 mg at weeks 0, 1, 2, and 4 (early regimen). The anti-inflammatory effect of the drug was supported by the demonstration that readministration of secukinumab at weeks 12 and 24 maintained clinical response [76]. In two phase 3, double-blind, 52-week trials, ERASURE (Efficacy of Response and Safety of Two Fixed Secukinumab Regimens in Psoriasis) and FIXTURE (Full Year Investigative Examination of Secukinumab vs. Etanercept Using Two Dosing Regimens to Determine Efficacy in Psoriasis), subcutaneous secukinumab at a dose of 300 mg or 150 mg (administered once weekly for 5 weeks, then every 4

weeks) reduced PASI 75 scores in patients with moderate to severe plaque psoriasis [77].

The therapeutic efficacy of IL-17 neutralization was confirmed by studies with ixekizumab, a humanized monoclonal antibody against IL-17A, and brodalumab. In randomized, placebo–controlled phase 2 trials, ixekizumab at doses of 25, 75, or 150 mg subcutaneously at baseline and weeks 2, 4, 8, 12, and 16 was clinically superior to placebo; the therapeutic effect of ixekizumab was evident by week 1 and maintained through week 20 [78]. Treatment with ixekizumab caused a broad downregulation of markers of epidermal pathology and of psoriasis-associated inflammatory molecules [79]. More recently, two phase 3 studies (UNCOVER-2 and UNCOVER-3) evaluated the clinical efficacy of ixekizumab compared with placebo or etanercept in patients with widespread moderate to severe psoriasis. Patients were randomized to receive subcutaneous placebo, etanercept, or one injection of ixekizumab every 2 weeks or 4 weeks. Both ixekizumab dose regimens had greater efficacy than placebo and etanercept over 12 weeks [80].

Brodalumab was administered subcutaneously at 70, 140, or 210 mg at baseline and weeks 1, 2, 4, 6, 8, and 10, or at 280 mg at baseline and weeks 4 and 8. Brodalumab was more effective than placebo in reducing mean PASI scores [81], significantly improved dermatology life quality index and reduced markers of disease pathology [82]. Two phase 3 studies (AMAGINE-2 and AMAGINE-3) compared the efficacy and safety of brodalumab with those of ustekinumab, an antibody against the p40 subunit shared by IL-12 and IL-23 approved for the treatment of psoriasis. Patients were assigned to receive subcutaneously brodalumab (210 mg or 140 mg every 2 weeks), ustekinumab (45–90 mg), or placebo for 12 weeks, and then continued with a maintenance dose through week 52. At week 12, both brodalumab regimens were superior to placebo and ustekinumab in controlling clinical activity of moderate to severe psoriasis [83].

22.8 IL-17A in Psoriatic Arthritis

Psoriatic arthritis (PsA) affects nearly one fourth of psoriasis patients and its onset occurs after cutaneous disease [84]. Th17 cells are increased in the circulation of PsA patients, and a high number of IL-17A-producing cells, including Th17 cells and c-Kit-positive mast cells, is seen in the synovial fluid of these patients [85–87]. Moreover, synovial fibroblasts isolated from PsA patients express high level of IL-17RA and produce enormous amount of IL-6, CXCL-8, and matrix metalloproteinase 3 in response to IL-17A [86].

The efficacy of secukinumab (10 mg/kg i.v. at baseline and week 3) in patients with active moderate to severe PsA was initially evaluated in a double-blind, placebo–controlled, proof-of-concept phase 2 study. The primary end point, American College of Rheumatology (ACR) 20 response at week 6, was not met. However, good European League Against Rheumatism responses and remission (Disease Activity Score using 28 joint count (DAS28) scores $\leq$2·6) at week 6 occurred more frequently in the secukinumab group (both 22% versus 9%), and significant decreases compared to placebo were seen for health assessment questionnaire–disability index (HAQ-DI) and the SF-36 physical component

score. Treatment with secukinumab also caused a significant reduction in serum concentration of β-defensin-2, a potential surrogate marker of psoriatic disease activity [88]. Data of phase 3 clinical trials in PsA with ixekizumab have not yet been published. Prespecified subanalyses of the phase 3 FIXTURE and ERASURE studies showed that secukinumab improves physical function in patients with psoriasis and concomitant PsA [77]. These preliminary results were confirmed by a phase 3 double-blind placebo–controlled trial, in which secukinumab was given intravenously (10 mg/kg) at week 0, 2, and 4, followed by subcutaneous doses of either 150 mg or 75 mg every 4 weeks or placebo. Response rates at week 24 were significantly higher in the group receiving secukinumab and improvements were maintained at week 52. However, infections and serious adverse events, such as strokes or myocardial infarction, were more frequent in secukinumab groups than placebo [89]. Interestingly, a double-blind, placebo–controlled trial of patients with another immune-mediated disease related to PsA, ankylosing spondylitis, showed that two intravenous infusions of 10 mg/kg secukinumab yielded favorable results [90].

22.9 IL-17A in Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease leading to synovial inflammation and destruction of cartilage and bone. Like many autoimmune diseases, RA is supposed to occur in genetically at risk individuals, in response to a trigger or triggers [91]. IL-17A-positive cells are abundant in synovial fluid of patients with RA and elevated levels of IL-17A in the synovia correlate with greater disease activity [92]. IL-17A exerts multiple biological functions that could be relevant for RA-associated joint inflammation, cartilage degradation, and bone erosion. For instance, IL-17A enhances the production of chemokines (e.g., CCL2, CCL20, and CXCL-8) and cytokines (e.g., TNF, IL-1β, and IL-6) from synovial fibroblasts, increases production of cartilage-degrading matrix metalloproteinases, blocks new matrix synthesis by chondrocytes, and stimulates bone reabsorption [93]. The pathogenic role of IL-17A in RA is supported by studies in animal models of arthritis. Neutralization of endogenous IL-17A with a soluble murine IL-17 receptor protein or anti-IL-17A suppresses synovial inflammation and joint damage in collagen-induced arthritis (CIA) in mice [94]. In contrast, IL-17A overexpression by gene transfer or intra-articular injection aggravates the disease course [95]. Consistently, the pathologic process characteristic of CIA is markedly reduced in IL-17A-null mice [96].

22.10 Clinical Findings

Secukinumab was the first IL-17 blocker used in RA. In a proof-of-concept study in patients with active RA despite stable methotrexate therapy, 10 mg/kg intravenous secukinumab at baseline and week 3 significantly improved the clinical and laboratory parameters of joint inflammation. The activity of secukinumab

was evident by week 1 and maintained until the final assessment at week 16 [74]. A subsequent randomized, placebo–controlled phase 2 trial showed a partial clinical benefit of subcutaneous secukinumab at doses of 75, 150, or 300 mg monthly. The clinical efficacy of secukinumab among responders was maintained through week 52, while patients who did not respond to secukinumab by week 16 derived little benefit from subsequent dose increase [97].

Ixekizumab was also evaluated initially in a proof-of-concept trial, which showed that the combined ixekizumab group (0.2, 0.6, or 2 mg/kg intravenous at baseline and weeks 2, 4, 6, and 8) was superior to placebo in reducing inflammatory scores in patients with active RA despite at least one conventional disease-modifying antirheumatic drug (DMARD) [98]. A subsequent phase 2 study confirmed the efficacy of ixekizumab in patients on background DMARD therapy either biologically naive or resistant to TNF blockers [99]. In contrast, a randomized, double-blind, placebo–controlled phase 2 trial showed the clinical inefficacy of subcutaneous brodalumab in biologically naïve RA patients [100]. Therefore, brodalumab is not being explored further in RA.

22.11 IL-17B, IL-17C, and IL-17D

The biological activities and role of IL-17B, IL-17C, and IL-17D in immune-inflammatory diseases remain largely unknown. These cytokines bind to IL-17RB and IL-17RE and share with IL-17A-F some proinflammatory effects [101]. IL-17B and IL-17C promote neutrophil recruitment and induce TNFα and IL-1β *in vivo* and *in vitro* [102,103]. IL-17C also stimulates epithelial cells to produce proinflammatory mediators and antimicrobial peptides and enhances tight junctions [104]. IL-17C is pathogenic in a murine model of skin inflammation but exerts a protective function in DSS colitis. This later finding is in accordance with the demonstration that IL-17C-deficient mice develop a more severe DSS colitis compared to wild-type mice [105]. IL-17C is synthesized by intestinal epithelial cells stimulated by intestinal microbiota (e.g., flagellin), but it is not yet known if such a production is dysregulated during IBD [106]. IL-17C is abundant in psoriatic skin lesion and overexpression of this cytokine in keratinocyte leads to psoriasis-like lesions [107]. Despite its ability to induce proinflammatory molecules and to mediate articular damage, IL-17B plays a protective role in DSS-induced colitis and *Ci*trobacter *rodentium*-driven intestinal inflammation [108]. IL-17D stimulates IL-6, IL-8, and GM-CSF *in vitro*, but its contribution in immune-mediated pathologies has yet to be clarified [109].

22.12 IL-25 (IL-17E)

Although originally identified as a member of the IL-17 cytokine family, IL-25 has just 20% homology with IL-17A and reveals unique biologic activities. IL-25 is highly produced by polarized Th2 cells and positively regulates Th2 cell responses. When injected into mice, IL-25 induces IL-4, IL-5, and IL-13 gene

expression, increases IgE production and determines pathological changes in different tissues such as liver, lungs, heart, and the digestive tract [110]. These lesions are characterized by inflammatory infiltration, hyperplasia, and eosinophilia [111]. IL-25 also enhances IL-9 synthesis [112]. The positive effect of IL-25 on Th2 cell responses is mediated by the ability of this cytokine to act directly on human Th2 cells, which bear a specific IL-25R, as well as on basophils, eosinophils, and mast cells, which indirectly promote Th2 cell differentiation [113,114]. In gut-associated lymphoid tissues, IL-25 promotes the accumulation of a lineage-negative cell population, which is involved in Th2 cell responses against parasites [115] and enhances responsiveness of innate lymphoid cells (ILC) type 2 to IL-33, thus contributing to helminth expulsion [116]. Consistently, IL-25-knockout mice infected with *Trichuris muris* fail to eradicate the infection, due to an impairment in type 2 immunity [117]. In *Nippostrongylus brasiliensis*-infected mice, intestinal epithelial cell-derived IL-25 stimulates ILCs to produce IL-13, thus leading to a rapid worm clearance [118].

IL-25 production by commensal bacteria-stimulated epithelial cells is, however, a physiologic phenomenon in the gut, which contributes to restrain detrimental immune responses. For example, intestinal epithelial cell-derived IL-25 targets IL-25R-expressing lamina propria dendritic cells and macrophages with the downstream effect of reducing Th1 and Th17 cell response and attenuating experimental colitis [119,120]. In colitic mice, the anti-inflammatory effect of IL-25 is associated with the enhanced expression of alternatively activated macrophages [121], a subset of macrophages with regulatory properties. This phenomenon is not organ-specific as in mouse models of renal ischemic/reperfusion injury, IL-25 significantly ameliorates renal function and such a protective effect is associated with increased levels of Th2 cytokines and induction of alternatively activated macrophages [122].

In the human gut, IL-25 is also produced by subepithelial macrophages and its synthesis is positively regulated by TGF-β1 [120,123]. Production of IL-25 is markedly reduced in the inflamed intestine of patients with CD and patients with UC. This decrease is not an epiphenomenon of the ongoing inflammation because IL-25 overproduction occurs in the duodenum of patients with celiac disease, another intestinal immune-mediated disorder [120]. The specificity of this decrease suggests that in IBD there might be factors that selectively downregulate IL-25. One of such factors is TNF-α, a proinflammatory produced in excess in IBD but not in celiac disease. Indeed, stimulation of normal colonic biopsies with TNF-α inhibits IL-25 expression, while anti-TNF-α restores IL-25 expression in IBD patients [123]. Defects in TGF-β1 activity, due to the increased expression of its intracellular inhibitor Smad7 [40], could also be responsible for IL-25 downregulation in IBD as knockdown of Smad7 with a specific antisense oligonucleotide in IBD mucosal explants increases IL-25 synthesis.

IL-25 production is constitutive in human and mouse liver and is reduced during fulminant hepatitis. In mouse models of fulminant hepatitis, IL-25 administration prevents and cures liver damage due to its capacity to enhance the accumulation of myeloid-suppressor cells that inhibit T-cell activation [124].

The IL-25-mediated promoting effects on Th2 cell responses well fit with the prominent role of this cytokine in allergic diseases. Genetic deletion of IL-25

decreases lung inflammation in a mouse model of Th2/Th9-driven allergic-induced asthma [112]. In the same model, overexpression of IL-25 in lung epithelial cells leads to mucus production and airway inflammation [113]. Both IL-25 and IL-25R are upregulated on epithelial cells of human asthmatic lung [114,125], and this cytokine may be involved in virus-induced exacerbations of the disease [126]. In experimental acute OVA-induced allergic asthma, blocking IL-25 prevents airway hyperresponsiveness during both the sensitization and the challenge phases leading to reduced levels of IL-5 and IL-13 production, eosinophil infiltration, goblet cell hyperplasia, and serum IgE secretion [127]. Chronic exposure of murine airways to IL-25 promotes angiogenesis and may contribute to bronchial vascular remodeling [128]. Moreover, IL-25 levels are increased in nasal lavages of patients with allergic rhinitis, compared to normal controls [129]. IL-25, together with IL-33, is also the pivotal cytokine inducing innate lymphoid cells type 2 in a mouse model of atopic dermatitis and plays a role in skin inflammation [130].

22.13 Conclusions

In recent years, a considerable amount of work has been done to clarify the expression and role of IL-17 cytokines in many immune-inflammatory pathologies, thus paving the way for the development of novel biologics to treat patients with such disorders. Some of these compounds have already reached the clinic and promising results have been documented in patients with psoriasis, PsA, and RA but not in patients with IBD. The cellular context in which IL-17 cytokines are produced, the stage or duration of disease, previous therapy, and the genetic architecture of the disease in individual patients could be factors influencing the therapeutic efficacy of IL-17 blockers in the various immune-mediated disorders. There may also be differences in proportions of subjects in these conditions who have IL-17-dependent pathways. The demonstration that such cytokines are constitutively produced in various organs highlights, however, their role both in physiologic processes (e.g., maintenance of epithelial barriers) and in the host defense against pathogens and neoplasia. So far, safety findings from clinical trials have indicated that IL-17A pathway inhibition results in higher infection rates compared to placebo. Therefore, further work is needed to better understand the physiologic role of IL-17 cytokines and provide insight into potential risks of IL-17 blockade.

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23

IL-6 Superfamily

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23.1 Introduction

Cytokine is a soluble protein that functions as a messenger to transmit intercellular signals to distant organs, to adjacent cells in local sites in a paracrine manner, or to identical cytokine-producing cells in an autocrine manner. Simply stated, many cytokines use multicomponent receptor systems, including cytokine-binding and signal transducing receptors on target cells to induce intracellular signals. Expression of cytokine-binding receptors on target cells dictates which organs and which cells respond to the cytokine. Binding of a cytokine to its receptor leads to the formation of homo- or heterodimerization of signaling receptors, and triggers activation of intracellular signaling pathways. Interleukin-6 (IL-6) is a prototypical cytokine featuring functional pleiotropy and redundancy, the latter often leading to overlaps with other members of the IL-6 family of cytokines. This chapter focuses on the signaling system of the IL-6 family of cytokines and recent progress in their clinical application.

23.2 IL-6 and its Family of Cytokines

23.2.1 Biological Activity and Signaling System of IL-6

IL-6 exerts pleiotropic effects on inflammation, immune response, and hematopoiesis [1–3]. It is produced at the inflammation site by infection or tissue damage. IL-6 induces production of acute phase proteins such as C-reactive protein (CRP), serum amyloid A, fibrinogen, and hepcidin in liver [4]. It also

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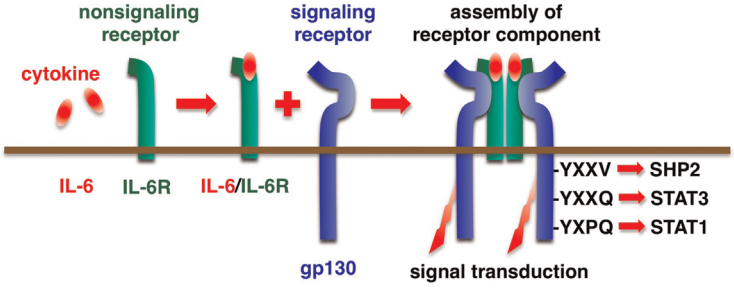


Figure 23.1 Nonsignaling and signaling receptor components. Visualization of the IL-6 receptor system representing the IL-6 family of cytokines. IL-6 binds to IL-6R to form the IL-6/IL-6R complex, which induces the gp130 homodimer to transduce intracellular signaling. Cytoplasmic tyrosine-based motifs of gp130, YXXV, or YXXQ (Y: tyrosine; Q: glutamine; V: valine; and X: any amino acid), select the SH2-containing molecule, SHP2, or STAT3 to lead the downstream signal.

plays an important role in acquired immune response to induce differentiation of activated B cells into antibody (Ab)-producing cells and to prolong survival of plasmablasts [5], while it promotes the development of Th17 cells and follicular helper T cells by naïve T cells and inhibits the differentiation into regulatory T cells (Treg) [6]. Moreover, IL-6 can promote differentiation or proliferation of several nonimmune cells, such as cardiac myocytes, neuronal cells, hematopoietic cells, vascular endothelial cells, and others. When IL-6 binds to its specific IL-6 receptor (IL-6R) with low affinity, the resultant IL-6/IL-6R complex associated with gp130, leading to homodimerization of gp130 to form a high-affinity IL-6-binding receptor complex, which in turn results in the formation of a hexamer structure consisting of two molecules each of IL-6, IL-6R, and gp130 (Figure 23.1) [7,8]. The cytoplasmic membrane-proximal domain of gp130 lacks enzymatic activity but is associated with JAK tyrosine kinases. Dimerization of gp130 allows JAKs to come close to each other, which then leads to phosphorylation in cytoplasmic tyrosine residues of gp130. The SH2 domain-containing molecules, the signal transducer and activator of transcription 3 (STAT3), and STAT1 and SH2 domain-containing protein-tyrosine phosphatase 2 (SHP2) are recruited to the tyrosine-phosphorylated motifs of gp130, YXXQ for STAT3, YXPQ for STAT1, and YXXV for SHP2 (Y: tyrosine, Q: glutamine, P: proline, V: valine, and X: any amino acid). STAT3 and STAT1 are then phosphorylated by JAK kinases and form STAT3-STAT3, STAT3-STAT1, or STAT1-STAT1 dimer via phosphorylated tyrosine and SH2 domain of each STAT molecule. They translocate to the nucleus, which produces the transcriptional output. SHP2 initiates activation of the Ras-MAP kinase pathway. Thus, the tyrosine-based motif in the cytoplasmic region of gp130 is a module for downstream signaling molecules [8–10]. The presence of a two-chain receptor system raises the possibility that the signaling chain is shared with some other cytokines. The above leads to the definition of the IL-6 family of cytokines as cytokines that are characterized by the use of gp130 as a common signal transducing receptor component, so that it is also known as the gp130 receptor cytokine family [11].

23.2.2 Original Members of IL-6 Family of Cytokines

The members of the IL-6 family of cytokines first identified comprised IL-6, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1), and IL-11. All of them feature a four-helix bundle structure and have similar effects on immune, hematopoietic, and nervous systems. The characteristic features of cytokines are the pleiotropic effect and functional redundancy. The pleiotropic property is explained by the wide distribution of signaling receptors on various types of cells. Functional redundancy resulting from different cytokines acting on the same cell to induce similar responses was observed among the first members of the IL-6 family of cytokines. These responses consisted of acute-phase protein induction in hepatocytes, differentiation of myeloid leukemia M1 cells, neurite growth in neuronal cells, and stimulation of cardiac myocytes. Later, cardiotrophin-like cytokine factor 1 (CLCF1) and viral IL-6 (vIL-6) were also included in the IL-6 family. CLCF1, referred to as neurotrophin-1 or B-cell-stimulating factor 3, features neurotrophic and B-cell-stimulating functions [12], while vIL-6 is a viral protein produced by human herpesvirus 8 (also referred to as Kaposi sarcoma-associated herpesvirus) with functions that overlap with those of many of IL-6 cytokines [13].

23.2.3 New Members of IL-6 Family of Cytokines

The two new members, IL-27 and IL-35, are structurally different from the original members of the IL-6 family. They are heterodimeric cytokines since IL-27 consists of p28 and EB virus-induced molecule 3 (EBI3) and IL-35 of p35 and EBI3. Originally, the IL-27p28 subunit was identified as the IL-6-related protein IL-30 [14] and the IL-35p35 subunit was cloned as a subunit of IL-12 and is homologous with IL-6. Both p28 and p35 have a four-helix bundle structure. Later, it was found that the complex of p28 or p35 with EBI3 produced the heterodimer cytokine IL-27 or IL-35, respectively. IL-27 and IL-35 belong to the IL-12 family of cytokines with a feature of heterodimeric structure. However, their subcomponent EBI3 is structurally related to the soluble IL-6R (sIL-6R), so that IL-27 and IL-35 are analogous with the IL-6/sIL-6R complex, and it is thought that they have evolved from the IL-6 family of cytokines and a soluble form of their original receptor. IL-27 and IL-35 are, therefore, included in the IL-6 family of cytokines, so that the current members of the IL-6 family consist of IL-6, LIF, OSM, CNTF, CT-1, IL-11, CLCF1, vIL-6, IL-27, and IL-35.

23.2.4 Receptor Components of IL-6 Family of Cytokines

Receptor components of the IL-6 family of cytokines are summarized in Table 23.1. IL-6, CNTF, IL-11, and CLCF1 first bind to nonsignaling receptors with short cytoplasmic domain and next to the ligand–receptor complex; IL-6/IL-6R, CNTF/CNTFR, IL-11/IL-11R, or CLCF1/CNTFR associates with and activates signaling receptors. While CNTF primarily binds to CNTFR, CNTF can also bind to IL-6R lower but with an affinity of 60 times lower than that of IL-6 binding to IL-6R [15]. For CT-1, a GPI-linked receptor has been postulated [16].

Table 23.1 IL-6 family of cytokines and their receptor components.

IL-6 Family of Cytokines	Nonsignaling Receptor	Signaling Receptor
IL-6	IL-6R	gp130 + gp130
LIF	—	gp130 + LIFR
OSM	—	gp130 + LIFR gp130 + OSMR
CNTF	CNTFR	gp130 + LIFR
CT-1	?	gp130 + LIFR
IL-11	IL-11R	gp130 + gp130
CLCF1 CLCF1+CLF	CNTFR	gp130 + LIFR
vIL-6	—	gp130 + gp130
IL-27 (p28+EBI3)	—	gp130 + IL-27R
IL-35 (p35+EBI3)	—	gp130 + IL-12Rβ2 gp130 + gp130 IL-12Rβ2 + IL-12Rβ2

IL-6 cytokine family members transduce their signals via the gp130 homodimer (gp130 + gp130) or gp130 heterodimer (gp130 + LIFR, OSMR, IL-27R, or IL-12Rβ2). IL-6, CNTF, IL-11, CLCF1, and CLCF1/CLF first bind to their respective nonsignaling receptor. IL-27 and IL-35 are heterodimeric cytokines and activate gp130-IL-27R and gp130-IL-12Rβ2 heterodimer and gp130-gp130 or IL-12Rβ2-IL-12Rβ2 heterodimer, respectively.
Abbreviations: LIF, leukemia inhibitory factor; OSM, oncostatin M; CNTF, ciliary neurotrophic factor; CT-1, cardiotrophin 1; CLCF1, cardiotrophin-like cytokine factor 1; CLF, cytokine-like factor; EBI3, EB-virus-induced molecule 3.

The cytokine/nonsignaling receptor complex assembles the gp130-gp130 homodimer or gp130-LIFR heterodimer to transduce signals. CLCF1 can form a heteromeric complex with cytokine-like factor-1 (CLF) that belongs to the cytokine receptor family. Both CLCF1 itself and the CLCF1/CLF complex bind to CNTFR and induce the gp130-LIFR heterodimer. In contrast, LIF, OSM, or vIL-6 can directly bind to and assemble the gp130-LIFR, the gp130-OSMR heterodimer, or the gp130-gp130 homodimer, respectively, without binding to a nonsignaling receptor [17]. The functional redundancy of these cytokines is clearly evidenced by the model in which signal transducing receptors gp130 and LIFR are shared among family members; moreover, the cytoplasmic regions of both gp130 and LIFR contain YXXQ, YXPQ, and YXXV motifs that recruit and activate the same set of signaling molecules, that is, STAT3, STAT1, and SHP2 [9].

IL-27 uses IL-27R (also referred to as WSX-1) and gp130 for its signal. Although IL-27 shares gp130 with IL-6, transgenic overexpression of IL-27p28 *in vivo* was found to reduce antigen-specific antibody production. It was shown that IL-27p28 by itself is able to inhibit IL-6-gp130 signaling independent of EBI3 [18]. The effect of IL-27 on T cells is also different from that of IL-6, since IL-27 inhibits differentiation of Th17 and induces IL-10-producing Treg-like populations. The opposite effects of IL-6 and IL-27 on T cells derive from the functional difference in signaling receptors of the gp130-gp130 homodimer and the gp130-IL-27R heterodimer. Although both IL-6 and IL-27

can induce activation of STAT3 and STAT1, analysis of transcriptional output by IL-6 and IL-27 revealed that transcriptional output of STAT3 is induced mainly by IL-6, whereas STAT1 is preferentially activated by IL-27 [19]. The difference in balance between STAT3 and STAT1 may thus determine the cellular response by cytokine stimulation [20]. IL-35, the most recently identified member of the IL-6 family of cytokines, is thought to be an inhibitory cytokine produced by Treg [21]. A receptor reconstitution study reported that IL-35 could induce the gp130-IL-12Rβ2 heterodimer, the gp130-gp130 homodimer, or the IL-12Rβ2-IL-12Rβ2 homodimer [22]. Although IL-35 uses gp130 as a receptor component, the effect of IL-35 on T cells is different from that of IL-6, since IL-35 inhibits the development of Th1 and Th17 cells.

23.2.5 Soluble Forms of Receptors of IL-6 Family of Cytokines

Soluble forms of cytokine receptor components in human serum regulate functions of their ligands under certain physiological and pathological conditions. Soluble forms of the nonsignaling receptors of the IL-6 family of cytokines, that is, sIL-6R, sIL-11R, and sCNTFR, can bind to their respective ligands and activate signaling receptors on target cells [23,24]. Proteolysis of the membrane-bound receptors and alternative splicing of receptor mRNA are the mechanisms by which soluble receptors are created. For example, sIL-6R occurs in serum at around 79 ng/ml, binds to IL-6, and transduces the IL-6 signal to cells that express gp130 but not cell surface IL-6R and are unresponsive to IL-6 alone (Figure 23.2). The IL-6/sIL-6R-mediated signal is known as the IL-6 trans-signaling, while the IL-6 signal mediated through cell surface IL-6R is differently classified as the classical signaling [25]. On the other hand, soluble forms of signaling receptors such as sgp130, sLIFR, and sIL-27R are natural inhibitors by functioning as antagonists to inhibit signal transduction. sgp130 occurs in human serum at around 390 ng/ml, can bind to IL-6/sIL-6R, and inhibit IL-6 function. sgp130 is also an antagonist to CNTF/sCNTFR, LIF, and OSM [23]. Similarly, sLIFR is present in human serum at around 4.3 ng/ml, binds to LIF, and inhibits functioning of LIF [26], while its quantity increases 12 times during pregnancy [27].

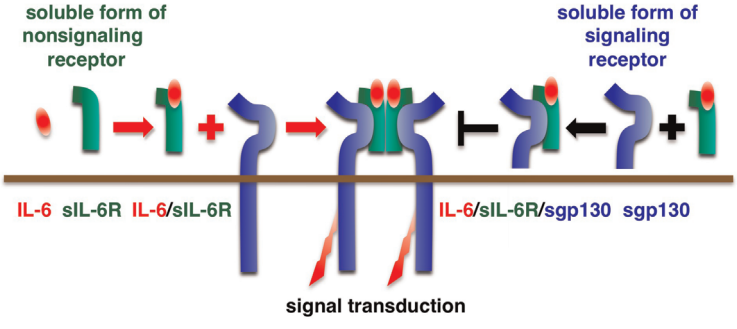


Figure 23.2 Different functions of soluble forms of nonsignaling and signaling receptors. The figure shows that IL-6 binds to sIL-6R and that the IL-6/sIL-6R complex associates with and activates cell surface gp130 in the form of IL-6 trans-signaling, whereas sgp130 blocks IL-6/sIL-6R to associate with gp130.

sIL-27R is found in human serum at around 10.3 ng/ml and inhibits IL-27 binding to its cell surface receptor and IL-27 function [28].

23.2.6 Redundant and Nonredundant Functions of IL-6 Family of Cytokines

Cytokine-deficient mouse or neutralizing antibody (Ab) against IL-6 family members was used for *in vitro* studies addressing which function of which member is compensated or unique for the respective cytokine. An IL-6 inhibitor has been successfully used for the treatment of inflammatory diseases in clinics. This inhibitor is tocilizumab, a humanized anti-IL-6R monoclonal antibody that binds to transmembrane IL-6R and sIL-6R and inhibits both IL-6 classical signaling and trans-signaling. Various clinical trials of tocilizumab for patients with chronic inflammatory diseases, such as Castleman disease, rheumatoid arthritis (RA), and idiopathic juvenile arthritis, have verified its outstanding effectiveness and tocilizumab is now clinically used for the treatment of these diseases [1–3]. Although *in vitro* studies using the hepatocyte cell line previously showed that members of the IL-6 family of cytokines overlap with IL-6 to induce acute-phase proteins, IL-6-deficient mouse or inhibition of IL-6 by administration of tocilizumab to patients demonstrated almost complete suppression of acute-phase protein production, indicating that IL-6 plays an essential role in acute-phase protein production and that other members of the IL-6 family do not offset this function. In fact, it was reported that circulating levels of IL-11 and LIF were not elevated in spite of the inflammation with high level of acute-phase protein [29].

In the case of Ab production, IL-6 induces differentiation of activated B cells into Ab-producing cells. Tocilizumab treatment corrects hypergammaglobulinemia in patients with Castleman disease, but the treatment dose not reduce immunoglobulin (Ig) concentration to abnormally low levels and appears not to impair Ab production in response to influenza and pneumococcal polysaccharide vaccination [30,31], suggesting that in humoral immune response the pathological elevation of Ig depends on IL-6, but other cytokines including those of the IL-6 family may interfere with physiological Ab production.

23.3 Clinical Application of Members of IL-6 Family of Cytokines and Their Inhibitors

23.3.1 Clinical Application of Members of IL-6 Family of Cytokines

Molecular characterization of the signaling system of the IL-6 family of cytokines and clarification of their biological activity and pathological roles raised expectations that a cytokine itself or its specific inhibitor might be a novel drug for a variety of diseases. So far, IL-11 is the only cytokine approved for the clinical treatment. Recombinant human IL-11, oprelvekin, stimulates megakaryocytes to increase platelet and prevents severe thrombocytopenia after myelosuppressive chemotherapy [32]. Clinical trials of LIF to evaluate its efficacy for chemotherapy-induced peripheral neuropathy, platelet recovery following chemotherapy, and

infertility in women were also performed, but the results were not promising [33]. The result of a clinical trial of CNTF for retinal degenerative diseases was reported [34]. CNTF was successfully delivered intraocularly for long term via intraocular encapsulated cell implant, but therapeutic benefit was not obvious.

23.3.2 Clinical Application of Inhibitors of Members of IL-6 Family of Cytokines

Gp130, a common signal transducer of the IL-6 family of cytokines, is an attractive target molecule for inflammatory diseases and cancers, and this led to the development of several gp130 inhibitors [35]. Targeted therapies against STATs are also being developed, while the JAK1/2 inhibitor ruxolitinib has been already approved for the treatment of psoriasis, myelofibrosis, and RA, the JAK3 inhibitor to facitinib has been approved for the treatment of psoriasis and RA [36,37]. Although a phase II clinical trial of a humanized anti-OSM Ab (GSK315234) in patients with RA showed potential therapeutic effect [38], so far IL-6 blockade strategy is the only successful clinical application of members of the IL-6 family of cytokines.

23.3.3 Present Status and Prospects of IL-6 Blockade Therapy

As explained before, IL-6 exerts various biological effects by acting on different target cells. When infections or tissue injuries occur, IL-6 is immediately produced from macrophages and monocytes by stimulation of pattern recognition receptors with pathogen- or danger-associated molecular patterns and then triggers immune, acute phase, and hematological responses [3]. IL-6 thus contributes to host defense against such emergent stresses. When the source of stress is removed from the host, the production of IL-6 is terminated and results in normalization of serum levels of IL-6 (less than 4 pg/ml) and CRP. However, dysregulated IL-6 production is involved in disease development in two ways [39,40]. First, during immune response against infectious agents or tissue injuries, excessive production of IL-6 causes fatal multiple organ failure that can be diagnosed as septic shock or systemic inflammatory response syndrome. In this case, serum IL-6 concentration can increase by a factor from one thousand to one million and the level is associated with disease severity and mortality rate. Second, chronic dysregulated and persistent IL-6 production by a particular cell population leads to the development of various chronic immune-mediated diseases.

The first demonstration that IL-6 was pathologically involved in disease development was observed in a patient with cardiac myxoma [41]. The culture fluid obtained from the myxoma tissue of this patient with unclassified connective tissue disease contained a large quantity of IL-6 and the myxoma tissues stained positively with anti-IL-6 Ab. The patient had presented with fever, polyarthritis, Raynaud phenomenon, increased serum CRP level, anemia, and hypergammaglobulinemia with positivity for antinuclear factor, rheumatoid factor, and anti-DNA Ab. Subsequent studies have also found excessive expression of IL-6 in synovial fluids of RA [42], germinal center B cells in swollen lymph nodes of Castleman disease [43], myeloma cells [44], and peripheral blood cells or

infiltrating cells in tissues involved in various other diseases, as well as in many tumor cells [1–3,39,40,45]. However, the mechanisms causing dysregulated IL-6 production remain unknown. The regulation of IL-6 gene expression is mediated by transcriptional factors, micro-RNAs and RNA-binding proteins [39,40]. Recently, several micro-RNAs and regulatory RNA-binding proteins such as Arid5a, regnase-1, and roquin have been identified [46–49], so that elucidation of these molecules is important for clarification of the pathogenesis of IL-6-related diseases.

The concept of IL-6 blockade treatment strategy for diseases has been supported by numerous experimental findings obtained with the use of IL-6 gene knockout mice or administration of neutralizing anti-IL-6 or anti-IL-6R Ab. For instance, IL-6 blockade resulted in a striking reduction in susceptibility to Castleman disease-like symptoms in IL-6 transgenic mice, and suppressed disease development in models of RA, systemic lupus erythematosus, systemic sclerosis, polymyositis, multiple sclerosis, uveitis, endotoxin shock, and various other diseases [2,3].

These findings led to the development of the humanized anti-IL-6R monoclonal Ab tocilizumab [50]. Starting with the first-in-man trial for a patient with refractory multiple myeloma at Osaka University Hospital, various clinical trials of tocilizumab have been performed worldwide. The results of these trials verified that tocilizumab is strikingly effective for the treatment of several chronic inflammatory diseases. Tocilizumab is now approved for the treatment of RA in more than 130 countries, as well as for systemic or polyarticular juvenile idiopathic arthritis in Japan, USA, EU and India, and for Castleman disease in Japan and India. Moreover, numerous clinical trials of tocilizumab for other refractory diseases and off-label use of tocilizumab for orphan diseases strongly suggest that it can be widely used for the treatment of various diseases. These include systemic and organ-specific autoimmune diseases, acute severe systemic inflammatory diseases with cytokine storm such as cytokine release syndrome and hemophagocytic histiocytosis, chronic inflammatory diseases including autoinflammatory syndromes, and other diseases such as myocardial infarction, atherosclerosis, diabetes mellitus, atopic dermatitis, sciatica, amyotrophic lateral sclerosis, schizophrenia, and various cancers and cancer-associated cachexia (Table 23.2) [2,39,40]. In particular, accumulated evidence provides strong indications that tocilizumab appears to be highly promising for the treatment

Table 23.2 IL-6 blockade therapy for a wide variety of diseases.

Autoimmune Disease
Rheumatoid arthritis (approval in more than 130 countries)
Systemic sclerosis[c]
Systemic lupus erythematosus
Vasculitis syndrome (Giant cell arteritis[b,c], Takayasu disease[c])
Polymyositis and dermatomyositis[b]
Sjogren's syndrome[b,c]
Relapsing polychondritis

- Autoimmune hemolytic anemia
- Acquired hemophilia
- Neuromyelitis optica and multiple sclerosis
- Cogan's syndrome

Inflammatory Disease

- Castleman disease (approval in Japan and India)
- Juvenile idiopathic arthritis (approval in Japan, USA, EU, and India)
- Cytokine storm (cytokine release syndrome, septic shock, systemic inflammatory response syndrome, hemophagocytic histiocytosis[b], and macrophage activation syndrome)
- Adult onset Still's disease
- Amyloid A amyloidosis
- Polymyalgia rheumatica[b] and remitting seronegative symmetrical synovitis with pitting edema
- Crohn's disease
- Behcet's disease and uveitis[a,b]
- Autoinflammatory diseases
- Graft versus host disease[a,b]
- IgG4-related diseases
- Pulmonary arterial hypertension
- Eosinophilic fascilitis

Endocrinal and Metabolic Disease

- Type I and II diabetic mellitus[b]
- Atherosclerosis
- Thyroid-associated ophthalmopathy[b]

Bone and Cartilage Disease

- Osteoarthritis[c]
- Fibrous dysplasia of bone[b]

Neuropsychiatric Disease

- Amyotrophic lateral sclerosis[b]
- Schizophrenia[b,c]
- Sciatica

Neoplastic Disease and Paraneoplastic Syndrome

- Multiple myeloma, solid carcinoma, B-cell chronic lymphocytic leukemia[a], Erdheim–Chester disease[b], and cachexia

Others

- Myocardial infarction[b], atopic dermatitis, kidney transplantation[a,b], and HIV infection[a,b]

Tocilizumab, a humanized anti-IL-6R monoclonal Ab, is currently approved for the treatment of rheumatoid arthritis, Castleman disease, and juvenile idiopathic arthritis. Off-label use of tocilizumab indicates that it will be widely applicable for the treatment of various diseases. [a], [b], or [c] denote, respectively, ongoing phase I, II, or III clinical trial of tocilizumab registered in ClinicalTrials.gov. For neuromyelitis optica, a phase III clinical trial of SA237, a new fully human IL-6R Ab generated from tocilizumab by techniques for structural antibody optimization, is in progress.

of systemic sclerosis, large-vessel vasculitis, neuromyelitis optica, adult-onset Still's disease, amyloid A amyloidosis, and cytokine release syndrome, for all of which clinical trials are in progress.

23.4 Concluding Remarks

IL-6, a prototypical cytokine with pleiotropic and redundant activity, belongs to the IL-6 family of cytokines, which also includes IL-11, LIF, OSM, CT-1, CNTF, CLCF1, IL-27, IL-35, and viral IL-6. This cytokine family uses a common signal transducing molecule, gp130, and shows some redundant but not uniformly identical biological activity. Molecular clarification of the signaling system of the IL-6 family of cytokines and recent progress in elucidation of their biological functions and pathological roles have facilitated the clinical application of the cytokines themselves or their inhibitors to a variety of diseases. The physiological and pathological role of IL-6 is the most characterized among the members of his family. IL-6 contributes to host defense against infections and tissue damages, but dysregulated excessive or persistent production of IL-6 plays a pathological role in various kinds of diseases. IL-6 blockade therapy is currently in clinical use for the treatment of several diseases including RA, juvenile idiopathic arthritis, and Castleman disease, while this procedure is expected to have a wide range of applications for a large variety of diseases.

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24

Type I and II Cytokine Superfamilies in Inflammatory Responses

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24.1 Introduction

Protective immunity relies on the decision-making capabilities of the cellular immune response. Is the microbe in question pathogenic, opportunistic, or commensal? Is the site of exposure conducive to repair, such as the skin, or will inflammatory responses damage nonregenerating tissues, such as neurons? Is the pathogen in question extracellular or intracellular, and which effector mechanisms need to be mobilized in response? When should the immune response switch from inflammatory to reparative processes? If these decisions are made in the correct fashion, the immune system can provide immunity to any number of pathogenic microorganisms. However, when these decision-making processes are aberrantly regulated, the host becomes susceptible to infections.

Central to these decision-making processes is the ability of leukocytes to communicate to each other via the expression of soluble signaling factors such as cytokines. Signal transduction events mediated by cytokine binding to membrane receptors help to guide these cells in lineage commitment decisions, activation, and/or resolution of inflammatory processes (Figure 24.1). Cytokines can function in autocrine, paracrine, and endocrine fashions to drive systemic immune responses to localized infections. Cytokines are expressed by multiple leukocyte lineages in generalized functional "families," with key cytokines synergizing to shape the quality of an inflammatory response to an infectious agent. Regulated cytokine expression is central to protective immunity and the development of immunological memory, while unchecked expression of proinflammatory mediators leads to devastating immunopathologies and autoimmune disease.

In this chapter, an overview of cytokine networks will be discussed with a focus on the inflammatory outcomes of cytokine expression. Following an overview of cytokine superfamilies organized by structure similarity, specific cytokine subfamilies will be organized based on shared receptor usage and listed based on nomenclature. The purpose of this chapter is to give a big-picture context to the

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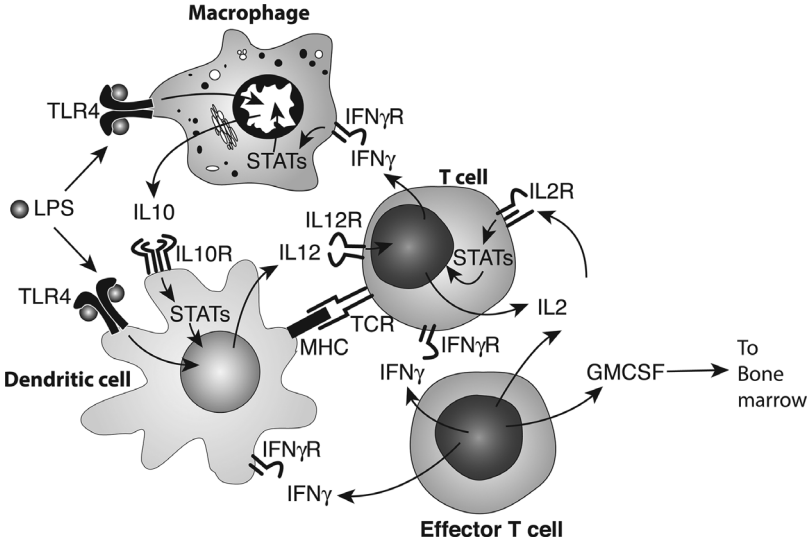


Figure 24.1 Signal transduction events mediated by cytokines.

complex, interdependent role of heterogeneous cytokine production in guiding specific immune responses to infectious agents.

24.2 Cytokine–Receptor Interactions

Cytokines are secreted proteins that serve to instruct both hematopoietic and nonhematopoietic cells during inflammatory responses. Cytokines can be delineated as a distinct class of signaling molecules from hormones based on two key factors. First, the kinetics of cytokine secretion (rapid, dramatic induction following specific extracellular stimuli) are quite different from that of hormone production, which is often prolonged at less dramatic concentrations to effect physiological changes. Second, cytokines can signal in autocrine, paracrine and endocrine fashions, whereas hormones function primarily through endocrine signaling. The rapid kinetics of cytokine production coupled with the ability to localize signaling to distinct sites of infection allows cytokines to tailor immune responses for specific pathogens at specific tissue sites.

Type I and type II cytokines exhibit a monomeric structure based on the grouping of four alpha-helix motifs. Certain members of the type I superfamily consist of combinations of shared protein subunits that associate following mRNA transcription, including the IL-12 subfamily. For example, association of IL-12α (p35) and IL-12β (p40) results in mature IL-12, whereas association of IL-12β with IL-23α (p19) results in mature IL-23 [1]. Type I and II superfamilies are translated as fully functional proteins (unlike the propeptides of the IL-1 family [2]) and are not normally anchored to the plasma membrane (as with the TNF family [3]). Subgroupings of type I and II cytokines are organized based on shared receptor usage and/or similarities in gene organization.

Cytokine receptors are remarkably heterogeneous, including simple single-pass transmembrane receptors of the immunoglobulin superfamily to complex multimeric receptors sharing common signaling chains (such as with the type I common β- and γ-chain cytokines). Cytokine receptors signal via protein kinase-dependent signal transduction cascades, often initialized by autophosphorylation of the cognate receptor followed by phosphorylation of downstream signal transduction proteins. Many of the type I and II cytokines utilize the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway for signal transduction [4]. Distinct cytokines will activate specific JAK/STAT pathways based on their receptor specificity, and in many cases cytokines with similar physiological functions will signal via common JAK/STAT pathways. For example, both type I and type II interferons (IFNs) participate in T-helper type 1 (T_H1) immune responses to intracellular pathogens by regulating expression of gene families regulated by STAT1 [5]. In this fashion, cytokines can have a regulatory effect on the function of multiple immune cell subtypes by regulating common gene expression pathways across cell lineages.

The cytokines to be discussed in this chapter will be grouped by similarities in either structure or receptor usage and will then be discussed in terms of their contribution to immunity. As the IL-17 superfamily of cytokines was discussed in previous chapters, the T-helper type 17 response to extracellular pathogens will be discussed only in terms of the contribution of other type I and II cytokines to the development of IL-17 producing cells.

24.3 Cytokine Superfamilies

24.3.1 Type I Superfamily

The type 1 cytokine superfamily includes the common γ-chain, common β-chain, and IL-12 subfamilies, as well as similar cytokine products with unique receptor characteristics (including IL-14, -16, -32, and -34). Included in the type 1 superfamily are the IL-6 and IL-6-like cytokines such as IL-11, -27, and -30; these cytokines will not be discussed in this chapter. In addition, type I superfamily members that belong in the IL-1, IL-17, and TNF subfamilies will not be discussed, as they have been reviewed in depth in previous chapters.

24.3.2 Common Gamma Chain

Cytokines that share the common γ chain for signal transduction include IL-2, -4, -7, -9, -13, -15, and -21 [6] (Figure 24.2). These cytokines all utilize the common γ chain (also known as IL2Rγ or CD132) for activation of JAK1/JAK3 and downstream STATs. The common γ chain does not exhibit any specificity for distinct type I cytokines; instead, it is responsible for the initiation of signal transduction following binding of cytokine to the cytokine receptor complex. Receptor specificity is conferred by the α and β subunits that complex with the common γ chain to make a functional type I cytokine receptor. For example, interaction of the common γ chain with IL-2Rα/CD25 and IL-2Rβ/CD122 results in a fully

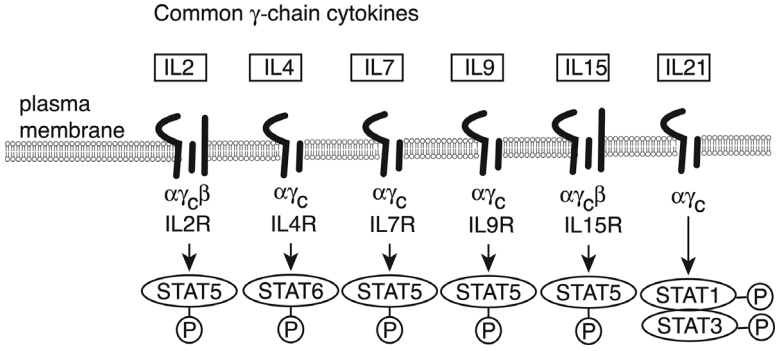


Figure 24.2 Common γ-chain cytokines.

functional IL-2 receptor [7]; however, replacement of the IL-2Rα subunit with IL-15Rα/CD215 results in functional IL-15 receptor [8], and interaction of the same γ subunit with only IL-7Rα/CD127 and no β subunit results in a functional IL-7 receptor [9]. As all three receptors share the same γ subunit with JAK3 signal transduction capability, these cytokines are all capable of activating STAT5a/b gene transcription in target cells [10]. In addition to STAT5 activation, cytokines within the common γ-chain family can also activate distinct STATs based on signal transduction capabilities provided by distinct cytokine receptor complexes, such as STAT1, STAT3, and STAT6 [10].

24.3.2.1 IL-2

IL-2, also known as T-cell growth factor, is responsible for guiding the development and proliferation of T cells. IL-2 is produced by activated T cells [11], certain myeloid lineage cells (e.g., dendritic cells [12]), and cells in the thymus [13] to support the proliferation of thymocytes and activated T cells. Upon activation with antigens bound to major histocompatibility complex (MHC), along with costimulatory ligand binding such as CD80 to T-cell-expressed CD28, activated T cells will produce IL-2 [14]. This IL-2 can signal through IL-2 receptors expressed in the IL-2-producing T cell (autocrine), as well as other T cells within the inflammatory microenvironment (paracrine) and T cells in distant sites (endocrine). IL-2 is critical for the development of immunological memory, and IL-2-dependent proliferative responses are central for the development of T-helper (T_H)-1, -2, and regulatory T-cell lineages [15]. Reports also suggest that IL-2 is essential for the development of T_H-9 through its ability to activate STAT5-dependent gene transcription [16]. However, IL-2 can suppress the development of T_H17 differentiation [17]. Animal models of IL-2 deficiency exhibit significantly decreased T-cell numbers in peripheral lymphoid organs [18]. As these animals age, they develop spontaneous autoimmune inflammation, indicating a loss of regulatory T-cell function and breakdown of peripheral tolerance [19].

24.3.2.2 IL-4

IL-4, also known as B-cell growth factor, links T- and B-cell activation during inflammatory responses. IL-4 promotes the proliferation of B cells and supports

class switching by activated B cells toward the production of immunoglobulin (Ig)-E [20]. IL-4 also drives MHC II expression by B cells, supporting antigen presentation [21]. IL-4 stimulation of activated CD4+ T cells results in differentiation toward the T_H2 lineage [22]; as T_H2 T cells can produce IL-4, this positive feedback loop promotes the development of T_H2 memory [23]. IL-4 also has potent effects on innate immunity; for example, macrophages stimulated with IL-4 exhibit alternative activation, characterized by a reduction in pathogen clearance phenotypes and an increase in reparative functions [24]. IL-4-deficient mice exhibit significantly decreased IgE production [25] and T_H2 T-cell function [26], resulting in resistance to the development of experimental allergy models [27].

24.3.2.3 IL-7

IL-7-dependent signals are critical for the differentiation of lymphoid progenitor cells from multipotent hematopoietic progenitors. IL-7 is produced in sites of lymphoid differentiation, particularly in the bone marrow and thymus [28]. Stromal cells in these tissues produce IL-7 to support lymphopoiesis and the development of T and B cells [29]. Stromal cells in other tissues can also produce IL-7, including hepatocytes [30]. IL-7 is also produced by structural cells in barrier tissues (e.g., keratinocytes [31] and epithelial cells [32]) for maintenance of lymphocytes in mucosal surfaces. The IL-7 receptor (IL-7R) is expressed by leukocytes and consists of the IL-7 receptor α chain (CD127) complexed with the common γ chain [33]. STAT5 activation by IL-7 binding to the IL-7R is a critical step in T-cell development; as such, IL-7 knockout animals exhibit an atrophied thymus with reductions in total thymocytes and peripheral T cells [34]. IL-7 is also important for the maintenance of memory T cells in the periphery; IL-7R expression can delineate effector from central memory T cells [35] and T-helper lineage memory cells from long-lived regulatory T cells [36].

24.3.2.4 IL-9

IL-9 is the primary cytokine produced by the T-helper lineage known as T_H9 T cells [37]. IL-9 is also produced by members of the innate lymphoid cell (ILC) lineage, most notably ILC2 cells [38]. The initial characterization of IL-9 indicated a role for this cytokine in promoting T_H2 responses, as IL-9 could synergize with other type 2 cytokines to promote class switching to I_gE and immunity to parasitic infections [39]. However, T_H9 T cells and ILC2 cells appear to have a much broader function in immune responses, both helping to shape inflammation through concomitant production of cytokines such as IL-10 and helping to skew type 1 responses toward type 2 [40]. The IL-9 receptor is a heterodimer consisting of the IL-9 receptor α chain (CD129) with the common γ receptor, and is expressed in lymphoid cells such as T cells, myeloid lineage cells such as eosinophils and mast cells, and certain epithelial cells [41]. IL-9 functions to promote the differentiation and effector function of target cells. For example, the type 2 promoting functions of IL-9 manifest through its ability to stimulate mast cell and eosinophil differentiation and accumulation in inflamed tissues [42]. IL-9's ability to promote generalized type 2 responses and mast cell function makes IL-9 an important mediator of immunopathology during food allergy responses [43].

24.3.2.5 IL-15

IL-15 shares structural and functional similarity with IL-2 and as such is an important cytokine for the proliferation and survival of T cells. IL-15 is often found expressed as either a membrane-bound protein or in a complex with the IL-15 receptor α (CD215) [44]. The trans-presentation of IL-15 through cell–cell interactions (membrane-bound IL-15 or IL-15/IL-15Rα complex) or through interactions with soluble IL-15/IL-15Rα complexes proceeds via the IL-15 receptor complex, consisting of the high-affinity IL-15Rα complexed with the β and (common) γ chains of the IL-2 receptor [45]. Downstream activation of JAK3 and STAT5a/b proceeds in a similar fashion as with IL-2, driving similar antiapoptotic and proliferative signals [46]. IL-15 supports memory CD8+ T cell and NK cell homeostasis in the periphery [47]. IL-15 production by epithelial cells can activate the cytotoxic activity of intraepithelial lymphocytes (IELs) that serve as a first line of defense against pathogens [48]. Due to its ability to support cytotoxic T and NK cell functions, IL-15 is a potent inducer of immune responses against intracellular pathogens.

24.3.2.6 IL-21

IL-21 is a potent proinflammatory cytokine produced by CD4+ T cells, including T_H2, T_H17, and T follicular helper (T_{FH}) cells [49]. Expression of the IL-21 receptor, consisting of the high-affinity IL-21 receptor (CD360) in a heterodimeric complex with the common γ chain, is largely restricted to lymphocytes, including T cells, B cells, and NK cells [50]. IL-21 stimulation proceeds through JAK1/JAK3, leading to STAT1 and STAT3 activation [51]. IL-21 drives B-cell class switching and plasma cell differentiation [52,53]. Animal models of IL-21 deficiency provide evidence for the importance of IL-21 in the maintenance of T_H1 versus T_H2 lineage differentiation [54], as well as negatively regulating eosinophil recruitment to inflamed tissues [55]. Paradoxically, IL-21 receptor-deficient animals exhibit increases in I_GE production, which is a hallmark of type 2 immune responses [56]. These results suggest that the physiology of IL-21 is more complex than simply a T-helper subtype cytokine. For example, IL-21 can potentiate the activation of macrophages, leading to increased phagocytosis [57] and production of macrophage elastase [58]. IL-21 can suppress the activation and survival of T_{reg} cells while stabilizing T_H17 T cells, suggesting an important role for IL-21 in driving inflammatory responses [59]. IL-21 can suppress antigen presentation by dendritic cells, which blunts antigen-specific responses by effector/memory T cells [60]. Ultimately, IL-21 has complex nonredundant effects on cellular immunity and has the potential to both support and suppress multiple types of inflammatory responses.

24.3.3 Common Beta Chain

Cytokines that share the common β chain for signal transduction include IL-3, IL-5, and granulocyte–monocyte colony-stimulating factor (GM-CSF) (Figure 24.3). All these cytokines utilize the common β chain (CSF2RB/CD131) for activation of the JAK/STAT pathway through interactions with JAK2 [61]. Common β-chain cytokine receptors often signal through STAT5a/b, with additional signals

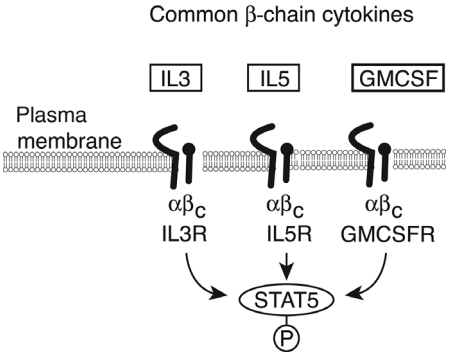


Figure 24.3 Common β-chain cytokines.

transmitted through PI3 K/AKT and RAS/MAPK pathways [62]. Cytokines in the common β-chain subfamily are involved in the growth and differentiation of leukocytes from hematopoietic precursors. However, all three of the cytokines in the common β-chain subfamily also play important roles in the initiation and maintenance of inflammatory responses.

24.3.3.1 IL-3

IL-3 plays a central role in hematopoiesis, helping to guide multipotent hematopoietic precursor cells to commit to either the myeloid or lymphoid lineage [63]. IL-3 is produced by stromal cells in hematopoietic tissues (e.g., bone marrow stromal cells [64] and thymic epithelial cells [65]). Activated T cells can also produce IL-3 to stimulate T-cell proliferation and differentiation in an auto- and paracrine manner, similar to the effects of IL-2 [66]. The IL-3 receptor consists of the high-affinity IL-3 receptor α chain (CD123) complexed with the common β chain, and signal transduction through the IL-3 receptor proceeds through PI3K/AKT and RAS/MAPK pathways in addition to STAT5 [67]. The effects of IL-3 stimulation are highly dependent on the local cytokine milieu. For example, stimulation of multipotent hematopoietic stem cells (HSCs) with IL-3 alone leads to differentiation to the myeloid lineage [68]. Conversely, stimulation of HSCs with IL-3 along with IL-7 results in differentiation to the lymphoid lineage [69]. Despite the important role for IL-3 in immune cell development, mice deficient in IL-3 production exhibit few changes to peripheral immune cell numbers compared to wild-type mice [70]. While eosinophil and mast cell development is negatively affected, the distribution of other leukocyte subsets is largely unaffected. These results suggest that IL-3 signals may be more important for guiding cell fate decisions, rather than being absolutely necessary for the initiation of hematopoiesis.

24.3.3.2 IL-5

IL-5, also known as eosinophil differentiation factor, is a key cytokine for the development of type 2 inflammation through its ability to stimulate eosinophil differentiation and chemotaxis. Activated T_H2 T cells and mast cells produce IL-5 [71]. The IL-5 receptor consists of the high-affinity IL-5 receptor α chain (CD125) complexed with the common β chain, and signal transduction through the IL-5 receptor is similar to the IL-3 receptor [72]. The IL-5 receptor is

primarily expressed by eosinophils, and IL-5 is a potent inducer of eosinophilia [73]. B cells can also express the IL-5 receptor, and IL-5 stimulation can synergize with IL-4 to drive class switching in these cells toward I_gE [74]. The IL-5 receptor is expressed by a subset of B cells known as B-1 cells, and IL-5 signaling helps to drive B-1 cell proliferation and survival [75]. IL-5 knockout mice exhibit decreased production of eosinophils [76] and resistance to experimental models of allergic airway disease [77]. Because of its central role in driving eosinophilia and B-cell activation, as well as its primary source in T_H2 T cells, IL-5 is considered to be a central mediator of type 2 responses.

24.3.3.3 GM-CSF

Functionally, granulocyte–monocyte colony-stimulating factor is often grouped with the colony-stimulating factors, including macrophage colony-stimulating factor (M-CSF) and granulocyte colony-stimulating factor (G-CSF). However, unlike the M- and G-CSF receptors, which are single-pass trans-membrane receptors, the GM-CSF receptor is a heterodimeric complex consisting of the GM-CSF receptor α chain (CD116) complexed with the common β chain [78]. GM-CSF signals through JAK2/STAT5a/b in addition to PI3 K, RAS/MAPK, and NF-κB [79]. GM-CSF receptor is expressed in a wide array of myeloid precursors and mature monocytes/granulocytes [80]. GM-CSF drives the differentiation of granulocytes and monocytes from precursor cells in the bone marrow [81]. GM-CSF can also drive the differentiation of monocytes into either macrophages with classical activation phenotypes [82] or myeloid-derived dendritic cells with potent antigen-presenting capabilities [83]. GM-CSF is also produced during inflammatory responses by activated T cells, especially T_H1 and T_H17 cells [84]. GM-CSF has important roles as both a hematopoietic factor and an inducible proinflammatory cytokine.

24.3.4 IL-12 Subfamily

The IL-12 subfamily cytokines (IL-12, -23, -27, and -35) consist of heterodimeric interactions between two of the five subunit proteins (including p19, p28, p35, p40, and Ebi3) and heterodimeric receptor complexes containing two of the five receptor chains (including IL-12Rβ1, IL-12Rβ2, IL-23R, IL-27R, and gp130). IL-12 subfamily cytokines can signal through multiple STATs, including STAT1, STAT3, and STAT4, and can have a wide range of effects on inflammatory responses, from highly stimulatory (IL-23) to inhibitory (IL-35) (Figure 24.4).

24.3.4.1 IL-12

IL-12 is a heterodimeric cytokine consisting of the IL-12α (p35) and IL-12β (p40) subunits. A homodimer of β subunits can also be formed (known as IL-12p40 homodimer or $p40_2$); certain studies have indicated a role for IL-12p40 in driving immune suppression [85]. Cells of the myeloid lineage produce IL-12, including monocytes, macrophages, and dendritic cells [86,87]. Activated neutrophils can also produce IL-12 in response to inflammatory stimuli, such as Toll-like receptor (TLR) ligands [88]. IL-12 promotes the differentiation of activated T cells into T_H1 T cells [89]. Stimulation of the IL-12 receptor, consisting of IL-12 receptor β1 (CD212) and β2 subunits, results in activation of STAT4, leading to the

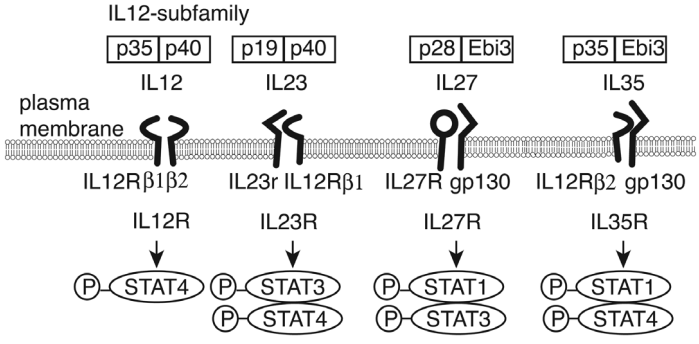


Figure 24.4 IL-12 subfamily cytokines.

production of IFNγ and TNFα in activated T cells and NK cells, as well as the production of the T_H1 lineage transcription factor T-bet [90]. IL-12 stimulation also drives the cytotoxic activity of activated CD8+ T cells [91]. IL-12 is a central cytokine for the development and maintenance of type 1 immune responses against intracellular pathogens.

24.3.4.2 IL-23

IL-23 is comprised of the IL-12p40 subunit complexed with the IL-23 α subunit (p19). IL-23 signals through the IL-23 receptor, a heterodimeric complex of the IL-12Rβ1 with IL-23R. The IL-23 receptor signals through STAT4 in a similar fashion as the IL-12 receptor, with IL-23 receptor subunit adding STAT3 signal transduction to the IL-23 receptor complex [92]. As IL-12 promotes T_H1 cells, IL-23 promotes the activation and differentiation of T_H17 T cells [93]. IL-23 is produced by activated macrophages and dendritic cells, and it can signal naïve T cells to differentiate to T_H17 in the context of antigen stimulation [94]. IL-23 can also mediate the activation of γδ and NK-T cells [95]. IL-23 has been implicated in numerous autoimmune diseases, including inflammatory bowel disorders [96], rheumatoid arthritis [97], and multiple sclerosis [98]. Studies highlighting the importance of IL-23 in autoimmune disease have helped to shift the accepted paradigm of T-helper subtype participation in autoimmunity toward a greater appreciation of the pathogenic role of T_H17 T cells.

24.3.4.3 IL-27

IL-27 is comprised of two distinct subunits in the IL-12 subfamily, IL-27p28 (also known as IL-27B or IL-30) and Epstein–Barr Virus induced gene 3 (Ebi3). IL-27 binds to the IL-27 receptor, a heterodimeric complex consisting of the IL-27 receptor α subunit (WSX-1) and gp130 (also known as IL6-β or CD130). Activation of the IL-27 receptor results in signal transduction through STAT1, STAT3, and MAPK [99]. IL-27 shares little similarity with the other IL-12 cytokine/receptor subfamily members, save for Ebi3 that is shared with IL-35; however, IL-27 functions in a synergistic fashion with other IL-12 subfamily cytokines to guide immune responses in both innate and adaptive immune cells [100]. IL-27 is produced by numerous immune cells, with dendritic cells being the primary source [101]. IL-27 stimulation of myeloid cells (e.g., monocytes) results not only in expression of

inflammatory cytokines such as TNFα and IL-6 but also in the upregulation of suppressive cytokines such as IL-10 [102]. For adaptive immune cells, IL-27 can help potentiate T_H1 responses through the promotion of IL12Rβ2 expression, which sensitizes T cells to respond to IL-12 [103]. However, IL-27 can also suppress IL-2 production, which in turn not only suppresses T_{reg} responses (indirectly promoting T_H1) but also enhances T_H17 responses (indirectly suppressing T_H1) [104]. STAT1 activation by IL-27 can drive T-bet expression in T cells, further supporting T_H1 differentiation [105]. IL-27 has pleiotropic effects on many facets of cellular immunity, with a preference toward the promotion of type 1 cytokine responses at the expense of type 2 responses and general immune cell proliferation.

24.3.4.4 IL-35

Of the IL-12 subfamily, IL-35 is the most immunosuppressive and has been linked with T_{reg} function and the induction of tolerance. IL-35 is a heterodimer consisting of the IL-12α subunit and Ebi3 from IL-27, and the IL-35 receptor consists of the IL12Rβ2 subunit complexed with gp130 from the IL-27 receptor [106]. Signal transduction through the IL-35 receptor proceeds though both STAT1 and STAT4, leading to the expression of immunosuppressive cytokines in target cells (i.e., IL-10 and TGFβ) [107]. IL-35 is produced by T_{reg} cells [108] and regulatory B cells [109] and expression of IL-35 receptor is found on T and B cells, suggesting that IL-35 can serve in an auto- and paracrine fashion to drive regulatory responses in peripheral tissues [110]. In addition, IL-35 produced by tolerogenic dendritic cells can also support the development of induced (i-) T_{regs} [111]. IL-35 can, therefore, drive the differentiation of iT- and B_{regs} that then produce IL-10 and TGFβ. As the subunits of both IL-35 and the IL-35 receptor are shared by other members of the IL-12 subfamily, it is challenging to selectively inhibit IL-35 in animal models; however, studies suggest that IL-35 blockade inhibits the conversion of inducible suppressive lymphocytes, especially B_{reg} induction [112].

24.3.5 Orphan Type I Cytokines

The remaining type I cytokines share basic structural similarities with other type I cytokines. However, these cytokines do not classify easily with other subfamilies, as they do not share common receptor or cytokine subunits with other members of the type I cytokines (Figure 24.5).

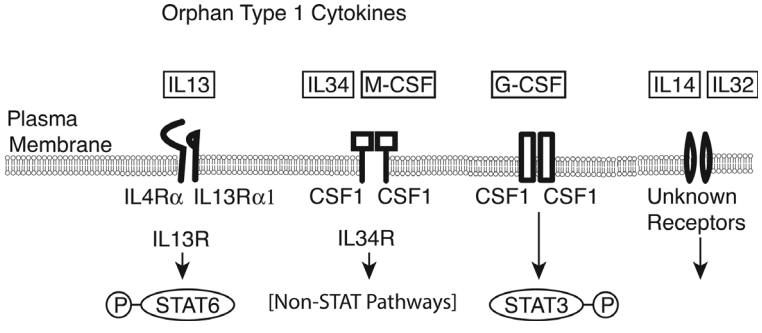


Figure 24.5 Orphan type I cytokines.

24.3.5.1 IL-13

IL-13 functions in concert with IL-4 and IL-5 to help drive type 2 inflammatory responses and T_H2 functions. IL-13 promotes mucus secretion by cells of the mucosal epithelium [113] and can enhance airway hyperreactivity through effects on bronchial/intestinal smooth muscle [114]. Interestingly, IL-13 knockout mice are still capable of generating T_H2 responses and I_gE antibodies, albeit at reduced levels, as long as IL-4 production remains unaffected [115]. The IL-13 receptor consists of the IL-4 receptor α subunit complexed with the IL-13 receptor α1 (CD213A), and binding of IL-13 to the mature IL-13 receptor results in signal transduction via STAT6 [116]. A second receptor for IL-13 also exists, known as the IL-13 receptor α2 (CD213A2); this receptor lacks an intracellular signal transduction domain and functions as a sequestering receptor [116]. IL-13 is grouped with IL-4 and IL-5 as the core cytokines driving type 2 inflammatory responses, which include the differentiation of both T_H2 T cells and alternatively activated/M2 macrophages.

24.3.5.2 IL-14

IL-14, also known as α-taxilin or high molecular weight B cell growth factor (HMW-BCGF), helps to drive proliferation of many different lineages of B cells. Activated T cells can produce IL-14 following activation [117]. Little is known about IL-14, save that the receptor is induced in B cells following activation, and IL-14 stimulation results in increases in cyclic AMP in activated B cells [118]. Studies of human patients suggest that IL-14 may be important in supporting B cell memory [119], and overexpression of IL-14 in animal models results in expanded B-cell populations, correlating with increased serum antibody levels and susceptibility to autoimmune disease [120].

24.3.5.3 IL-32

Little is understood about the biological functions of IL-32, also known as natural killer transcript 4 (NK4), as there are no rodent homologues of this cytokine, making experimental design challenging. Studies of human leukocyte responses have identified six isoforms of IL-32, all of which appear to have similar functions [121]. IL-32 can be expressed by numerous cells, including hematopoietic (e.g., NK, T cells, monocytes, and mast cells) and nonhematopoietic (e.g., epithelial cells and keratinocytes) cell types, and its production can be induced by IL-18 [122,123]. IL-32 helps to drive expression of proinflammatory cytokines in target cells, including IL-1β and IL-6 [124]. IL-32 stimulation can also drive expression of IL-17 in CD4+ T cells [125]. Studies have implicated IL-32 as an important proinflammatory cytokine in numerous immunopathologies, including autoimmune diseases such as rheumatoid arthritis [126].

24.3.5.4 IL-34

IL-34 is a homodimer expressed by a wide range of cell types. IL-34 signals through the CSF-1 receptor (CD115) that also binds macrophage colony-stimulating factor [127]. IL-34 stimulation through CSF-1 results in signal transduction primarily through non-STAT pathways, such as PI3 K and Ras/Erk [127]. IL-34 affects the differentiation and activation of cells responsive to M-CSF, specifically

microglia and Langerhans cells [128], and can induce the differentiation of blood monocytes [129]. These results suggest that IL-34 signals are critical for the survival of terminally differentiated myeloid-lineage innate immune cells in peripheral tissues.

24.3.5.5 G-CSF

Granulocyte colony-stimulating factor, also known as colony-stimulating factor 3 (CSF3), is the primary cytokine for the differentiation of neutrophils. G-CSF signals through the monomeric G-CSF receptor (CD114) that contains both extracellular ligand-binding domains and intracellular signal transduction domains [130]. Binding of G-CSF to its receptor induces signal transduction through STAT3, PI3K, and ERK [131]. G-CSF receptor is found on hematopoietic precursor cells, and stimulation with G-CSF results in their differentiation to neutrophils [132]. Animal models of G-CSF deficiency exhibit neutropenia, supporting the central role for G-CSF in neutrophil development [133]. G-CSF can be produced by hematopoietic (e.g., macrophages [134]) and non-hematopoietic (e.g., endothelial [135]) cells during inflammatory responses, and G-CSF production during the initial stages of microbial infection is critical for neutrophil-mediated immunity [136].

24.3.5.6 M-CSF

Macrophage colony-stimulating factor (also known as CSF1) signals through a monomeric receptor (CSF1R/CD115) to support myeloid cell development [137]. M-CSF is produced by bone marrow stromal cells [138] and activated T cells [139] to support the survival and effector function of monocytes/macrophages in peripheral tissues. CSF1R is expressed on myeloid cells, and binding of M-CSF to CSF1R results in activation of RAS/MAPK, ERK, and PKC pathways [140]. M-CSF signals also regulate the balance of osteoclast and osteoblast functions, with paracrine signaling by activated osteoblasts having a stimulating effect on the opposing functions of osteoblasts [141]. M-CSF ligand [142] and receptor [143] deficient animals exhibit osteopetrosis (excessive bone formation) due to the loss of osteoclast regulation.

24.3.6 Type II Superfamily

The type II superfamily, which contains the interferons and the IL-10 subfamily, has many similarities to type I cytokines, including the use of multimeric receptors with shared subunits. The major difference between type I and II superfamily cytokines is based on the sequence of the extracellular portion of the receptor, with type I cytokine receptors exhibiting a common WSXWS amino acid motif that type II cytokines do not exhibit. Within the type II superfamily, there are two major subfamilies, the IFNs (in three classes) and the IL-10 subfamily (including IL-10, -19, -20, -22, -24, and -26). The IFNs are responsible for inducing antiviral responses in both hematopoietic cells and structural cells, serving as an essential mediator of cross-talk between the immune system and host physiology during viral infections. The IL-10 subfamily contains both pro- and anti-inflammatory cytokines; this subfamily grouping is primarily due to

commonalities with receptor composition. Type III IFNs are often classified as members of the IL-10 subfamily and assigned interleukin identities – IFNλ1 as IL-29 and IFNλ2/3 as IL-28A/B – and these classification schemes differ based on the specifics of the subgroup's chromosomal location and gene organization (i.e., the use of multiple exons for mRNA transcription). In this chapter, these cytokines will be organized under the type III IFNs.

24.3.7 Interferons

24.3.7.1 Type I: IFN-α, -β, and Variants

Type I IFNs are produced by numerous cells, with IFNα primarily produced by leukocytes and IFNβ produced by fibroblasts [144] (Figure 24.6). A subset of dendritic cells known as plasmacytoid dendritic cells (pDCs) are particularly robust producers of type I IFN [145]. The type I IFN receptor consists of a heterodimeric complex containing IFNAR1 and IFNAR2, and signaling through this receptor results in activation of multiple STATs, NF-κB, P38/JNK, and ERK1/2 [146]. This extensive signal transduction cascade helps to drive the potent proinflammatory effects of type I IFNs. The two major subtypes of type I IFN are IFNα and IFNβ. "IFNα" is a class of at least 12 different proteins, expressed as single exons arising from a common IFNA gene cluster [147]. In contrast, the function of IFNβ is primarily mediated by IFNβ1 [148]. Other variants include IFNε, -κ, -δ, -ζ, -ω, and possibly -ν; these are expressed at various levels (if at all) in different mammals, and are not as well described as IFNα and β [149]. Type I IFN can stimulate antiviral responses by immune cells and can synergize with type II interferon (i.e., IFNγ) to potentiate T_H1 lineage commitment by T-helper cells [150] and cytotoxic activity by CD8+ "killer" T cells [151]. Type I IFNs signal nonhematopoietic cells in the body to initiate an antiviral program meant to limit viral replication. Type I IFN signaling drives expression of antiviral genes such as 2′-5′ oligoadenylate synthase, protein kinase R, and the Mx proteins (Mx1 and Mx2); these genes have broad downregulatory effects on protein synthesis, meant to limit viral replication in infected cells [152]. Type I IFNs can also drive the febrile response [153]. This global suppressive effect on protein synthesis can have a suppressive effect on immune responses, and type I IFN production during

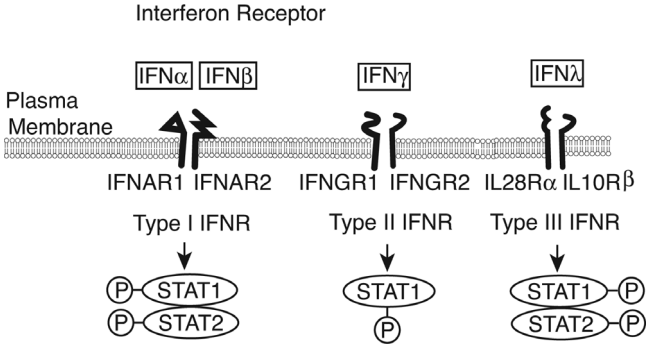


Figure 24.6 Interferon receptors.

chronic infection has the potential to be detrimental to immunity [154]. Animal models of type I IFN deficiency, such as the IFNAR1 targeted deletion in mice, results in significant impairments in antiviral [155] and antitumor [156] immune responses.

24.3.7.2 Type II: IFNγ

The only cytokine in the type II interferon subfamily is IFNγ, which mediates adaptive–innate immune cell cross-talk. IFNγ is produced by activated lymphocytes, especially CD4+ and CD8+ T cells and NK and NK-T cells [157]. The IFNγ receptor is similar to the type I IFN receptor in that it is a heterodimer consisting of IFNGR1 and IFNGR2; however, the IFNγ receptor primarily signals through STAT1, unlike the multiple STATs activated by the type 1 IFN receptor [158]. The IFNγ receptor is expressed by lymphocytes (both B and T cells) and macrophages, and IFNγ stimulation of these cells drives type 1 immune responses [159]. IFNγ promotes class switching of B cells toward the production of IgG2a, an important antibody isotype for Fc-mediated phagocytosis and complement activation [160]. IFNγ also promotes cytotoxic T-cell activity and T_H1 cell differentiation by promoting the expression of the transcription factor T-bet [161]. IFNγ promotes the upregulation of both class I and class II MHC molecules on antigen-presenting cells, helping to stimulate adaptive immune responses to antigens [162]. IFNγ promotes classical activation of macrophages, resulting in enhanced antimicrobial functions such as increased phagocytosis and killing of microorganisms [163]. Animals that are deficient in IFNγ signaling (IFNGR1 knockout) are extremely susceptible to intracellular infections [164].

24.3.7.3 Type III: IFNλ-1, -2, and -3

The Type III interferons walk a fine line between subfamilies in the type II cytokine superfamily. For example, IFNλ2 and IFNλ3, also known as IL-28A and IL-28B, respectively, regulate the expression of many type I IFN responsive antiviral genes, such as 2′-5′ oligoadenylate synthase and Mx1/2, highlighting their functional similarity to other interferons [165]. However, these two cytokines are expressed from gene loci containing multiple exons, unlike the type 1 IFNs that are typically encoded by a single exon [166]. In addition, the IL-28 receptor complex contains both an IL-28 receptor α chain and a β chain shared with the IL-10 receptor (IL10Rβ/CDW210B), which links these cytokines with the IL-10 subfamily [167]. IFNλ1 shares a high degree of sequence similarity with the other type III IFNs, and also signals through the IL-28 receptor [168]. Regardless of the classification, the type III IFNs function in a similar fashion to the type I IFNs. Signal transduction through the IL-28 receptor leads to STAT1 and STAT2 activation, and serves to induce antiviral responses in numerous cell types, albeit not to the same widespread effect as the type I IFNs [168].

24.3.8 IL-10 Subfamily

The IL-10 subfamily shares the same common receptor subunit as the type III IFNs, IL10Rβ (Figure 24.7). The subfamily is named after its founding member, a potent anti-inflammatory cytokine that can suppress the downstream expression

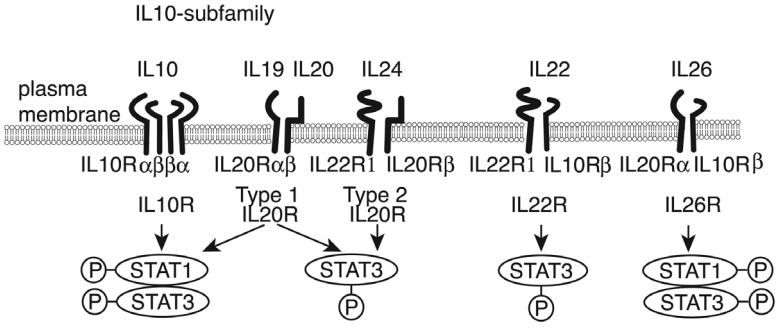


Figure 24.7 IL-10 subfamily receptors.

of many proinflammatory genes in immune cells. Despite the shared receptor subunit, the other members of the IL-10 subfamily do not all function as anti-inflammatory cytokines. In fact, many of the other IL-10 subfamily cytokines both promote or suppress lineage-specific inflammatory cytokine responses and mediate cross-talk between the immune system and structural cells during wound healing and scar tissue formation.

24.3.8.1 IL-10

IL-10 is a potent anti-inflammatory cytokine, as evidenced by its alias as cytokine synthesis inhibitory factor (CSIF). IL-10 is a homodimer that binds to a multimeric receptor made up of pairs of IL-10Rα and IL-10Rβ molecules, for a total of four subunits per receptor [169]. IL-10 signals through STAT1, STAT3, PI3K, and p38 MAPK pathways [170,171]. IL-10 is produced by monocytes and macrophages, along with T_H2 and T_{reg} cells [172]. IL-10 has strong immunosuppressive functions and directly antagonizes T_H1 cytokine production, classically activated/M1 macrophage inflammatory gene expression, and costimulatory ligand expression in antigen-presenting cells [173]. Despite its anti-inflammatory properties, IL-10 expression is primarily induced by microbial stimuli, including TLR signaling [174] and cross-linking of Fc receptors [175]. This implicates IL-10 as an important regulatory cytokine to help restrain inflammatory responses and limit collateral damage to healthy cells and tissues. IL-10 is also critical for gut homeostasis, and IL-10 production in the intestine plays a role in tolerance induction against commensal bacteria. As evidence of this, animals lacking the IL-10 gene develop spontaneous colitis due to unchecked inflammatory responses to gut bacteria [176].

24.3.8.2 IL-19

IL-19 is produced by activated monocytes and macrophages, B cells, and structural cells such as epithelial cells and keratinocytes [177]. IL-19 signals through the IL-20 receptor, consisting of the IL-20Rα chain complexed with the IL-10Rβ chain, and results in signaling through STAT1 and STAT3 [178]. IL-19 shares functionalities with IL-10, such as suppression of macrophage inflammatory responses and production of TNFα and IL-1β [179]. IL-19 deficiency results in increased disease severity in animal models of colitis [178]. IL-19 also drives

expression of T_H2 cytokines such as IL-4, IL-5, and IL-13 [180]. However, IL-19 has also been shown to drive inflammatory responses in psoriasis, which is considered to be a T_H1-driven inflammatory response [181]. IL-19 drives the differentiation and proliferation of keratinocytes, and these cells can also produce IL-19; this positive autocrine feedback loop may drive psoriasis pathology through aberrant keratinocyte proliferation [182]. IL-19 is difficult to slot as strictly a T_H1, T_H2, or suppressive cytokine. It is clear that IL-19 plays an important role in immune cell–structural cell cross-talk at mucosal or barrier surfaces.

24.3.8.3 IL-20

IL-20 shares similar functions with IL-19 and IL-24 as a proliferative signal for keratinocytes. IL-20 can signal through two distinct receptor complexes that each use the IL-20 receptor β chain, the type 1 IL-20 receptor (complex with IL-20 receptor α chain), and the type 2 IL-20 receptor (complex with IL-22 receptor α1) [183]. IL-20 signals result in activation of STAT3 in target cells, including keratinocytes, adipocytes, and endothelial cells [183]. IL-20 is produced in response to microbial stimuli (e.g., TLR ligands) and inflammatory cytokines such as IL-1β and TNFα [184]. IL-20 can be produced by activated macrophages and T_H1 T cells, yet appears to counteract type 1 cytokine responses through the promotion of T_H2 cytokines [185]. IL-20 plays a central role in psoriasis, using a similar keratinocyte-activating mechanism to IL-19 and IL-22 [186]. IL-20 production has been implicated both in the pathophysiology of chronic inflammatory responses and in wound healing following tissue damage [187].

24.3.8.4 IL-22

IL-22 is produced by inflammatory lymphocytes, such as T_H17 T cells and activated NK cells [188]. The IL-22 receptor is composed of the common IL-10 receptor β chain complexed with the IL-22 receptor α1; this receptor is specific for IL-22 and signals through STAT3 [189]. IL-22 signals support the proliferation of epithelial cells and keratinocytes [190], along with supporting systemic inflammatory responses. IL-22 drives the production of acute-phase proteins from hepatocytes [191] and antimicrobial defensins from epithelial cells [192]. IL-22 production supports protective inflammatory responses in mucosal surfaces and barrier tissues during microbial infection [193].

24.3.8.5 IL-24

IL-24, or melanoma differentiation-associated 7 (mda-7), shares similar functions and receptor complexes to IL-19 and IL-20 (type I and type II IL-20 receptors). Unlike other IL-10 family cytokines that are produced by "classic" inflammatory cells such as M1 macrophages or T_H1T cells, IL-24 is primarily produced following stimulation with T_H2-associated cytokines such as IL-4 [194]. Keratinocytes, T_H2 T cells, and activated monocytes stimulated with IL-4 can produce IL-24 [195]. IL-24 functions in an opposite fashion to other IL-10 subfamily cytokines, as it restricts rather than promote proliferative responses during wound healing [196]. In this way, IL-24 may play a role in the resolution of wound healing through its ability to restrict keratinocyte proliferation after

successful repair of localized tissue damage. IL-24 can also stimulate the apoptosis of cancer cells, possibly by utilizing the same antiproliferative molecular mechanisms that are activated in IL-24 responsive structural cells [197].

24.3.8.6 IL-26

IL-26 signals through a receptor consisting of the IL-20 receptor α chain complexed with the common IL-10 β chain, expressed in structural cells such as epithelial cells [198]. Stimulation with IL-26 results in signal transduction through STAT1 and STAT3 [198]. IL-26 is produced by T cells, and T_H17 T cells in particular [199], although it can also be produced by NK cells following activation [200]. IL-26 serves as a proinflammatory cytokine at mucosal surfaces through its ability to induce CXCL8 secretion and subsequent accumulation of T_H17 T cells [201]. This makes IL-26 arguably the most proinflammatory member of the IL-10 superfamily, as its expression can have direct effects on neutrophil influx into barrier surfaces [202].

24.4 Summary: Cytokine Networks in Cellular Immunity

It is important to note that this discussion of the alpha-helix cytokines omitted cytokine families discussed in previous chapters (i.e., IL-1, IL-6, and IL-17 cytokines). Many other soluble factors can also have drastic effects on immune cell function and inflammatory responses, including chemokines, growth factors, and hormones. There is little doubt that in the future, the physiological effects ascribed to specific cytokines will be changed to other previously described or novel cytokines. As our understanding of the molecular biology of cytokines grows, so will our understanding of the functions of specific cytokines in specific inflammatory contexts.

It is also important to remember that the functions of these signaling molecules are dependent upon the tissue microenvironement and the inflammatory milieu. Cytokines that function as proinflammatory mediators in one environment may function to suppress inflammation in others. This is especially important when discussing lineage-specific cytokine expression, such as classical versus Alternatively activated macrophages, or T_H1 versus T_H2 T cells. A cytokine that might drive one type of inflammatory response (e.g., IFNγ for M1/T_H1) could be viewed as suppressive or anti-inflammatory in the contrasting context (M2/T_H2). "Inflammation" is a catch-all term that encompasses distinct immunological mechanisms geared toward protection against disparate pathogens (e.g., intracellular viruses versus multicellular parasites). It is, therefore, important when studying cytokine functions in disease pathology to consider the effects of the specific cytokine of interest especially as it relates to the overall immune response.

Cytokines provide the necessary signals for tailoring the inflammatory response for optimal protection against specific pathogens, through their signals meant to promote the differentiation and proliferation of specific leukocyte subsets, their ability to tailor the effector functions of activated leukocytes as needed to promote protective immunity, and their capacity to suppress inflammation following clearance of the pathogen, promoting tissue repair and a return to homeostasis.

Productive immune responses rely on the coordinated expression of specific cytokines from both innate and adaptive immunities; conversely, aberrant production of cytokines can lead to autoimmune disease, immunopathology, and hypersensitivity reactions. Understanding the molecular mechanisms underlying cytokine expression and cytokine receptor-mediated signal transduction and gene expression is central to the study of immunology and the continued search for better treatments and therapies for inflammatory disorders.

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25

Chemokines and Chemotaxis

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25.1 Introduction

While the term "chemokine" is coined from chemotactic cytokine, the chemokine superfamily is a group of mostly basic, heparin binding, 8–14 kDa secretory proteins, whose main functions are to induce directed migration (chemotaxis) in cells expressing their receptors [1–7]. Chemokines have four well-conserved cysteine residues and, based on the arrangement of the N-terminal two cysteine residues, they are further divided into CXC, CC, (X)C, and CX_3C subfamilies (X indicates other amino acids). The XC or C subfamily has only the second and fourth cysteines. Humans are now known to have at least 44 ligands (Table 25.1) and 24 receptors including 5 scavenger receptors (Table 25.2) [6–8]. All chemokine receptors belong to the family of seven-transmembrane G protein-coupled receptors (GPCRs), which represent the largest gene family in the human genome (~800) and react to a wide variety of external stimuli including light, taste, smell, peptide hormones, bioactive amines, lipid mediators, autacoids, and various chemoattractants including chemokines. The so-called "classical chemotactic factors," which were known before the discovery of chemokines such as formyl peptides (bacteria derived), C5a, and C3a (the complement cleavage products), prostaglandins and leukotrienes (the arachidonic metabolites), and platelet-activating factor (a phospholipid), also use this type of receptors [9,10]. Indeed, GPCRs are the universal receptor system employed by the cell for quick and transient responses to external stimuli. The unique characteristics of the chemokine system that are quite different from the conventional cytokines are likely to be closely related to the nature of the GPCR system.

Chemokines can be produced by a wide variety of tissue cells such as epithelial cells, endothelial cells, and stromal cells. Furthermore, leukocytes often produce chemokines that act on themselves, thus generating a feedforward loop for recruitment of the same types of leukocytes in inflammation [1–7]. Production of many chemokines is strongly induced by inflammatory stimuli. Such

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Table 25.1 Summary of human and mouse chemokines.

Systematic Name (active form)	Other Names		Category	Agonistic Receptor (Human)	Antagonistic Receptor (Human)	Chromosome (Human)	NCBI Database	
	Human	Mouse					Human	Mouse
CC subfamily								
CCL1	I-309	TCA-3	I	CCR8		17q12	P22362	P10146
CCL2	MCP-1	JE	I	CCR2		17q11.2-q12	P13500	P10148
CCL3	MIP-1α		I	CCR1 and CCR5		17q12	P10147	P10855
CCL4	MIP-1β		I	CCR5		17q12	P13236	P14097
CCL5	RANTES		I	CCR1, CCR3, and CCR5		17q12	P13501	P30882
Mouse Ccl6	None	C10	Unknown	Unknown				P27784
CCL7	MCP-3		I	CCR2, CCR1 and CCR3	CCR5	17q11.2–q12	P80098	Q03366
CCL8	MCP-2	None	I	CCR2, CCR1, and CCR5		17q11.2	P80075	
Mouse Ccl8	None	Mcp-2	I	Ccr8		NA		Q9Z121
Mouse Ccl9	None	MIP-1γ		Unknown		NA		P51670
CCL11	Eotaxin-1		D	CCR3 and CCR5	CCR2, CXCR3	17q12	P51671	P48298
Mouse Ccl12	None	Mcp-5	Unknown	Ccr2		NA		Q62401
CCL13	MCP-4	None	I	CCR2 and CCR3		17q11.2	Q99616	
CCL14 (9–74)	HCC-1	None	P	CCR1, CCR3, and CCR5		17q11.2	Q16627	
CCL15 (24,25,28–92)	HCC-2	None	P	CCR1 and CCR3		17q11.2	Q16663	
CCL16	LEC	Pseudogene	P	CCR1, CCR2, and CCR5		17q11.2	O15467	

CCL17	TARC		D	CCR4		16q13	Q92583	Q9WUZ6
CCL18	PARC	None	D	CCR8	CCR3	17q11.2	P55774	
CCL19	ELC		H	CCR7		9p13	Q99731	O70460
CCL20	LARC		D	CCR6		2q36.3	P78556	O89093
CCL21	SLC		H	CCR7		9p13	O00585	P84444
CCL22	MDC		D	CCR4		16q13	O00626	O88430
CCL23 (25–99)	MPIF-1	None	P	CCR1		17q12	P55773	
CCL24	Eotaxin-2		D	CCR3		7q11.23	O00175	Q9JKC0
CCL25	TECK		H	CCR9		19p13.2	O15444	O35903
CCL26	Eotaxin-3	Pseudogene	I	CCR3 and CX3CR1	CCR1, CCR2, and CCR5	7q11.23	Q9Y258	Q5C9Q0
CCL27	CTACK		H	CCR10		9p13	Q9Y4X3	Q9Z1X0
CCL28	MEC		H	CCR10 and CCR3		5p12	Q9NRJ3	Q9JIL2
CXC subfamily								
CXCL1	GROα	KC	I, ELR	CXCR2		4q21	P09341	P12850
CXCL2	GROβ	MIP-2	I, ELR	CXCR2		4q21	P19875	P10889
CXCL3	GROγ	MIP-2β	I, ELR	CXCR2		4q21	P19876	Q6W5C0
CXCL4	PF-4		H, non-ELR	CXCR3-B		4q12–q21	P02776	Q9Z126
CXCL5	ENA-78	LIX	I, ELR	CXCR2		4q13.3	P42830	P50228
CXCL6	GCP-2	None	I, ELR	CXCR1 and CXCR2		4q13.3	P80162	
CXCL7	NAP-2		I, ELR	CXCR2		4q12–q13	P02775	Q9EQI5
CXCL8	IL-8	None	I, ELR	CXCR1 and CXCR2		4q13-q21	P10145	
CXCL9	MIG		I, non-ELR	CXCR3	CCR3	4q21.1	Q07325	P18340

(*continued*)

Table 25.1 (*Continued*)

Systematic Name (active form)	Other Names		Category	Agonistic Receptor (Human)	Antagonistic Receptor (Human)	Chromosome (Human)	NCBI Database	
	Human	Mouse					Human	Mouse
CXCL10	IP-10		I, non-ELR	CXCR3	CCR3	4q21	P02778	P17515
CXCL11	I-TAC		I, non-ELR	CXCR3	CCR3, CCR5	4q21.2	O14625	Q8R392
CXCL12	SDF-1		H	CXCR4		10q11.1	P48061	P40224
CXCL13	BLC		H	CXCR5 and CXCR3		4q21	O43927	O55038
CXCL14	BRAK		H	Unknown		5q31	O95715	Q6AXC2
Mouse Cxcl15	None	Lungkine	Unknown	Unknown		NA	NA	Q9 WVL7
CXCL16	SR-PSOX		D	CXCR6		17p13	Q9 H2A7	Q8BSU2
CXCL17	DMC		H	CXCR8		19q13.2	Q6 UXB2	Q8R3 U6
CX3C subfamily								
CX3CL1	Fractalkine		D	CX3CR1		16q13	P78423	O35188
XC subfamily								
XCL1	Lymphotactin/SCM-1α		D	XCR1		1q23	P47992	P47993
XCL2	SCM-1β	None				1q24.2	Q9 UBD3	

Category: D, dual function chemokine; ELR, ELR motif chemokine; H, homeostatic chemokine; I, inflammatory chemokine; non-ELR, non-ELR chemokine; P, plasma chemokine.
Abbreviations: BLC, B-lymphocyte chemoattractant; BRAK, breast and kidney; CTACK, cutaneous T-cell-attracting chemokine; ELC, EBI1-ligand chemokine; ENA-78, epithelial-derived neutrophil attractant-78; GCP-2, granulocyte chemotactic protein-2; GROα, growth-related gene product α; HCC-1, human hemofiltrate CC chemokine-1; IL-8, interleukin-8; IP-10, interferon-inducible protein-10; I-TAC, interferon-inducible T-cell α chemoattractant; LARC, liver and activation-regulated chemokine; LEC, liver-expressed chemokine; LIX, LPS-induced CXC chemokine; MCP-1, monocyte chemotactic protein-1; MDC, macrophage-derived chemokine; MEC, mucosae-associated epithelial chemokine; MIG, monokine induced by interferon γ; MIP-1α, macrophage inflammatory protein-1α; MPIF-1, myeloid progenitor inhibitory factor-1; NAP-2, neutrophil activating protein-2; PARC, pulmonary and activation-regulated chemokine; PF-4, platelet factor-4; RANTES, regulated upon activation, normal T expressed and secreted; SCM-1α, single C motif-1α; SDF-1, stromal cell-derived factor-1; SLC, secondary lymphoid-tissue chemokine; SR-PSOX, scavenger receptor that binds phosphorylated and oxidized lipoprotein; TARC, thymus and activation-regulated chemokine; TCA-3, T-cell activation gene-3; TECK, thymus-expressed chemokine.

Table 25.2 Summary of human chemokine receptors.

Systematic Name	CD No.	Agonist	Antagonist	Major Expressing Cells	Human Chromosome	NCBI Database	
						Human	Mouse
CC chemokine receptors							
CCR1	CD191	CCL3, CCL3L1, CCL5, CCL7, CCL8, CCL14, CCL15, CCL16, and CCL23	CCL26	Mo, memory T, iDC, and mNeu	3p21	P32246	P51675
CCR2	CD192	CCL2, CCL7, CCL8, CCL13, and CCL16	CCL11 and CCL26	Mo, memory T, and iDC	3p21.31	P41597	P51683
CCR3	CD193	CCL3 L1, CCL5, CCL7, CCL11, CCL13, CCL14, CCL15, CCL24, CCL26, and CCL28	CCL18, CXCL9, CXCL10, and CXCL11	Eo, Bas, iDC, and some Th2	3p21.3	P51677	P51678
CCR4	CD194	CCL17 and CCL22		Th2, CLA^+ T, Treg, Th17, and Plt	3p24	P51679	P51680
CCR5	CD195	CCL3, CCL3L1, CCL4, CCL5, CCL8, CCL11, and CCL16	CCL7, CCL26, and CXCL11	Mo, activated T, Th1, and iDC	3p21.31	P51681	P51682
CCR6	CD196	CCL20		B, Th17, and iDC	6q27	P51684	O54689
CCR7	CD197	CCL19 and CCL21		Naïve T, T_{CM}, B, and activated DC	17q12–21.2	P32248	P47774
CCR8	CDw198	CCL1, CCL16?, mouse Ccl8		Mo, skin T	3p22	P51685	P56484
CCR9	CDw199	CCL25		SI-homing T, IEL, and IgA-APC	3p21.3	P51686	Q9 WUT7
CCR10		CCL27 and CCL28		CLA^+ T, IgA-APC	17q21.1–21.3	P46092	Q9 JL21

(*continued*)

Table 25.2 (*Continued*)

Systematic Name	CD No.	Agonist	Antagonist	Major Expressing Cells	Human Chromosome	NCBI Database	
						Human	Mouse
CXC chemokine receptors							
CXCR1	CD181	CXCL6 and CXCL8		Neu, $CD8^+$ T, and NK	2q35	P25024	Q810 W6
CXCR2	CD182	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, and CXCL8		Neu, $CD8^+$ T, and NK	2q35	P25025	P35343
CXCR3	CD183	CXCR4?, CXCL9, CXCL10, CXCL11, and CXCL13	CCL11	Activated T, Th1, NK, and some B	Xq13	P49682	O88410
CXCR4	CD184	CXCL12		B, naïve T, memory T, DC, and Plt	2q21	P61073	P70658
CXCR5	CD185	CXCL13		B, T_{FH}	11q23.3	P32302	Q04683
CXCR6	CD186	CXCL16		Th1, NK, NKT, and PC	3p21	O00574	Q9EQ16
CXCR8		CXCL17		Mac, DC, and Neu	2q37.3	Q9 HC97	Q9ES90
CX3C chemokine receptor							
CX_3CR1		CX3CL1, CCL26		Patrolling Mo, Mac, DC, $CD8^+$ T, and NK	3p21.3	P49238	Q9Z0D9
XC chemokine receptors							
XCR1		XCL1 and XCL2		$CD11c^+CD141^+$DC (human) $CD8\alpha^+$DC (mouse)	3p21.3	P46094	Q9R0 M1

Atypical chemokine receptors						
ACKR1	CD234/DARC	CCL2, CCL5, CCL7, CCL11, CCL13, CCL14, CCL17, CXCL5, CXCL6, CXCL8, and CXCL11	RBC, HEV	1q21–q22	Q16570	Q9QUI6
ACKR2	D6	CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17, and CCL22	Lymphatic En, DC, and some B	3p21.3	O00590	Y12879
ACKR3	RDC1 and CXCR7	CXCL11 and CXCL12	B, En, and stromal cells	2q37.3	P25106	P56485
ACKR4	CCRL1, CCX CKR	CCL19, CCL21, and CCL25	Keratinocytes, SLO stroma cells, and thymic Ep	3q22	Q9 NPB9	Q924I3
ACKR5	CCRL2	CCL19	Mo, DC, Neu, T, B, and NK	3p21	O00421	O35457

Abbreviations: APC, antibody-producing cells; B, B cells; Bas, basophils; CLA, cutaneous lymphocyte antigen; DC, dendritic cells; En, endothelial cells; Eo, eosinophils; Ep, epithelial cells; iDC, immature dendritic cells; IEL, intraepithelial lymphocytes; HEV, high endothelial venule; Mac, macrophages; Mo, monocytes; Neu, neutrophils; mNeu, mouse neutrophils; NK, natural killer cells; NKT, natural killer T cells; Plt, platelets; RBC, red blood cells; SI, small intestine; SLO, secondary lymphoid organ; T_{CM}, central memory T cells; Th1, type 1 helper T cells; Treg, regulatory T cells.

chemokines usually attract neutrophils, monocytes, eosinophils, and effector lymphocytes and play important roles in acute or chronic inflammation. In addition, some chemokines are constitutively expressed by specific tissue cells and are involved in homeostatic migration of lymphocytes and dendritic cells. Yet some chemokines play important roles in organogenesis during the development [1–7]. Chemokines also contribute to cancer stroma formation and metastasis [11]. Moreover, certain chemokine receptors are known to directly contribute to the infection of human immunodeficiency virus (HIV) by acting as entry coreceptors [12]. Thus, the chemokine superfamily is involved in a wide range of biological phenomenon. In this chapter, we provide an overview of the chemokine superfamily with a particular focus on their role in the immune system.

25.2 Chemokine and Chemokine Receptor Nomenclature Systems

Concerning the chemokine receptor nomenclature, it was impractical to give a name based on its ligand because of the existence of multiple ligands for a single receptor. It was, however, noted that ligand chemokines of a receptor belong to the same subfamily. Therefore, in 1995, a method was adapted in which "R" was affixed to the ligand subfamily such as CXC and CC to indicate a receptor followed by a sequential number in the order of identification [2]. Similarly, the names of ligands were quite confusing particularly in the case of recently identified chemokines because of multiple names given to a single chemokine. Thus, in 1999, a similar nomenclature system was adopted in which "L" was affixed to the subfamily name to indicate a ligand and a sequential number [3]. However, the sequential numbers were not in the order of discovery but were the same numbers used by the gene symbols, which consist of SCY indicating small cytokine; A, B, C, or D indicating the subfamily; and a sequential number in the order of chromosomal gene mapping. As for the chemokines to be reported in the future, however, the sequential numbers will be in the order of discovery and those of gene symbols will be matched [3].

25.3 Extravasation of Leukocytes: The Multistep Model

Inflammation is a tissue reaction to accumulate fluid and leukocytes upon microbial infection or injury. Therefore, infiltration of leukocytes along with increased vascular permeability is a common sign of inflammation. Neutrophils are the main infiltrating cells in acute inflammation, while monocytes and lymphocytes represent the majority in chronic inflammation. In allergic inflammation, eosinophils and lymphocytes are the predominant cells. A combination of specific cell adhesion molecules and chemokines/chemokine receptors cooperatively promote tissue infiltration of select leukocytes [13,14]. The extravasation of leukocytes takes place in the postcapillary venules through several steps (Figure 25.1): (i) circulating leukocytes in the blood transiently stick to and

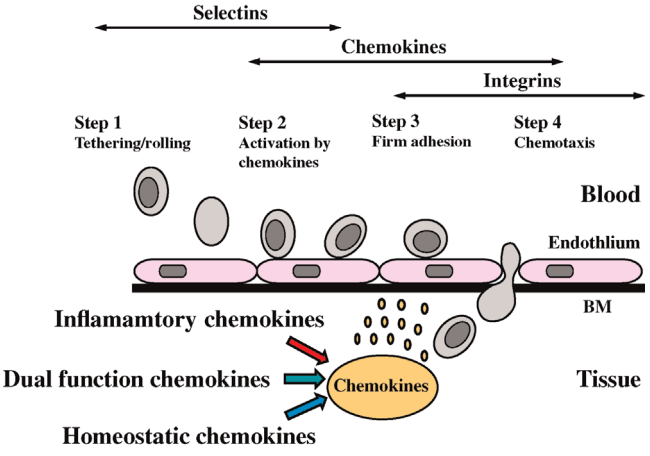


Figure 25.1 The multstep model of leukocyte extravasation.

slowly roll on the surface of endothelium through weak interactions involving selectins and selectin ligands or integrin VLA4 and its ligand VCAM-1 (tethering and rolling), (ii) if chemokines or other chemoattractants are produced and presented on the surface of endothelial cells, leukocytes expressing their receptors are activated and firmly bind to the endothelium by interactions between integrins (LFA-1 and Mac-1) and their ligands (inside-out signaling and firm adhesion), (iii) attached leukocytes further polarize their morphology and start crawling on the endothelial surface by ameboid movement (outside-in signaling, polarization, and diapedesis), and (iv) leukocytes extravasate mainly via paracellular route and sometimes via transcellular route penetrate the basement membrane and migrate within tissue by sensing gradients of chemotactic factors/chemokines. As the combinations of cell adhesion molecules and chemokines/chemokine receptors specify the kind of leukocytes to extravasate, the model is also referred to as the "address code model."

25.4 Inflammatory Chemokines and Homeostatic Chemokines

The field of chemokines started in 1987 when interleukin-8/CXCL8 was first discovered as a chemotactic factor for neutrophils. This was followed by reports in 1989, wherein monocyte chemotactic protein-1/CCL2 was described as a chemotactic factor for monocytes [15]. The comparison of these molecules quickly revealed that they form a family with some other secretory proteins of unknown function. Specifically, the four well-conserved cysteine residues served as a useful molecular signiture to identify the members of this family and new chemokines were rapidly identified among genes whose expression was strongly induced by inflammatory and other stimuli [1,4]. Subsequently, the chemokine paradigm was formed: (i) chemokines were divided into two subfamilies based on the motif

formed by the N-terminal two conserved cysteine residues: CXC (one other amino acid exists between the two cysteine residues) and CC (the two cysteine residues are adjacent), (ii) CXC chemokines mainly attract neutrophils and their genes are clusterd in chromosome 4 in humans, (iii) CC chemokines mainly attract monocytes and their genes are clustered in chromosome 17 in humans, and (iv) chemokines have highly promiscous ligand–receptor relationships: one ligand acts on multiple receptors and one receptor interacts with multiple ligands [1,4].

However, these chemokines could not sufficiently explain the immune cell migrations such as homeostatic recirculation of mature lymphocytes, migration of dendritic cells to regional lymph nodes upon antigen loading, and tissue infiltration of effector lymphocytes upon various immune responses. Therefore, the existence of chemokines that attract immune cells was postulated [16]. Meanwhile, the EST (expressed sequence tag) databases consisting of partial sequences of randomly assorted cDNAs were rapidly expanding and became publicly available. It thus became relatively easy to search for new members of a gene family using bioinformatics. Specifically, chemokines had advantages for this kind of search because of their small sizes and the molecular signiture of four conserved cysteine residues [3,4]. Thus, a new wave of chemokine discovery started around the mid-1990s with surprising revelations: (i) most of the newly discovered chemokines are expressed in some normal tissues (constitutive expression), (ii) most of them attract lymphocytes and dendritic cells (new target cells), (iii) their genes are mostly mapped to chromosomal sites different from the classical major chemokine clusters (evolutionarily distant), and (iv) they have relatively specific ligand–receptor relationships (low redundancy). Thus, an entirely new group of chemokines that are evolutionarily and functionally distinct from the previous chemokines emerged [3,4].

In this way, in addition to the classical CXC chemokine group that mainly acts on neutrophils and the CC chemokine group that mainly acts on monocytes, the new CXC and CC chemokine groups that mainly act on lymphocytes and dendritic cells, along with new molecular species of (X)C and CX_3C chemokines, have been identified (Table 25.1) [3,4,6]. Furthermore, since the entire sequence of the human genome has been determined, we now know almost all the members of the chemokine superfamily [6]. The classical chemokine groups are now referred to as "inflammatory chemokines," because (i) they are strongly induced by inflammatory stimuli, (ii) mainly attract neutrophils and monocytes, and (iii) play crucial roles in acute and chronic inflammatory reactions. On the other hand, the new groups of chemokines are often referred to as "homeostatic chemokines" since (i) they are constitutively expressed in some normal tissues, (ii) mainly attract lymphocytes and dendritic cells, and (iii) play essential role both in homeostasis of lymphoid tissues and in immune responses. However, in reality, many of the new chemokines are also strongly induced by various inflammatory and other stimuli and could have a role in acute and chronic inflammatory reactions. Thus, such chemokines are often referred to as "dual function chemokines" [6]. However, it should be noted that these classifications are not mutually exclusive and at most tentative.

Now, the question is why the inflammatory chemokines have such ligand redundancy and receptor promiscuity? This can be explained as follows. The ancestral chemokine genes in chromosomes 4 and 17 rapidly increased in number

by repeated gene duplications that occurred relatively recently in the evolutionary term. As a result, multiple ligands binding to a single receptor had been generated. However, the ancestral receptor genes also increased in number by repeated gene duplications. Thus, complex relationships involving multiple ligands and multiple receptors had been created and evolved [4,5]. However, even when multiple ligands act on a single receptor, the binding affinities and signaling functions may be different among the ligands. In addition, due to differences in expressing cells and tissues along with other molecular characteristics, each ligand can have a unique role in the body. Nevertheless, why did such large-scale gene duplication events involving the CXC and CC chemokines in chromosomes 4 and 17 occur in the evolution? This is probably related to the fact that these CXC and CC chemokines attract cells such as neutrophils, monocytes, and eosinophils in inflammatory reactions where it is necessary to rapidly recruit a large number of select leukocytes to a local inflammatory site in response to microbial infection or tissue injury. Therefore, concerted activities involving multiple chemokines and their receptors may be advantageous for such large scale reactions [4,5]. On the other hand, the chemokines that act on lymphocytes and dendritic cells are evolutionary older and their ligand–receptor relationships are more restricted [4,5]. This can be understood by the facts that the migration and homing of specific subsets of lymphocytes and dendritic cells in homeostasis and immune responses need to be tightly regulated. For example, migration of T cells to different target sites such as the secondary lymphoid organs, the gastrointestinal tract, and the skin are mostly determined by the combinatorial expression of specific adhesion molecules and specific chemokine receptors [3,4,6]. In other words, the expression of chemokine receptors specifies the migratory property of each T-cell subset and as such, chemokine receptors often serve as useful surface markers of various functional T-cell subsets (Table 25.3) [3,4,6].

Table 25.3 Chemokine receptor expression by T-cell subsets.

Subset	Major Chemokine Receptors
Naïve T cells	CCR7
Central memory T cells (T_{CM})	CCR7
Type 1 helper T cells (T_H1)	CXCR3, CCR5, and CXCR6
Type 2 helper T cells (T_H2)	CCR4 and CCR3
IL-17-producing T cells (T_H17)	CCR6 and CCR4
IL-22-producing T cells (T_H22)	CCR6
Regulatory T cells (T_{REG})	CCR4
Follicular helper T cells (T_{FH})	CXCR5
Gut-homing T cells ($\alpha4\beta7^+T$)	CCR6
Skin-homing T cells (CLA^+T)	CCR4 and CCR10
Small intestine T cells	CCR9
Skin resident T cells	CCR8
Cytotoxic effector T cells (T_{CT})	CX_3CR1

25.5 The Structure of Chemokines

The structures of many chemokines have been solved both by NMR and X-ray crystallography, revealing that all chemokines have a similar tertial structure that are stabilized by two disulfide binds formed between the first and third and the second and forth cysteine residues (Figure 25.2) [17,18]. The C chemokines have only one disulfide bond and is in equilibrium between a monomer chemokine structure and a dimer nonchemokine glycosaminoglycan (GAG)-binding structure [19]. Some chemokines such as CCL1, CCL15, CCL23, and CCL28 have two extra cysteine residues, one between the second and the third residues and another in the C-terminus, forming the third disulfide bond [17]. The N-terminal sequence prior to the two conserved cysteine residues exhibits a disorganized structure and is important for receptor activation [17,18]. The central part stabilized by the two disulfide bonds consists of a long N-terminal loop and three antiparallel β sheets connected by two short loops. A short loop connects the third β strand and the C-terminal sequence that forms an overlaying α-helix (Figure 25.2) [17,18]. Some chemokines such as CXCL9, CCL24, CCL16, CCL21, CCL25, and CCL28 have extra long C-terminal sequences, often highly basic, suggesting some biological functions [17]. The CXC chemokines with the Glu-Leu-Ag (ELR) motif prior to the first cysteine residue (CXCL1, CXCL2, CXCL3,

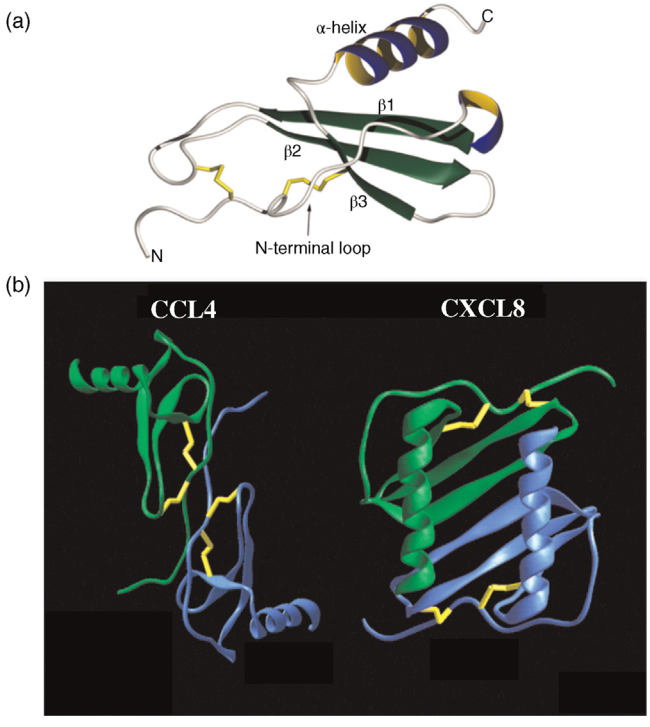


Figure 25.2 Ribbon diagrams of a typical chemokine monomer (a) and dimers (CCL4 and CXCL8) (b). The disulfide bonds are indicated by yellow. The monomer subunits in dimer are indicated by green and blue.

CXCL5, CXCL6, and CXCL7) that commonly signal via CXCR2 are also known to be antiogenic, while non-ELR CXC chemokines such as CXCL4, CXCL9, CXCL10, and CXCL11are angiostatic [20]. Many CXC and CC chemokines have a proline residue at the second position of their N-termini and can be cleaved between the second and the third resides by a membrane-associated serine protease CD26 (dipeptidyl peptidase IV), mostly resulting in impaired activity [17,21]. However, some chemokines are also capped with N-terminal pyroglutamate and thus resistant to the cleavage by CD26 [17].

In solution, chemokines are in equilibrium between monomers and dimers or even higher oligomers [18]. Monomers are the primary forms of receptor activation, while dimers are the form for GAG binding. The binding to GAG is also considered to promote the dimer formation of chemokines [18]. A BBXB (B, basic) motif, a common heparin-binding motif for several proteins, is also found in many CC chemokines close to the fourth cysteine residue [17] and has been shown to be the principal heparin-binding site of CCL5 [22]. The basic residues involved in GAG-binding has also been shown to be aligned in proximity on the dimer interface of CCL3 and CCL4 [23,24]. Similarly, positively charged residues are aligned at the dimer interface of CXCL12 and involved in the binding of heparan sulfate [25]. The binding to GAGs is considered to be important for locally sequestering chemokines on cell surfaces and tissues to establish immobilized chemokine gradients for haptotactic cell migration [26]. Importantly, however, dimers of CXC and CC chemokine subfamilies have distinct quaternary conformations (Figure 25.2) [17,18]. CXC chemokines tend to dimerize into a more globular structure with the interface made up of residues largely from the first β-strand. On the other hand, CC chemokines associate into a relatively elongated structure with the subunit interface composed of residues from part of the extended N-terminus [17,18]. Thus, CXC chemokines have many residues involved in receptor binding still on the surface of the protein, while CC chemokines bury such residues within the dimer interface. Consistently, a covalent CXCL8 dimer is still able to bind CXCR1 and CXCR2, albeit with lower affinity than the wild-type protein [27]. Similarly, CXCL12 monomer and dimer both induce intracellular Ca^{2+} mobilization but only monomeric CXCL12 efficiently induces chemotactic cell migration [28]. In contrast, CCL4 dimer is unable to bind or activate its receptor [29].

In the case of two transmembrane-type chemokines CX3CL1 and CXCL16, an N-terminal chemokine domain is connected to a muchin-like stalk with a transmembrane α-helix and a short cytoplasmic tail [17,30]. Thus, these chemokines are primarily membrane associated and function as a soluble chemokine upon cleavage at membrane proximal sites by disintegrin and metalloproteinases ADAM10 and ADAM17 [21]. By the membrane-bound form, CX3CL1 acts as an efficient signal-independent cell adhesion molecule under flow conditions [31,32], while CXCL16 may also act both as a cell adhesion molecule and as a scavenger receptor for oxidized LDL [30]. Of note, monocytes are divided into two major subtypes: $CX3CR1^{low}$ classical monocytes ($CD14^{+}CD16^{-}$ in humans and $Ly6C^{+}CD43^{-}$ in mouse) and $CX3CR1^{high}$ nonclassical monocytes ($CD14^{dim}CD16^{+}$ in humans and $Ly6C^{-}CD43^{+}$ in mouse) [33]. The $CX3CR1^{high}$ nonclassical monocytes are characterized by their unique ability to patrol the

vascular endothelium independent of the direction of blood flow (~12 μm/min) under homeostatic and inflammatory conditions [33]. The adhesive property of the CX3CL1-CX3CR1 axis appears to play a role in this patrolling behavior of nonclassical monocytes [33].

25.6 Chemokine Receptors

All chemokine receptors are seven-transmembrane-type receptors and form a subgroup within the class A rhodopsin-like family of GPCRs [1–7]. Their structures consist of a relatively long N-terminal extracellular domain enriched with acidic amino acids and tyrosine-sulfated, the relatively well-conserved seven-transmembrane α-helices that are connected by three intracellular loops (ICL1-3) and three extracellular loops (ECL1-3), and a C-terminal cytoplasmic region forming α-helix and enriched with serine and threonine residues (Figure 25.3). The receptors are also divided into CC, CXC, (X)C, and CX3C subfamilies based on the subfamily of their ligands (Table 25.2). At present, 19 receptors are known that mediate chemotactic and calcium flux responses in responding cells [6–8]. They are commonly coupled with pertussis toxin-sensitive $G\alpha_i$-type trimeric G proteins. Moreover, five atypical chemokine receptors (ACKRs) that do not signal via trimeric G proteins have been identified [6,7]. Their primary functions

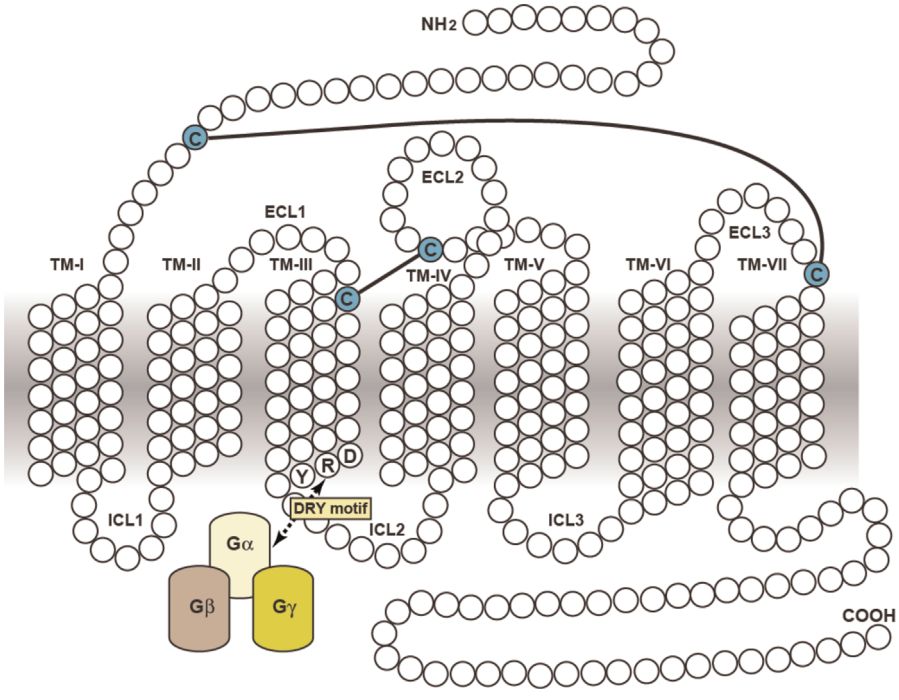


Figure 25.3 A schematic depiction of a typical chemokine receptor. Abbreviations: DRY, aspartic acid–arginine–tyrosine; ECL, extracellular loop; ICL, intracellular loop; TM, transmembrane.

are to scavenge chemokines. Similar to the case of ligands, there exists a large gene cluster consisting of mainly CC chemokine receptors on chromosome 3 in humans [5].

Most GPCRs are known to form homodimers or heterodimers, and the chemokine receptors are no exceptions [34]. The formation of homodimers or even heterodimers with other chemokine receptors or even with different GPCRs adds a new dimension to the complex regulatory mechanisms of the chemokine receptor system through mechanisms such as changes in ligand-binding affinity due to allosteric effects, changes in signal transduction efficiency due to competition for G proteins, and passive internalization by ligand binding to the partner receptor [34]. Furthermore, chemokine receptors may associate with membrane molecules other than GPCRs. The best studied examples are the association of CCR5 and CXCR4 with CD4, which explains why CCR5 and CXCR4 are preferentially used as the major coreceptors of HIV along with the primary receptor CD4 for target cell infection [35,36]. In particular, CCR5 is strongly expressed by activated T cells, intestinal T cells, and monocytes/macrophages, the major target cells of HIVs, and constitutively associated with CD4 with a stoichiometry of 5 to 1 [36]. In addition, the affinity of CCR5 to HIV-1 gp120 is much higher than that of CXCR4 (Kd: <10 nM versus 200–500 nM) [35]. These facts explain why R5-type strains using CCR5 are initially dominant in HIV-infected patients, while the X4-type strains using CXCR4 appear only at the terminal stages of the disease when $CD4^+$ T cells are almost depleted [12].

25.7 The Two-step Model of Chemokine Receptor Activation

Chemokines activate their receptors through a two-step binding process [18,34,37]. First, the structurally rigid central part of a chemokine docks on a chemokine recognition site 1 (CRS1) of its receptor mainly formed by the free N-terminus and ECL2, the longest extracellular loop forming a β-hairpin structure. Since chemokines are generally rich with basic amino acids and thus highly cationic, the electrostatic interactions are considered to play the major role in high-affinity binding. In particular, the N-terminal-free structures of chemokine receptors, which are rich with acidic amino acids and are also tyrosine sulfated, are likely to be involved in initial trapping of positively charged chemokine molecules. Subsequently, if the flexible N-terminal short sequence of a chemokine fits into the binding pocket buried in the cavity formed by the outer parts of the seven-transmembrane helices (CRS2), the interactions cause a drastic conformational change in the receptor, thereby resulting in its activation [18,34,37]. Thus, the N-terminal short amino acid sequence of a chemokine is crucial for receptor activation and can be regarded as a functional mimic of a conventional GPCR ligand, which is usually a small molecule. Accordingly, proteolytic processing of the N-terminal sequence of a chemokine can drastically alter its activity, often from an agonist to an antagonist, without changing its binding affinity to the receptor [21]. Conversly, N-terminal peptides of a chemokine may act as agonists

or antagonists, albeit with low potency [38]. It also explains why some chemokines act as natural antagonists (Tables 25.1 and 25.2); a chemokine may bind to CRS1 of a receptor with high affinity without fitting to its CRS2. For example, CXCL9, CXCL10, and CXCL11 (the CXCR3 ligands) function as antagonists to CCR3, while CCL11 (the CCR3 ligand) behaves as an antagonist to CXCR3 [39]. Given that CXCR3 is mainly expressed by Th1 cells while CCR3 is expressed by eosinophils, basophils, and some Th2 cells, the mutual antagonism may further polarize immune responses. Furthermore, some chemokines such as CCL14, CCL15, CCL16, and CCL23, often present at high concentrations in the blood, have a relatively long N-terminus compared to those of other chemokines and become a potent antagonist only after the N-terminal sequences are processed by proteases such as neutrophil-derived catepsins at inflammatory sites [6]. Thus, these "plasma chemokines" can be regarded as the precursor-type chemokines [6].

25.8 Signal Transduction

The mechanism of signal transduction by chemokine receptors follows the general rule of GPCR activation (Figure 25.4) [9,40]. When a chemokine binds to its receptor, GDP bound to the α-subunit of trimeric G proteins (consisting of the three subunits of α, β, and γ) coupled to the receptor is exchanged for GTP (the GDP–GTP exchange reaction). Thus, the ligand-activated receptor functions as a GDP–GTP exchange factor (GEF). This results in release of the trimeric G proteins from the receptor and dissociation of the α-subunit and the βγ-subunit.

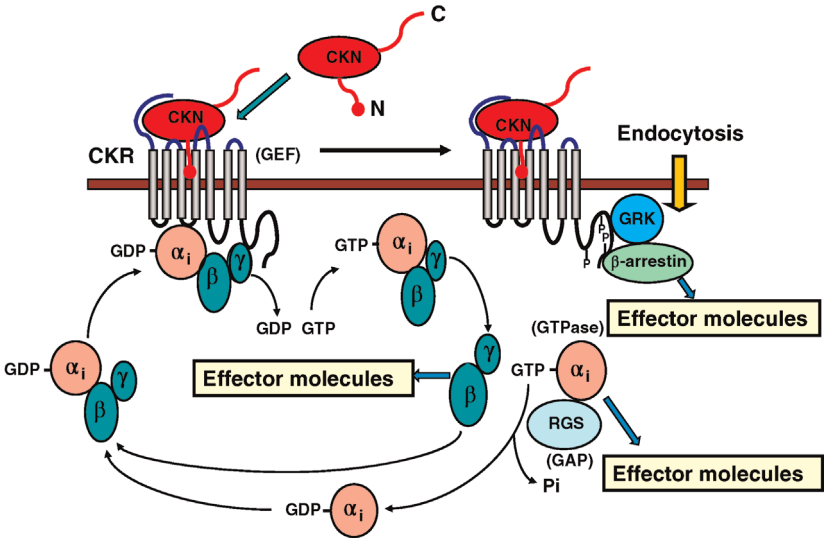


Figure 25.4 The activation cycle of GPCR. Abbreviations: CKN, chemokine; CKR, chemokine receptor; GAP, GTPase activation protein; GEF, GDP-GTP exchange factor; GRK, G protein-coupled receptor kinase; RGS, regulator of G protein signaling.

The dissociated subunits have been shown to activate various downstream effector molecules. However, because the α-subunit has its own GTPase activity, the bound GTP is quickly converted to GDP, thereby the α-subunit and βγ subunits reassociate and signal transduction stops. The GTPase activity of α-subunits is also regulated by a group of proteins referred to as regulators of G protein signaling (RGS), which can be regarded as GTPase-activating proteins (GAPs). Thus, a GPCR can function as a quick molecular on–off switch in response to its ligand. Moreover, ligand-bound chemokine receptors are quickly phosphorylated at the serine/threonine residues in the C-terminus by G protein-coupled receptor kinases (GRKs). This results in association of β-arrestins with the C-terminus and internalization of chemokine receptors into the cells by endocytosis. Internalized receptors may be degraded in the lysosomes or recycled back to the cell surface after releasing bound ligands under endosomal low pH conditions. As for the downstream signaling pathways, activation of molecules such as phosphoinositide 3-kinase γ (PI3 kinase γ), phospholipase C, protein kinase C, Mitogen-activated kinase (MAP) pathways, and small G proteins Rac-1 and Rap-1 have been reported [9,40,41].

25.9 Atypical Chemokine Receptors

There are five atypical chemokine receptors (ACKRs) [6,7,42]. While they have the seven-transmembrane structures similar to the standard chemokine receptors, they do not transduce signals via the trimeric G proteins. This is because the Asp-Arg-Tyr (DRY) motif (typically DRYLAIV) that is present in ICL2 of the standard chemokine receptors and is necessary for efficient coupling with the Gαi class G proteins is missing or substantially altered. However, the signaling via β-arrestins may still occur. It is now known that ACKRs play essential roles in the chemokine biology primarily by acting as chemokine scavengers [6,7,42].

ACKR1/DARC is expressed by erythrocytes and binds many inflammatory CXC and CC chemokines. It is thus considered that ACKR1 regulates chemokine concentrations in the blood by functioning as a chemokine sink. Moreover, ACKR1 is expressed by venular endothelial cells and transports chemokines from tissues to the vascular surface by transcytosis [7,43]. ACKR2/D6 also binds many inflammatory CC chemokines and continuously recycles between the cell surface and the cytoplasm, while internalized chemokines are dislodged from the receptor by low pH in endosomes and destroyed in lysosomes. ACKR2 is strongly expressed by lymphatic endothelial cells and functions as a chemokine scavenger receptor for lymphatic fluids, thereby suppressing unnecessary inflammatory reactions within the lymphatic vessels [7,42]. ACKR3/CXCR7 is a chemokine scavenger receptor of great interest in both development and disease [44]. Along with CXCL12 and CXCR4, ACKR3 is one of the oldest and highly conserved members of the chemokine superfamily in vertebrates [5]. ACKR3 binds CXCL11 and CXCL12 [6,7]. In particular, ACKR3 is closely involved in some developmental processes that are controlled by the CXCL12-CXCR4 axis (see below). Moreover, ACKR3 is often expressed by tumor cells and tumor vessels and promotes tumor cell growth and angiogenesis by activating MAPK cascades and

other signaling pathways [44,45]. ACKR4/CCRL1 binds CCL19 (a CCR7 ligand) and is likely to modulate the function of the CCR7 axis, which plays an essential role in homeostatic migration of lymphocytes and dendritic cells. Similarly, ACKR5/CCRL2 binds CCL19 and CCL21 (the CCR7 ligands) as well as CCL25 (the CCR9 ligand). Thus, it may modulate the functions of not only the axis of CCR7 but also that of CCR9, which is involved in mucosal immunity [7,42].

25.10 Organ Development and Chemokines

CXCL12, CXCR4, and ACKR3 are the oldest members of the chemokine superfamily, being present even in the jawless fish sea lamprey [5]. From the analyses of the respective KO mice, it has been revealed that CXCL12 and CXCR4 are critically involved in various developmental processes during the fetus such as B lymphopoiesis in the liver, colonization of hematopoietic stem cells in the bone marrow, vascularization of the gastrointestinal tract, formation of the cardiac ventricular septal, and migration of granule cells in the cerebellum and interneurons in the neocortex [46]. Ventricular septal defects are also observed in ACKR3 KO mice [47]. This is probably due to the necessity of ACKR3 for modulating the CXCL12-CXCR4 axis both by scavenging excess CXCL12 and by promoting internalization of CXCR4 through heterodimer formation. Among others, ACKR3 also plays an important role in the migration of embryonic primordial germ cells to the future gonads [48,49]. In this case, ACKR3 expressed by somatic cells along the migratory pathway scavenges CXCL12 in the rear side to maintain the CXCL12 gradient, thereby enabling the primordial germ cells to proceed [48,49]. ACKR3 is also involved in the formation of lateral lines in fish, which comprise the mechanosensory system to perceive the water flow [50]. In this case, the precursor cells migrate as a single mass along the lateral lines during the process of neuromast formation. Migrating cells at the front express CXCR4 and proceed toward CXCL12, while cells in the rear stop migration by upregulating ACKR3 and form neuromasts [50].

25.11 The Primary Lymphoid Organs and Chemokines

The CXCL12-CXCR4 axis that plays crucial roles in the fetal hematopoiesis remains to be involved in bone marrow functions thereafter. For example, the CXCL12-abundant reticular (CAR) cells form the niche in which hematopoietic stem cells and other hematological cells adhere, grow, and differentiate [51]. In addition, the CXCL12–CXCR4 interactions are necessary for retention of immature hematological cells in the bone marrow. Evidence is also provided by a “nature’s experiment” WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome, an autosomal dominant immunodeficiency disease caused by mutations in the CXCR4 gene resulting in C-terminal truncations, which is characterized by enhanced bone marrow retention of mature hematological cells and peripheral pancytopenia [52]. The mutations cause truncations of

the C-terminal region of CXCR4, leading to enhanced CXCR4 activity due to impaired ligand-induced internalization and desensitization. In the same vein, AMD3100 (plerixafor), a low molecular weight antagonist of CXCR4, has been clinically approved for mobilization of hematopoietic stem cells from the bone marrow in order to perform autotransplantation in patients with non-Hodgkin lymphomas and multiple myeloma [7]. Conversely, aged neutrophils in the peripheral blood return to the bone marrow for destruction via upregulating CXCR4 [53].

In the case of T cell development, thymic precursor cells released from the bone marrow home to the thymus primarily via CCR7 and CCR9, while medullary epithelial cells produce CCL19/CCL21 (the CCR7 ligands) and CCL25 (the CCR9 ligand) [54,55]. CCR7 is also involved in medullary entry of positively selected single-positive thymocytes from cortex [55,56], while CCR4 facilitates negative selection in the medulla where medullary dendritic cells produce CCL17 and CCL22 (the CCR4 ligands) [57]. After positive and negative selections, mature thymocytes increase the surface expression of sphingosine-1-phosphate receptor type 1 ($S1P_1$) and leave the thymus by sphingosine-1-phosphate (S1P), which is present at high concentrations in the peripheral blood [58].

25.12 The Secondary Lymphoid Organs and Chemokines

The secondary lymphoid organs (SLOs) such as regional lymph nodes, spleen, and Peyer's patches in the small intestine have common histological characteristics: (i)high endothelial venules (HEVs) functioning as the gateway for lymphocytes to enter from the bloodstream (note that HEVs do not exist in the spleen; instead, sinusoids, which exist in the marginal zone between the red pulp and the white pulp, function as the gates), (ii) the specialized T-cell area (paracortex) and B-cell areas (follicles), (iii) the backbone network structures formed by fibroblastic reticular cells in the T-cell area where dendritic cells and macrophages are positioned as well as the distinct network structures formed by follicular dendritic cells in the B-cell areas, (iv) afferent lymphatics by which antigen-loaded dendritic cells flow in from peripheral tissues (note that these do not exist in the spleen or Peyer's patches), and (v) efferent lymphatics by which lymphocytes flow out of the SLOs [59,60]. Mature T and B cells continuously recirculate between the blood and the SLOs [16,59]. On the other hand, dendritic cells distributed in the peripheral tissues initiate maturation upon antigen loading and home to the T-cell areas of regional lymph nodes via afferent lymphatics, where they function as professional antigen-presenting cells that efficiently activate naïve T cells [4,61]. Thus, SLOs serve as the crossroads for circulating lymphocytes and antigen-presenting dendritic cells.

Naïve T cells express CD28 and CD40L, while mature dendritic cells express their respective ligands CD80/CD86 and CD40. In addition to the T-cell receptor (TCR) signals, naïve T cells require the costimulatory signals via CD28 and CD40L for full activation, making their activation dependent on mature dendritic cells functioning as professional antigen-presenting cells in the SLOs [62]. On the other hand, the activation of effector/memory T cells is less dependent on the

costimulatory signals, which enables their activation in tissues other than SLOs. Therefore, their migratory routes include peripheral tissues such as the skin and mucosa, where they are more likely to encounter their cognate antigens [62]. In the case of B cells, immature B cells are released from the bone marrow and migrate into the white pulp of the spleen where most B cells are short-lived and only a portion of B cells form primary follicles to become a pool of circulating long-lived mature naïve B cells [63]. Circulating naïve B cells also enter the SLOs via HEVs. Upon antigenic stimulation within SLOs, B cells grow and differentiate within extrafollicular areas to become short-lived plasma cells, which localize in the medullary areas of the SLOs and produce low-affinity IgM antibodies, or migrate to the B-cell areas where they form germinal centers, undergo somatic hypermutation and affinity maturation, and differentiate into long-lived plasma cells that produce high-affinity IgG or IgA antibodies [64]. These plasma cells home to the bone marrow or submucosal tissues via the blood flow. To form germinal centers, costimulatory signals through the interaction between CD40 expressed by B cells and CD40 L expressed by activated T cells are necessary [64]. Moreover, follicular dendritic cells in the germinal centers retain and present immune complexes by Fc receptors and complement receptors, thereby enhancing the selection of B cells producing high-affinity antibodies [64]. On the other hand, B cells that undergo apoptosis in the germinal centers produce CX3CL1 as an "eat me" signal and are removed by macrophages expressing its receptor CX3CR1 [65].

After homing to the SLOs, T cells migrate on the network structure formed by fibroblastic reticular cells with a fast ameboid movement and repeatedly make brief contacts with dendritic cells on the network [66,67]. If the TCRs recognize cognate antigen peptides presented by dendritic cells in association with the MHC molecules, T cells stop migration and firmly adhere to the dendritic cells. This lasts from several minutes to hours and results in the activation of T cells [66,67]. On the other hand, T cells that fail to encounter antigenic peptides upregulate their surface expression of $S1P_1$ and leave the SLOs via efferent lymphatics where the concentration of its ligand S1P in the lymphatic fluid is high [58]. The time from entering to leaving the SLOs is approximately 6–24 h [66,67].

The combined expression of L-selectin and CCR7 endows circulating T cells and B cells to home into SLOs via HEVs [4]. On the other hand, immature dendritic cells express various receptors for inflammatory chemokines, which supports their migration from the bone marrow to various peripheral tissues via the blood [4]. In particular, immature dendritic cells express CCR6, while epidermal keratinocytes and mucosal epithelial cells produce its ligand CCL20 [4]. Upon antigen loading, dendritic cells start maturation and increases the expression of CCR7, while decreasing other chemokine receptors including CCR6, thus enabling their emigration via afferent lymphatics where endothelial cells constitutively express its ligand CCL21 [4,68]. In the T-cell areas of SLOs, mature dendritic cells produce CCL19 (another CCR7 ligand) to facilitate their interactions with CCR7-expressing naïve T cells and central memory T cells [4,68]. Interestingly, CCL19 and CCL21 that similarly act on CCR7 in terms of chemotaxis induction *in vitro* play different roles *in vivo* [68]. Only CCL21 is

abundantly produced by the afferent lymphatic endothelium. The endothelial cells of HEVs also constitutively produce CCL21, while CCL19 produced by dendritic cells and stroma cells in the SLOs can be presented by HEVs through transcytosis. CCL21 also possesses a positively charged 40 amino acid long C-terminus that enables its strong binding to GAGs. On the other hand, only CCL19 efficiently induces internalization and desensitization of CCR7. Therefore, T cells and B cells home to the SLOs primarily via CCL21. Within the SLOs, CCL19 produced by dendritic cells can override CCL21-mediated migration and eventually downregulates the expression of CCR7, allowing T cells to be localized in the T-cell areas [68]. Dowregulation of CCR7 is also necessary for B cells to redirect their migration to the B-cell areas where follicular dendritic cells produce CXCL13, while B cells express its receptor CXCR5 [4,69]. Follicular B helper T (T_{FH}) cells that both provide costimulatory signals via CD40L and ICOS for B-cell activation and produce IL-21 to promote B-cell differentiation into antibody-producing cells also express CXCR5 to be localized in the B-cell areas and produce CXCL13 to attract B cells [4,69].

25.13 Memory/effector T Cells and Chemokine Receptors

Memory/effector T cells are divided into two major subpopulations according to the surface phenotypes of L-selectin$^+$CCR7$^+$ and L-selectin$^-$CCR7$^-$. The former represents central memory T cells (T_{CM}), which, like naïve T cells, home to the SLOs and efficiently activate dendritic cells but are slow to exert effector functions. The latter represents effector memory T cells (T_{EM}), which migrate to peripheral tissues and quickly exert effector functions [4,70]. Furthermore, memory/effector T cells are subdivided into several functional subsets based on their cytokine signature [4,71]. This is because activated naïve T cells differentiate into each functional subset in accordance with the cytokine milieu during their differentiation (cytokine imprinting). Th1 cells that produce IL-2 and IFN-γ are involved in cell-mediated immunity and granuloma formation. IL-12 promotes Th1 differentiation by inducing T-bet, the Th1 master regulator transcription factor [72]. Th1 cells thus induced express CXCR3 as one of the downstream target genes of T-bet, while IFN-γ is a potent inducer of CXCL9, CXCL10, and CXCL11 (the CXCR3 ligands) [4], thereby amplifying Th1 responses by further recruiting Th1 cells expressing CXCR3. Th1 cells may also express other chemokine receptors such as CCR5 and CXCR6 [4]. Th2 cells produce IL-4, IL-5, IL-13, and so on and are involved in humoral immunity and allergic reactions. IL-4 promotes Th2 differentiation by inducing the Th2 master regulator transcription factor GATA-3 [72]. Th2 cells predominantly express CCR4, while Th2 cytokines such as IL-4 and IL-13 are potent inducers of CCL17 and CCL22 (the CCR4 ligands) in various cells [4], thereby amplifying the Th2 responses by further recruiting Th2 cells expressing CCR4. Th2 cells may also express receptors such as CCR3 and CRTH2, a G protein-coupled receptor for prostaglandin D_2 [4,71]. Th17 cells produce IL-17 and IL-22 and are involved both in immune responses toward extracellular pathogens and in various autoimmune diseases. The combination of IL-6 and TGF-β promotes Th17

differentiation by inducing the expression of RORγt, the Th17 master regulator transcription factor [71]. While Th17 cells express CCR6 as one of the downstream target genes of RORγt, IL-17 induces the production of CCL20 (the CCR6 ligand) in various cells [73], thereby amplifying Th17 responses by further recruiting Th17 cells expressing CCR6. Th17 cells may also express chemokine receptors such as CCR4. T_{FH} cells are localized in the B-cell areas of SLOs and produce CXCL13 (the CXCR5 ligand) along with IL-21, thereby both attracting B cells expressing CXCR5 and promoting their differentiation into antibody-producing cells by IL-21 [74]. Treg cells are important for immunosuppression and express FOXP3, the master regulator transcription factor of Treg [75]. While natural Treg cells are generated in the thymus, the combination of TGF-β and IL-2 promotes differentiation of Treg cells in the periphery by inducing FOXP3 [75]. Treg cells predominantly express CCR4 and are recruited by CCL17/CCL22 produced by cells such as dendritic cells and tumor cells [76]. Treg cells may also express other chemokine receptors such as CCR5 and CXCR3 to regulate a wide range of immune responses by colocalizing with diverse effector T cells. Similarly, effector $CD8^+$ T cells and natural killer cells express a wide range of chemokine receptors such as CCR2, CCR5, CXCR3, CXCR1, and CX3CR1 in accordance with their differentiation and activation states [77,78].

25.14 Mucosal Immunity and Chemokines

Mucosal tissues form the boundary between the inside and the outside of the body. For instance, the small intestine is covered with a monolayer of columnar epithelial cells, with a surface area of 200–300 m^2 in adults. Thus, the mucosal immunity is vital for the host defense. The intestinal immune system is composed of organized lymphoid tissues and free lymphoid cells. The former includes Peyer's patches and isolated lymphoid follicles, while the latter consists of IgA-producing plasma cells, effector T cells, and intraepithelial T cells. The organized lymphoid tissues are the inductive sites of the immune response. On the other hand, free lymphoid cells constitute effector cell populations in intestinal immunity [4]. In particular, Peyer's patches are covered by follicle-associated epithelium (FAE) where specialized cells referred to as microfold cells (M cells) exist. While FAE epithelial cells produce CCL20 and recruit dendritic cells expressing its receptor CCR6 underneath the FAE, M cells actively take up intestinal antigens and pathogens (antigen sampling) and transport them to underneath dendritic cells and macrophages [4]. In addition, intestinal epithelial cells express CX3CL1, while subepithelial dendritic cells extend their cellular processes through the epithelial cells by expressing its receptor CX3CR1, thereby directly sampling antigens in the lumen [79].

T cells that home to Peyer's patches express α4β7 integrin and L-selectin, while the HEVs of Peyer's patches express their common ligand MAdCAM-1 [80]. Once T cells are activated by antigenic stimuli in Peyer's patches, they start differentiation and migrate to the mesenteric lymph nodes via efferent lymphatics. After returning to the blood, most of these cells redistribute to the lamina propria of the intestinal mucosa to become effector cells [80]. Moreover,

intraepithelial lymphocytes (IELs) exist within the intestinal mucosa with a frequency of one to every three to five epithelial cells. IELs are locally generated from the precursor cells present in the intestinal mucosa and express αEβ7 integrin, while epithelial cells express its ligand E-cadherin [4,81]. Intestinal epithelial cells also express CX3CL1, while IELs express its receptor CX3CR1 [4].

The epithelial cells of the small intestine produce CCL25 at high levels, while $\alpha4\beta7^{+}$gut-homing T cells in the blood express its receptor CCR9 [4,80]. Moreover, effector T cells, IgA-producing B cells, and IELs present in the small intestine express CCR9 [4,80]. Thus, the CCL25–CCR9 axis plays a pivotal role in localization of immune cells in the small intestine. In this context, dendritic cells present in the mesenteric lymph nodes produce retinoic acid, the metabolite of vitamin A, which is a potent inducer of α4β7 integrin and CCR9 expression in activated T cells [82]. Thus, gut-associated dendritic cells are able to imprint gut tropism to activated T cells.

In contrast to CCL25 production by the small intestine, other mucosal tissues in the body such as the large intestine, airway mucosa, salivary glands, and lactating mammary glands produce CCL28 at high levels, while its receptor CCR10 is widely expressed by mucosal IgA-producing B cells [83,84]. Therefore, the CCL28–CCR10 axis is considered to play an important role in the pan-mucosal immune system by redistributing locally generated IgA-producing B cells throughout the body. Moreover, CCL28 is secreted in the mucosal fluids and exerts a strong antimicrobial activity [85]. In fact, a number of chemokines have been shown to possess a significant antimicrobial activity [86]. The mechanism of how chemokines carry out antimicrobial action is not clear at present but may be related to the molecular features similar to classical antimicrobial peptides, the natural antibiotics in multicellular organisms, such as disulfide bridges, high canionic amino acid contents, and amphipathic stretches [86].

25.15 Skin Immunity and Chemokines

Like the mucosal tissues, the skin functions as an interface between the inside and the outside of the body. In the case of skin, however, the keratinized stratified squamous epithelium provides a strong physical barrier. While no organized lymphoid tissues are present in the skin, there exist large numbers of epidermal and dermal dendritic cells, which upon activation by antigen-loading upregulate the expression of CCR7 and home to regional lymph nodes via afferent lymphatics to become professional antigen-presenting cells in the T cell area [4,68]. Epidermal dendritic cells are specifically referred to as Langerhans cells, which express CCR6 and are considered to be recruited to the epidermis by CCL20 produced by epidermal keratinocytes [4]. The CCL1–CCR8 axis may also regulate the localization of dendritic cells in the skin [87]. On the other hand, cutaneous lymphocyte antigen (CLA)-positive skin-homing memory/effector T cells present in human peripheral blood express CCR4, while the CCR4 ligands CCL17 and CCL22 are produced in inflamed skin by microvascular endothelial cells and epidermal dendritic cells, respectively [4,88]. Thus, skin-homing memory/effector T cells extravasate into inflamed skin by the guidance of CCL17 and further

migrate within the dermis by the guidance of CCL22. This is possible because, although both CCL17 and CCL22 similarly induce chemotaxis via CCR4 *in vitro*, only CCL22 efficiently induces internalization and desensitization of CCR4 [88]. Thus, CCR4-expressing T cells can migrate into and within the skin by responding to CCL17 and CCL22 sequentially. Furthermore, CLA-positive skin-homing memory/effector T cells express CCR10, while epidermal keratinocytes produce CCL27 (a CCR10 ligand) [4,89]. Therefore, after CCR4, CCR10 may take over the migration of T cells toward the epidermis by the guidance of CCL27. In parallel to retinoic acid in the gut, vitamin D_3 is produced in the skin epidermis from 7-dehydrocholesterol by the action of ultraviolet B radiation and converted into the active form 1, 25-$(OH)_2$-vitamin D_3 by dermal dendritic cells. 1, 25-$(OH)_2$-vitamin D_3 induces the expression of CCR10 and suppresses the expression of α4β7 integrin and CCR9 in activated T cells [90]. This suppression of α4β7 integrin expression by 1, 25-$(OH)_2$-vitamin D_3 explains why CCR10-expressing T cells are unable to home to the mucosal tissues where CCL28, another CCR10 ligand, is highly expressed. In addition, the vast majority of T cells present in the normal human skin express CCR8, while subcutaneous microvascular endothelium and epidermal dendritic cells produce its ligand CCL1 [91]. CXCL14 is also produced in the skin by keratinocytes, macrophages, and mast cells and exhibits a potent antimicrobial activity against various microbes [92]. Like CXCL12, CXCL14 is a chemokine highly conserved among the vertebrate species. No major abnormalities are, however, observed in CXCL14-deficient mice and its receptor has not yet been identified [93]. Of note, the N-terminal sequence of CXCL14 prior to the first cysteine residue consists of only two amino acids (Ser-Lys), which may be too short to activate a GPCR receptor [17].

25.16 Clinical Applications

The chemokine superfamily plays important roles in both innate and acquired immunity by inducing tissue infiltration of select leukocytes [1–7]. However, tissue accumulation of leukocytes is a "double-edged sword" and could be a cause of disease. Therefore, drugs targeting the chemokine superfamily are expected to be therapeutically useful for diseases involving excess inflammatory reactions. Furthermore, given that many successful drugs in use are known to target GPCRs, the chemokine receptors are considered to be highly promising molecular targets for drug development. Moreover, since hematological tumors are derived from leukocytes at particular stages of differentiation, the expression patterns of chemokine receptors often reflect both their cellular origins and their preferred sites of invasion. Thus, the chemokine receptors are also expected to provide excellent target molecules for diagnosis and treatment of hematological tumors. In addition, chemokines are involved in various clinically important problems such as cancer growth and metastasis, angiogenesis, and HIV infection [11,12].

Despite such promising features, however, the list of successful drugs targeting chemokine receptors still remains small [7,94]. At present, only three drugs are clinically approved: an orally available CCR5 inhibitor maraviroc as an entry

inhibitor of R5 type HIV-1, a low molecule CXCR4 inhibitor AMD3100 (plerixafor) as a mobilizer of hematopoietic stem cells from the bone marrow for stem cell autotransplantation, and a defucosylated humanized anti-CCR4 antibody (mogamulizumab) developed in Japan for the treatment of CCR4-expressing adult T-cell leukemia/lymphoma and peripheral T-cell lymphoma [7,88]. The unexpected difficulties in the development of drugs targeting chemokine receptors may be explained by reasons such as the intrinsic redundancy of the chemokine system and mainly charge-driven and less conformation-driven interactions of chemokines and chemokine receptors in the first step of the two-step receptor activation. Other reasons may include the considerable species differences in the chemokine system even between humans and mice, making it often difficult to use the same compounds in animal studies or to directly translate results from animal studies to human diseases. However, the recent progress in the elucidation of crystal structures of GPCRs including chemokine receptors will now open new possibilities for rational design of drugs having improved inhibitory profiles and mechanisms of action [95–97].

Meanwhile, the chemokines are also expected to be useful biomarkers of various disease conditions due to their excessive production by some tissue cells in the event of inflammation and immune responses. For instance, the blood concentrations of CCL17 and CCL22 are highly elevated and serve as sensitive markers of disease activity in patients with atopic dermatitis [88]. This has led to the approval of a CCL17 measurement kit (Alaport TARC) in Japan to monitor the disease activity and therapeutic efficacy of atopic dermatitis [88]. There are many studies demonstrating significant elevations of some chemokines in association with particular diseases [87,98–100].

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26

Lipid Mediators in Inflammation

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26.1 Introduction: Biosynthesis, Degradation, and Receptors of Lipid Mediators

Lipid mediators reviewed in this chapter include prostaglandins (PGs), leukotrienes (LTs), endocannabinoids, platelet-activating factor (PAF), lysophosphatidic acid (LPA), sphingosine-1-phosphate (S1P), and others. Biosynthesis, degradation, and receptors of these lipid mediators are depicted in Figure 26.1. PGs and LTs are produced from carbon 20 unsaturated fatty acids, mainly arachidonic acid (AA) in mammals fed on normal diet. AA is esterified to *sn*-2 position of phosphoglycerides in resting cells and is cleaved out from them on cell activation [1]. The classic pathway catalyzing the AA release is phospholipase A_2 (PLA_2), either cytosolic PLA_2 ($cPLA_2$) or secretory PLA_2 ($sPLA_2$), which acts on glycerophospholipids and release AA. More recently, it was found that monoacylglycerol lipase acts on 2-arachidonyl glycerol, one of the endocannabinoids, to liberate AA and that this pathway operates in the brain, liver, and lung [2]. AA liberated is then converted to PGs and LTs by the action of cyclooxygenase (COX) and 5-lipoxygenase (5-LOX), respectively. COX converts AA to PGH_2, which is then converted to PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, thromboxane (TX) A_2, and 12-hydroxyheptadecatrienoic acid (12-HHT) by respective synthases. These PGs are released out of the cells and exert autocrine and/or paracrine functions by acting on the cognate receptor on the surface of target cells [3]. The PG receptors include PGD receptor (DP or DP1), four subtypes of PGE receptor (EP1–EP4), PGF receptor (FP), PGI receptor (IP), and TXA receptor (TP). These receptors are G protein-coupled receptors (GPCRs), with seven transmembrane domains, and constitute a subfamily in the GPCR superfamily. There is another GPCR responding to PGD_2 outside this subfamily, which is called CRTH2 or DP2 and belongs to the chemokine receptor family. PGs are inactivated by being taken up by the cell via PG

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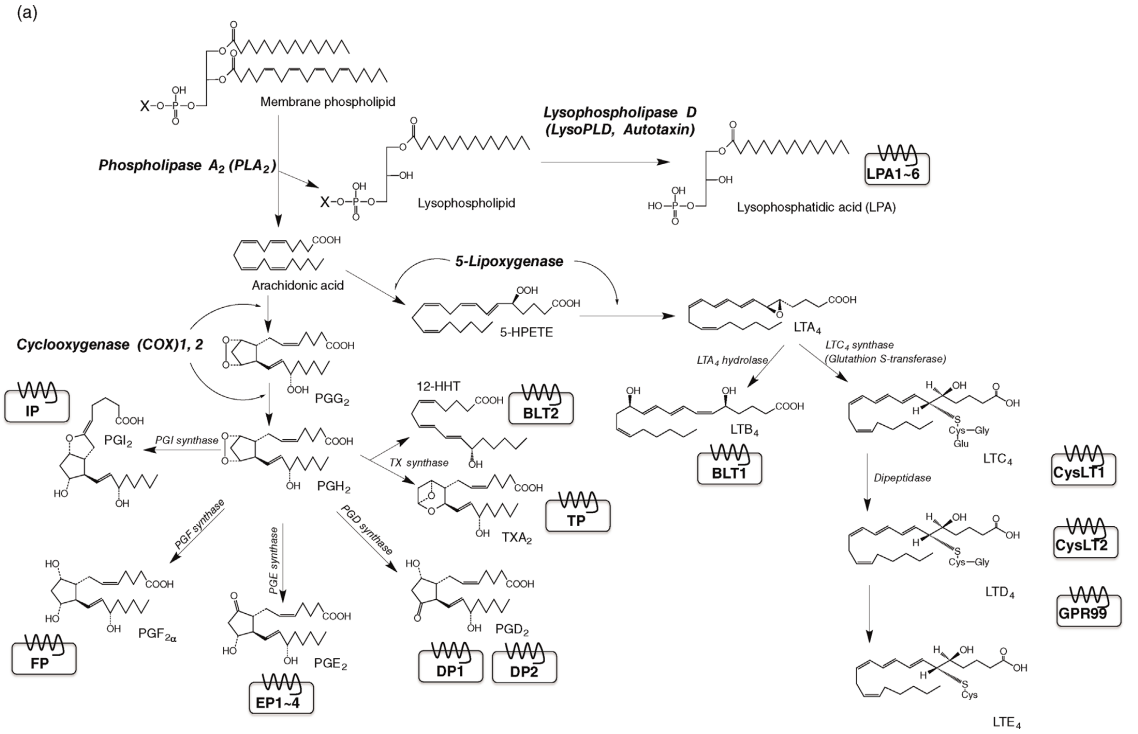


Figure 26.1 Biosynthesis, degradation, and receptors of lipid mediators. (a) Biosynthesis of prostaglandins (PGs), leukotrienes (LTs), and lysophosphatidic acid from membrane phospholipids. Italics indicate enzymes and bold characters in boxes show the nomenclature of receptors. (b) General scheme of inactivation of prostaglanidns. Hydroxyl group at C15 is dehydrogenated by a dehydrogenase (PGDH), and the double bond at C13, 14 is reduced by a reductase (PGR). In addition to this pathway, omega and beta oxidation of PGs is reported.

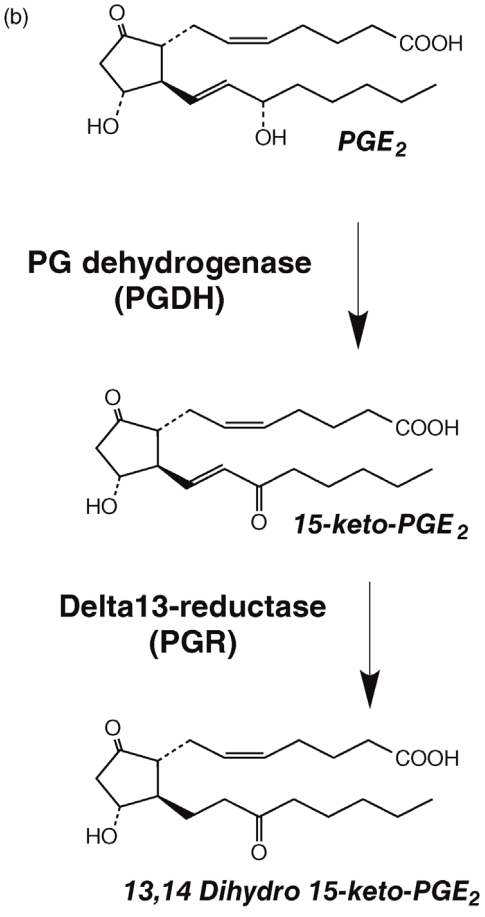


Figure 26.1 (*Continued*)

transporter and conversion to 13, 14-dihydro-15-ketoPGs by 15-hydroxyPG dehydrogenase (PGDH) and PG Δ^{13}-reductase (Figure 26.1b) [4].

In some cells, such as granulocytes, eosinophils, and macrophages that express 5-LOX, released AA is converted by dual enzymic activities of 5-LOX into an unstable intermediate, leukotriene A_4 (LTA_4) without any known biological activities. LTA_4 is hydrolyzed into a dihydroxyLT, leukotriene B_4 (LTB_4) by LTA_4 hydrolase [5]. In addition to LTB_4-producing activity, LTA_4 hydrolase has an aminopeptidase activity, which was recently reported to cleave and inactivate an inflammatory peptide Pro-Gly-Pro (PGP) derived from collagen [6]. Thus, LTA_4 hydrolase exhibits both pro inflammatory and anti-inflammatory functions. In mast cells, LTA_4 is conjugated with a glutathione into a peptideLT, leukoctiene C_4 (LTC_4) by LTC_4 synthase. LTC_4 is transported to extracellular space by multidrug-resistant protein 1 (MRP1) [7], because MRP1 recognizes glutathione-modified molecules. Extracellular peptidases cleave amino acids from LTC_4 and generate LTD_4, the most important bronchoconstrictor in bronchial asthma, and LTE_4. In contrast to the limited expression of 5-LOX and LTC_4 synthase in phagocytes, LTA_4 hydrolase is ubiquitously expressed in various tissues and cells.

Now, the concept "transcellular biosynthesis of LTB_4" in which LTA_4, produced in inflammatory cells, is transferred to the others cells to be converted to LTB_4 is widely accepted [8].

26.2 PGs in Inflammation

26.2.1 PGs in Acute Inflammation

Rubor (redness), calor (heat), tumor (swelling), and dolor (hyperalgesia) are cardinal signs of acute inflammation. Acute inflammation also causes systemic symptoms such as fever, activation of the hypothalamo–pituitary–adrenal (HPA) axis, anorexia, lethargy, and hypersomnia, which are collectively termed sickness behavior. Since aspirin-like, nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit COX and suppress PG production lessen these signs of acute inflammation, it has been thought that PGs are involved in elicitation of these signs and symptoms [9]. Further, since pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and proinflammatory cytokines such as interleukin (IL)-1β induce two pivotal enzymes for PGE_2 synthesis, a COX isoform, COX2, and membrane-type PGE synthase (mPGES) [10], PGs are thought to function downstream of these molecules in innate immunity as terminal mediators of acute inflammation.

Local redness and heat are caused by increased blood flow in microcirculation and local swelling is induced by increased permeability of postcapillary venules and resultant plasma exudation. It is generally believed that PGs induce vasodilation and other inflammatory mediators such as histamine, bradykinin, and leukotriene C_4 act on dilated venules and induce increased vascular permeability. Identities of PGs and receptors involved in these processes have been examined by subjecting mice deficient in each PG receptor and applying receptor type/subtype-specific antagonists to various models of acute inflammation. Murata *et al.* [11] injected carageenan into the mouse foot and found that edema formation in IP-deficient mice was suppressed to the level found in indomethacin-treated wild-type mice, suggesting that PGI_2-IP pathway plays a major role in edema formation in this model. On the other hand, effusion induced by carageenan injection to the pleural cavity was significantly decreased in not only $IP^{-/-}$ mice but also $EP2^{-/-}$ and $EP3^{-/-}$ mice [12]. Furthermore, EP2 deficiency and an EP4-selective antagonist suppressed edema formation in UV-irradiated skin in an additive manner by inhibiting irradiation-induced vasodilation and increase in blood flow [13]. In addition, Morimoto *et al.* [14] recently studied AA or PGE_2-induced edema formation in the skin and found that PGE_2 activates mast cells there through EP3 and induces histamine release for edema formation. These results suggest that either PGE_2 or PGI_2 or both are involved in inflammatory microcirculation responses in a context-dependent manner through receptors, EP2, EP3, EP4, and IP, and can be involved indirectly in vascular permeability change.

PGs also cause inflammatory hyperalgesia by sensitization of the nociceptive neurons mostly in the periphery. The primary sensory afferents have their cell

bodies in the dorsal root ganglion, and IP, EP1, EP3, and EP4 mRNAs were found in these neurons [15,16]. Studies subjecting mice deficient in these receptors to various pain models revealed that all of these receptors are involved in mediating pain sensation in a context-dependent manner. For example, $IP^{-/-}$ mice showed markedly decreased pain responses in acetic acid writhing test to a level observed in indomethacin-treated wild-type mice. While this study indicates that the hyperalgesic response in this model is evoked by endogenous PGI_2 acting on the IP receptor, LPS priming induced EP3-dependent hyperalgesia in the same test [17], indicating that hyperlagesic response can be induced either by PGI_2 or by PGE_2 or both, depending on what species of PGs are produced in the context. The TRPV1 cation channel detects heat and pH for pain sensation. Moriyama *et al.* [18] found that both PGE_2 and PGI_2 can augment thermal hyperalgesia mediated by TRPV1 through EP1 and IP, respectively, by lowering the threshold of the TRPV1 channel to temperature and pH in a manner dependent on protein kinase C (PKC)-mediated phosphorylation (Figure 26.2a). The channel can be activated also through the protein kinase A (PKA)-dependent manner by the EP4 signaling, which may be consistent with the finding that the selective EP4 antagonist can attenuate thermal hyperalgesia after complete Freund' adjuvant-induced peripheral inflammation [19]. In addition to these hyperalgesic actions in the periphery, PGE_2 added into the rat spinal cord dorsal horn reduces inhibitory glycinergic synaptic transmission in pain transmission through EP2 to induce central hyperalgesia, thus facilitating the transmission of nociceptive input through there to higher brain areas [20]. Interestingly, EP2-deficient mice exhibit only short lasting hyperalgesia after a peripheral inflammatory stimulus and lack a second sustained hyperlagesic phase of spinal origin [20].

Systemic symptoms of acute inflammation including fever and HPA axis activation are driven by the central nervous system and induced by blood-borne factors such as LPS and proinflammatory cytokines including IL-1β, IL-6, and TNF-α. These factors induce COX-2 and mPGES-1 in brain microvessels, and resultant PGE_2 is believed to enter the brain regions poor in blood–brain barrier and mobilize relevant neural circuits [21]. Ushikubi *et al.* [22] found that both LPS and IL-1β failed to induce febrile response in $EP3^{-/-}$ mice, suggesting EP3 in the brain for fever generation. A later work by Nakamura *et al.* [23] revealed that EP3 is expressed in GABAergic neurons in the preoptic area (POA) and that EP3 activation inhibits activity of these neurons. Combined with other studies, it is now proposed that PGE_2 suppresses EP3-expressing POA neurons, thus disinhibiting two downstream neural pathways, one directly projecting to Raphe pallidum neurons to control skin vasoconstriction and the other projecting indirectly to Raphe pallidum via dorsomedial hypothalamus to control heat generation in brown adipose tissue, which in combination induce the elevation of body temperature [24] (Figure 26.2b).

Like fever, the HPA axis activation is also evoked by LPS and IL-1β, which release corticotropin releasing factor (CRF) from neurons in the paraventricular hypothalamus (PVH). CRF then evokes ACTH release from the pituitary, which in turn stimulates glucocorticoid production and secretion from the adrenal cortex. This response measured as ACTH release in mouse consists of two components, one being of long duration and insensitive to NSAIDs and the other

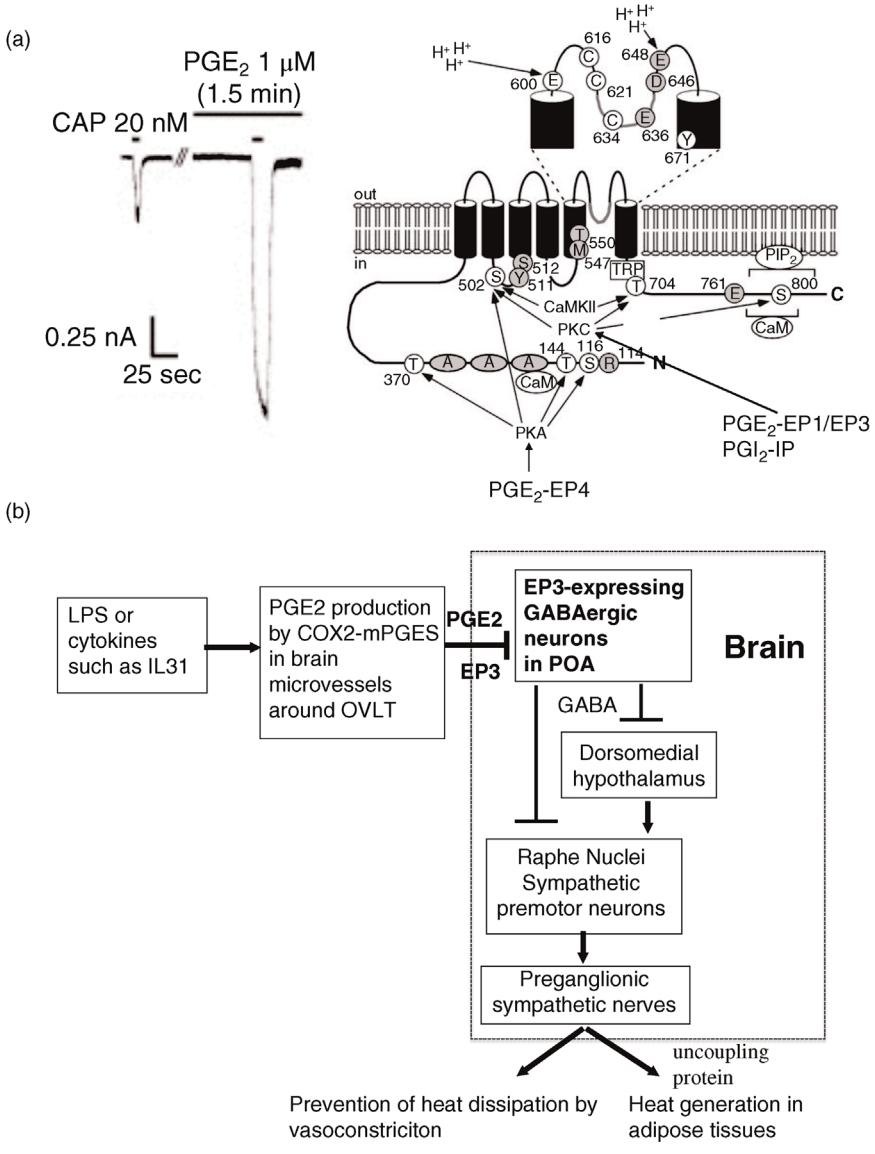


Figure 26.2 Roles of PGs in nervous system. (a) PG-mediated sensitization of TRPV1. (*Left*) Incubation with 1 μM PGE_2 augmented the TRPV1 current caused by 20 nM capsaicin (CAP) (reproduced with permission from Ref. [14]). (*Right*) PG-mediated modification of TRPV1 channel. Stimulation of PG receptor, EP1, EP3, IP, or EP4, induces phosphorylation of the cytoplasmic structure of TRPV1 channel through activation of PKC, CaMKII, or PKA (modified from Figure 2 of Ref. [81]). (b) Fever generation pathway. PGE_2 formed in brain microvessels acts on EP3 of GABAergic POA neurons and disinhibits downstream neural pathways for fever generation.

being transient and sensitive to NSAIDs. The latter response is absent in mice deficient in either EP1 or EP3 [25], suggesting that PGE_2 acts on EP1 and EP3 in an interdependent manner for HPA activation. c-fos mapping suggested that EP1- and EP3-mediated neural pathways converge to activate PVH neurons [25]. Since EP1 is localized at presynaptic structures on PVH neurons and PGE_2 injection to the PVH evokes ACTH release [26], it is proposed that EP1 facilitates neurotransmitter release from the terminals to CRF-containing neurons in PVH and facilitates CRF release.

Sickness-induced lethargy and anorexia can also be attenuated by NSAIDs, indicating the involvement of PGs in these processes. EP1 may be involved in the former process, because EP1 regulates impulsivity through modulation of dopaminergic system and $EP1^{-/-}$ mice exhibit extensive aggression behavior even after LPS administration [27]. On the other hand, PGE_2-EP4 system is implicated in anorexigenic responses to complement C3a agonist peptides and angiotensins [28]. Furthermore, although PGD_2 can induce slow-wave sleep and is suggested to be involved in regulation of physiological sleep [29], PGD_2-DP signaling appears not to be involved in inflammatory hypersomnia [30].

26.2.2 PGs in Chronic Inflammation

Chronic inflammation is inflammation of prolonged duration (weeks to months to years) in which active inflammation, tissue injury, and healing proceed simultaneously, and is histologically characterized by infiltration of mononuclear cells including macrophages, lymphocytes, and plasma cells, tissue destruction by products of the inflammatory cells, and repair involving angiogenesis and fibrosis [31]. Chronic inflammation underlies various chronic diseases including autoimmune diseases, cancer, neurodegenerative diseases, vascular diseases, and metabolic syndrome. Recent studies using animal models of these diseases indicate that PGs are involved in various phases of chronic inflammation.

26.2.2.1 PGs Act as Cytokine Amplifiers and Form Positive Feedback Loops of Inflammation Through Induction of COX-2

Recent studies revealed that, in addition to their short-lived actions in acute inflammation, PGs exert long-term inflammatory actions by acting on messenchymal, epithelial and immune cells and regulating gene expression at the transcription level. In these actions, PGs often cooperate with various cytokines and enhance their actions in gene expression. Honda *et al.* [32] used collagen-induced arthritis of mice as a model and found that PGI_2-IP signaling facilitates arthritis through augmentation of IL-6 content in arthritic paws. By analyzing the mechanism of this action *in vitro* in IL-1β-stimulated synovial fibroblasts, they found that IL-1β induces production of PGI_2 in synoviocytes, which acts on IP and amplifies the IL-1β-induced gene expression; affected genes include those related to inflammation such as IL-6 and IL-11, those related to cell proliferation such as fibroblast growth factor (FGF) and vascular and endothelial growth factor (VEGF), and those related to tissue remodeling such as HIF-1α and RANKL. Since PGI_2 alone did not induce expression of these genes, these results suggest that PGI_2 can function as an amplifier of IL-1β signaling. Intriguingly, PGI_2-IP

signaling augmented expression of IL-1 receptor (IL1R1) itself, suggesting that induction of the receptor for relevant cytokine is one of the mechanisms of PGI_2-mediated amplification. As described below, induction of relevant cytokine receptor is seen also in PG-mediated facilitation of differentiation and expansion of helper T-cell (Th) subsets in acquired immunity. Amplification by PGs is also seen in actions of innate immunity molecules such as PAMPs and DAMPs. For example, LPS-induced expression of COX-2, IL-1β, and IL-6 in cultured macrophages was ameliorated by the addition of a COX-2 inhibitor or an EP4 antagonist [33]. PGE_2-EP2 signaling was reported to amplify TNF-α-induced gene expression in macrophages and neutrophils (see below). In these actions, PG signaling and signaling from PAMPs and cytokines converge at the level of transcription factors such as NFκB for synergy. These amplifying mechanisms by PGs combined with cytokine-induced COX-2 expression make a self-amplification loop for inflammation.

Several studies have demonstrated the formation and contribution of such PG-mediated positive feedback loops in various animal models of chronic inflammation. Intracranial aneurysm (IA), a major cause of subarachnoid hemorrhage, is a regional bulging at bifurcation sites of intracranial arteries and is chronic inflammation histologically characterized by degenerative changes and inflammatory cell infiltration mainly of macrophages in the arterial wall. Aoki *et al.* [34] demonstrated induction of COX-2 and EP2 in endothelial cells at prospective site of IA formation in an animal model of IA. Importantly, COX-2 inhibition by celecoxib significantly suppressed EP2 expression and EP2 deficiency suppressed COX-2 induction, and both suppressed inflammation in IA walls and prevented IA formation *in vivo*. PGE_2-EP2 signaling activates NFκB and stimulates NFκB-mediated expression of various proinflammatory genes including COX-2 itself and MCP-1, a macrophage chemokine, in endothelial cells and macrophages *in vitro* [34]. These findings led the authors to suggest that the positive feedback loop composed of COX-2-PGE_2-EP2-NFκB-COX-2 is formed in endothelial cells and recruited macrophages to amplify inflammatory responses (Figure 26.3). Macrophage infiltration recruited by MCP-1 plays a crucial role in the pathogenesis of IA, because administration of chlodronate liposome to deplete macrophages and gene deletion of MCP-1 prevented IA formation [35,36]. Recruited macrophages themselves also form the above amplification loop, and produce MCP-1 in addition to various cytokines and tissue-destructive proteinases, thus

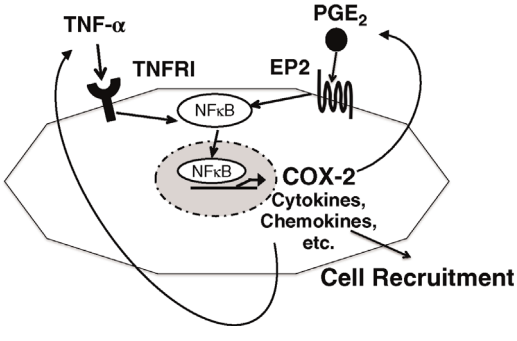


Figure 26.3 EP2-mediated positive feedback pathway of inflammation.

making an autocrine loop for sustained macrophage accumulation and further exacerbation of inflammation in the lesion [35,37].

Chronic inflammation also underlies cancer development and is typically characterized by COX-2 expression in tumor lesion [38]. Pharmacological inhibition of COX by NSAIDs is long known to reduce incidence of colorectal cancer (CRC) in humans [39], and genetic deletion of COX-2 in mice reduced intestinal adenoma formation in animal model of human familial adenomatous polyposis coli [40]. Ma *et al.* [41] used azoxymethane-dextran sodium sulfate treatment as a model of colitis-associated colon cancer and examined the identity of PG receptor and its role in inflammation of CRC. They found that EP2 deficiency remarkably reduced inflammatory infiltrates and suppressed tumorigenesis in this model. Notably, EP2 was expressed in both neutrophils, the major infiltrating cells in lesions, and tumor-associated fibroblasts surrounding tumor cells and worked synergistically with TNF-α to produce various cytokines and chemokines through the self-amplification loop of COX-2-PGE_2-EP2 to promote tumorigenesis. One of the genes whose expression is amplified by this loop is CXCL1, a neutrophil chemokine. Intriguingly, infiltrating neutrophils expressed both EP2 and CXCL1, and EP2 deficiency suppressed neutrophil infiltration and CXCL1 expression. Further, EP2 stimulation of primary culture of neutrophils augmented CXCL1 expression synergistically with TNF-α. These results suggest that neutrophils self-amplify their recruitment through the PGE_2-EP2-CXCL1 pathway, which critically contributes to tumorigenesis in their model [41]. Similarly, Oshima *et al.* [40] used *Helicobacter pylori*-infected gastric tumor as a model and found that bacterial colonization and PGE_2-EP4 signaling cooperatively induced MCP-1 expression to recruit macrophages to promote gastric tumors. These findings clearly show that PG signaling sustains infiltration of inflammatory cells under different inflammatory settings through induction of various chemoattractants in a positive feedback manner and makes inflammation long-lasting.

26.2.2.2 PGs in Autoimmune and Allergic Inflammation

In response to invasion of foreign pathogens, the adaptive immune system is activated by engagement of T-cell receptor (TCR) on T cells with antigenic substances presented by MHC-II on antigen-presenting cells (APCs). Activated APCs also produce various cytokines to prime activated T cells to differentiate into different helper T-cell (Th) subsets, IL-12, IL-4, and TGF-β plus IL-6 for Th1, Th2, and Th17 cell differentiation, respectively. Resultant Th1, Th2, and Th17 cells produce subset-specific cytokines, interferon (IFN)-γ, IL-4 and IL-5, and IL-17 (also called IL-17A), and IL-17 F and IL-22. While these Th subsets fight against external pathogens through producing these cytokines, uncontrolled differentiation and activation of these cells also trigger immune diseases [42]. Th1 cell and Th17 cells mediate development and progression of autoimmune inflammatory diseases such as multiple sclerosis (MS), inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and psoriasis, while Th2 cells mediate development of allergic inflammation such as atopic dermatitis and asthma. PGs appear to function in opposite ways in Th1/Th17-mediated autoimmune inflammation and Th2-mediated allergic inflammation.

26.2.2.3 PGs in Th1/Th17-mediated Autoimmune Inflammation

It was long known that PGE_2 added to naïve T cells in culture inhibits T cell responses, including antigen-driven TCR activation, T-cell proliferation and cytokine (e.g., IL-2, IFN-γ, etc.) production, through cAMP signaling [43]. Based on these findings, it was thought that PGE_2 suppresses Th1 cell development and function. However, there is now accumulating evidence that stronger physiological stimulation of T cells conquers this suppressive effect and PGE_2 can facilitate both IL-12-induced Th1 differentiation under the Th1 skewing conditions and Th17 expansion under stimulation with IL-23. Yao *et al.* [44] showed these PGE_2 actions in mouse T cells and revealed that they are mediated through EP2/EP4-cAMP-PKA signaling. They further showed that the former Th1-facilitative action of PGE_2 is elicited through induction of receptors involved in Th1 differentiation, namely, IL-12Rβ2 and IFN-γ-R1, by the cAMP-PKA-CREB/CRTC2 pathway [45]. PGE_2-mediated facilitation of Th17 cell expansion was also seen in human Th17 cells stimulated with IL-23 and/or IL-1β [46]. Interestingly, PGE_2 acts on EP2 in human cells and upregulates expression of IL-23R and IL-1R, suggesting that PGE_2 also amplifies the cytokine action here by inducing the relevant cytokine receptors. Intriguingly, IL-23 production by activated APCs was also enhanced by PGE_2. This PGE_2 action was observed in DCs stimulated with various TLR ligands, that is, LPS, Poly-I-C, CpG, and proteoglycan [47] and those stimulated with anti-CD40 antibody. Interestingly, treatment with indomethacin or an EP4 antagonist almost completely suppressed the IL-23 production in the latter [44], suggesting the presence of the PGE_2-mediated self-amplification cycle for IL-23 production in activated DCs. EP2 and EP4 mediate the former PGE_2 action through interaction of NFκB activated by TLR pathway and CREB and C/EBP-β activated by cAMP signaling [48]. Interestingly, whereas this PGE_2 action in TLR-stimulated cells is transient, peaking at 1 h, the one by CD40 stimulation lasts over 36 h, in which the early phase is mediated canonical and the late phase is mediated by noncanonical NFκB signaling, both being similarly enhanced by the PGE_2-EP4 signaling [49]. Thus, PGE_2-EP2/4 signaling facilitates IL-23 production, Th1 differentiation, and Th17 expansion for immune inflammation (Figure 26.4).

Several animal models for autoimmune inflammatory diseases supported these *in vitro* findings and revealed the importance of PGE_2-EP2/4 signaling in immune inflammation mediated by Th1 and Th17 cells. In experimental autoimmune encephalomyelitis (EAE), an animal model for human MS, EP4 deficiency, or administration of an EP4-selective antagonist during T cell priming stage after immunization markedly suppressed EAE development, which is accompanied by reduction of IFN-γ and IL-17 production in lymph node cells. EP2 deficiency also showed inhibition of EAE development and production of Th1 and Th17 cytokines, which was additive to that of EP4 antagonism, while mice deficient in other PG receptors developed EAE similar to WT mice [44,50]. Blockade of EP4 signaling also reduced skin swelling and downregulated Th1/Th17 cell function in contact hypersensitivity [44]. Further, selective deletion of EP4 in T cells substantially suppressed IFN-γ-producing Th1 cell development and colonic inflammation in a T-cell transfer colitis model [45]. EP4 antagonism also ameliorated CIA progression with concomitant suppression

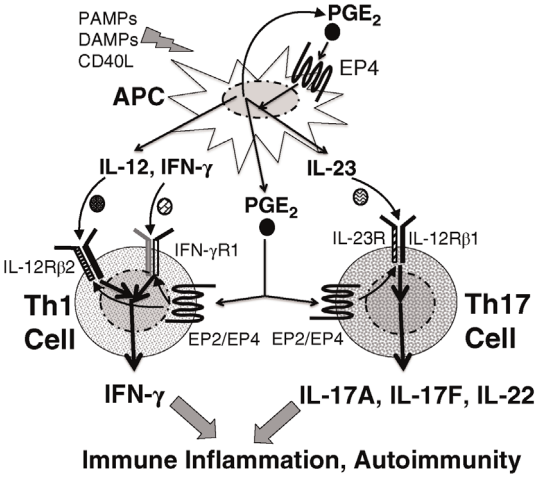


Figure 26.4 PGE_2-EP2/EP4-mediated facilitation of Th1 differentiation and Th17 expansion for immune inflammation.

of IFN-γ and IL-17 production by lymph node cells [51]. Conversely, intraperitoneal administration of PGE_2 or an EP3/EP4 agonist, misoprostol, exacerbated CIA and 2,4,6-trinitrobenzene sulfonic acid-induced colitis accompanied by the increase of IL-23 p19 and IL-17 in the lesions [52]. Such Th1 activating function of PGs is not limited to PGE_2 and is also seen in PGI_2-IP signaling, which couples to cAMP elevation like EP2/EP4. Nakajima *et al.* demonstrated that PGI_2-IP signaling facilitates Th1 differentiation *in vitro* and that IP deficiency impairs CHS [53].

Clinical relevance of these findings to human autoimmune diseases was indicated by recent genome-wide association studies (GWAS), which have identified *Ptger4* (EP4) as a susceptible locus in a number of autoimmune diseases including Crohn's disease [54], multiple sclerosis [55], and allergy [56]. Further, causal single-nucleotide polymorphisms (SNPs) related to these autoimmune diseases are enriched in enhancer region of *Ptger4* locus and these SNPs well correlate with EP4 expression [57]. Further, Kofler *et al.* [58] found overexpression of EP2 in T cells of patients of multiple sclerosis. EP2 simulation of these T cells induces highly pathogenic T-cell phenotype expressing both IL-17 and IFN-γ. Taken together, these findings strongly suggest that PGE_2 promotes autoimmune inflammation through facilitating proinflammatory Th1 and Th17 cells.

26.2.2.4 PGs in Th2-mediated Allergic Inflammation

On the contrary to Th1 differentiation and Th17 expansion that PGs can facilitate, PGs appear to inhibit Th2 differentiation. For example, Nakajima *et al.* [53], who found the IP-mediated facilitation of Th1 differentiation, reported that the same IP stimulation concentration-dependently suppresses Th2 differentiation of native $CD4^+$ T cells from BALB/c mice under the Th2-skewing condition. If this operates *in vivo*, the inhibition of this pathway may lead to enhancement of Th2 response. Indeed, a study subjecting mice deficient in either

COX-1 or COX-2 to ovalbumin (OVA)-induced allergic asthma model found that allergic inflammation of the lung was significantly enhanced by the deficiency of either COX isoform with significantly higher serum IgE levels compared to wild-type mice [59]. Consistent with these findings, $IP^{-/-}$ mice subjected to OVA-induced allergic asthma model developed higher IgE levels and exhibited exacerbation of lung inflammation [60]. Furthermore, Kunikata *et al.* [61] examined lung inflammation of mice deficient in each EP subtype and found that $EP3^{-/-}$ mice developed enhanced inflammation compared to wild-type mice with the serum IgE level similar to that in wild-type mice. They further examined expression and action mechanism of this EP receptor and reported that EP3 is expressed in airway epithelial cells and activation of this receptor with an EP3-selective agonist suppressed expression of a variety of genes induced by OVA challenge to the lung. These results suggest that PGE_2-EP3 signaling negatively regulates allergic inflammation by downregulating inflammation-related genes in the epithelium. Similar action of the PGE_2-EP3 signaling was reported in allergic conjunctivitis and delayed-type hypersensitivity in the skin [62,63]. On the other hand, Birrell *et al.* [64] reported that lung inflammation induced by various stimuli including OVA, LPS, and cigarette smoke was suppressed in $EP4^{-/-}$ mice. Since $EP4^{-/-}$ mice were in mixed genetic background of C57BL/6 and 129/ola, their results are difficult to interpret. However, they also found that EP4 stimulation inhibits expression of various inflammation-related genes including TNF-α and IL-6 in human monocyte cell line, THP-1, and suggested that this might be a responsible inhibitory mechanism. It is enigmatic that the same EP4 signaling and the similar signaling of EP2 often amplify inflammatory gene expression in macrophages in different contexts and exacerbate inflammation. Whether this difference is due to macrophage subclasses remains to be examined.

Mast cell activation by IgE binding initiates allergic inflammation in most cases. Mast cells are, therefore, obviously the therapeutic target for antiallergy treatment including asthma. Mast cells express four EP subtypes differentially in individual subsets, but EP2 and EP3 generally predominate [65]. Contrary to the findings by Kunikata *et al.* in C57BL/6 mice [61], the de Mora's group reported that an EP3-selective agonist was without effect in house dust mite-sensitive asthma in Balb/c mouse strain and found that EP2 agonist was effective in this model [65]. They attributed this effect to EP2-mediated inhibition of mast cell activation observed in different mast cell preparations. Since mast cells release PGD_2 upon activation, the pathophysiological role of this PG has been studied. One role is to induce bronchoconstriction by acting TP in bronchial smooth muscle [66]. In relation to allergic inflammation, Matsuoka *et al.* [67] reported suppression of OVA-induced allergic inflammation in $DP^{-/-}$ mice. Since DP is induced in the bronchial epithelial cells, they ascribed the effect of DP deficiency to its role in the epithelium. Although this phenotype may be in part due to impaired mast cell differentiation found recently in $DP^{-/-}$ mice [68], allergic inflammation in allergic rhinitis, conjunctivitis, and asthma models was attenuated by administration of a selective DP1 antagonist, S-5751, to wild-type mice, validating the role of DP1 in elicitation of allergic inflammation [69]. Furthermore, genetic association of two SNPs of DP1 with higher risk of asthma was noted in Caucasians and African-

Americans but not in Japanese, Han Chinese, and Hispanics [70]. PGD_2 acts also on DP2/CRTH2. Because this receptor is expressed in Th2 cells and eosinophils, and exerts chemotactic function, CRTH2 is thought to play an important role in allergic inflammation. Although the CRTH2 deficiency did not suppress allergic inflammation in acute asthma model, it dampens the infiltration of inflammatory cells including eosionophils in BALF of mice subjected to chronic asthma model [71].

26.2.2.5 PGs in Tissue Remodeling (Fibrosis, Angiogenesis, Lymphangiogenesis, etc.)

Tissue remodeling including tissue metaplasia, fibrosis, angiogenesis, and granulation follow destruction of affected tissues in chronic inflammation. PGs either facilitate or suppress these processes in a context-dependent manner. For example, in the OVA-induced allergic asthma model, OVA challenge induced expression of genes involved in tissue remodeling such as Gob-5, Munc5ac, MMP-12, and ADAM-8, and stimulation of PGE_2-EP3 potently suppresses this induction as mentioned [61]. On the other hand, Amano *et al.* [72] implanted a Matrigel sponge or tumor cells in mice and found the same PGE_2-EP3 signaling facilitates angiogenesis associated with chronic inflammation in the sponge and tumors. Their bone marrow transfer experiment indicates that EP3-bearing bone marrow-derived cells mediate VEGF expression in the stroma around the implants and recruitment of VEGFR-1^+/VEGFR-2^+ cells [73]. Katoh *et al.* [74] used implants of tumor cells and micropore chamber and found that PGE_2-EP3/4 signaling at the site of implant induces expression of CXCL12 around the implants, which recruits $CXCR4^+S100A4^+$ fibroblasts from bone marrow to the site for granulation. The PGE_2-EP3/4 signaling was also suggested to mediate lymphangiogenesis by inducing VEGF-3 and VEGF-4, growth factors for lymph endothelial cells [75].

Lastly, tissue fibrosis occurs in various organs including lung, heart, liver, and kidney and is characterized by proliferation of fibroblasts and excessive deposition of extracellular matrix proteins. Oga *et al.* [76] used bleomycin-induced pulmonary fibrosis as a model of idiopathic pulmonary fibrosis in humans and found that $PGF_{2\alpha}$-FP signaling mediates collagen synthesis independent of TGF-β without affecting inflammation. On the other hand, Lovgren *et al.* demonstrated that the loss of IP augmented lung fibrosis in the same pulmonary fibrosis model [77]. PGI_2-IP signaling appears to exert similar antifibrotic action in the heart of mice subjected to pressure overload [78], which was antagonized by TXA_2-TP signaling [79]. Protective action of PGs was also reported for the PGE_2-EP4 signaling, which protects mice from tubulointerstitial fibrosis in the kidney in unilateral ureteral obstruction [80].

26.2.3 Anti-inflammatory Actions of PGs

Although PGs usually exert proinflammatory actions, they also elicit anti-inflammatory actions as discussed above in "their actions in Th2-mediated allergic inflammation." In addition, Kabashima *et al.* [81] showed that PGE_2-EP4 signaling protects mouse from dextran sodium sulfate (DSS)-induced colitis and that

this action of EP4 may be due to increasing barrier function of epithelial cells in the gut. This protective function of PGE_2-EP4 signaling in intestinal epithelium has been confirmed recently by the study inhibiting PG-degrading enzyme, 15-PGDH, in mice [82]. This study found that this procedure elevates the levels of endogenous PGs and potentiates PG-mediated tissue regeneration including intestine, bone marrow, and liver. Furthermore, Soontrapa *et al.* [83] examined the mechanism of UV irradiation-induced systemic immunosuppression, which is known to be sensitive to NSAIDs. They found that this immunosuppression is due to induction of regulatory T cells, which is mediated by PGE_2 produced in response to UV irradiation in the skin. PGE_2 acts on EP4 in keratinocytes, induces RANKL, which activates $DEC205^+$ dendritic cells for regulatory T cell induction. Thus, PGs can exert protective actions in some contexts. Therefore, when clinical application of manipulation of PG signaling is considered, it is utmost important to identify whether PG signaling is pro- or anti-inflammatory in that context.

26.3 LTs in Inflammation

26.3.1 LT Receptors

So far, four G protein-coupled receptors for LTs have been isolated, and several GPCRs are reported for their involvement in peptide LT signaling [84]. BLT1 is the high-affinity LTB_4 receptor [85] and expressed in granulocytes, eosinophils, dendritic cells, and macrophages. BLT1 is not expressed in naïve T cells, but it is induced in differentiated T cells including CD4-positive Th1, 2, 17 and CD8-positive effector cell [86–88], not in central memory cells. BLT2 was originally reported as a low-affinity LTB_4 receptor [89], but later reported as a high-affinity receptor for 12-HHT that is produced simultaneously with thromboxane A_2 by activated platelets [90,91] (Figure 26.1a). BLT2 is expressed in the cells that are present at the border of the body such as intestinal epithelial cells and keratinocytes, maintains barrier function of intestine and skin [92], and accelerates skin wound healing [93]. Recently, we identified one BLT1-like and two BLT2-like receptors in zebrafish [94], showing that BLT1 and 2 have been conserved during evolution. Two subtypes of CysLT receptors have been pharmacologically characterized and two GPCRs were cDNA cloned as CysLT1 [95,96] and CysLT2 [97,98] receptors. Molecular identity of these two receptors is established, but several additional GPCRs were reported to modify peptide LT signaling. LTE_4 was reported to act through P2Y12 [99] and GPR17 was reported as a dual receptor for peptide LTs and nucleotides [100]. Recently, GPR99 was reported as an important receptor for LTE_4-dependent edema using GPR99-deficient mice [101].

26.3.2 LTB4 and BLT1 in Acute and Chronic Inflammation

As LTB_4 has been known as a potent chemoattractant for granulocytes, the peritonitis model was used to evaluate the contribution of BLT1 in acute inflammation. Two independent groups reported the attenuated peritoneal inflammation

in BLT1-KO mice with much reduced numbers of eosinophils [102–104]. Interestingly, BLT1 deficiency caused a small reduction in granulocytes number in the peritoneal exudate, suggesting the presence of other neutrophil chemoattractants in addition to LTB_4. The importance of BLT1 in eosinophil attraction was confirmed by a nematode infection model, and the lack of BLT1 resulted in the reduced eosinophil accumulation and attenuated nematode killing in mice [105]. The importance of LTB_4/BLT1 axis in acute inflammation was confirmed in several disease models such as allergic skin inflammation [106,107] and in neutrophil swarming phenomenon after bacterial infection [108].

In addition to acute inflammation, LTB_4/BLT1 plays important roles in subacute and chronic inflammation. BLT1 expressed in macrophages activate macrophage chemotactic protein-1 (MCP-1) expression through NF-kB signaling, leading to atherosclerosis [109]. Recently, a BLT1 antagonist BIIL284 was shown to decrease atherogenesis in ApoE-null mice [110]. BLT1 was also reported to enhance the formation of abdominal aortic aneurysm caused by continuous infusion of angiotensin II [111]. In addition to peptide LTs, LTB_4 is also involved in bronchial asthma. We showed that BLT1-deficient mice showed much reduced eosinophil migration and production of Th2 cytokines in peribronchial lymph node cells and reduced airway hypersensitivity [112]. The attenuated asthmatic phenotype was confirmed by the other groups, and BLT1 expressed in dendritic cells [113] and effector T cells [114] was responsible for the asthmatic reactions. LTB_4/BLT1 signaling is involved in Th17 reaction, because BLT1-deficient mice showed attenuated phenotype in a Th1/17-biased disease model experimental autoimmune encephalomyelitis with much attenuated accumulation of granulocytes, mononuclear cells, and monocytes into the spinal cord [115]. LTB_4/BLT1 axis also participates in insulin resistance, in which LTB_4/BLT1 signals in macrophages, hepatocytes, and myocytes augment insulin resistance induced by high fat diet [116]. BLT1 is also expressed in osteoclasts, and osteoclastic activity was enhanced by LTB_4. BLT1-deficient mice were protected from osteoporosis induced by ovariectomy [117], suggesting the possible application of BLT1 antagonist to protect osteoporosis in the future. BLT1 is also involved in inflammatory pain [118] and itch behavior in mice [119–122] possibly by recruiting inflammatory cells.

26.3.3 Roles of BLT2 in Inflammation and Tissue Homeostasis

Despite the well-characterized roles of BLT1 in acute and chronic inflammation, the roles of BLT2 are still under investigation and debate [123,124]. BLT2 is expressed in mast cells [90] and allergen stimulation increased the expression leading to the enhanced production of Th2 cytokines [125]. We also found that BLT2 is also expressed in human and mouse T lymphocytes [126] and negatively regulates allergic airway eosinophilia [127]. In mice, high BLT2 expression is observed in intestinal epithelial cells and epidermal keratinocytes [128]. BLT2-deficient mice were more susceptible to inflammatory colitis induced by oral administration of dextran sodium sulfate and exhibited severe loss of body weight and overproduction of inflammatory cytokines in the gut [92], suggesting the anti-inflammatory role of BLT2 in intestine. The roles of BLT2 in keratinocytes

were also investigated. *In vitro* study showed that UVB radiation-induced apoptosis [129] of keratinocytes and matrix metalloproteinase (MMP)-1 expression [130] in HaCaT cells were dependent on the production of reactive oxygen species through BLT2. Based on the finding that BLT2 couples to pertussis toxin-sensitive Gαi protein that generally mediates cell movement, we focused on the role of BLT2 in keratinocyte migration and skin wound healing using BLT2-deficient mice. BLT2-deficient mice showed much retarded wound healing after skin punching. Aspirin-treated wild-type mice also showed retarded wound healing, and this aspirin-dependent delay of wound healing disappeared in BLT2-deficient mice, clearly showing that COX-dependent production of 12-HHT stimulate wound healing by stimulating BLT2 in keratinocytes. Stimulation of BLT2 on keratinocytes enhanced the expression of MMPs that was required for keratinocyte migration and skin wound healing. Topical application of a synthetic BLT2 agonist to punched skin greatly accelerated wound healing, showing the possible application of BLT2 agonists as therapeutic tools for intractable skin ulcers [93]. We also found that skin barrier function is impaired in BLT2-deficient mice with increased water loss and antigen uptake through the skin. As a result, BLT2 maintains epidermal barrier function by upregulating several tight-junctional proteins including claudin 4 and enhancing tight junction [131]. We and others have reported the possible roles of BLT2 in oncogenesis, cancer metastasis [132–138], and angiogenesis [139]; however, extensive studies are required to confirm these actions of BLT2.

26.3.4 CysLTs in Acute and Chronic Inflammation

In contrast to BLT1 mainly expressed in inflammatory cells and important for recruitment of immune cells to the site of inflammation, CysLT receptors are expressed in vascular and smooth muscle cells in addition to inflammatory cells. Thus, the phenotypes of knockout mouse of CysLT receptors are different from those of BLT1-deficient mouse. LTD_4 has been known as a potent bronchoconstrictor, and LTD_4/CysLT1-dependent contraction of bronchialsmooth bronchial smooth muscle are known to last longer than that of histamine [84]. Blockage of LTD_4/CysLT1 is beneficial for the asthmatic patients, and several CysLT1 antagonists have been clinically used to prevent and treat bronchial asthma, and more recently allergic rhinitis. CysLT1 antagonists were developed before the molecular identification of CysLT1 [95]. After the molecular identification of human [95,140], mouse CysLT1 [96], and CysLT2 [96,98], mice deficient in either CysLT1 or CysLT2 were generated and analyzed for their phenotype using several inflammatory disease models.

CyLT1-deficient mice showed decreased vascular leakage in IgE-dependent passive cutaneous anaphylaxis [141], and attenuated allergic lung inflammation by the house dust mite [142], showing that CysLT1 mediates Th2 type immune responses in addition to its role as a local inflammatory mediator. Attenuated zymosan-induced peritoneal vascular permeability and IgE-dependent passive cutaneous anaphylaxis were also confirmed in mice deficient in LTC_4 synthase [143]. Thus, acute inflammatory responses caused by peptide LTs seem to be mediated mainly by CysLT1 receptor. Although the *in vivo* roles of CysLT2

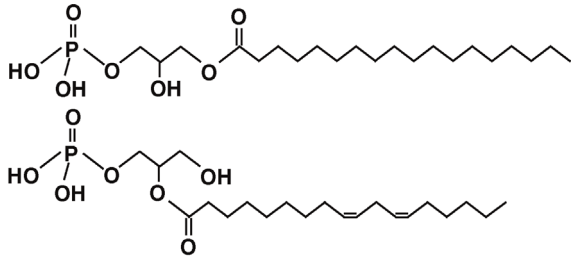
had not been clarified, CysLT2-deficient mice showed reduction in IgE/antigen-mediated microvascular permeability [141]. Although the involvement of peptide LTs in tissue fibrosis was reported [144], CysLT2 was reported as an important receptor involved in peptide LT-dependent chronic inflammation and subsequent tissue fibrosis. Bleomycin-induced lung fibrosis [141] and skin fibrosis in atopic dermatitis model [145] were much attenuated in CysLT2-deficient mice, but not in CysLT1-deficient mice, showing the fibrosis-promoting effects are mainly mediated by CysLT2 receptor.

According to the results obtained by the analyses of CysLT1/CysLT2 double-knockout studies, the presence of other CysLT receptors was predicted [146]. Several GPCRs (GPR17, GPR99, and P2Y12) were found to bind or modify classical CysLT receptors 1 and 2. GPR17, an orphan GPCR that was reported to be activated by both uracil nucleotides and peptide LTs [147], was later reported as a negative regulator of CysLT [100]. Kanaoka *et al.* identified a LTE_4 signaling in mice lacking both CysLT1 and CysLT2 and identified that GPR99 as an LTE_4 receptor [101]. GPR99 has been known as a GPCR for a ketone body 2-oxoglutarate and is expressed in skeletal muscle and kidney [148]. CysLT1/CysLT2/GPR99 triple knockout mice lost the vascular leak caused by LTE4, suggesting that GPR99 is a high-affinity LTE_4 receptor (CysLT3?) [101]. In mast cells, CysLT2 interacts with CysLT1 and downmodulates CysLT1 signaling [149]. Thus, the identity of CysLT3 and interplay of CysLT receptors are still puzzling [150] and further studies are required to clarify the roles of peptide LTs and their receptors in acute and chronic inflammation.

26.3.5 Lysophospholipid, Lysophosphatidic Acid, and Sphingosine 1-Phosphate

Lysophospholipids (LPLs) are phospholipids with only one acyl chain. They include LPA, S1P, lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI), lysophosphatidylserine (LysoPS), and lysophosphatidylglycerol (LPG). LPLs had long been thought to be merely byproducts of the arachidonate cascade and cholesterol metabolism. However, they have been shown to induce multiple cellular responses such as stimulation of proliferation, migration, and mast cell degranulation and suppression of apoptosis. Moreover, in the past two decades, a number of studies have identified multiple receptors and synthetic enzymes specific to each LPL. From studies on their receptors and producing enzymes and studies on LPLs themselves, the pathophysiological roles have been identified, especially for LPA and S1P. The structures of LPA and S1P (Figure 26.5) are similar. LPA generally has a glycerol backbone to which both a phosphate group and a fatty acid are linked by an ester bond and phosphodiester bond, respectively. It should be noted that a fatty acid is linked to either the *sn*-1 or *sn*-2 hydroxy group of the glycerol backbone (Figure 26.5). By contrast, S1P has shingosine base in its structure. The two ester bonds of LPA and the one ester bond of S1P are the targets of degrading enzymes. In this chapter, we review the receptors and synthetic enzymes for LPA and S1P and summarize their pathophysiological functions.

Lysophosphatidic acid (LPA)



sphingosine 1-phosphate (S1P)

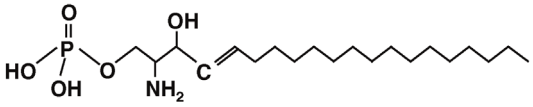


Figure 26.5 Structure of lysophosphatidic acid and shingosine-1-phosphate.

26.3.6 Receptor for LPA and S1P

Like PGs and LTs, LPA, and S1P exert their functions via G protein-coupled receptors. Eleven GPCRs specific either to LPA (6 GPCRs) or to S1P (5 GPCRs) have been identified [151] (Table 26.2). LPAs also act as ligands for other types of receptor such as peroxisome proliferator-activated receptor γ (PPARγ) [152] and transient receptor potential V1 (TRPV1) [153], but we shall not consider them in this chapter. After their discovery, multiple nomenclatures have been proposed. In this chapter, we principally follow the nomenclature proposed recently by Kihara *et al.* [151] (Table 26.2).

26.3.6.1 Receptors for LPA

In 1989, van Corven and coworkers reported that LPA-induced cell proliferation is G protein (Gi)-dependent and indicated that the LPA receptor was GPCR [154]. In 1996, Hecht *et al.* first reported that endothelial differentiation gene 2 (EDG2/LPA_1) was a cellular receptor for LPA [155]. After the discovery of EDG2/LPA_1, the second and third LPA receptors, EDG4 (LPA_2) and EDG7 (LPA_3), were discovered by virtue of their homology to EDG2 [156,157]. More lately, Noguchi *et al.* reported that P2Y9/GPR23 (LPA_4) reacted specifically with LPA [158]. GPR92/LPA_5 and P2Y5/LPA_6 are close homologues of LPA_4/P2Y9/GPR23. Accordingly, they reacted specifically with LPA and thus were classified as LPA receptors [159–161]. Thus, there are currently six GPCRs for LPA. They have been classified into two families, EDG and P2Y. EDG family members consist of LPA_1/EDG2, LPA_2/EDG4, and LPA_3/EDG7, and P2Y family members consist of LPA_4/P2Y9, LPA_5/GPR92, and LPA_6/P2Y5.

Each LPA receptor shows different substrate specificities; LPA_3 and LPA_6 are activated specifically by LPA with unsaturated fatty acid at the *sn*-2 position of the glyerol backbone [161,162]. LPA_5 is more strongly activated by alkyl-LPA than by

Table 26.1 Receptors for PG and LT.

Ligand	Receptor	Expression	Key Functions	Possible Diseases
PGE_2	EP1	Widespread; lung, stomach, kidney, and brain	Gastric protection	Hyperalgesia, neuropathic pain, and stress behavior
	EP2	Bronchial and vascular smooth muscle, myometrium, cumulus cells, T cells, leukocytes, macrophages, microglia, and endothelium	Bronchodilation, tocolytic action of pregnant uterus, regulation of fertilizaiton, and Th1/Th17 differentiaiton	Th1/Th17-mediated immune inflammation, inflammatory cancer, and vascular inflammation
	EP3	Widespread; brain, dorsal root ganglion, heart, platelets, kidney, urinary bladder, stomach, lung, and myometrium	Regulation of neurotransmission, gastric acid secretion, and urinary osmolality	Fever generation, hyperalgesia, and allergic inflammation
	EP4	Widespread; myocardium, ductus arteriosus, stomach, uterus, gastrointestinal tract, bone, synoviocytes, T cells, and dendritic cells	Ductus arteriosus closure, gstrointestinal homeostasis, Th1/Th17 differentiaiton, dendritic cell migration, and bone resorption	Immune inflammation, bone remodeling, hyperalgesia, inflammatory bowel disease, and cancer
$PGF_2\alpha$	FP	Corpus luteum, uterine smooth muscle, endometrium, eye, kidney, and fibroblasts	Regulation of parturition	Lung fibrosis and inflammatory tachycardia
PGD_2	DP1	Ileum, stomach, lung, and uterus; platelets (human), vascular smooth muscle, mast cells, and dendritic cells	Vasodilatation, sleep induction, mast cell maturation, and inhibition of Langerhans cell migration	Allergic inflammation
	DP2 (CRTH2)	Widespread; stomach, small intestine, heart, thymus, colon, and spinal cord; eosinophils and Th2 cells	Th2 lymphocyte activation, chemotaxis, and degranulation of eosinophils	Allergic inflammation
PGI_2	IP	Endothelial cells; vascular smooth muscle, heart, kidney, dorsal root ganglion, T cells, and synoviocytes	Cardiovascular homeostasis, gastroprotection, and pain sensation	Inflammatory edema, hyperalgesia, and immune inflammation
TxA_2	TP	Platelets, vascular and bronchial smooth muscles, and thymocytes	Hemostasis and thrombosis	Atherosclerosis, asthma, and hepatic microcirculatory failure
LTB_4	BLT1	Granulocytes, macrophages, differentiated T cells, and dendritic cells	Chemotaxis	Arthritis, multiple sclerosis, and asthma

(*continued*)

Table 26.1 (*Continued*)

Ligand	Receptor	Expression	Key Functions	Possible Diseases
12-HHT LTB_4	BLT2	Keratinocytes, intestinal epithelial cells, and endothelial cells	Migration, adhesion, and wound healing	Colitis and skin ulcer
LTC_4 LTD_4	CysLT1	Leukocytes, spleen smooth muscle, and intestine	Migration contraction	Asthma
	CysLT2	Leukocytes, spleen adrenal medulla, lung, and heart	?	?
LTE_4	GPR99	Kidney	?	?

Table 26.2 Receptors for LPA and S1P.

LPLs	Receptor	Expression	Key Functions	Possible Diseases
LPA	LPA_1/Edg2	Ubiquitous, neurons, fibroblast, osteoblast, and chondrocyte	Neural cell survival, migration, chondrogenesis, and myelination	Fibrosis, neuropathy pain, dyschondroplasia, and hydrocephalus
	LPA_2/Edg4	Lymphocytes intestine	Protection of cell death	UV- or anticancer drug-induced intestinal cell death
	LPA_3Edg7	Uterus, testis, and lung	Implantation	Endometriosis
	LPA_4/P2Y9	Ubiquitous, endothelial cells, and stromal cells	Lymphovessel formation and hematopoiesis	?
	LPA_5/GPR92	Blood cells	Immunosuppression	?
	LPA_6/P2Y5	Epithelial cells, endothelial cells, and macrophages	Hair follicle formation	Congenital alopecia
S1P	$S1P_1$/Edg1	Endothelial cells and lymphocytes	Embryonic blood vessel formation, lymphocyte egress, and endothelial barrier	Autoimmune disease
	$S1P_2$/Edg5	Ubiquitous	Vascular tone, endothelial barrier, inner ear maintenance, and endothelial barrier function	Loss of hearing and balance, and seizures
	$S1P_3$/Edg3	Ubiquitous	Endothelial barrier function	?
	$S1P_4$/Edg6	Lymphocytes	?	?
	$S1P_5$/Edg8	Oligodendrocytes and NK cells	?	?

acyl-LPA [163]. While LPA_5 does react with LPA, it also responds to a dietary protein hydrolysate (peptone) at nanomolar concentrations [164] and to farnesyl pyrophosphate [165]. These LPA receptors are expressed by particular cells in different organs and activate multiple intracellular signaling pathways, which accounts for their many biological functions.

26.3.6.2 Receptor for S1P

In 1998, two years after the discovery of the first LPA receptor, LPA_1/EDG2, Hla and coworkers showed that S1P was the ligand for EDG1 [166]. Because there were six other EDG family members (EDG3-8) at that time, they were tested for their reactivity to LPA and S1P. To date, five S1P receptors, namely, EDG1, EDG3, EDG5, EDG6, and EDG8, have been identified and named $S1P_1$, $S1P_3$, $S1P_2$, $S1P_4$, and $S1P_5$, respectively [151]. Thus, the eight EDG receptors are divided into two groups: EDG LPA receptors (EDG2/LPA_1, EDG4/LPA, and EDG7/LPA_3) and EDG S1P receptors (EDG1/$S1P_1$, EDG5/$S1P_2$, EDG3/$S1P_3$, EDG6/$S1P_4$, and EDG8/$S1P_5$) (Table 26.2). Unlike LPA, S1P does not have any known receptors within the P2Y family.

26.3.7 Synthetic Pathways and Enzymes for LPA and S1P

LPA is produced under various conditions both in cells and in biological fluids, where multiple synthetic reactions occur [167]. Historically, at least two pathways have been postulated. In serum and plasma, LPA is mainly converted from lysophospholipids. By contrast, in platelets and some cancer cells, LPA is converted from phosphatidic acid (PA). Each pathway requires at least two phospholipase activities. One pathway involves phospholipase A_1 (PLA_1)/PLA_2 plus lysophospholipase D (lysoPLD) activities and the other pathway involves phospholipase D (PLD) plus PLA_1/PLA_2 activities [167] (see Figure 26.6). It should be noted that all these enzymes are extracellularly secreted enzymes, as shown by signal sequences at the N-terminus. This feature is unique to LPA-producing enzymes because all the other lipid mediators covered in this chapter such as PGs, LTs, and S1P are produced intracellularly and are then extracellularly delivered by transporters specific to each lipid mediator. S1P is produced intracellularly, in which sphingosine is converted to S1P by a kinase reaction [168]. We should keep it mind that energy (i.e., ATP) is required for S1P production, while it is not for LPA production. The most marked difference between the productions of S1P and LPA is that the former requires energy (i.e., ATP), while the latter does not.

26.3.7.1 Synthetic Pathways and Enzymes for LPA

At least two pathways have been postulated for bioactive lipid LPA production [167]. In the first pathway, lysophospholipids are produced by phospholipase A_1 (PLA_1) or phospholipase A_2 (PLA_2) and then converted to LPA by lysoPLD. In the other pathway, phosphatidic acid is hydrolyzed by PLA_1 or PLA_2, generating LPA. Extracellular LPA production mainly involves two phospholipases, autotaxin (ATX)/lysoPLD and phosphatidic acid-selective phospholipase $A_1\alpha$ (PA-$PLA_1\alpha$). Inside the cell, LPA is generated as intermediates by addition of fatty

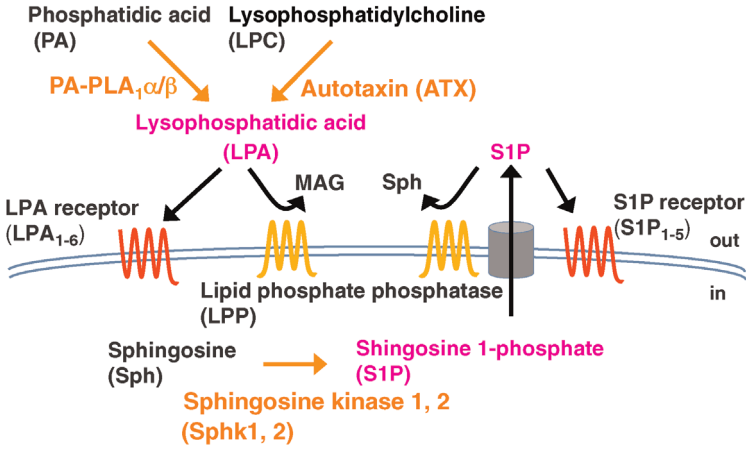


Figure 26.6 Synthetic, degrading, and action of lysophosphatidic acid and shingosine-1-phosphate. There are two pathways for LPA production. First, lysophospholipids generated by phospholipase A_1 (PLA_1) or PLA_2 reaction are subsequently converted to LPA by lysophospholipase D (lysoPLD) reaction. Second, phosphatidic acid is converted to LPA by PLA_1 or PLA_2 reaction. In extracellular LPA production, mainly two phospholipases, autotaxin (ATX)/lysoPLD, and phosphatidic acid-specific phospholipase $A_1\alpha$ (PA-$PLA_1\alpha$) and β (PA-$PLA_1\beta$) are crucial. LPA has six cognate cellular receptors. S1P is produced from sphingosine by two kinases called sphingosine kinase 1 and 2 (Sphk1 and 2) and acts on five receptor specific to S1P. Both LPA and S1P are degraded to monoacyl glycerol and sphingosine, respectively, by phosphatases called lipid phosphate phosphatases (LPP).

acyl-CoA to glycerol-3-phosphate in the course of *de novo* phospholipid biosynthesis. This LPA-producing pathway is conserved in lower organisms such as *Escherichia coli* that do not have LPA receptors. At present, there is no evidence that this pathway contributes to the extracellular functions of LPA production. In this section, we describe the latest findings in LPA-producing enzymes, especially ATX and PA-$PLA_1\alpha$.

LPA Production by ATX

ATX is a member of the nucleotide pyrophosphate phosphodiesterase (NPP) family and is also referred to as NPP2. Some NPP family members such as NPP1 and NPP3 hydrolyze nucleotides such as ATP [169]. ATX was originally found as an autocrine motility-stimulating factor identified in the supernatant of human malignant melanoma cell cultures [170]. However, the mechanism by which ATX stimulated cell motility was unknown. In 2002, we and others showed that an enzyme (lysoPLD) that acts on various LPLs and produces LPA by its phosphodiesterase activity is identical to ATX [171,172]. ATX is a secretory glycoprotein with a molecular weight of about 100 kDa. Multiple ATX isoforms are generated by alternative splicing sites [173]. ATX activity depends on divalent cations such as Co^{2+} or Mn^{2+}. The preferred substrates of ATX are LPLs such as LPC with a short fatty acid (12: 0, 14: 0, and 16: 0) or an unsaturated fatty acid (18:2 and 20: 4). Recently, the crystal structure of ATX was determined and showed that these substrate specificities can be explained by the structure of the catalytic

pocket [174,175]. ATX is unique among NPP family members in having a deep hydrophobic pocket for substrates. LPA acyl chains are accommodated in this hydrophobic pocket. Unsaturated fatty acids bend sharply at the unsaturated bonds, confirmong that highly unsaturated fatty acids bind more stably.

LPA Production by PA-PLA$_1\alpha$

Phosphatidic acid-selective phospholipase A_1 (PA-PLA$_1\alpha$) has sequence similarity to phosphatidylserine-specific phospholipase A_1 (PS-PLA$_1$). These enzymes, both with molecular weights of about 55 kDa, are classified into pancreatic lipase family, which includes hepatic lipase and lipoprotein lipase [176]. Although PA-PLA$_1\alpha$ has a signal sequence at the N-terminus like other lipases, it is mainly detected in the membrane fraction in both tissues and cultured cells, indicating that PA-PLA$_1\alpha$ is a membrane-binding protein rather than a secreted protein. PA-PLA$_1\alpha$-expressing cells constantly produce LPA from phosphatidic acid. Thus, the LPA produced by PA-PLA$_1\alpha$ is an LPA with an unsaturated fatty acid at the *sn*-2 position of the glycerol backbone. Interestingly, LPA_3 and LPA_6 receptors are more strongly activated by 2-acyl LPA than by 1-acyl LPA [161,162]. Therefore, PA-PLA$_1\alpha$ might supply the ligand for these LPA receptors.

PA-PLA$_1\alpha$, which is encoded by *LIPH*, is the gene responsible for autosomal recessive hypotrichosis [177]. Many mutations in *LIPH* have been identified in some family lineages with hypotrichosis [178–180]. A gene encoding LPA_6 receptor (*LPAR6*) has also been identified as a causative gene for the same disorder [181,182], which suggests that LPA produced by PA-PLA$_1\alpha$ promotes the formation of hair follicles. In 2011, PA-PLA$_1\alpha$ KO mice showed a hair abnormality (wooly hair) as was observed in human [183]. That study also demonstrated that, in wild-type mice, PA-PLA$_1\alpha$ and LPA_6 receptors are highly expressed in epidermal cells in the inner root sheath of hair follicles. Together, these findings show that PA-PLA$_1\alpha$ and LPA_6 receptors regulate growth or differentiation of epidermal cells in the follicles. Interestingly, similar abnormal hair (wooly hair) was also found in TACE or TGFα knockout mice [184,185] and in mice with mutations in EGFR genes [186]. The activation of LPA_6 induces TACE-dependent ectodomain shedding of TGFα followed by activation of EGFR, as described above. It is now accepted that a PA–PLA$_1\alpha$–LPA–LPA_6 axis regulates the differentiation and maturation of hair follicles via a TACE-TGFα-EGFR pathway.

26.3.7.2 Synthetic Pathways for S1P

As stated above, S1P is produced intracellularly from sphingosine (Sph) by a kinase reaction. Two such kinases, named shingosine kinase 1 and 2 (SphK1 and 2), have been identified. In 1998, Kohama and coworkers identified SphK1 by purifying it from kidney extract [187]. SphK2 was subsequently identified by its sequence homology to SphK1 [188]. S1P produced intracellularly is delivered to the extracellular millieu by transporters specific to S1P. ABC-type transporters and another transporter, named Spns2, have been identified [168]. Endothelial cells and red blood cells release S1P, which is picked up and chaperoned by ApoM on high-density lipoprotein particles and albumin [189]. *In vitro*, S1P can be converted from sphingosylphosphorylcholine (SPC) by ATX [190]. However, unlike LPC, which is present in the blood at several hundred micromolar level, the

SPC concentration is below 1 μM. Thus, ATX does not appear to be involved in S1P production.

26.3.8 Degradation Pathway for LPA and S1P

LPA and S1P are also known as lipid mediators, indicating that they are locally produced and degraded rapidly. In fact LPA and S1P are highly unstable. Although several routes for the degradation of LPA and S1P have been postulated, the most probable route is a dephosphorylation reaction. Several membrane-bound enzymes responsible for degradation of LPA and S1P have been identified. Lipid phosphate phosphatases (LPPs or PLPPs) are tetra-spanning membrane proteins that remove the phosphate groups from LPA and S1P [191]. LPA and S1P are also referred to as lipid phosphates. Importantly, these enzymes degrade lipid phosphates extracellularly and, thus, negatively regulate LPA and S1P signaling by degrading LPA and S1P. S1P is also degraded to hexadecenal and ethanolamine phosphate by a cytosolic enzyme called S1P lyase [192]. S1P lyase is evolutionally conserved from yeast to mammals and has another role in the sphingolipid to glycerophospholipid metabolic pathway.

26.3.9 Pathophysiological Roles of LPA and S1P in Inflammation

26.3.9.1 Role of LPA and S1P in Angiogenesis

ATX-deficient mice are embryonic lethal due to severe vascular defects at embryonic days (E) 9.5–E10.5 [193]. None of the LPA receptor-deficient mice have shown such a phenotype, although LPA_4-deficient mice are partially embryonic lethal with bleeding [194]. Therefore, in mammals, it was unclear which LPA receptors are involved in embryonic vascular development and how embryonic vascular development is regulated by ATX. Yukiura *et al.* reported that ATX as well as six LPA receptors are highly conserved in zebrafish and that gene knockdown with antisense morpholino oligonucleotides showed that ATX also has a critical role in embryonic blood vessel formation [195]. They also suggested that LPA_1, LPA_4, and LPA_6 have a redundant role. As stated above, the level of LPA is also regulated by degradative pathways that involve three lipid phosphate phosphatases (LPP1–3). LPP3-deficient mice are also embryonic lethal with vascular defects [196]. This suggests that an ATX–LPA–$LPA_{1,4,6}$ axis is involved in embryonic vascular formation and that appropriate degradation of LPA mediated by LPP3 is essential for development. Yukiura *et al.* also showed that in cultured endothelial cells (HUVEC), LPP3 is predominantly expressed in cell–cell contact sites and inhibits LPA signaling mediated by LPA_6 in the contact sites, thereby restricting LPA_6 signaling in noncontact sites [197]. The precise role of LPA in embryonic blood formation (probably vasculogenesis) remains to be determined. Interestingly, ATX is often overexpressed in cancer tissues. Thus, ATX may promote cancer progression by promoting blood vessel formation in cancer tissues.

Like LPA, S1P is essential for vascular maturation during embryonic development. $S1P_1$ was shown to be essential for vascular maturation during embryonic development. Disruption of the *S1P1* gene in mice caused embryonic lethality because of massive hemorrhage at embryonic days (E) 12.5–E14.5 [198]. $S1P_1$ is

expressed in both endothelial cells and vascular smooth muscle cells. However, endothelial cell-specific $S1P_1$ knockout mice showed a similar vascular defect, indicating $S1P_1$ expressed in endothelial cells has a major role in regulating embryonic blood vessel formation in this period [199]. Unlike $S1P_1$ knockout mice, neither $S1P_2$ knockout mice nor $S1P_3$ knockout mice exhibited significant embryonic lethality or obvious phenotypic abnormalities. $S1P_1/S1P_2$ double-null and $S1P_1/S1P_2/S1P_3$ triple-knockout embryos displayed a substantially more severe vascular phenotype than did embryos with only $S1P_1$ deleted [200]. Thus, the $S1P_1$, $S1P_2$, and $S1P_3$ receptors have redundant or cooperative functions for the development of a stable and mature vascular system during embryonic development. *In vitro*, S1P strongly strengthens the cell–cell adhesion of HUVEC [201], indicating that S1P is involved in the maturation of preformed vessels. Mice with single knockouts of SphK1 and SphK2 appear to be normal, although each has decreased blood S1P levels [202,203]. Interestingly, however, SphK1 and SphK2 double-knockout mice, like $S1P_1$ knockout mice, were embryonic lethal [203].

26.3.9.2 Fibrosis

Fibrosis is a cell-mediated abnormal wound healing and extracellular matrix remodeling response. It manifests itself as a combination of overgrowth of cells and hardening and/or scarring of tissues, which together can cause the failure of multiple organs including lung, kidney, liver, and skin. The principal cell type involved is the fibroblast, although injured epithelial cells are implicated in fibrosis of the lung and kidney and stellate cells are specifically associated with liver fibrosis. Myofibroblasts differentiate from fibroblasts in response to profibrotic mediators that are released during the early inflammatory response following tissue injury [204]. Unlike fibroblasts, myofibroblasts express α-smooth muscle actin (α-SMA) and produce extracellular matrix (ECM), which, under chronic inflammatory conditions, is produced in excess, leading to the formation of a fibrotic lesion. Several lines of evidence support the idea that LPA and S1P are involved in the progression of various types of fibrosis.

Recently, LPA-LPA_1 signaling has been shown to have a critical role in the progression of bleomycin-induced pulmonary fibrosis [205]. LPA_1 knockout mice were found to be completely resistant to treatment with bleomycin. LPA was initially identified as a fibroblast chemoattractant in bronchoalveolar lavage (BAL) fluid from injured mice. In mice, LPA_1 knockout dramatically attenuated bleomycin-induced fibroblast accumulation and vascular leakage, but did not change fibroblast proliferation, expression of matrix components, or leukocyte recruitment. Idiopathic pulmonary fibrosis (IPF) patients have elevated levels of LPA in BAL fluids and the BAL fluid-induced fibroblast migration *in vitro* [206]. The migration-stimulating activity was attenuated by Ki16425 (a potent LPA_1 and LPA_3-specific antagonist). These results show that the LPA-LPA_1 axis is critically involved in pulmonary fibrosis in humans. An LPA-LPA_1 axis is also involved in renal fibrosis [207]. Unilateral ureteral obstruction (UUO)-induced renal TIF, for which a mouse model has been developed, was found to be associated with an increased release of LPA by the kidney. In addition, expression of LPA_1 mRNA was significantly increased after UUO. In mice, LPA_1 knockout significantly

attenuated UUO-induced elevations of collagen type III and α-smooth muscle actin mRNA (a fibrosis marker) and F4/80 mRNA (an inflammatory marker) in the kidney. LPA_1 knockout also decreased the expressions of connective tissue growth factor (CTGF) and transforming growth factor β (TGFβ), which are thought to play crucial roles in tubulointerstitial fibrosis (TIF). These results were also obtained in mice treated with Ki16425.

The serum ATX level correlates significantly with liver fibrosis stage in humans and is a better predictor of cirrhosis than either the serum hyaluronic acid level or the aminotransferase-to-platelet ratio index, which is an established marker of liver fibrosis. In another cohort, the serum ATX level correlated significantly with liver stiffness, a novel reliable marker of liver fibrosis [208]. Liver stiffness was the second-best predictor of fibrosis in males (area under receiver-operating characteristic curve (AUROC), 0.799) and females (AUROC, 0.876), the best predictor of cirrhosis in males (AUROC, 0.863), and the third-best predictor of cirrhosis in females (AUROC, 0.872).

26.3.9.3 Neuropathic Pain

Neuropathic pain is also associated with morphological and biochemical abnormalities, such as demyelination and upregulation of the expressions of the γ-isoform of protein kinase C (PKCγ) [209] and the α2δ1 subunit of the voltage-gated calcium channel (Caα2δ1) [210]. Peripheral nerve injury can lead to neuropathic pain that is characterized by allodynia (pain as a result of nonnoxious stimuli) and hyperalgesia (an increased response to normally painful stimuli). Morphological and biochemical abnormalities such as demyelination and upregulation of the γ-isoform of protein kinase C (PKCγ) and the α2δ1 subunit of the voltage-gated calcium channel (Caα2δ1) expression are also associated with neuropathic pain. Intrathecal injection of LPA induced neuropathic pain with the above-mentioned abnormalities [211]. Interestingly, BoTXC3 (a Rho inhibitor) and Y-27632 (a ROCK inhibitor) attenuated the LPA-induced neuropathic pain. Pharmacological and genetic deletion of LPA_1 also completely abolished neuropathic pain induced by not only intrathecal LPA but also by partial sciatic nerve ligation (general neuropathic pain model). These data suggest that LPA–LPA_1 signaling has a crucial role in the initiation of neuropathic pain. Lysophosphatidylcholine (lysolecithin) was also found to induce neuropathic pain-like symptoms [212]. These behaviors were completely abolished in LPA_1 knockout mice and partially rescued in ATX heterozygous mice. ATX heterozygous mice were also more resistant to abnormalities induced by partial sciatic nerve ligation. Therefore, LPA may be synthesized from LPC by ATX at the site of the nerve injury. These results suggest that inhibition of the ATX–LPA–LPA_1 axis is a potential drug target for treatment of the initial phases of neuropathic pain.

26.3.9.4 Roles of S1P and LPA in Lymphocyte Circulation

After T cells are activated by antigen-presenting cells in lymphoid organs, they must return to the circulation to reach sites where antigens are present. It was unclear how the exit of T cells from lymphoid organs is regulated. Cyster and coworkers showed that $S1P_1$-deficient hematopoietic cells, especially T cells, are

not present in the periphery because they can not exit the thymus [213]. Although $S1P_1$-deficient B cells are present in peripheral lymphoid organs, they are severely deficient in blood and lymph. Now, it is well accepted that $S1P_1$ is essential for lymphocyte recirculation and that it regulates the egress of T cells from both thymus and peripheral lymphoid organs. An immunosuppressant drug, FTY720, inhibits lymphocyte emigration from lymphoid organs by downregulating $S1P_1$, creating a temporary pharmacological $S1P_1$-null state in lymphocytes. Now, FTY720 (Fingolimod) is a promising drug for reducing the rate of relapses in relapsing-remitting multiple sclerosis [214].

On the other hand, LPA has been proposed to stimulate the entry of lymphocytes into high endothelial venules (HEVs). Bai *et al.* reported that lymphocyte transmigration across the basal lamina of the HEVs is at least partly regulated by LPA produced by ATX [215]. ATX is selectively expressed by HEVs. Attenuation of ATX *in vivo* impaired the extravasation of lymphocytes from HEVs, inducing the accumulation of lymphocytes in endothelial cells. Interestingly, the accumulation was abrogated by LPA. Although the LPA receptors involved in LPA-regulated lymphocyte extravasation from HEVs are not known, LPA_2 and LPA_5 are the dominant LPA receptors in lymphocytes [216]. These LPA receptors are also targets for drugs that modulate the distribution of lymphocytes.

26.4 Concluding Remarks

Here, we summarized recent progress on bioactive lipid mediators including prostaglandins, leukotrienes, and lysophospholipids. In clear contrast to peptide hormones, the structures of lipid mediators are the same among various animal species, which prompts us to develop new drugs targeting biosynthetic enzymes and receptors for lipid mediators based on the findings obtained from animal disease models. Many subtype-specific agonists and antagonists for receptors for lipid mediators are currently under development, and we will evaluate and develop new drugs targeting lipid mediators in the near future.

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27

Free Radicals in Inflammation

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27.1 Introduction

Acute inflammation is a universal protective response that serves to combat noxious infectious agents and to orchestrate the healing process following an injury. While saving life in the wild and therefore highly conserved across species, the association of chronic inflammatory events with contemporary diseases of affluence is likely the cost/benefit trade-off (i.e., the price we pay) for retaining a once essential physiological response that evolved in, and was optimized for, a more hostile natural environment [1]. Inflammatory processes are generally associated with changes in cellular status toward a more oxidized redox tone compared to a normal cell, a situation characterized by an increased production of *r*eactive *o*xygen *s*pecies (ROS) and accompanying "oxidative stress." In much of the clinical and some of the biochemical literature, oxidative stress is equated with the production of "free radicals" and tissue damage. As this can undoubtedly be the result of an ongoing process associated with enhanced free radical formation and an inadequate cellular capacity for defense to deal with these chemical threats, the range of reactive species formed are not limited to oxygen but can also be derived from nitrogen/nitrogen oxides and sulfur. As we will see throughout the course of this chapter, not all species are particularly reactive, "free" in terms of their ability to move about, or "radicals" by chemical definition. Moreover, the negative connotation of "stress" implies the involvement of something rather unfavorable that ought to be curbed (in the case of reactive oxygen species by antioxidants, for example) in order to hasten recovery from the stressful situation. The last two decades have taught us that this is a gross oversimplification of the situation at hand, that free radical production is part of normal life and needed for proper cellular functioning, and that tampering with any of its key regulatory elements comes at a price. This chapter attempts to shed some light on the

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enigmatic world of "free radicals" as it relates to human physiology and disease, with particular emphasis on inflammation. We will briefly review the properties of some important classes and key representatives and look at their role in the intricate network of regulatory processes that fuel our cellular machinery, allowing cells and tissues/organs to quickly adapt to changes in environmental conditions, energetic and metabolic demand, and nutritional status.

27.2 What Are Free Radicals?

A free radical is any atom or molecule with a single unpaired electron. This property makes free radicals particularly reactive. The single electron can be lost to another molecule, an "oxidation" reaction; or equally the free radical can gain a second electron off another molecule to form an electron pair, a "reduction" reaction. These "redox" (a catchy term signifying coupled *red*uction/*ox*idation) reactions always occur together as electrons move from one molecule to another [2].

Although indirect measures had inferred the existence of free radicals in processes such as the action of ultraviolet and/or ionizing radiation and carcinogenesis earlier, their first direct observation in biological systems was not until 1954 when Barry Commener, Jonathan Townsend, and George Pake demonstrated that free radical production dramatically increases in response to increased metabolic activity in yeast preparations and germinating seeds; they also detected free radicals in other living materials including plant leaves, various animal tissues, eggs, and flies [3,4]. In many cases, these radicals were surprisingly stable and appeared to be associated with proteins and polymeric compounds. This advance discovery was made possible by the availability of a new technique called electron paramagnetic resonance (EPR) or electron spin resonance (ESR) spectroscopy, a nondestructive technique using microwave radiation in a magnetic field. Two years later, Denham Harman proposed his theory that free radical-induced damage (now known as oxidative stress) could underlie the physiological process of aging – a theory that is still being actively investigated today [4,5]. Yet despite these developments, even in the late 1960s free radicals were believed by many to be simply too reactive to serve any physiological role in normal cellular processes [6]. Since then, successive discoveries of new free radicals and their widespread cellular effects have not only disproved this view but also opened up new areas of cellular biology and created novel fields of medical research. Arguably this process began in 1968 when Joe McCord and Irwin Fridovich discovered the enzyme superoxide dismutase (SOD) and described its widespread tissue distribution [7]. We now know that superoxide ($O_2^{\bullet -}$) is central to many physiological and pathophysiological cellular functions by acting as a signaling molecule. In contrast to the protein-associated radicals detected by Commoner and colleagues, superoxide is very small, mobile, and highly unstable, decomposing spontaneously to form other reactive species (see below). It is formed not only as a by-product of aerobic respiration in mitochondria but also intentionally by a group of enzymes called NADPH oxidases (NOX) [2]. It has also become clear that superoxide is not the only transient free radical produced inside

cells [6]. Nitric oxide (NO) was the next small molecule containing an unpaired electron demonstrated to fulfill important cell messenger functions. It is formed in virtually all mammalian cells via oxidation of the amino acid L-arginine by a family of enzymes called nitric oxide synthases (NOS) [8–10]. In 1990, Beckman *et al.* suggested that superoxide and nitric oxide could react with each other to form peroxynitrite ($ONOO^-$) [11]. Peroxynitrite itself is not a radical but a powerful prooxidant and also a source of nitrogen dioxide ($^\bullet NO_2$) and the extremely reactive hydroxyl radical ($^\bullet OH$), which reacts with most other compounds in the cell including DNA and membrane lipids [6,11]. While this can obviously have disastrous consequences (e.g., hydroxyl reactions may underlie some of the toxic side effects caused by chemotherapeutic agents such as doxorubicin), it also makes hydroxyl radicals powerful antimicrobial agents with important roles in phagocytosis and possibly also in potentiating the effects of antibiotics [2,12–14].

Within a short period of time, several other reactive molecules were being discovered and an interesting metamorphosis took place; changing their role from unavoidable harmful waste products of aerobic respiration that are better kept at bay to the latest hype of cellular messengers. New small-molecule mediators (some of which are gases at normal pressure and temperature and therefore termed "gasotransmitters") were discovered in quick succession. These included carbon monoxide (CO), hydrogen sulfide (H_2S), and sulfur dioxide (SO_2), with several other candidates including nitrous oxide (N_2O) and ammonia (NH_3) joining in [15]. Almost "overnight," a new, incredibly complex and intricate cellular signaling network emerged with many different interactions, resulting in a functional cross-talk between different signaling pathways. This unique cellular signaling system is now known to be part of the "redox code," a wider set of principles governing interwoven regulatory networks that orchestrate cellular and bodily functions. From the way energy is derived from food to how we adapt to changes in environmental conditions, the redox code has significant implications for health and disease and is likely to become increasingly important as we practice more "personalized medicine" over the coming years [16].

27.3 Oxidative Stress and the Redox Code

Given that redox reactions occur millions of times every day throughout the body and are essential to normal cellular processing in mitochondria, for example, it is not surprising that three of the four most common elements in the universe (hydrogen, oxygen, and nitrogen – with the fourth common element being carbon) all easily form free radicals even though they are not radicals themselves [2,17]. For example, oxygen reacts with hydrogen to form the hydroxyl ion ($^\bullet OH$), with nitrogen to form nitric oxide ($NO^\bullet$) and it can also form superoxide ($O_2^{\bullet -}$). These, along with secondary reaction products such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) or related reactive compounds such as nitrogen dioxide (NO_2) are collectively known as reactive oxygen species and *r*eactive *n*itrogen *s*pecies (RNS), respectively. However, while most of these compounds (see Table 27.1) are indeed rather reactive, many of them are not

Table 27.1 Overview of the general properties of exemplary ROS, RNS, and RSS.

	ROS	RNS	RSS
Examples	Superoxide Hydrogen peroxide Hydroxyl Radical	Nitric oxide Nitrogen dioxide Peroxynitrite	Hydrogen sulfide Thiyl radicals Sulfite Polysulfides
Production enzymes	Mitochondrial respiratory chain; Cyt P-450; NOX1-5	nNOS, iNOS, eNOS	CSE, CBS, MST; SOX
Exemplary Actions	Oxidative burst	Vasodilation	Redox regulation
Major Clearance Pathways	SOD/Cat, Peroxidases, reaction with biological targets, leading to, for example, lipid oxidation	Reactions with metals, oxygenated heme proteins, oxygen (slow) and thiols	Reactions with metals, heme proteins and oxygen (slow); mitochondrial oxidation

Abbreviations: Cyt P-450: cytochrome P_{450}; NOX: NADPH oxidase; SOD: superoxide dismutase; Cat: catalase; nNOS/iNOS/eNOS: neuronal/inducible/endothelial nitric oxide synthase; CSE: cystathionine y-lyase; CBS: cystathionine ß-synthase; MST: 3-mercaptopyruvate sulfurtransferase; and SOX: sulfur oxidases.

actually radicals. More recently, a third class of compounds, *r*eactive *s*ulfur *s*pecies (RSS), has come to the fore, but much less is known about their specific pathways of formation and overall biological relevance at present [18].

Most of these reactive species are believed to fulfill very specific and highly localized signaling functions, but they can also interact with unwanted cellular targets such as lipids in the cell membrane, essential cellular proteins, or even DNA itself [2]. This may both lead to local cell/tissue damage and enzyme inactivation and give rise to the formation of harmful by-products, which can impair cellular functioning or even result in cell death if production rates exceed the natural clearance capacity. In the case of ROS, this became known as the hallmark of oxidative stress (a situation in which the production of ROS exceeds the cellular antioxidative defense capacity) in the mid-1980s and this term has since become one of the most frequently used terms in redox biology, with a PubMed search at the end of 2015 finding well over 150 000 results [2,19].

The phrase "oxidative stress" incorrectly suggests that a shift in balance between ROS formation and inactivation has universally negative effects on cells and wrongly implies that excess free radical production is typically unintended, which is not the case. More importantly, this term makes no suggestion as to the important physiological role of free radicals, particular in the context of cellular signaling.

In 2015, Helmut Sies and Dean Jones proposed a new and much improved framework, aptly called the redox code, to describe how redox reactions help with both temporal and spatial cellular organization. They suggest that while the cell uses its genetic code to store and transfer information (in the form of DNA), the redox code is essential to how the cell processes, uses, and manipulates that

information to appropriately respond to (and cope with) changes in environmental conditions. As a corollary of this new concept, free radicals are yet another essential way of transferring cellular information, just like DNA [16].

The redox code has four main principles:

1) High flux electron transfer pathways (i.e., oxidation/reduction reactions) organize metabolism through linking different metabolic pathways to each other.
2) Redox pathways also link metabolism to protein structure due to proteomic switches sensitive to redox status, affecting protein activity including enzymatic reactions and molecular trafficking (discussed in more detail below).
3) Differing levels of activation and deactivation of redox pathways (particularly hydrogen peroxide pathways, H_2O_2) allow rapid and precise spatiotemporal cellular signaling.
4) Being able to use the above properties of redox pathways is essential for cells and cellular microcompartments to quickly adapt to changing environmental stimuli and remain healthy – failure of these processes is a major contributory factor to the progression of a wide range of disease processes [16].

To better understand these principles, we first need to look at how free radicals are produced and investigate their normal role in cellular metabolism and aerobic respiration, which occurs deep inside the mitochondria, before looking at each group of reactive species in more detail.

27.4 Free Radical Production: The Mitochondrion

Aerobic respiration – producing cellular energy in the form of adenosine triphosphate (ATP) from oxygen and either glucose or fatty acids – is essential to mammalian life. This process is constantly being performed in almost every cell of the human body (a notable exception is the red blood cell that has no mitochondria) at the level of the mitochondrial inner membrane. Successive redox reactions release energy at each stage of the electron transport chain, and >90% of all ROS in the cell are estimated to be produced as a result of electron leakage during this process, affecting mitochondrial coupling efficiency and thus ATP production. Besides the basal electron leak, which is unregulated and believed to be largely a consequence of the activity of mitochondrial anion carriers, there is an inducible leak that is controlled by a family of uncoupling proteins (the physiological role of UCP1 in brown adipose tissue, for example, is heat production). The escape of electrons at Complexes I and III, that is, prior to the reduction of oxygen to water at the level of Complex IV (cytochrome c oxidase) of the mitochondrial respiratory chain, causes superoxide production [20]. Oxygen normally undergoes four reduction steps (uptake of four electrons and successive reaction with protons) to ultimately produce water. When this process is interrupted at any point, then superoxide, hydrogen peroxide, or hydroxyl radicals will be left depending on whether only one, two, or three reduction reactions have occurred, respectively (see Figure 27.1).

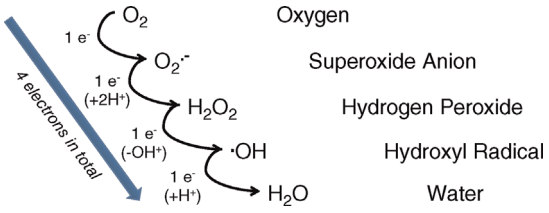


Figure 27.1 In mitochondria, oxygen is subjected to controlled reduction to water in four stages, with free radical intermediate stages (superoxide and the hydroxyl radical) (reproduced with permission from Ref. [2].)

These molecules are collectively known as reactive oxygen species and of particular relevance to the pathophysiology of inflammation.

27.5 Reactive Oxygen Species

Oxygen is the most abundant element in the Earth's crust. It has an unusual electron configuration with two unpaired electrons, making it formally a diradical. Although in its ground state (as the triplet $^3O_2$) it is only a relatively weak oxidant, however, if given more energy it can easily be converted into more reactive molecules [21]. As well as regulating essential cellular processes such as cell differentiation and proliferation, these ROS are hugely important to both the innate immune response and the development of inflammation [22]. One of the most important ROS is superoxide.

27.5.1 Superoxide

Superoxide ($O_2^{\bullet -}$) anions are produced by enzymes that oxidize the cofactor nicotinamide adenine dinucleotide (NAD^+ or NADH, depending on whether it is oxidized or reduced, respectively). This cofactor is essential for respiration as it links the citric acid cycle to Complex 1 at the start of the electron transport chain. NAD^+ can also be phosphorylated itself to form NADPH, and together these two pathways form one of the most important elements of the redox code. Indeed, the discovery of NADPH oxidases (NOX enzymes) was one of the strongest pieces of evidence to suggest that redox reactions have important cellular signaling functions in their own right. They were first discovered as components of phagocytic membranes, forming an important part of innate immunity, but are now known to be expressed widely throughout cells/tissues. Five distinct NOX enzyme isoforms (NOX1-5) have now been identified – all are outward-facing multisubunit complexes that are assembled in the plasma membrane to become functionally active and have only one known function – making either superoxide or hydrogen peroxide [2].

Superoxide can also be generated through other enzymatic reactions. For example, xanthine oxidase reduces oxygen to form ROS such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), or hydroxyl radicals ($^{\bullet}OH$) as xanthine is converted to uric acid [2,23]. Equally, cytochrome P-450, cyclooxygenase (COX), and

lipoxygenase enzymes have all been suggested to also produce ROS, often as unwanted by-products when functioning abnormally in pathological states. Perhaps unsurprisingly, therefore, many of these enzymatic pathways are now also widely targeted in clinical practice with pharmacological inhibitors such as cyclooxygenase inhibitors (e.g., nonsteroidal inflammatory drugs such as ibuprofen) or xanthine oxidase inhibitors (e.g., allopurinol).

By far the largest source of superoxide production, however, remains the mitochondrion. Superoxide can be produced on the outer mitochondrial membrane, the mitochondrial matrix, and on both sides of the inner mitochondrial membrane. Complexes I, II, and III all leak electrons to react with oxygen and form superoxide as a relatively stable intermediate. While Complex III appears to be the main source of superoxide production in heart and lung mitochondria, Complex I produces most superoxide in the brain under physiological conditions and also appears to be most important in different pathological states such as Parkinson's disease and also in aging [24].

27.5.2 Hydrogen Peroxide, Hydroxyl Radical, and Oxidative Damage

Although hydrogen peroxide is not technically a free radical as it does not have a free electron, it is still a physiologically important ROS as it can easily be converted into very reactive hydroxyl radicals, particularly in the presence of iron via either the Fenton mechanism ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH$) or the Haber–Weiss reaction ($O_2^- + H_2O_2 = Fe^{3+} \rightarrow {}^{\bullet}OH + {}^{-}OH + O_2$) [2].

Hydroxyl ions are, in fact, so reactive (and thus very short-lived) that they will react with almost anything they come across in the cell. Being completely nonselective, they often cause cellular damage by oxidizing lipids, reacting with structural proteins or DNA itself. Some of these oxidation products are widely used as biomarkers of oxidative stress, and their circulating and urinary concentrations tend to be markedly elevated under inflammatory conditions. Importantly, several of these products are not only biomarkers of ROS but also have signaling properties in their own right.

The ease with which lipids undergo peroxidation (by reacting with ROS or RNS) depends on the number of double bonds they have – for example, arachadonic acid is relatively easy to oxidize with four double bonds, while oleic acid only has one double bond and is rather resistant to oxidation (but can be nitrated, see below). The peroxidation process is initiated when lipids (LH) react with ROS or RNS to form lipid radicals ($L^{\bullet}$), which can quickly react further with molecular oxygen to form a lipid peroxyl radical ($LOO^{\bullet}$). Once this second stage has occurred, a self-propagating chain reaction develops as the peroxyl radicals ($LOO^{\bullet}$) abstract hydrogen from further lipid molecules (LH) to produce more lipid radicals ($L^{\bullet}$), which are then peroxidized again ($LOO^{\bullet}$), propagating the chain reaction. This vicious cycle stops only when either the oxygen supply is exhausted or an antioxidant molecule (e.g., vitamin E) can supply a hydrogen atom instead of another lipid molecule and terminate the process [2,25].

Of note, the level of cellular damage caused by oxidative reactions very much depends not only on the amount but also on the type of reactive species present.

For example, the hydroxyl radical can react with all four DNA bases (adenine, guanine, cytosine, and thymine) possibly resulting in transition mutations (e.g., AT → GC or vice versa), impairing gene expression, altering downstream protein synthesis, and ultimately changing cellular function. Meanwhile, hydrogen peroxide does not react with DNA bases at all and does not cause any of these effects. Equally, superoxide cannot react with DNA bases alone, although it does so in the presence of nitric oxide – another reactive species with very important physiological functions but derived from nitrogen instead of oxygen [2].

27.6 Reactive Nitrogen Species

27.6.1 Nitric Oxide

Nitric oxide ($NO^{\bullet}$) was originally known as "endothelial derived relaxing factor" (EDRF) because it was first discovered in the cardiovascular system, where it acts as a potent endogenous vasodilator. Soon thereafter, it was found to also play important roles in the central and peripheral nervous system, the gastrointestinal tract, lung, and kidneys, as a modulator of mitochondrial function and as a crucial component of innate host defense responses [26]. The discovery that EDRF was actually NO, a deceivingly simple free radical and one of the 10 smallest molecules in nature, earned three American pharmacologists (Robert Furchgott, Louis Ignarro, and Ferid Murad) the Nobel Prize in 1998 [27,28]. This controversial decision received widespread attention by the scientific community inasmuch as the Honduran–British pharmacologist Salvador Moncada, one of the most cited scientists in the world whose research made a major contribution to our understanding of how NO is formed and acts, was curiously excluded from consideration by the Nobel committee [29].

However, it was not this sort of attention that fascinated, and continues to attract, so many research scientists, biomedical researchers, and medical doctors to NO, but rather its widespread significance for physiology across the microbial, plant, and animal kingdoms as well as its unusual character as a biological signaling molecule. Unlike many other cellular transmitters, it was not stored in specific vesicles to be released on demand but rather enzymatically produced as needed (although bound forms of NO later became known to serve specialized functions); most peculiarly, NO is a gas at normal pressure and temperature that could cross cell membranes by simple diffusion. It also did not interact with specific cell surface receptors in a lock-key fashion, a principle pharmacologists were most accustomed to at the time, but rather by binding to the heme group of the soluble isoform of guanylate cyclase (sGC) and other heme proteins. This intracellular enzyme converts GTP into the second messenger cyclic GMP, which not only exerts direct downstream effects on phosphodiesterases and ion channels but also causes protein phosphorylation through protein kinase G (PKG) [30]. We now know that NO is just one of several representatives of a growing family of "gasotransmitters," although so far the only radical species.

27.6.2 Enzymatic and Nonenzymatic Production of Nitric Oxide

NO is produced by a family of iso-enzymes called nitric oxide synthases. NOS converts the amino acid L-arginine into L-citrulline and NO in a two-stage process, whereby electrons flow from the reductase domain to the oxidase domain of the enzyme. First, a two-electron oxidation produces *N*-hydroxy-L-arginine as an enzyme-bound intermediate before a second three-electron reaction converts this to L-citrulline and NO. Citrulline can be recycled back to arginine by the cell. Like superoxide production by NOX enzymes, both processes depend on NADPH – however, unlike NOX, NOS is also dependent on the presence of oxygen. In fact, half of each molecule of oxygen is incorporated into the final product, NO. As well as oxygen and NADPH, this process requires a number of other cofactors such as calcium, calmodulin, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH_4), linking NO production to other intracellular signaling pathways.

Three different isoforms of NOS exist in mammalian systems. Named either by the tissue in which they were first noted to be expressed or by the chronological order of their successful cloning, all are coded by separate genes on different chromosomes and have different physiological functions. Neuronal nitric oxide synthase (nNOS or NOS-1) and endothelial nitric oxide synthase (eNOS or NOS-3) are both calcium-dependent enzymes regulated by cytokines, hormones, microbial products (e.g., LPS), and other micromilieu factors. As nNOS and eNOS are both constitutively expressed, they are sometimes called "cNOS" and typically only produce pico- to nanomolar concentrations of NO lasting for seconds to minutes (as long as intracellular [Ca^{2+}] is elevated). Meanwhile, inducible nitric oxide synthase (iNOS or NOS-2) is a homodimeric enzyme predominantly found in smooth muscle cells and macrophages; unlike nNOS and eNOS, its activity is calcium-*i*ndependent and *i*nduced by *i*mmunogical/*i*nflammatory stimuli – hence the name. In contrast to the constitutive NOS isoforms, iNOS produces much higher fluxes of NO, depending on the level of expression leading to micromolar concentrations and lasting for hours to days [2,30,31]. Earlier literature also described a specific mitochondrial isoform (mtNOS), but since no DNA encoding for a fourth NOS could be identified this was likely a splice variant of either eNOS or nNOS that was imported into mitochondria under specific experimental conditions.

Besides the level of expression and intracellular localization of NOS, its interaction with other proteins (e.g., caveolin for eNOS) and post-translational modification at specific phosphorylation sites all play a major role for NO output. Other major determinants of NO production in cells and tissues are the availability of cofactors and substrate. L-arginine is taken up through specific transporters, while intracellular NOS substrate concentrations are additionally influenced by the concomitant expression of arginase, an enzyme important for protein formation that utilizes arginine for the production of ornithine and urea. Although the K_m for oxygen has been determined for all three NOS isoforms *in vitro*, how important tissue oxygenation is for NO output *in vivo* is not so clear. Nevertheless, acute hypoxia (which is typically associated with increased ROS production), enhanced arginase expression (a hallmark of inflammation), and

decreased BH_4 availability (secondary to folate deficiency or oxidative stress) all have the potential to reduce NO availability and/or synthesis.

Some NO may also be produced by the spontaneous decomposition of nitrous acid (HNO_2), which arises as a product of protonation of swallowed nitrite (NO_2^-), in the stomach. Some nitrite may be ingested with the diet, but the major proportion of it is the result of commensal bacterial reduction of nitrate (NO_3^-) in the oral cavity. Nitrate is the final oxidation product of NO and a dietary constituent (particularly rich in green leafy vegetables); approximately 20–25% of the circulating nitrate is actively secreted into our saliva where the commensal bacterial flora reduces it to nitrite, which is subsequently swallowed with the saliva to end up in the stomach. Most of this nitrite is absorbed and contributes to circulating nitrite levels in blood, but a small amount may arise in the form of NO as described above. Microbial organisms are well known to reduce nitrate and nitrite through a stepwise respiratory process called denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Although NO is produced as an intermediate, denitrification ultimately results in the formation of nitrous oxide (N_2O) and nitrogen (N_2), in particular under anaerobic conditions [31,32]. More recently, xanthine oxidoreductase has been shown to also act as a mammalian nitrate reductase [33]. These interconversions of RNS by a combination of bacterial and mammalian enzymes have become known as the "mammalian nitrogen oxide cycle" [34].

There is another route through which RNS can be produced nonenzymatically. When solar radiation is shone on the skin, both UVA (315–400 nm) and blue light (420–460 nm) can bioactivate preformed stores of *S*-nitrosothiols and nitrite in the uppermost layer of the skin (the epidermis) to release RNS including NO or peroxynitrite into the circulation [35,36]. The physiological outcome of this process seems to depend on the redox state of the skin tissue being irradiated. Under unstressed (more reducing) conditions, this process seems to favor NO production, which is associated with beneficial hemodynamic effects such as vasodilation and reduced blood pressure; it may also make NO equivalents available to exert preconditioning-like, protective effects on internal organs including the heart [37]. When more oxidative conditions are present, this process seems to favor the production of peroxynitrite and other potentially harmful RNS enhancing radiation-mediated cell death [35].

27.6.3 Nitrogen Dioxide, Peroxynitrite, and RNS Metabolism

Like NO, nitrogen dioxide (NO_2) is also a radical. It is the first oxidation product of NO, and as long as NO is in excess it immediately reacts further with another molecule of NO to form dinitrogen trioxide (N_2O_3), which hydrolyzes to nitrous acid (HNO_2; at physiological pH, it exists largely in the form of nitrite, NO_2^-). *In vivo*, NO and nitrite are both oxidized to nitrate (NO_3^-) in red blood cells, and the latter is used as a marker of whole body NO production [38]. NO_2 also forms in combustion engines and wherever fossil fuels are burned, but human exposure can also be from cigarette smoke. As a strong oxidant, NO_2 is a pulmonary irritant that can lead to permanent lung damage when inhaled chronically or at high levels. *In vivo*, NO_2 and N_2O_3 may also be formed by the action of methemoglobin

(and possibly other hemoproteins) on nitrite, and as part of the downstream chemistry associated with the formation of peroxynitrite [39].

Peroxynitrite ($ONOO^-$) is an important RNS that principally arises as a direct reaction product of the combination of the two radicals, NO and $O_2^{\bullet-}$ [11]. It is a potent prooxidant that can both oxidize sulfhydryl groups to sulfenic and sulfinic acids and entertain nitrosation reactions, giving rise to *S*-nitrosothiols; in addition, tyrosin is oxidized to dityrosine. Moreover, peroxynitrite is (via generation of NO_2) a powerful nitrating species that leads to formation of nitrotyrosine and nitrotryptophan residues in proteins. Nitrotyrosine (NO_2Tyr) formation is a hallmark of inflammation and considered a footprint of enhanced combined ROS and RNS production. However, it may also be indicative of the peroxidative chemistry in the presence of nitrite since $ONOO^-$ can also be formed by the action of myeloperoxidase (MPO) and other peroxidases on nitrite – an action that requires the additional presence of H_2O_2 [40]. Whether this post-translational protein modification contributes to cell toxicity or rather helps limiting local peroxynitrite concentrations and therefore serves an anti-inflammatory role remains unclear [41]. Similarly unclear is whether the nitration of different targets (tyrosine versus tryptophan residues) is associated with different outcomes. Much less is currently known about nitrotryptophan compared to nitrotyrosine, but this is likely merely a reflection of the specificity of detection. While NO_2Tyr formation has traditionally been associated with oxidative damage and pathophysiological processes, more recent evidence suggests it is also a post-translational protein modification involved in physiological cell signaling and organ development [42].

As with oxidative stress, not all nitration is necessarily bad. NO and nitrite-dependent nitration of unsaturated fatty acids (e.g., oleic acid and conjugated linoleic acid both in endogenous lipids and in extra virgin olive oil), for example, leads to formation of various nitrated fatty acids (NO_2FA). These species have recently been demonstrated to exert potent anti-inflammatory and beneficial metabolic regulatory effects by acting, at nanomolar concentrations, as specific ligands to peroxisome proliferator-activated receptor-gamma (PPARγ) [43].

27.6.4 RNS as Anti-infectious Agents

Although now widely known as "inducible" NOS, this particular isoform was originally named iNOS because it was first characterized in immunological macrophages; we today know that it is also expressed at lower levels by other myeloid (e.g., dendritic, neutrophils, and eosinophils) and nonmyeloid cells (e.g., hepatocytes). As well as having similar roles to ROS in phagocytosis, RNS including NO also have both direct and indirect antimicrobial actions and exert regulatory control over different immune cells, particularly T cells [31]. To this end, iNOS-derived RNS can directly interrupt microbial functioning and reproduction through reactions with respiratory chain and cell cycle components, nucleic acids, and metabolic (e.g., Krebs cycle) enzymes as well as by interfering with iron uptake and transport. They can also directly inhibit virulence factors associated with many pathogenic organisms [44,45]. New targets for direct RNS antimicrobial action are still being discovered – some result in immediate

microbial cell death, others delay microbial growth just enough for other components of the immune system to successfully target and clear infectious agents [31].

NO is also associated with cell death through different indirect effects. Interferon gamma (IFN-γ)-induced production of NO by iNOS has also been shown to indirectly trigger apoptotic cell death in macrophages infected with *Mycobacterium tuberculosis* [46]. And in murine macrophages infected with *Listeria*, ß-interferon triggered iNOS activity/NO production has been shown to contribute to cell death through cellular necrosis [47]. NO also indirectly inhibits microbial virulence factors such as *enterohemorrhagic Escherichia coli*'s (EHEC) cardinal virulence factor, Shiga-toxin [48], and indirectly mediates bacterial biofilm dispersal [49]. The realization of this effect on *Pseudomonas aeruginosa* colonies have led NO to being used in clinical trials as an adjunctive therapy in cystic fibrosis patients who are often chronically colonized with difficulty to clear *Pseudomonas* infections [50,51]. This effect of NO on bacterial biofilm formation may also be important for other pathogens, particularly those that have become resistant to multiple antibiotics as dispersal from biofilm into planktonic phenotype renders bacteria considerably more susceptible to the bacteriostatic or bactericidal actions of antibiotics.

Many of the indirect anti-inflammatory effects of NO appear to be mediated by an elaborate redox cross-talk of the three nuclear transcription factors, HIF-1, Nf-kB, and Nrf2, which coordinate oxygen-dependent responses, pro- and anti-inflammatory processes, and cellular antioxidant defense systems, respectively. All three transcription factors are under strict redox control and therefore sensitive to minute changes in cellular redox status, which is influenced by ROS and RNS and additionally modulated by reactive sulfur species.

27.7 Reactive Sulfur Species

Redox biology has focused on effects mediated by ROS and RNS for many years, but reactive sulfur species including thiyl radicals (R-S$^{\bullet}$), hydrogen sulfide (H_2S) and its longer-chain cousins, the per- and polysulfides (R-SSH, R-S_xSH) are increasingly being implicated in important physiological and pathophysiological processes and constitute a significant component of the redox code. They are principally formed from thiols (organic compounds that contain a sulfhydryl (–SH) group) [16,18]. However, because of the instability of RSS (and the associated challenges in detecting and studying them), and with interest in this area of free radical biology having only developed relatively recently compared to both ROS and RNS, the biological implications of RSS reactions are currently much less well understood [18].

27.7.1 Thiyl and Other Sulfur-Based Radicals

Alkylthiyl radicals (R-S$^{\bullet}$) can be formed from thiols physiologically in three main ways: hydrogen abstraction, enzymatic oxidation, or reaction with ROS. For

example, they are commonly produced enzymatically through extremely rapid peroxidase-catalyzed thiol oxidation reactions or via reactions involving the reduction of carbon-based radicals during DNA repair mechanisms. They can also be formed by reactions of both hydroxyl radical and peroxynitrite with thiols – particularly under conditions of increased oxidative stress. Although thiol oxidation is undoubtedly one of the most common mechanisms of RSS production, already-oxidized sulfur-containing compounds can often be oxidized further to produce other sulfur-based radicals [18]. Thiyl radicals are also generated during the homolytic cleavage of S-nitrosothiols (RSNO), i.e. when these compounds release NO.

Thiyl radicals are very unstable and typically react further with other biological targets immediately after they have been formed. For example, oxidation produces either sulfenic or sulfinic acids, they can be reduced back to thiol compounds, or dimerization results in the formation of disulfides ("R-S-S-R"-containing compounds). As disulfides are key structural elements of many cellular proteins and enzymes, changes in cellular redox status have the potential to alter protein structure and enzyme function [16,18].

Of the four common sulfur-containing amino acids (methionine, cysteine, homocysteine, and taurine), only methionine and cysteine are involved in protein synthesis. Methionine is normally the initiating amino acid in the synthesis of every eukaryotic protein; however, it is often buried deep inside the hydrophobic core of the protein once the protein folds into its tertiary structure. Cysteine, which is important for determining a protein's secondary and tertiary structures, often forms and breaks disulfide bonds very easily in response to changes in cellular redox status [52]. This structural and functional plasticity, an integral part of the redox code, allows cells to constantly adjust protein structure and enzymatic function through structural conformational changes induced by the oxidation or reduction of cysteine thiol groups [16,18,52].

Cysteine (and other thiol) groups also play important roles in other methods of cellular communication as they are essential to the production of hydrogen sulfide (H_2S) [18,53].

27.7.2 Hydrogen Sulfide

H_2S is produced from cysteine, cystine, and/or homocysteine by three enzymes of the transsulfuration pathway: cystathionine γ-lyase (CSE – also known as CTH), cystathionine β-synthase (CBS), and mercaptopyruvate sulfurtransferase (MST), depending on substrate availability and tissue type [53,54]. It shares with NO its affinity to metals (i.e., it binds to heme proteins), but unlike NO, it is not a radical. H_2S is inactivated either through heme-mediated reactions or through mitochondrial oxidation into sulfite and thiosulfate. At high concentrations, it inhibits mitochondrial respiration, but at lower levels it also serves as a mitochondrial substrate; this process not only makes H_2S the first noncarbon respiratory fuel to be characterized in mammals [55] but also means that H_2S concentrations are explicitly linked to cellular respiration. The products of sulfide respiration can either go on to exert various physiological effects themselves or be further oxidized into sulfate by sulfur oxidases (SOX) [53].

Although the realization that H_2S may be an important signaling molecule is a rather recent event, pharmacological concentrations of H_2S/HS^- have already been shown to have an astonishing variety of protective or beneficial physiological actions, and inhibition of its formation has been linked to pathophysiology [54,56–58]. H_2S appears to directly interact with various protein structures, have potent antioxidant properties, and modulates the effects of some RNS [53,54]. Of particular significance in this context appears to be its interaction with the NO signaling pathway, a growing area of research that has become known as the "NO/H_2S cross-talk." This interaction occurs both at a functional level and by direct chemical reaction; the latter gives rise to formation of several new products including sulfur/nitrogen hybrid molecules (e.g., nitrosopersulfide, $SSNO^-$) and longer-chain sulfur species (S_x^{2-}, polysulfides) [59]. The latter have recently come to the fore as potential mediators of the action of H_2S as a signaling molecule [60], and their cysteine and glutathione adducts were found in surprisingly high concentrations in murine tissues [61]. These longer-chain sulfur species are of interest not only because they are considerably more reactive than regular thiols but also due to the ease with which their sulfur–sulfur bonds can break to form two sulfur radical species (e.g., a pentasulfide anion falls apart spontaneously to generate one trisulfur radical and one persulfide radical: $S_5^{2-} \rightarrow {}^{\bullet}S_3^- + {}^{\bullet}S_2^-$) [62].

27.7.3 Could RSS have been Mistaken for ROS?

An interesting development in the free radical/oxidative stress field is the recent realization of commonality in chemical reactivity between ROS (derived from the reduction of oxygen) and RSS (originating from the oxidation of H_2S) (see Figure 27.2). This was first pointed out in the context of toxicity from dietary thiols [63], and more recently has been discussed in a review about the possible role of H_2S in evolution [64], to be followed up by a specific report on the cross-reactivity of RSS with five probes/techniques commonly used to detect ROS [65]. All those reagents and detection methods that are widely used to ascertain the production of ROS and/or to quantify the extent of oxidative stress in biological systems were also found to react with RSS, in some cases with considerably higher sensitivity to polysulfides than to H_2O_2. The authors of this work also pointed out the similarity; these findings suggest that RSS may be considerably more prevalent in biological systems than hitherto assumed, calling for a careful reassessment of the significance of ROS in cell signaling and questioning the role of ROS as the most biologically important oxidants. Moreover, this discovery may also be of relevance to the development and treatment of prooxidative conditions with antioxidants.

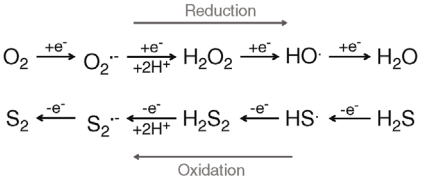


Figure 27.2 Similarity in intermediary products of redox interconversion of ROS (from oxygen, O_2) and RSS (from hydrogen sulfide, H_2S) [65].

27.8 Making Redox Regulation Work: ROS, RNS, and RSS Acting in Concert at the Level of Thiols

As we have seen, ROS, RNS, and RSS can interact with a variety of biological targets, depending on the chemical reactivity of individual species. By far the most important targets in terms of redox signaling are free sulfhydryl (–SH) groups in peptides and proteins as these represent the "nanotransducing" regulatory elements integral to a proper functioning of the redox code [66]. This is made possible by the versatility of sulfur to exist in many different oxidation states and molecular forms, as illustrated in Figure 27.3.

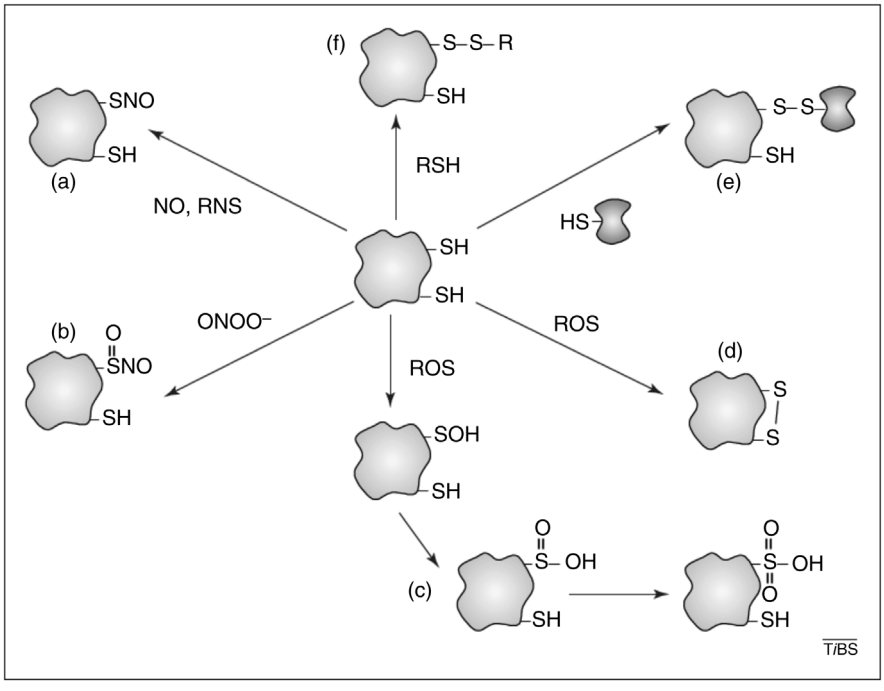


Figure 27.3 The many faces of cysteine as signal transducing element. (a) Reactive nitrogen species including nitric oxide can mediate S-nitrosylation to yield an *S*-nitrosothiol (SNO). (b) RNS, including peroxynitrite ($ONOO^-$), can also mediate S-nitration to yield *S*-nitrothiols. (c) Sequential oxidation of cysteine thiols yields sulfenic (–SOH), sulfinic ($–SO_2H$), or sulfonic ($–SO_3H$) acid derivatives. Alternatively, (d) oxidation by ROS and RNS (peroxynitrite, *S*-nitrosothiols, etc.) can result in a disulfide bridge (cystine) between two adjacent thiols in a protein. (e) Similar disulfide bridges can also be formed between different proteins as well as between a protein and a hydrogen sulfide (H_2S) metabolite (not depicted) to form a mixed disulphide (persulfide). (f) Reaction between protein thiols and low molecular weight thiols (e.g., glutathione, cysteine etc.) and other reactive sulfur species) can yield mixed disulfides (R-S-S-R′). Each of these thiol modifications has the possibility of eliciting different biological responses (reproduced with permission from Ref. [66]).

27.9 Antioxidants/Free Radical Scavengers

The term "antioxidant" originates from industrial contexts in the early nineteenth and twentieth centuries where it was used to describe compounds that prevented oxygen consumption. Their manufacturing uses ranged from preventing rusting of ferrous metals to minimizing the damage petroleum fuels caused to combustion engines. In the biological context, the term is now used to describe any molecule able to donate an electron to a reactive free radical (i.e., one single electron) and thereby successfully neutralize it before it can do any damage. Although it is possible to get an idea about the relative reactivity of certain molecules towards free radicals by calculating and comparing one-electron reduction potentials based on their physicochemical properties (and thus to predict a reaction hierarchy or "pecking order") [67], this is an oversimplification of the situation *in vivo* and usually of little help to determine which is the most important antioxidant. Not all antioxidants are equally well suited to scavenge free radicals everywhere, and their efficiency to act as antioxidants depends on many other factors including their local concentration, water versus fat solubility (which determines cell/tissue distribution), the nature of the free radical it is supposed to protect against, and the ease with which it can be produced and regenerated. Most antioxidants have more or less specific enzyme systems in place that serve to quickly regenerate the reduced form with the help of reducing equivalents, such as NADH and NADPH, once it has reacted with an oxidant. In fact, an elaborate network of interconnected antioxidant generating/replenishing systems exists, acting together to protect nuclear constituents, intermediary enzymes, structural proteins, lipids, hormones, and other messengers from damage.

Many antioxidants are sourced from our diets, such as vitamin C (ascorbic acid) or vitamin E (α-tocopherol), although many of them are also produced endogenously during normal metabolic processes, including albumin, glutathione, melatonin, and uric acid. In addition to the finite amounts of small-molecule (and protein) antioxidants, a number of endogenous enzymes such as SOD and catalase fulfill important antioxidant functions as part of the bodily antioxidative defense system [25].

27.9.1 Dietary Antioxidants

Vitamin C (ascorbic acid) is a potent water-soluble electron donor giving it antioxidant properties. It also functions as an essential cofactor in several metabolic processes; unusually, and although it is ubiquitous to many animal and plant species, it cannot be synthesized in humans and hence must be part of our diet. Even though direct experimental evidence that vitamin C supplementation can reduce oxidative DNA damage is very limited, vitamin C-rich diets (e.g., those high in fruit and vegetables) have repeatedly been associated with a lower incidence of cardiovascular disease in epidemiological studies [25,68,69]. The importance of vitamin C for health has been well documented throughout history because of scurvy – a disease caused by vitamin C deficiency resulting in lethargy, petechia, and bleeding from mucous membranes. Scurvy is one of the oldest

diseases known to man – ancient Egyptians noted that onions and vegetables (now known to be rich in vitamin C) reverse its symptoms, as described in the Papyrus of Ebers in 1550 BC – yet it has remained endemic to mariners, prisoners of war, and polar explorers throughout the eighteenth, nineteenth, and twentieth centuries [70].

Vitamin E is a collection of four tocopherols and four tocotrienols. All are lipid soluble, but α-tocopherol is the most abundant and probably the most important form in humans as it also has the highest bioavailability. It plays an important role in terminating the lipid peroxidation chain reaction (see above) by directly reacting with lipid radicals [25,71,72]. Dietary polyphenols seem to serve a similar role.

27.9.2 Endogenous Antioxidants

Melatonin is a naturally occurring hormone with powerful antioxidant effects. Being lipid soluble, it can easily cross cell membranes and the blood–brain barrier, but unusually once it has been oxidized it cannot be reduced again, making it a terminal antioxidant[25].

Uric acid is one of the most common antioxidants found in the blood stream, and a considerable portion of the total antioxidant capacity of plasma is due to uric acid [25]. Another abundant extracellular antioxidant is serum albumin. It has long been used as a resuscitation fluid in critical illness and known to fulfill many important physiological functions (e.g., osmotic control, binding and transport of metals, hormones, and drugs); its free radical scavenging properties are linked to its amino acid content and tertiary structure. In addition to a few tryptophan and methionine residues, (which can also confer antioxidant protection), it contains a single reduced thiol group at Cys-34 that appears to be the major target of ROS, RNS, and RSS and responsible for the majority of its antioxidant/anti-inflammatory properties.

NO is not only an important cell signaling molecule but also an efficient "chain-breaking antioxidant" by virtue of its efficient reaction with lipid radicals before they have a chance to react with oxygen [73]. As a radical, NO can also intercept other oxygen-based radicals such as superoxide (to form peroxynitrite) and the hydroxyl radical (to form nitrous acid, HNO_2) – these radical–radical reactions are among the fastest reactions known in biology.

Glutathione is a cysteine containing tripeptide (Glu, Cys, and Gly) synthesized *in vivo* from constituent amino acids. The free sulfhydryl (SH) group in its constituent cysteine gives it powerful antioxidant properties allowing it to be reversibly oxidized and reduced many times. Together with its relatively high (millimolar) concentration inside cells, this property makes it one of the most important cellular antioxidants and a critical element of the redox code [25]. By contrast, extracellular glutathione concentrations (and that of other low molecular weight thiols) are very low – this is where the free SH group of serum albumin dominates as thiol protectant of other plasma constituents and cell surface constituents.

Glutathione is also particularly important clinically for the treatment of paracetamol overdoses. Paracetamol is one of the most commonly used analgesic agents in the world. Unlike NSAIDs, it has relatively little anti-inflammatory

activity and is normally exceptionally well tolerated with minimal side effects in the normal therapeutic range. However in excess (i.e., >4 g per day), it can rapidly be fatal through severe hepatotoxicity and liver failure. More than 30 000 patients are hospitalized in the United States of America every year due to paracetamol toxicity with up to 50% of these overdoses being accidental [2,74]. Although the majority of paracetamol is metabolized into nontoxic breakdown products, 5% is converted by cytochrome P_{450} enzymes into *N*-acetyl-*p*-benzoquinoneimine (NAPQI). NAPQI is a very reactive electrophilic intermediate that causes hepatotoxicity through covalently binding to cysteine-containing proteins in the liver, producing ROS- and RNS-mediated mitochondrial dysfunction – hence rapid clearance is essential for health. Normal detoxification of NAPQI is through reacting with glutathione to form nontoxic cysteine and mercapturic acid conjugate products. However, large doses of paracetamol (either as one large dose or as multiple staggered doses that accumulate) can quickly exhaust hepatic glutathione stores, at which point the rate of NAPQI clearance is limited by the rate that hepatocytes can synthesize glutathione. The treatment for paracetamol overdose, *N*-acetylcysteine, works by restoring hepatic levels of glutathione to allow NAPQI clearance to continue [2,75].

27.9.3 Cells Respond to Oxidative Stress through Increased Antioxidant Gene Expression

The redox code proposes that proteomic functioning can be controlled kinetically through adjusting redox potentials and the effect they have on sulfur switches [16]. Indeed, an increased antioxidant production is the natural response to acute and prolonged exposure to oxidants through exactly this mechanism. Under normal cellular conditions, kelch-like ECH-associated protein (Keap1) binds to and stabilizes nuclear erythroid 2-related factor (Nrf2) stabilizing it in the cytoplasm. However, increased ROS, RNS, or RSS production oxidizes sulfur residues in the cysteine components of Keap1 causing transformational changes that induce its release from Nrf2. Once free from Keap1, the Nrf2 transcription factors can migrate into the nucleus and bind to the so-called antioxidant response elements (ARE), activating the transcription of antioxidant genes to upregulate the production of SOD, glutathione reductase/peroxidase, heme oxygenase, and many other components of the antioxidative defense network [2].

27.9.4 Antioxidants: Harmful or Beneficial?

For some time, dietary supplementation of antioxidants was proposed as a safe way of reducing the harmful effects caused by excess cellular free radicals, yet many studies that tried to prove this effect have failed to do so. A meta-analysis of many of those studies suggests that antioxidant supplementation may worsen outcomes even further and actually increase all-cause mortality [76]. This finding may now not be that surprising once we acknowledge that these compounds are an integral part of and important control elements to the redox code, which help balance redox potentials and modulate cellular signaling systems appropriately to environmental challenges. Interfering with the natural

ARE system by curbing oxidant signaling inputs will fool the system into believing its natural defense systems are appropriately balanced, leading to an unintentional downregulation of antioxidant poise. Since one particular dietary antioxidant cannot fulfill all the functions of the many constituents of this heterogenous redox network, an inevitable result is increased damage of regulatory elements with eventual failure of important signaling pathways. Together with lifestyle-related factors, including an unfavorable diet and inappropriately low physical activity, this will give rise to what may soon be called "redox diseases."

27.10 The Redox Code in Health and Disease

We have known for some time about the essential role free radicals play in physiological processes such as the innate immune response and aging. However, as our understanding of the redox code improves, we are seeing that alterations in free radical production are also important to disease progression under an increasing number of chronic inflammatory conditions, from atherosclerosis to cancer.

27.10.1 Inflammation and the Innate Immune Response

The early stages of inflammation are characterized by innate immune responses such as "the respiratory burst," with free radical (particularly ROS) formation by phagocytic cells such as neutrophils, monocytes, and eosinophils forming a key part of this process.

Phagocytes surround and engulf material labeled as foreign by the innate immune system (e.g., invading bacteria labeled by complement), encapsulating it in phagocytic vacuoles. NADPH oxidases are then activated in the wall of the vacuole, producing superoxide and hydrogen peroxide inside the vacuole. Hydrogen peroxide converts to hydroxyl ions via the Fenton mechanism ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{*}OH + {}^{-}OH$) or the Haber–Weiss reaction ($^{\bullet}O_2^- + H_2O_2 \xrightarrow{Fe^{3+}} {}^{*}OH + {}^{-}OH + O_2$), as discussed in Section 27.5 on ROS. Second, MPO (an enzyme secreted into the phagocytic vacuole) catalyzes H_2O_2 to convert chloride, bromide, or iodide ions into the respective hypohalous acids, for example, chloride into hypochlorous acid (HOCl). Together, these reactive species then kill the invading organism inside the vacuole [2]. This mechanism, in concert with the peroxynitrite-mediated chemistry discussed above, is what kills invading pathogens and forms a vital part of the innate immune response. Given the aggressiveness of this part of intracellular "chemical warfare," it is not difficult to see how this could become problematic if inappropriately activated and/or prolonged in the absence of a pathogen to combat because the redox-related "switch-off signal" is lacking or because the set-point for redox control moved to an inappropriate level due to enhanced ROS/RSS tone secondary to impaired NO production.

27.10.2 Aging

As organisms grow older, they become more susceptible to disease, many of their biological functions deteriorate and their chance of dying within a given period of

time increases significantly [77]. It is now almost 60 years since Denham Harman first proposed that this process of aging (which he defined as "growth, decline, and death") could be due to the "deleterious effects of free radicals on cell constituents" [5].

Lipid peroxidation, DNA oxidation, and protein oxidation are all known to increase with advanced age. The redox balance of thiol/disulfide groups has also been shown to shift meaning that redox changes in one compartment can result in transformational changes in other cellular compartments through structural and conformational changes, giving one potential mechanism to underlie the increasing functional impairment seen with aging [77]. The redox status of a cell's microenvironment may also be one of the key determinants in how quickly that cell becomes senescent (stops replicating) – a natural cellular process that is clearly associated with aging. For example, hydrogen peroxide and increased ROS production caused by exposure to UV light or $p21^{ras}$ have all been shown to accelerate cellular senescence in experimental models *in vitro* [77–80].

Different drosophila lines with mutations to increase their resistance to oxidative stress and increased expression of both SOD and catalase have also all been shown to have longer than expected life spans in experimental studies [81–83]. Similar effects have been shown in other model experimental species with mitochondrial DNA being particularly important to the free radical aging theory. Mitochondrial DNA appears to be particularly susceptible to free radical-mediated damage during aging and accumulating deletion mutations may also directly contribute to the generation of sarcopenic phenotypes in skeletal muscle – a phenotype particularly common and well associated with advanced age [77] as well as chronic hypoxic situations, from living at high altitude over COPD to critical illness.

However, despite almost 60 years of work investigating this free radical theory of aging, all of the above evidence only shows an association between free radical production and aging, and often only *in vitro* – a "cause and effect" relationship *in vivo* has yet to be demonstrated.

27.10.3 Diabetes Mellitus

The hallmarks of diabetes are hyperglycemia and increased insulin resistance – both of which are associated with increased free radical formation. Hyperglycemia itself both causes, and is also caused by, increased ROS production. Increased glucose oxidation, nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins result in production of disproportionately high levels of ROS, which in turn results in further glucose oxidation setting off a vicious cycle [84]. The resultant cell damage caused by this enhanced oxidative stress, including lipid peroxidation and damage both to cellular organelles and to intracellular proteins and enzymes, also contributes to increased insulin resistance [84].

Type I diabetes, however, is caused not by increased resistance but by a lack of insulin production caused by the destruction of pancreatic beta cells. This process is caused by immune-mediated targeting of insulin-producing beta cells, and this

appears to be at least partially mediated through increased ROS production by white immune cells [85].

ROS are also associated with the development of complications in diabetes. Extensive animal studies have demonstrated that oxidative stress may be a final common pathway in the development of diabetic neuropathy and that antioxidants can prevent or reverse hyperglycemia-induced nerve dysfunction [86]. Hyperglycemia also increases the ability of vascular cells to oxidize low-density lipoproteins (LDL) by lowering the bioavailability of endothelial NO, increasing the pathogenesis of atherosclerosis [77,87].

27.10.4 Cardiovascular Disease: Atherosclerosis

Atherosclerosis is a common multifactorial inflammatory condition characterized by thickening and hardening of arterial vessel walls and the formation of fatty plaques under their endothelium. Diabetes, hypertension, and hyperlipidemia are all well-known risk factors for the development of atherosclerotic lesions, but excessive ROS production and resulting oxidative stress may provide the common link associated with the development and progression of atherosclerosis [77].

Oxidative stress increases cellular invasion of the arterial wall by increasing the expression of cellular adhesion molecules (e.g., ICAM-1) and protein kinases, resulting in migration of monocytes, macrophages, and T cells. Both monocytes and macrophage cells in particular possess scavenger receptors for oxidized LDL and increase manganese SOD (Mn-SOD) expression, resulting in enhanced H_2O_2 production in response to oxidized LDL binding. This increase in ROS not only causes further lipid peroxidation, but also results in more oxidized LDL binding, propagating this process further; it also increases apoptotic cell death of macrophages, further destabilizing the plaque [77].

Besides leading to a progressive narrowing of the intravascular lumen one of the biggest risks posed by atherosclerosis is that the plaques rupture and release fatty acids and inflammatory products into the blood. Immediate thrombus formation occurs to wall off this break in the endothelium, but this thrombus will also cause either partial or total occlusion of the vessel lumen, resulting in downstream tissue ischemia or infarction, respectively, for example, a myocardial infarction (MI) caused by a thrombus over an atherosclerotic plaque causing occlusion of a coronary artery. Interestingly, reperfusing these tissues after a period of ischemia may also cause free radical- mediated injury [2].

27.10.5 Ischemia Reperfusion Injury

Ischemia and reperfusion result in increased free radical formation through a number of different mechanisms. First, tissue ischemia results in cellular ATP depletion. Lack of oxygen reduces forward flux through the electron transport chain (which produces 34 molecules of ATP for every molecule of glucose) and instead the cell is forced to rely on glycolysis for ATP production, which only produces two molecules of ATP from every glucose molecule. Lack of flow through the electron transport chain increases the proton gradient across the

mitochondrial membrane increasing the likelihood of mitochondrial ROS production as this proton-motive force leaks back across the membrane but without enough substrate to result in complete oxidation to water [2,77].

Ischemia also encourages the conversion of the xanthine dehydrogenase enzyme into xanthine oxidase (XO). As ATP production is decreased under hypoxic conditions (see above), concentrations of catabolic breakdown products such as ADP, AMP, and adenosine accumulate. Ultimately, ATP is catabolized to hypoxanthine, which XO converts into uric acid, with huge amounts of superoxide and hydrogen peroxide produced as by-products of this reaction [2,77,88]. Finally, ischemic conditions also increase the release of free metal ions such as Fe^{2+} and Fe^{3+}, which can cause increased ROS production through the Fenton mechanism or the Haber–Weiss reaction, as discussed earlier [2].

These reperfusion-induced free radical effects have numerous important clinical implications, such as in transplant surgery when a removed organ is rapidly reperfused after being surgically fixed in the recipient, potentially resulting in cellular necrosis and subsequent organ failure or graft rejection [2]. Similar harmful free radical-mediated reperfusion effects have also been described in stroke medicine where thrombolysis treatment may paradoxically cause further injury in some cases [89].

27.11 Looking to the Future: Newly Discovered Signaling Roles, the Reactive Species Interactome, and an Increasing Need to Assess the Role of Free Radicals in Personalized Medicine

With our increasing knowledge of free radical species interactions, it has become increasingly clear that ROS, RNS, and RSS have broad signaling effects well beyond individual cells. For example, it has recently been demonstrated that changes to extracellular redox status can result in downstream alterations to cellular metabolic function, with a more reduced extracellular state maximally increasing hepatocellular mitochondrial activity [90]. The stable breakdown products of reactive species present in extracellular fluids such as blood or lymph could conceivably link individual cells and provide a particular microenvironment that has the potential to modulate immune cell function and that of other circulating blood cells; equally, it may provide a read-out of the redox tone and stress or energetic status of cells in distant organ systems.

Free radicals also signal between host tissues and commensal organisms or invading pathogens. For example, subtle changes in oxygen gradients may also govern how our gut microflora interacts with our intestinal cells [91]; the enterosalivary recirculation pathway (where commensal bacteria in the mouth and GI tract sequentially reduce dietary nitrite and nitrate) is an important component of the mammalian *N*-oxide cycle contributing to an elevation of circulating levels of nitrite and a mild but notable reduction in blood pressure [92–94]. Similarly, microbial generation of H_2S (e.g., as a result of sulfate reduction) may significantly contribute to total body sulfide content and also

lower blood pressure, although this microbial sulfide circulatory pathway is currently much less clearly defined [95,96]. Recent data from a large epidemiological cohort study demonstrating the modulation of nitrate's effects on blood pressure by sulfate intake [97] are testament to the potential for interaction between different reactive species and highlight the complexity of these interactions in the context of the host/microbial cross-talk *in vivo*.

Reactive species could link physiological, environmental or pharmacological stressors to the development of particular disease states. With the current drive to increasingly "personalize" medicine, free radicals that characterize specific disease activity (disease-specific "biomarkers") could be utilized to target clinical interventions. Trying to identify and produce reliable assays for these markers has consequently become a major focus of current research.

27.11.1 Prognostic Markers in an Increasingly Personalized World of Medicine

Emerging evidence suggests that certain reactive species, in particular RSS, could turn out to be excellent clinical biomarkers – of interest in this context are the total concentration of circulating protein thiols as well as free and protein-bound low-molecular-weight thiols [98].

Interestingly, the ratio of reduced to oxidized small aminothiols (e.g., cysteine and glutathione) never reaches equilibrium – even in a dynamic and constantly changing redox system – and shows diurnal variation [99]. The relative proportion of reduced over oxidized thiols also decreases with age [100]. These observations suggest that this is a tightly regulated system even though we currently know little about its maintenance and regulation [98]. Even more unexpectedly, given the overall complexity of intra- and extracellular redox systems, the concentration of total free thiol groups in plasma or serum alone has been associated with clinical outcomes in a number of widely varied and seemingly unrelated clinical conditions [98]. These include blood dyscrasias, malignancies, endocrine disorders such as diabetes mellitus and thyroid disease, cardiovascular conditions, renal pathologies, neurological complaints, dermatological conditions, respiratory diseases, and other inflammatory disorders [101–107]. Known disease risk factors such as smoking, aging, alcoholism, and obesity all correlate with the degree of thiol oxidation [108].

Given that the single free cysteine residue of human serum albumin accounts for almost two-thirds of all free thiols [109], accounting for the total protein level will also indirectly account for total thiol content, and protein-adjusted free thiols can therefore approximate for the ratio of reduced:oxidized thiols [98]. Measured in this way, protein-adjusted serum free thiol levels closely predicted outcome in both kidney transplant recipients and patients with congestive cardiac failure [110,111] as well as correlated independently with overall life span [112].

27.11.2 The Reactive Species Interactome

We started this chapter by introducing the concept of the "Redox Code" – a set of principles that place redox species (e.g., thiols, NAD^+, or ROS/RNS) at the heart

of cellular reactions and define how redox systems enable and protect certain biological functions in both space and time [16]. However, even this concept does not fully explain the complexity and integrative nature of redox systems; how free radical species interact with multiple downstream physiological targets to produce such varied effects; why antioxidants seem to have such small effects on an organism's function, or why free thiols are of prognostic value in terms of clinical outcome.

A new concept has recently been proposed that goes even further in recognizing this higher level of complexity, organization, and integrative function – the *Reactive Species Interactome* [98]. This concept explains how ROS, RNS, and RSS (probably alongside other small reactive species yet to be identified and investigated) interact among themselves and with other biological targets in a highly adaptable, flexible, and controlled manner, thereby enabling effective sensing of and rapid adaptation to changes in environmental conditions and stressors. To this end, the *Reactive Species Interactome* can be understood as a stress adaptation system that confers stability and resilience to cells and tissues in a world of constant change. This system allows energetic demand and supply to be matched by fine-tuning intermediary metabolism to ensure the whole organism stays fit-for-purpose and copes with daily stresses. In the *Reactive Species Interactome*, blood and lymph act as the central communication conduit between cells and organs and provides a readout of whole-body redox status, offering opportunities for biomarker-guided personalized medicine once we understand the significance of each metabolite (the concentrations of which can be measured in blood). Remarkably well preserved through evolution across different species and kingdoms, the complexity of this system has evolved to confer a high level of reserve capacity and redundancy to protect the organism against a variety of stressors. This explains its importance in the development of different disease states when it is allowed to become unbalanced or other factors intervene [98]. It also offers opportunities to use a combination of appropriate biomarkers to interrogate the status of the entire system for risk stratification and to guide interventions aimed at restoring health. Clearly, this novel concept requires further investigation but free radical chemistry and redox systems biology undoubtedly hold much promise for the future of clinical medicine.

27.12 Conclusions: Free Radicals, the Reactive Species Interactome, and Its Importance to the Inflammatory Response

Although it is now over 60 years since their first discovery, it is only relatively recently that we have started to see how important free radicals are to cellular signaling. While we have understood their importance to immune processes and inflammation for much longer, our understanding of the cellular redox code at the systems level is still at its infancy.

Free radicals are essential to the successful functioning of our innate immune response but ROS, RNS, and RSS conspire to carry out many other important

cellular signaling functions. Nevertheless, unopposed excess free radical formation is undoubtedly harmful with oxidative stress being associated with a number of inflammatory conditions and processes from diabetes to aging. The emerging concept of an overarching redox code offers a useful framework to explain how these interactions impact both beneficial and deleterious processes. These range from organizing catabolism, anabolism, and metabolism through high-flux electron carrier systems such as NAD(P)H, to linking energy usage to protein structure and to cysteine-based redox reactions, and forming signaling networks both by dividing the cell into smaller functional intercommunicating microcompartments and by arranging organizational networks between cells and even across different tissues. The redox code offers the cell an intricate, precise, and functioning chemical signaling system, which neatly links information storage to information processing while allowing the flexibility to meet varying metabolic demands depending on environmental conditions and challenges. Extending this idea further with the emerging concept of the "Reactive Species Interactome," a highly flexible, adaptive, and rapidly responsive integrative system with free radicals and redox status at its heart offers unprecedented potential for disease diagnosis, guidance of therapeutic interventions, and risk stratification.

Free radical production is essential for normal life and needed for proper cellular functioning. Arguably "inflammation" could be considered simply to be the result not of a bodily malfunction of some sort but rather the consequence of or "price to be paid" for a necessary adjustment of key regulatory elements of the whole-body redox web to keep all systems still functioning. As our understanding of these processes grows, the *Reactive Species Interactome* will almost certainly offer many new potential therapeutic targets and become an increasingly important component in the personalized medicine of the future.

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28

Proteases

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28.1 Introduction

Increased proteolytic activity at inflammatory sites has been described for decades. However, the role of proteases in inflammation was underestimated, due to the belief that proteases were merely involved in debris clearance from inflammatory sites and tissue remodeling. A number of studies have now involved proteases as true signaling molecules that have ubiquitous roles in inflammatory response, ranging, for instance, from antimicrobial activities to permeability functions and inflammatory mediator processing.

Herein, we will discuss the origin and function of proteases at inflammatory sites, and the relevance that this knowledge brings to possible intervention in chronic inflammatory disorders.

28.2 Host Proteases and Inhibitors: Origin and Distribution

Proteases, which represent up to 2% of the human genome, are defined as proteins able to cleave specifically other proteins, either at their extremities (for exopeptidases) or at the core of the protein (for endopeptidases, also named proteinases). The proteolytic mechanism of proteases involves specific residues, which define the class of proteases, thereby called serine, cysteine, metallo-, aspartic, or threonine proteases (Figure 28.1 and Table 28.1). Some proteases reside and act from the intracellular milieu, while others can be secreted (see Figure 28.1). Upon inflammation, increased proteolytic activity is detected at the inflammatory site, and the origin of these proteases can be either eukaryotic or prokaryotic. Whether originating from microbes or from the host, protease hyperactivity could initiate the inflammatory response or could be a consequence of tissue inflammatory response. From the host side, upon inflammation, proteases can be released either by inflammatory cells or by resident noninflammatory cells.

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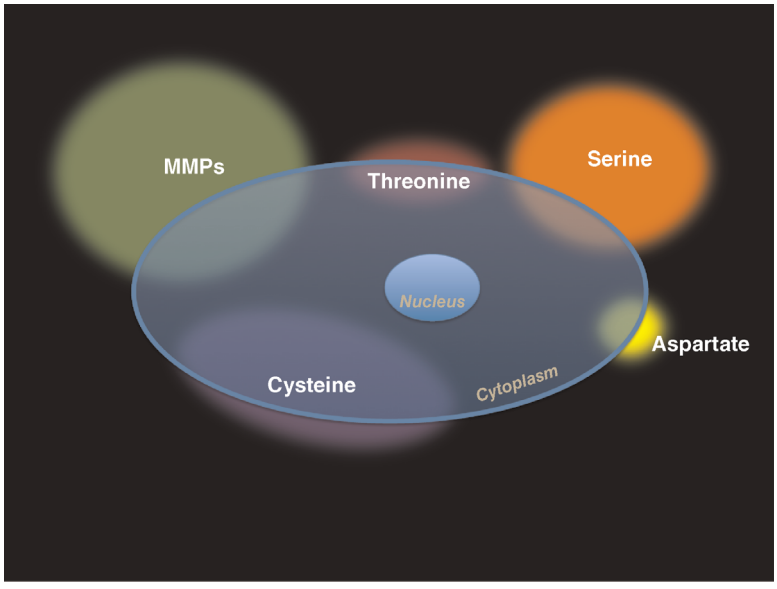


Figure 28.1 Eukaryotic cell proteases according to their catalytic mechanisms and their intracellular/extracellular representation. The size of the protease circles is proportional to their cellular representation.

28.2.1 Inflammatory Cells

Among infiltrated leukocytes, neutrophils constitute a major source of proteases. Within their granules, large amounts of elastase, proteinase-3, and cathepsin G are contained and released upon cell activation [1]. Macrophages are also rich in proteases, producing metalloproteinases (MMPs), in particular MMP-12, and also in caspases and cathepsins such as cathepsins L and D [2]. Proteases also constitute the major protein content of mast cells, which release tryptase, chymase, cathepsin G, or granzyme B [3].

28.2.2 Resident Cells

At mucosal surface, epithelial cells constitute an important source of proteases: mesotrypsin, matryptase, and even elastolytic activity have been associated with epidermal or intestinal epithelium [4–8]. In the case of elastolytic activity, this was clearly associated with chronic inflammation of the gut [5]. All other resident cells express intracellular cysteine proteases, such as the large family of intracellular caspases, but they can also release serine proteases upon damage. This has been demonstrated for endothelial cells, keratinocytes, or even neurons or fibroblasts. Finally, all cell types express intracellular proteases of the proteasome system, which are crucial regulators of the intracellular protein turnover. These proteases, which belong to the families of the cysteine or metalloproteinases, target ubiquitinylated proteins and change their degradation fate inside all cells. The proteasome system is a major component of cellular homeostasis and is not

Table 28.1 Host proteases identified in inflammation and possible sites of action (M, matrix; P, plasma; I, intracellular) and association with diseases (IBD, inflammatory bowel diseases; LI, lung inflammation; A, arthritis; SI, skin inflammation; LF, liver fibrosis; B, brain injury; PANC: pancreatitis.

Family	Proteases	Cellular Location	Possible sites of Action	Disease Association
Serine proteases	Elastases	Intra/extra	M, P, and I	IBD, LI, SI, A,
	Proteinase-3	Intra/extra	M, P, and I	LF, and PANC
	Neutrophil Serine protease-4	Intra/extra	M, P, and I	IBD and A
	Chymase	Extra	M and P	IBD
	Kallikreins	Extra	M and P	IBD, SI, and B
	Granzymes	Intra/extra	M and I	IBD
	Tryptase	Extra	M and P	IBD, A, and LI
	Plasminogen	Extra	M and P	IBD and PANC
	Activator	Extra	M and P	IBD, A, LI, and
	Trypsins	Intra/extra	M, P, and I	B
	Cathepsin G	Extra	M and P	IBD
	Thrombin	Extra	M and P	LI
	Factors V and VIII	Membrane-bound	M and P	SI and A
	FactorXa	Extra	M and I	LI
	Matryptase	Membrane-bound, Extra		LI
	Granzymes			
	Human airway trypsin-like protease			
Cysteine proteases	Caspases	Intra	I	IBD, LF, and LI
	Cathepsins (B, L, and K)	Extra	M, P	IBD, LI, obesity, and diabetes
	Autophagins	Intra	I	
	Calpains	Intra	II	IBD
	Deubiquitinases	Intra		
Aspartate proteases	Cathepsin D	Intra	I	IBD and obesity
	Renin	Intra/extra	M, P, I	IBD
Metalloproteinases	MMPs	Intra/extra	M, P, I	IBD, LF, LI, A,
	ADAM(TS)	Extra	M, P, II	and SI
	Deubiquitinases	Intra		LI

specifically associated with inflammation, but in an inflammatory context, changes in the proteasome functions have been described (see Section 28.3).

28.2.3 Protease Inhibitors

Protease inhibitors have coevolved with proteases, with the aim of controlling the destructive nature of proteases. Protease inhibitors can either be circulating inhibitors produced for the most part in the liver and released within the circulation or could be produced on site, by resident cells, or infiltrated inflammatory cells (see Table 28.2). Serpins are mostly systemic protease inhibitors that

Table 28.2 Endogenous protease inhibitors associated with inflammation: source (S, systemic; L, local) and possible sites of action: M, matrix; P, plasma; and I, intracellular.

Family	Protease Inhibitor	Targeted Proteases	Source	Possible Sites of Action	Regulation by Inflammation
Serpins	Serpin A1	Trypsin/chymase/tryptase/elastase/proteinase-3/cathepsin G/thrombin/kallikreins	S	M and P	↘
	Serpin A3	Chymotrypsin/chymase/cathepsin G	S	M and P	↗
	Serpin A4	Kallikreins	S	M and P	↘
	Serpin E1	Plasminogen activator	S	M and P	↗
	Serpin C1	Thrombin	S	M and P	↘
Chelonianin	SLPI	Elastase/cathepsin G/trypsin/chymotrypsin/tryptase/chymase	L	M, P, and I	↘
	Elafin	Elastase and proteinase-3	L	M, P, and I	↘
Kazal	LEKTI	KLK5/KLK7/KLK14	L	M	↘
	SPINK6	KLK2/KLK4/KLK5/KLK6/KLK7/KLK12/KLK13/KLK14			↘
	LEKTI2	KLK5			
α-Macroglobulin	A2 M	Plasmin, KLK1, trypsin, thrombin, and collagenase	S	P	↗
	A2 M-like 1	Chymotrypsin, papain, thermolysin, subtilisin A, and KLK7	L	M	
TIMPs	TIMP-1	MMP-1/MMP-2/MMP-3/MMP-4/MMP-6/MMP-19/ADAM10/ADAM17	S and L	M and P	↗
	TIMP-2	MMP-1, MMP-2, and MMP-14		M and P	
	TIMP-3	Membrane-bound MMPs	L	M, P	
	TIMP-4	MMP-1/MMP-2/MMP-3/MMP-4/MMP-6/MMP-19	S	M, P	
	c-IAP2	Caspase-9	L	I	↘
Cystatin	Cystatin M/E	Cathepsin V, L, Legumain	L	M	↘

Upregulation concomitantly to inflammation is represented by ↗, while downregulation by ↘.
A2 M: α2-macroglobulin; ADAM: A disentegrin and metalloproteinase; IAP: Inhibitor of Apoptosis; KLK: kallikrein; LEKTI: lympho-epithelial Kazal-type-related inhibitor; MMP: matrix metalloproteinase; SLPI: Secretory Protease Inhibitor; SPINK: serine protease inhibitor Kazal type; serpin: serine protease inhibitor; TIMP: tissue inhibitor of metalloproteinases.

inhibit a wide range of extracellular serine proteases. Locally released protease inhibitors such as secretory leukocyte protease inhibitor (SLPI) or Elafin are produced *in situ* both by resident cells and by infiltrated leukocytes, and inhibit elastases and proteinase-3, in addition to trypsin, chymotrypsin, cathepsin G, and tryptase for SLPI. Tissue inhibitors of metalloproteinases (TIMPs) are ubiquitous and inhibit a wide range of MMPs (see Table 28.2). A family of protease inhibitors has been uncovered in the skin, the Kazal family, and it seems to be specific for the inhibition of kallikreins (KLKs). This family of protease inhibitors includes LEKTI (lympho-epithelial Kazal-type inhibitor), LEKTI2, and SPINK6 (serine protease inhibitor of Kazal type 6) (see Table 28.2).

28.3 Host Protease's Functions in Inflammation

Association of dysregulated proteolytic activity with inflammation has been largely described and has often been associated with the degradative functions of proteases. However, proteases dispose of a very large arsenal of mechanisms to intervene in inflammatory processes and to participate in symptoms generation (see Figure 28.2).

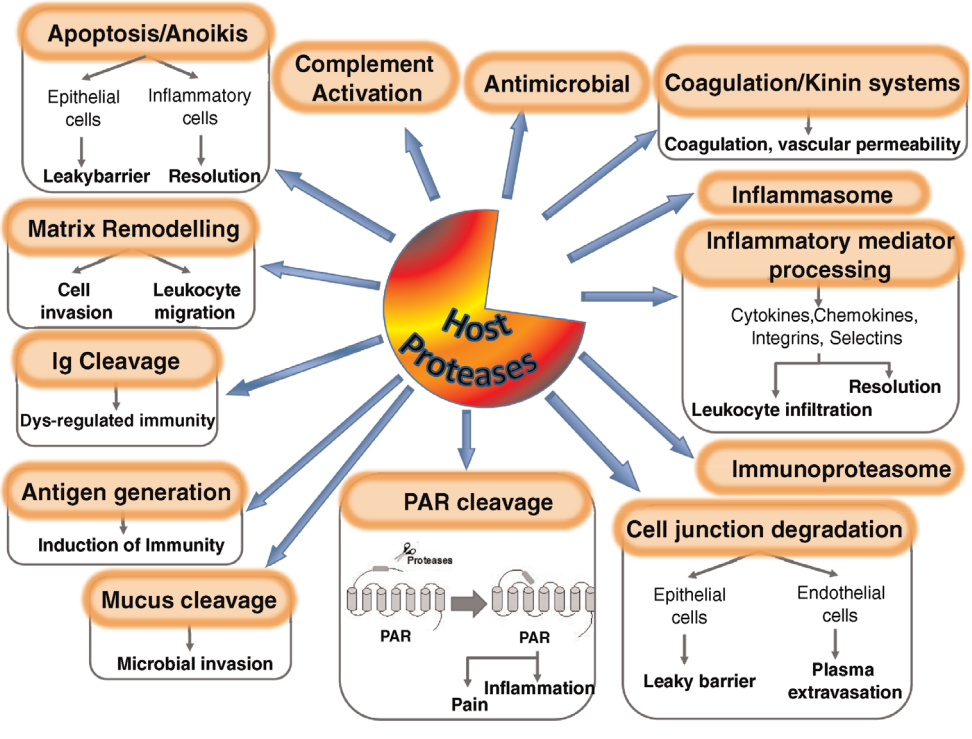


Figure 28.2 Mechanisms of action of host proteases at inflammatory sites and their consequences.

28.3.1 Antimicrobial Functions

Proteases and in particular those from neutrophils (including neutrophil elastase, proteinase-3, cathepsin G, and neutrophil serine proteinase-4) display strong antimicrobial functions. They can (1) directly kill bacteria, they can (2) activate by cleavage host immune proteins to generate active antimicrobial peptides, they can (3) target and inactivate bacterial virulence factors, resulting in attenuated microbial potency. and finally, (4) they participate in the formation of neutrophil extracellular traps (NET) (see Chapter 10).

Neutrophil elastase can directly kill bacteria such as *Klebsiella pneumonia* or *Escherichia coli* by cleavage of the outer membrane protein A (OmpA), resulting in the loss of bacteria membrane integrity and cell death [9]. Neutrophil proteases can also act in combination to enter the bacteria capsules and provoke membrane holes. This is what was observed with the Gram-positive *Streptococcus pneumonia*, killed by the concerted action of neutrophil elastase, cathepsin G, and proteinase-3 [10].

Extracellular cleavage of the host protein hCAP-18 by proteinase-3 or KLK5 and KLK7 from epithelial cells generates the antimicrobial peptide LL-37 [11–13]. Similarly, cleavage of the complement protein C3 or of the tissue-factor pathway inhibitors (TFPI-1 and TFPI-2) by neutrophil elastase leads to the release of powerful antimicrobial peptides that kill a wide range of bacteria [14].

Proteases can also target microbial virulence factors. For example, neutrophil elastase cleaves the invasins and the mobility protein IscA of *Shigella flexneri*, preventing thereby bacterial dissemination. Cathepsin G cleaves the *Staphylococcus aureus* adhesion clumping factor A [15].

Finally, neutrophil elastase is absolutely required for NET formation (see Chapter 10), where it cleaves histones to facilitate DNA decondensation. Within the NET, neutrophil proteases can exert the same antimicrobial actions described above, being in direct and privileged contact with microbes, while limiting damage in adjacent healthy tissues.

28.3.2 Inflammatory Mediator Processing

By their ability to cleave other proteins, proteases can either activate or deactivate protein targets. Indeed, they can transform an inactive precursor protein or a repressed receptor into an active mediator or a derepressed receptor. On the contrary, they can also degrade proteins and thereby inactivate active proteins. In an inflammatory context, proteolytic cleavage of some chemokines and cytokines preforms is necessary to transform them into active molecules. Proteinase-3 and cathepsin G are known to cleave CXCL-8 and CXCL-5, respectively. The cleaved fragments of these two chemokines acquire through proteolytic cleavage a higher chemotactic activity toward neutrophils. KLK7 is able to cleave the proform of Interleukin (IL)-1β (IL-1β) into an active form of IL-1β [16]. Proteinase-3 also activates IL-1, IL-18, and tumor necrosis factor-alpha (TNFα). In both cases, proteinase-3 clearly exerts proinflammatory effects, amplifying the activity of proinflammatory cytokines and chemokines. However, proteinase-3 can also exert anti-inflammatory effects by degrading the proinflammatory cytokine

IL-6. Cathepsin G can also be anti-inflammatory, degrading and inactivating mature TNFα and IL-6. The net effect of proteolytic modifications on chemokine and cytokine activation or degradation is still unclear. The overall pro- or anti-inflammatory effect of proteolytic activity depends on the nature and quantity of proteases released in the inflammatory microenvironment and on the kinetic of cytokine and chemokine release. Selectins and integrins also constitute proteolytic targets. Chymotrypsin, collagenase, stromelysin, and a disintegrin and metalloproteinase (ADAM)-17 metalloproteinase are able to shed selectins at cell surface. Cathepsins cleave integrins, thereby inhibiting the attachment of migrating cells to extracellular matrix components. These evidences demonstrate that proteases can regulate the initial steps of leukocyte recruitment in an inflammatory context, controlling rolling and adhesion events through the cleavage of selectins and integrins.

28.3.3 Junction Molecule Degradation

Cell–cell junctions constitute an important element of tissue homeostasis, which is disrupted in inflammation. These cell–cell junctions are disrupted in blood vessels, between endothelial cells, thereby allowing microvascular leakage, leukocyte transmigration, plasma extravasation, and edema (one of the cardinal signs of inflammation). Upon inflammation, cell–cell junction disruption also occurs at mucosal surfaces between epithelial cells, thereby leading to a leaky mucosal barrier that allows the passage of luminal content and further innate immune response. Proteases, by degrading cell junction proteins, are associated with both microvascular and mucosa barrier leakages.

Neutrophil elastase and cathepsin G cleave the vascular E-cadherin adherent junction protein on endothelial cell, thereby participating in the disruption of endothelial barrier and the transendothelial passage of leukocytes toward inflammatory sites [17,18]. Thrombin is also able to disassemble claudin-5 from the endothelial cell junction [19].

A number of studies have reported the disrupting effects of proteases on barrier function. However, evidence that proteases are acting through a direct degradation of junction proteins is scarce. Chymases are able to alter tight junction proteins such as occludins and zonula occcludens (ZO)-1 in intestinal epithelial cells [20]. KLK5, a form of trypsin produced by the epidermis, is able to degrade desmoglein-1, thereby causing barrier defects [21–23]. Indeed some members of the KLK family are expressed in the most superficial layer of epidermis, where they are involved in maintaining barrier integrity and function. Among the KLK family composed of 15 serine proteases, only KLK5, KLK7, KLK8, and KLK14 are known to be present as active forms in the *stratum corneum* [24]. In normal skin, these proteases are known to play prominent roles. In particular, it has been well documented that KLK5, KLK7, and KLK14 contribute to desquamation by degrading desmosomal cadherins, such as desmoglein1 and desmocollin1 [25], whereas caspase-14 is needed to complete the processing of filaggrin [26,27]. However, in the absence of inhibition as illustrated in Netherton syndrome, or after skin injury, proteolytic activity could be increased and detrimental. Hyperactivity of KLK5 through desmoglein1 degradation leads to hyperdesquamation [21,23], and

elastase-2 hyperactivity leads to skin dehydration [6]. This detrimental effect of proteases is not solely mediated by junction protein degradation. Activation of protease-activated receptors (PARs) is largely implicated (see Section 28.3.8).

28.3.4 Mucus and Surfactant Protein Cleavage

An important element of the mucosal barrier is the mucus layer. This is a matrix produced by mucosal epithelial surface and consists in a protective coating. It is made of glycoproteins known as mucins, which are secreted by specialized epithelial cells. The role of the mucus layer is to physically restrain microorganisms in the lumen, blocking their access to the underneath tissues. Mucins are large highly glycosylated proteins. A defective mucus layer leads to severe pathologies associated with chronic inflammation and infection, such as cystic fibrosis or inflammatory bowel diseases (IBD). Bacterial proteases have been largely studied for their properties to degrade mucus (see Section 28.4). In contrast, few studies have investigated the role of host proteases in mucus properties, but a recent study has demonstrated that mice deficient for the metalloproteinase meprin-β have a mucus layer strongly attached to epithelial cells in the intestine. This attachment can be reversed by exposure to meprin-β. Whether this plays a positive or negative role in a context of inflammation is not clear yet. Neutrophil elastase is known to induce mucus instability at lung surfaces. In chronic obstructive pulmonary diseases, rapid consumption of secreted proteases by an increased presence of protease inhibitors enhances mucin gel stability and possible mucus retention [28]. These two examples suggest that host proteases are important for mucus detachment from the epithelial mucus producing cells and that protease deficiency might lead to mucus retention. Host proteases are also important in signaling mechanisms to induce mucus production [29]. Indeed, neutrophil elastase is one of the most potent mucus secretagogue, not only inducing globlet cell degranulation and secretion of mucins but also acting at the transcriptional and post-transcriptional level, to increase the expression of mucins [29]. Although their mechanism of action has been less studied, proteinase-3 and cathepsin G also stimulate mucus secretion. Neutrophil elastase, Human airways trypsin-like protease and ADAM-17/TACE all display sheddase activity on the epidermal growth factor (EGF) receptor ligand transforming growth factor (TGF)-α, leading to downstream production of mucins [29].

Along with mucins, surfactant proteins are also important protective molecules present on the lung epithelial surface and can be targeted by host proteases. Neutrophil elastase, proteinase-3, and cathepsin-G are able to degrade surfactant proteins, resulting in the loss of biophysical and immunomodulatory properties of surfactant proteins [30].

28.3.5 Immunoglobulin Cleavage

Immunoglobulins (Ig) are highly sensitive to proteolytic cleavage by proteases. Several studies have reported the effects of microbial proteases on Ig degradation (see Section 27.4) and the subsequent modification of immune response. It is

not known yet whether host proteases are also capable of degrading Ig in an inflammatory context. However, considering the very large amount of host proteases that are released at inflammatory sites, and the large spectrum of their proteolytic actions, it is conceivable that host proteases could also cleave Ig or Ig receptors and thereby modify the immune response. Indeed, *in vitro* studies suggest that host proteases are capable of Ig degradation. MMP-3, MMP-12, cathepsin G, and neutrophil elastase are able to cleave Fab fragments and the amino-terminal amino acid sequence of the Fc fragment of human IgG. Similar fragments to the ones obtained by *in vitro* cleavage induced by these host proteases were recovered in synovial fluids of rheumatoid arthritis patients, suggesting that proteolysis of IgG in the joints by host proteases could occur *in vivo* and could contribute to joint inflammation [31].

28.3.6 Immunoproteasome, Antigen Presentation, and Processing

In lysosomal compartment of cells lies a very acidic network of vesicles, where proteases (aspartic and cysteine proteases) are the proteins the most represented. These proteases are constitutively expressed in most cell types, but a particular strong representation exists in antigen-presenting cells: macrophages and dendritic cells [32]. These proteases consist mostly of cathepsins and an endopeptidase named asparagine endopeptidase (AEP).

The main function of these intracellular proteases in antigen-presenting cells is to participate in the two well-established roles of class II major histocompatibility complex (MHC) pathways: (1) initiation of the removal of the invariant chain chaperone from class II MHC molecules and (2) the generation of suitable peptides for class II MHC binding [32]. Indeed, the uptake of exogenous antigens by macrophages or dendritic cells is followed by proteolytic degradation in endo/lysosomal compartments. Little is known about the precise identity of the specific enzymes that generate particular MHC class-II-bound peptides *in vivo* [33]. However, AEP has a central role in the generation of specific T-cell epitopes from several antigens. The protease initiates proteolytic events presumably, eliciting unfolding of the antigen to generate substrates for other enzymes, such as cathepsin D and cathepsin E [34,35]. Cathepsin L and cathepsin S are the best characterized proteases involved in the endosomal processing of the invariant chain, a chaperone molecule, which is associated with MHC class II molecules in the endoplasmic reticulum [35]. Cathepsins are also involved in antigen cross presentation of pathogen antigens, which are internalized, degraded, and presented by MHC class I, a crucial step to prime CD8 T cell responses. Numerous mechanisms can regulate the activity of endolysosomal cysteine proteases. Transcriptional regulation, trafficking, environmental pH, and activation of the proenzyme by the presence or absence of inhibitors are among those mechanisms [32,36]. There is no consensus on the transcriptional level of cathepsins after exposure to an inflammatory stimulus such as interferon-gamma (IFNγ), but the regulation of cathepsins mRNA seems to be highly sensitive to environmental changes, such as the presence of cytokines in the milieu, cell adhesion to substrates, and cell–cell contact [37–40].

Proteases other than the endolysosomal proteases are able to influence the initiation and polarization of adaptive immune responses. Studies have shown that neutrophil elastase may cleave the dendritic cell costimulatory molecules CD40, CD80, and CD86, which are essential for the stimulation of adaptive immunity. This proteolytic cleavage blocks the maturation of dendritic cells in response to lipopolysaccharide, reducing T-lymphocyte stimulatory capacity [41]. Together with neutrophil elastase, cathepsin G can cleave T-cell surface proteins that are involved in cell–cell interactions and activation, which may lead to reduced T-lymphocyte functionality in neutrophil-rich areas [42].

28.3.7 Matrix Remodeling

The extracellular matrix is a highly dynamic structure that is strongly remodeled upon inflammation. It is remodeled by the infiltration of microbial agents in the case of nonsterile inflammation, by the infiltration of inflammatory cells, and by tissue damage and destruction at inflammatory sites. In the resolution phase, active mechanisms also remodel the extracellular matrix, in order to reestablish tissue functions and homeostasis (see Chapter 60). The extracellular matrix interacts with cells to regulate migration, proliferation, and differentiation, and cleavage of extracellular matrix constitutes the main regulatory process of these functions. Clearly, MMPs, ADAMs, and ADAMTS are the main proteases responsible for matrix remodeling. They are controlled by the presence of TIMPs (see Table 28.2). Excessive extracellular matrix degradation is often associated with chronic inflammatory diseases, and could lead to a loss of organ function. Excess deposition of collagen-rich extracellular matrix and inappropriate inhibition of MMPs could also lead to fibrosis, a debilitating condition that can occur in most organs exposed to chronic inflammation. Therefore, it is crucial that the role of proteases in extracellular matrix remodeling could be tightly controlled in chronic inflammatory disorders.

28.3.8 Receptor Activation

Proteases may also modulate the inflammatory response by acting on receptors, and several studies report the involvement of receptor activation in proteases-induced effects on inflammation.

Cathepsin G is known to interact with the G protein-coupled formyl peptide receptor, and this interaction leads to the activation of MAP kinase pathways and further chemotactic effects for neutrophils [43]. However, the exact mechanism of action of cathepsin G on the formyl peptide receptor is unknown.

Neutrophil elastase also seems to have receptor-dependent effects involving the Gram-negative bacteria receptor Toll-like receptor(TLR)-4. Proteolytic intervention on this receptor leads to interleukin-8 upregulation and, therefore, potential chemoattraction of inflammatory cells. Proteolytic activity is necessary for this effect, but the mode of activation of the receptor is still unknown [44].

The mechanism by which proteases activate cell receptors that has been the most studied and fully characterized is the activation of the protease-activated receptor family (see Figure 28.3). Four members belong to this family of receptors,

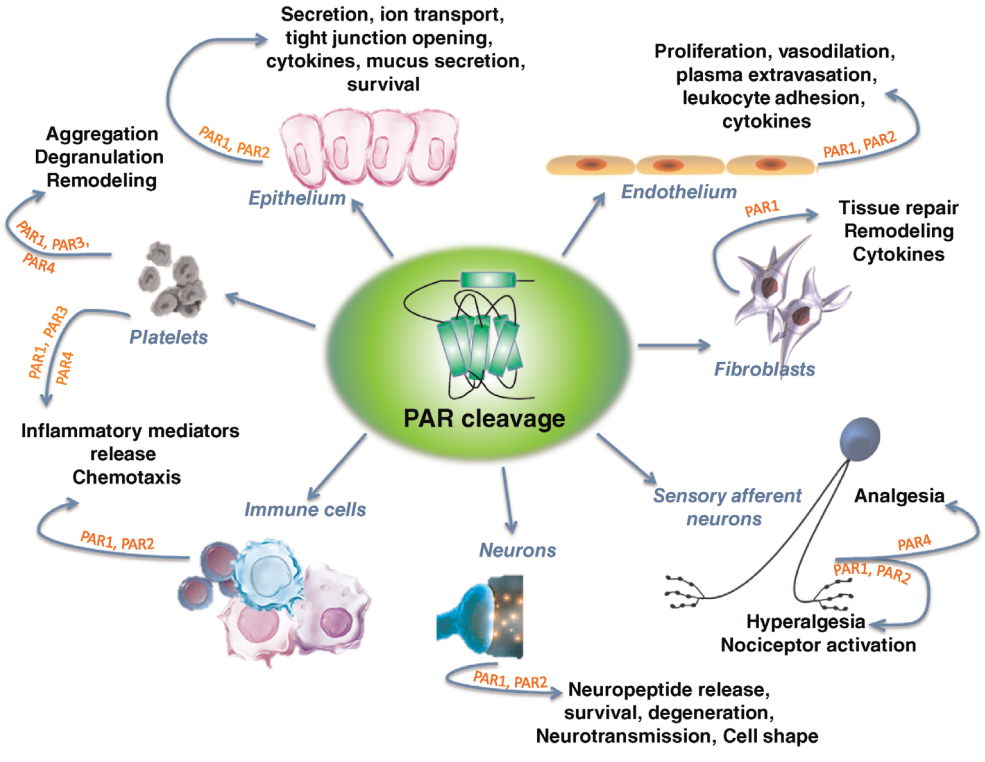


Figure 28.3 Inflammation-related effects of PAR activation on cells.

PAR1–4, and they are activated by the proteolytic cleavage of their extracellular N-terminal domain [45]. This proteolytic cleavage leads to the release of a new N-terminal domain that acts as a tethered ligand, which then binds and activates the receptor for further intracellular signaling (see Figure 28.3). Initially, canonical sites of activation have been described and were thought to be the only sites of activation. Small peptides corresponding to these canonical cleavages could be synthesized and used as pharmacological agonist tools. However, more recent studies have determined that cleavage sites other than the canonical sites are present on the N-terminal domain of these receptors, and cleavage at these noncanonical sites could induce different signaling pathways, therefore, identifying possible biased agonism for these receptors [46]. While the first receptor of this family was identified in the field of vascular research for its role in platelet aggregation (PAR1 was identified as the receptor responsible for thrombin-induced platelet aggregation in humans) [47], early studies have also uncovered the role of these receptors in inflammation. PAR1 and PAR2 activation are leading to all the cardinal signs of inflammation: *rubor et tumor, cum calor et dolor* [48,49]. Further studies have demonstrated that the expression of these receptors is highly ubiquitous and activation of these receptors on different cell types leads to a wide range of inflammatory features (see Figure 28.3). They are present on endothelial cells, where their activation leads to microvascular leakage [45]. They are present in epithelial cells (lung, intestine, bladder, skin, etc.), where activation of PAR1

and PAR2 affects epithelial barrier function by a mechanism involving not only the mobilization of myosin light chain kinase and the relocalization of junction proteins but also the induction of apoptosis in the case of PAR1 [50–54] and the induction of cell survival in the case of PAR2 [55]. Activation of PARs on epithelial cells also induces the release of chemokines such as interleukin-8, and modifies ion exchanges [56,57]. For instance, PAR2 activation on keratinocytes leads to the release of IL-8, TNFα, and thymic stromal lymphopoietin (TSLP) [58,59]. PARs are present on sensory neurons. The activation of PAR1 and PAR2 on primary afferents provokes the release of neuropeptides such as substance P or calcitonin gene-related peptide [60,61] and activation of pain pathways [62], while PAR4 activation seems to be analgesic [63,64]. PARs are also present in inflammatory cells, where their cleavage leads to cell activation. This has been particularly studied in lymphocytes and mast cells, where their activation could lead to the release of proinflammatory cytokines [45]. In the case of PAR2 activation on mast cells, studies have demonstrated that it induces the release of histamine, playing a role in scratching and itch [65]. Overall, PAR activation seems to coincide with proinflammatory effects, although some studies in specific conditions and tissues have also described discrete anti-inflammatory properties [45]. However, most of the studies investigating the consequences of PAR activation have used small synthetic peptides corresponding to the canonical site of cleavage or proteases known to cleave at these sites. The pharmacology of these receptors and their function in inflammation would need to be further studied taking into account biased agonism and proteolytic cleavage at sites other than the canonical site. The corollary of these studies would be the determination at sites of inflammation of the proteases that are present and that activate the different PARs.

28.3.9 Apoptosis or Anoikis

Programmed cell death is an important step of chronic inflammatory disorders (see Chapters 10 and 18), and it is tightly controlled by proteolytic activity. Intracellular caspases and autophagins are obviously major actors of these processes. Apoptosis is induced by the activation of cysteine proteases, including caspases 8, 9, and 3 [66]. Other caspases are also known for their pro-inflammatory properties, for instance, caspase 1 and 4. Overall, caspases through their role in inducing cell death regulate inflammatory and immune responses [66]. Proteases other than caspases are also capable of inducing apoptosis or anoikis. Among them, thrombin and granzymes are proteases that are released in the extracellular milieu at inflammatory sites. If apoptosis is induced in inflammatory cells such as neutrophils, this process favors the resolution of inflammation. Protease-triggered neutrophil apoptosis would, therefore, be protective in chronic inflammatory disorders. In contrast, if apoptosis occurs in tissue cells, it could lead to excessive tissue destruction and potential loss of function. There, protease-triggered apoptosis would further feed inflammatory processes and be detrimental to tissue repair. The proteolytic mechanisms involved in caspase intracellular signaling associated with apoptosis have been largely described [66]. The mechanisms of extracellular proteases-induced apoptosis have been less investigated,

but some studies describe a role for PAR activation, and in particular PAR1 activation, which is able to induce apoptosis in intestinal epithelial cells, contributing both *in vitro* and *in vivo* to a loss of barrier function [50].

28.3.10 Coagulation and Fibrinolysis

Apart from their role in hemostasis, coagulant and fibrinolytic proteases are important mediators of inflammation (see Chapter 32) (Figure 28.4) [67]. Coagulation proteases circulate in the blood under their zymogen form (Factor X and plasminogen). They enter damaged tissues as a consequence of vascular rupture, and there, they become activated and contribute to extracellular coagulation and fibrinolysis. These proteases can act through the activation of PARs, evoking cell-mediated actions on blood and vascular cells (endothelium and platelets), on structural cells (fibroblasts, smooth muscles, neurons, etc.) or on inflammatory cells (macrophages). PAR1 is the prototypical receptor of thrombin and Factor Xa [68], although PAR4 can also be activated by thrombin, and most recently thrombin signaling in PAR2 has been discovered [69]. Coagulation proteases can

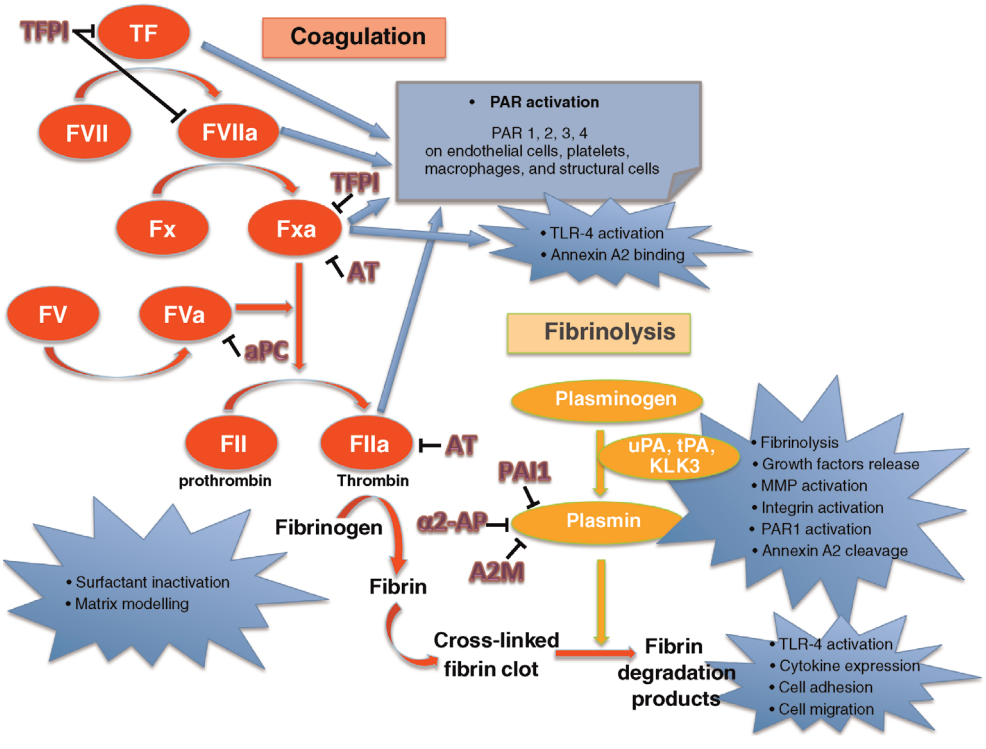


Figure 28.4 Intervention of coagulant and fibrinolytic proteases in inflammatory processes. In tissue injury and disease, the proteases FXa, FVIIa, tissue factor (TF), thrombin, plasmin, uPA, and tPA not only participate in extravascular coagulation (red pathway) or fibrinolysis (orange pathway) but also influence inflammation (blue background). These proteases are controlled by protease inhibitors (in purple), such as antithrombin (AT), tissue factor protease inhibitor (TFPI), activated protein C (aPC), and plasmin activator inhibitor (PAI1)

signal through PARs by binding the receptor at hirudin-like sites and further cleaving PAR extracellular domain, as it was demonstrated for thrombin and PAR1. Fibrinolysis proteases can also use cofactors, such as integrins; plasmin by binding $\alpha_9\beta_1$ integrin is localized at the cell surface, where it is protected from inhibitors, and gets closer to PAR1, increasing its affinity for this receptor. Finally, coagulation proteases also use receptor dimerization, as illustrated by the PAR1–PAR4 cooperation in platelets [70].

Coagulation proteases also act by proteolytic cleavage of other proteases and growth factor transactivation. Plasmin catalyzes the proteolytic activation of MMPs and releases latent forms of growth factors such as EGF and TGFβ. Plasmin activates MMP-1, MMP-2, MMP-3, MMP-9, MMP13, and MMP-14. The effects of plasmin on EGF receptor signaling involve heparin-binding EGF, an EGF receptor ligand, which is released from cell surface by MMP proteolysis [71]. Similarly, plasmin is able to mobilize matrix-bound TGFβ, contributing to collagen synthesis [72].

Urokinase (uPA) and tissue-type plasminogen activator (t-PA), the proteases that convert plasminogen into plasmin, also signal through other receptors: the uPAR and the LDL receptor-related protein 1 [67]. This signaling, in an inflammatory context, involves integrin recruitment and conveys remodeling. By interacting with uPAR, uPA activates coreceptors, including the formyl-peptide receptor 2 (see Chapters 57 and 58), the EGF receptor, and integrins. This signaling regulates migration, chemotaxis, and cytokine production [67].

Coagulation and fibrinolysis systems also contribute to inflammation by their role in the formation and degradation of fibrin. Fibrinogen cleavage by thrombin releases fibrinopeptides A and B, which are potent chemoattractants for neutrophils, monocytes, and macrophages [73]. Fibrin and fibrin degradation products activate cells at inflammatory sites (resident or infiltrated cells), via the binding of TLR-4 or CD11b/CD18 integrins [74]. Fibrin D dimers stimulate the production of cytokines by peripheral blood monocytes, and fibrin E fragment induces both cytokine expression in leukocytes and leukocyte migration [75,76]. Fibrin degradation products are potent chemoattractants for neutrophils and fibroblasts, but they also induce cytokine expression. Other fragments have anti-inflammatory and immunosuppressive effects. Factor Xa involves for its signaling annexin A2 binding and is required for adenoviral activation of TLR4/MyD88 signaling in infected cells. By activating TLR-4, annexin A2 regulates proliferation in smooth muscle cells and cytokine production in macrophages [71,77].

Overall, coagulant and fibrinolytic proteases evoke mostly proinflammatory and remodeling actions in inflammation, and targeting the inflammatory effects of these proteases, without disturbing their role in hemostasis, could constitute a beneficial strategy for the treatment of inflammatory diseases.

28.3.11 Kinin–Kallikrein System

The kinin–kallikrein system is an endogenous multiprotein cascade, which triggers the enzymatic hydrolysis of kininogens, ultimately ending into the release of bradykinin-related peptides (Figure 28.5). Those peptides constitute potent vasoactive peptides that act on bradykinin receptors (B1 and B2) and play essential roles in inflammatory features, including vasodilation, vascular

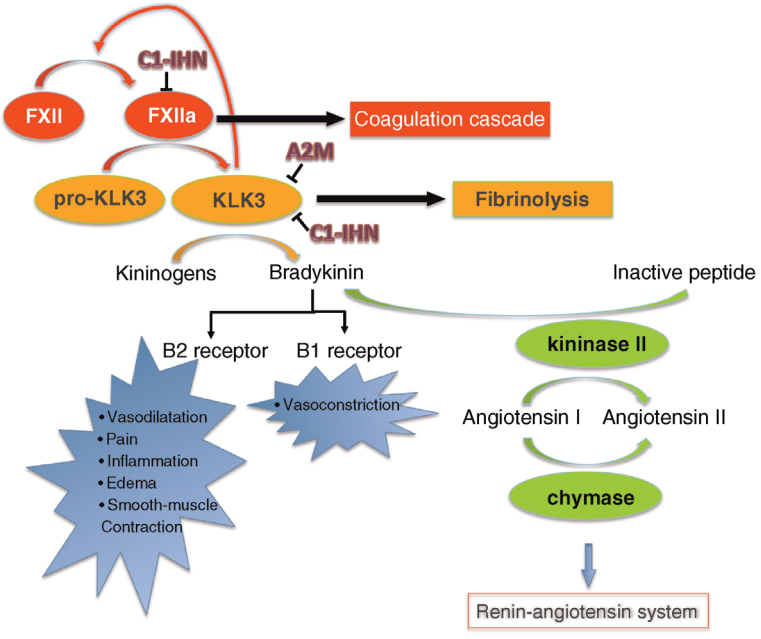


Figure 28.5 The plasma kallikrein–kinin system is linked to inflammation (blue), thrombosis (red), fibrinolysis (orange), and the renin–angiotensin system (green). It is controlled by inhibitors (purple): A2 M, alpha 2-macroglobulin, and C1-IHN, complement 1 inhibitor. Abbreviations: KLK: kallikrein; F: factor.

permeability, smooth muscle contraction, and pain [78]. Indeed, bradykinin induces the four classical signs of inflammation, heat, redness, swelling, and pain, when injected into animal tissues. After tissue injury, bradykinin is among the very first mediators to be released, taking part in the silent phase of inflammation. Therefore, even during the very early events of inflammation, well before the recruitment of inflammatory cells, proteases of the kinin–kallikrein system are playing a significant role in the initiation of inflammation. B2 receptor activation seems to play a prominent role in acute inflammatory response, inducing vasodilation, edema, and hyperalgesia, while B1 receptor seems to substitute the effects of B2 receptor in chronic inflammatory processes [79].

28.3.12 Complement System Activation

Another first-line defense machinery in which proteases play a preponderant role is the complement system (see Chapter 30). Similarly to the kallikrein–kinin system, this pathway starts with inactive precursors produced by the liver and present in normal condition in the blood stream. The activation of this system relies on a proteolytic cascade, which ultimately leads to active proteins playing a role in important defense mechanism of the host against pathogens: opsonization, chemotaxis, cell destruction, and pathogen agglutination. During the proteolytic cascade, the complement factors C3a and C5a are produced from the proteolytic conversion of their precursors C3 and C5 by the C3 and C5 convertases,

respectively. Both C3a and C5a display anaphylatoxin activity by inducing mast cell degranulation, smooth muscle contraction, and powerful chemoattraction of neutrophils and monocytes. Therefore, all proteases able to convert C3 and C5 into active C3a and C5a might potentially initiate or enhance the inflammatory host response. Complement proteases have very narrow specificity and low substrate turnover. The structure and activities of complement proteases have been extensively reviewed. Interestingly, not all complement proteases have a natural endogenous inhibitor. Much attention has been focused on controlling the formation or activity of complement protease complexes C3bBb and C4b2a, as these generate the inflammatory peptides C3a and C5a. However, the development of selective and potent inhibitors for complement proteases is still ahead of us.

28.3.13 Inflammasome

Inflammatory caspases include caspase-1, caspase-4, caspase-5, and caspase-11. All inflammatory caspases participate in the activation of the inflammasome, which drives the induction of an inflammatory form of programed cell death, also known as pyroptosis (see Chapter 20).

Caspase-1 is activated by different pathways: the nucleotide-binding domain (NOD), the leucine-rich repeat, and the NOD-like receptor pyrin domain-containing protein (NLRP)-1, as well as NLPR3 and CARD-containing protein 4. The caspase 1 inflammasome orchestrates antimicrobial effector functions, tissue repair, metabolic regulation, and cell survival [80], but the most investigated of its roles is its ability to cleave interleukin-1β and interleukin-18. Caspases 4, 5, and 11 interact with and activate caspase 1 [81]. They activate the NLRP3 inflammasome in response to Gram-negative bacteria and intracellular presence of LPS, and it is believed that this is through this activation that caspase 1 is further activated to cleave interleukin-1β and interleukin-18. Direct cleavage of IL-1β and IL-18 by caspase 4 has been proposed, but it still has to be confirmed. Activation of caspases 4, 5, and 11 also leads to pyroptosis and to the release of interleukin1α independent of caspase 1.

Caspase 8 is not really considered as a protease of the inflammasome, but it is recognized for its role in apoptosis and in the control of inflammation. Indeed, it is required for the activation of NF-κB signaling and is involved in both direct and indirect cleavage of pro-IL1 and pro-IL-18 into bioactive proteins [82]. This effect could either be exerted together with the caspase 1 inflammasome or independently [83]. In contrast, other evidence shows that caspase 8 can inhibit inflammation, through the inhibition of the inflammasome [84], the inhibition of interferon responses [85], and the activation of necroptosis [86].

28.4 Microbial Proteases and Inflammation

Different inflammatory pathologies are associated with infection, and this diversity depends both on the pathogen species and on their specific site tropism

within the host. Proteases delivered by pathogens are believed to take part in inflammatory-driven processes, and some of them are specifically designed to help the microbes resisting the hostile host environment. Pathogen-derived proteases are, therefore, often considered important virulence factors. The strategies that pathogens have developed to hamper host effectors, modulating proinflammatory pathways, impact all major innate immune host responses as illustrated in this section (Table 28.3).

Table 28.3 Major identified pathogen-associated microbial proteases.

Protease category	Microbial Proteases	Examples of Pathogens
Aspartic	Type 4 prepilin peptidase	EHEC
	Preflagellin	Archaeal bacteria
	PLA	*Y. pestis*
	Sap5p	*C. albicans*
Cysteine	Sortases	*E. faecalis*
	Gingipains	*P. gingivalis*
	Staphopain	*S. aureus*
	Derp 1	*D. pteronyssinus*
	Cruzipain	*T. cruzi*
	Falcipains (2 and 3)	*P. falciparum*
	Clostripain	*C. histolyticum*
Serine	Subtilisin	*Clostridium difficile*
	Protease IV	*P. aeruginosa*
	HtrA	*S. flexneri*, *C. jejuni*, and *H. pylori*
	ASP	*A. sobria*
	SCP	*Stretococcus* group *A*
	EspP-alpha	EHEC
Metalloproteinases	Fragilysin	*Enterotoxigenic* and *B. fragilis*
	Gel E	*E. faecalis*
	Elastase B	*P. aeruginosa*
	Alkaline protease	*P. aeruginosa*
	Collagenase	*Salmonella thyphimurium*
	HA/P	*V. cholerae* and *H. pylori*
	56 K	*S. marcescens*
	ZmpB	*B. cenocepacia*
	SSlE	EPEC and EHEC
	Vibriolysin	*Vibrio*
	IgA1 protease	*S. pneumoniae*

28.4.1 Effects on Barrier Function

Microbial proteases are able to modify the three components of mucosal barrier function: the IgA presence, the mucus, and the epithelial junction integrity.

IgA's function at mucosal surfaces is to block the access of antigens and pathogenic microorganisms to their epithelial receptors, in order to facilitate the luminal elimination of those microorganisms. This process, known as immune exclusion, is of high importance in mucosal homeostasis. For instance, *Blastocystis ratti*, an enteric protozoan parasite, is able to degrade IgA via an aspartic protease activity [87]. In lung infection, IgA is degraded by elastase B of *Pseudomonas aeruginosa* [88]. *S. pneumoniae* express IgA1 protease, which restricts bacterial agglutination by polymeric and secretory IgA and interferes with IgA1-mediated bacterial killing [89]. *Burkholderia cenocepacia*, a microorganism causing lung infection in 4–7% of cystic fibrosis patients, has a metalloprotease ZmpB that cleaves IgA [90]. For all these microbial species, IgA-degrading activities favor host colonization/infection through host defense evasion.

Another important element of the mucosal barrier is the mucus layer. The nematode *Trichuris muris* causing trichiriasis (diarrhea, abdominal pain, and fatigue) expresses a serine protease able to degrade Muc2 [91]. Similarly, *E. histolytica* displays cysteine protease activity, which is able to degrade Muc2 in its C-terminal domain and to break down the colonic mucus layer [92]. Both enteropathogenic *E. coli* (EPEC) and entero-hemorrhagic *E. coli* (EHEC) express SslE, a mucin-degrading enzyme [93,94]. Mucus proteolytic digestion by the *Helicobacter pylori* hemagglutinin/protease (HA/P) has been suggested as an important mechanism by which this pathogen could drive its pathogenic effects, that is, gastric inflammation and adenocarcinoma [95]. Finally, *Vibrio cholerae* also produces HA/P, which is required for vibrio translocation through a mucin gel, suggesting an important role of this protease in cholera pathophysiology [96]. *Trichomonas vaginalis*, a parasite colonizing the vaginal mucosa, produces mucin-degrading cysteine proteases [97]. All these pathogen-derived mucinase activities might help the microbes to reach the underlying epithelium and promote an efficient infection of the host.

Cell–cell epithelial junctions constitute the last physical impediment element of mucosal barrier function. Among junctional proteins, E-cadherin of adherent junction represents a particularly appealing target for microbial proteases. This applies to a number of food-borne pathogens involved in enteric pathologies such as EPEC, *S. flexneri*, *Campylobacter jejuni*, and *H. pylori* through the action of a conserved serine protease named HtrA [98]. The zinc protease fragilysin from *Bacteroides fragilis* also cleaves E-cadherin, directly disrupting adherent junction, thereby also impacting tight junction integrity [99]. An additional colitogenic bacterium, *Enteroccocus faecalis* secretes a gelatinase activity enzyme, GelE, which has the capacity to degrade E-cadherin [100]. Finally, *Candida albicans*, the causal agent of oral or genital infection called candidiasis, also produces an E-cadherin-degrading protease, Sap5p, which contributes to mucosal tissue invasion [101]. With regard to the tight junction proteins, occludin extracellular domain is cleaved by the *V. cholerae* metalloprotease HA/P, also described above

for its mucinase activity. This induces conformational changes of occludin intracellular domain and subsequent ZO-1 dissociation from tight junction [102]. In the airway infection context, elastase B expressed by *P. aeruginosa* has been shown to degrade TJ components in lung epithelial cells [103]. Furthermore, tight junction degradation is also operated by *Der p* 1, the major allergenic cysteine protease of *Dermatophagoides pteronyssinus* house dust mite (HDM) [104]. Recent evidence also indicates the presence of *Der p 1* in the gastrointestinal tract with functional consequences on the integrity of epithelial barrier. Likely, *Der p 1* is also able to alter the barrier function in intestinal epithelial cells by degrading ZO-1 [105]. Several gingipains, cysteine-like proteases from *P. gingivalis*, are thought to degrade junctional proteins of both adherent junctions and tight junctions, that is, E-cadherin and occluding. This mechanism may help bacterial penetration through the deep connective gum tissue [106].

Through their IgA-, mucin-, and cellular junction-degrading properties, the above-mentioned microbial proteases are suggested to play a prominent role in the local disruption of mucosal barrier. This obviously impacts epithelial permeability, pathogen invasion, and subsequent host immune response modulation. Upon mucosal barrier impairment and once within the tissue, pathogens are able to activate different host immune/inflammatory response pathways in which microbial proteases also play a direct role. Among them are the control of vascular permeability, anaphylatoxin production, and cytokine secretion.

28.4.2 Effects on Vascular Permeability

In addition to host proteases being able to activate the kallikrein–kinin cascade, microbial proteases have been shown to participate in proteolysis at several steps and thereby to induce increased vascular permeability, plasma protein extravasation, and further infection-associated edema. The vast majority of them belong to the group of bacterial proteases. For instance, *S. aureus* is capable of kinin production via the cysteine protease staphopain [107]. Like *S. aureus*, *Streptococcus* groupA and *Clostridium histolyticum* also express a cysteine protease able to generate kinins [108]. More specifically, the cysteine protease clostripain has been shown to exert kinin-releasing activity [109]. In the context of gingival tissue infection, *P. gingivalis* gingipains are able to perform the conversion of prekallikrein into active kallikrein and to convert kininogen into kinin [110]. Additional bacterial species such as *Serratia marcescens* have been shown to generate kinins through metalloprotease activities (56 K zinc protease) [111]. Vibriolysin proteases produced by *Vibrio* bacteria have also been shown to activate the kallikrein–kinin cascade *in vitro* [112] and *in vivo* [113].

Enzymes from protozoan parasites and arthropod species have also been shown to play a role in this pathway. This is illustrated by the cysteine protease cruzipain, produced by *Trypanosoma cruzi*, causing agent of the Chagas disease [114]. The malaria-causing parasite, *Plasmodium falciparum*, secretes cysteine proteases falcipain-2 and -3 that can produce vasoactive kinins from human kininogen [115]. Finally, literature reports the kinin-generating ability of a serine protease from the house dust mite *Dermatofagoides farina (Df)* [116] and its effect on vascular permeability enhancement in guinea pigs [117].

28.4.3 Effects on Complement Proteins

Pathogens produce some proteases that enhance or impede the complement system pathway. These functions might participate to the modulation of inflammation as well as pathogen subsistence within the host tissue.

In addition to its kinin-producing ability mentioned above, the cysteine protease gingipain R is able to convert C5 into active C5a, adding another potential pathophysiological function to this *P. gingivalis* protease in the progress of periodontitis [118]. A similar C5a-generating activity is attributed to ASP, a serine protease from *Aeromonas sobria* [119]. This pathogenic bacterium is associated with gastroenteritis and skin infection. Moreover, the Df serine protease from house dust mites displays C3a- and C5a-producing activity and this protease is believed to play a dual role as an allergen and an anaphylatoxin-triggering agent [120]. The microbial protease-generated anaphylatoxins, C5a and C3a, by binding to the corresponding receptors on mast cells trigger the degranulation of mast cells and the release of granule contents.

In contrast to proteases with C3a and/or C5a prodelivering action, some pathogens also express proteases, which degrade components of the complement cascade. This feature might appear as a way for the pathogens to counteract the host system immune defense. This escape mechanism may give advantage to the pathogen and favor its survival within the host milieu. Among the complement factor-degrading enzymes, three of them belong to the *P. aeruginosa*. The C3 complement factor is a substrate for two metalloproteases, alkaline protease and elastase B, as well as for a serine protease named protease IV [121,122]. Elastase B is also able to inactivate C1, C5, C8, and C9, while protease IV degrades C1q and alkaline protease cleaves C2, thereby impeding downstream complement effector functions. In *Streptococcus* group A, *S*treptococcal *C*5a *P*eptidase (SCP) is secreted and remains bound to the surface of the bacteria, where it is able to degrade C5a [123]. Furthermore, C3 and C5 are also the targets of EspPα protease that belongs to the serine protease autotransporters of Enterobacteriaceae family produced by EHEC [124]. The colitogenic bacterium *E. faecalis* secretes a gelatinase activity GelE, which has the capacity to degrade C3 [125]. Finally, in *Yersinia pestis*, the causative agent for plague, the aspartic acid surface protease PLA, is an important virulence factor with both C3-degrading and plasminogen activator functions [126].

28.4.4 Effects on Cytokine/Chemokine Production

Several studies report the effect of pathogen proteases on the production of CXCL8 with evidence or not of PAR2 or TLR implication. In the human macrophage cell line Thp1, *P. gingivalis* gingipains have been shown to increase CXCL8 expression, with synergetic effects between TLR and NOD1/2 signaling pathways [127]. In addition to gene expression regulation, the biological activity of CXCL8 is directly modulated by proteolytic activity of gingipains. Soluble forms of gingipain R and K increase CXCL8 biological function, while the bacterial surface-bound gingipain has an opposite destructing function on CXCL8 [128]. This CXCL8-digesting effect could help the bacteria biofilm to

maintain within the plaque. PAR2 pathway is stimulated by gingipain RgpB protease and this is associated with the production of another important proinflammatory cytokine, IL6 in an oral epithelial cell line [129]. In addition, proinflammatory neuropeptides CGRP and substance P are also upregulated by RgpB [130].

Regarding intestinal inflammation, a number of enteric microbes are able to modulate the production of CXCL8. As an illustration, a diarrhea-causing enteric protozoan parasite mentioned above, *E. histolytica*, produces cysteine proteases that induce CXCL8 release from human colonic epithelial cells and possess IL1-beta-converting enzyme activity [131]. In addition, colitogenic EPEC and EHEC strains produce two proteases with opposite effects on CXCL8 production; while the zinc protease NleC is immunosuppressive through direct interference with the NF-κB pathway and CXCL8 production in Hela cells [132], SslE is a secreted protease able to upregulate this chemokine by intestinal epithelial cells [94]. CXCL8 secretion is also stimulated by fragilysin, a zinc protease from *B. fragilis* bacteria [133].

In the context of respiratory tract infection, several microbial proteolytic activities have been shown to modulate CXCL8 secretion. This includes a serine protease from *S. aureus* [134] and LepA and elastase B from *P. aeruginosa* [135,136]. In addition, metalloprotease activities from this pathogen are able to degrade chemotactic molecules such as human regulated on activation normal T-cell exrepssed and secreted (RANTES), monocyte-chemoattractant protein (MCP)-1, and epithelial neutrophil-activating protein 78 (ENA-78) [137]. Finally, the *D. pteronyssinus* HDM produces a cysteine protease *Der p 1* that releases CXCL8 from respiratory epithelial cells [138]. In conclusion, the ability of proteases to control the expression level of cytokines/chemokines and to degrade them represent an important mechanism by which the pathogen may stimulate or decrease host inflammatory response.

28.5 Proteases as Targets in Inflammatory Pathologies

Considering the multitude of mechanisms by which proteases influence the inflammatory response, they constitute obvious targets for inflammatory pathologies. Overall, more pro- than anti-inflammatory effects of proteases have been considered so far for therapeutic options, and delivery of protease inhibitors has been considered for the treatment of several inflammatory diseases. However, depending on the tissues, proteases might have different mode of action and potentially important physiological functions that would have to be conserved. Moreover, the use of large-spectrum inhibitors versus more specific inhibitors is still an open question. Finally, different pharmacological options have to be considered: (i) the use of small synthetic inhibitors, (ii) the delivery or enhancement of natural endogenous protease inhibitors, or (iii) the use of blocking antibodies.

28.5.1 Gut

The use of large spectrum inhibitors in gut inflammatory pathologies would bear a number of side effects, when considering the physiological functions of

proteases in the intestine. However, severe increased proteolytic activity is associated with inflammatory bowel disease [139], as well as with low-grade inflammation irritable bowel syndrome (IBS) [140], suggesting that protease inhibition is an important possible therapeutic avenue to consider in gut pathologies. From all the families of proteases that are upregulated in IBD, MMP inhibitors have been extensively considered. First, because synthetic inhibitors suitable for use in humans have been developed for cancer. Second, because strong anti-inflammatory properties have been demonstrated for MMP inhibitors in animal models of colitis. However, in humans, MMP inhibitors appear to be more efficient at helping mucosal healing and extracellular matrix restructuration than exerting anti-inflammatory effects. Indeed, the use of MMP inhibitors has revealed anti-inflammatory effects of MMPs, thereby identifying MMP inhibitors as anti-targets for inflammation [141].

Upon active protease identification, studies have identified some interesting proteolytic targets for IBD. Elastase and trypsin activities seemed particularly upregulated [4], and as a corollary, elastase inhibitor Elafin and trypsin inhibitor serpin A1 are downregulated in tissues of IBD patients [4,140], associated for the latter with a more aggressive disease [142]. The delivery of Elafin, the natural endogenous inhibitor of elastase and proteinase-3 in the intestine of IBD patients has been proposed, as positive results have been obtained in animal models of colitis [5]. The use of genetically modified food-grade bacteria has been used to deliver such endogenous protease inhibitors. Commensal or probiotic bacteria colonizing the gut have been genetically transformed to express human epithelium-derived protease inhibitors such as Elafin or SLPI. Strong anti-inflammatory properties have been described for lactic acid bacteria genetically modified for the expression of protease inhibitors [5,143]. Treatments with these recombinant bacteria were drastically more effective than treatments with bacteria recombinant for other anti-inflammatory mediators such as TGF or IL-10 [143]. In addition, local delivery of protease inhibitors by using food-grade bacteria probably also decreases the possible side effects of protease inhibition therapeutic intervention.

Polymorphisms of several genes of the ubiquitine–proteasome system have been identified in IBD patients [144,145]. Proteasome inhibitors, such as bortezomib, which was successfully developed for cancer treatment, could be shortly tested for IBD.

In low-grade intestinal inflammation such as IBS, trypsin and tryptase activities are upregulated. Supernatants from IBS patients signal to sensory neurons in a protease- and PAR-dependent manner, suggesting that proteases, through PAR activation, are major mediators of sensory neuron hyperactivity and possibly of visceral hypersensitivity associated with this disease [140]. The forms of trypsin present in tissues from IBS patients have yet to be determined, and consensus is not reached on whether this activity originates from the host, from the microbiota, or from both. A better knowledge on the nature of the proteases responsible for such activities constitutes a necessary step to further consider and determine specific protease inhibitors for the treatment of IBS symptoms.

28.5.2 Airways

MMPs have been implicated in asthma, and polymorphisms in MMP genes may contribute to a predisposition to asthma [146]. Altered levels of MMPs are

reported, with, for instance, an increased level of MMP-9 in lung tissues and sputum. MMP-1, -2, -7, -10, -12, and 19 have also been implicated [147]. However, when MMP inhibitors such as tetracyclines have been investigated as potential treatments for asthma, the results obtained were not clear-cut. Doxycycline, for example, was reported to decrease eosinophilic inflammation in some models but was reported ineffective in others [147]. While some *in vivo* studies may have found some interest for MMP broad-spectrum inhibitors, their valuable clinical use is still unclear. The disappointment faced in other pathologies (such as the gut) has lowered the enthusiasm to test these drugs in an inflammatory context, off-target effects with these broad-spectrum MMP inhibitors being expected. Evidences from animal models of chronic obstructive pulmonary disease (COPD) suggest that targeting MMPs may be more valuable in the context of alveolar destruction. The effectiveness of MMP inhibitors as treatment of COPD in humans remains unknown, since few clinical trials have been conducted so far. A recent trial of 55 patients with stable moderate to severe COPD report that treatment with a selective MMP-9/MMP-12 inhibitor was well tolerated, but only limited clinical outcomes were noticed [148]. Further studies will be required to conclude on the value of MMP inhibitors.

The main thrust of protease inhibitors for the treatment of asthma has concentrated on mast cell proteases and in particular on tryptase. This interest was driven by data showing that tryptase can contribute not only to airway bronchoconstriction and hyper responsiveness but also by the fact that tryptase may have a major role in fibrosis and matrix remodeling [149]. However, phase I clinical trials have been discontinued in development for the treatment of asthma, since at certain doses, tryptase inhibitors exhibited signs of bronchospasms, while in phase IIa studies, the compounds did not reveal any bronchospasm activity and was on the contrary quite efficient at preventing airway response in asthmatics [150].

A form of alpha-1-anti-trypsin (AAT) has been available for the treatment of AAT deficiency since 1987. Intravenous administration of plasma-purified AAT augments serum and lung levels of AAT, while it increases antielastase capacity in patients deficient in the expression of AAT. In cystic fibrosis patients, AAT administration induces a fall in neutrophil elastase activity, although the overall bacterial loading in the sputum of those patients is not modified (for review, see Ref. [151]). The delivery of AAT in right amounts and at the right place (diseased versus less diseased areas) has been questioned and still has to be improved in cystic fibrosis patients to gain better efficacy.

Recombinant SLPI has been administered to individuals with cystic fibrosis and results in a decreased neutrophil elastase activity and decreased IL-8 expression at the epithelial surface of the lung [152]. Safe delivery of Elafin has also been reported in healthy individuals (Proteo Biotech AG), but phase II reports have never been reported yet. For both natural protease inhibitors, local delivery might be important to establish therapeutic benefits, and in the lung, such delivery has been proven to be challenging.

Neutrophil elastase inhibitors have been developed to treat lung inflammatory diseases. The molecules developed were mostly small synthetic molecules. All were potent and selective neutrophil elastase inhibitors, and yet, none of these compounds have demonstrated sufficient promises to make it to the market.

These compounds were developed on the assumption that neutrophil elastase was the major driving mechanism of lung inflammation and did not take into account the beneficial effects of neutrophil elastase as an antimicrobial agent. This fact might explain the low efficacy and side effects neutrophil elastase inhibitors have demonstrated in clinical trials.

Bortezomib, a proteasome inhibitor, can rescue CFTR, resulting in the appearance of mature CFTR and functional chloride channels. A reduction of IL-8 expression levels due to the proteasome inhibition was also demonstrated [153]. The effects of Bortezomib in lung inflammatory diseases could be shortly investigated and would bring some new knowledge on the use of proteasome inhibitors. The side effects that this drug might have, in particular on the normal proteasomal degradation, are yet to be studied.

28.5.3 Skin

Several studies point to aberrant expression and enhanced enzymatic activity of kallikreins, in atopic dermatitis [154–157]. Based on that, topical administration of protease inhibitor could represent a new strategy to reestablish skin barrier function and thereby limit antigen entry and inflammation (patent reference WO2005117955 A1). However, no clinical trial using kallikrein inhibitors for atopic dermatitis treatment has been reported yet.

Individuals with the inflammatory skin disease rosacea show high baseline levels of cathelicidin and KLK5 [13]. Interestingly, subcutaneous injection of KLK5 in mice, with a dose corresponding to the amount of serine protease activity that is found in lesional skin of individuals with rosacea, induces an increased proportion of the mature cathelicidin. This was accompanied by erythema and leukocyte infiltration [13]. In addition to its direct antimicrobial effects, LL-37 has the capacity to be both proinflammatory and angiogenic [158]. These properties are not shared by other forms of active cathelicidin in the healthy skin. The reason why KLK5 protease activity and cathelicidin are increased in the skin of rosacea patients is still unknown, but this illustrates the importance of the large web of epidermal KLK proteases in skin inflammation. To date, a clinical trial was initiated to treat patients suffering from rosacea to modulate KLK5 and hCAP18 expression. Patients were treated by topical application of azelaic acid 15% gel to the involved skin, twice daily for 16 weeks. KLK5 and cathelicidin mRNA decreased in rosacea skin after several weeks of this treatment and this correlated with clinical improvement, that is, reduction of erythema and inflammatory papules [159].

Netherton syndrome is a severe genetic skin disease due to the absence of LEKTI inhibitor leading to an uncontrolled kallikrein activity. Two different pathways but both leading to inflammation are triggered in this pathology. First, LEKTI deficiency causes premature detachment of corneocytes from the rest of epidermis. Excessive activity of KLK5 and KLK7 leads to desmosome cleavage in the upper granular layer through desmoglein-1 degradation. KLK5 is a protease processing proelastase-2 into proteolytic active enzyme. In Netherton syndrome skin, elastase-2 hyperactivity participates also to epidermal permeability. Barrier disruption by itself stimulates keratinocyte proliferation, as well as cytokine and

chemokine production (IL-1β, IL8, TNFα, granulocyte macrophage colony-stimulating factor (GM-CSF), and TSLP), which are all major features of skin inflammation [160–162]. In addition, barrier breach causes dehydration and facilitates allergen and microbe penetration and inflammation due to sensitivity of keratinocyte to UV.

Finally, KLK5 also initiates a proallergic signaling cascade, leading to overexpression of the pro-Th2 TSLP, CXCL8, and TNFα cytokines via the direct activation of PAR2 on keratinocytes. This proinflammatory context leads to the development of a broad range of allergic manifestations including atopic dermatitis with elevated serum IgE levels [23,162,163]. However, here again, no clinical trial has started yet to investigate the potential effects of KLK inhibitors.

28.5.4 Joints

Different proteases are determinant to cartilage degradation and inflammation in osteoarthritis. These proteases include metalloproteinases, which degrade not only the cartilage macromolecules and the collagen but also serine and cysteine proteases. From animal studies, and in particular studies performed with genetically engineered mouse models, three proteases appear to be major targets for osteoarthritis treatment: ADAMTS-5, MMP-13, and cathepsin K [164]. Animal model studies have proven efficacy of these protease inhibitors both on progression of structural damage to the joint and on osteoarthritis-associated pain [165–169]. Phase I clinical trials have been completed and did not raise any safety issues for ADAMTS-4/5 inhibitors (NCT00427687 and NCT00454298, www.clinicaltrials.gov). GlaxoSmithKline also recently reported a ADAMTS-5 neutralizing antibody. Therapeutic protocols have not yet been reported for MMP-13 inhibitors, but Novartis has reported three phase II clinical trials testing a cathepsin K inhibitor (NCT00170911, NCT00100607, and NCT00371670). Results from these studies have yet to be posted.

MMPs seem to be important in rheumatoid arthritis, as increased MMP activity was reported from synovial tissues of patients [170]. Only animal models have been used to investigate the potential efficacy of MMP inhibitors, with some level of success [171]. However, here again, the anti-inflammatory effects discovered for some MMPs have lowered the enthusiasm for further clinical studies. A more accurate knowledge on the enzymes and their different cellular origin, together with their precise role in disease processes, is required for MMP inhibition approaches to be successful in rheumatoid arthritis clinics.

Calpain-1 is an important player of neutrophil motility, and the discovery of the recent series of alpha-mercaptoacrylate inhibitors that are able to target regulatory domains of calpain-1 has raised considerable interest in possible therapies. These compounds are now considered as potential future oral therapies for rheumatoid arthritis, but bioavailability still seems to be an issue [172].

As for IBD, inhibition of the immunoproteasome is considered for rheumatoid arthritis, as it plays a critical role in T-cell expansion, cytokine production, and T helper differentiation [173]. In animal models of arthritis, inhibition of the immunoproteasome ameliorated disease symptoms [174]. The unique role of the immunoproteasome subunit low molecular mass polypeptide-7 (LMP7) in

controlling pathogenic immune response and chronic inflammation provides a strong therapeutic rationale for targeting LMP7 in autoimmune disorders such as rheumatoid arthritis.

28.5.5 Pancreatitis

Pancreatic digestive enzymes are stored as inactive precursors in pancreatic zymogen granules. Under physiological conditions, digestive enzyme activation is strictly controlled to prevent autodigestion of the pancreas. SPINK1 is considered to be the first-line safeguard system that can inactivate about 20% of total trypsin activity if trypsinogen is accidentally converted into trypsin in acinar cells. Hereditary chronic pancreatitis is a very rare form of early onset of chronic pancreatitis. Gene mutations of cationic trypsinogen (*PRSS1*), anionic trypsinogen (*PRSS2*), cystic fibrosis transmembrane conductance regulator (*CFTR*), chymotrypsinogen C (*CTRC*), and calcium-sensing receptor (*CASR*) have been identified as being associated with hereditary chronic pancreatitis. In the case of the *PRSS1* gene, pancreatitis can be caused by various molecular mechanisms. Mutations in this gene can lead to inhibition of trypsin degradation, to increased autoactivation of trypsinogen into active trypsin, to disturbance of intracellular transport resulting in colocalization of trypsinogen with lysosomal enzyme cathepsin B, or to triplication and duplication of the copy number of trypsinogen gene. Because the intrapancreatic balance between trypsin and SPINK1/PSTI (pancreatic secretory trypsin inhibitor) activity is important for pancreatitis, mutations in the *SPINK1* gene that affect SPINK1 binding with trypsin are considered to contribute to the onset of pancreatitis. There have been many reports on the association between mutations in the *SPINK1* genes and pancreatitis in patients, since excessive amounts of pancreatic trypsinogen are transformed into active trypsin, leading to activation of other zymogens and autodigestion of the pancreas [175]. Aprotinin was the first protease inhibitor used in clinical trials for acute pancreatitis. However, no benefit was observed when administered either intravenously or intraperitoneally. Numerous clinical trials were conducted using a small chemical compound as serine protease inhibitor such as gabexate mesilate, nafamostat mesilate, and ulinastatin. They have demonstrated a reduction in complications and mortality, but the trials are very small and meta-analysis failed to show benefits of these medications significantly reducing the rate of death in moderate to severe pancreatitis [176–179].

28.5.6 Traumatic Brain Injury

Traumatic brain injury is a major cause of mortality and an important medical challenge. To date, despite improvements in acute intensive care, no specific therapies to ameliorate the effects of traumatic brain injuries are available. The kallikrein–kinin system is a promising therapeutic target for traumatic brain injury as it mediates key pathologic events of traumatic brain damage, such as edema formation, inflammation, and thrombosis. Plasma kallikrein or Factor-XII has an essential role in thrombosis [180]. Factor-XII depletion protected

knockout mice from pathogenic thrombus formation without increased risk of bleeding. Aprotinin is known to inhibit several serine proteases, including plasma kallikrein. A reduced protease activity has been associated with a lower mortality rate [181]. There are also promising results from recent studies in murine models suggesting a therapeutic potential for the serine protease inhibitor C1-inhibitor (inhibition of activated plasma kallikrein) and cystein protease/calpain inhibitor [182,183].

28.6 Conclusions

Protease inhibition has reached the level of "hot topic" for the development of new therapeutic strategies to treat chronic inflammation. Initially considered for cancer treatment or antiviral therapies, and initially focused on large spectrum inhibitors, protease inhibition strategies have evolved and are now embracing the challenge of targeting specific proteases. Several approaches are nowadays conceived: protease inhibition by small synthetic molecules, biological therapies, and natural protease inhibitors' delivery or enhancement are among them. All these strategies aim at reequilibrating proteolytic homeostasis in diseased tissues and are based on the multiple proinflammatory roles described for most proteases (see Figures 28.2 and 28.6). However, a strong need to characterize the proteolytic

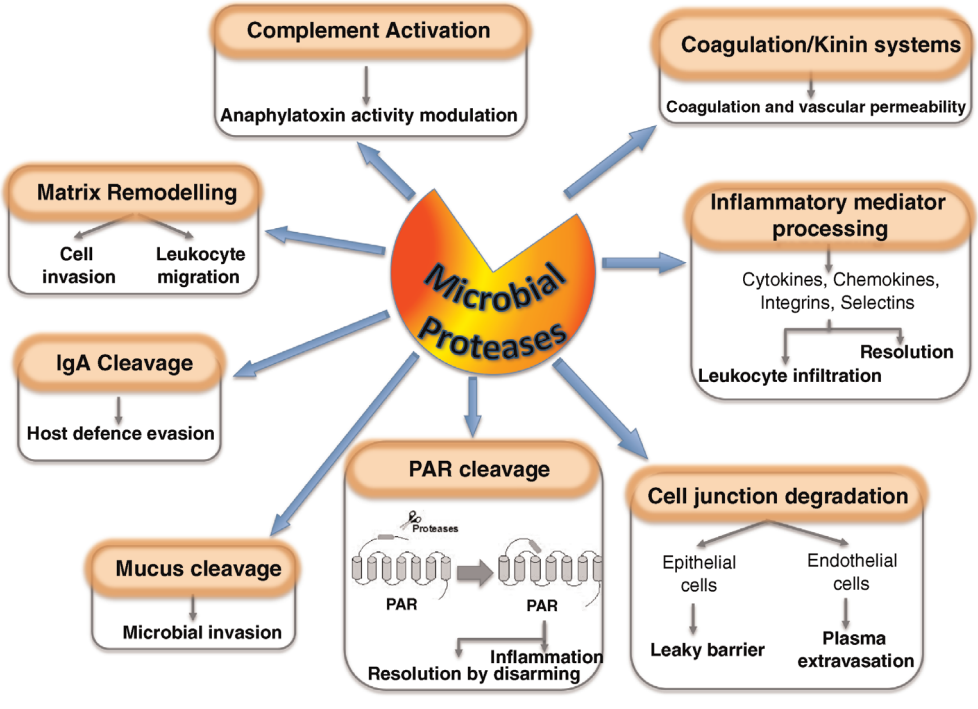


Figure 28.6 Mechanisms of action of microbial proteases at inflammatory sites and their consequences.

profiles associated with chronic inflammatory pathologies, and even within the same pathology between patient's subgroups, has emerged. Such knowledge will first allow determining the role of the identified proteases in pathologies, and based on this, could point to the relevance of considering specific proteases as pharmacological targets. Targeting specific proteases and specific activities in the context of chronic inflammatory disorders should avoid therapeutic failure associated with strong side effects or weak efficacy. Determining the proteolytic profiles associated with inflammatory pathologies should also point to other roles for some proteases, including potential anti-inflammatory effects. Finally, in future studies, by further establishing disease-associated proteolytic profiles, to a given profile, adapted therapeutic strategies could be applied, targeting individual or a combination of proteases.

List of Abbreviations

AAT	alpha-1 antitrypsin
ADAM	a disintegrin and metalloproteinase
AEP	asparagine endopeptidase
CARD	caspase activation and recruitment domain
CASR	calcium-sensing receptor
CFTR	cystic fibrosis transmembrane conductance regulator
CGRP	calcitonin gene-related peptide
CTRC	chymotrypsinogen C
CXCL	chemokine C-X-C motif ligand
EGF	epidermal growth factor
EHEC	enterohemorrhagic *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ENA	neutrophil activating protein
GM-CSF	granulocyte macrophage colony-stimulating factor
HDM	house dust mite
IBD	inflammatory bowel disease
IBS	Irritable bowel syndrome
Ig	immunoglobulin
IL	interleukin
KLK	kallikrein
LEKTI	lympho-epithelial Kazal-type inhibitor
MAP	nitogen-activated protein
MCP-1	monocyte chemoattractant protein-1
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
NET	neutrophil extracellular traps
NLRP	NOD-like receptor pyrin domain protein
NOD	nucleaotide-binding oligomerization domain
Omp A	outer membrane protein A
PAR	protease-activated receptor
RANTES	regulated on activation normal T-cell expressed and secreted
SLPI	secretory leukocyte inhibitor
SPINK	serine protease inhibitor of Kazal type

TFPI	tissue factor pathway inhibitor
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TLR	toll-like receptor
TNF	Tumor necrosis factor
t-PA	tissue-type plasminogen activator
TSLP	thymic stromal lymphopoietin
uPA	urokinase
ZO	zonula occludens

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29

Psychiatric Disorders and Inflammation

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29.1 Introduction

In most psychiatry textbooks, psychiatric disorders are described in terms of alterations in neurotransmitter systems that psychotropic drugs aim at normalizing. All current antipsychotic drugs target the brain dopaminergic system, whereas antidepressants aim to increase deficient serotoninergic and noradrenergic neurotransmission. The skillful use of these drugs has greatly improved quality of life for patients with psychiatric disorders by helping them control their symptoms.

However, symptom control does not equate with cure. Since the introduction of most psychotropic drugs in the 1950s–1960s, no progress has been made in the eradication of psychiatric disorders and the elucidation of their pathophysiology. In addition, the stagnation of drug discovery in psychiatry has forced many pharmaceutical companies to put an end to their investment in research and development. In this gloomy context, some faint rays of hope are slowly emerging, thanks to the progress made in molecular and cellular neurobiology and the knowledge generated by the powerful steamroller of genomics and postgenomics. In a multistage genome-wide association study of about 37 000 patients with schizophrenia and 113 000 controls, expected associations were found between schizophrenia and genes expressed in the brain, including the gene coding for the dopamine receptor D2 (the target of all effective antipsychotic drugs) and several genes involved in glutamatergic neurotransmission and synaptic plasticity [1]. However, strong associations were also identified with genes expressed in tissues with important immune functions, particularly B-lymphocyte lineages. In another genome-wide association study carried out in 60 000 patients with schizophrenia, major depressive disorders, or bipolar disorder, associations between these conditions and multiple immune and neuronal signaling pathways were found [2].

The possible role of immune-mediated events in the pathophysiology of psychiatric disorders is not a totally new story. A long tradition of research into the possible viral causes of schizophrenia has resulted in the discovery of circulating autoantibodies against various neurotransmitter receptors and

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synaptic proteins [3–5]. A modern version of this scenario is the reactivation of human endogenous retroviruses in response to early infection by banal viruses, for example, herpes and influenza viruses [6]. Other scenarios are also possible, including reactivation of latent infection by the intracellular parasite *Toxoplasma gondii* [7].

The positive findings obtained in many studies on the association between inflammation and symptoms in patients with psychiatric disorders have aroused neurobiologists' interest in the expression and action of inflammatory mediators in the brain. Surfing on the same wave, clinicians have been quick to investigate the possible therapeutic action of anti-inflammatory compounds, including nonsteroidal anti-inflammatory agents and biopharmaceuticals. Although these treatments are still far from being curative, much has been learned in the process. The objective of this chapter is to review this literature and examine its limitations.

29.2 Association Studies between Inflammation and Psychiatric Disorders

If inflammation plays a role in the symptoms presented by patients with psychiatric disorders, there should be evidence for associations between symptoms and biomarkers of inflammation. Several recent meta-analyses of the published literature point to such a positive association.

Schizophrenia is a progressive neurodevelopmental disorder, meaning that it has its roots in alterations in early brain development [8]. The possibility that inflammatory processes occurring during development participate in the pathogenesis of schizophrenia has been examined in studies relating increased maternal levels of C-reactive protein (CRP) and tumor necrosis factor (TNF)-α to schizophrenia in adult offspring (reviewed in Ref. [9]). In a prospective study from the Avon Longitudinal Study of Parents and Children, children with high serum levels of interleukin (IL)-6 at 9 years of age were found to have a twofold increased risk for psychotic disorder at 18 years of age, independent of typical confounders such as sex, body mass, and psychological and behavioral problems [10].

A meta-analysis specifically focused on CRP identified eight studies, of which five were included in a pooled analysis. There was a significant increase in CRP in patients compared to controls, although the possible interfering role of metabolic alterations in response to treatment and smoking was not systemically controlled for [11]. An earlier meta-analysis of cross-sectional studies of cytokine alterations in patients with schizophrenia had already identified increases in IL-1 receptor antagonist (IL-1ra), IL-6, and soluble IL-2 receptor (sIL-2r) and decreases in IL-2, with the same limitations as the meta-analysis on CRP [12]. A meta-analysis of 26 cross-sectional or longitudinal studies showed a positive correlation between CRP levels and the severity of positive, but not negative, symptoms of schizophrenia [13]; CRP levels were also positively correlated with body mass index. These immune alterations are already present in medication-naïve first-episode psychosis, as evidenced by highly significant effect sizes for elevated serum levels of IL-1β, sIL-2r, IL-6, and TNF-α [14]. No increase in cytokine levels is apparent

with the repetition of psychotic episodes [13]. These results suggest that symptoms of schizophrenia are associated with inflammation.

The immune features of bipolar disorders are grossly similar to those of schizophrenia. Patients with bipolar disorders have increased levels of sIL-2r, TNF-α, soluble TNF receptor 1 (sTNFr1), sIL-6r, and IL-4, but not IL-6, compared to healthy control subjects [15–17]. Most of the reported alterations occur during manic episodes [17]. Euthymic patients generally have lower levels of TNF-α and sTNFr1 than do manic patients [17].

Most of the literature on cytokines and psychiatric disorders focuses on major depressive disorders. CRP, IL-6, IL-1β, and IL-1ra have repeatedly been found to be associated with depression [18], although the evidence for IL-1 and IL-1ra is far from consistent and that for TNF-α is mixed [19]. Antidepressants reduce the levels of IL-1β and IL-6 but not TNF-α [20]. Reductions in levels of CRP and IL-6 in response to antidepressants were also reported in another meta-analysis [21]. The differences in sIL-2r, TNF-α, and IL-1β between patients with major depressive disorders and controls appeared to be more marked in European than in non-European subjects, although there was no clear explanation for this finding [22]. A systematic search for the causes of heterogeneity between studies revealed an influence of the mode of definition of depression and the presence of comorbid somatic pathologies: differences in IL-6 levels between depressed subjects and healthy controls were maximized when patients were diagnosed according to Diagnostic and Statistical Manual of Mental Disorders Fourth Edition criteria or when melancholic features corresponding to a more severe form of depression were taken into consideration [23]. Smaller effect sizes for IL-6 were detected when patients had cardiovascular disease or another comorbidity such as Parkinson's disease or fibromyalgia. High body mass index was associated with higher effect sizes for IL-6, whereas age, sex, and smoking status had no apparent effect [23]. Longitudinal studies of the associations between biomarkers of inflammation and depression confirmed the robustness of the association with CRP compared to IL-6 [24].

In addition to meta-analyses that assess the current status of a hypothesis on the basis of results that are available in the literature, cumulative meta-analyses evaluate the extent to which further studies are unlikely to change the already-reached conclusions. In a cumulative meta-analysis, the results from individual studies are added chronologically and the aggregate effect estimate is recalculated after each study is incorporated. An analysis of this type has provided further support for a robust association between elevated IL-6 or CRP levels and depression, with little supplementary information resulting from studies on this topic carried out after 2006 for IL-6 or 2012 for CRP. In contrast, the data available on TNF-α and IL-1β were not consistent enough to confirm or refute the hypothesis of an association with major depression [25].

In summary, much evidence exists to suggest an association between inflammation and psychiatric disorders that is independent of diagnostic category. It is certainly remarkable that this association is apparent despite the heterogeneity of the studies and their relatively low methodological quality. Most of the studies are cross-sectional and lack consideration of the possible role of confounding factors such as age, obesity, lifestyle factors, comorbidities, and current medication. In

addition, serum or plasma cytokines were measured without any consideration being paid to the sensitivity of the assays and the fact that the cytokines are not hormones but paracrine or autocrine communication factors. This certainly explains why only circulating inflammatory biomarkers such as CRP and IL-6 provide reliable data.

Whether inflammation is another reflection of psychiatric disorders or a contributing factor to the disease process is not always clear, as most association studies are cross-sectional rather than longitudinal. The few longitudinal studies that have been carried out reveal that the two possibilities coexist. Repeated measures of inflammatory markers and mental disorders in an adult occupational cohort, the Whitehall II cohort study, revealed that subjects with high IL-6 had a greater likelihood of common mental disorders (mainly major depressive disorders and dysthymia, on the basis of response to the General Health Questionnaire) 6–11 years later and higher odds of new-onset mental disorders 11 and 16 years later [26]. There was no evidence of sex difference in this relationship, and it was little affected by adjustments for acute inflammation, obesity, smoking, and drug treatments. As emphasized by the authors, these findings support the hypothesis that persistently elevated levels of IL-6 contribute to the development of common mental disorders.

In the Avon Longitudinal Study of Parents and Children mentioned earlier in this section, children with elevated IL-6 levels at 9 years of age had a higher chance of presenting with depression and psychotic experiences at age 18 years, meaning that inflammation was a nonspecific predictor [10]. It is important to note that there is also evidence that mental disorders are predictors of inflammation. In the prospective population-based Great Smoky Mountains Study covering children in the community aged 9–16, 19, and 21 years, cumulative depression episodes predicted later CRP levels, whereas early CRP did not predict later depression [27]. These findings indicate that depression can promote inflammation. However, there is also the possibility that the inflammatory status underlying elevated CRP levels at an early age favors the development or reoccurrence of depressive disorders later in life, although this possibility was not tested [28].

29.3 Mechanisms of the Association between Inflammation and Psychiatric Disorders

Once inflammatory cytokines were cloned and characterized, it was not long before they were found to be expressed and active in organs other than those of the immune system – in particular, in the brain. For instance, IL-1β was quickly identified as one of the endogenous pyrogens that are released by activated monocytes and macrophages and that act on the brain during an infection to alter the set point for temperature regulation, resulting in the mounting of a fever response [29]. IL-1β was also demonstrated to be able to activate the hypothalamic–pituitary–adrenal axis and induce the stress response to infection described by Hans Selye several decades ago [30,31]. In the same manner, IL-1β and TNF-α were demonstrated to be responsible for behavioral alterations that develop in ill individuals during an infection [32,33]. This so-called “sickness

behavior" response is seen at the onset of febrile infectious diseases, when sick individuals are lethargic, depressed, and anorexic. They frequently isolate themselves from the rest of the group and they rarely engage in environment-directed activities. These drastic changes in behavior are commensurate with the necessity of sparing energy for mounting a fever, and they are part of an adaptive strategy to overcome the disease process [33].

Physiologists who engaged in studying the brain effects of cytokines were soon faced with the question of how cytokines that were supposed to be released in the general circulation during the host response to infection could reach the brain to cause fever, neuroendocrine activation, and sickness behavior. The initial hypothesis, at least in the context of endogenous pyrogens and fever, was that peripheral cytokines act on brain circumventricular organs, such as the organum vasculosum laminae terminalis, where the blood–brain barrier is leaky [34]. There, cytokines were thought to induce the synthesis and release of prostaglandin E2, which can easily penetrate the brain because of its lipophilic nature and then act on thermosensitive neurons in the hypothalamus. However, this hypothesis was invalidated by subsequent findings showing that (i) peripheral cytokines induce the synthesis and release of cytokines in the brain, as demonstrated initially by bioassays and confirmed subsequently by molecular biology techniques and immunohistochemistry [35–39], (ii) cytokine receptors are expressed in the brain [40–43], and (iii) antagonism of brain cytokines abrogates the brain effects of peripheral cytokines [44]. A major step forward was the demonstration that cytokines induced in the brain in response to peripheral cytokines are mainly produced and released by meningeal and perivascular macrophages and microglial cells within the brain parenchyma [39]. Astrocytes can also play a role, for instance, by producing IL-6 in response to microglial IL-1β and TNF-α [45].

If peripheral cytokines are able to induce the synthesis and release of the same cytokines in the brain, communication pathways must exist between the immune system and the brain that enable brain cells to monitor peripheral cytokines and respond to them in a coordinated manner. Circulating cytokines cannot play this role, because their release in the circulation corresponds to an overspill and they are not geared to act as endocrine factors (with the exception of IL-6, which targets the liver for the synthesis of acute-phase proteins). In contrast, afferent nerves that innervate the site of the body in which the inflammatory response takes place can play this role, because their activation is responsible for the symptoms of pain and heat – two of the four cardinal features of inflammation.

The neural pathway hypothesis was submitted to test by nerve sections. The initial experiments were carried out in vagotomized rodents, as studies of the brain effects of cytokines are usually based on intraperitoneal injections of lipopolysaccharide (LPS) or recombinant cytokines, and most of the sensitivity of the visceral cavity is conveyed to the brain by vagal nerves. Subdiaphragmatic vagotomy was found not only to block hyperalgesia and sickness behavior induced by LPS and IL-1β [46–49] but also to abrogate the induction of IL-1β expression in the brain [50] as well as the expression of c-Fos, the product of the early activation gene *c-fos*, in the brain neurocircuitry recruited by systemic inflammation [51]. The results of vagotomy experiments are not always easy to interpret, however, because this procedure impairs several important

physiological functions that can affect behavior. A more rigorous demonstration of the role of vagus nerves in the sickness response to systemic inflammation was achieved by reversible inactivation of the dorsal vagal complex, the brain area in which vagal sensory nerves terminate [52]. This was done by injecting a local anesthetic into the dorsal vagal complex of LPS-injected rats. This treatment abrogated LPS-induced decreases in social interactions and c-Fos expression in key immune response areas of the brain. Experiments directly comparing sickness-behavior and fever responses to LPS in vagotomized rats showed that the neural pathway of immune to brain communication is more important for the sickness-behavior response to LPS than for the fever response [53,54].

Transporters allowing circulating cytokines to enter the brain have also been described at the level of the blood–brain barrier [55]. Although their molecular nature has not yet been characterized, they are likely represented by cytokine membrane receptors that internalize their ligand. The role of the transporter that enables IL-1α to enter the brain was demonstrated in a very ingenious experiment based on the memory-impairing effects of this cytokine [56]. Human recombinant IL-1α injected intravenously into mice induced sickness behavior and at the same time impaired the retention of an avoidance/escape response learned just before the injection. This effect of IL-1α was mediated in the posterior division of the septum of the brain. Intraseptal injection of a blocking antibody specific to human IL-1α abrogated the memory-impairing effect of IL-1α injected intravenously, demonstrating that the human IL-1α needs to be transported from the blood into the brain to exert its effect.

The emphasis has recently shifted to brain vascular cells as key cells in the transduction of inflammatory signals between the periphery and the brain. In response to peripheral immune stimuli, these cells express receptors for IL-6 and IL-1 and prostaglandin-E2-synthesizing enzymes [57]. Genetic deletion of IL-1 or IL-6 receptors in brain vascular cells abrogated the fever response to LPS [58,59]. The same effect was obtained with blockage of cyclo-oxygenase-2 induction or microsomal prostaglandin E synthase 1 specifically in brain endothelial cells [60]. However, the sickness-behavior response to LPS was not abrogated in these mice, which agrees with the previously mentioned data showing predominance of the neural pathway of immune to brain communication for the sickness-behavior response to LPS [53,54]. In the event that inflammation takes place in the liver, the liver to brain communication pathway involves chemokine (C-C motif) receptor-like 2 (CCRL2)-positive monocytes rolling on the brain endothelium in a P-selectin-dependent manner and possibly trafficking into the brain in response to activation of microglia expressing monocyte chemoattractant protein-1 [61]. Inhibition of the interactions between monocytes and brain endothelial cells was sufficient to attenuate microglial activation and development of sickness behavior [62].

Inflammatory stimuli can also exert their effects on the brain by activating metabolic pathways. This is particularly the case of the tryptophan/kynurenine metabolism pathway, which plays a key role in the development of immune tolerance by depleting the microenvironment of the essential amino acid tryptophan, generating cytotoxic kynurenine metabolites, and favoring the formation of regulatory T cells [63]. The tryptophan/kynurenine pathway is dependent on the

activation of indoleamine 2, 3 dioxygenase (IDO1) in response to interferon (IFN)-γ and TNF-α. Activation of IDO1 metabolizes tryptophan into kynurenine, which is further metabolized into kynurenine metabolites by a complex chain of enzymatic reactions. Tryptophan serves as a precursor in the synthesis of serotonin, and major depressive disorders are supposedly associated with a deficit in serotoninergic neurotransmission. Most of the clinical studies on the role of IDO1 in inflammation-associated depression have been carried out in cancer patients with melanoma or kidney cancer treated with IFN-α or IL-2 and in patients with hepatitis C virus infection treated with IFN-α. In these quasi-experimental models of inflammation-induced depression, symptoms of sickness (fatigue, pain, decreased appetite, and sleep disorders) developed in all patients as early as immediately after the first immunotherapy treatment, whereas depression occurred only in one-third to one half of the patients and took several days to several weeks to develop [64,65]. These findings indicate that depression develops on a background of sickness in the presence of risk factors still to be identified. The distinction between sickness and clinical depression was further solidified by the demonstration that pretreatment with the antidepressant paroxetine blocked the symptoms of depression but not those of sickness in patients with cancer [66]. Immunotherapy-induced depression was associated with activation of IDO1 [67,68], with a linear relationship between the resulting decrease in circulating levels of tryptophan and the intensity of symptoms of depression [68].

In terms of mechanisms, it was initially proposed that activation of IDO1 is responsible for the development of depression in IFN-α-treated patients, because it decreases the bioavailability of tryptophan for the synthesis of serotonin while at the same time generating more kynurenine for the formation of neurotoxic kynurenine metabolites [69], including 3-hydoxy-kynurenine, a potent generator of reactive oxygen species, and quinolinic acid, an agonist of the *N*-methyl-D aspartate (NMDA) receptor. Experimental studies in mouse models of depression confirmed that pharmacological inhibition of IDO1 or genetic deletion of *ido1* abrogated inflammation-induced depression without interfering with sickness behavior [70,71]. Further studies in patients with hepatitis C treated with IFN-α showed that inflammation-induced depression was associated with decreased levels of tryptophan at the periphery but not in the cerebrospinal fluid [72], confirming the results from the brains of mice injected with LPS [71]. An increased formation of neurotoxic kynurenine metabolites was also observed in the cerebrospinal fluid of IFN-α-treated patients [72] and in the brains of LPS-treated mice [73]. Furthermore, blockade of NMDA receptors by ketamine abrogated the depression-like behavior induced in mice by LPS, confirming the role of quinolinic acid [73].

These findings, together with the results of other studies on the transport of circulating kynurenine into the brain during systemic inflammation [74], led to a reformulation of the original hypothesis of a role for IDO1 in the pathophysiology of inflammation-induced depression [75]. It is now thought that the development of symptoms of depression during systemic inflammation is mediated by the transport of excess peripheral kynurenine into the brain and its local metabolism in neurotoxic kynurenine metabolites, including quinolinic acid, which act in synergy with glutamate released by activated microglia [76]. In this scenario,

decreased brain serotoninergic neurotransmission would proceed not from decreased levels of plasma tryptophan but from an immune-mediated activation of the serotonin transporter [77]. Support for this possibility was provided by neuroimaging studies showing a positive correlation between plasma levels of TNF-α and brainstem serotonin transporter availability in neurologically healthy subjects and in patients with psoriasis [78].

Activation of the tryptophan/kynurenine metabolic pathway has also been implicated in the pathophysiology of schizophrenia, but with an emphasis on kynurenic acid rather than on quinolinic acid [79]. The production of kynurenic acid is increased in the brain of schizophrenia patients [80,81]. Kynurenine is metabolized either by kynurenine amino transferases (KATs) that are present in astrocytes or by kynurenine mono-oxygenase (KMO) that resides in microglial cells. Astrocyte activation is associated with a predominance of the first pathway, whereas microglia activation is associated with a predominance of the second pathway that leads to the formation of neurotoxic kynurenine metabolites. Kynurenic acid acts as an antagonist at NMDA receptors and α7 nicotinic acetylcholine receptors that modulates glutamate, dopamine, and cholinergic neurotransmission [82], The enhanced production of kynurenic acid could be a result of astrocyte activation or the consequence of decreased KMO expression. In either case, it leads to cognitive deficits characteristic of those observed in patients with schizophrenia. These cognitive deficits can be characterized in animal models of kynurenic-acid overproduction and corrected by KAT inhibitors.

It is not yet clear how the same nonspecific inflammatory process can lead to divergent effects, in this case activation of the KAT pathway in schizophrenia and the KMO pathway in major depressive disorders. A possible explanation still to be elaborated resides in the neurodevelopmental aspect of schizophrenia that is unique to this disease. It could be speculated that the long-term consequences of an experience of inflammation during early development differ from the shorter-term consequences of an episode of inflammation taking place in an already mature organism.

29.4 Other Causal Factors for the Relationship between Inflammation and Psychiatric Disorders

The high prevalence of major depressive disorders in physically ill patients with chronic inflammatory processes [83] can be explained by the mechanisms described above, that is, activation of immune to brain communication pathways. However, this does not easily apply to cases of major depressive disorders in which there is no evidence of intercurrent inflammatory process. Another set of causal factors for activation of the cytokine network needs to be identified.

Stress is a well-recognized causal factor in psychiatry, especially in the case of major depressive disorders and post-traumatic stress disorders [84,85]. An experience of social threat or adversity can upregulate the innate immune system, even in the absence of any physical or microbial injury [86]. Both early-life stress and stress during adulthood are associated with elevated inflammation. This

phenomenon is sufficiently robust to be studied in the laboratory, using, for instance, the Trier social stress test. In this test, participants are asked to prepare and present an impromptu speech and to perform a difficult mental arithmetic task in front of a nonresponsive intimidating audience. The presence of an audience is a necessary component of the stressfulness of this test, which has been found to lead to stimulated production of TNF-α [87]. In a different study, the inflammatory activity detected in participants who were exposed to the Trier social stress test was found to be associated with greater neural sensitivity to social rejection, as measured by activation of the dorsal anterior cingulate cortex and anterior insula [88].

Multiple pathways lead from stress to inflammation. The most obvious one is represented by the effect of stress mediators on innate immune cells. Increased cortisol levels are well known to be anti-inflammatory rather than proinflammatory. However, under some conditions, endogenously released glucocorticoids are capable of potentiating neuroinflammatory processes by priming microglia in an inflammasome-dependent manner [89]. This occurs in particular when stress takes place well in advance (24 h in the rat) of the immune event.

The involvement of the sympathetic nervous system in peripheral immune responses to stress is not mediated by circulating adrenaline or noradrenaline but by the noradrenergic innervation of primary and secondary lymphoid organs. Stress-induced activation of the sympathetic outflow to the peripheral immune system recruits immature glucocorticoid insensitive myeloid $CD11b^+$ cells in the blood and spleen in rodent models of repeated social defeat [90]. These myeloid cells can traffic to the brain and contribute to the excessive microglial activation that is observed in response to stressors. This results in enhanced anxiety-like and depression-like behavior. The effects of noradrenaline are mediated by activation of β-adrenergic receptors.

Bacterial translocation and alterations in the microbiome can also contribute to the inflammatory response to stress. Studies in laboratory animals indicate that exposure to stressors can significantly change microbial populations residing in the gastrointestinal tract and in particular reduce the relative abundance of bacteria in the genus *Bacteroides*, while increasing the relative abundance of bacteria in the genus *Clostridium* [91,92]. This effect of stress on the gut microbiome is not without consequence: exposure of antibiotic-treated mice to repeated social defeat abrogated the increased inflammatory response normally displayed by these animals [92]. Bacterial translocation has been proposed to drive inflammatory responses, oxidative and nitrosative stress, and autoimmune responses directed against neoantigenic determinants generated by oxidative and nitrosative stress that are present in depressed subjects [93,94].

29.5 Relations between Inflammation and Neuropathology

Despite the large number of studies published on the neuropathology of schizophrenia, there is little consensus among neuropathologists on the main histologic features of the brains of patients with schizophrenia. The wide distribution of patchy, fibrillary gliosis in the brains of these patients was interpreted to suggest

the occurrence of a low-grade inflammation process [95]. The development of neuroimaging approaches allowing acquisition of volumetric measurements of specific brain areas and assessment of structural and functional connectivity between brain areas has greatly facilitated the task of pathologists, as correlations between gray-matter and white-matter pathology and neuroinflammation can now be studied *in vivo*. In a systematic review of the existing neuropathological and neuroimaging studies, Najjar and Pearlman reported consistent evidence of an association between schizophrenia and microglial activation, particularly in white-matter rather than gray-matter regions [96]. In these studies, positron emission tomography was used to measure microglial activation by increased binding of the peripheral benzodiazepine receptor ligand PK11 195. Microglial activation mainly occurred near dystrophic and apoptotic oligodendrocytes, demyelinating axons, and swollen and vacuolated astrocytes. In contrast to what is seen in neurodegenerative disorders, astrogliosis is absent in the brain of patients with schizophrenia. The main feature appears to be oligodendroglial pathology that leads to impairment of myelination and limitation of normal development of white matter.

White-matter pathology is also observed in major depressive disorders, although the evidence is much more scarce. This anomaly consists of decreased oligodendrocyte density and function and is particularly apparent in the prefrontal brain region. However, these findings could be biased by the old age of the patients who have been examined and the occurrence of vascular lesions and impaired cerebral blood flow [97]. Major depressive disorders are also associated with reductions in the volume of brain areas such as the prefrontal and anterior cingulate cortices and subcortical structures such as the hippocampus, amygdala, hippocampus, and basal ganglia [98–100]. These alterations are thought to reflect dendritic atrophy, decreased neurogenesis, and impaired neurotrophic function. Activation of the KMO branch of the kynurenine pathway as measured by circulating levels of kynurenine metabolites was found to be associated with decreased striatal, hippocampal, and amygdalar volumes in patients with major depressive disorders [101–103]. These findings likely reflect inflammation-driven neurotoxic events in the brains of these patients.

29.6 Conclusions

Evidence for a possible role of low-grade inflammation in the pathophysiology of psychiatric disorders has been steadily accumulating over the years. Whether the inflammatory response is primary or secondary to the brain alterations that occur in patients with psychiatric disorders is still uncertain. Preclinical and clinical studies of the brain effects of inflammatory mediators have revealed that the brain is programmed to respond to these signals by switching on a hard-wired sickness-behavior response that enables the ill individual to cope with the constraint of the inflammatory response on the organism physiology. Whether psychiatric disorders correspond to alterations in the functioning of this protective response or in distorted fragments of it remains to be established. In the case of major depressive disorders, there is clear evidence for a primary role of inflammation in driving symptoms that can be found in other disease entities independent of the

clinical diagnosis (e.g., anhedonia and fatigue). However, it is still too early to ascribe specific symptoms to inflammation and to use this feature as a guide to personalized treatment in psychiatry. Progress in this field will be possible only when psychiatrists pay more attention to dimensional aspects of psychiatric disorders and their relationship with alterations in well-characterized neurobehavioral processes.

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30

Complement System

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30.1 Introduction

The complement system, originally described as defense system limited to pathogen removal, is now an acknowledged critical driver of inflammation. Sensing of pathogens or other irritants leads to complement activation with generation of complement activation products and subsequent engagement of receptors for complement activation fragments on innate immune cells and induction of the acute inflammatory responses and pathogen clearance. However, complement also modulates T- and B-cell responses and, when complement activation is dysregulated, this may contribute to persistent inflammation, which is mostly driven by adaptive immunity. Furthermore, recently discovered new modes and locations of complement activation suggest that the impact of complement in immunity is more extensive than previously thought. There is need to conduct an intensive study to determine the intracellular location of complement activation products and the biological meaning of such data. This chapter provides an overview of the recently defined roles of complement in the inflammatory response, which is rigorously regulated, and appears to be in disarray in certain inflammatory disorders such as autoimmune diseases.

30.2 Pathways of Complement Activation

30.2.1 Complement Activation

The complement system is a critical part of innate immunity and a major initiator of the inflammatory reaction. This evolutionarily old system is required for the detection and removal of invading pathogens that have breached the protective epithelial borders separating the host from the environment [1,2]. The complement system is comprised of over 50 blood-circulating and membrane-bound proteins

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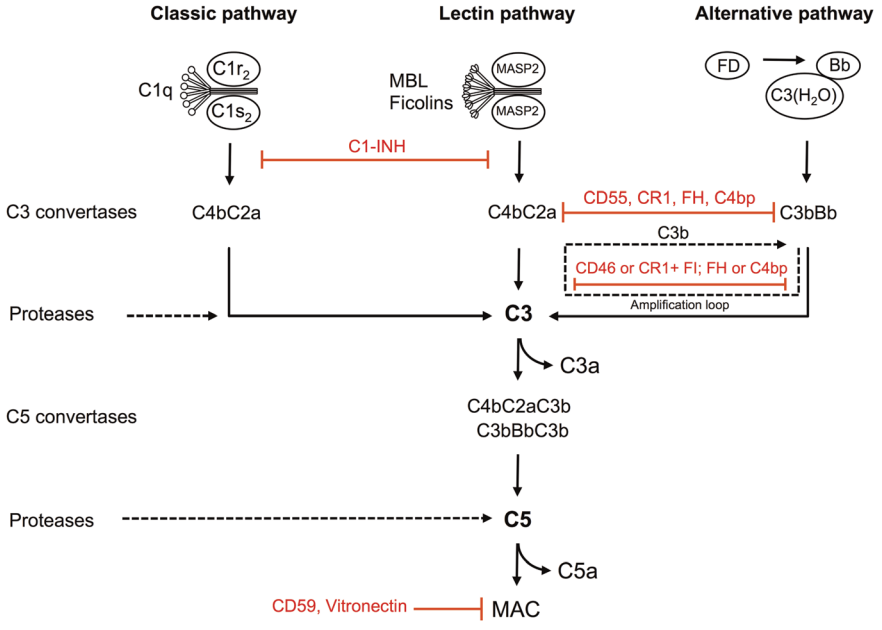


Figure 30.1 Complement activation and regulation. Complement can be activated through the classical, lectin, and alternative pathways with the initial deposition of C3b on a surface also initiating a feedback amplification loop. C3 convertase formation (C4bC2a for the classical and lectin pathways and C3bBb for the alternative pathway) leads to the generation of the opsonin C3b and the anaphylatoxin C3a and subsequent C5 convertase formation (C4bC2aC3b for the classical and lectin pathways and C3bBbC3b for the alternative pathway) generates C5b and the anaphylatoxin C5a, with C5b initiating the formation of the MAC in target membranes. Convertase-independent C3 and C5 activation also occurs via activating proteases. Several fluid phase and cell-bound regulators protect host tissue from unwanted complement deposition: C1 inhibitor (C1-INH) impedes the activity of C1r and C1s and MASP2 and deposited C3b (and C4b) are inactivated by serine protease Factor I and one of the several cofactors (cell-bound CD46 (MCP) and CR1 or fluid phase Factor H and C4b-binding protein, C4bp). Convertases are regulated through disassembly by regulators that have DAA (membrane-bound CD55 (decay accelerating factor, DAF) or CR1, Factor H, and C4bp) and the formation of the MAC is restricted by CD59 and vitronectin (protein S).

and regulators with the effector molecules existing mostly in a precursor state that can be activated rapidly in a cascade-like fashion following activation of the system. Complement activation occurs via three distinct pathways: the classical, the lectin, and the alternative pathway (Figure 30.1) [3,4]. The classical pathway is activated when the C1 complex (C1q, $C1r_2$, and $C1s_2$) binds to the Fc portion of IgG or IgM antibodies that bind to surfaces of pathogens, injured cells, or other antigens, resulting in cleavage of C2 and C4 to form the classical pathway C3 convertase. The lectin pathway is triggered by the recognition of pathogen-derived carbohydrates by mannose-binding lectin (MBL) or ficolins in complex with MBL-associated serine proteases (MASPs), which also cleave C2 and C4, leading to the formation of the lectin pathway C3 convertase. The alternative pathway activation is initiated when C3b-like molecules that are continuously generated in blood in low amounts via spontaneous hydrolysis ($C3[H_2O]$) bind covalently to a target surface and recruit

factors B and D to then form the alternative pathway convertase. The alternative pathway is also called "amplification pathway or loop" since it potentiates the activity of all three pathways [5] (Figure 30.1). C3 convertases cleave the central component C3 into the active C3a and C3b fragments and also "seed" the formation of C5 convertases, which process C5 into the biologically active C5a and C5b fragments. C5b assembles with circulating complement components C6–C9 to generate the pore-forming membrane attack complex (MAC) that promotes direct lysis of microorganisms [6].

It is increasingly acknowledged that convertase-independent cleavage of C3 and C5 by specific proteases is of greater physiological importance than previously thought. For example, several proteases of the coagglutination system [7,8] and of the cathepsin and granzyme families [9–12] process C3 and C5 into bioactive fragments, and these alternative modes of complement activation play key roles in trauma and sepsis [8,13].

30.2.2 Complement Regulation

Activated C3b and C4b (and C3[H_2O]) not only deposit on pathogens but also bind nondiscriminatively to host cells via hydroxyl and amino groups and, therefore, have the potential to induce tissue damage and inflammation [14]. Thus, complement activation is tightly regulated through a variety of complement inhibitors and regulators (Figure 30.1) that prevent uncontrolled complement activation in the fluid phase (no target) or on host cells (wrong target) [15–17]. Much of the control is focused on the alternative pathway amplification loop and involves proteolytic cleavage and inactivation of deposited C3b and C4b (cofactor activity), disassembly of formed C3 and C5 convertases (decay-accelerating activity, DAA), and inhibition of MAC formation and insertion (Figure 30.1) [18]. Interestingly and further discussed in detail below, several complement regulators not only protect host cells from complement attack but also transmit intracellular signals upon activation and participate actively in effector responses of immune cells during inflammatory processes [19].

30.3 Activation Products of Complement

C3, C4, and C5 are homologue proteins of about 200 kDa in size. C3 and C5 consist of two subunits (α and β chains) connected by disulfide bonds, while C4 is composed of three chains (α, β, and γ) [20]. Complement activation induces the proteolytic cleavage of these components at the N-terminus of their α-chains, generating the biologically active C3a, C4a, and C5a and C3b, C4b, and C5b fragments, respectively [1,2]. C3b and C4b (and their further degradation products) are *opsonins*. Following fragmentation of complement proteins, there are often physicochemical associations of certain complement fragments, that can result in new enzymatic activities. Opsonization of pathogens or other targets induces MAC (C5b-9) formation as detailed above and direct lysis but also "tags" these antigens for phagocytic uptake and removal by scavenger cells expressing

C3b- and/or C4b-binding receptors [21,22]. C3a and C5a are of approximately 12kDa size and are called *anaphylatoxins* as the interaction with their receptors, which are expressed in a mast immune cells, are indispensable in mediating the inflammatory reaction [23,24]. Of note, a receptor for C4a remains unidentified and C4a is, therefore, generally not considered an anaphylatoxin.

Although C3a and C5a often elicit similar functions, C5a is more potent and widely seen as the body's most effective chemoattractant functioning in the low nanomolar range [24]. Importantly, C5a-mediated functions are influenced by the amounts of C5a generated as low concentrations of this anaphylatoxin induce migration of myeloid cells to the site of complement activation, while higher concentrations of C5a provoke generation of superoxide, which can be converted to H_2O_2. In addition C5a can cause degranulation in myeloid cells [25]. Once generated, C3a and C5a are rapidly cleaved by several serum carboxypeptidases to C3adesArg and C5adesArg forms that lack the C-terminal amino acid arginine [26]. Initially, this reaction was thought to neutralize, control, and/or fine-tune the proinflammatory actions of the anaphylatoxins but it is now recognized that C3a and C5a and their respective desArg forms can mediate distinct immunological functions [27]. However, in general, most studies show that C5adesArg (lacking the C-terminal arginine) has little biological activity. The desArg form of C5a is generated in plasma by carboxypeptidase B and C5a loses most of its biological activity in the C5adesArg form [28]. It is not clear to what extent carboxypeptidase B is present in tissues.

Although opsonins and anaphylatoxins are considered the most critical among the complement activation products driving inflammatory processes, recent work suggests that sublytic MAC formation is an activator of the NLRP3 inflammasome and contributes substantially to the production of proinflammatory cytokines in activated cells [12,29].

30.4 Receptors for Complement Activation Products

Virtually all immune cells express receptors for complement activation fragments and signals triggered by the activation of these receptors are mediators of the inflammatory reaction.

30.4.1 Opsonin Receptors

Scavenger cells including monocytes, macrophages, and neutrophils express receptors for the opsonins C3b and C4b (or their degradation products iC3b, C3dg, and C3d) and are critical in the removal of opsonized pathogens and other antigens [24]. For example, engagement of CD35 (complement receptor 1, CR1), a large (~200kDa) transmembrane glycoprotein composed of 30 so-called short consensus repeats (SCRs), on neutrophils or macrophages by C3b and C4b deposited onto microbial surfaces induces the phagocytic uptake of these pathogens [30,31], an event that is synergistically supported by C5aR1 activation [32]. CD35 specifically expressed on erythrocytes also mediates "immune adherence clearance" that refers to the ability of CD35 to bind and transport

opsonized immune complexes or other inflammatory particles to the sinusoids of the liver and spleen for removal by resident macrophages (Kupffer cells) [33]. The complement receptors 3 (CR3, CD11c-CD18) and 4 (CR4, CD11b-CD18) are composed of two proteins each and belong to the β2 integrin family of adhesion molecules. They are present on neutrophils, monocytes, and macrophages and are also expressed in low amounts on activated T, B, and NK cells [24,34,35]. CR3 and CR4 bind the inactivated form of C3b (iC3b), and activation of CR3 via iC3b has recently been shown critical to the induction of phagocytosis in neutrophils, monocytes, and macrophages. C1q is not a complement activation product per se but is, aside from being the recognition component of the C1 complex, the main opsonin to specifically tag apoptotic cells for their removal [36]. Different cell surface receptors exist for both the C1q globular head domain (e.g., gC1q-R) and the collagen-like stalk domain (e.g., cC1q-R, also known as calreticulin – C1qRP) [37], and the C1q receptor activation on monocytes and macrophages can stimulate macropinocytosis [38,39].

Importantly, engagement of these complement "opsonin receptors" initiates not only phagocytic uptake of antigens but also additional cell-specific effector responses, contributing to inflammation such as the neutrophil oxidative burst and proinflammatory cytokine production.

30.4.2 Anaphylatoxin Receptors

The anaphylatoxins mediate their effects through engagement of their respective receptors [23]. Whereas the C3aR binds only C3a, the C5aR1 can bind both C5a (K_d~2.5nm) and the desArg form of C5a (K_d~660nm) and the C5aR2 binds C5a with similar affinity to the C5aR1 but binds C5adesArg with a 10-fold higher affinity (K_d~12nm) compared to C5aR1 [40–42]. All three receptors are seven-transmembrane receptors, but only the C3aR and C5aR1 are G protein-coupled receptors [43]. The human C3aR shares 37% sequence homology with the C5aR1 and has a distinctive 175 amino acid insert in the second extracellular loop, while the C5aR1 and C5aR2 share 38% sequence homology [42]. The size of all three receptors ranges from about 42 to 61kDa and displays diversity depending on the cells on which the receptors are expressed (possibly due to different post-translational modifications) [44]. The C3aR has been detected on monocytes, macrophages, neutrophils, eosinophils, and basophils and in low amounts on T cells [24,45,46]. C5aR1 and C5aR2 seem to be expressed in tandem not only on most cells including immune cells of lymphoid and myeloid origin but also on endothelial and epithelial cells and vascular smooth muscle cells [47,48], and a "propensity" for cell surface expression of C5aR1 but mostly intracellular expression of C5aR2 has been reported [49].

C3aR1 and C5aR2 stimulation results in activation of heterotrimeric guanine nucleotide-binding proteins (G proteins) and induction of key signaling events that generally drive (proinflammatory) cell effector functions, such as modulation of cyclic adenosine monophosphate (cAMP) [50], calcium mobilization, and protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3 kinase) activation [51], and, ultimately, activation of mitogen-activated protein (MAP) kinases [21,52]. Because the C5aR2 lacks the structural motifs required for G protein

coupling, it was long considered a decoy receptor, in line with the early observation that C5aR2-deficient mice have increased lung injury in an immune complex-induced injury model [53]. C5aR2's potential to negatively regulate C5aR1-driven signals is still controversial and appears to operate via competition for C5a/C5adesArg binding [54] and/or inhibition of C5aR1-mediated responses through direct interactions with β-arrestin, which inhibits G protein binding to receptors [49]. In contrast to suggestions that C5aR2 functions to reduce inflammatory injury, especially after immune deposition of IgG immune complexes, according to a recent report [55] dealing with acute lung injury (ALI) caused by airway deposition of IgG immune complexes, C5a of LPS shows strikingly reduced ALI intensity in either C5aR1$^{-/-}$ or C5aR2$^{-/-}$ mice. This suggests that engagement of both receptors is necessary in "full-blown" ALI. However, an increasing body of work suggests distinct and specific roles for C5aR2 with clear proinflammatory functions as defined in C5aR2$^{-/-}$ mice that show strongly attenuated house dust mite (HDM)-induced asthma [56]. Blockade of C5aR2 also has led to increased survival in sepsis [57] and, contrasting with earlier observations, reduced lung injury in an immune complex-based model performed by another group [48]. These discrepancies remain unresolved, but a picture emerges where C5aR2 function is complex, possesses potential plasticity, and is likely dictated by the cell type on which C5aR2 is expressed, the nature of the antigen encountered, and the general microenvironment in which immune cell activation occurs.

30.5 Role of Complement in Innate and Adaptive Immunity

The normal inflammatory response driven by complement-activated innate immune cells is characterized by a rapid (acute) onset, a fairly short "effector phase," and a clear termination point that is accompanied by the initiation of tissue repair pathways; thus, the acute inflammatory response is normally mild and self-limiting. However, in situations where the inflammation-causing insult is not eliminated or where complement activity is dysregulated, inflammation may persist and can become chronic. Chronic inflammation is characterized by a more prominent adaptive immune response and complement activation fragments can directly support B- and T-cell activation in these settings. A recent review describes in-depth linkages between the complement and the innate immune system [58].

30.5.1 Complement, Innate Immunity, and Acute Inflammation

Anaphylatoxins and complement-derived opsonins orchestrate the rapid arrival and activation of innate immune cells to the site of insult (Figure 30.2), events that are accompanied by the visual signs of inflammation: redness, swelling, and heat, along with pain. Neutrophils and mast cells are among the first responders during inflammation [59] and C3a induces migration of mast cells and eosinophils to inflamed tissues [60], while specifically C5a functions as key chemoattractant to recruit neutrophils, mast cells, monocytes, macrophages, and eosinophils to the

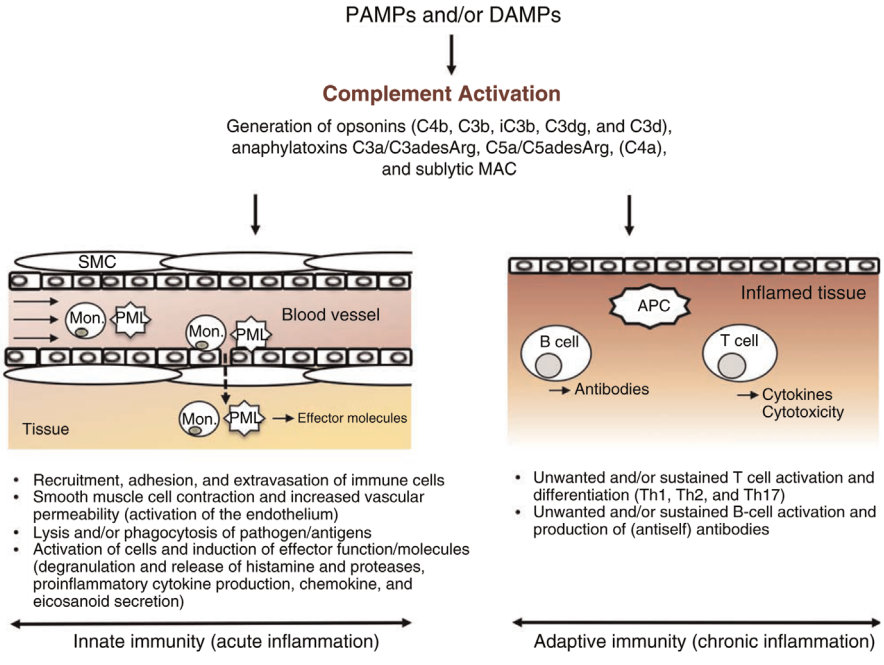


Figure 30.2 Key contributions of complement activation to the innate (acute) and adaptive (chronic) inflammatory response. Model summarizing the key effects of opsonins and anaphylatoxins on immune and endothelial cells during the acute inflammatory response and on T and B cells during chronic inflammation. Other complement activation fragments contributing to inflammation and mentioned in the chapter (e.g., C1q, CR1, CR3, and CR4) are not shown to keep the schematic focused. Abbreviations: APC, antigen-presenting cell; DAMP, danger-associated molecular pattern; Mon, monocyte/macrophage; PAMP, pathogen-associated molecular pattern; PML, polymorphonuclear leukocyte; SMC, smooth muscle cell.

site of infection [14,61]. However, anaphylatoxin receptor stimulation not only mediates immune cell mobilization and migration but also activates neutrophils, mast cells, and eosinophils and induces the degranulation and release of proinflammatory molecules such as tumor necrosis factor (TNF), histamine, and chemokines (IL-8) from these cells [62–64]. Further, C5aR1 signaling activates the lipoxygenase pathway and arachidonic acid metabolism [65] leading to increased eicosanoid production in neutrophils and monocytes. Moreover, C5aR1 activation is a strong inducer of reactive oxygen species production in neutrophils and mediates killing of phagocytosed bacteria [66]. Both C3aR and C5aR1 activation induces increased expression of adhesion receptors and secretion of proinflammatory TNF and IL-1β in neutrophils and monocytes, and C3aR and C5aR2 activation has been shown to regulate IL-6 production in these cells [63,67]. The "opsonin receptors" also contribute directly to the inflammatory response as CD35 engagement on macrophages induces IL-12 [68], while CR3 and CR4 stimulation increase LPS-induced TNF and IL-6 secretion in monocytes [69].

TNF, IL-1β, IL-6, and IL-12 are central to the inflammatory response, because they are critical activators of innate and adaptive immune cells and of the

endothelium (and induce, sustain, or increase anaphylatoxin receptor expression on many cells). In conjunction with this, activation of C3aR and C5aR1 on vascular endothelial cells induces increased expression of adhesion molecules such as E-cadherin and vascular cell adhesion protein 1 (VCAM-1) [70]. An enhanced expression of chemokines (monocyte chemoattractant protein 1, MAC-1, and macrophage inflammatory protein-2, MIP-2) [71] also occurs resulting in amplified attachment and subsequent extravasation of blood leucocytes into tissue surrounding the vessels. In addition to promoting vascular permeability (which also allows for the penetration of protective antibodies or other larger proteins into the tissue), the anaphylatoxins increase the contraction of smooth muscles in some vessels, thereby leading to increased blood flow and cell movement [72].

Recently, a direct link has been described between C3aR stimulation and the activation of the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3, also known as NALP3) inflammasome, involving monocytes [73]. In addition, sublytic activation of the MAC mediates NLRP3 activation in human lung epithelial cells by triggering release of Ca^{2+} from the endoplasmic reticulum [74], and C1q has been identified as an NLRP3 activator (but also a negative regulator in macrophages [75]) in human retinal epithelial cells. Given the central roles for complement and the inflammasomes in innate immunity, delineating the functional crosstalk(s) between these two systems will likely help to better define the mechanisms regulating inflammation in health and disease.

30.5.2 Complement, Adaptive Immunity, and Chronic Inflammation

30.5.2.1 Humoral Immunity

The complement system is now acknowledged as vital bridge between innate and adaptive immunity [76]: CD21 (complement receptor 2, CR2) is expressed in B cells and follicular dendritic cells (FDCs), two main players in humoral immunity. Engagement of CD21 by iC3b, C3dg, or C3d reduces the threshold for B-cell receptor (BCR) activation by several magnitudes and is required for optimal protective antibody production and antigen shuttling to B-cell germinal centers [77]. Although the underlying mechanisms are not fully understood, CD21 also contributes to the maintenance of B-cell tolerance and memory [77]. Uncontrolled or continued complement activation in chronic inflammation, therefore, adds to unwanted antibody responses that underlie a range of chronic inflammatory diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and so on [78]. Of note, pathologically reduced complement levels are associated with chronic vessel wall inflammation (vasculitis) as a result of decreased complement-mediated clearance of immune complexes and uncontrolled *in situ* immune complex deposition [79].

30.5.2.2 T-cell Immunity

Complement activation products also impact T-cell activation and regulation, either indirectly by influencing the maturation and cytokine production of antigen-presenting cells (APCs) or directly by engaging complement receptors/regulators expressed by T cells [80]. Combined signaling by the C3b/C4b-binding

complement regulator CD46 (membrane cofactor protein, MCP) and the C3aR expressed in $CD4^+$ T cells has been established as a prerequisite for IFN-γ production and human Th1 induction [9,81,82] and dysregulation of CD46-mediated signaling events in T cells have been identified in chronic disease settings including multiple sclerosis (MS) [83] and RA [84]. The role of C3a/C5a/C5adesArg in T-cell immunity has mainly been studied in mouse models and demonstrated that the anaphylatoxin receptors are critical to the induction and regulation of Th1, Th2, and Th17 and natural regulatory T-cell (Treg) responses [85,86]. While C3aR expression is required for Th2 induction in a HDM-driven model of asthma, C5a in general seems to negatively regulate both Th2 responses and the induction and function of induced Tregs [80]. C5a also modulates Th17 cell differentiation by altering the local production of IL-23, IL-6, and TGF-β by dendritic cells (DCs) or macrophages and can either enhance or inhibit Th17-cell differentiation depending on the inflammatory context, and both C5aR1 and C5aR2 contribute to Th17 regulation [85]. Furthermore, C1q, possibly via binding to its receptors calreticulin or C1qRp and CD35 [39,87], regulates T-cell responses mostly in a negative fashion (by reducing cell proliferation and cytokine secretion), suggesting that C1q deficiencies may directly lead to pathologically increased T-cell responses observed in patients with SLE. In sum, the idea that complement not only contributes to acute but also chronic inflammation (Figure 30.2) is now becoming more mainstream, as is the view that complement activation needs to be controlled tightly "in either direction" to prevent inflammatory disease.

30.6 Evidence for Intracellular Activation of Complement

Although the liver is the main source of serum-circulating (systemic) complement [1,2], epithelial cells, endothelial cells, adipocytes, microglial cells, astrocytes, and immune cells can produce complement components [12,88], and complement activation fragments generated locally by cells impact heavily on their respective (effector) functions [12,46]. Specifically, during the cognate interaction between T cells and APCs, both partners not only upregulate C3aR and C5aRs expression but also produce several complement components including C3 and C5 and Factors B and D [46,89,90]. This leads to subsequent C3 and C5 convertase formation on activated T- and APC-cell surfaces and generation of complement activation fragments that then engage T-cell and/or APC-expressed complement receptors (Figure 30.3). Such autocrine complement production impacts the DC maturation and not only mediates upregulation of MHC class II molecules and costimulatory proteins CD40, CD86, and cytokine production but also induces directly Th1 cells [46,91]. Local complement production by epithelial cells contributes to the inflammatory response in ischemia/reperfusion injury after transplantation or myocardial infarction [92], where restoration of blood flow in oxygen-deprived organs induces cell damage mediated mainly by C3a, C5a, and MAC formation. Furthermore, generation of complement activation fragments locally is involved in inflammatory pathologies of the brain and the central nervous system including Alzheimer's disease and multiple sclerosis (MS) [93].

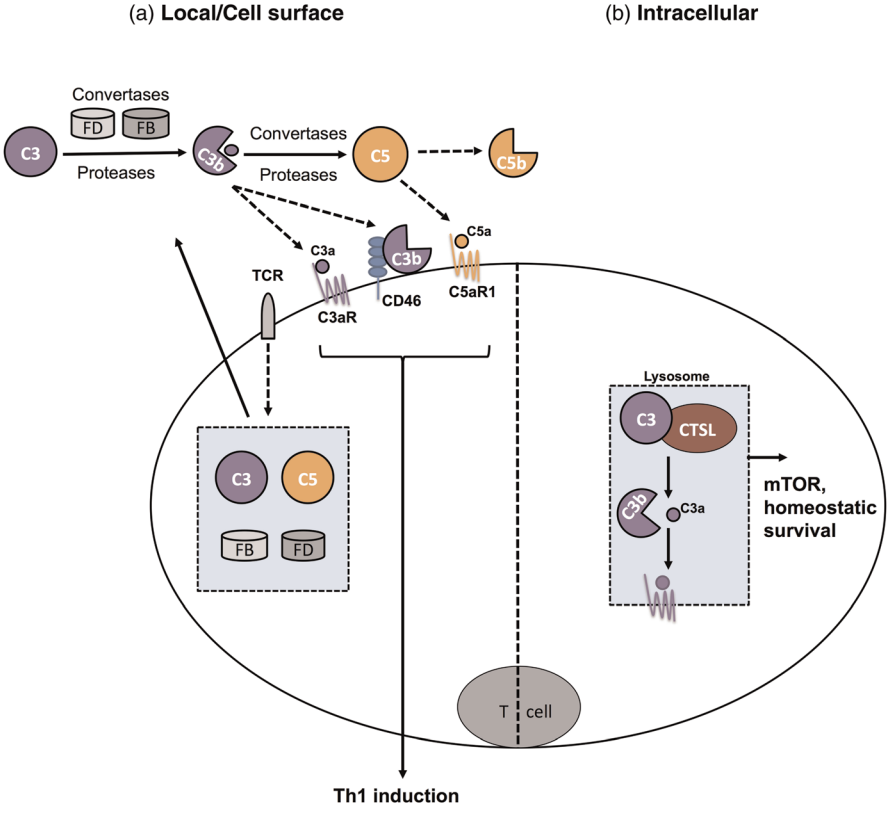


Figure 30.3 Local and intracellular complement C3 activation and function. (a) Locally occurring complement is triggered when the stimulating signal (e.g., TCR stimulation) initiates the generation and secretion of C3, C5, and Factors B and D leading to C3 and C5 convertase formation in the extracellular space and on the cell surface and ultimately the generation of the complement activation fragments C3a, C3b, C5a, and C5b. These bind to their respective receptors on the T cell and induce cellular responses. Note that this local activation mode has also been described for APCs (where it induces maturation and cytokine production). (b) Intracellular complement activation in $CD4^+$ T cells occurs through continuous cleavage of C3 by the protease cathepsin L. The resulting bioactive C3a stimulates intracellular C3aR for tonic mammalian target of rapamycin (mTOR) activation and homeostatic cell survival. Intracellular C3 activation also occurs in a wide range of myeloid and nonmyeloid cells; however, the C3-activating protease differs depending on the cell type (not shown). Abbreviations: CTSL, cathepsin L; FB, Factor B; FD, Factor D.

It has recently been demonstrated that C3 activation occurs intracellularly via cathepsin L-mediated cleavage of C3 and that C3a generated via this "pathway" stimulates intracellular C3aR signaling that then drives a specific cellular response (homeostatic survival) distinct from the engagement of cell surface-expressed C3aR (IFN-γ production) [80] (Figure 30.3). Further, as dysregulation of intracellular complement activation partakes in the sustained T-cell response and chronic inflammation in RA [80], one can envision that changes in the intracellular complement activation machinery will also contribute to the uncontrolled responses of innate immune cells in acute inflammatory settings.

Thus, the roles of complement in the inflammatory response involve complex mechanisms, distinct locations, and are considerably more multifaceted than previously thought.

30.7 Roles of C5a, C5aRs, and C3aR in Inflammatory Disorders

30.7.1 Acute Respiratory Distress Syndrome (ARDS)

This disease was described in the 1960s and is accompanied by bilateral granular densities in lungs as detected by radiographs or CT scans. There are usually severe problems with gas exchange, with hypercapnia (elevated pCO2 in blood), along with reduced pO2 in blood. Lung lesions of ARDS (acute respiratory distress syndrome) are associated with a mortality rate of 40%, characterized by edema fluid in alveolar walls and in spaces, together with large numbers of polymorphonuclear (PMN) and red blood cells (RBCs), and deposits of fibrin along alveolar walls ("hyaline membranes") and in alveolar spaces [94,95]. The integrity of vascular and alveolar epithelial barriers has clearly been breached. It has been known for some time that C3 activation products (C3b, iC3b, etc.) and C5a are present in bronchoalveolar lavage fluids (BALFs), along with the presence of activated factor B [96]. Fundamentally, there are few clues as to what has triggered ARDS, what has caused complement activation, and there are no US Food and Drug Administration (FDA) approved drugs to treat ARDS. Anti-inflammatories (steroids, NSAIDS, etc.) have not been reliably effective.

30.7.2 Sepsis

Sepsis caused by infectious agents (bacteria, viruses, and protozoa) has long been known to be associated with robust activation of complement, resulting in consumptive depletion of both complement proteins and clotting factors [97]. Over the past two decades, the incidence of fatal outcomes in sepsis has dropped from 50–60% to 20%, due entirely to improved supportive care: reduced barotrauma by modifying ventilation procedures that employ reduced inflation pressures, aggressive fluid resuscitation treatment, and early employment of broad-spectrum antibiotics [97]. Evidence for complement activation is reflected in reduced CH50 values in plasma and the presence of C5a in plasma. Not surprisingly, C5aR1 levels in blood PMNs often drop dramatically in septic patients, and the plasma contains cell membrane-defined microparticles that are positive for granule content (from PMNs) together with C5aR1 [98].

In the setting of polymicrobial sepsis in mice, administration of a neutralizing antibody to the middle portion or to the C-terminal regions of C5a greatly improved survival (e.g., from 20 to 80%) and reduced evidence of multiorgan failure [99–101]. To what extent similar interventions in septic humans will improve the outcome of sepsis in humans will depend on current phase II clinical trials in Germany.

It should be noted that there has been a clinical focus on the role of Toll-like receptor (TLR) 4 in sepsis. TLR4 is a well-known receptor for lipid A from bacterial endotoxin (LPS), and there has been an assumption that sepsis in humans might be effectively treated with a compound that blocks TLR4. The FDA-approved drug, Eritoran, is an analog of lipid A, but it lacks one of the four fatty acids of lipid A. It binds with high affinity to TLR4 but does not activate the receptor, competing with the agonist, lipid A, in plasma. Unfortunately, clinical trials in patients with sepsis were cut short after 6 months because of lack of efficacy of the drug [102]. Another clinical trial using activated protein C (Xigris) in patients with sepsis failed to show clinical efficacy and was subsequently withdrawn from use in humans with sepsis [103]. Obviously, there is intense ongoing activity to define a complement inhibitor that can interfere with the activation cascade leading to blockade of C5a or targets essential for its appearance in sepsis.

30.7.3 Acute Lung Injury

This disorder occurs both in humans and in experimental animal models of acute lung injury (ALI), chiefly involving rodents. At a recent consensus conference in Berlin, it was decided to avoid use of the term ALI in human lung disorders, restricting the classification to ARDS [104]. Experimental ALI shares many of the physiological features of ARDS (severe defects in gas exchange in lungs, resulting in reduced arterial pO2 and elevated pCO2). In addition, experimental ALI has been associated with evidence of "consolidation" of lungs, with diffuse bilateral radiographic densities caused by lung edema and PMNs in alveolar walls and spaces. These changes are associated with a series of physiological abnormalities: greatly increased respiratory rate together with increased minute ventilation and inspiratory flow rate. Values for inspiratory and expiratory times and total respiratory cycles fell dramatically [55]. Most of these parameters were reversible in animals surviving ALI.

Experimental ALI was induced in wild-type (WT) mice by airway deposition of IgG immune complexes, recombinant mouse C5a (essentially endotoxin free), or LPS, the latter two being given intratracheally. Lung injury peaked 6–8h later. There was a striking appearance of extracellular histones in BALFs. Histone presence was PMN-dependent and resulted in an intensely phlogistic local environment [55]. Histones appeared to be the most immediate cause of intense lung injury involving both alveolar epithelial and vascular endothelial cells. Histone appearance in BALFs of mice with ALI required availability of caspase 1 and NLRP3 [105]. It seems likely that C5a activated the expulsion of formed neutrophil extracellular traps (NETs) that resulted in the appearance of extracellular histones. Histones also had the capacity to activate the NLRP3 inflammasome [105,106], resulting in IL-1β release from phagocytes, further amplifying the inflammatory response [105]. These features are described in Figure 30.4. The extent to which these events occur in human ARDS seems high. Histones have been found in BALF obtained from patients with ARDS [55]. When $C5aR1^{-/-}$ or $C5aR2^{-/-}$ mice were used, dramatic reduction in lung injury was found, with reduced albumin leak into lungs, varying from 80 to 94%.

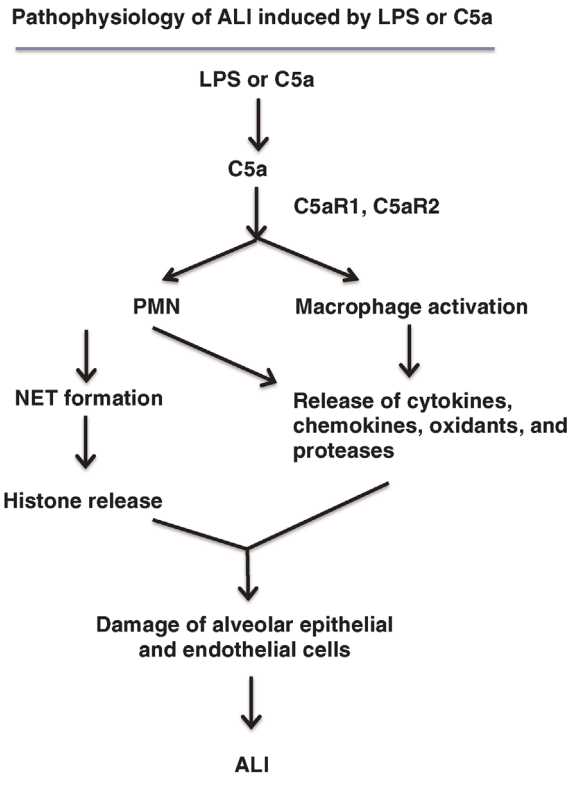


Figure 30.4 Pathophysiology of ALI induced by LPS or C5a. Acute lung injury (ALI) can be induced by airway deposition of LPS or C5a, resulting in an acute inflammatory response in the distal airway (alveolar compartment). Complement C5a itself or after complement activation via LPS results in C5a interacting with its receptors (C5aR1 and C5aR2) on lung macrophages, alveolar epithelial cells, and other cell types results in recruitment of PMNs. The end result is endothelial cell and alveolar epithelial damage, resulting in the loss of the vascular and airway barriers. Activation of recruited PMNs results in their formation of NETs that not only capture or kill bacteria but also release histones that are highly proinflammatory and procoagulative. This sequence of events in ALI often results in severe structural damage, which is usually reversible.

There were accompanying reductions in PMN appearance in BALF from mice lacking C5a receptor. We also found the reductions in levels of extracellular histones in BALF from mice that lacked one of the C5a receptors. When we examined BALF from humans with ARDS, extracellular cellular histones were also present [55]. When recombinant purified histones were injected into mouse airways, intense inflammatory responses ensued, associated with alveolar edema and the presence of PMNs and fibrin deposits, together with thrombi in pulmonary veins, which indicates that, in lung, histones are intensely prothrombotic.

Based on the data described above, the pathogenesis of ALI is described in Figure 30.4. The three initiators of ALI C5a, LPS, and IgG immune complexes triggered the early activation of complement, resulting in generation of C5a. C5a interacted with C5aRs, leading to NET formation in PMNs. This also resulted in

the appearance in BALF of extracellular histones. We recently showed that extracellular histones directly activated the NLRP3 inflammasome, which could be largely responsible for the appearance of both IL-1β and IL-1β in the BALFs from mice developing ALI. In addition, histone appearance apparently resulted from NET formation by PMNs, resulting in the release of extracellular histones [105].

30.7.4 Biological Roles of C3a and C5a Receptors

Substantial effort has been focused on defining the roles of C3a and C5a receptors in a variety of biological responses, most involving some type of inflammatory response. Several strategies have been employed, most using mice with genetic deficiency of the receptor under study. Only one C3a receptor, C3aR, has been identified. In the case of C5a, two C5a receptors have been identified, C5aR1 and C5aR2, the latter previously known as C5L2. While it is clear that C3aR and C5aR1 are surface associated, C5aR2 was originally described on the surfaces of PMNs. A controversy ensued, suggesting that when human blood PMNs were very carefully and gently purified, most of C5aR2 was intracellular. Furthermore, it was suggested that the ligand for C5aR1 was C5a, while C5adesArg was the ligand for C5aR2. There has also been a controversy related to the biological functions of each of the two C5 receptors. At present, those issues remain unresolved.

In the setting of polymicrobial sepsis in mice, participation of both C5aR1 and C5aR2 appears to be required for the full development of the complications of sepsis (the "cytokine storm," development of multiorgan failure, appearance of plasma histones, and compromised survival) [57]. For development of ALI in mice, induced by airway instillation of C5a, LPS, or IgG immune complexes, full development of inflammatory injury requires availability of both C5a receptors. The absence of either C5a receptor resulted in nearly complete blockade of lung injury leading to>90% reduction of injury [55], suggesting that there may be sequential engagement of both receptors.

In terms of allergic asthma in mice sensitized to ovalbumin or recombinant cockroach antigen, aerosol delivery of the antigen results in an effector response characterized by prompt "airway hyperresponsiveness" with airway constriction, followed by peribronchial edema mucous secretion, all resulting in inflamed and dysfunctional upper airways. Earlier studies suggested that global deficiency of the C3aR attenuated this string of events [107], as did the absence of C5aR1. In early studies with C5a null mice, these animals were protected from development of the series of events leading to asthma. The conclusions of these studies suggested that C3a and C5a via their receptors promoted proallergic functions required in the effector phase of asthma development. Together, these studies suggest a regulatory role for C5a and C3aR in asthma, while C5a and its receptor(s) is/are required for "allergic sensitization." C5a and C5aR1 promote the full-blown effector phase of asthma, all of which makes it very difficult for research groups to develop therapeutic interventions targeted at the complement in the setting of human asthma.

30.8 Role of Complement in Selected Human Diseases

30.8.1 Vasculitis

There are many forms of vasculitis affecting arterioles in humans. However, medium-sized arteries and large arteries may also be affected. The inflammation process appears in the walls of vessels, associated with PMNs, fibrin deposits, and sometimes necrosis (referred to as necrotizing vasculitis). In patients with SLE, sera often contain DNA–anti-DNA immune complexes, and deposits of IgG and C3 are frequently seen in the affected vessels [108,109]. There is also emerging evidence in SLE that patients develop autoantibodies to citrullinated histones, which may intensify vasculitis [110]. Lesions of vasculitis also occur in arterioles of individuals who have developed reactivity to drugs, but details of the precise cause of the vasculitis are scant and, importantly, there are no reliable biomarkers of vasculitis except for those that are nonspecific (elevated serum levels of C-reactive protein).

30.8.2 Examples of Complement-related Glomerulopathies in Humans

Listed below are three examples of complement-related glomerulopathies occurring in humans. These disorders are rather common, and each features the presence of complement activation products in the kidney. Figure 30.5 contains three examples of complement-related glomerulopathies (Figure 30.5b–d) that are described below, together with an example of a normal human kidney tissue section (Figure 30.5a).

30.8.2.1 Membranous Glomerulonephritis

Membranous glomerulonephritis (MGN) is a slowly progressive glomerular disease featuring focal segmental glomerulosclerosis (fibrosis and capillary obliteration), occurring in adults between the ages of 30 and 50 years (Figure 30.5b). Most cases of this disorder are of unknown cause. Autoantibodies to the phospholipase (PLA) 2 receptor on podocytes of glomeruli have been identified in 85% cases of MGN [7]. MGN may also be secondary to autoimmune disorders, infections, drugs, heavy metals, and malignant tumors. Glomerular deposits of C3 and C4 are found, along with deposits of the complement-derived membrane attack complex (C5b-9, MAC). These deposits occur on surfaces of podocytes, which play a key role in regulating glomerular filtration of proteins. The presence of these complement activation products appears to be correlated with the inability of podocytes to restrain filtration of albumin and larger proteins in the glomerular filtrate. An autoantibody to the PLA2 receptor on podocytes may cause local complement activation and glomerular dysfunction. At present, there is an interest in using rituximab, which depletes B cells, thereby reducing the levels of the autoantibody [82].

30.8.2.2 C3 Glomerulonephritis

C3 Glomerulonephritis (C3GN) features extensive C3 deposits in glomeruli (in mesangial and subepithelial locations) (Figure 30.5c) [111]. Heavy deposits may occur in which case the process has been referred to as “dense deposit disease.”

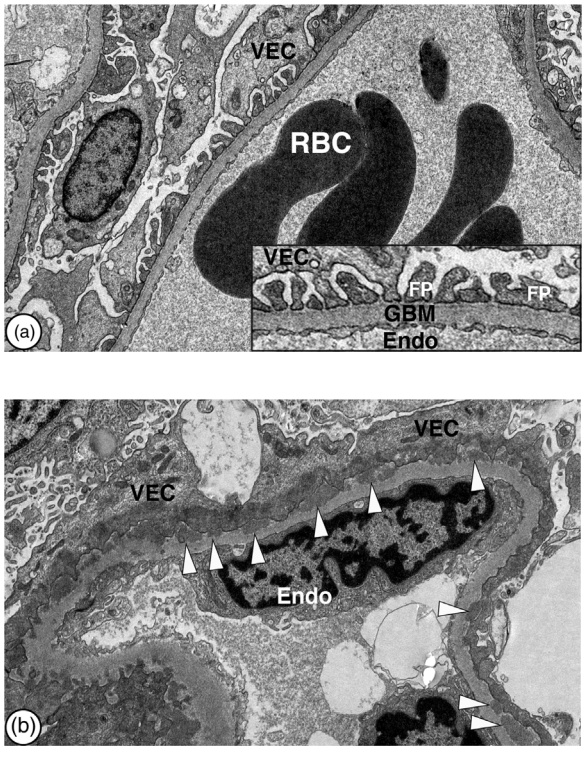


Figure 30.5 Transmission electron micrographs of human renal biopsies. (a) Normal. The renal glomerulus is the site of urinary filtrate production. Composed of three layers: visceral epithelial cells (VEC) or podocytes, fenestrated endothelial cells (Endo), and a thick (~350nm) glomerular basement membrane (GBM). The podocytes have interdigitating cell processes or foot processes (FP) which adhere to the glomerular basement membrane and have specialized intercellular junctions, (slit diaphragms), which restrict the permeability of macromolecules such as large molecular weight plasma proteins. (b) Membranous Glomerulopathy. Idiopathic membranous glomerulopathy is most frequently due to the binding of an autoantibody directed against a VEC antigen, the phospholipase A2 receptor. Binding of the autoantibody, leads to shedding of the antigen into the subepithelial space, between the epithelial cell and the glomerular basement membrane (arrowheads), where the antigen-antibody lattice activates complement. The terminal complement components bind to the VEC cell surface causing sublethal injury resulting in increased protein in the urine and ultimately, increased basement membrane accumulation or sclerosis. (c) C3 Glomerulopathy – dense deposit disease. Seen as a rare complication of hereditary defects in complement regulation or an acquired defect due to C3 nephritic factor, there is a distinctive morphologic abnormality in which an electron dense deposit forms a ribbon-like band in the GBM (arrowheads) with unregulated C3 activation and attendant recruitment of inflammatory cells (WBC) in the glomerular vasculature. (d) Atypical hemolytic uremic syndrome. aHUS is a rare but devastating form of complement-mediated glomerular injury leading to extensive endothelial injury with marked swelling of the endothelial cells that partially or completely exclude the capillary lumen, with consequent fragmenting of RBCs forming schistocytes (SC) and extensive platelet (*) aggregation with resulting thrombocytopenia.

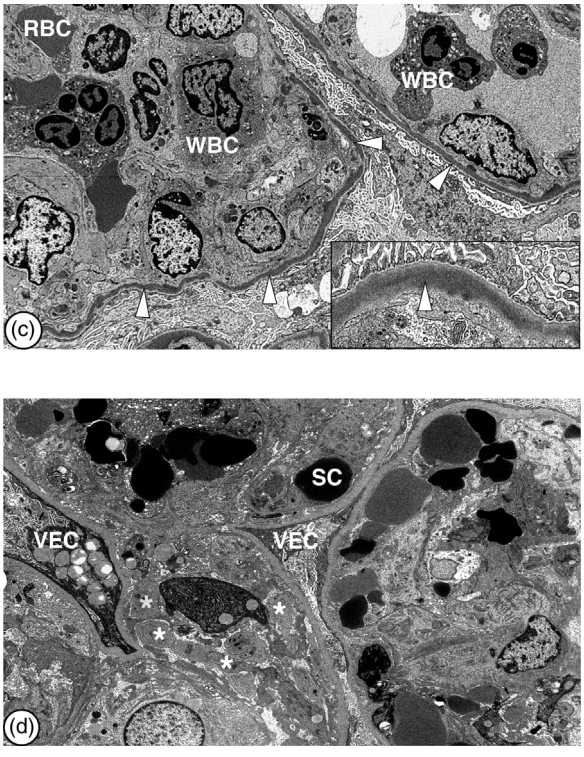


Figure 30.5 (*Continued*)

Causes of these responses are poorly understood, but in some cases defective Factor H (a regulatory factor for the complement system) has been linked to C3GN. C3GN also occurs in patients with persistent infectious diseases (e.g., group A Streptoccocal infections, meningococcal infections, etc.). There may be progressive proteinuria and renal dysfunction. In patients treated with a renal transplant, C3GN develops in >50% of the transplanted kidneys. There is no effective clinical intervention available for C3GN.

30.8.2.3 Hemolytic Uremic Syndrome and Related Disorders

Thrombotic microangiopathies include the hemolytic purpura and hemolytic uremic syndrome (HUS) (Figure 30.5d) [112]. In all cases, inflammatory glomerular injury develops, together with microvascular injury, often with thrombosis, along with glomerular and interstitial tubular injury. Typical HUS often occurs in children following shigella infection. Atypical HUS often develops in children and may be associated with autoimmune diseases involving complement-activating antibodies (antifactor H, antiphospholipid antibody, etc.) that trigger complement activation [113,114]. It has been suggested that the terminal complement pathway, featuring the generation of the MAC, results in acute glomerular injury and vasculitis in atypical HUS. HUS may lead to progressive renal dysfunction and interstitial fibrosis. A mAb to human C5 has been FDA-approved for treatment of patients with HUS [115].

30.8.2.4 Systemic Lupus Erythematosus and Lupus Nephritis

SLE is one of the more common autoimmune diseases known to be associated with consumptive depletion of serum complement (C1q, C3, and C4). At least 50% of patients with SLE develop progressive renal dysfunction [116]. The morphology of lupus nephritis is associated with diffuse proliferation of glomerular mesangial cells, dense matrix deposition in glomeruli together with dense subendothelial deposits of IgG, IgA, and IgM in glomerular capillary walls. Lupus nephritis may advance to glomerular sclerosis (fibrosis) and progressive renal dysfunction. As is well established, patients with SLE often have systemic manifestations featuring inflammation involving skin, joints, kidneys, and the central nervous system. Vasculitis often develops, along with deposits of immune complexes, especially in renal glomeruli. Renal dysfunction is a major problem in patients with SLE and evidence of consumptive depletion of plasma complement often develops. At present, clinical therapy includes immunosuppressive drugs as well as antibodies (rituximab) that suppress B-cell function. Typically, 25% of SLE patients develop progressive renal dysfunction that results in end-stage renal disease.

30.9 Strategies to Block Complement Activation and Complement Activation Products

30.9.1 Compstatin

Compstatin is a cyclic trideca peptide that inhibits the C3 convertase converge, preventing downstream generation of biologically active split products of C3 as well as the downstream C5 and its activation products, C5a, C5b, and the MAC (C5b-9). Compstatin, over the past decade, has been subject to numerous modifications that have improved its solubility in biologic solvents, its inhibitory potency for complement blockade, and its pharmacokinetics, resulting in a water-soluble peptide with subnanomolar binding affinity [117]. In its most recent version, it has a half-life approximately 12h in the circulation of subhuman primates of and appears to be nontoxic. With its primary target being blockade of C3, compstatin blocks C3 cleavage that results in its active fragments, C3a and C3b [108]. The latter is a major opsonic factor promoting phagocytosis of microorganisms bearing C3b on their surfaces. Theoretically, excessive blockade of C3 could compromise the phagocytosis-promoting properties of C3b, thus impairing the protective opsonic-promoting properties of C3. However, such problems can be overcome by applications of compstatin to clinical settings in which there is little evidence that an infectious agent is at play. Clinical considerations currently under assessment include age-related macular degeneration, hemodialysis that is often associated with complement activation, and preservation of organs to be used for transplantation [60].

30.9.2 C1 Esterase Inhibitor

The C1 esterase inhibitor (C1INH) blocks C1r, C1q, and MASP2, thereby reducing activation of the classical pathway of complement. However, it is

acknowledged that C1INH has broader blocking activities as demonstrated by reduced generation by kallikrein of bradykinin, diminished activation of factor XII of the clotting system, and suppressed activation of matrix metalloproteinases [59]. While C1INH has been used in patients with severe sepsis, ischemia reperfusion injury and a variety of other conditions, where there were suggestions of clinical improvement, the FDA has restricted its clinical use in hereditary angioedema [118].

30.9.3 C5 Blocking Antibodies

There is abundant evidence that C5 is a major source of powerful proinflammatory products (C5a, C5b-9) in a variety of conditions (e.g., sepsis, excessive inflammatory responses to infectious agents, ischemia reperfusion injury, hemolysis occurring in a variety of situations, etc.) and it has therefore been postulated that targeting C5 should yield protective outcomes. Several anti-C5 antibodies have been produced in a variety of forms: intact IgG, Fab fragments, and single-chain Fv molecules. The humanized Fab and scFv versions block lysis of chicken RBCs and porcine aortic endothelial cells upon complement activation. With some antibody preparations, generation of C5a and C5b was also blocked [119]. The FDA approved a humanized mAb to human C5 ("Eculizumab") for treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH). These patients present with MAC-induced activation of the clotting system and platelet aggregation in arterioles of the kidney. Further, increased MAC activation in this setting has been connected with reduced blood flow to glomeruli, resulting ultimately in renal dysfunction. To date, the use of the C5 blocking mAb appears to be safe and reports of incidences of increased infections are, somewhat unexpected, rare [120]. However, a major problem with the mAb is its cost. In the setting of PNH, the annual cost of treatment with Eculizumab may exceed $500 000 per year. There are experimental peptides that bind to human C5 and block its activation, but these compounds have not reached the point of clinical trials, and there is a question of whether such drugs might be in violation of patents held by Alexion, the producer of the mAb described above that has been approved for use in PNH.

30.9.4 C5a Antibodies

Based on extensive evidence that sepsis often leads to consumptive depletion of complement (CH50 levels), there is a body of evidence in the case of polymicrobial sepsis in rodents that generation of C5a and its interaction with C5a receptors is linked to adverse outcomes of sepsis (multiorgan damage and failure and mortality) [121]. Development of neutralizing rabbit antibodies (IgG) to C5a led to protection against adverse outcomes in sepsis in monkeys [122], but the number of animals included into the study was too small to generate data with robust statistical significance. At present, phase II clinical trials in humans with sepsis are being carried out in Germany (refer to InflaRx, www.inflarx.com/). The clinical efficacy of this mAb in septic humans remains to be determined.

30.10 Conclusions for Role of C3aR and C5aR

Published data emphasize that C5a and C5aR promote proinflammatory outcomes, especially including activation and recruitment of neutrophils and monocytes to extravascular sites where cellular injury and disruption of extracellular matrix occur. In the case of C5aR2, there continues to be controversy as to whether C5a interaction with this receptor is proinflammatory or anti-inflammatory. It is difficult to reconcile the current literature. With respect to C3aR, it appears that the bulk of the current literature would suggest that its interaction with C3a suppresses inflammatory responses, at least in the setting of asthma triggered by extrinsic antigens and in ischemia reperfusion of the intestine. Clearly, much more needs to be done before we have an understanding of how these agonists and receptors function biologically.

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31

Heat Shock Proteins

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31.1 The Molecules (HSP Families)

In every living cell in every tissue, HSPs carry out important housekeeping functions and often are molecular chaperones. Their protein folding and transport functions make them crucial for the survival of both prokaryotic and eukaryotic cells. Their expression is strongly upregulated under a variety of stress conditions, that is, high/low temperature, oxidative stress, nutrient deficiency, infection, inflammation, and in general conditions that lead to protein unfolding.

Based on their monomeric molecular size, HSPs can be categorized into six families, the s(mall)Hsp (HSPB), DNAJ (Hsp40), Hsp70 (HSPA), Hsp90 (HSPC), Hsp100 (HSPH), and the chaperonins (HSPD/E and CCT), each family having at least one member but often many more [1], as a result of gene duplications during evolution. Also between species, the number of members of a specific family varies widely. Some families also comprise constitutively expressed members that under normal, unstressed conditions contribute to protein transport and folding (e.g., in most eukaryotic cells highly expressed HSPA8 (Hsc70)).

In general, the HSP families are evolutionary strongly conserved and some have members that are immunologically highly cross-reactive with their microbial homologues (see Table 31.1). These families are the subject of the following sections.

Other less conserved HSP families (such as the HSP90 family, the HSP100 family, and the small HSP family, which includes HSP27 and α-B crystallin) have been reviewed elsewhere [2,3].

31.2 Biological Function of HSPs (Chaperones)

31.2.1 The Markers of Cellular Stress

Events in cells under stress are the result of phosphorylation and dephosphorylation-based protein alterations. These lead to a stress-specific

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Table 31.1 Strongly conserved HSP families in mammals and typical microbial homologues.

HSP Family	Family Members in Mammals	Cellular Location	Functions	Typical Microbial Homologues
HSP70 (HSPA)	HSP70 Hsc70 BiP/Grp78 mHSP70/Grp75	Cytosol, nucleus Cytosol, nucleus ER Mitochondrion	Chaperoning nascent polypeptide chains, (re)folding, and transport over subcellular organelle membranes	DnaK (*E. coli*), Ssa1-4, Kar2 (*S. cerevisiae*)
HSP60 (HSPD)	HSP60/cpn60	Mitochondrion	Chaperone activity: (re)folding, assembly of multimeric protein structures	GroEL (*E. coli*)
DNAJ (HSP40)	Hdj1 and Hdj2	Cytosol	Cochaperone for HSP70 activities	DnaJ (*E. coli*), Ydj1 (*Saccharomyces cerevisiae*)
HSP10 (HSPE)	HSP10/Cpn10	Mitochondrion	Cochaperone for HSP60 activities	GroES (*E. coli*)

pattern of protein modifications. Subsequently, as part of a general survival strategy of cells, there will be inhibition of translation initiation and the cell will move toward translation of only mRNA essential for the production of proteins needed for survival [4]. In other words, genes that become activated are meant to assist the repair of cellular damage. Different stressors will lead to the activation of distinct sets of stress-related genes. Above all, upregulated heat shock proteins are the markers of a cell that is responding to stress in an adequate manner.

31.2.2 Molecular Basis of the Heat Shock Response

The transcriptional component of the HSP response is directed by a family of so-called heat shock factors (HSF). HSF-1 is the main HSF. HSF-1 is present normally in a latent monomeric (nonstressed) state and is converted into a DNA-binding trimer when cells are stressed [5]. It has been of interest to elucidate the mechanisms that control the monomeric nature of latent HSF. Besides the originally described intramolecular interactions between amino- and carboxy-terminal leucine zippers that regulate this activity [6], there is now convincing evidence that the primary reason for equilibrium between repressed and active HSF-1 is competition for the chaperoning activity of HSP90, HSP70, and HSP40. HSF-1 seems to remain partially in the latent state due to interactions with these three chaperones. This equilibrium is disrupted by sudden appearance of nonnative client proteins that result from cell stress, whereby the chaperones move into their sequestering activities. As a result, HSF-1 self-associates and forms DNA-binding trimers. The actual binding will occur with the heat shock elements (HSEs) that are located upstream of the HS genes. This leads to elevated transcription of HS genes [7]. Besides HSF-1, the presence and activity of which is conserved in humans, *Drosophila*, *Caenorhabditis elegans*, and yeast, there are three additional HSFs (HSF-2, HSF-3, and HSF-4) present in vertebrates and

these exhibit diverse functional properties during development, stress, and life span [7]. It is known that the trimerization of HSF-1 is impeded during aging, leading to a reduction in the heat shock response and therefore production of heat shock proteins in elderly individuals. It is possible that this in itself contributes to the higher incidences of protein deposition diseases and some inflammatory diseases in later life.

31.2.3 Triggers for the Heat Shock Response

The stressors that initiate the heat shock response can be of both environmental and physiological origin. Besides heat shock, other environmental initiators include heavy metals, UV irradiation, inhibitors of energy metabolism, and certain amino acid analogs. Physiological triggers may include factors involved with growth and development, but more relevant for this chapter, the triggers may also include factors involved with pathophysiological states or diseases. Most relevant in this context are fever, the stress of ischemia, infection, tissue damage, and stress caused by the mediators of inflammation. The latter may include reactive oxygen species (ROS) that at higher concentrations lead to oxidative stress that may damage many cellular components [8] and lipid mediators of inflammation [9]. Proinflammatory cytokines, such as IL1 and TNFα, may lead to the heat shock response in an indirect manner, through their induction of the lipid mediators of inflammation and also ROS [8].

As a result of this, inflammatory diseases will lead to induction of the stress response in cells. Other diseases that induce stress responses are diseases associated with protein folding malfunctions, such as Alzheimer, Parkinson, ALS, Huntington, some metabolic diseases, and cancer (see Figure 31.1).

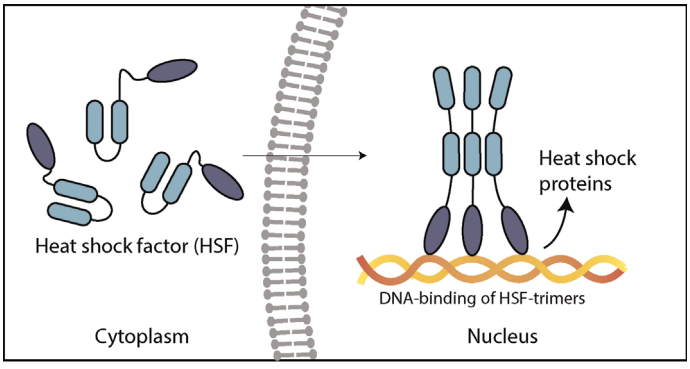


Figure 31.1 Induction of the heat shock response can result from the factor in the environment or factors connected to pathophysiology, leading to trimerization of HSF and subsequent DNA binding in het HSP promoter region.

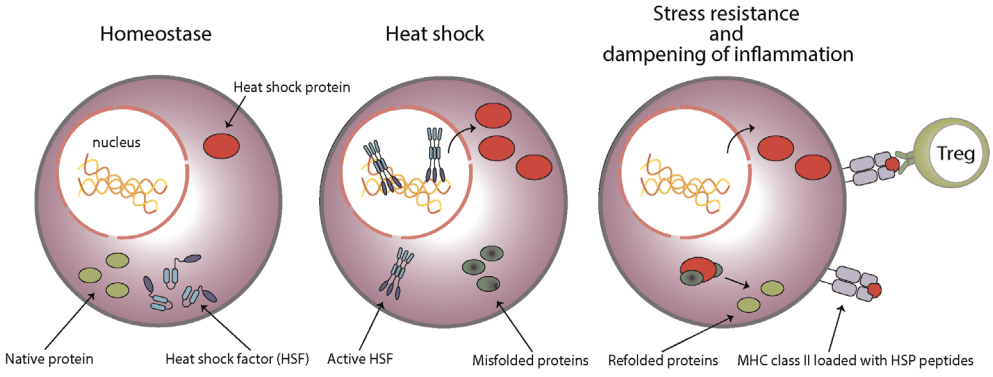


Figure 31.2 The various molecular and cellular events associated with homeostasis, heat shock (stress), and immune regulation.

31.2.4 The Chaperone Functions of HSP Families

The primary function of heat shock proteins is their chaperoning activity. A great deal of pioneering work was done for GroEL, which is the prokaryotic (*Escherichia coli*) equivalent of HSP60 in mammals [10]. GroEL is a folding catalyst, of which the monomers are assembled into a 7-mer-ring structure. Two of the rings stacked upon each other form together a subcellular structure that becomes capped by two GroES (HSP10) molecules at either site. Unfolded or misfolded proteins enter this structure and by the transient interactions that occur with the HSP60 molecules in the inside of the structure, proteins can become properly folded. The activity of HSP60 is ATP dependent. In mammals, the HSP60 molecules are present in this formation in the mitochondria where proteins become refolded after having entered this subcellular organelle. Intuitively, one may well accept the unique degree of evolutionary conservation of HSP60, because of the delicate and complex structure in which HSP60 functions. Only subtle changes in primary structure seem to occur, which leads to the presence of large areas of sequence conservation between HSP60 homologues present in plants, microbes, and vertebrates. Knockout mice do not exist for most HSP due to the vital functions of HSP.

Similar levels of conservation are seen for members of some other HSP families, such as HSP70. The HSP70 family (HSPA1A/B) in conjunction with DNAJB1 (HSP40) are the strongest stress inducible proteins. The human genome sequencing revealed that there are encoded 13 different HSP70 molecules. The majority of them are present in the cytosol and it seems that they have their specialized functions. The HSP70 family chaperones act in an ATP-dependent manner and interact with the proteins that are handed over to them by HSPB and DNAJ molecules. The substrate-binding domains of HSP70 interact only with short linear polypeptides; therefore, the molecule can bind and assist the folding of a large variety of substrates. HSPA8 (Hsc70) is omnipresent in all cell types and is, therefore, held to be an essential housekeeper that takes care of the folding and chaperoning of molecules that are transported over intracellular membranes. Similar activity may have HSPA5 (BiP) in the endoplasmatic reticulum. While

some HSP70 members are exclusively dependent on stress for their presence, some of the HSP70 family members are constitutively expressed in cells.

The HSPH (HSP110) molecules are nucleotide exchange factors for the HSPA family of molecules. HSPC (HSP90) molecules are proteins that function as ATP-dependent molecular chaperones that regulate the signaling conformation and expression of multiple client proteins. Among these client proteins are receptors, such as the corticosteroid hormone receptors and kinases, and also critical inflammatory mediators.

31.2.5 HSP70 as a Prominent Peptide Donor for the MHCII Ligandome

Cells under stress move into a strategy that enables them to economize their exogenous protein needs. Autophagy (or autophagocytosis) (from the Greek auto, "self" and phagein, "to eat") is the basic catabolic mechanism that involves cell degradation of redundant or dysfunctional cellular components through the actions of lysosomes [11]. The breakdown of cellular components promotes cellular survival during starvation by maintaining cellular energy levels. Autophagy allows the degradation and recycling of cellular components. During this process, targeted cytoplasmic constituents are isolated from the rest of the cell within a double-membrane vesicle known as autophagosome. The autophagosome then fuses with a lysosome and its cargo is degraded and recycled. There are three different forms of autophagy, namely, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). In this last form of autophagy, there is a functional role of HSP70 family members. CMA is a pathway that involves the recognition of peptides by a HSP70 (Hsc-70)-containing complex. This means that a protein must contain the recognition site for this complex that will allow it to bind to its chaperone, forming the CMA–substrate/chaperone complex. This complex then moves to the lysosomal membrane-bound protein that will recognize and bind with the CMA receptor. Upon recognition, the substrate protein gets unfolded and it is translocated across the lysosomal membrane with the assistance of the lysosomal HSP70 chaperone. The frequent presence of HSP70 peptides in MHCII molecules has already been documented [12]. Given the role of HSP70 in CMA it may well be no surprise that cell stress forms one of the drivers of the loading of HSP70 fragments into MHCII molecules. A telling example was presented by Dengjel *et al.* [13], when they analyzed the MHCII ligandome of human B cells, stressed by nutrient deprivation, and found multiple HSP70-derived peptides. Among the detected peptides was a peptide already discovered in the mouse as a prominent MHCII-restricted target antigen for anti-inflammatory regulatory T cells [14–16].

31.3 The Immunology of HSPs

31.3.1 Immunodominance of Prokaryotic HSP

Already before the actual discovery of HSP in bacteria, it was known that the immune response (antibodies) against prokaryotic microorganisms was dominated by the response against a molecule with the descriptive name: common

antigen of Gram negatives [17]. Upon cloning of GroEL (HSP60) from *E. coli*, it became clear that this so-called common antigen was HSP60. The driving force behind such immunodominance may be trivial. Given the conservation of this molecule, it is possible that the repetitive and frequent contact with these molecules, as they are present in all bacterial species, leads to a certain level of immunological memory at the level of B and T cells with specificity for these molecules. And understandably, the focus of such responses would then be on the more conserved antigenic epitopes, as these would be encountered most often.

Alternative explanations are, however, also possible. Irun Cohen has argued that, highly evolved as the immune system is, there exists a hierarchy in the way the immune system scans the outside world of antigens [18]. In other words, the immune system has a built-in capacity to put focus on recognition of stress proteins. If this would be true, one could speculate about the evolutionary forces that direct such an immunological focus on these proteins. The interest of the immune system in recognizing HSP must stem from the fact that it is advantageous to respond to HSP. And indeed, in the case of infection, adaptive immune responses directed against microbial HSPs, including responses to epitopes shared among various pathogens, seem to have an effective role in protective immunity [19].

31.3.2 Cross-reactivity with Self-HSP

The high level of immunogenicity associated with microbial HSP is, however, difficult to explain given the high degree of cross-reactivity with self-HSP. Self-tolerance would theoretically put a break on immune responses directed to such conserved molecules. Nonetheless, B- and T-cell-mediated cross-reactivity of the adaptive immune response has been observed in several experimental systems. Immunization with bacterial HSP60 protein or peptides has been found to lead to the production of self-HSP cross-reactive antibodies in models of induced atherosclerosis [20]. However, this was not the case in all studies. For example, comparison of epitope specificities of anti-HSP60 IgG-type antibodies in the sera of healthy subjects, patients with coronary heart disease, and inflammatory bowel disease has, however, not shown a great deal of microbial self-cross-reactivity [21].

Interestingly, some studies documented that self-antigens including HSP, myosin, tubulin, and aldolase with highly conserved structures show similar patterns of binding with natural antibodies and form a well-defined cluster as demonstrated by cluster analysis of immunoreactivity data [22]. The results of the latter study favor the hypothesis that HSP belongs to a subset of highly conserved and immunodominant self-antigens that are the primary target for natural autoantibodies in normal human IgG. A more recent discussion on the role of HSP60 and HSP70 as natural biomarkers and critical antigens in autoantibody repertoires can be found in [23].

For T cells, it was found that immunization with mycobacterial HSP60 induced T-cell responses to several HSP60 T-cell epitopes (peptides), among which there were some very conserved sequences [24]. In this study on inbred Lewis rats, there were nine major histocompatibility complex class II-restricted epitopes

recognized in mycobacterial HSP60. Of these, there turned out to be one, covering the 256–270 sequence, which primed for cross-reactive T-cell responses to the corresponding region of rat HSP60. Interestingly, this peptide containing the 256–270 epitope was the only one able to confer protection against adjuvant-induced arthritis. Similarly, administration of a T-cell line specific for this epitope protected against this disease. Preimmunization with the 256–270 epitope induced T cells that not only responded to heat-shocked syngeneic antigen-presenting cells but also protected against CP20961-induced arthritis, indicating that activation of T cells, recognizing an epitope in self-HSP60, can protect against arthritis induced without mycobacteria [24].

Also for HSP70 T-cell cross-reactivity was observed. A conserved mycobacterial HSP70 sequence was found to be immunogenic and to induce T cells that cross-reacted with the rat homologue sequence. Upon analysis of cytokines produced by these peptide-specific T cells, high IL-10 production was found, as was the case with T cells responsive to whole HSP70 protein. Nasal administration of this peptide was found to lead to inhibition of subsequent adjuvant arthritis induction. The data presented here shows the intrinsic capacity of conserved bacterial HSP to trigger self-HSP cross-reactive T cells with the potential to downregulate arthritis via IL-10 [25].

In a more recent study, we have discovered a conserved T-cell epitope in mycobacterial HSP70 in Balb/c mice. Also in this case, the peptide (called B29) synthesized on the basis of this epitope sequence, induced T-cell responses that cross-recognized multiple homologous peptides present in endogenous HSP70 family members [14]. Interestingly, this peptide was found to trigger the development of regulatory T cells (Tregs) and the transfer of such induced Tregs into naïve recipient mice showed the *in vivo* activation of the cells. Apparently, the cross-recognition of the endogenous HSP70 homologues led to their activation. One of the induced markers was lymphocyte activation gene 3 (LAG3) and selection of the LAG3 positive cells by cell sorting produced a population a Tregs that at the extremely low number of only 4000 cells suppressed inflammation upon transfer.

31.4 HSPs Protect in Models of Inflammatory Disease

First observations on the role of HSP in inflammatory disease models were made in the model of adjuvant arthritis in rats. In this model, disease is induced with heat-killed mycobacteria in oil, and Holoshitz and coworkers had developed mycobacteria (*Mycobacterium tuberculosis*) specific arthritogenic T cell lines and clones by repeated Mt stimulations *in vitro*. Depending on the conditions, the lines were also capable of protecting the animals against induced disease upon *in vivo* transfer. The latter observation was the basis of a series of studies that led to the development of the so-called T-cell vaccination as a possible therapeutic approach for autoimmune diseases. This approach has progressed into the phase of clinical trials, in multiple sclerosis, for example. T-cell vaccination employs a classic vaccine strategy of injecting an attenuated form of the disease-causing agent – in this case T cells – that have been selected and expanded from patients

and then reinjected after irradiation to induce protective immunity. This anti-T-cell immunity consistently results in selective deletion or regulation of the targeted pathogenic T cells *in vivo* [26].

Although in the above-mentioned T-cell-induced adjuvant arthritis some fractionation of mycobacterial antigens was done, there was no solid clue on the precise nature of the mycobacterial antigen that was recognized by the cells. By mere coincidence, the antigen was discovered to be HSP60 when molecular cloning of the first mycobacterial genes in *E. coli* was done and one of the successfully cloned proteins was seen to stimulate the cells [27]. When the same cloned protein was found to protect rats against adjuvant arthritis, it became clear that an interesting relationship between HSP and inflammatory disease could exist.

Induction of disease with the HSP60 molecule itself was, however, not possible. And epitope mapping with an overlapping series of HSP60 peptides showed that the arthritis producing T cells were recognizing a nonconserved (180–188) epitope of mycobacterial HSP60. Although a certain degree of molecular mimicry was found for this epitope with cartilage proteoglycans [28], which could possibly explain the arthritogenic nature of the cells, there was no evidence for any cross-recognition of the endogenous mammalian HSP60 associated with the adjuvant arthritis cell lines and clones. Thus, although seminal observations around HSP and inflammation were made in the adjuvant arthritis model, the mechanisms of HSP-mediated dampening of inflammation were elucidated in other experimental models (see Section 31.6).

Nonetheless, as the anti-inflammatory protective effects of HSP were attributed to induction of self-homologue cross-reactive T-cell responses, the question was raised as to whether this protective effect could be extended to other highly conserved and immunodominant microbial antigens with mammalian homologues. Therefore, we immunized Lewis rats with conserved bacterial antigens (superoxide dismutase, aldolase, GAPDH, and HSP70). Although all antigens appeared highly immunogenic, we only found a protective effect in experimental arthritis after immunization with bacterial HSP70. The protective effect of HSP70 was accompanied with a switch in the subclasses of HSP70-specific Abs, suggesting the induction of Th2-like response. The most striking difference between immunization with HSP70 and all other immunodominant antigens was the expression of IL-10 found after immunization with HSP70. Even more, while immunization with HSP70 led to antigen-induced production of IL-10 and IL-4, immunization with aldolase led to increased production of IFN-gamma and TNF-alpha. Thus, the protective effect of conserved immunodominant proteins in experimental arthritis seems to be a specific feature of HSP. Therefore, HSP may offer unique possibilities for immunological intervention in inflammatory diseases [29].

Immunomodulation with other HSP, such as glucose-regulated protein 96 (gp96), was also documented in the model of NOD diabetes in mice and in MBP or proteolipid protein-induced EAE [30]. Although transfer experiments showed that the observed protection was T-cell mediated, there was no evidence for a role of Treg since the active population was not in the CD25+ T-cell population.

Thus, altogether HSPs have many biological functions, including the signaling of inflammatory stress and the induction of regulatory control through the adaptive immune response.

31.5 HSP as Inflammatory Mediators

31.5.1 HSP are DAMPs?

For inflammation, in a generalized sense, two main types of activators are known: nonself-antigens (known as pathogen-associated molecular patterns (PAMPs)), which are present in or released from infectious invaders, and self-antigens known as damage-associated molecular patterns (DAMPs), which are host molecules (such as high-mobility group box 1 protein (HMGB1)) released from damaged cells under necrotic but not apoptotic conditions. In many ways and under many different experimental conditions, HSPs are frequently mentioned as prime examples of DAMPs. There are, however, several qualities inherent to HSPs that disqualify them as DAMPs. We have reviewed the arguments recently [31,32]. First, DAMPs are intracellular molecules normally hidden from recognition by the immune system, whereas HSPs are freely present in the extracellular fluids and are also frequently exposed at the outer surface of cells (both eukaryotic and prokaryotic cells). Second, Toll-like receptor 2 (TLR2) and TLR4 are seen as two of the main receptors involved in the recognition of HSP60 and HSP70. These HSPs are released from cells under necrotic conditions, but it has been shown that TLR2 and TLR4 are not required for the host response to DAMPs that are derived from necrotic cells. Moreover, other receptors for HSPs, such as the HSP70 scavenger receptor SRA1, have been shown to confer a suppressive rather than an activating signal to host cells. Third, *in vitro* cultured dendritic cells have been shown to adopt a tolerizing phenotype, rather than a mature or activated phenotype, in the presence of HSPs. Fourth, as already argued in this chapter, in experimental models of autoimmunity and of tissue or tumor transplants, immunization with HSPs was shown to lead to the induction of regulatory T cells, which suppressed disease or transplant rejection.

One major obstacle to reach a proper interpretation of the findings made in this area is that much of the original experimental work was carried out with recombinant HSPs that were contaminated with remnants of *E. coli* LPS and other stimulatory components of bacterial origin. One may assume that much of the proinflammatory effects seen in the earlier work was caused by the presence of these, difficult to remove, contaminants [33].

Unfortunately, this may have contributed to the existence of a number of possibly conflicting or difficult to reconcile reports. For example, recombinant human HSP70 was reported to promote the antigen-presenting function of DCs and to convert T-cell tolerance to autoimmunity (Millar *et al.*, 2003). Around the same time, others reported that an LPS-free recombinant mycobacterial HSP70 induced IL-10 production and immunomodulation through a delayed maturation of bone marrow-derived DCs [35].

31.6 HSP are DAMPERs of Inflammation

Given the risks associated with unregulated forms of inflammation, many inflammation-associated factors have their built-in function in the dampening

of inflammation. As we have seen, the models have provided ample evidence for the anti-inflammatory effects of HSP. A large variety of mechanisms may be responsible for this.

31.6.1 Effects of HSP on Mediators of Inflammation

Rothbard *et al.* reported that systemic administration of one of the smaller HSPs (HSPB5) had a downmodulating effect on inflammation through the temperature-dependent binding of acute-phase proteins such as complement factors [36].

Another central component of inflammation is, of course, NF-κB. Translocation of this transcription factor to the nucleus leads to expression of inflammation-promoting genes, such as those coding for inflammatory cytokines IL-6 and IL-12. HSPs have been found to mediate the degradation of the p65 subunit of NF-κB. In LPS-treated dendritic cells, HSP70 was shown to have an active role in transport of the NF-κB-PDLIM2 (an ubiquitin E3 ligase that targets the p65 subunit) to the proteasome for degradation. In other words, HSP70 inhibits proinflammatory NF-κB signaling by acting as a bridge between an ubiquitin E3 ligase and the proteasome ([37], ra119). Long before the elucidation of the exact molecular events leading to this NF-κB inhibition, the phenomenon was known already. One of the earlier observations was on HSP70 and its suppressive effect on astroglial-inducible nitric oxide synthase by decreasing NF-κB activation [38]. More general claims that the heat shock response blocked Iκ-B degradation and NF-κB nuclear translocation were also made already long before [39]. A further review on how the heat shock response and the NF-κB signaling pathway interact at multiple levels is available ([40], S89–95). This review mentions that the heat shock response inhibits TNFα, IL-1β, IL-8, RANTES, complement factor 5, and ICAM-1 in mononuclear cells.

It can be debated whether these effects of the heat shock response on inflammatory effector mechanisms are specific or just the result of gene expression prioritization for those genes that are essential for survival. Several findings, however, have argued in favor of specificity of the heat shock response and its anti-inflammatory effects. For instance, overexpression of HSP70 by stable transfection was shown to inhibit cytokine-mediated expression of NO synthase in cultured cells [38] and HSF-1 was found to repress the proIL-1 promoter [41].

31.6.2 HSP are Targets for a Default Regulatory Immune Response

For the functional activity of Tregs to control inflammation, it has already been argued by others that the recognition of an antigen by the very small number of autoantigen specific T cells in a polyclonal T-cell population would probably not suffice. A better scenario would be the activity of a collection of polyclonal Tregs that recognize complexes of MHC class II loaded with ubiquitous self-peptides [42]. As already mentioned above, HSP peptides are abundant in the MHCII ligandome, especially at sites of inflammation. Therefore, the repertoire of T cells with TcRs that enable such T cells to detect the presence of HSP peptides are well positioned to react adequately and to mitigate the inflammatory process.

31.6.3 HSP and Tregs

The mycobacterial HSP70 peptide B29, mentioned above, was found to induce very effective Tregs [14]. The responding CD25+, Foxp3+ T-cell population, when injected into mice, suppressed induced arthritis through its cross-recognition of endogenous B29 homologues present in multiple members of the HSP70 family of molecules. The transferred T cells were positive for the CD90.1 congenic marker, and when transferred these cells were easy to differentiate from the CD90.2 positive host cells. In this manner, the continued presence of the transferred Tregs was documented for at least 50 days and when during suppression of disease the cells were killed *in vivo* by the infusion of a cytotoxic CD90.1-specific antibody, disease was seen to reappear. These experiments are well indicative of the fact that endogenous HSPs can be effective targets for Tregs in inflammatory conditions. Also during active disease, the transfer of B29-specific CD25+ Tregs was seen to fully inhibit disease.

Direct relevance of HSP70 for the function of Tregs and Foxp3 was shown by the manipulation of histone/protein deacetylases (HDACs) in Tregs by the use of HDAC inhibitors. The inhibitors were shown to prevent and suppress induced colitis in mice and the effect was shown to be associated with increased numbers and function of Tregs. Subsequent experiments in HDAC9−/− mice showed that they were resistant to colitis and had increased HSP70 levels. With immunoprecipitation, a direct interaction between HSP70 and Foxp3 was demonstrated [43].

31.6.4 HSP Coinducing Substances

Endogenous upregulation of HSP, as argued above, does promote the presence of HSP peptides in MHCII molecules, which are then recognized by CD4+ cells, and possibly with some preference by Tregs. In this manner, such endogenous upregulation can contribute to the control of inflammation. For this reason, HSP coinducers are of interest. HSP coinducers are substances that promote the synthesis of HSPs when cells are subjected to stressors. HSP coinducers are not stressors themselves and are, therefore, not capable of inducing a stress response in cells by themselves. A well-known HSP coinducer is the compound geranylgeranylacetone, despite its sometimes less pronounced activities [44]. Interesting coinducers are hydroxylamine derivatives such as bimoclomol. Some of them were developed and tested for their capacity to induce control of disease through their cytoprotective effects, such as in cardiovascular diseases [45].

In our laboratory, we have uncovered the HSP coinducing activity of carvacrol, one of essential oils present in Oregano species. Carvacrol was already known as an antimicrobial agent that was used for food conservation purposes. When tested on mammalian cells, human and mouse, HSP70 was found to be upregulated when the cells were exposed to carvacrol followed by heat stress. Interestingly, when carvacrol was orally administered in mice, the animals were found to show an increased T-cell response to HSP70 as tested in an *ex vivo* T-cell proliferation assay. In addition, with quantitative RT-PCR, an enhanced presence of mRNA coding for HSP70 was detected in the Peyer's patches of carvacrol-treated animals.

Apparently, the harsh environment of the gut tissues or the rapid replication of intestinal cells created already the stress needed for a further effective coinduction of HSP by carvacrol. Similarly, oral carvacrol prior to arthritis induction in the mouse model inhibited the disease. This inhibitory effect was shown to be T-cell-mediated by the successful suppression of disease following transfer of CD3+ T cells obtained from carvacrol-treated mice [46].

31.7 The Clinical use of HSP in Chronic Inflammatory Diseases

31.7.1 HSP Peptides and Proteins

Peptides and proteins for immunotherapy are usually selected from proposed autoantigens that are held responsible for initiating the pathological reactivity directed against self. However, at the time of actual diagnosis, human autoimmune diseases are already characterized by a secondary inflammatory process in which multiple antigens are targets of the immune system (a process known as epitope spreading). The antigens that are specifically upregulated at the site of inflammation and are immunologically dominant (bystander antigens) are, therefore, attractive candidates for antigen-specific immunotherapy. Induced tolerogenic immune responses to such bystander antigens could lead to a local downregulation of the ongoing inflammatory process that propagates the disease. Peptides developed from HSPs have the potential to serve as effective bystander antigens.

Until now, only few clinical trials with HSP peptides were reported. Vaccination with HSP60-derived peptide p227 protected against type 1 diabetes in both NOD mice and humans, as shown in a successful phase II clinical trial [47].

The first HSP peptide tested in RA in a phase I trial was dnaJP1 in patients with early RA. Mucosal administration of dnaJP1 led to a shift in T cells from a proinflammatory to a regulatory phenotype [48]. There were no relevant side effects seen. More recently Koffeman *et al.* performed a placebo–controlled phase II/III trial with dnaJP1 in RA patients [49]. After oral administration, clinical improvement was reported. The short-term effects of intravenous administration of HSP10 were investigated in a randomized, double blind, multicenter study. In this study with 23 treatment-resistant RA patients, disease activity scores improved significantly during the 12 weeks of therapy in the high-dose group. Most adverse events were mild [50].

At present, a first phase I/IIa clinical trial with BiP in RA patients has been carried out and the results will be made public soon. The primary end point was very successful, there was no toxicity in any patient at any time over the three months monitored that was attributable to BiP. Some of the tested biomarkers were seen to respond favorably to the treatment (Corrigall, personal communication).

31.7.2 TolDC Loaded with HSP Peptides

Dendritic cells (DCs) play a central role in the immune system, they launch effective immunity against invading pathogens and simultaneously preserve

tolerance to self and harmless antigens encountered daily. DCs contribute to maintenance of immunological homeostasis by promoting peripheral tolerance, among others, via the induction of antigen-specific regulatory T cells and induction of energy or deletion in effector T cells. These unique traits make them possible targets for tolerance-inducing cellular therapies. DCs are indispensable for tolerance induction as ablation of DCs in mice results in spontaneous autoimmunity [51]. Indeed, several studies have shown that transfer of autologous dendritic cells, *in vitro* modulated to obtain tolerogenic capabilities, that is, tolerogenic DC (tolDC), can prevent the induction of autoimmunity in animal disease models such as arthritis, EAE, and diabetes [52]. Moreover, first clinical phase I trials in diabetes and RA using *ex vivo* manipulated human tolDCs have been performed showing their safety *in vivo* and the practical feasibility of DC transfer therapy [53].

Recently, we have started to study the functionality of tolDCs in our experimental arthritis model. Dendritic cells were cultured from bone marrow from wild-type Balb/c mice (BMDCs). To create tolerogenic DCs, dexamethasone and the active form of vitamin D3 (1,25-dihydroxyvitamin D3) were added to the culture and LPS was used to induce maturation. The tolDCs were loaded with HSP70 peptide B29 and put in coculture with CD4+ T cells isolated from a mB29b (HSP70 epitope)-TCR transgenic mouse.

The tolerogenic characteristics were shown by a reduction in maturation markers, a reduced production of IL-12p70 and the production of IL-10. Furthermore, after stimulation with tolDC, CD4+ T cells developed a Treg-like character, indicated by an increased IL-10 production. These results indicate that HSP peptide-loaded TolDCs are able to induce a regulatory phenotype in HSP-specific CD4+ T cells. Given this result, it may be clear that this use of tolDC may offer attractive therapeutic chances. HSP peptides can be produced under GLP conditions and are not restricted to a certain autoimmune disease, requiring the use of a disease-specific autoantigen.

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Part Four

Inflammation and Host Response

32

Inflammation and Coagulation

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32.1 Introduction

Virtually all patients with severe inflammatory conditions have coagulation abnormalities. These abnormalities range from subtle activation of coagulation that can be detected only by sensitive markers for coagulation factor activation to somewhat more stronger coagulation activation that may be detectable by a small decrease in platelet count and subclinical prolongation of global clotting times. In its most extreme form, coagulation activation my lead to fulminant disseminated intravascular coagulation (DIC), characterized by simultaneous widespread microvascular thrombosis and profuse bleeding from various sites [1,2]. Patients with severe forms of inflammation-associated coagulopathy may present with manifest thromboembolic disease or clinically less apparent microvascular fibrin deposition, which predominantly presents as multiple organ dysfunction [2,3]. Alternatively, severe bleeding may be the leading symptom [4], but quite often a patient with DIC has simultaneous thrombosis and bleeding. Bleeding is caused by consumption and subsequent exhaustion of coagulation proteins and platelets, due to the ongoing activation of the coagulation system [5]. In its most severe form, this combination may present as the Waterhouse–Friderichsen syndrome, commonly seen during fulminant meningococcal septicemia, although many other microorganisms may cause this clinical state [6].

32.2 Incidence of Inflammation-associated Coagulopathy

Clinically relevant coagulation abnormalities may occur in 50–70% of patients with severe systemic inflammation, whereas about 35% of patients will meet the criteria for DIC (see further) [7,8]. In general, the incidence of thrombocytopenia (platelet count $<150 \times 10^9$/l) in critically ill medical patients is 35–50% [9,10]. Typically, the platelet count decreases during the first four days in the intensive

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care unit [11]. Systemic inflammatory states, for example, as associated with severe sepsis, is a clear risk factor for thrombocytopenia in critically ill patients and the severity of sepsis correlates with the decrease in platelet count [12]. Main factors that contribute to thrombocytopenia in patients with severe systemic inflammatory states are impaired platelet production, increased consumption or destruction, or sequestration in the spleen or at the endothelial level. Impaired production of platelets in the bone marrow may seem contradictory to the high levels of platelet production-stimulating proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, and high concentration of circulating thrombopoietin in patients with inflamation, which theoretically should stimulate megakaryopoiesis in the bone marrow [13]. However, in a substantial number of patients with systemic inflammation marked hemophagocytosis may occur, consisting of active phagocytosis of megakaryocytes and other hematopietic cells by monocytes and macrophages, hypothetically due to stimulation with high levels of macrophage colony-stimulating factor (M-CSF) [14]. Platelet consumption probably also plays an important role, due to ongoing generation of thrombin. Platelet activation, consumption, and destruction may also occur at the endothelial site as a result of the extensive endothelial cell–platelet interaction that may vary between different vascular beds in various organs (see further) [15]. A prolonged global coagulation time (such as the prothrombin time (PT) or the activated partial thromboplastin time (aPTT)) occurs in 14–28% of patients [16]. Other coagulation test abnormalities in patient with severe systemic inflammatory states include high fibrin split products (in up to 99% of patients) [17,18] and low levels of coagulation inhibitors, such as antithrombin and protein C [18,19].

32.3 Clinical Relevance of the Interaction between Inflammation and Coagulation

There is evidence that activation of coagulation in concert with inflammatory activation can result in microvascular thrombosis and thereby contribute to multiple organ failure [20]. First, there are several reports of post-mortem findings in patients with systemic inflammatory conditions with coagulation abnormalities and DIC [21,22]. These autopsy findings include diffuse bleeding at various sites, hemorraghic necrosis of tissue, microthrombi in small blood vessels, and thrombi in mid-size and larger arteries and veins. The demonstration of ischemia and necrosis was associated with fibrin deposition in small and mid-size vessels of various organs [23]. Importantly, the presence of these intravascular thrombi appears to be clearly and specifically related to the development of organ dysfunction. Second, experimental animal studies of inflammation-elicited coagulopathy show fibrin deposition in various organs. Experimental bacteremia or endotoxemia causes intra- and extravascular fibrin deposition in kidneys, lungs, liver brain, and various other organs. Amelioration of the hemostatic defect by various interventions in these experimental models appears to improve organ failure and, in some but not all cases, mortality [24–27]. Interestingly, some

studies indicate that amelioration of the systemic coagulation activation will have a profound beneficial effect on resolution of local fibrin deposition and improvement of organ failure [4,28]. Last, clinical studies support the notion of coagulation as an important determinant of clinical outcome. DIC has shown to be an independent predictor of organ failure and mortality [3,29]. In a consecutive series of critically ill patients, the mortality of patients with DIC was 43%, as compared to 27% in those without DIC. In this study, mortality was also directly related to the severity of the coagulopathy [30].

32.4 Pathogenetic Pathways in the Coagulopathy of Sepsis

In recent years, the mechanisms involved in the pathological derangement of coagulation in patients with inflammatory conditions have become increasingly clear. Apparently, various mechanisms at different sites in the hemostatic balance act simultaneously toward a procoagulant state. It has become clear that the most important mediators that orchestrate this imbalance of the coagulation system elicited by inflammation are cytokines [31]. Increasing evidence points to extensive cross-talk between these two systems, whereby inflammation leads to activation of coagulation and coagulation also considerably affects inflammatory activity [32]. Interestingly, systemic activation of coagulation and inflammation may have some organ-specific manifestations, which are relevant for the specific organ dysfunction (see further) [33].

32.4.1 Initiation of Inflammation-induced Activation of Coagulation

Tissue factor plays a central role in the initiation of inflammation-induced coagulation [34]. Blocking tissue factor activity completely inhibits inflammation-induced thrombin generation in models of experimental endotoxemia or bacteremia [26,35]. The vast majority of cells constitutively expressing tissue factor are found in tissues not in direct contact with blood, such as the adventitial layer of larger blood vessels. However, tissue factor comes into contact with blood when the integrity of the vessel wall is disrupted or when endothelial cells and/or circulating blood cells start expressing tissue factor. The *in vivo* expression of tissue factor seems mostly dependent on IL-6, as demonstrated in studies showing that inhibition of IL-6 completely abrogates tissue factor-dependent thrombin generation in experimental endotoxemia, whereas specific inhibition of other proinflammatory cytokines had less or no effect [31,36]. Inflammatory cells in atherosclerotic plaques produce abundant tissue factor and upon plaque rupture there is extensive tissue factor exposure to blood [37]. In severe sepsis, mononuclear cells, stimulated by proinflammatory cytokines, express tissue factor, which leads to systemic activation of coagulation [38]. Even in experimental low-dose endotoxemia in healthy subjects, a 125-fold increase in tissue factor mRNA levels in blood monocytes can be detected [39]. A potential alternative source of tissue factor may be endothelial cells, polymorphonuclear cells, and other cell types. It is hypothesized that tissue factor from these sources is shuttled between

cells through microparticles derived from activated mononuclear cells [40]. It is, however, unlikely that these cells actually synthesize tissue factor in substantial quantities [38,41].

Upon exposure to blood, tissue factor binds to factor VIIa. The complex of tissue factor–factor VIIa catalyzes the conversion of factor X to Xa, which will form the prothrombinase complex with factor Va, prothrombin (factor II), and calcium, thereby generating thrombin (factor IIa). One of the key functions of thrombin is to convert fibrinogen into fibrin. The tissue factor–factor VIIa complex can also activate factor IX, forming a tenase complex with activated factor IX and factor X, generating additional factor Xa, thereby forming an essential amplification loop. The assembly of the prothrombinase and tenase complex is markedly facilitated if a suitable phospholipid surface is available, ideally presented by activated platelets. In the setting of inflammation-induced activation of coagulation, platelets can be activated directly by endotoxin or by proinflammatory mediators, such as platelet activating factor. Thrombin itself is one of the strongest platelet activators *in vivo*.

32.4.2 Role of Platelets in Inflammation-induced Coagulation

Activation of platelets may also accelerate fibrin formation by another mechanism. The expression of TF on monocytes is markedly stimulated by the presence of platelets and granulocytes in a P-selectin-dependent reaction [42]. This effect may be the result of nuclear factor kappa B (NF-kB) activation induced by binding of activated platelets to neutrophils and mononuclear cells [43]. This cellular interaction also markedly enhances the production of Il-1b, Il-8, MCP-1, and TNF-α [44]. The expression of P-selectin on the activated platelet membrane will mediate the adherence of platelets to endothelial cells and leukocytes [45].

Upon disruption of the integrity of the vessel wall or upon endothelial cell perturbation, as may occur in the systemic inflammatory response in critical illness, a swift and complex interaction between circulating platelets, endothelial cells, and subendothelial structures may occur [46]. The result of this interaction is platelet adhesion to the vessel wall and forming aggregates with each other, thereby materializing microvascular obstruction. The interaction between the platelets and the vessel wall is mediated by cellular receptors on the surface of platelets and endothelial cells, such as integrins and selectins, and by adhesive proteins, such as von Willebrand factor and fibrinogen. Von Willebrand factor is a large polymer of disulfide-linked subunits, each comprising 2050 amino acid residues and up to 22 carbohydrate chains [47]. The molecular mass of the multimers range from about 500 kDa to more than 10 000 kDa. The multimers may form coiled molecules or thin filaments up to 1250 nm long (which means as large as a platelet). Data from clinical studies indicate that large von Willebrand factor multimers may be hemostatically more active than smaller molecules [48]. The biochemical basis for this observation probably relies on the fact that they contain a relatively large number of the domains that will support multiple interactions between the vessel wall, subendothelial matrix, and cellular receptors on platelets. Besides playing a role in platelet vessel wall interaction, von Willebrand factor may also be a ligand for platelet receptors IIb/IIIa, thereby

competing with fibrinogen. Under normal circumstances, the size of von Willebrand factor multimers is regulated by ADAMTS13, also known as von Willebrand factor-cleaving protease. It has been suggested that a relative insufficiency of ADAMTS13 in critical illness contributes to disturbed platelet–vessel wall interaction [49]. Hypothetically, endothelial disruption with massive release of ultra large von Willebrand factor and relative insufficient cleavage due to consumption of ADAMTS13 play a role in the increased platelet–vessel wall interaction in patients with sepsis. Several authors studied the relation between ADAMTS13 activity and the extent of sepsis in patients. They found an association between decrease in ADAMTS13 activity and increase in both renal failure as well as DIC caused by sepsis. Ultra large von Willebrand factor multimers were detected in patients with severe sepsis-induced ADAMTS13 deficiency [48,50,51]. Others have confirmed the association between the low ADAMTS13 levels and the severity of sepsis [52].

32.4.3 Downregulation of Physiological Anticoagulant and Fibrinolytic Pathways during Inflammation

Procoagulant activity is regulated by three important anticoagulant pathways: antithrombin (AT), the protein C system, and tissue factor pathway inhibitor (TFPI). During inflammation-induced activation of coagulation, the function of all three pathways can be impaired [53] Antithrombin appears to be incapable of adequate regulation of thrombin activity during inflammation for several reasons. Antithrombin levels are continuously consumed by the ongoing formation of thrombin and other activated proteases that are susceptible to antithrombin complex formation and antithrombin is degraded by elastase released from activated neutrophils. In addition, impaired synthesis of antithrombin because of liver failure and extravascular leakage of this protease inhibitor as a consequence of capillary leakage further contribute to low levels of antithrombin. Similarly, there are several reasons for severe injury to the protein C system in inflammatory states. Enhanced consumption, impaired liver synthesis, and vascular leakage may also result in low circulating levels of protein C. Second, activation of the cytokine network, in particular high levels of tumor necrosis factor-α (TNF-α), results in a marked downregulation of thrombomodulin in endothelial cells, thereby prohibiting adequate protein C activation [54]. The third mechanism contributing to the inflammation-induced prothrombotic state is impaired fibrin degradation, due to high circulating levels of PAI-1, the main physiological inhibitor of fibrinolysis. In clinical studies in patients with severe infection and inflammation, a high plasma level of PAI-1 was one of the strongest predictors of mortality [34].

32.4.4 Antithrombin and Inflammation

The serine protease inhibitor antithrombin is the main inhibitor of thrombin and factor Xa. Without heparin, AT neutralizes coagulation enzymes in a slow, progressive manner [55]. Heparin induces conformational changes in AT that result in at least a 1000-fold enhancement of AT activity. Thus, the clinical

efficacy of heparin is attributed to its interaction with AT. Endogenous glycosaminoglycans, such as heparan sulfates, on the vessel wall also promote AT-mediated inhibition of thrombin and other coagulation enzymes. Besides the mechanisms by which inflammation leads to low levels of antithrombin (see above) proinflammatory cytokines can cause reduced synthesis of glycosaminoglycans on the endothelial surface, which will also contribute to reduced AT function, since these glycosaminoglycans can act as physiological heparin-like cofactors of AT [56].

Antithrombin possesses anti-inflammatory properties, many of which are mediated by its actions in the coagulation cascade [57]. Most importantly, thrombin inhibition by antithrombin blunts activation of many inflammatory mediators. For example, thrombin activates platelets and endothelial cells, which in turn contribute to local inflammation [58]. Activated platelets secrete inflammatory mediators such as IL-1, which stimulate leukocyte activity. In particular, recruitment and adhesion of neutrophils and monocytes to blood vessels within the microcirculation promote inflammation. Increasing evidence suggests that antithrombin possesses potent anti-inflammatory properties independent of its anticoagulation activity [58]. Most of these effects have been demonstrated *in vitro* or *in vivo* at high concentrations. Nevertheless, these mechanisms may be important in clinical settings that are driven by a combined activation of inflammation and coagulation. Perhaps most importantly, antithrombin induces prostacyclin release from endothelial cells [59–61]. Prostacyclin inhibits platelet activation and aggregation, blocks neutrophil tethering to blood vessels, and decreases endothelial cell production of various cytokines and chemokines [62]. Additional anti-inflammatory actions of antithrombin are mediated by direct interaction with leukocytes and lymphocytes. Antithrombin binds to receptors, such as syndecan-4, on the cell surfaces of neutrophils, monocytes, and lymphocytes and blocks the interaction of these cells with endothelial cells [34]. Inhibition of leukocyte–endothelial cell interactions by antithrombin may be mediated by prostacyclin release, downregulation of P-selectin, or prevention of leukocyte activation. Thus, antithrombin directly hinders leukocyte migration and adhesion to endothelial cells, which in turn impacts the severity of capillary leakage and subsequent organ damage. Given the wide-ranging impact of antithrombin on coagulation and inflammation, there are multiple potential clinical applications of antithrombin in different clinical settings that encompass thrombotic states generally not associated with inflammation (e.g., pregnancy) and in coagulation-related disease states with powerful proinflammatory elements (e.g., sepsis)

32.4.5 Activated Protein C and Inflammation

Activated protein C (APC) appears to play a central role in the pathogenesis of systemic inflammation and associated organ dysfunction [54]. There is ample evidence that an insufficient functioning of the protein C pathway contributes to inflammation-associated coagulopathy [63,64]. In patients with severe inflammation, the protein C system is found malfunctioning at virtually all levels. First, plasma levels of the zymogen protein C are low or very low, due to impaired

synthesis, consumption, and degradation by proteolytic enzymes, such as neutrophil elastase [65–67]. Furthermore, a significant downregulation of thrombomodulin, caused by proinflammatory cytokines such as TNF-α and IL-1, has been demonstrated, resulting in diminished protein C activation [68,69]. Low levels of free protein S may further compromise an adequate function of the protein C system. In plasma, 60% of the cofactor protein S is complexed to a complement regulatory protein, C4b binding protein (C4bBP). Increased plasma levels of C4bBP as a consequence of the acute-phase reaction in inflammatory diseases may result in a relative protein S deficiency, which further contributes to a procoagulant state during sepsis. Although it has been shown that the β-chain of C4bBP (which mainly governs the binding to protein S) is largely unaffected during the acute-phase response [70], support for this hypothesis comes from studies showing that the infusion of C4bBP in combination with a sublethal dose of *Escherichia coli* into baboons resulted in a lethal response with severe organ damage due to DIC [71]. Finally, but importantly, in sepsis the EPCR has shown to be downregulated, which may further negatively affect the function of the protein C system [72]. Apart from these effects, sepsis may cause a resistance toward APC by other mechanisms, which are partly dependent on a sharp increase in factor VIII levels (released from endothelial cells), but partly occur by yet unidentified mechanisms [73].

There is compelling evidence that besides their role as an important regulator of coagulation activity, components of the protein C system also have an important function in modulating inflammation [74,75]. APC plays an important role in attenuating the systemic inflammatory response in sepsis as demonstrated in experiments showing that blocking the protein C pathway in septic baboons exacerbated the inflammatory response. In contrast, administration of APC ameliorated the inflammatory activation upon the intravenous infusion of *E. coli* [27]. Similar experiments in rodents showed identical results and demonstrated a beneficial effect on inflammatory effects in various tissues [76]. Support for the notion that APC has anti-inflammatory properties comes from *in vitro* observations, demonstrating an APC-binding site on monocytes, which may mediate downstream inflammatory processes [77,78] and from experiments showing that APC can block NF-kB nuclear translocation, which is a prerequisite for increases in proinflammatory cytokines and adhesion molecules [79]. These *in vitro* findings are supported by *in vivo* studies in mice with targeted disruption of the protein C gene. In these mice with genetic deficiencies of protein C, endotoxemia was associated with a more marked increase in proinflammatory cytokines and other inflammatory responses compared to wild-type mice [80,81].

It is likely that the effects of APC on inflammation are mediated by the EPCR [74]. Binding of APC to EPCR influences gene expression profiles of cells by inhibiting endotoxin-induced calcium fluxes in the cell and by blocking NF-κB nuclear translocation [78,79]. Blocking the EPCR with a specific monoclonal antibody aggravates both the coagulation and the inflammatory response to *E. coli* infusion [72].

Apart from its effect on cytokine levels, APC has been shown to inhibit leukocyte chemotaxis and adhesion of leukoyctes to activated endothelium [82,83]. This notion was confirmed in a hamster endotoxemia model at

concentrations of recombinant human APC (rhAPC) that preclude a significant anticoagulant effect [84]. Morevover, in a human model of endotoxin-induced pulmonary inflammation, systemic administration of rhAPC resulted in significant local anti-inflammatory effects [85]. A potential mechanism is that APC inhibits expression of platelet-derived growth factor in the lung [86]. In addition, it has been shown that APC protects against the disruption of endothelial cell barrier in sepsis, probably by interfering with EPCR and PAR-1 on endothelial cells [87–89].

32.4.6 Thrombomodulin and Inflammation

Thrombomodulin (CD141) is a transmembrane multidomain glycoprotein receptor that is expressed predominantly not only on vascular endothelial cells but also on monocytes, neutrophils, osteoblasts, synovial cells, and dendritic cells [90–92]. Thrombomodulin exhibits several properties in coagulation, fibrinolysis, innate immunity, and inflammation that are based largely on its distinct structural domains. Thrombomodulin is a membrane protein with several domains, including a lectin-like domain, six epidermal growth factor (EGF)-like repeats, a transmembrane domain, and a short cytoplasmatic tail [74]. The binding of thrombin to thrombomodulin occurs at the site of the EGF repeats [93]. This binding not only results in an about 100-fold increase in the activation of protein C but also blocks the thrombin-mediated conversion of fibrinogen into fibrin and inhibits the binding of thrombin to other cellular receptors on platelets and inflammatory cells. The central domain of thrombomodulin is comprised of six EGF-like repeats, three of which provide critical cofactor activity for thrombin-mediated generation of APC and of activated thrombin activatable fibrinolysis inhibitor (TAFI). Protein C activation by thrombin-thrombomodulin is further augmented by the endothelial protein C receptor (EPCR). In addition to its pivotal role in activating PC and TAFI [94], thrombomodulin also has direct anti-inflammatory properties that are mediated at least in part by its N-terminal C-type lectin-like domain [91,95]. This structure interferes with neutrophil adhesion, complement activation, and cytokine generation, and mice lacking the lectin-like domain of thrombomodulin (thrombomodulinLeD/LeD mice) exhibit increased sensitivity to tissue injury in models of endotoxemia, lung and myocardial ischemia–reperfusion, and inflammatory arthritis. Importantly, deletion of the lectin-like domain of thrombomodulin did not influence the capacity of thrombomodulin to activate PC, indicating that the anti-inflammatory effects of this part of thrombomodulin are not mediated by APC. A recent study in experimental pneumococcal pneumonia showed that mice lacking the terminal lectin-like domain had a markedly better survival compared to wild-type mice, associated with lower bacterial loads in blood and liver and less inflammatory changes in various organs [90].

Thrombomodulin plays a central role in the pathogenesis of sepsis [91,96]. First, thrombomodulin is essential for the activation of protein C to activated protein C; as such, thrombomodulin plays a key role in the anticoagulant and anti-inflammatory properties of the protein C system. Second, thrombomodulin binds thrombin, thereby preventing it from exerting prothrombotic and

proinflammatory effects. Third, thrombomodulin-bound thrombin activates TAFI, which has been demonstrated to suppress bradykinin activity and complement activation. In sepsis, the expression of thrombomodulin is largely downregulated [68]. The proinflammatory cytokines TNF-α and interleukin-1 may significantly down regulate the cellular expression of thrombomodulin, as suggested by cell culture experiments. Observations in patients with severe Gram-negative septicemia indeed confirmed the downregulation of thrombomodulin *in vivo* and impaired activation of protein C [69]. In this study, histological analysis of skin biopsies from patients with meningococcal sepsis showed decreased endothelial expression of thrombomodulin, both in vessels with and without thrombosis. However, *in vivo* studies suggest that EPCR is not downregulated in sepsis but upregulated and that this effect is mediated by thrombin. The formation of thrombin may induce the shedding of EPCR by the endothelium, due to activation of metalloproteinases by thrombin. It is presently unknown whether thrombomodulin is also cleaved by similar mechanisms.

32.4.7 TFPI and Inflammation

The third inhibitory mechanism of thrombin generation involves TFPI, the main inhibitor of the tissue factor–factor VIIa complex. The role of TFPI in the regulation of inflammation-induced coagulation activation is not completely clear. Experiments showing that administration of recombinant TFPI (and thereby achieving higher than physiological plasma concentrations of TFPI) blocks inflammation-induced thrombin generation in humans, and the observation that pharmacological doses of TFPI are capable of preventing mortality during systemic infection and inflammation suggests that high concentrations of TFPI are capable of importantly modulating tissue factor-mediated coagulation [24,97].

32.5 Modulation of Inflammation by Coagulation *in vivo*

Communication between inflammation and coagulation is bidirectional, such that coagulation can also modulate inflammatory activity. Coagulation proteases and protease inhibitors not only interact with coagulation protein zymogens but also with specific cell receptors to induce signaling pathways (Figure 32.1). In particular, protease interactions that affect inflammatory processes may be important in critically ill patients. *In vivo* evidence for a role of coagulation-protease stimulation of inflammation comes from experiments showing that the administration of recombinant factor VIIa to healthy human subjects causes a small but significant three–fourfold rise in plasma levels of IL-6 and IL-8 [98].

A pivotal mechanism by which coagulation proteases modulate inflammation is by binding to protease activated receptors or PARs. Four types (PAR 1–4) have been identified, all belonging to the family of transmembrane domain, G protein-coupled receptors [99]. A typical feature of PARs is that they serve as their own ligand. Proteolytic cleavage by an activated coagulation factor leads to exposure of

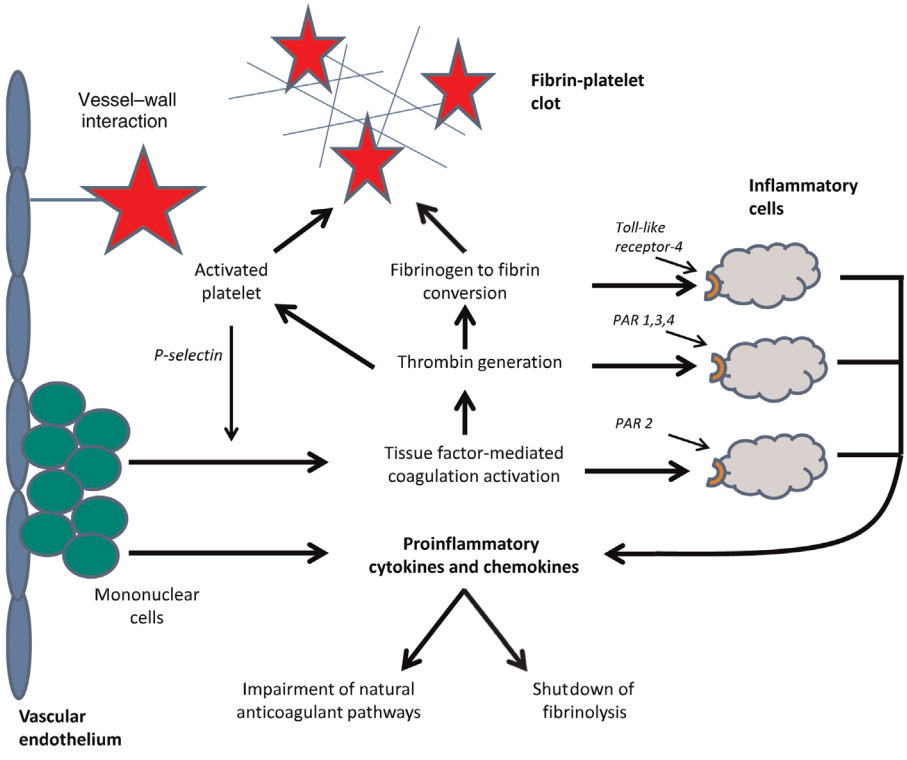


Figure 32.1 Communication between inflammation and coagulation.

a neo-amino terminus, which activates the same receptor (and possibly adjacent receptors), initiating transmembrane signaling. PARs are localized in the vasculature on endothelial cells, mononuclear cells, platelets, fibroblasts, and smooth muscle cells [99]. PAR 1, 3, and 4 are thrombin receptors and PAR-1 can also serve as receptor for the tissue factor–factor VIIa complex and factor Xa. PAR-2 cannot bind thrombin, but can be activated by the tissue factor–factor VIIa complex or factor Xa. Binding of thrombin to its cellular receptor may induce the production of several cytokines and growth factors. Binding of tissue factor–factor VIIa to PAR-2 also results in upregulation of inflammatory responses (production of reactive oxygen species and expression of MHC class II and cell adhesion molecules) in macrophages and was shown to affect neutrophil infiltration and proinflammatory cytokine (TNF-α and IL-1β) expression. The *in vivo* relevance of PARs has been confirmed in various experimental studies using PAR inhibitors or PAR-deficient mice [100–102].

32.5.1 Extracellular DNA, Histones, and Neutrophil Extracellular Traps

Recent observations have pointed to an important role of extracelluar DNA and DNA-binding proteins (such as histones and high-mobility group box 1 protein) in the pathogenesis of DIC. This cell-free DNA and DNA-binding components are released from nucleosomes of degraded cells and may form a surface on which

assembly of activated coagulation factor complexes may be greatly facilitated [103]. In addition, histones activate platelets and therby stimulate thrombin generation [104]. Activation and binding of neutrophils by DNA components results in the formation of neutrophil extracellular traps (NETs) that have recently been identified as important contributors to vascular thrombosis and inflammation [105]. NETs may provide the availability of inflammatory cells expressing tissue factor [106]. Activation of coagulation is further enhanced by the proteolytic cleavage of physiologic anticoagulants by abundant neutropilic elastase in NETs [107]. NETs may also induce endothelial cell death and detrimental inflammatory activity, an effect likely mediated by NET-associated proteases or cationic proteins, including histones [105,108].

32.6 Injured Endothelium and Microparticles

Microparticles may play a role in endothelial dysfunction. Elevated levels of circulating endothelium-derived microparticles have been observed in patients with sepsis. Microparticles are circulating fragments of cell membranes released from activated or perturbed cells. They appear to be able to shuttle membrane-associated receptors from cell to cell and may provide a suitable surface on which plasma protease interactions can take place [109]. Microparticles have procoagulant activity resulting in thrombin generation *in vivo* via a tissue factor/factor VIIa-dependent mechanism. Microparticles may play a role in the pathogenesis of sepsis in several ways. They are potent vascular proinflammatory and procoagulant mediators, can interfere in inflammatory processes, and are involved in maintenance of vascular tone [110]. Recent observations demonstrated that leucocyte – but even more strongly – endothelial cell-derived microparticles were associated with an early and severe derangement of coagulation during severe inflammation, suggesting that endothelium-derived microparticles can be considered as relevant biomarkers of early vascular injury and DIC under inflammatory conditions. The utility and accuracy of measuring microparticles in the setting of systemic inflammatory states needs some further research and prospective validation; however, the test seems promising and deserves further exploration.

32.7 Systemic versus Localized Responses

Although the mechanisms mentioned above have been demonstrated to occur *in vivo* as a general response upon proinflammatory stimuli, it is likely that marked differences in the procoagulant response as well as the underlying pathogenetic pathway may exist between cells and tissues [33]. This may be caused by differences in cell-specific gene expression, environmental factors, and organ-specific differences. First, localization of coagulation activity may relate to a cell-specific gene expression. For example, inflammatory mediators enhance PAI-1 gene expression in a complex and tissue-specific way [111]. Recent studies have

demonstrated that the von Willebrand factor promotor contains cell-specific elements, and similar response elements may be involved in protein synthesis in cells in general [112]. Second, the tissue environment may determine whether specific gene transcription occurs [113]. It is not completely clear why specific sites and organs are at greater risk of developing microvascular thrombosis and also local differences in the consequences of (micro)thrombosis are still poorly understood. Environmental factors underlying the inflammatory response are thought to play a role in this differential coagulative response as well. In mice with disturbances in the plasminogen–plasmin system subjected to hypoxia, the formation of fibrin is induced and is particularly evident in the lungs [114]. In contrast, these same mice respond to endotoxemia with fibrin deposition in the microvasculature of the kidney in particular. Similarly, mice with a functional thrombomodulin deficiency had a marked increase in pulmonary fibrin deposition after hypoxic challenge [115]. In addition, when mice with a functional defect in the thrombomodulin gene were challenged with endotoxin in sublethal amounts, fibrin formation was apparent in the lungs, but not in any other organ studied. In the latter model, fibrin was only temporarily present and disappeared after 24 h [116]. These models illustrate the assumption that fibrin formation is a localized phenomenon, rather than a generalized process.

Lastly, various organ systems may markedly differ in their endothelial cell response toward inflammation and injury. In general, endothelial cells play a central role in the coagulation response upon systemic inflammation [117]. The endothelium plays a central role in all major pathways involved in the pathogenesis of hemostatic derangement during severe inflammation. Endothelial cells appear to be directly involved in the initiation and regulation of thrombin generation and the inhibition of fibrin removal. Endothelial cells may express tissue factor, which is the main initiator of coagulation. In addition, physiological anticoagulant pathways, such as antithrombin, the protein C system, or tissue factor pathway inhibitor, are mostly located on endothelial cells and endothelial cell dysfunction is directly related to impaired regulation of coagulation. Also, endothelial cells are the main storage site of plasminogen activators and inhibitors and can acutely release these factors, thereby importantly mediating fibrinolytic activity or inhibition. Proinflammatory cytokines are crucial in mediating these effects on endothelial cells, which themselves may also express cytokines, thereby amplifying the coagulative response [31]. Although not completely clear, various organs may differ in all these endothelial cell-related factors influencing local coagulation activation and fibrin deposition.

32.8 Organ-specific Responses by Endothelial Cells

In their excellent overview, Rosenberg and Aird postulate that endothelial cells integrate different extracellulair signals and cellular responses in different regions of the vascular bed [112]. Various exogenous stimuli, such as shear stress, inflammatory mediators, and growth factors, exert their action on endothelial cells and the response of the endothelial cells to transduce the signal may vary between various tissues and even from endothelial cell to endothelial cell within a

tissue. As a result, the pro- or anticoagulant response of endothelial cells may differ between organs. Experiments in mice with a targeted deletion of the anticoagulant part of the thrombomodulin gene showed abundant fibrin formation in lungs, heart, and spleen [118]. Mice with homozygous deficiencies of plasminogen activators form clots in liver, heart, and lungs, but not in brain or kidneys [119]. Plasminogen activator inhibitor-deficient mice have fibrin deposition predominantly in kidneys [111]. Rosenberg and Aird state that various mechanisms play a role in the individual response of endothelial cells. Cell to cell communication may have important effects, as evidenced by the fact that PAI-1 expression in endothelial cells is upregulated if the culture is incubated with medium from aorta or umbilical vein cell culture but, in contrast, is downregulated by addition of conditioned medium from vascular smooth muscle cells [120,121]. In addition, cell signaling pathways may vary between endothelial cell subtypes. For example, hemodynamic changes may have opposite reactions in nitric oxide mRNA expression in endothelial cells from the aorta or from the pulmonary artery [122]. Another example is provided by the experiment showing that endothelial cells from renal and cerebrovascular vessels have decreased prostacyclin production and more apoptosis when exposed to plasma of patients with thrombocytopenic thrombotic purpura, whereas endothelial cells derived from lungs and liver do not respond to this same stimulus [123]. Lastly, also at the level of transcription a differential phenotype of endothelial cells between various organs can be demonstrated. Coagulation proteins, such as von Willebrand factor, have been shown to respond to various promoters by expressing this factor in different tissues, for example, exclusively in heart and skeletal muscle or in brain [113].

32.9 Diagnostic Approach to the Inflammation-induced Coagulopathy

According to the current understanding of inflammation-associated coagulation abnormalities, the determination of soluble fibrin in plasma appears to be crucial [124,125]. In general, the sensitivity of these assays for severe coagulation activation or DIC is relatively higher than the specificity. Indeed, initial clinical studies indicate that if the concentration of soluble fibrin has increased above a defined threshold, a diagnosis of DIC can be made [17,126]. Most of the clinical studies show a sensitivity of 90–100% for the diagnosis of DIC but a rather low specificity [127]. Fibrin degradation products (FDPs) may be detected by specific ELISAs or by latex agglutination assays, allowing rapid and bedside determination in emergency cases [128]. None of the available assays for fibrin degradation products discriminates between degradation products of cross-linked fibrin and fibrinogen degradation, which may cause spuriously high results [129]. The specificity of high levels of fibrin degradation products is, therefore, limited and many other conditions, such as trauma, recent surgery, inflammation, or venous thromboembolism, are associated with elevated FDPs. More recently developed tests are specifically aimed at the detection of neo-antigens on

degraded cross-linked fibrin. One of such tests detects an epitope related to plasmin-degraded cross-linked γ-chain, resulting in fragment D-dimer. These tests better differentiate degradation of cross-linked fibrin from fibrinogen or fibrinogen degradation products [130].

Consumption of coagulation factors leads to low levels of coagulation factors in patients with inflammation-associated coagulopathy. In addition, impaired synthesis, for example, due to impaired liver function or a vitamin K deficiency, and loss of coagulation proteins, due to massive bleeding, may play a role as well [131]. Although the accuracy of the measurement of one-stage clotting assays in this situation has been contested (due to the presence of activated coagulation factors in plasma), the level of coagulation factors appears to correlate well with the severity of the coagulopathy [131]. Measurement of fibrinogen has been widely advocated as a useful tool for the diagnosis of DIC but, in fact, is not very helpful to diagnose DIC in most cases [5,132]. Fibrinogen acts as an acute-phase reactant and despite ongoing consumption plasma levels can remain well within the normal range for a long period of time. In a consecutive series of patients, the sensitivity of a low fibrinogen level for the diagnosis of DIC was only 28% and hypofibrinogenemia was detected in very severe cases of DIC only. Sequential measurements of fibrinogen might be more useful and provide diagnostic clues.

Thrombelastography (TEG) is a method that has been developed decades ago and provides an overall picture of *ex vivo* coagulation. Modern techniques, such as rotational thrombelastography (ROTEM), enable bedside performance of this test and have again become popular recently in acute care settings [133]. The theoretical advantage of TEG over conventional coagulation assays is that it provides an idea of platelet function as well as fibrinolytic activity. Hyper- and hypocoagulability as demonstrated with TEG was shown to correlate with clinically relevant morbidity and mortality in several studies [134,135], although its superiority over conventional tests has not unequivocally been established [136]. Also, TEG seems to be overly sensitive to some interventions in the coagulation system, such as administration of fibrinogen, of which the therapeutic benefit remains to be established. There are no systematic studies on the diagnostic accuracy of TEG for the diagnosis of coagulation-induced coagulopathy; however, the test may be useful for assessing the global status of the coagulation system in critically ill patients.

For the diagnosis of the most extreme form of coagulation activation in critically ill patients, disseminated intravascular coagulation, a simple scoring system has been developed [137]. The score can be calculated based on routinely available laboratory tests, that is, platelet count, prothrombin time, a fibrin-related marker (usually D-dimer), and fibrinogen. Tentatively, a score of 5 or more is compatible with DIC, whereas a score of less than 5 may be indicative but is *not* affirmative for nonovert DIC. By using receiver-operating characteristics curves, an optimal cutoff for a quantitative D-dimer assay was determined, thereby optimizing sensitivity and the negative predictive value of the system [127]. Prospective studies show that the sensitivity of the DIC score is 93%, and the specificity is 98% [138,139]. The severity of DIC according to this scoring system is related to the mortality in patients with sepsis [30]. Linking

prognostic determinants from critical care measurement scores such as Acute Physiology and Chronic Health Evaluation (APACHE-II) to DIC scores is an important means to assess prognosis in critically ill patients. Similar scoring systems have been developed in Japan [140].

32.10 Supportive Treatment of Coagulation Abnormalities during Severe Inflammation

The keystone of the treatment of hemostatic abnormalities in patients with severe inflammation is the specific management of the underlying disease. However, in many cases additional supportive treatment, aimed at circulatory and respiratory support and replacement of organ function, is required. Coagulation abnormalities may proceed, even after proper treatment has been initiated. In those cases, supportive measures to manage the coagulation disorder may be considered and may positively affect morbidity and mortality. The increase in the insight into the various mechanisms that play a role in the coagulation abnormalities associated with sepsis has indeed been accommodating in the development of such supportive management strategies.

Low levels of platelets and coagulation factors may increase the risk of bleeding. However, plasma or platelet substitution therapy should not be instituted on the basis of laboratory results alone; it is indicated only in patients with active bleeding and in those requiring an invasive procedure or otherwise at risk for bleeding complications [141]. The presumed efficacy of treatment with plasma, fibrinogen, cryoprecipitate, or platelets is not based on randomized controlled trials but appears to be rational therapy in bleeding patients or in patients at risk for bleeding with a significant depletion of these hemostatic factors [142]. It may be necessary to use large volumes of plasma to correct the coagulation defect. Coagulation factor concentrates, such as prothrombin complex concentrate, may overcome this obstacle, but these compounds may lack essential factors, such as factor V. Moreover, in older literature, caution is advocated with the use of prothrombin complex concentrates in DIC, since it may worsen the coagulopathy due to small traces of activated factors in the concentrate. It is, however, not clear whether this is still relevant for the concentrates that are in use. Specific deficiencies in coagulation factors, such as fibrinogen, may be corrected by administration of purified coagulation factor concentrates.

Experimental studies have shown that heparin can at least partly inhibit the activation of coagulation in severe inflammatory conditions [143]. Uncontrolled case studies in patients with sepsis and DIC have been claimed to be successful. However, a beneficial effect of heparin on clinically important outcome events in patients with DIC has never been demonstrated in controlled clinical trials [144]. Interestingly, withdrawal of heparin was found to be detrimental to the prevention of severe thrombotic events in patients with sepsis (such as cerebral stroke, myocardial infarction, or pulmonary embolism) in patients with sepsis [145]. Therapeutic doses of heparin are indicated in patients with clinically overt thromboembolism or extensive fibrin deposition, like purpura fulminans or acral

ischemia. Patients with sepsis may benefit from prophylaxis to prevent venous thromboembolism, which may not be achieved with standard low-dose subcutaneous heparin [146].

In view of the deficient state of physiological anticoagulant pathways in patients with severe infammation, restoration of these inhibitors may be a rational approach [53]. Since activated protein C and antithrombin are the most important physiological inhibitors of coagulation and based on successful preclinical results, the use of concentrates of these factors in patients with sepsis has been studied relatively intensively. Most of the randomized controlled trials concern patients with sepsis, septic shock, or both. All trials, showing some beneficial effect in terms of improvement of laboratory parameters, shortening of the duration of DIC, or even improvement in organ function, however, failed to reduce the 28-day mortality.

The most promising intervention at this moment is recombinant soluble thrombomodulin. Recombinant human soluble thrombomodulin binds to thrombin to form a complex that inactivates thrombin's coagulant activity and activates protein C. Several preclinical studies in experimental sepsis models have shown that soluble thrombomodulin is capable of improving the derangement of coagulation and may restore organ dysfunction [53]. In phase I–II clinical studies, the pharmacokinetic profile of recombinant soluble thrombomodulin was determined [147]. In a subsequent phase III randomized double-blind clinical trial in patients with DIC, administration of the soluble thrombomodulin had a significantly better effect on bleeding manifestations and coagulation parameters than heparin, but the mortality rate at 28 days was similar in the two study groups [148]. DIC patients ($n = 234$) were randomized to receive soluble thrombomodulin (ART-123) at a daily dose of 0.06 mg per kg for 30 min, or unfractionated heparin (8 U per kg per hour for 24 h) for 6 days, using a double-dummy method. The primary efficacy end point was DIC resolution rate. DIC was resolved in 66.1% of the thrombomodulin group, compared to 49.9% of the heparin group (difference 16.2%; 95% confidence interval (CI) 3.3–29.1). Patients in the thrombomodulin group also showed more marked improvement in clinical course of bleeding symptoms. The incidence of bleeding-related adverse events up to 7 days after the start of infusion was lower in the thrombomodulin group than in the heparin group (43.1% versus 56.5%, $P = 0.0487$) [148]. When limiting these results to patients with severe infection and sepsis, DIC resolution rates were 67.5% in thrombomodulin-treated patients and 55.6% in the control group, and 28-day mortality rates were 21.4 and 31.6%, respectively. Mortality rates of patients who recovered from DIC were 3.7% in the thrombomodulin group and 15% in the heparin group [149]. Recently, soluble thrombomodulin was evaluated in a phase II/III clinical study in 750 patients with sepsis and disseminated intravascular coagulation. Twenty-eight-day mortality was 17.8% in the thrombomodulin group and 21.6% in the placebo group. Markers of coagulation activation were lower in the thrombomodulin group than in the placebo group. There were no differences between groups in bleeding or thrombotic events.

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33

Fever: Mediators and Mechanisms

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33.1 Introduction

Fever is a frequently observed sign in critically ill patients, which occurs in response to many types of infectious or noninfectious stimuli. It is just one component of a complex array of host defense responses, which develop during the time course of systemic inflammation. Collectively termed acute-phase response (APR), there is a pronounced systemic reaction to disturbances of homeostasis by infections, tissue injury, trauma/surgery, neoplastic growth, or immunological disorders; fever is one of the most prominent components of the APR, a hallmark of disease [1–3]. Depending on the severity of a given inflammatory insult or environmental conditions, phases of fever and phases of hypothermia can alternate during systemic inflammation [4–6]. Recently, the hypothermic component of body temperature (T_b) alterations during systemic inflammation gained some attention in experimental research [7,8]. Still, the main focus of this chapter is the febrile response, the regulated and actively established increase of T_b, which occurs along with all the other components of the APR. Special emphasis will be directed to putative exogenous and endogenous mediators of fever and to the transfer of peripherally generated inflammatory signals to those brain structures, which are implicated in the manifestation of the febrile response.

In this context, it has to be considered that fever is not an isolated brain-mediated modification, which occurs during systemic inflammation. There is rather a characteristic pattern of disease-related and brain-controlled symptoms, which have collectively been termed sickness behavior. Some of these symptoms (see also Chapters 32 and 37) are anorexia and adipsia, hyperalgesia, anxiety, fatigue and sleep disturbance, lethargy, loss of interest in usual activities, social withdrawal, signs of depression, listlessness, malaise, and cognitive dysfunction [9–11]. Although exogenous and endogenous mediators of fever and sickness behaviors are – at least in part – overlapping or identical, the brain sites

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and neural circuitries that are employed to generate fever or to establish behavioral and cognitive modifications are distinct [10,12–14].

According to the classical view, fever develops in a characteristic sequence of steps starting with the appearance of the pathogenic agent, the "exogenous pyrogen" in the afflicted host. In case of infection, the exogenous pyrogen frequently belongs to a family of molecules termed pathogen-associated molecular patterns (PAMPs, see Chapter 2). This exogenous pyrogen, in turn, causes the release of fever-producing substances by the host's polymorphonuclear leukocytes and by other cells. These substances are, therefore, called "endogenous pyrogens" [1,15,16]. Traditionally, a number of cytokines (see, for example, Chapters 23, 24, and 26), namely, interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), interferons (IFNs), and others were identified and characterized as the endogenous pyrogens [1,17–20]. Nowadays, it is clear that there are further peripherally elaborated substances, other than cytokines, which fulfill criteria of an endogenous pyrogen, depending on the experimental model used to investigate fever. For example, lipopolysaccharide (LPS), the most frequently studied experimental fever-inducing agent, employs the complement (C) fragment C5a (see also Chapter 33) and peripherally generated prostaglandin E_2 (PGE_2) for the initiation of the rapidly developing first phase of the febrile response [21–24]. The most important endogenous pyrogens are introduced in detail in Section 33.2 of this chapter, after a brief look at the physiological and neuroanatomical background for the phenomenon fever. Thereafter (Section 33.3), the putative mechanisms for the transfer of the inflammatory message across the blood–brain barrier to the critical brain sites for the generation of fever are dealt with. Some new discoveries on this issue were published in the past few years [25–29]. The substances, which might influence the brain-intrinsic neuronal circuitries to activate efferent pathways to those peripheral effector organs that trigger fever, are discussed in Section 33.4 of this chapter. These are called centrally acting endogenous pyrogens. Some, but not all of them, are identical to peripherally generated endogenous pyrogens, but classical neurotransmitters such as noradrenaline (NA) are included as well [3]. Indeed, for most fevers a sustained brain-intrinsic formation of PGE_2 seems to be essential for the maintenance of an increased T_b.

Finally, a look at clinical fevers and possibilities for and outcome of fever treatment will close this chapter. Within this section, the arguments for fever being a friend or rather a foe will also be introduced [30].

33.2 Fever: Physiological and Neuroanatomical Considerations

33.2.1 Fever Defined

In the latest version of the "glossary of terms for thermal physiology" [31], fever is defined as "*a state of elevated core temperature which is often, but not necessarily, part of the defensive responses of organisms to the invasion of live or inanimate matter recognized as pathogenic or alien by the host. The rise in core temperature*

is usually designated as due to a change in the thermocontroller characteristics resulting in an elevation of the set point of body temperature. The higher temperature is actively established and defended by the operation of heat-producing and heat-conserving thermoeffectors . . . core temperature is eventually reduced when the set point of temperature regulation returns to its normal level. While any rise in body temperature may be due to fever, those rises which are not accompanied by supportive changes in thermoeffector activities, are termed hyperthermia. The humoral mechanisms underlying fever consist of the alteration of the neuronal control of body temperature by agents named pyrogens." Indeed, there is evidence from numerous experimental studies that the rising phase of fever is accompanied by appropriate thermoeffector activities such as increase in metabolic heat production by shivering or nonshivering thermogenesis (NST), reduced peripheral blood flow, or depressed panting ([3], for review). On the other hand, there is a problem with this physiological definition of fever; this problem is related to the term "elevation of the set point of body temperature." According to the classical concept [31–33], the "set point" is defined as a reference temperature that is compared with the regulated value of T_b. Any difference between this reference value and the actual T_b is an error signal that elicits corrective thermal responses proportional to the deviation with the aim to restore the T_b to its proper reference level. A neuronal correlate to such a reference signal has never been detected and obviously does not exist.

Another model for the physiological manifestation of fever was suggested that does not require a set point and a reference in the form of a neuronal signal [34]. According to this model, fever is achieved by the modification of thresholds and gains for the various thermoregulatory effectors, as shown schematically in Figure 33.1.

In this context, "threshold temperature" is defined as a given T_b, which activates appropriate and available thermoeffector responses, when a deviation from this specific T_b occurs [31]. As illustrated in the upper part of Figure 33.1, the rate of heat production of a nonfebrile subject begins to increase at a T_b slightly below 37 °C, while heat dissipation mechanisms are activated at a T_b of slightly above 37 °C. After challenge with a pyrogen (lower part of Figure 33.1), these threshold temperatures are symmetrically shifted to values of 39.3 °C (heat production) and 39.7 °C (heat dissipation).

Different thermoeffectors are activated at distinct threshold temperatures. In most cases, cutaneous vasoconstriction in humans is activated at a higher mean T_b compared to the T_b at which shivering or NST is initiated. During the course of fever, these thresholds can change, so that actual T_b responses can vary, for example, according to environmental conditions ([3,6], for review).

According to current concepts of the organization of the thermoregulatory system, the efferent neuronal pathways for the activation of individual thermoeffectors (skin vasomotor tone, shivering, NST, salivary secretion, panting, or warm- and cold-seeking behaviors) form independent loops that operate in a coordinated manner [35–38]. It was, therefore, suggested that the "set point"-based definition of the fever should be replaced by a "balance point"-based definition [6,39]. Accordingly, the balance point during fever would be above that during normothermia [6]. Based on these concepts, the question arises how a

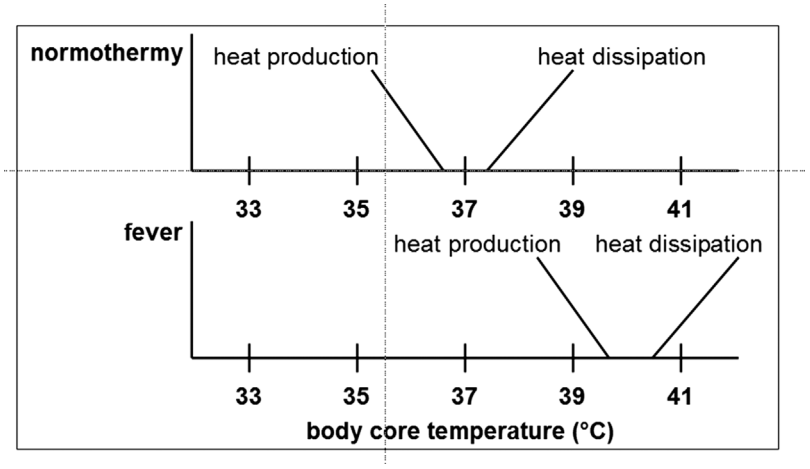


Figure 33.1 Thermoregulatory thresholds under normal conditions (normothermy, upper part) and during the rising phase of fever (fever, lower part). In normothermic subjects, heat production increases in a linear fashion when T_b is decreasing below 36.8 °C; heat dissipation is increasing when T_b is climbing above 37.2 °C. During the rising phase of fever, both thermoregulatory thresholds are symmetrically shifted to higher values, meaning that heat production is activated when T_b is decreasing below 39.3 °C, while heat dissipation is activated at a T_b higher than 39.7 °C.

given pyrogen interferes within the identified components of the thermoregulatory system, which consist of a sensory afferent part, a central integrative part, and an efferent part terminating at the distinct effector organs [38]. PGE_2 is a critical terminal mediator of many fevers and it exerts direct effects on neurons. In the following section, we therefore use PGE_2 as a representative example for a pyrogen directly acting at critical relay stations of the neuronal thermoregulatory circuitries.

33.2.2 Possible Sites of Action for Putative Pyrogens within the Neuronal Thermoregulatory Circuitries

Rapid thermoregulatory responses occur when changes in environmental or ambient temperature (T_a) are monitored by thermosensors in the skin. For example, skin cooling is monitored by ion channels of cold-sensitive nerve fibers, and this information is transmitted as a feedforward signal to the central thermoregulatory structures located within the preoptic anterior hypothalamic area (POA) of the brain [39]. We recently showed that a subpopulation of these cold-sensitive neurons is also stimulated by PGE_2 [40]. By acting on peripheral sensory nerves (Figure 33.2), PGE_2 could thus contribute to the manifestation of cold defense responses, which are observed when fever is initiated.

Depending on the pyrogenic stimulus, its strength, and the route of its administration, there is some evidence that PGE_2 can contribute to the initiation of fever ("early phase," see Sections 33.3 and 33.4) by activating afferent nerve fibers from the skin [41,42], from the vagus nerve [3,43–45], or from the trigeminal nerve [46].

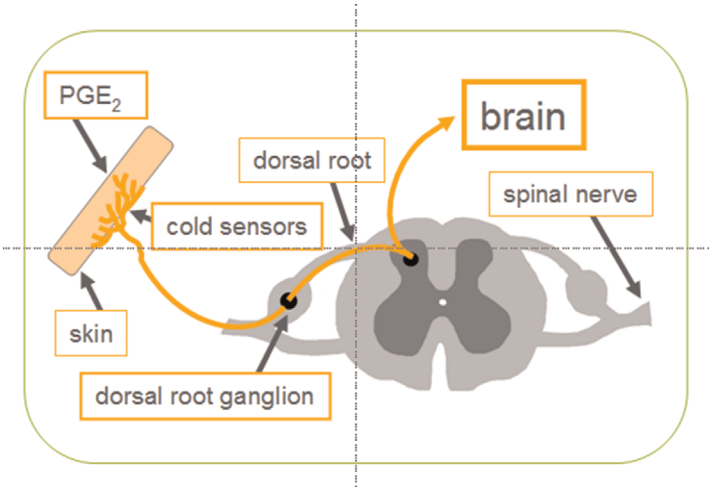


Figure 33.2 Schematic illustration of the first part of the initial components of the thermoafferent pathway, starting from the cold-sensitive nerve fibers in the skin and reaching the dorsal horn of the spinal cord. Recently, a population of neurons from dorsal root ganglia was detected, which is activated by cold/menthol and by PGE_2 [40]. Since the activation of cutaneous cold sensors evokes appropriate cold defense responses, a stimulation of these fibers by PGE_2 could moderately contribute to the rapidly developing rising phase of fever.

The information generated by peripheral thermosensors is transmitted to the spinal dorsal horns (Figure 33.2), where secondary neurons receive and transport the signals via collaterals to the lateral parabrachial nucleus (LPB) in the pons. LPB neurons, in turn, project to the critical site within the POA, the median preoptic nucleus (MnPO), which is activated via glutamatergic neurotransmission ([47], for review). MnPO and the closely located medial preoptic area (MPO) seem to be essential for the manifestation of fever (Figure 33.3, Sections 33.3 and 33.4). These structures represent the so-called "pyrogenic zone" within the POA, where smallest amounts of PGE_2 cause a rise of T_b [48].

The neurons within the MnPO, which receive the glutamatergic input from the LPB and are responsive to skin cooling, use, in part, gamma amino butyric acid (GABA) as neurotransmitter [47,49,50]. It was proposed that these GABAergic neurons inhibit warm-sensitive neurons within the POA, which in turn exert a tonic inhibitory influence on thermogenic neurons located in the dorsomedial hypothalamus (DMH) and in the rostral raphe pallidus nucleus (rRPa, Figure 33.3). After disinhibition by external cooling or by the appearance of PGE_2 within the POA, appropriate thermoeffector responses are activated via DMH/rRPa and descending neuronal pathways to brown adipose tissue (BAT), blood vessels, or skeletal muscles ([38,47], for review). Within these central thermoregulatory pathways, receptors of the subtype EP3 for PGE_2 (and possibly receptors for other pyrogenic or thermogenic substances, see Section 33.4) are present not only in the MnPO but also in the rRPa [51]. It was, therefore, speculated if the rRPa represents a subsidiary fever center in addition to the MnPO/MPO area, but injections of PGE_2 into the rRPa had no pyrogenic effect [52].

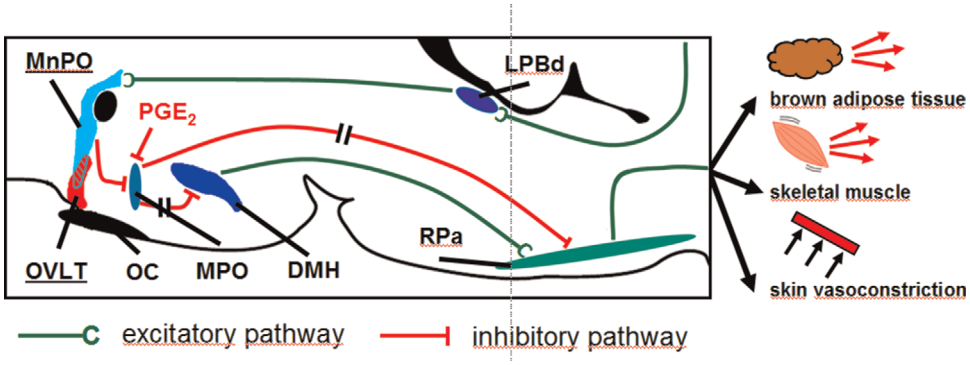


Figure 33.3 The neurons in the dorsal horn of the spinal cord, which receive the afferent input from cutaneous cold sensors (see Figure 33.1), project to the LPB. From there, the thermoafferent information is transmitted to the MnPO. In a cold environment, neurons within the MnPO are activated via glutamatergic neurotransmission. These neurons are also influenced by PGE_2 in a manner that nearby MPO neurons are inhibited by a GABAergic mechanism, resulting in a disinhibition of cold-defensive or fever-generating responses. The efferent pathways to brown adipose tissue (nonshivering thermogenesis), skeletal muscles (shivering, "chills"), or peripheral blood vessels (vasoconstriction) are routed via the DMH or the raphe pallidus nucleus (RPa). In summary, disinhibition of heat-generating and heat-conserving responses can be achieved either by the peripheral cooling or by the appearance of PGE_2 within the MnPO during fever (modified according to [38,47,49]). OC = optic chiasm; OVLT = organum vasculosum laminae terminalis.

33.2.3 Experimentally Induced Fever

Experimental fever research aims to generate reproducible, stereotypic febrile responses of more or less constant height and duration. This approach allows the evaluation of the physiological processes that underlie the distinct phases of fever (see Section 33.2, Figure 33.4). Experimental fever further enables researchers to identify and characterize putative endogenous mediators produced and released outside and inside the brain during the course of the febrile response. A variety of experimental fever models have been developed for these reasons ([3], for review). Many substances ("exogenous pyrogens"), which evoke fever when injected into experimental animals, are ligands of Toll-like receptors (TLRs, see Chapter 8). Depending on the nature of these substances, they are called "bacterial, viral, or fungal mimetics," although these synthetic molecules do not completely reflect a natural infection. These experimental approaches have in common that specific components of innate immunity are readily activated by the injection of all these molecules. With regard to putative endogenous mediators or mechanisms responsible for the manifestation of distinct febrile responses, there is thus some analogy among the various models of experimentally induced fevers, but there are also some differences [3]. In the beginning of experimental fever research, the rabbit was frequently used as animal model due to the high pyrogenic sensitivity of this species [1]. Nowadays, the preferred pyrogenic agent is LPS and animal subjects used in the majority of current experimental fever research are rats and mice. More recently, genetically modified mice were

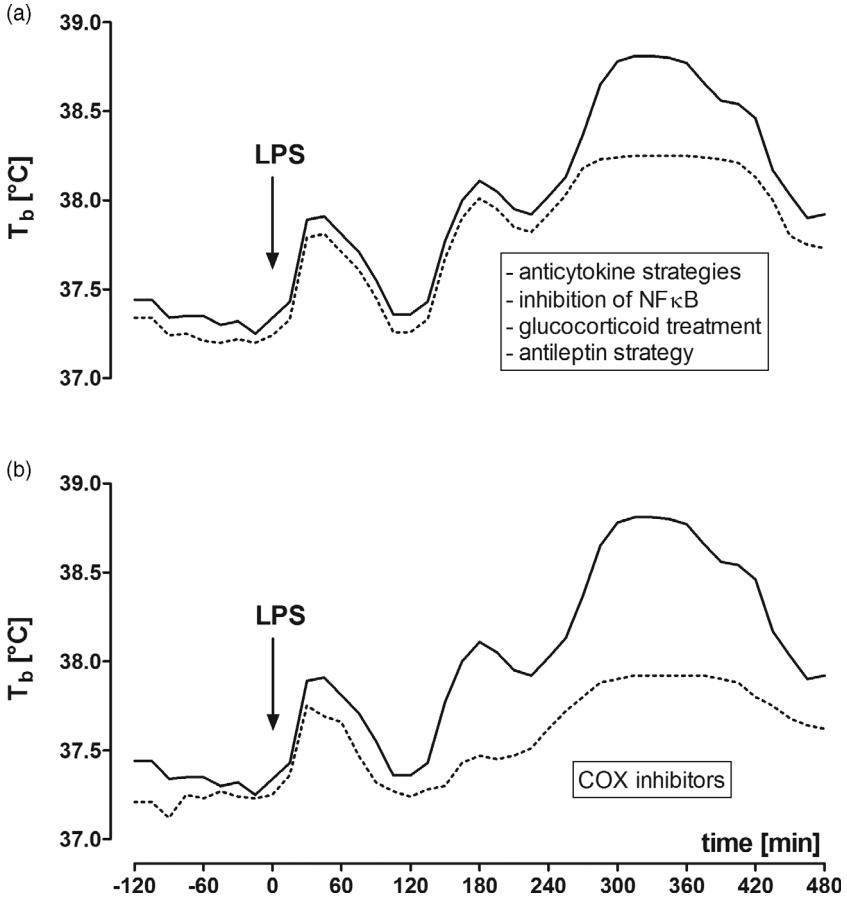


Figure 33.4 Schematic illustration of the course of experimental fever in rats in response to intraperitoneal injection of a moderate LPS dose. At time "0," LPS was injected either alone (continuous lines) or in combination with a drug that reduces fever (interrupted lines). From 0 to 60 min, rats show a stress-induced increase in T_b due to the injection procedure, followed by a biphasic febrile response. Drugs that inhibit production or biological action of cytokines, or leptin cause an attenuation of the longer second fever phase (a). Drugs, which inhibit formation of PGE_2 (COX inhibitors) cause an attenuation of both fever phases (b) (3,19,58–61] for further details).

employed to characterize specific molecules, even specific cell types or organs, in the manifestation of fever or other brain-controlled illness responses [22,25,26,28,53–57]. Again, in most of these studies, LPS was used as the exogenous trigger of fever.

At low to moderate doses, LPS evokes a prototypic and reproducible fever that consists of several sequential phases [1,3,6,24] usually with a total duration of about 6–8 h. At high doses, LPS can cause a T_b fall followed by a rise, which can last several days [5,62]. Characteristic fevers, evoked in rats by injections of a moderate dose of LPS and their modulation by distinct pharmacological interventions, are shown and discussed in Section 33.2 (Figure 33.4).

33.3 Putative Peripherally Generated Endogenous Pyrogens

In 1948, Beeson introduced the concept of the "endogenous pyrogen." He obtained a heat labile substance from isolated peritoneal macrophages, which induced fever in healthy rabbits [15]. The assumption of this concept was that after injection of an "exogenous pyrogen" (e.g., LPS), the "endogenous pyrogen" would appear in the circulation of the LPS-stimulated animal to induce fever. Later, it turned out that this substance is represented by a family of small proteins termed cytokines ([1,17,20]; see also Chapters 23–27). With a few exceptions, circulating cytokines are not detected in healthy individuals, but appear in the blood during various states of disease to regulate and coordinate immune responses. Pathogens share highly conserved microbial components called "pathogen-associated molecular patterns" (PAMPs). These activate the innate immune system via TLRs (see Chapter 8). Employing a number of intracellular adapter proteins, activation of the transcription factor nuclear factor-κB (NF-κB) occurs, translocating it into the nucleus of the PAMP-stimulated cell [63–65]. Here, the transcription of a number of target genes is initiated, some of which are related to fever. These include cytokines (TNF-α, IL-1β, IL-6, and others), chemokines, adhesion molecules, receptors, and the inducible (i) forms (as opposed to constitutive) of inflammatory enzymes including nitric oxide synthase, cyclooxygenase-2 (COX-2), and microsomal prostaglandin E synthase-1 (mPGES-1). COX-2 and mPGES-1 are strongly coupled in their induction after an inflammatory insult and considered to be the critical enzymes for the generation of PGE_2 during fever [66]. LPS, the most potent exogenous pyrogen, employs a second, faster way for peripheral formation PGE_2 by activation of the complement cascade via the alternative pathway ([67], see Section 33.2.2). In addition to TNF-α, IL-1β, IL-6, and PGE_2, there are more peripherally produced molecules with intrinsic pyrogenic properties upon stimulation of TLRs by a given PAMP ([3], for review).

33.3.1 The Classical Pyrogenic Cytokines: TNF-α, IL-1β, and IL-6

Following the systemic injection of LPS or other agonists of TLRs, TNF-α, IL-1β, and IL-6 appear in the blood in a characteristic sequence, which made them appropriate candidates to act as circulating pyrogens. TNF-α is the first of these cytokines to appear in the bloodstream, followed by a rather small elevation of IL-1β and a longer lasting and bigger rise of IL-6 ([1,3,19,20], for review). These cytokines, together with other molecules (PGE_2), influence the production of each other or the inhibition of it. These interrelationships underlie the so called "cytokine cascade," this is the self-amplification and the generation and release of other cytokines in a complex, integrated network. In this context, studies employing mice deficient in a given cytokine ("knockout mice, KO mice) have to be interpreted with caution due to redundancies so that the lack of one cytokine may be compensated by another cytokine [53]. Another critical issue is that KO mice lack a given cytokine not only in the periphery but also in the brain, where several cytokines seem to participate in the manifestation of fever (see Sections 33.3 and 33.4).

The following further arguments support a role of peripheral TNF-α, IL-1β, or IL-6 for the generation of fever after appearance of an exogenous pyrogen. Systemic injections of all the three of these cytokines can evoke strong (IL-1β) to moderate (IL-6) fevers in man or experimental animals, although pharmacological rather than physiological doses are sometimes required to evoke a febrile response [1,3,19]. Several tools have been employed, which aimed to neutralize, antagonize, or block the formation of single circulating cytokines after injection of a given exogenous pyrogen [1,5,19,58,68–71]. In most cases, these interventions selectively modified (attenuated) the so called "late phase" but not the initial phase of the febrile response (Figure 33.4a).

Similarly, systemic inhibition of the early inflammatory transcription factor nuclear factor kappa B (NFκB), which is involved in the stimulus-induced formation of the cytokine cascade, exerted an inhibitory effect on LPS fever. This inhibition was restricted to the late phase of the febrile response [59]. The same effect occurs after treatment of experimental animals with glucorticoids [60], which also depress production of cytokines. Interestingly, treatment of rats with an antiserum against the cytokine-like hormone leptin also attenuates the late phase of LPS fever. This observation suggested a role for leptin as mediator of inflammation and fever [72,73].

Based on all these observations, the classic endogenous pyrogens (cytokines) seem to contribute to the maintenance of experimentally induced fever, while there has to be a trigger for the initiation of the "early phase" of fever. At least in response to intravenous injections of LPS, the onset of fever indeed seems to precede the appearance of pyrogenic cytokines in the blood [3,23,45]. This temporal discrepancy as well as the outcome of many anticytokine approaches (Figure 33.4b) inspired the search for another trigger for the onset of fever, which would have to be produced rapidly after an exogenous pyrogen appears in the given experimental animal.

33.3.2 Putative Peripheral Triggers for the Early Phase of Experimental Fever: PGE2, Complement Fragment C5a, or Oxygen Radicals

PGE2 is a derivative of arachidonic acid, which is cleaved from membrane phospholipids by phospholipase A2 (PLA2). In the brain, an alternative pathway to produce arachidonic acid for PGE2 synthesis seems to operate ([27,28]; see also Section 33.3). In a second step, arachidonic acid is converted to PGH2 by constitutive (COX-1) or inducible (COX-2) cyclooxigenase. Finally, PGH2 is isomerized to PGE2 by prostaglandin E synthase (PGES). COX-2 and microsomal PGES-1 are inducible enzymes that are regulated transcriptionally by NFκB and STAT3. These transcription factors are activated by numerous exogenous and endogenous pyrogens [26,59,70,74]. In contrast to the various "anticytokine strategies" (Figure 33.4a), systemic treatment with COX inhibitors, thereby preventing formation of PGE2, attenuates not only the late phase but also the early phase of fever induced by LPS (Figure 33.4b) or other exogenous pyrogens [60,61,75,76]. Based on the observation that peripheral, but not central, administration of antibodies against PGE2 selectively reduced the first phase of the febrile response, it was suggested that peripherally derived PGE2 is the trigger

for the early phase of fever [24]. Macrophages of the lung and the liver have been identified as the cells, which produce PGE2 via induced expression of COX-2 and mPGES-1 to trigger fever [77].

For the experimental LPS–fever model, several studies provided evidence for a central role of liver Kupffer cells (Kc, hepatic macrophages) in the formation of PGE_2 as a peripheral trigger of the febrile response ([3], for review). According to these studies, the febrigenic process is initiated by the arrival of LPS in the liver and its uptake by Kc [78], causing virtually immediately the activation of the complement (C) cascade and thereby the generation of the complement fragment C5a [21,22]. C5a, in turn, stimulates the Kc very rapidly to release constitutive COX-1- and COX-2-dependent PGE_2 [23,79]. Indeed, via this mechanism, peripheral PGE_2 is elevated at the onset of fever [79] and thus a likely candidate to trigger the first phase of fever (Figure 33.4b). It should, however, be noted that the rapid activation of C5a is a specific feature of LPS but not of other exogenous pyrogens [3].

The production of free oxygen radicals by neutrophils and macrophages is enhanced immediately after LPS challenge. It has, therefore, been suggested that these products could play a role in fever production. Methylene blue, an inhibitor of superoxide and hydroxyl radical formation, was administered to rabbits prior to a challenge with LPS. The usual febrile response to LPS was, indeed, consequently depressed in these animals, apparently supporting a role for oxygen radicals in the manifestation of fever [80,81]. The authors of these studies, therefore, suggested that the LPS-induced rise of T_b could normally result from the rapid and excessive production of reactive oxygen species that, in turn, could be sensed by redox-sensitive sites of glutamate receptors in the brain. They also suggested that the antipyretic activity of aspirin could, therefore, be due to its antioxidant activity rather than to its inhibition of prostaglandin formation. A major objection to this hypothesis is its rejection of PGE_2 as a critical mediator of fever [82], whereas most data support its essential role not only as a neuronal mediator in the brain but also as a peripheral trigger of the febrile response (see above).

Having introduced the central thermoregulatory circuitries in the brain and some of the putative peripheral mediators of fever, the question how the peripherally generated pyrogenic message is transferred into the brain is addressed.

33.4 Transfer of Fever-generating Signals Across the Blood–Brain Barrier

As discussed in Section 33.1 and shown in Figure 33.3, the structures, where PGE_2 (or any other pyrogenic substance) causes the febrile shift of the thermoregulatory balance point to a higher level, are located inside the blood–brain barrier (BBB). Intensive research was directed to the possible mechanisms of how a peripherally appearing or generated pyrogen might transport a message to the relevant thermoregulatory structures. Here, three possible routes for the transfer of febrile signals to the brain are going to be introduced: (i) the circumventricular organs

lacking a tight BBB, (ii) cells forming the BBB and acting as relay stations for the transfer of febrigenic signals to the brain, and (iii) the activation of afferent nerves by peripherally generated pyrogens (Figure 33.2).

33.4.1 Sensory Circumventricular Organs (Cvos)

Sensory Cvos form a subgroup of the CVOs and include the *area postrema* (AP), the *organum vasculosum laminae terminalis* (OVLT), and the subfornical organ (SFO). The OVLT and SFO are located within the lamina terminalis in the anterior wall of the third ventricle (AV3 V), while the AP is situated in the dorsal vagal complex in the medulla oblongata [3,83]. The parenchyma capillaries of the sensory CVOs possess a fenestrated endothelium, thus allowing circulating hydrophilic signal molecules, including endogenous pyrogens, to enter the brain. The parenchyma of the CVOs is composed of neurons and glial cells, and bidirectional neuronal connections between sensory CVOs and preoptic anterior hypothalamic nuclei have been identified. Thus, the neural signal transfer of inflammatory information into the brain, that is, the MnPO (Figure 33.3), is possible [84]. With regard to fever, special attention has been directed to the OVLT due to its proximity to the MnPO/MPO. The hypothesis that the OVLT may serve as a portal of entry for circulating pyrogens based on the classical observation that electrolytic ablation of the OVLT prevented fever in response to the peripheral injection of LPS [85]. Although there are some problems with the interpretation of lesion studies [86], there are other arguments in support of a role for sensory CVOs in the detection of circulating cytokines or even PAMPs. There is an extensive expression of receptors for cytokines and even TLRs in all of the three sensory CVOs ([3,83,87], for review). This means that cells within sensory CVOs can directly respond to PAMPs with the expression of inflammatory genes, thereafter with production of a second pool of cytokines, and other cellular responses [88]. Clearly, the sensory CVOs belong to the most responsive sites within the CNS to systemic inflammatory stimulation [89]. However, their role as essential or exclusive central sensors of cytokines or PAMPs is challenged by the fact that cells of the BBB in the surrounding regions including MnPO/MPO, endothelial cells, and perivascular macrophages are equipped with receptors for PAMPs and cytokines ([3], for review) and are also highly responsive to inflammatory stimulation [90,91].

33.4.2 Cells Forming the Blood–Brain Barrier

Cells forming the BBB (vascular endothelial cells and perivascular cells) within the entire brain have the capacity to secrete cytokines (IL-6), PGE_2, and other mediators into the brain parenchyma after their respective inflammatory stimulation from the luminal (blood) side [90–92]. These cells thus seem to play an important role in the transduction of inflammatory signals from the blood to the brain [93]. With regard to LPS-induced fever, the expression on BBB-associated cells of TLR4 and of receptors for pyrogenic cytokines on brain endothelial cells is especially relevant. Consequently, all these pyrogens have the capacity to directly activate the brain endothelium. In brain endothelial cells, and also in cells within

the sensory CVOs, activation of inflammatory transcription factors occurs during systemic inflammation. NF-κB is induced either directly by PAMPs (LPS and others) binding to their respective TLRs or by the pyrogenic cytokines IL-1β and TNF-α. Circulating IL-6 employs the transcription factor signal transducer and activator of transcription-3 (STAT3); pronounced nuclear translocation of STAT3 occurs in brain endothelial cells and astrocytes of sensory CVOs upon stimulation with IL-6 ([3,74], for review). More recently, the activation of a third transcription factor, nuclear factor IL-6 (NF-IL-6), was demonstrated in brain cells during LPS-induced fever, again predominantly in sensory CVOs and brain endothelial cells [94]. In contrast to NF-κB and STAT3, the nuclear translocation of NF-IL-6 coincides with the late phase of fever, so that its putative role in the maintenance or even the termination of fever was suggested [29,95].

In the context of fever, the target genes of NF-κB and STAT3 include COX-2 and mPGES-1. Some new studies suggest that in the brain not phospholipase A2 (PLA_2) but rather monoacyglycerol lipase, through hydrolysis of the endocannabinoid 2-arachidonoylglycerol, provides the source of arachidonic acid and thereby the substrate for COX-2/mPGES-1 [27,28]. As already mentioned in Section 33.2, the coupled induction of COX-2 and mPGES-1 is important for inflammatory formation of PGE_2. The co-localized expression of both these inducible enzymes in brain endothelial cells is regarded as the crucial prerequisite for PGE_2 synthesis in the brain during fever [25,93,96]. The activation of NF-κB or STAT3 in brain endothelial cells is indeed associated with the appearance of COX-2 and mPGES-1 in these cells [70,97–99]. Consequently, the formation of PGE_2 specifically by brain endothelial cells has been suggested to be essential for the production of LPS-induced fever [100]. Further evidence for a critical role of the brain endothelium in fever production is derived from studies in which mice with brain endothelial-selective deletions of IL-1-receptors, IL-6-receptors, or COX-2/mPGES-1 were employed [25,26,101]. In all these studies, the febrile response of mice was impaired, and the lack of IL-1- or IL-6 receptors further resulted in an inhibition of NFκB (IL-1 receptor deletion) or STAT3- (IL-6 receptor deletion) mediated induction of COX-2.

Within the MnPO/MPO, one could assume that the PGE_2 produced by endothelial cells is released abluminally and might diffuse to the responsive preoptic neurons (Figure 33.3). However, it has to be mentioned that the inflammatory induction of COX/mPGES-1 does not occur exclusively within the brain sites, which are implicated in fever and other components of the brain-controlled illness responses. For example, a strong inflammatory genomic activation occurs in endothelial cells of vessels located in the cortex [67]. It has recently been shown that COX-2-derived PGE_2 production in the cortex contributes to changes of neurovascular coupling by inducing hyperemia [102]. Still, the functional consequences of the inflammatory activation of brain intrinsic PGE_2 formation in so many different brain areas requires more attention in future research. Taken together, all these studies indicate that the genomic activation of brain endothelial cells by PAMPs or cytokines and the subsequent formation of PGE_2 via inducible COX-2/mPGES-1 are essential for the manifestation of fever, at least for the longer second fever phase (Figure 33.4). A faster signal transfer mechanism might be required to account for the early initiation of fever [3].

33.4.3 A Rapid Signal Transfer from the Periphery to the Brain via Sensory Nerves

During the 1990s, intensive research was directed toward a possible transmission of peripheral immune signals to the brain via sensory nerves with special focus on the vagus nerve [9–11,19,45]. The question whether such an alternative and rapid communication pathway between the activated immune system and those parts of the brain, which are involved in fever induction, makes a quantitative contribution to the febrile response was later on discussed with some controversial arguments [3,103,104]. The evidence for a participation of afferent sensory nerves in the manifestation of fever is summarized in this section.

33.4.3.1 The Afferent Vagus

Initial evidence for a role of the vagus nerve based on observations that bilateral truncal vagotomy attenuated or abrogated various inflammation-induced sickness responses including fever [3,9,10,19,43,44]. Subsequently, some controversy arose with regard to this issue [103,104]. Some studies showed that just fevers induced by low doses of pyrogens could be blocked by vagotomy, while the febrile responses to a challenge with high doses of LPS or pyrogenic cytokines were unimpaired [103,105]. The reason for this discrepancy may be caused by the fact that high concentrations of circulating pyrogens will readily activate the humoral inflammatory signal transfer to the brain (Sections 33.3.1 and 33.3.2), so that the neuronal contribution of the vagus nerve is overridden and difficult to detect [106]. It turned out that the involvement of vagally transmitted signals predominantly contributed to the early phase of fever induced by moderate doses of pyrogens and that this effect is mediated by the hepatic branche of the vagus nerve [103]. In this context, it should be reconsidered that the onset of LPS-induced fever shows a temporal relation to the uptake of LPS by liver Kupffer cells [78] and that the C5a-dependent rapid production of PGE_2 by these cells is a putative trigger for the early LPS-induced fever phase [22,23,79]. Kc-derived PGE_2 was, therefore, proposed to activate afferent fibers of the hepatic branches of the vagus nerve, which in turn forward this inflammatory message to the afferent projection areas of the vagus nerve within the nucleus of the solitary tract (NTS) [3,45]. The further processing of these neuronal signals, which have to reach the fever-inducing areas within the MnPO/MPO (Figure 33.3), will be addressed in Section 33.4.2.

33.4.3.2 Afferent Neuronal Signals from Other Nerves

Earlier studies provided some evidence that cutaneous afferent nerve fibers can, to a small degree, contribute to the manifestation of fever in an experimental model of localized subcutaneous inflammation. Injections of a low dose of LPS into a subcutaneous compartment resulted in a febrile response that was attenuated by coadministration of a local anesthetic [41]. It was proposed that some inflammatory mediator might have activated cutaneous cold-sensitive afferent nerve fibers. The activated cold sensory neurons, in turn, could have evoked the characteristic pattern of cold defense reactions, which are typical for the rising phase of fever [3]. PGE_2 turned out to be a likely candidate for an effect

like this [40], Figure 33.2). The febrile response, which was induced by a local subcutaneous injection of LPS, could be attenuated by coadministration of a low dose of a COX inhibitor into the subcutaneous site of inflammation. The same amount of the COX inhibitor was ineffective when injected into another site remote from the locus of LPS injection [42]. Similar observations were reported by another group, in this case for fever induced by injections of LPS into periodontal tissue. An inflammatory stimulation of the respective afferent fibers of the trigeminal nerve by locally produced PGE_2 seemed likely, due to the fact that fever was attenuated by injections of local anesthetics or COX inhibitors into the inflamed tissue [107].

Neural transmission of peripheral inflammatory signals to the brain has the advantage to operate faster than the humoral transmission, which requires transcriptional and translational processes in the periphery and/or at the BBB. With regard to a localized inflammation, another benefit of the neural signal transfer is that nerve terminals within the inflamed tissue can directly transmit the inflammatory status from the critical site of action to the brain [106]. Neural pathways may have the task to provide a first alarm-like response that originates at the site of a local inflammatory reaction and triggers almost immediately some nonspecific host defense responses including the initiation of an early phase of fever [3].

33.5 Centrally Acting Pyrogenic Mediators and Processing of Febrile Signals in the Brain

33.5.1 The Role of PGE2 in the MnPO

Within the MnPO, PGE_2 is thought to be released from brain endothelial cells into the brain parenchyma (see Section 33.3.2) and would there exert effects on neurons, which are compatible with generation of fever. Such neuronal effects could include direct inhibition of warm-sensitive neurons [47,49]. Four subtypes of PGE_2 receptors (EP_1–EP_4) have been described so far, which are all G protein-coupled receptors employing distinct signal transduction pathways [108]. In the MnPO, there is pronounced expression of EP_3 and EP_4 [108]. The EP3 receptor is critical for PGE_2-mediated fever since EP3 receptor-deficient mice fail to produce fever in response to centrally injected PGE_2 and to peripheral or central injections of other pyrogens [109,110]. Furthermore, the selective deletion of EP3 receptor genes in the MnPO of mice results in the depression the febrile responses to LPS or PGE_2 [55], confirming that this brain site is the presumptive site of the pyrogenic action of PGE_2. Indeed, EP3-expressing MnPO neurons are the origin of descending neuronal chains, which activate heat-generating and heat-conserving responses [49–51].

33.5.2 The Role of Noradrenaline in the POA

Intra-POA released noradrenaline (NA) seems to be an important central mediatior of the early phase of LPS-induced fever in connection with PGE_2-mediated stimulation of afferent fibers of the vagus nerve [3]. The noradrenergic

A1 and A2 cell groups in the medulla oblongata receive excitatory inputs from afferents from the abdominal (hepatic) vagus nerve. This excitation is transmitted to the POA via the ventral noradrenergic bundle, where a rapid increase in NA release can be monitored after peripheral injection of LPS [111]. Another major noradrenergic brain stem nucleus, the locus coeruleus, has also been proposed to participate in the mediation of fever [112]. The suggested mode of action of NA on neurons within the POA is similar to that, which was postulated for PGE_2, an inhibition of warm-sensitive neurons and thereby disinhibition of thermogenesis [3,111]. Indeed, in guinea pigs, intrahypothalamic microinjections of NA cause an increase of the thermoregulatory balance point by shifting the threshold T_b for heat production to a higher level [113]. Intra-POA release of NA might thus contribute to fever, for instance, during the rapidly developing first fever phase [3].

33.5.3 Other Centrally Acting Endogenous Pyrogens (Cytokines, Chemokines, and Other Substances)

There are a number of other substances, which cause an increase in T_b, supported by appropriate thermoeffector responses, when injected into the cerebral ventricles (intracerebroventricular, i.c.v.) of experimental animals. These substances include cytokines, chemokines, and other substances. The fact that i.c.v. injections of all these molecules evoke a fever-like response alone does not prove that one or several of these substances play a significant role within the brain to establish fever induced by a given exogenous pyrogen. The putative centrally acting pyrogenic substance should be produced and released in the brain after injection of an exogenous peripheral trigger of fever (LPS or other PAMPs, living bacteria, or other stimuli). Finally, drugs, which inhibit production or biological action of the centrally acting pyrogen, should have an inhibitory influence on fever.

IL-6 belongs to those cytokines, which are produced by cells forming the BBB during systemic inflammation and released into the brain ([20,106]; for review). I. c.v. injections of IL-6 in IL-6-KO and in other mice cause pronounced fever [1,114,115], while an i.c.v. injection of IL-6-antibodies attenuated the febrile response to experimental *E. coli* infection [116]. Other cytokines/chemokines including IL-1β, IL-8, MIP-1, or CINC-1 also fulfill the criteria of being centrally acting pyrogenic cytokines [71,117–120]. Central IL-8 and MIP-1 even induce fevers, which develop independent of PGE_2.

Other centrally acting substances with intrinsic pyrogenic properties include endothelin [121], endocannabinoids [57,122], opiods [123], and others ([3], for review). For some of these centrally acting pyrogenic substances (NA, IL-1β, and IL-6), the mechanism of action seems to be similar to that employed by PGE_2, that is an inhibition of warm-sensitive preoptic neurons [3,124–126]. Still, the precise roles of all these centrally acting pyrogenic molecules remain to be further characterized.

33.6 Clinical Aspects of Fever

Fever is one consequence of the complex process of inflammation. It usually occurs during various kinds of infections, but almost 50% of all clinical fevers are

of noninfectious origin (trauma, surgery, cerebral bleeding, tumors, thromboembolic events, autoimmune diseases, and others) [127]. The temporal patters of infectious or noninfectious clinical fevers are not comparable to the courses of experimental fevers as shown for LPS-induced fever in Figure 33.4. Some of the most prominent clinical febrile courses have recently been reviewed [3]. In brief, some fevers are characteristic of prolonged elevations of T_b with just small fluctuations ("continuous fever," for example, seen in patients with pneumonia [128–130). Fevers with larger fluctuations are called "remittent." When longer febrile episodes are interrupted by longer intervals without elevated T_b, these fevers are called "recurrent," malaria being a prominent example of this pattern ([3], for review). "Biphasic" clinical fevers have nothing to do with the two phases of LPS-induced fever (Figure 33.4). They rather consist of two phases lasting several days with two peaks, interrupted by a phase of reduced fever, which is observed in dengue and yellow fever [130]. For further patterns of clinical fevers and more details on the diseases that are typical for a given pattern, some review articles are recommended [128–130]. The causes of all these different patterns are still speculative. There are just few studies aiming to obtain insight into molecular mechanisms of clinical fevers [131], although some experimental fever models have been developed, which might better reflect naturally occurring fevers than systemic bolus injections of LPS or other PAMPs [116,132,133].

33.6.1 Is Fever Beneficial or Harmful? Some Classical Arguments

It is not that only species with the abilities for neuronal/autonomic thermoregulation, mammals, and birds develop infectious fever. Using appropriate behaviours to elevate T_b, infected invertebrates and poikilothermic vertebrates adjust T_b to higher levels [134]. Hasday *et al.* [135] concluded that a fever-like response must have developed about 600 million years ago together with heat shock and innate immune responses. The energetic costs of fever are remarkable [3,136]. The fact that a febrile response persisted for so many millions of years despite its metabolic costs has frequently been used as an argument that fever must be of adaptive value [134,135]. Another putative valuable component of fever relates to a direct effect of enhanced T_b on growth or survival rates of infectious microorganisms. There are just a few examples for such an effect, but in the majority of all infectious diseases T_b will not increase to levels that inhibit growth of a given pathogen or even kill it [2,30].

Still, in classical studies performed by Kluger and colleagues [137,138], the behaviorally established fever of lizards made them resistant to usually lethal bacterial infections, whereas the suppression of this kind of fever by treatment with antipyretics reduced the animals' survival rates. The mechanistic background for Kluger's observations is predominantly based on the stimulating effects of febrile temperatures on various components of the innate and adaptive immunity ([1,3], for review). In a recent review, Evans *et al.* worked out all the cellular components of immune responses, which are thermally regulated by febrile temperatures [139]. The cells, which are positively influenced by febrile temperature, include dendritic cells, macrophages, natural killer cells, B cells, T cells, neutrophils, and vascular endothelial cells. Further

molecular components include heat shock proteins and IL-6-signaling and adhesion molecules required for coordinated trafficking of immune cells to the site of inflammation or into tumor tissue [139]. Another argument, explaining why lizards, rabbits, and other species of experimental animals [134,139] show higher mortality, when fever is suppressed by COX inhibitors, relates to the drastic influence of these drugs on the entire inflammatory process ([30], for review). PGE_2, the synthesis of which is suppressed under the influence of COX inhibitors, not only is an important mediator of inflammation but also regulates the strength of the inflammatory response as part of a negative feedback loop, for example, by inhibiting excessive formation of TNF-α [30,61]. Thus, there are possible explanations for repeatedly observed adverse effects of fever suppression under experimental conditions in laboratory animals, induced by treatment with nonsteroidal anti-inflammatory drugs (NSIADs). Here, the question arises whether a direct transfer to the clinical setting is possible or not. This question is important in so far as the enhanced immune functions under the influence of a febrile T_b can be associated with both positive and negative consequences. The most important positive consequence would be an accelerated pathogen clearance. A negative effect could be collateral tissue damage at a given site of infection due to excessive formation of reactive oxygen and other radicals by cells of the innate immune system [1–3,30,134,135].

33.6.2 Is Fever Beneficial or Harmful? No Clear Response from Clinical Studies

Critically ill patients frequently show abnormalities in T_b, not only fever but also hypothermia, especially during septic blood infections ([30], for review). Several independently performed clinical studies aimed to determine association between the T_b abnormalities and the final outcome (survival). The worst prognosis was determined for hypothermic patients [140–143]. This observation is not surprising in so far as the condition of septic patients with severe hypothermia is rather poor in most cases [4]. For febrile patients, the prognosis was better but not for those who showed very high fevers (>39.5 °C) of noninfectious origin [142]. As there may be groups of patients, who may benefit from antipyretic therapy, a brief look at clinical studies seems warranted, in which fever was reduced either by physical cooling or pharmacologically by use of NSIADs. There are single clinical studies, which report an improved outcome for septic patients when fever was controlled and depressed by means of external cooling [144]. Other studies reported higher mortality rates of critically ill patients when fever was treated physically or pharmacologically [145]. Due to these sharp inconsistencies, Niven *et al.* [146] provided a systematic review and meta-analysis of the outcome of antipyretic therapy in critically ill patients. Just 5 out of 755 published reports fulfilled the strict criteria for the meta-analysis, among them the two studies cited above [144,145]. The analysis resulted in a tie: it could not be determined whether treatment of fever is harmful or beneficial [146]. It seems that this question has to be addressed by using selected groups of critically ill patients. Indeed, it might be the case that fever can have negative impact on the outcome in ischemic stroke, traumatic brain injury, or diseases of the cardiovascular and pulmonary

systems [127,129], while it may be beneficial in many other infectious diseases ([30], for review).

Although there are mechanistic arguments that support the view of an adaptive value of fever, the transfer to the clinical situation is still difficult and complicated due to the large number of distinct infectious or noninfectious diseases, which are accompanied by fever.

33.7 Concluding Remarks

Intensive experimental research on fever in the past 25 years provided a broader understanding not only of the phenomenon fever per se but also of several other aspects of how a peripherally generated inflammatory process propagates into the brain. Research in the 1990s focused on the roles of single cytokines in the manifestation of the febrile response [1,3,18–20]. Later on, the discovery of TLRs and the presence of this family of receptors at the interface between blood and brain challenged the exclusive role of cytokines as circulating pyrogens [11,64,65,87]. Other components of innate immunity, which are relevant specifically for LPS-induced fever, include complement fragments [3,21,22] and, perhaps, oxygen radicals [80,81]. The participation of afferent nerves in the early induction of fever is discussed controversially, but an inflammatory activation of afferent fibers of the vagus and other nerves by peripheral PGE_2 is well established [3,9–11,40]. Starting in the 1990s and continuing to present times, the critical role of the coupled induction of COX-2/mPGES in brain endothelial cells was intensively studied [25,70,93,96–100]. More recently, the source of arachidonic acid in the brain for the brain-intrinsic formation of PGE_2 has been identified [27,28]. A transcription factor (NF-IL-6) with early pro- and late anti-inflammatory properties was characterized; the properties of this transcription factor might reflect the natural reprogramming of inflammatory processes within the brain during infectious diseases [29,74,94,95].

In spite of all this progress, a direct transfer of the knowledge derived from experimental research on fever is difficult to be transferred to the clinical situation. Namely, the question of whether fever is a friend or foe for the critically ill patient remains unresolved [30,127,135,146]. A possible strategy for the clinician to obtain some responses to this question might be a detailed analysis of groups of individuals with specific disease states to define whether fever and the strength of the disease-associated inflammatory processes are beneficial or harmful for the given condition [30]. For experimental researchers, on the other hand, it is recommended to develop experimental models of fever, which better reflect the conditions of natural infections or other diseases of noninfectious origin, which are known to be associated with fever.

List of Abbreviations

AA	arachidonic acid
AP	area postrema

APR	acute-phase response
BAT	brown adipose tissue
BBB	blood–brain barrier
C	complement
COX	cyclooxygenase
DMH	dorsomedial hypothalamus
DRG	dorsal root ganglion
EP3	prostaglandin E2 receptor subtype 3
GABA	gamma amino butyric acid
ICV	intracerebroventricular
IFN	interferon
IL	interleukin
Kc	Kupffer cells
KO	knockout
LPB	lateral parabrachial nucleus
LPBd	lateral parabrachial nucleus, dorsal subregion
LPS	lipopolysaccharide
MIP-1	macrophage inflammatory protein
MnPO	median preoptic nucleus
MPO	medial preoptic area
NA	noradrenaline
NF-IL-6	nuclear factor IL-6
NFκB	nuclear factor kappa B
NOS	nitric oxide synthase
NST	nonshivering thermogenesis
OVLT	organum vasculosum laminae terminalis
PAMP	pathogen-associated molecular pattern
PGE_2	prostaglandin E2
PGES	prostaglandin E synthase
PLA_2	phospholipase A2
PLC	phospholipase C
POA	preoptic anterior hypothalamic area
rRPa	rostral raphe pallidus nucleus
SFO	subfornical organ
STAT3	signal transducer and activator of transcription 3
T_a	ambient temperature
T_b	body core temperature
TLR	Toll-like receptor
TNF-α	tumor necrosis factor-α
WT	wild type

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34

Pain

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34.1 Introduction

The classic description of an inflammatory reaction consists of four cardinal signs *rubor*, *tumor*, *calor*, and *dolor* (redness, swelling, heat, and pain). Inflammation is an essential component of a large (if not the largest) group of painful syndromes including arthritis, musculoskeletal and visceral disorders, neuropathy, cancer, wounds, and surgery [1]. Since chronic diseases with an inflammatory component represent our greatest health threat [2], pain management is bound to play an increasingly important role in the treatment of acute and chronic illness.

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" [3]. Pain is always subjective and a sensation in parts of the body. At the same time, it is unpleasant and therefore also an emotional experience. Nociception is neurophysiological activity in peripheral sensory neurons (nociceptors) and higher nociceptive pathways, and is defined by the IASP as the "neural process of encoding noxious stimuli." Nociception is not synonymous to pain. Pain is always a psychological state, even though it often has a proximate physical cause. This chapter will discuss mechanisms underlying the generation and inhibition of inflammatory pain.

34.2 Basic Concepts of Pain

Pain may be roughly divided into two broad categories: physiological and pathological pain. Physiological (acute, nociceptive) pain is an essential early-warning sign that usually elicits reflex withdrawal and thereby promotes survival by protecting the organism from (further) injury. In contrast, pathological (chronic) pain is an expression of the maladaptive operation of the nervous

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system and is considered a disease in its own right [4]. The IASP defines it as "persistent or recurrent pain lasting longer than three months" [5]. It is "pain that persists past normal healing time and hence lacks the acute warning function of physiological nociception" and affects an estimated 20% of people worldwide [5].

34.2.1 Excitatory Mechanisms

Physiological pain (nociception) is mediated by a sensory system consisting of primary afferent neurons, spinal interneurons, and ascending tracts and several supraspinal areas. Trigeminal and dorsal root ganglia (DRG) give rise to high-threshold Aδ- and C-fibers innervating peripheral tissues (skin, muscles, joints, and viscera). These specialized primary afferent neurons, also called nociceptors, transduce noxious stimuli into action potentials and conduct them to the dorsal horn of the spinal cord (Figure 34.1). When peripheral tissue is damaged, inflammation usually ensues and primary afferent neurons are directly activated and/or sensitized by chemical, mechanical, and/or thermal stimuli. Examples are protons (acidosis) (Table 34.1), sympathetic amines, adenosine triphosphate (ATP), glutamate, neuropeptides (calcitonin gene-related peptide, substance P), nerve growth factor, prostanoids, bradykinin, lipids, proinflammatory cytokines, or chemokines [6–9] (see also Chapters 22–33 on inflammatory mediators and Chapter 68 on nonsteroidal anti-inflammatory drugs). Many of these agents lead to opening of cation channels in the membrane of primary afferent neurons. Such channels include the capsaicin-, proton-, and heat-sensitive transient receptor potential vanilloid-1 (TRPV1), acid-sensing ion channels (ASICs) or the ATP-gated purinergic $P2X_3$ receptor. These channels enable an inward current of Na^+ and Ca^{++} ions into the peripheral nociceptor terminal. If this depolarizing current is sufficient to activate voltage-gated Na^+ channels (e.g., Nav1.8), they too will open, further depolarizing the membrane and initiating a burst of action potentials that are then conducted along the sensory axon to the dorsal horn of the spinal cord [7,10].

Transmission of input from nociceptors to spinal neurons that project to the brain is mediated by direct monosynaptic contact or through multiple excitatory or inhibitory interneurons. The central terminals of nociceptors contain excitatory transmitters such as glutamate, substance P (sP), and neurotrophic factors. These activate postsynaptic *N*-methyl-D-aspartate (NMDA), neurokinin (NK1), and tyrosine kinase receptors, respectively. Repeated nociceptor stimulation can sensitize both peripheral and central neurons (activity-dependent plasticity; "wind up") [51]. Later, sensitization can be sustained by transcriptional changes in the expression of genes coding for various neuropeptides, transmitters, ion channels, receptors, and signaling molecules (transcription- dependent plasticity). Important examples include the NMDA receptor, cyclooxygenase-2 (COX-2), Ca^{++} and Na^+ channels, cytokines, and chemokines expressed by neurons and/or glial cells [52]. In addition, physical rearrangement of neuronal circuits by apoptosis, nerve growth, and sprouting occurs in the peripheral and central nervous system [4], and spinal microglial cells release mediators (e.g., brain-derived neurotrophic factor, cytokines, etc.) that increase neuronal excitability

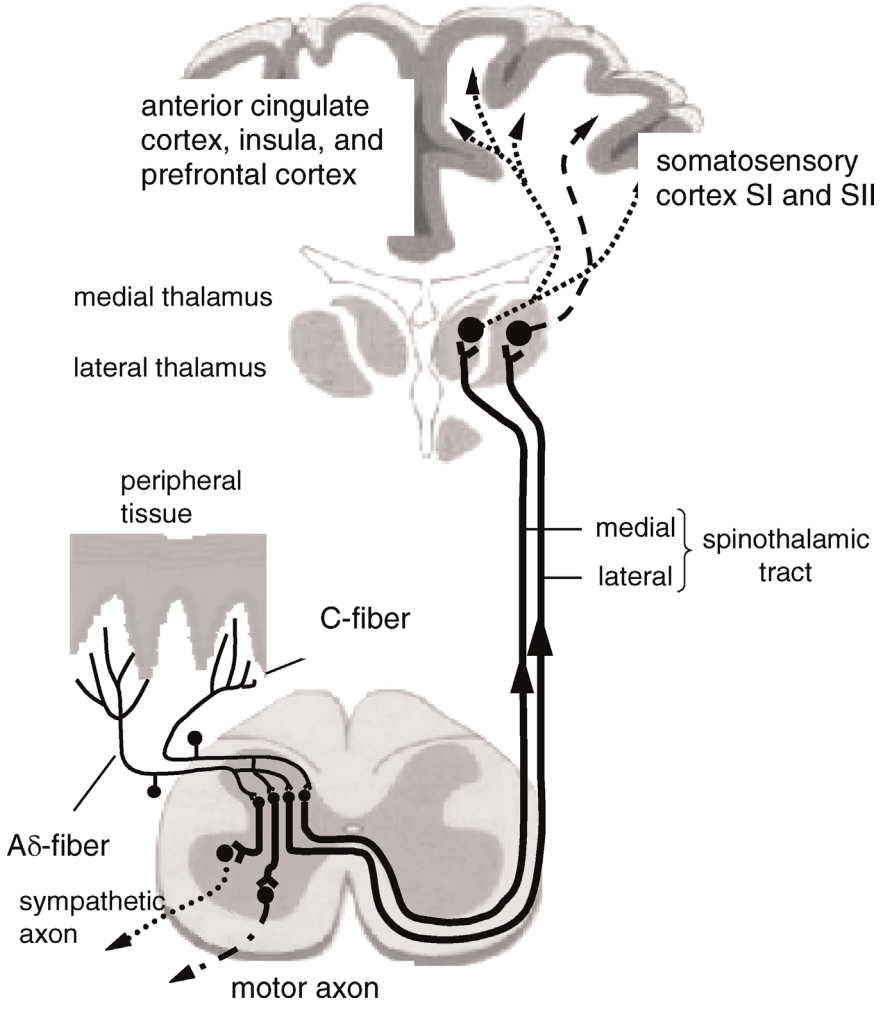


Figure 34.1 Nociceptive pathways (adapted with permission from Ref. [11]).

and enhance nociception [52]. Both induction and maintenance of central sensitization are critically dependent on the peripheral drive by nociceptors, indicating that therapeutic interventions targeting such peripheral neurons may be particularly effective, even in chronic pain syndromes [51].

After synaptic processing, the peripheral input is conveyed to higher centers via the spinothalamic, spinoreticulothalamic, and spinoparabrachial pathways. This activity relays through the brain stem and thalamus to the somatosensory cortex and associated areas (Figure 34.1). The parabrachial region of the brain stem connects to the ventromedial nucleus of the hippocampus and the central nucleus of the amygdala, brain regions involved in the affective/aversive components of pain. There is no single brain area essential for pain but rather activation of a distributed group of structures. Many impulses from supraspinal centers are

Table 34.1 Acidosis in inflamed tissues: pH measurements *in vivo/ex vivo*.

Tissue	Lowest pH	Method	Reference
Human: abscess	5.4	Pinkussen: hydrogenperoxide	[12]
Guinea pig: intraperitoneal bacterial inoculation	5.6	Clark–Lubs color indicator	[13]
Mouse: s.c., i.p. bacterial inoculation	5.8		
Dog: turpentine-induced pleural exudates	6.6	Hastings and Sendroy: bicolor system	[14]
Rat: implanted tumors	6.82	Glass electrode, Northrop potentiometer	[15]
Dog: turpentine-induced pleural exudates	6.5	Hastings and Sendroy: bicolor system	[16]
Human: malignant tumors, inflamed tissues	5.44	Beckman electrode	[17]
Human: malignant tumor	5.7	Hartman and Braun glass electrode	[18]
Dog: turpentine-induced pleural exudates	6.0		[19]
Human: osteoarthritis, joint injury, synovial fluid	6.5	Marconi pH meter T.F. 5117	[20]
Human: astrocytoma	5.85	Pusl 1260 glass electrode	[21]
Human: melanoma	6.4	Voegtlin and Kahler glass electrode	[22]
Human: rheumatoid arthritis synovial fluid	7.08	Radiometer pHM4 + glass electrode; Beckman glass electrode	[23]
Human: rheumatoid arthritis synovial fluid	6.0	Radiometer + antimony electrode	[24]
Human: rheumatoid arthritis synovial fluid	6.4	Radiometer + antimony electrode	[25]
Human: rheumatoid arthritis	6.84	Model 113 blood gas analyzer, Instrumentation Lab. Watertown, MA	[26]
Human: arthritis	6.60	Radiometer P27b pH meter, pH.4 927b gas monitor, E5021 microelectrode	[27]
Rabbit: diabetic skin wounds	6.9	Microelectrode	[28]
Rabbit: brain, wounds, ischemia	5.0	Glass electrode	[29]
Rat: ischemic heart, intracellular	5.7	^{31}P NMR	[30]
Rat: seminiferous tubules, and epididymis	6.57±0.08	Antimony microelectrode	[31]
Mouse mammary carcinoma	5.8	Hebert and Hinke glass electrode	[32]
Human: rheumatoid and osteoarthritis synovial fluid aspirated	6.85	ABL Acid Base Analyzer	[33]
Rat: air pouch granuloma induced by carrageenan, dextran, *Staphylococcus aureus*	6.87	Roche glass microelectrode	[34]

Table 34.1 (*Continued*)

Tissue	Lowest pH	Method	Reference
Human: exercised muscle, intracellular pH	6.1	^{1}H and ^{31}P NMR spectroscopy	[35]
Human: exercised muscle, calculated intracellular pH	6.31±0.09	^{31}P NMR spectroscopy	[36]
Human: rheumatoid arthritis synovial fluid	7.03	ABL 300 Radiometer	[37]
Horse: *S. aureus*-induced arthritis, synovial fluid aspirated	6.2	Fisher Accumed pH meter	[38]
Nude mouse: implanted tumors	6.65	MI 408b glass electrode	[39]
Human: abdominal abscess	6.0	AVL 995-S blood gas analyzer	[40]
Mouse: implanted tumor	6.66	^{31}P-MRS of 3-APP	[41]
Rat: carrageenan inflammation, aspirated	6.94	BMS3MK2 radiometer	[42]
Human: exercised muscle, intracutaneous pH	6.67	Needle electrode	[43]
Rat, mouse: implanted tumors	6.3	^{31}P- and ^{19}F-MRS; 3-APP	[44]
Mouse: implanted tumors	6.0	^{31}P- and ^{19}F-MRS; 3-APP and ZK-150 471	[45]
Rat: BSA-induced arthritis	5.66	^{14}C-DMO distribution	[46]
Rat: plantar/gastrocnemius incision	6.54 ± 0.12	Glass electrode	[47]
Mouse: implanted subcutaneous lymphoma	6.0	^{13}C-MRI	[48]
Rat: Freund's adjuvant paw inflammation; paw incision	6.8 7.02	Glass electrode	[49]
Rat: Freund's adjuvant paw inflammation	6.82	Glass electrode	[50]

Abbreviations: s.c., subcutaneous; i.p., intraperitoneal; NMR, nuclear magnetic resonance; MRS, magnetic resonance spectroscopy; MRI, magnetic resonance imaging; 3-APP, 3- aminopropylphosphonate.

integrated in the midbrain periaqueductal gray, a pivotal region for descending modulation of nociception [52,53].

34.2.2 Inhibitory Mechanisms

Concurrent with the events described above, powerful endogenous inhibitory mechanisms unfold both in the periphery and in the central nervous system. Two families of neuromodulators binding to class A inhibitory G protein-coupled receptors (GPCRs) have been extensively investigated, the opioids and the cannabinoids [9,54–56]. In inflamed peripheral tissue, nociception can be suppressed by interactions between leukocyte-derived opioid peptides (OP) and

peripheral nociceptor terminals carrying opioid receptors (OR) [55, 57–59], by lipid endocannabinoids derived from primary afferent neurons and neighboring nonneuronal cells [9,55], and/or by anti-inflammatory cytokines [6]. In the central nervous system, inhibition of nociception is mounted by opioids, cannabinoids, GABA, or glycine, which activate their respective presynaptic receptors to reduce excitatory transmitter release [52,54]. In addition, the opening of postsynaptic K^+ or Cl^- channels by opioids or GABA, respectively, evokes hyperpolarizing inhibitory potentials in spinal dorsal horn neurons. During ongoing nociceptive stimulation, spinal interneurons upregulate gene expression and production of opioid peptides [60,61]. Powerful descending inhibitory pathways from the brain stem also become active by operating mostly through noradrenergic, serotonergic, and opioid systems. A key region is the periaqueductal gray. It connects to the rostral ventromedial medulla, which projects along the dorsolateral funiculus to the spinal dorsal horn [62]. The integration of signals from excitatory and inhibitory pathways, cognitive, emotional, and environmental factors results in the central perception of pain.

Besides disease-modifying drugs (e.g., steroids, biologicals, etc., see Chapters 59 and 63), two classes of analgesic compounds are mainly used in the treatment of inflammatory pain, COX inhibitors and opioids (see Section 3.2). The COX inhibitors (nonsteroidal anti-inflammatory drugs) are covered in Chapter 68. The following section will discuss the opioid system in more detail.

34.2.2.1 Opioid Receptors and Signaling

Opioid receptors are expressed by central and peripheral neurons, by neuroendocrine (pituitary and adrenals), immune, and ectodermal cells (reviewed in Refs [56,59,63]). Three main types of opioid receptors are defined, the mu-, delta-, and kappa receptors [64,65]. After binding of a ligand, conformational changes allow intracellular coupling of heterotrimeric Gi/o proteins to the C-terminus of the receptor. At the Gα subunit, GTP replaces GDP and dissociation of the trimeric G protein complex into Gα and Gβγ subunits ensues. The former inhibit adenylyl cyclases and cAMP production, whereas the latter directly interact with different ion channels in the cell membrane [66,67]. All three opioid receptors can modulate pre- and postsynaptic Ca^{++} channels, suppress Ca^{++} influx, and thereby attenuate the excitability of neurons and/or reduce the release of pronociceptive neuropeptides [68]. In addition, opioid receptor activation leads to opening of G protein-coupled inwardly rectifying K^+ (GIRK) channels, thereby preventing neuronal excitation and/or propagation of action potentials [63,67,69]. Opioids also inhibit Na^+-, Ih-, TRPV1, and ASICs in DRG neurons, as well as excitatory postsynaptic currents evoked by glutamate receptors in the spinal cord [70–74]. The result is decreased transmission of nociceptive stimuli at all levels of the neuraxis and profoundly reduced perception of pain.

34.2.2.2 Plasticity of Opioid Receptors and Signaling Pathways

Persistent inflammatory pain is associated with multiple adaptations in the nervous, endocrine, and immune systems [6,60,61,75]. In the late 1980s, evidence began to accumulate that antinociceptive effects can be mediated by opioid receptors localized on peripheral sensory neurons [57,76]. Initial studies showed

that the systemic or local application of opioid agonists elicited significantly more pronounced analgesic effects on inflamed than noninflamed tissue of animals and humans (reviewed in Refs [76,77]). This intriguing finding stimulated extensive research into the underlying mechanisms.

Alterations in opioid receptor expression occur at the mRNA and protein levels and can be subject to epigenetic regulation [64]. With regard to opioid receptor synthesis, it was found that peripheral tissue inflammation can induce differential upregulation of opioid receptor types and their mRNAs in DRG neurons. This upregulation is dependent on neuronal electrical activity, on cytokine production in the inflamed tissue, and may be related to cytokine-induced binding of transcription factors to opioid receptor gene promoters (reviewed in Refs [59,78]). Furthermore, it was shown that the peripherally directed axonal transport of opioid receptors was augmented and opioid receptor expression on DRG membranes was enhanced [79,80]. The axonal transport was stimulated by cytokines and nerve growth factor produced within the inflamed tissue [81,82], resulting in increased density and antinociceptive functionality of opioid receptors on peripheral nerve terminals [57,80,83]. On the other hand, short-lasting inflammatory stimuli do not significantly alter the number of opioid receptors on peripheral sensory nerve terminals (reviewed in Ref. [59]). Inflammation is also accompanied by a disrupted perineural barrier facilitating the access of opioid agonists to their receptors [84,85].

In cultured sensory neurons, bradykinin was found to stimulate the trafficking of intracellular opioid receptors to the plasma membrane [86]. Furthermore, priming with bradykinin, PGE2, or a protease-activated receptor agonist led to more potent inhibition of neuropeptide release and of cAMP accumulation by opioid agonists [86,87]. Also, painful paw inflammation and activation of sensory neurons by capsaicin or P2Y receptor agonists enhanced membrane recruitment of opioid receptors [88,89]. On the other hand, pretreatment of cultured DRG neurons with proinflammatory chemokines resulted in internalization and functional inhibition of opioid receptors [90]. *In vivo*, local pretreatment with bradykinin or arachidonic acid enabled [91], whereas the genetic ablation of bradykinin receptors reduced [92] peripheral opioid antinociception (reviewed in Ref. [59]).

With regard to signaling pathways, G protein coupling of opioid receptors was shown to be augmented during peripheral tissue inflammation [80]. Earlier studies had shown that low extracellular pH (as seen in inflammation, see Table 34.1) increased opioid agonist efficacy, possibly by altering the interaction of opioid receptors with G proteins and adenylyl cyclase [93,94]. This may augment inhibition of cAMP, Ca^{++}, Na^{+}, and TRPV1 and/or ASIC currents, and/or neuronal hyperpolarization via opening of GIRK channels [59,69–72]. Under conditions of elevated cAMP (as seen in inflammation), morphine can inhibit TRPV1 translocation to the plasma membrane [95]. Furthermore, extracellular recordings from sensory nerve fibers supplying injured tissue demonstrated opioid inhibition of spontaneous and stimulus-evoked action potentials [96–99]. A recent study reported the computer-assisted development of a pH-dependent opioid analgesic (NFEPP) that selectively activates peripheral opioid receptors in injured (acidotic) tissue without eliciting adverse side effects (apnoea, reward, motor disturbance, constipation) in noninflamed tissues (brain, intestinal wall) [49].

Opioid receptors are also expressed by immune cells (reviewed in Refs [59,100]). There is evidence that such opioid receptors contribute to analgesia [101], and they were shown to modulate various immune cell functions. However, the latter effects were mostly examined *in vitro*, they were often contradictory and dependent on specific experimental conditions (e.g., cultured cell types, duration of cultures, doses, and timing of opioid exposure) (reviewed in Ref. [100]). Based on such studies, immunosuppressive effects of opioids were frequently proposed, but they have not been verified in clinical or epidemiological studies [102,103]. In addition, opioid receptors were shown to modulate cellular growth, inflammation, and wound healing (reviewed in Refs [104,105]).

In summary, the expression, accessibility, axonal transport, and signaling of opioid receptors on DRG neurons are modulated by inflammation and these mechanisms apparently contribute to enhanced analgesia [59,104]. This is consistent with the notion that tissue injury "unmasks" peripheral opioid effects, whereas the local application of opioids in normal tissue does not reliably produce analgesia [76,106,107].

34.2.2.3 Endogenous Opioid Receptor Ligands

Endogenous opioid receptor ligands are derived from the precursors proopiomelanocortin (encoding β-endorphin), proenkephalin (encoding Met-enkephalin and Leu-enkephalin), and prodynorphin (encoding dynorphins). These peptides contain the common Tyr-Gly-Gly-Phe- Met/Leu sequence at their amino terminals, known as the opioid motif. Beta-endorphin and the enkephalins are antinociceptive agents acting at mu- and delta-opioid receptors. Dynorphins can elicit both pro- and antinociceptive effects via *N*-methyl-D-aspartate receptors and κ-opioid receptors, respectively. A fourth group of tetrapeptides (endomorphins) with yet unknown precursors do not contain the pan-opioid motif but bind to mu-receptors with high selectivity. Opioids are expressed throughout the central and peripheral nervous system, in neuroendocrine tissues, and in immune cells [63,78,108]. Opioid peptide-containing immune cells extravasate and accumulate in inflamed tissue. These cells upregulate the gene expression of opioid peptide precursors and the enzymatic machinery for their processing into functionally active peptides [108–110]. In response to stress, catecholamines, corticotropin releasing factor, cytokines, chemokines or bacteria, and leukocytes secrete opioids, which then activate peripheral opioid receptors and produce analgesia by inhibiting the excitability of nociceptors, the release of excitatory neuropeptides, or both [59,111] (Figure 34.2). The clinical relevance of these mechanisms has been confirmed in studies demonstrating that patients with knee joint inflammation express opioid peptides in immune cells and opioid receptors on sensory nerve terminals within synovial tissue [112–114]. After knee surgery, pain and analgesic consumption were enhanced by blocking the interaction between endogenous opioids and their receptors [112,115], and pain was reduced by stimulating opioid secretion [116].

Interestingly, endogenous opioids from inflammatory cells appear to produce additive/synergistic interactions rather than tolerance at peripheral opioid receptors [113,117,118]. After release, extracellular opioid peptides are susceptible to rapid enzymatic inactivation by aminopeptidase N and neutral endopeptidase ("enkephalinases"). Both are expressed in the central nervous system, peripheral

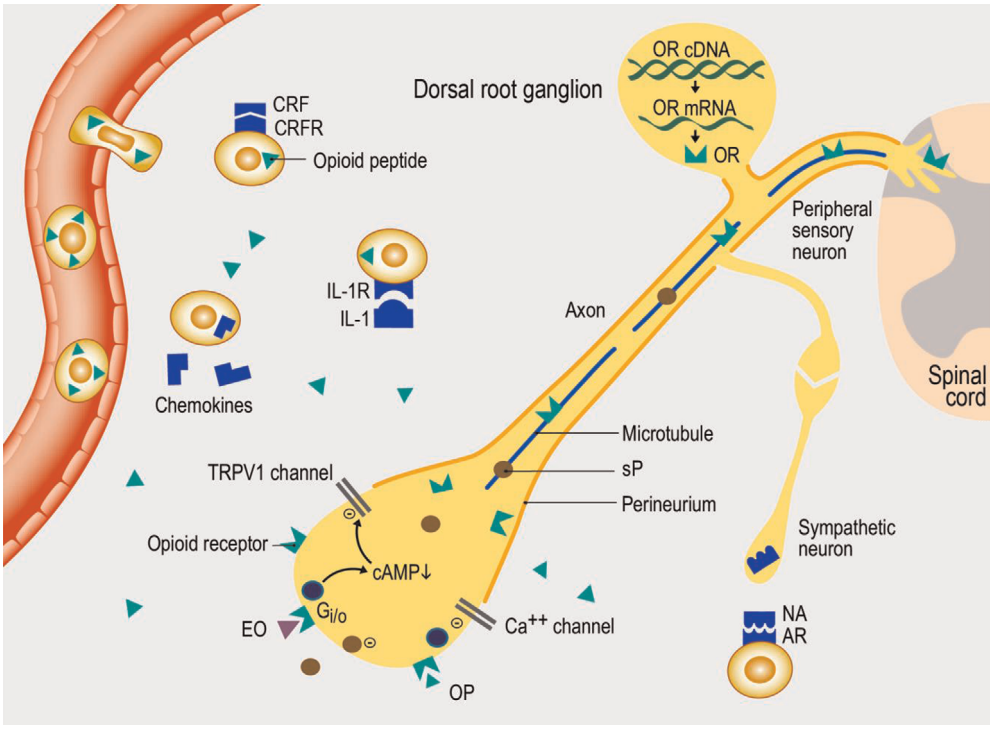


Figure 34.2 Endogenous antinociceptive mechanisms within peripheral inflamed tissue (adapted with permission from Ref. [59]). Opioid peptide-containing circulating leukocytes extravasate upon activation of adhesion molecules and chemotaxis by chemokines. Subsequently, these leukocytes are stimulated by stress or releasing agents to secrete opioid peptides. For example, corticotropin-releasing factor (CRF), interleukin-1β (IL-1), and noradrenaline (released from postganglionic sympathetic neurons) can elicit opioid release by activating their respective CRF receptors (CRFR), IL-1 receptors (IL-1R), and adrenergic receptors on leukocytes. Exogenous opioids (EO) or endogenous opioid peptides (green triangles) bind to opioid receptors that are synthesized in dorsal root ganglia and transported along intraaxonal microtubules to peripheral (and central) terminals of sensory neurons. The subsequent inhibition both of ion channels (e.g., TRPV1, Ca^{++}) and of substance P release results in antinociceptive effects.

nerves, and leukocytes. Preventing the extracellular degradation of endogenous opioid peptides by enkephalinase inhibitors, both in central and in peripheral compartments, has been shown to produce analgesic effects in many animal models and in some human trials [55,119]. This strategy avoids unphysiologically high concentrations of exogenous agonists at the receptor and, thus, diminishes the risk for development of receptor downregulation, tolerance, desensitization, off-site, or paradoxical excitatory effects [55,119].

34.3 Clinical Concepts of Pain

There is a tradition to distinguish between acute (e.g., intra- or postoperative), chronic malignant (related to cancer and its treatment) and chronic

nonmalignant (e.g., arthritic and neuropathic) pain. Little evidence exists to suggest that different somatic or psychological mechanisms warrant such a distinction and overlaps apparently exist. For example, cancer invasion can elicit inflammatory pain and/or neuropathic pain from invasion of the tumor into tissues innervated by primary afferent (nociceptive) neurons (e.g., pleura, peritoneum, etc.). Inflammation can contribute to both acute (e.g., postoperative) and chronic (e.g., arthritic) pain [5].

34.3.1 Biopsychosocial Concept and Multidisciplinary Management

All chronic (but also acute) pain syndromes have in common the complex influences of biological (tissue damage), cognitive (memory and expectations), emotional (anxiety and depression), and environmental factors (reinforcement and conditioning). The interplay between biological, psychological, and social factors results in the persistence of pain and illness behaviors [120,121]. Treating only one aspect of this complex syndrome is obviously insufficient, particularly in chronic pain. Therefore, a biopsychosocial concept of pain has been adopted. In a multimodal approach, pain management addresses physical, psychological, and social skills and underscores the patients' active responsibility to regain control over life by improving function and well-being [121,122]. This encompasses various pharmacological and nonpharmacological (psychological and physiotherapeutic) treatment strategies (for review, see Ref [123]).

34.3.2 Analgesic Drugs

Besides disease-modifying drugs (e.g., steroids and biologicals, see Chapters 59 and 63), an important component of pain therapy is the use of traditional analgesic drugs. Such compounds interfere with the generation and/or transmission of impulses following noxious stimulation in the nervous system (nociception). This can occur at peripheral and/or central levels of the neuraxis. Analgesics aim at modulating either the formation of noxious chemicals (e.g., prostaglandins) or the activation of neuronal receptors/ion channels transducing/transmitting noxious stimuli (e.g., opioid receptors, NMDA receptors, and Na^+ channels). The most widely used compounds are nonsteroidal anti-inflammatory drugs (see Chapter 68) and opioids. Other classes such as adrenergic receptor (AR) agonists, neurotrophin antagonists, neuropeptide (e.g., calcitonin gene-related peptide) receptor antagonists, kinin receptor antagonists, prostaglandin E receptor antagonists, cannabinoids, and ion channel (e.g., TRP, P2X) blockers are under investigation but not in routine use. Local anesthetics block Na^+ channels and are applied in local and regional anesthetic techniques. Mixed drugs combine different mechanisms, for example, noradrenaline (NA) reuptake inhibition and opioid agonist effects (tramadol and tapentadol), or opioid agonist and NMDA antagonist effects (ketamine). Various routes of drug administration (e.g., oral, intravenous, subcutaneous, intrathecal, epidural, topical, intraarticular, and transmucosal) can be used. In addition, placebo treatments have shown impressive analgesic effects, mediated by opioid and nonopioid mechanisms (for review, see Refs. [123,124]).

34.3.2.1 Opioids

Consistent with the expression of opioid receptors at all levels of the neuraxis, opioid agonists can effectively inhibit clinical pain after peripheral (topical and intraarticular), neuraxial (intrathecal, epidural, and intracerebroventricular), or systemic (intravenous, oral, subcutaneous, sublingual, and transdermal) administration [56,63,125–128]. The commonly available opioid drugs (morphine, codeine, methadone, fentanyl, and derivatives) are primarily mu-receptor agonists [125,129,130]. Systemically and spinally administered opioids can produce similar side effects (e.g., respiratory depression), depending on dosage and rostral/systemic redistribution. All three opioid receptor types mediate analgesia but have differing side effects [56,63]. Tolerance and physical dependence may occur with prolonged administration of pure agonists, and abrupt discontinuation or antagonist administration can result in a withdrawal syndrome [63,71].

The long-term use of opioids in chronic pain is increasingly scrutinized. While conventional opioid agonists are undisputed in the treatment of severely acute and cancer pain, their long-term use in chronic nonmalignant (e.g., neuropathic, musculoskeletal, and inflammatory) pain has not proven effective [131]. Instead, addiction, overdoses, death rates, and abuse of prescription opioids have reached epidemic proportions and have become a public health problem [132–135]. Meta-analyses show clinically insignificant reduction of pain scores, and epidemiological data suggest that quality of life or functional capacity are not improved [131,136]. Adverse side effects (nausea, sedation, constipation, dizziness, respiratory depression, cardiac arrhythmia, cognitive deficit, and endocrinopathy) and lack of analgesic efficacy have led to the dropout of high numbers of subjects in long-term studies [131,134,137–141]. Instead, chronic pain requires a multidisciplinary approach encompassing various pharmacological and nonpharmacological (psychological and physiotherapeutic) treatment strategies [75,123].

Experimental and clinical studies have demonstrated that peripheral opioid receptors mediate a substantial proportion of analgesia produced by systemically applied opioids (reviewed in Refs [56,104]. A recent clinical trial showed that the selective blockade of peripheral opioid receptors led to a 55% increase in systemic morphine requirements for sufficient pain relief during the first 4 h after surgery [142]. Peripheral application of opioids became of considerable interest because many pain syndromes depend to a significant degree on the peripheral activation of DRG neurons and side effects may be reduced [51]. The most extensively studied and well-established regimen is intraarticular morphine administration in inflammatory pain following surgery [126,128,143,144]. Meta-analyses showed that its analgesic efficacy is similar to local anesthetics [145]. In many small clinical trials, locally applied opioids (e.g., dermal formulations, gels, etc.) have shown analgesic actions in inflammatory pain associated with skin ulcers, cystitis, cancer-related oral mucositis, corneal abrasion, neuropathic pain, chronic arthritis, and bone injury (reviewed in Ref. [127]). For systemic administration, novel opioid receptor ligands are being developed. While earlier attempts to demonstrate peripheral opioid analgesia in healthy

tissue failed, potent antinociception was consistently detected in models of nerve damage, inflammatory, visceral, cancer, and bone pain (reviewed in Refs. [56,59,76]). Current research pursues the development of systemically applicable opioid agonists and enkephalinase inhibitors that do not permeate blood–brain, intestinal, or placental barriers [50,55,56,104,119,146,147] or do not activate central and intestinal opioid receptors [49]. In addition, gene therapeutic approaches enhancing the expression of peripheral opioid receptors and peptides are being investigated [148,149]. Thus, the selective activation of peripheral opioid receptors promises advantages such as anti-inflammatory actions and the absence of side effects typical for conventional opioids (sedation, nausea, apnea, fetal/neonatal depression, and addiction), nonsteroidal analgesics (gastrointestinal ulcers, bleeding, stroke, and myocardial infarction), or disease-modifying drugs (steroids and biologicals, see Chapters 59 and 63) [125,150].

34.3.3 Translation of Basic Research into Clinical Applications

Basic research on pain continues at a rapid pace, but its translation into clinical applications has been difficult [151,152]. Many obstacles have been discussed, including overinterpretation of data, reporting bias toward neglecting negative results, and flawed design of experimental and clinical studies [137,152,153]. Animal studies are indispensable, continue to be improved, and have successfully predicted adverse side effects of drug candidates [151,153]. However, for ethical reasons, many models are restricted to days or weeks, while human chronic pain can last for months or years. Therefore, animal models do not mirror the truly chronic clinical situation and should be more cautiously termed as reflecting "persistent pain" [137,151,153,154].

Brain imaging is an area of intense research in patients with various pain syndromes, but such studies have not yet provided reproducible findings specific for a disease or a pathophysiological basis for individual syndromes [154]. Similarly, imaging opioid mechanisms in the human brain has been limited mostly to single-dose studies in healthy volunteers and has not substantially advanced our understanding of pain relief or opioid use in patients [155]. Neuroimaging can only detect alterations associated with nociceptive processes, whereas clinical pain encompasses a much more complex subjective experience that critically relies on self-evaluation. Thus, imaging cannot provide an objective proxy biomarker or predictor for pain [156].

Personalized pain therapy based on genetics is an attractive concept. The mu-opioid receptor gene *OPRM1* was among the first genes screened for functional relevance with regard to analgesia. The human single-nucleotide polymorphism (SNP) *OPRM1* 118 A>G is the most thoroughly investigated candidate to date. However, as emerged from meta-analyses, this SNP appears to be without major clinical relevance as a solitary variant [157,158]. Thus, although basic research has produced some evidence for a genetic control of pain, such findings are not expected to serve as a guide to individualized ("personalized") clinical pain therapy any time soon [154,159]. Nonetheless, efforts continue to find other genetic variants predicting analgesic efficacy and side effects [160–162].

34.4 Conclusions and Outlook

Pain is a symptom and an essential component of inflammation. Depending on the duration of pain, treatment must be tailored toward both pathophysiological mechanisms and individual needs of the patient. In keeping with the biopsychosocial concept, pain treatment comprises not only interventions or drugs but also physio- and psychotherapeutic strategies. Besides disease-modifying drugs (e.g., steroids and biologicals, see Chapters 59 and 63), the most widely used compounds are COX-inhibitors (see Chapter 68) and opioids. However, the long-term use of opioids in chronic nonmalignant pain has not proven effective and carries the risk of addiction, overdosage, and abuse [131,132,135]. Thus, it is crucial to select suitable patients and to consider alternative (e.g., nonpharmacological) therapeutic approaches.

Several avenues of research (e.g., peripheral opioid receptors, biologicals, and cannabinoids) are being pursued to find new treatment approaches reducing current risks. A field of intensive investigation is the endocytic trafficking and processing of opioid receptors in sensory neurons, particularly the influence of injury and the implications for tolerance development and efficacy and potency of opioid agonists. Furthermore, the recent flurry of studies on GPCR structures enables novel approaches to elucidate ligand-biased signaling and allosteric, oligo-, and heteromeric modulation of opioid receptor function. A recent example is the computer-assisted development of a novel opioid analgesic (NFEPP) that selectively activates peripheral opioid receptors in injured (inflamed) but not in noninflamed tissues (brain, intestinal wall), thereby precluding adverse side effects such as respiratory depression, addiction potential, motor disturbance, and constipation [49]. Whether genetic or epigenetic variations in opioid receptor expression play a significant role in clinical efficacy of opioid analgesics needs further investigation [64]. For example, species differences in the expression of GIRK channels and opioid effects on DRG neurons have been demonstrated, raising questions about the most suitable animal species to model humans [69].

In addition, the role of leukocytic opioid receptors in pain modulation needs to be explored further [101]. An area that has not received much attention is the influence of opioids on inflammation and wound healing. Although there is ample evidence of beneficial effects from basic research, clinical studies are lacking to date [104]. Similarly, clinical studies on the augmentation of effects of endogenously released opioid peptides by inhibiting their degrading enzymes are needed [55,119].

The epidemic of opioid misuse in chronic pain illustrates the persistent problems resulting from nonselective activation of ubiquitous opioid receptors throughout central and peripheral compartments. Thus, the potential of peripheral actions is increasingly recognized by researchers and clinicians [51,55,59,126,128,144,147]. Peripheral opioid receptor activation can reduce pain and inflammation, while avoiding sedation, respiratory depression, addiction, and adverse effects typical of biologicals, steroids, and nonsteroidal analgesics [49,50]. Beyond the described ongoing efforts, technology-oriented research (e.g., nanocarriers) is needed to find novel approaches to peripheral restriction of opioids [50]. For oral application, such compounds should be able to penetrate the gastrointestinal but not the blood–brain or placental

barriers [163]. For topical application, they should sustain slow release of the active drug. Manipulating the perineurium of peripheral sensory neurons is another interesting approach [84,85]. In contrast to available anti-inflammatory agents (steroids, NSAIDs, and biologicals) or local anesthetics, peripherally acting opioids carry little risk for organ toxicity or infection, making them interesting candidates for development of novel drugs and formulations with a wider benefit to risk window.

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35

Inflammation, Hormones, and Metabolism

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35.1 Introduction

Inflammation is usually a self-limiting and protective response to infection or tissue damage. However, dysregulated inflammation can progress to multiorgan dysfunction and, ultimately, death. Precise pathophysiological mechanisms remain elusive [1] due in part to the immense complexity of the host response, with variation in the presentation, progression, and response to treatment. There is considerable cross-talk between neuroendocrine and immune systems [2]. They regulate each other and share common receptors, peptide signaling, and functional pathways. Immune function is controlled through the hypothalamic-pituitary–adrenal, –gonadal, and –thyroidal axes [3], the renin–angiotensin–aldosterone system, and adipokines [4,5]

Another aspect of the interaction between metabolism and inflammation is the requirement for an adequate energy supply. Some immune cells rely to a greater extent on oxidative metabolism, whereas others, such as neutrophils, are more glycolytic in nature. These metabolic differences influence the expression of cytokines and, ultimately, the immune response.

This chapter will describe hormonal influences on the immune response, and the influence of bioenergetics on metabolism in immune cell function.

35.2 Thyroid Hormones

Thyroid hormones play critical roles in metabolism and immune regulation [6–8]. Thyroxine (T4; 3,5,3′,5′-tetraiodothyronine) constitutes approximately 90% of the circulating thyroid hormone; the remainder is the much more biologically active T3 (3,5,3′-triiodothyronine) [7]. Only a small proportion (0.03% of T4 and 0.3% of T3) is nonprotein bound, determining activity. Regulation is by negative feedback involving TRH, TSH, and thyroid hormones themselves [8]. During acute illness, hormone levels fluctuate, relating to both severity and duration of

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illness. In severe or prolonged illness, T3, T4, and TSH fall while reverse (inactive) T3 rises [7]. This "sick euthyroid" or "nonthyroidal illness" (NIST) syndrome relates to a poor prognosis.

35.2.1 Macrophages

Thyroid hormones influence macrophage function by regulating chemosis, phagocytosis, and production of inflammatory markers and reactive oxygen species (ROS). Hypothyroid rats demonstrated increased phagocytosis and ROS release upon administration of *Streptococcal* cell wall [9], while the reverse was true for T4-induced hyperthyroid rats [10]. However, murine peritoneal showed no effect from T3 incubation but increased phagocytosis on incubation with T4 [11]. Supplementation of T3 or T4 enhanced phagocytosis in mice given *Neisseria meningitides* by increasing macrophage binding to bacteria and increasing their bacteriocidal ability [12]. T3 administration to mice induced unpolarized macrophages to display an M1 phentoype, as revealed by expression analysis of surface proteins, gene markers, and cytokine release in response to Toll-like receptor (TLR) ligands including lipopolysaccharide (LPS) and interferon (IFN)-γ [13]. Anti-inflammatory M2 activation was reversed [13]. In rat Kupffer cells, high doses of T3 increased proinflammatory functions, including the respiratory burst, nitric oxide synthase activity, and tumor necrosis factor (TNF)-α expression [8].

35.2.2 Neutrophils

Thyroid hormones increase neutrophil bacteriocidal activity by stimulating cytochrome P450, myeloperoxidase and NADPH oxidase to increase superoxide generation [14,15]. Oxidative injury is normally limited via superoxide dismutase (SOD) activity that converts superoxide to hydrogen peroxide. Thyroid hormones, particularly T3, regulate SOD-1 activity and thus oxidative stress [16]. Neutrophils from patients with low T3 levels had impaired phagocytosis [17]. TSH administration increased neutrophil phagocytic activity within the reticuloendothelial system [18].

35.2.3 Natural Killer Cells

Thyroid hormones have a biphasic action on natural killer (NK) cells; low dietary levels of T3 enhanced IL-2 receptor expression, whereas higher levels decreased expression [19]. This may be due to cellular apoptosis, decreased receptor expression, or loss of cellular membrane components [20]. Intraperitoneal thyroxine enhanced murine NK cell activity without increasing IFN-γ; however, *in vitro* administration of thyroxine with IFN markedly enhanced NK activity [21].

35.2.4 Lymphocytes

Hypothyroidism induces splenic and lymph node involution and impairs humoral and cell-mediated immune responses [22] These effects are reversible with

thyroid hormone [23]. Hyperthyroid states may, however, enhance or suppress primary antibody responses and T and B lymphocyte proliferation [22,23]. T4 promotes IgM production in mice and stimulates the humoral response to *Salmonella typhi* [8]. In aged mice, T4 treatment promotes cellularity of thymus, spleen, lymph nodes, and bone marrow, increases peripheral blood leukocyte numbers and T lymphocyte mitogenesis, and enhances phagocytic function of peritoneal exudate cells [24]. T lymphocytes cultured with T3 and T4 also demonstrate increased apoptosis and ROS production [25]

35.3 Steroid Hormones

Activation of the hypothalamic–pituitary–adrenal (HPA) axis induces hypothalamic CRH release into the hypophyseal portal blood supply [26]. Centrally secreted CRH has an indirect anti-inflammatory effect via release of glucocorticoids (GCs), while peripherally secreted CRH has a direct proinflammatory action [27,28]. Central CRH stimulates pituitary ACTH (adrenocorticotropic hormone) release that, in turn, induces expression of GCs, mineralocorticoids, and androgenic steroids. GCs regulate metabolic processes and both innate and adaptive immune responses including cell-mediated lytic activities and cytokine production by monocytes/macrophages, T lymphocytes, and NK cells. GCs bind to specific DNA motifs and alter expression of transcription factors [29]. They either regulate activation of target genes by direct protein–protein interactions with transcription factors such as NF-κB [30] or act synergistically with PPAR-γ and PPAR-α to suppress NF-κB- and IRF-3-dependent target genes [31]. Proinflammatory cytokines are suppressed, while anti-inflammatory cytokines are upregulated [32]. Aldosterone also activates immune responses [33], expression of NADPH oxidase (which increases production of superoxides and ROS in vascular tissue), and secretion of mediators such as prostaglandins and vascular endothelial growth factor [34].

35.3.1 Macrophages

CRH increases secretion of proinflammatory cytokines and ROS in macrophages [35,36], an effect mediated by TLR-4 [37]. A combination of ACTH and IFN-γ reduced monocyte cytotoxicity that could be reversed with substance P [38]. GCs modulate leukocyte function at different levels including gene expression, enzymatic activity, and cellular migration. GCs repress expression of proinflammatory cytokines, chemokines, inducible nitric oxide synthase (iNOS), and COX-2 [30,39]. While these reduce oxidative stress and phagocytotic activity, macrophage function may be enhanced at low corticosterone concentrations [40]. GCs prevent leukocyte migration and accumulation at inflammatory sites due to decreased CCR-7 expression [41]. Aldosterone can induce inflammation by decreasing expression of glucose-6-phosphate dehydrogenase that reduces $NADP^+$ to NADPH and increases NF-κB activation [42]. This, in turn, increases production of adhesion molecules, chemokines and proinflammatory cytokines [43]. Macrophages possess mineralocorticoid receptors,

expression of which increases in response to IFN-γ secreted by T lymphocytes. Aldosterone treatment increased cardiac expression of adhesion molecules, COX-2, osteopontin, and MCP-1 and promoted inflammatory arterial lesions with perivascular macrophages [44].

35.3.2 Neutrophils

GCs increased blood neutrophils by impairing endothelial cell adhesion and migration to inflammatory sites by reducing neutrophil attractants and suppressing endothelial adhesion molecules [45–47]. Aldosterone upregulated proangiogenic factors produced by neutrophils (e.g., VEGF-A) in a dose- and time-dependent manner [46,47].

35.3.3 NK Cells

GCs adversely affected NK cell activity [48]; however, chronic GC treatment increased IFN-γ and IL-6 levels via histone acetylation [49].

35.3.4 Lymphocytes

CRH induced IL-2 receptor expression and chemotaxis of mononuclear leucocytes to inflammatory sites [36] and stimulated lymphocyte proliferation and secretion of IL-1 and IL-2 [50,51]. GCs caused lymphopenia by inducing apoptosis and synthesis of nonlysosomal endonucleases [52] and reduced proliferation and activation of lymphocytes by directly inhibiting IL-2 and IL-2 receptor production [45]. GCs also suppress production of IL-12, the main inducer of Th-1 responses, thereby leading to a Th-2 shift. GCs also upregulated Th2-induced production of anti-inflammatory cytokines [53], reduced splenic and lymph node B-cell numbers, attenuated B-cell progenitor proliferation, and suppressed IgG production [54,55]. On the other hand, GCs can exert an immunostimulatory response, for example, induction of acute-phase proteins, enhancement of delayed-type hypersensitivity responses, and stimulation of T-cell mitogenesis [40,49].

35.4 Sex Hormones

Sex hormones such as progesterone, estrogen, and testosterone play key physiological roles in reproduction, inflammation, and metabolism [56]. These regulate activity of various immune cells including lymphocytes, macrophages, granulocytes, and mast cells [57]. Sexual dimorphism in both humoral and cell-mediated immune responses is well documented. In general, postpubertal females are immunologically more active due to higher innate, antibody-mediated, and cellular responses [58,59]. Males are more susceptible to infections and have poorer outcomes [60]. Pregnancy increases susceptibility to infection due to reduced cell-mediated immunity. Autoimmune disease such as rheumatoid arthritis and multiple sclerosis may be ameliorated in pregnancy [61,62].

35.4.1 Macrophages

Testosterone reduces TLR-4 expression by macrophage/monocytes, thereby reducing the proinflammatory response [63]. Testosterone also inhibits NF-κB [64] and increases synthesis of anti-inflammatory cytokines such as IL-10 [65]. Estrogens enhance both cell-mediated and humoral immune responses. Estradiol increases the number of granulocyte progenitor cells [66] that contribute to resistance against infections by stimulating synthesis of proinflammatory cytokines while inhibiting anti-inflammatory cytokine production [67]. Low concentrations of estrogens enhance proinflammatory cytokine production in monocytes, while high concentrations reduce production [68]. Estradiol can also enhance TLR-4 expression on the surface of peritoneal macrophages and increase TNF-α production [69]. Progesterone is typically immunosuppressive; it inhibits TLRs, regulates miR-155 and NF-κB activation [70,71], and inhibits NO production in murine macrophages [72].

35.4.2 Neutrophils

Estrogen, progesterone, and testosterone either do [73] or do not [74] influence superoxide generation. Estrogen decreased production of neutrophil chemoattractants at inflammatory sites, with resulting decreases in neutrophil accumulation [75]. Similarly, estradiol limited release of the potent neutrophil chemoattractant CXL-8 from human monocytes *ex vivo* [76]. Estrogens limited neutrophil adhesion to activated endothelial cell monolayers under shear stress conditions, directly affecting neutrophil transmigration and tissue infiltration [75]. Estrogen also reduced proinflammatory mediator release from neutrophils [77].

35.4.3 NK Cells

Females have lower levels of NK cell activity [78], which declines even further during pregnancy [79]. High doses or prolonged administration of estradiol results in decreased NK cell number and activity [80]. However, low doses or short exposures had no significant effect [81]. Several reports indicate higher NK cell activity in men [82], while progesterone reduces NK cell activity [70].

35.4.4 Lymphocytes

Cell-mediated immune responses differ between genders. The T lymphocyte count is reduced in males, but no difference was seen in TH-1- or -2-mediated cytokine production [83]. There are currently no data available on any gender differences in B-lymphocyte count. Estrogens increase bone marrow progenitor B cells in mice by protecting them from apoptosis, inhibiting estrogen-mediated inhibition of suppressor T cells and increasing survival in splenic B cells [66]. *In vitro* studies suggest that estrogen increases immunoglobulin production in both sexes [84], whereas testosterone is inhibitory [85]. Androgens may suppress the proliferation of immature thymocytes, accelerate thymic apoptosis, and limit B-cell lymphopoiesis. Progesterone inhibits the TH-1 response

and proinflammatory cytokine production, while promoting TH-2 responses, including synthesis of anti-inflammatory cytokines [68,83]. Progesterone also suppresses antibody production [86].

35.5 Pituitary Hormones

35.5.1 Arginine Vasopressin

Arginine vasopressin (AVP) or antidiuretic hormone is a nonapeptide produced in the paraventricular and supraoptic nuclei of the hypothalamus, stored in axonal terminals, and transported to the pituitary gland where it is released on demand. AVP is a critical stress response hormone that increases rapidly in septic shock, but it then falls to inappropriately low levels relative to the degree of hypotension [87]. AVP is found in thymic epithelial cells and in B lymphocytes in rat spleen where its expression increases in adjuvant arthritis [88,89]. AVP binds to (i) V1a receptors to increase vascular tone and thus blood pressure, (ii) to V2 receptors to mediate its antidiuretic effect and to increase von Willebrand factor, and (iii) to V1b receptors in the central nervous system, the effects of which are unclear [87]. V1b may play a role in the acute response to stress. AVP and CRH have synergistic effects on the release of ACTH. V1b knockout mice have an impaired acute stress response because of a blunted ACTH response [90]. Proinflammatory cytokines downregulate V1a receptors in lung, liver, kidney, and heart [91]. AVP also decreases NF-κB activation and, subsequently, IL-6 levels [92]. AVP action on renal V2 receptors inhibited TLR-4 and NF-κB activation and downstream molecules in the collecting ducts of endotoxic rats [93]. AVP also regulates immune function using hypothalamic receptor systems.

35.5.1.1 Macrophages

AVP receptors are present on human PBMC [94]. AVP is reported to be both proinflammatory [95] and anti-inflammatory [96] in activated macrophages. It inhibits CD14 expression, which is essential for endotoxin binding by macrophages and thus NF-kB activation [96]. AVP also inhibits endotoxin-induced upregulation of MAP kinases and AP-1 [97] and can stimulate prostaglandin synthesis [98].

35.5.1.2 Lymphocytes

AVP is expressed in peripheral mononuclear cells including T and B cells [89,99]. AVP modulates helper signaling and activates T cells to increase IgM production, but it may be a negative regulator of B-cell receptor signaling [100,101]. V1a knockout mice have a shift in immunoglobulin balance from IgM (high)/IgD (high) to the more mature IgM (low)/IgD (high) expression in B cells [101].

35.5.1.3 NK Cells

Brattleboro rats with AVP deficiency have increased NK cell activity [87].

35.5.2 Prolactin

Prolactin (PRL) is a 24 kDa, single-chain peptide secreted by the anterior pituitary [102]. Prolactin has pronounced effects on growth, reproduction, and osmoregulation in many species. Within the immune system, PRL is produced by T and B lymphocytes, macrophages, thymocytes, mononuclear, and NK cells [103]. However, in PBMC, PRL production is mainly associated with the T lymphocyte fraction. PRL receptors are ubiquitously expressed by hematopoietic cells in murine thymus, spleen, lymph nodes, and bone marrow [104]. PRL modulates cells in the immune system by altering their potential to proliferate, differentiate, and/or undergo apoptosis [105]. PRL also activates the Janus family of tyrosine kinases and signal transducers, as well as transcription activators and cytokines [106].

35.5.2.1 Macrophages

In vitro treatment of murine peritoneal macrophages with PRL resulted in the production of cytokines, NO, and chemokines such as RANTES [107].

35.5.2.2 Neutrophils

PRL can stimulate neutrophils in a dose-dependent fashion [108].

35.5.2.3 NK Cells

Studies demonstrate both decreased NK cell activity in hyperprolactinaemia or no change [105]. Resting and the IL2-activated cytotoxic functions of NK cells were also depressed by high doses of PRL *in vitro* [105].

35.5.2.4 Lymphocytes

Pharmacological suppression of PRL impaired normal lymphocyte function and lymphocyte-dependent immune host defenses against Listeria and mycobacteria [109]. Lymphocytes can also produce a PRL-like protein that promotes lymphocyte proliferation [110]. In T lymphocytes, IL-2, IL-1β, and IL-4 could reduce PRL mRNA, while IL-10 and IFN-γ had no effect [111]. PRL can increase ornithine decarboxylase activity, an enzymatic step integrally involved in initiating the immune response [112]. PRL activates protein kinase C pathways and regulates expression of immunologically relevant genes such as C-myc and IFN regulatory factor-1 [113]. Hypoprolactinemic animals are deficient in mounting various B- and T-cell-mediated immune responses, while high PRL inhibits suppressor (CD8+) T cells [104].

35.6 Growth Hormone

Growth hormone (GH) is produced by the pituitary under stimulation by growth hormone-releasing hormone and inhibition by somatostatin [107].

35.6.1 Macrophages

Human mononuclear cells can synthesize GH and this appears to be directly involved in immune regulation [107]. GH acts on GH receptors on monocytes

and macrophages to increase proliferation [114]. GH primes phagocytes for enhanced production of ROS, hydrogen peroxide, and proinflammatory cytokines [107,115]. GH also decreases macrophage NF-κB activity [116]. *In vivo* studies demonstrated beneficial effects of GH and IGF-1 pretreatment on survival and bacterial killing capacity in animals challenged with infectious bacteria [117].

35.6.2 NK Cells

NK cell activity is decreased in GH-deficient patients but enhanced by IGF-1 [117].

35.6.3 Lymphocytes

Human lymphocytes synthesize and secrete GH and can bind IGF-1. IGF-1 aids the differentiation of lymphoid precursor cells, thymic T-cell progenitors, and bone marrow pro-B lymphocytes [117,118]. IGF-I also induces immunoglobulin production and proliferation of human plasma cell lines [119]. Human GH can act directly, increasing adhesion molecule binding by human T cells to murine matrix proteins *in vitro* [119]. GH increases trafficking and localization of T-cell progenitors from bone marrow to thymus. It can also directly promote immunoglobulin production and *in vitro* proliferation of human plasma cell lines [119].

35.7 Adipokines

Obesity is a growing epidemic in developed countries. Chronic energy overload leads to cellular damage by mitochondrial dysfunction, endoplasmic reticulum stress, and ROS production. This leads to a chronic state of inflammation known as meta-inflammation [120]. The link between obesity and inflammation was first established when a positive correlation was found between adipose mass and TNF-α expression [121]. Adipose tissues are a heterogeneous group of adipocytes, stromal preadipocytes, immune cells, and endothelium [122]. White adipose tissue (WAT) acts as an endocrine organ that stores fat and produces several adipokines such as adiponectin, leptin, resistin, and visfatin, cytokines (such as TNF-α, IL-1, and IL-6), mediators of clotting such as PAI-1, and certain complement factors [123]. Brown adipose tissue (BAT) is the site of nonshivering thermogenesis [124]. Nutritional excess, in particular consumption of a high-fat diet, can directly alter gut microbiota, increasing endotoxin-producing bacteria that can then activate TLRs and nucleotide-binding oligomerization domain-like receptors (NLRs). These detect damage-associated molecular patterns (DAMPs), increasing production of proinflammatory cytokines and recruitment of immune cells. NLRs can contribute to meta-inflammation by inducing inflammasome-dependent caspase-1 activity; this leads to maturation and further secretion of proinflammatory cytokines [125]. Another mechanism implicating adipocytes in meta-inflammation is the production of hypoxia-induced factors, which results from reduced oxygen tension in adipocytes. This may be due to adipocyte hypertrophy or an increased proportions of saturated fatty acids, increasing

oxygen consumption due to uncoupling of mitochondrial respiration away from ATP production. Compromised adipose tissue vascularization, thereby restricting oxygen availability, is a further mechanism [126]. Changes in oxygen tension are detected by HIF-1α that, in turn, increases chemokine and leukotriene B4 production by adipocytes, macrophages, and endothelial cells.

35.7.1 Leptin

Leptin, a 16 kDa pleiotropic protein encoded by the obese gene (Ob) [127], belongs to the type I helical cytokine family, which also contains growth hormone, prolactin, oncostatin M, and cytokines such as IL-6, IL-12, and IL-15 [128,129]. Leptin is mainly produced by white adipose tissue in proportion to body fat mass [130]. Immune cells such as monocytes express mRNA for the ObRa and ObRb isoforms of the leptin receptor, while neutrophils express mRNA for the ObRa isoform only [131]. Activation of the ObRb by leptin sets off a cascade of intracellular signal transduction pathways [132,133]. Leptin deficiency is associated with impaired cell-mediated immunity, an increased incidence of infectious disease, and an associated increase in mortality [131].

35.7.1.1 Macrophages

Leptin promotes phagocytosis and production of leukotriene B4, COX2 and nitric oxide. It is a chemoattractant, increasing secretion of cytokines and chemokines [128,134]. Leptin increases proliferation of circulating cells and reduces apoptosis in a dose-dependent manner [135]. Leptin also increases the expression of monocyte surface markers such as HLA-DR, CD11b, and CD11c [136].

35.7.1.2 Neutrophils

Leptin is a neutrophil chemoattractant, inducing chemotaxis without causing cell activation [137]. The ObRa activates p38/MAPK pathway and SRC kinases without JAK2/STAT3 activation, and it can induce neutrophil locomotion [131] and ROS production [128,138].

35.7.1.3 NK Cells

Leptin is involved in all processes of NK cell development, differentiation, proliferation, activation, and cytotoxicity [128,136]. NK cells express both ObRa and ObRb. STAT3 influences NK cell cytotoxicity by transcription of genes encoding IL-2 and perforin [128]. Leptin also mediates activation of NK cells indirectly by modulation of proinflammatory cytokines by monocytes and macrophages [136].

35.7.1.4 Lymphocytes

Leptin maintains thymic parenchyma via direct antiapoptotic effects on T cells through stimulating expression of IL-7, a thymocyte growth factor [135]. Lymphocytes express ObRb receptors; leptin binding increases secretion of proinflammatory cytokines via JAK2/STAT3 and p38/MAPK/ERK1/2 pathways. Leptin inhibits memory T-cell proliferation and stimulates naïve T-cell proliferation, exacerbating the inflammatory response. Leptin polarizes naïve T-cell

cytokine production toward a proinflammatory phenotype [135] and increases expression of adhesion molecules, for example, ICAM-1 [128].

35.7.2 Adiponectin

Adiponectin is produced mainly by adipocytes and also by skeletal muscle, cardiac myocytes, and endothelial cells [123,124,139]. Adiponectin acts on AdipoR1, AdipoR2, and T-cadherin receptors; AdipoR1 is found ubiquitously, whereas AdipoR2 is commonly found in the liver [140].

35.7.2.1 Macrophages

AdipoR1 and AdipoR2 are expressed in macrophages. AdipoR1 is more abundant initially and decreases upon differentiation into mature macrophages, whereas AdipoR2 remains constant [141]. On LPS stimulation, adiponectin suppresses expression of T lymphocyte chemokines [142], reducing macrophage differentiation, migration, and phagocytosis [123]. Adiponectin switches the macrophage phenotype from (M1) to the anti-inflammatory, alternatively activated M2 [143]. Adiponectin also enhances the ability of macrophages to remove early apoptotic bodies, crucial in preventing inflammation and immune system dysfunction. Adiponectin also reduces induction of ICAM-1, VCAM-1, E-selectin, and IL-8 [144].

35.7.2.2 Dendritic Cells

Adiponectin reduces expression of major histocompatibilty complex class II, CD80 and CD86, and production of IL-12/p40, but increases expression of the inhibitory programmed death-1 ligand (PDL-1) [145]. Activation of AdipoR1 receptors stimulates IL-10 production via AMPK and p38/MAPK pathways; IL-10 promotes further anti-inflammatory actions that are dependent on STAT3 and SOCS3 pathways [146]. Stimulation of these pathways inhibits NF-κB activation, thereby attenuating DC ability to stimulate antigen-specific T-cell responses.

35.7.2.3 NK Cells

NK cells preferentially express AdipoR1, but they can express both types either on the cell surface or intracellularly. Adiponectin also inhibits TLR ligand-induced IFN-γ production, but has no effect on degranulation or cytotoxicity [139].

35.7.2.4 Neutrophils

Adiponectin exerts anti-inflammatory effects by inhibiting production of superoxide and chemokines [147] and decreasing phagocytosis [148].

35.7.2.5 Lymphocytes

All lymphocytes secrete small amounts of adiponectin, whether they are dormant or activated [149]. This can induce significant inhibitory effects on hematological precursors leading to neutropenia [149]. Adiponectin acts on mice splenocytes to inhibit T-cell proliferation and cytokine production [150]. In humans, it also affects apoptosis [151]. Adiponectin promotes $CD4^+$ T cell differentiation toward

a TH-1 lineage, through activation of p38/MAPK and STAT4, and induction of T-beta [152].

35.7.3 Ghrelin

Ghrelin is mainly secreted from the oxyntic mucosa cells in the stomach, though small quantities are expressed by immune cells [153]. Ghrelin levels are increased by fasting, low BMI, and by hormones such as leptin, GHRH, thyroid hormones, testosterone, and parasympathetic activity [154,155], and disease states such as cancer-induced cachexia, renal failure, and sepsis [118,156,157]. In inflammatory diseases, ghrelin expression correlates with disease severity and sequelae. The physiological functions of ghrelin include control of energy homeostasis [158], promotion of adipocyte survival, differentiation and metabolism [159], cardioprotection [160], and skeletal muscle regeneration [161]. The function most relevant to this chapter is its potent anti-inflammatory action on lymphocytes, monocytes, and dendritic cells (DCs) [162,163]. Ghrelin, via GHS-R1a, reduces mortality and attenuates the inflammatory effects of LPS [164,165].

35.7.3.1 Macrophages

Monocytes are the main targets of the anti-inflammatory action of ghrelin [164]. Ghrelin inhibits oxidative stress, cellular apoptosis, cell adhesion, and proinflammatory cytokine expression [162–164]. Tissue macrophages only express the GHS-R in inflammatory conditions [166]. Ghrelin reduced stress-induced increases in phagocytic activity via inducible NO synthase [167]. Delayed administration of ghrelin had a protective effect in endotoxemia and sepsis by decreasing the secretion of high-mobility group box 1 (HMGB1) [168].

35.7.3.2 NK Cells

In septic mice, ghrelin increased NK cell recruitment [169]. Beneficial effects occurred within 24 h, with the reverse occurring thereafter, leading to diminished cellular defenses against bacteremia and reduced survival.

35.7.3.3 Neutrophils

Ghrelin suppressed neutrophil accumulation and production of the neutrophil chemoattractant IL-8 plus TNF-α and MPO in airways of patients with chronic respiratory infections [163]. Ghrelin also decreased adhesion molecule expression on vascular endothelial cells [170]. These effects reversed after 24–48 h with suppression of the anti-inflammatory response [169].

35.7.3.4 Lymphocytes

The GHS-R, acetylated ghrelin, and GH levels decline with progressive thymic aging [171]; this was partially reversed by administration of acetylated ghrelin in aged mice. Ghrelin infusions increased progenticor cell numbers in the bone marrow and thymus [172]. This was associated with improved peripheral TCR diversity of both CD4+ and CD8+ T cells [172]. Caloric restriction promoted a rise in ghrelin levels, correlating directly with greater T-cell output [173].

35.7.4 Other Adipokines

35.7.4.1 Resistin

Resistin belongs to a family of secretory proteins, called resistin-like molecules (RELMs) or found in inflammatory zone (FIZZ). RELM-α (FIZZ1) is present mainly in adipose tissue, RELM-β (FIZZ 2) in the gastrointestinal tract and tumors, while RELM-γ is found in hematopoietic tissue where it has cytokine-like functions [174,175]. Mouse resistin is highly expressed in WAT by macrophages and monocytes, while human resistin is expressed predominantly in bone marrow [175].

The role of resistin in inflammation is not completely understood; however, elevated levels are seen in both septic and nonseptic critical illness and other inflammatory conditions [176,177]. Human resistin is predominantly expressed in macrophages and neutrophils [178], and in human adipose tissue mainly by nonadipocyte-resident inflammatory cells [179]. Expression and secretion of resistin by monocytes is induced by inflammatory stimuli and cytokines [176,180,181]. Resistin drives expression of cell adhesion molecules, matrix metalloproteinases, and pentraxin-3, which promote endothelial cell–monocyte adhesion and infiltration [182]. Resistin impairs the ability of dendritic cells to uptake antigens and produce proinflammatory cytokines [183], and the effector function of T cells, such as proliferation and differentiation into TH-1 cells [183]. Resistin indirectly induces expression of FoxP3, a key regulator of the development of regulatory T cells in PBMCs [183]. Resistin inhibited PMNL chemotaxis in uremic patients and decreased the oxidative burst to *Escherichia coli*, but did not influence phagocytosis of opsonized *E. coli* [184]. The exact role and mechanism of resistin in inflammation, however, remains elusive

35.7.4.2 Visfatin

Visfatin is an adipokine that modulates insulin activity by binding to the insulin receptor [185]. Visfatin is upregulated in acute lung injury, sepsis, trauma, and other conditions [186]. During inflammation, visfatin levels increase in immune cells such as macrophages, DCs, neutrophils, and lymphocytes [187]. Proinflammatory cytokines increase visfatin levels via NF-κB- and AP-1-dependent mechanisms [188]. In turn, visfatin upregulates proinflammatory cytokines in PBMCs and CD14+ monocytes [188]. Visfatin also induces expression of cell adhesion molecules, metalloproteinases-2 and -9, and monocyte atttractant protein-1 [186,189]. Visfatin inhibits neutrophil apoptosis and prolongs neutrophil survival in sepsis [190]. Visfatin also stimulates pre-B lymphocyte activation and increases expression of CD80 and CD40 in monocytes [188]. These provide important signals that augment and sustain T-cell responses. Visfatin affects primary lymphocyte responses by causing a dose-dependent proliferative response in monocytes [188]. Visfatin induces granulocytic differentiation of CD34+ hematopoietic progenitor cells through sirtuin activation and G-CSF upregulation. Visfatin also acts as a potent chemotactic factor, particularly for CD14+ monocytes and CD19+ B cells [188].

35.8 Melanocortins

Melanocortins – including α-, β-, and γ-melanocyte-stimulating hormone (MSH), and adrenocorticotropic hormone – are involved in several physiological functions, including inflammation and immunomodulation [191] and energy homeostasis [192]. The MC1 and MC3 melanocortin receptors play a role in immune regulation and may be expressed by several immune cells including neutrophils, monocytes, dendritic cells, and B lymphocytes [193,194]. The MC5 receptor also has immunomodulatory effects in B lymphocytes and mast cells [195,196]. Melanocortins, in particular α-MSH, can inhibit in various inflammatory conditions, for example, experimental bowel disease [197], brain inflammation [198], and ischemia [199].

35.8.1 Macrophages

Melanocortin receptors (MCRs), especially MC1, MC3, and MC5 are found on several macrophage cell lines [200]. Addition of α-MSH to monocytes significantly reduced proinflammatory cytokine release [201] and upregulated anti-inflammatory factors [202,203]. α-MSH reduced expression of CD86 and CD40 on monocyte-derived DCs [204] and is also involved in the inhibition of chemotactic migration of neutrophils [205]. *In vivo* α-MSH reduced endotoxin-induced liver cell infiltration by macrophages and neutrophils [201].

35.8.2 NK Cells

Intracerebroventricular administration of α-MSH, which blocks many IL-1 effects, significantly blunted shock-induced suppression of both T lymphocyte proliferation and NK cytotoxicity [206].

35.8.3 Neutrophils

In addition to the above effects, exogenous α-MSH administration inhibited cytokine-induced chemotaxis via a direct effect on MC1 receptors [207].

35.8.4 Lymphocytes

B lymphocytes express MC1 and MC3 receptors, while T lymphocytes express no MCRs [208]. α-MSH suppressed antigen-stimulated lymphocyte proliferation, specifically the anti-CD3/CD28-induced proliferation of spleen cells and CD4+CD25 T lymphocytes [209]. α-MSH also downregulated CD86, a major T-cell costimulatory molecule, in LPS-stimulated monocytes [203].

35.9 Neuropeptide Y

Neuropeptides act as neurotransmitters, hormones, and paracrine factors involved in control of all components of the immune system. Examples of

neuropeptides include calcitonin gene-related peptide, substance P, adrenomedullin, and neuropeptide Y [210]. Neuropeptide Y (NPY) is the most abundant and widely distributed neuropeptide present in the mammalian central and peripheral nervous system. NPY influences the regulation of multiple physiological and psychological processes, including energy homeostasis [211] and neuroendocrine secretion [212]. NPY treatment prolonged survival in endotoxemic rats [213].

35.9.1 Macrophages

NPY binds to Y1, Y2, and Y5 receptors on macrophages to increase adhesion, chemotaxis, phagocytosis, and production of cytokines and superoxide anions [214,215]. However, in old mice, NPY decreases chemotaxis and phagocytosis [215]. High cAMP and low PKC correlate with a decrease in chemotaxis and phagocytosis [214,216].

NPY administration inhibited IL-6 release from splenic macrophages; this was potentiated by norepinephrine but reversed by the β-agonist, isoproterenol [217]. NPY thus induces either an increase or decrease in cAMP formation depending on the catecholaminergic pathway activated [217]. NPY increased *ex vivo* production of pro-inflammatory cytokines from healthy volunteer blood samples [218] but decreased TNF-α release in response to LPS, and IL-1β and IL-2 release in response to concavalin A in murine peritoneal macrophages [214]. NPY directly increased ROS release in murine peritoneal monocytes [219,220].

35.9.2 NK Cells

NPY inhibits NK cells activity directly in an age- and dose-dependent manner [221,222]. Increased sympathetic activity and elevated NPY levels correlate with reductions in NK cell activity [222].

35.9.3 Neutrophils

Human neutrophils have a low-level presence of NPY receptors [223]. NPY stimulation inhibited phagocytosis through activation of the Y5 receptor-potentiated ROS production [223]. NPY modulated the fMLP-evoked respiratory burst in a dose-dependent manner [224]. NPY also influences the adhesion abilities of leukocytes in a time- and dose-dependent manner via activation of Y2 receptors [223,225,226].

35.9.4 Lymphocytes

NPY attenuated LPS-induced alterations of T lymphocytes by specifically modulating trafficking patterns [225]. NPY elevates IL-4 and reduces IFN-γ production in splenic lymphocytes [227]. *In vitro* studies show a shift toward TH-2 cells by NPY stimulation [227,228]. NPY thus alters the commitment of T-helper cells to a specific phenotype, leading to potentially altered immune function *in vivo* [228].

35.10 Orexin

Orexins are neuropeptides of which there are two isoforms, orexin A (hypocretin-1) and orexin B (hypocretin-2) [229,230]. Orexin A is suppressed by bacterial LPS and by TNF, which, in turn. reduces food intake and causes lethargy [231,232]. In murine sepsis, orexigenic activity was decreased within the hypothalamus [233].

35.10.1 Macrophages

Orexins modulate peritoneal macrophage function, with Orexin B being more potent. They also activate phagocytosis of mouse peritoneal macrophages [234].

35.10.2 Neutrophils

Orexin A decreases ischemia-reperfusion-induced lipid peroxidation and myeloperoxidase activity, an index of neutrophil infiltration [235]. Endogenous orexin A has a suppressor effect on inflammatory processes by modulating production of free radicals and cytokines that, in turn, attenuate leukocyte infiltration or direct tissue damage [235,236].

35.11 Bioenergetics + Metabolism

Cellular energy metabolism is a crucial part of the machinery that ensures proper functioning of immune cells [237] Mitochondria play the principal role in providing sufficient energy in most cells, in addition to their vital functions in heme and steroid biosynthesis, cell cycle regulation, apoptosis, calcium signaling, and redox homeostasis and signaling. Induction of an inflammatory response may lead to mitochondrial dysfunction via several mechanisms including direct inhibition and/or damage by reactive oxygen and nitrogen species and an overall decrease in mitochondrial biogenesis including decreased expression of genes encoding for the mitochondrial electron transport chain, yet an increase in uncoupling proteins [238],

Consistent with their evolutionary origin as probacteria, DNA from mitochondrial represents a source of mitochondrial DAMPs, which also activate pattern recognition receptors (PRRs). Proinflammatory mediators may initially increase mitochondrial membrane potential, Ca^{2+} buffering capacity and resistance to apoptotic signaling. However, prolonged exposure increases ROS production leading to further oxidative stress and cell damage [239].

35.11.1 Dendritic Cells

DC immunogenicity is positively influenced by PRRs and negatively by signals that prevent DC activation and full functional maturation. The anti-inflammatory transcription factor PPAR-γ is a negative regulator of DC maturation and function; it profoundly inhibits IL-12 expression and inhibits DC capacity to prime naive CD4+ T cells *in vitro* [240]. Resting DCs derive ATP predominantly

from oxidative phosphorylation within their mitochondria; however, activation by inflammatory mediators shifts this to a greater dependence on glycolysis [241]. This shift provides the additional energy required for DC activation and cytokine production [241]. This process involves signaling through PI3 K/Akt [242].

35.11.2 Macrophages

PPAR-γ is also expressed by macrophages and T cells, where it inhibits macrophage activation and the production of monocyte inflammatory cytokines [240]. In addition, hypoxia-inducible factor-1 (HIF-1) plays an important role in the commitment to glycolysis by orchestrating the decision to commit pyruvate to either acetyl-CoA or lactate [243]. Macrophages contain high levels of HIF-1α as they often reside at sites of low oxygen tension. HIF-1α, which is also induced by TLR agonists and proinflammatory cytokines, promotes glycolysis that is essential for macrophage activation [244]. The polarization of macrophages into its two functional states (M1 and M2) is influenced by its bioenergetic state: M1 macrophages possess higher glycolytic activity, which is required to produce proinflammatory cytokines, while IL-4 activated M2 macrophages use oxidative glucose metabolism as their main metabolic pathway to provide a sustained source of energy for tissue remodeling and repair [245].

35.11.3 Neutrophils

Neutrophils also depend on glucose for ATP production via glycolysis; their rate of mitochondrial respiration is very low. This O_2-independent mechanism for ATP generation is beneficial in that it allows neutrophils to function at inflamed or infected sites where local O_2 tensions may be very low [246]. Upon activation by TLR agonists, or phagocytosis of antibody-coated particles, neutrophils use large quantities of molecular oxygen to generate O_2^- and other oxidants via a respiratory burst catalyzed by NADPH oxidase [246]. The presence of nonfunctional mitochondria may lead to the loss of mitochondrial membrane potential that, in turn, may release cytochrome C and initiate apoptosis [247]. However, under inflammatory conditions such as sepsis, neutrophil apoptosis is delayed [190]. Leukocytes exposed to endotoxin or isolated from septic patients showed both immunoparalysis and a generalized metabolic defect affecting both glycolysis and oxidative metabolism that were restored on patient recovery [248]. Neutrophil mitochondria maintain their membrane potential through the glycerol 3-phosphate shuttle, a pathway that allows receipt of electrons from glycolysis by complex III of the electron transport chain to simultaneously contribute to redox balance and avoid apoptosis [242].

35.11.4 Lymphocytes

In vitro inhibition of mitochondrial oxidative phosphorylation or glycolysis diminished T-cell proliferation, indicating that mitochondrial metabolism and glycolysis support T-cell proliferation [242]. Similarly, B-cell receptor activation is regulated by ROS and inactivation of receptor-coupled phosphates, allowing

activation of downstream signaling pathways. Plasma cells produce higher levels of phospholipids compared to naive B cells, and plasma cell differentiation is dependent on mitochondrial citrate conversion to acetyl-CoA and oxaloacetate in the cytoplasm [249]. In prolonged sepsis, the initial shift to glycolysis offers host defense through enhanced proinflammatory cytokine production [248]. Blocking mitochondrial activity diminished cytokine production and increased mortality from fungal infection.

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36

Microenvironmental Regulation of Innate Immune Cell Function

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36.1 Introduction

The innate immune system is the first line of defense against invading pathogens. It has a dual role to provide initial control of the infection and to initiate an adaptive immune response. The lack of specificity of the mediators of the innate response mean that a balance must be struck between effective immunity without excessive "self" injury. It is becoming increasingly clear that the cells of the innate immune system have the capacity to fine-tune the response and that much of this tuning depends on the context in which cellular activation occurs. In this chapter, we will discuss the factors in the microenvironment that may regulate and augment the innate immune response.

The innate immune system consists of physical barriers such as epithelial layers and mucus in the airways, soluble factors such as the complement system, and cells such as neutrophils, macrophages, and dendritic cells. We will focus on the role of neutrophils and macrophages and on the coordinated response that they generate to infection or injury. This response depends on many factors in the environment including oxygen availability, availability and utilization of metabolites, pathogen factors, and host damage signals and is mediated via an array of inflammatory (and anti-inflammatory) mediators. Crucially, the balance of all of these factors will change throughout the course of the immune response and as we will see, the outcome of any immune response is highly context dependent (Figure 36.1).

Neutrophils are the most abundant leukocyte in the peripheral blood. In the steady state, only mature neutrophils are released from the bone marrow and their release into the bloodstream depends on a balance of retention signals and release signals as well as growth factor signals from other immune cells, such as dendritic cells and T cells. In sepsis, however, there is a dramatic recruitment of immature neutrophils from the bone marrow to the circulation ("left shift") [1]. The half-life of a circulating neutrophil is short (only a few hours) and, unusually, they undergo

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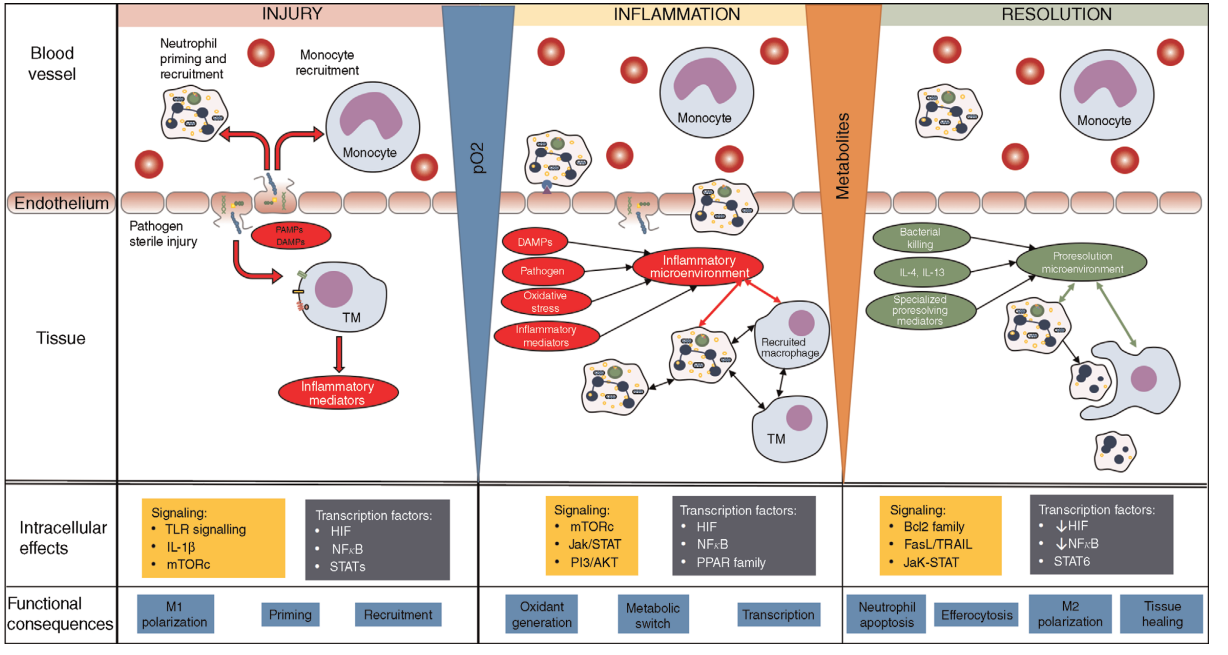


Figure 36.1 The role of microenvironmental factors in regulating innate immune cell function.

constitutive apoptosis in the absence of activating stimuli. Neutrophils are rapidly recruited to areas of tissue injury. Monocytes and macrophages represent a diverse population of cells. Tissue macrophages (TM) are a heterogeneous population of cells with specific phenotypes and roles suited to their anatomical and physiological niche. They include Langerhans cells in the liver, alveolar macrophages in the lung, and microglial cells in the brain. Increasing evidence suggests that these cells are long-lived and are embryonically derived. They are able to self-renew, rather than recruiting replacements from the circulating monocyte population. Tissue macrophages have many roles, but predominantly they act as immune sentinels, surveying for infection or injury. Monocytes are released from the bone marrow and circulate in the peripheral blood. They are recruited to the sites of infection and inflammation and depending on other factors in the microenvironment can differentiate into activated macrophages with variable phenotypes.

The interactions between neutrophils and macrophages change dramatically over the course of infection: Tissue macrophages identify a pathogen or other danger signal. They, along with damaged tissue and endothelial cells, will produce proinflammatory cytokines and chemokines that recruit other immune effector cells, particularly neutrophils and circulating monocytes. Recruited neutrophils and monocytes, once activated, will carry out bacterial killing and generate inflammatory mediators that may result both in host cell damage and in pathogen eradication. Once the infection is cleared, rapid resolution of the inflammatory response is key in minimizing further host cell damage. Resolution of inflammation is increasingly recognized as an active and carefully coordinated process, not just a defervescence of the inflammatory response. Resolution requires apoptosis of the large population of infiltrating neutrophils and efferocytosis of these cells to safely remove them and their toxic granule contents from the site of injury. Resolution also requires the production of anti-inflammatory mediators such as lipoxins and a phenotypic switch in macrophage population with increased activation of the "anti-inflammatory" M2 macrophage population.

Through the action of neutrophils and monocyte/macrophages, the innate immune response is rapidly built up and, upon successfully completing its job, is carefully deconstructed without the release of harmful inflammatory products.

36.2 The role of Hypoxia in Regulating Innate Immune Cell Function

36.2.1 Physiological Response to Hypoxia

Cells of the innate immune system may function within a wide range of hostile environments. Both experimental models and clinical studies have confirmed profound hypoxia in a range of inflammatory and infective scenarios including colitis, arthritic joints, and pleural infection [2–5] (Figure 36.2).

For effective immunity, neutrophils and macrophages must, therefore, be adapted to function in these hypoxic environments. Cellular responses to hypoxia are predominantly mediated by a family of transcription factors called hypoxia-

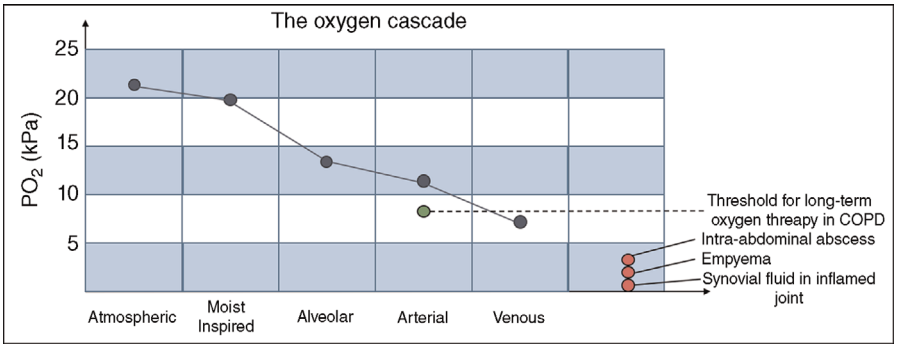


Figure 36.2 The oxygen cascade demonstrating levels of hypoxia under inflammatory conditions.

inducible factors (HIFs). The HIF consists of a heterodimer comprising a single constitutively expressed beta subunit (the aryl hydrocarbon receptor nuclear translocator, ARNT) and an α subunit whose oxygen-dependent degradation determines activity. To date, three α subunits have been identified, HIF-1α, HIF-2α, and HIF-3α corresponding to isoforms with distinct but overlapping activity. In its active form, HIF binds hypoxic response elements, upregulating transcription of genes with diverse functions required for adaptation to hypoxia including erythropoiesis, angiogenesis, glucose transport and metabolism, and control of proliferation and apoptosis. Hydroxylation of prolyl residues within the oxygen-dependent degradation domain (ODDD) of HIFα is catalyzed by a family of prolyl hydroxylases (PHD 1, 2, and 3, also called EGLN 2, 1, and 3 respectively). These 2-oxaloglutarate (2OG)-dependent hydroxylases have an absolute requirement for molecular oxygen, thus providing the "oxygen sensor" controlling HIF activity. Following hydroxylation, HIFα binds with increased avidity to the von Hippel Lindau protein (VHLp) that targets it for destruction through its E3 ubiquitin ligase activity. HIF activity is also regulated by the factor inhibiting HIF (FIH) that hydroxylates an asparagine residue in the HIFα subunit and thus prevents interaction with the cofactor p300 that is required for transcriptional activity. HIFα can also be stabilized by inflammatory factors such as LPS (a TLR-4 ligand) and plays a key role in linking hypoxia and inflammation.

36.2.2 Hypoxia and Innate Immune Cell Survival

Hypoxia has been shown to enhance neutrophil survival [6], mediated by HIF-1α-dependent NFκB activity [3]. The impact of hypoxia on monocyte/macrophage survival is more complicated. A number of studies in human and murine macrophage cell lines have shown hypoxia-induced apoptosis [7,8]. However, in both cell lines subpopulations of hypoxia-resistant macrophages were induced by either prolonged hypoxia or repeated cycles of hypoxia. Furthermore, studies of murine bone marrow-derived macrophages (BMM) show increased survival in hypoxia compared to normoxia when the survival stimulus CSF-1 was depleted [9]. In the same study, human monocytes also showed enhanced viability in hypoxia, but the same was not true of human monocyte-derived macrophages

(MDM s) that were able to survive in the absence of CSF-1 with no enhanced viability seen in hypoxia. These data suggest that hypoxia may have pro- or antiapoptotic effects on monocyte/macrophage populations and that this is context and cell-type specific. The role of HIF in mediating monocyte/macrophage hypoxic survival responses is also less clear than in neutrophils. Circulating monocytes show increased cytoplasmic levels of HIF-1α under hypoxic conditions, but there is no increase in nuclear HIF-1α levels until the cells are differentiated into MDMs [10] suggesting that monocytes may utilize alternative pathways to mediate hypoxic adaptation. This difference may reflect the usually adequate oxygen supply that monocytes experience in peripheral blood.

Clearly hypoxic survival is advantageous in that it allows these cells to function where they are most needed to avoid or minimize disseminated infection. In keeping with this, key innate immune functions are preserved under hypoxic conditions.

36.2.3 Chemotaxis in Hypoxia

In *in vitro* studies, neutrophil chemotaxis has been shown to be maintained or increased in hypoxia. Chemotaxis in response to formyl-Met-Leu-Phe (fMLP) and expression of key integrins LFA-1 and Mac-1 were found to be increased in hypoxia [11]. There was no shape change and chemotaxis in response to IL-8 was unchanged in hypoxia compared to normoxic conditions [12]. Preserved chemotaxis also seems likely *in vivo*; for example, inflammatory bowel disease mucosa is noted to have areas of marked hypoxia [13] and to have high numbers of infiltrating neutrophils. Monocytes and macrophages also migrate effectively to hypoxic areas. Hypoxia in macrophages leads to increased surface expression of CD11b/CD18 (Mac-1) [14], which may aid their ability to migrate into ischemic and inflammatory tissues. Hypoxia also alters endothelial cell expression of chemoattractants such as VEGF and endothelin [15], facilitating enhanced monocyte migration. Hypoxic sites such as tumors and inflammatory lesions have high numbers of myeloid-derived cells. Once monocytes have migrated to tissues, distinct differences in their cytokine profile are induced by hypoxia. These essentially upregulate cytokines that act on and attract neutrophils such as CXCL2, CXCL8, and CXCL5, while simultaneously retaining monocytes by downregulating chemokines that promote monocyte migration and motility, namely, CCL2(MCP-1), CCL8, and CCL23, among others [16]. While this change in profile of chemokines differs to some degree in macrophages compared to monocytes, upregulation of CXCL8 with simultaneous downregulation of CCL2 has also been demonstrated in macrophages [17]. Variations in the expression of matrix metalloproteinases (MMPs) may also impact monocyte retention at sites of inflammation. MMPs degrade the extracellular matrix to facilitate immune cell migration and may be up- or downregulated by hypoxia depending on the cell type. MMP9 is expressed at lower levels in hypoxic human monocytes and this results in reduced migration toward CCL-2 [16]. MMP1, however, is upregulated in macrophages in hypoxia [18]. The body of evidence suggests that once monocytes have been attracted to the site of inflammation/hypoxia, certain key changes are induced by this environment to ensure their tissue retention.

36.2.4 Secretory Function in Hypoxia

Neutrophils undergo a carefully orchestrated process of secretion and degranulation in order to carry out their effector functions. This involves early mobilization of secretory vesicles whose contents include surface receptors, such as CD11b, CD16, and the fMLP receptor, and late mobilization of azurophillic granules whose contents are cytotoxic and include neutrophil elastase and myeloperoxidase. *In vitro* assessment of hypoxic neutrophils has shown preserved cell surface expression of CD11b, preserved IL-8 secretion, and enhanced elastase production in fMLP-stimulated neutrophils [12].

36.2.5 Phagocytosis in Hypoxia

This key immune cell function is highly adapted to cope under the stress of hypoxic environments and the phagocytic function of macrophages is increased in hypoxic conditions. Hypoxia improves not only the number of phagocytosing macrophages but also the quantity of particles ingested by each macrophage. Interestingly, this rate increases with time spent in hypoxia. This has been confirmed to be HIF dependent and studies suggest it relates to a combination of HIF-1α and p-38 MAPK activity [19]. Neutrophil phagocytosis depends on expression of cell surface receptors including CD11b and CD16. As detailed above, the expression of these receptors is maintained in hypoxia. Furthermore, studies of human neutrophils cultured in hypoxia show a significant increase in the uptake of heat-inactivated *Streptococcus pneumoniae* compared to those cultured in normoxia [20]. Using flow cytometry, phagocytosis of *Escherichia coli* [11] was also found to be unchanged in hypoxia compared to normoxia. Innate immune cells, therefore, demonstrate enhanced or at least maintained phagocytosis in hypoxic environments.

The role of the hypoxic signaling pathways is highlighted by the study of neutrophils from patients with mutations in the VHLp that would normally target HIFα for degradation. Neutrophils from these patients demonstrate enhanced phagocytosis in normoxia equivalent to the effect of hypoxia on healthy control neutrophils. In macrophages, silencing of PHD3 (and again subsequent increased HIFα activity) also results in increased phagocytosis [21].

36.2.6 Bacterial Killing

Bacterial killing is, however, less well preserved in hypoxia than other innate functions. The respiratory burst generated by NADPH oxidase is essential for the killing of certain bacteria. This multicomponent enzyme generates reactive oxygen species (ROS) through electron transfer from cytosolic NADPH to intraphagasomal O2. Oxygen is also required for the generation of reactive nitrogen species (RNS) that are part of the neutrophil and macrophage armory against pathogens. These ROS and RNS are highly toxic to invading pathogens. The critical importance of the NADPH oxidase is highlighted by the overwhelming infections that characterize the clinical course of patients with chronic granulomatous disease, in which there is a mutation in one of the components of

the NADPH oxidase. Given the requirement for molecular oxygen to generate ROS, it was predicted that hypoxia would limit the neutrophil's ability to generate an effective respiratory burst. This was confirmed in *in vitro* experiments that showed not only that hypoxic neutrophils had reduced capacity to generate intra- and extracellular ROS but also that this resulted in impairment of *Staphylococcus aureus* killing [12]. Killing of other pathogens, however, can occur independent of ROS activity as demonstrated by the preserved killing of *E. coli* in the study.

The reduction in ROS production may also impair bacterial killing by macrophages, but again this depends on the pathogen. There have been reports of improved *E. coli* bacterial killing in macrophage cell lines under hypoxic conditions *in vitro*, in a HIF-dependent manner [22]. However, *in vivo* experiments by Wiese *et al.* revealed impaired killing of *E. coli* and *S. aureus* in hypoxia. As would be expected, this was largely due to reduced NADPH oxidase and NO synthase activity [23].

The impact of hypoxia on neutrophil energetics and metabolism will be discussed later.

36.2.7 Antigen Presentation

While macrophages are key players in the innate immune system, they also have a major role interfacing between the innate and the adaptive immune responses. As professional antigen-presenting cells, their interaction with T cells is crucial in triggering adaptive immunity. Hypoxic macrophages have been shown to effectively interact with the TCR of CD8+ T cells and induce signal transduction to activate these cells.

Cellular adaptation to hypoxia may also play a role in inflammatory pathologies with prolonged inflammation leading to increasing tissue damage. Chronic obstructive pulmonary disease (COPD) is a chronic lung disease, most often related to cigarette smoking. It is characterized by chronic lung inflammation, recurrent infections, and local and sometimes systemic hypoxia. Both neutrophils and macrophages are implicated in this chronic inflammation and their persistence in COPD airways may be in part due to the alterations in their function in response to hypoxia. Neutrophil degranulation persists despite hypoxia, as detailed above, and this can lead to further tissue damage with neutrophil elastase being a well-recognized cause of lung damage in COPD [24]. Despite the evidence above that macrophage phagocytosis is preserved in the hypoxic environment, other factors including cigarette smoke, have been shown to have a deleterious effect on efferocytosis of apoptotic cells, resulting in failure of resolution [25]. We can see then that in some pathologies, the adaptations the innate immune system has evolved for hypoxia, which may be useful in the context of acute infection, can have deleterious effects over the longer term.

36.3 Metabolism and Nutrient Availability

The microenvironment plays a role in directing cellular metabolism in both macrophages and neutrophils with wide-ranging implications for immunity. In

addition to traversing a wide range of oxygen tensions, immune cells will also be exposed to highly variable availability of nutrients during their life span.

Many immune cells and tumor cells can carry out glycolysis even under conditions of oxygen availability, avoiding oxidative phosphorylation as their main route for ATP formation. In highly proliferative tumor cells and lymphocytes, this can be advantageous as it allows generation of the key components required for generation of a new cell [26]. Tissue resident macrophages are able to both proliferate and maintain their population in this way. Circulating monocytes are also able to proliferate but not to the extent of tumor cells or T cells as described. Activated macrophages and neutrophils are terminally differentiated with little or no capacity to proliferate. Despite this, monocytes, macrophages, and neutrophils are all able to carry out aerobic glycolysis. The benefits of this are twofold: not only does myeloid cell aerobic glycolysis make these cells well adapted to hypoxic environments but it also allows the cells to utilize metabolic intermediates and enzymes for other key functions. Both circulating neutrophils and monocytes change from relatively inert/inactive cells patrolling the body to highly active effectors of the immune response characterized by high rates of protein synthesis and membrane recycling that require generation of proteins and lipids. This is facilitated by the increased availability of metabolic building blocks achieved by alterations in metabolism [27]. The mammalian target of rapamycin (mTOR) is a key player in integrating metabolic and inflammatory signals to regulate innate immune cell function. The mTOR pathway may be activated by cytokines, growth factors, or TLR ligation. Activation results in active mTOR complexes (mTORc1 and 2) that in turn have wide-ranging effects on neutrophils and macrophages. mTORcs regulate translation and post-translational modifications, and the high number of new proteins generated early in macrophage activation is partially dependent on post-translational modifications mediated by PI3-K/mTOR activity. mTOR has been shown to activate HIF, PPARγ (peroxisome proliferator-activated receptor gamma) and MYC that in turn drives synthesis of lipids, proteins, and nucleic acids. mTORc1 is also able to sense amino acid and energy (i.e., ATP) levels and alter cellular metabolism accordingly.

In addition to their role in biosynthesis, it is becoming increasingly clear that metabolic enzymes and intermediates play key roles in modulating the innate immune response. As such, Krebs cycle intermediates that are not required for ATP generation can function to modulate the immune response (Figure 36.3).

Succinate is a key inflammatory signal in M1 macrophages through its ability to stabilize HIF-1α and thus upregulate IL-1β [28]. Succinate competitively inhibits 2OG-dependent enzymes including the PHDs to prevent HIFα degradation and may also indirectly affect HIFα stability via its effects on ROS production [29]. Elevated succinate levels in neutrophils of patients with succinyl dehydrogenase B mutations are also associated with enhanced survival, but this is independent of HIFα. Instead, the impaired function of SDHB results in impaired mitochondrial complex II function and reduced oxidative stress [30]. Finally, succinate accumulation may alter cellular metabolism through post-translational modification (i.e., succinylation) of proteins involved in glucose and lipid metabolism [31].

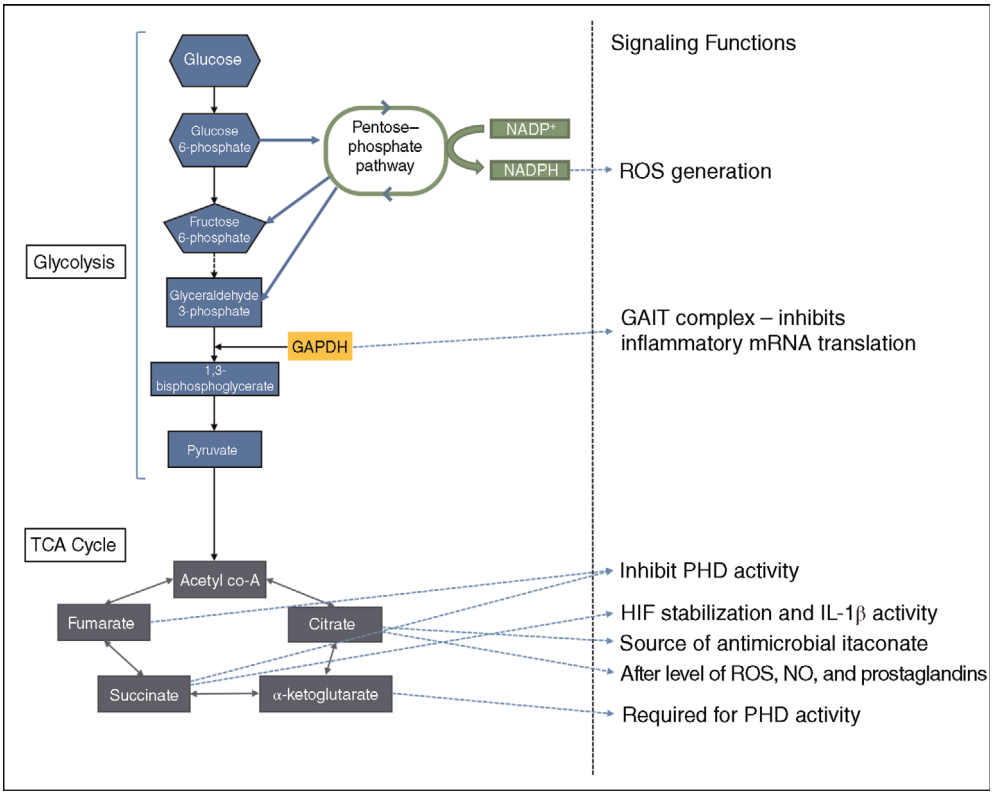


Figure 36.3 The role of metabolic intermediates in augmenting innate immunity.

The mitochondrial citrate transporter CIC transports citrate from the mitochondria to the cytosol and influences level of inflammatory molecules in macrophages: CIC knockdown results in reduced levels of ROS, NO, and prostaglandins [32]. Citrate metabolism is also important in the generation of the antimicrobial metabolite itaconate (ITA). Although traditionally thought of as a bacterial product, ITA has also been shown to be produced by macrophages through the decarboxylation of *cis*-aconitate (which is generated from citrate in the Krebs cycle) [33]. The immune responsive gene 1 (Irg-1) has been identified as the *cis*-aconitate decarboxylase and has been shown to be elevated in inflammatory M1 macrophages. Intriguingly, macrophages activated with LPS show a distinct metabolic phenotype from hypoxic macrophages, although both phenotypes show increased HIFα stabilization. LPS-activated macrophages show persistent metabolism of pyruvate to generate a pool of citrate that is then available for itaconate production [34]. Silencing of Irg-1 (and thus reduction in itaconate production) in macrophages has been shown to inhibit their antimicrobial activity. Thus Irg-1 represents another link between metabolism and immunity [35].

Enzymes involved in glucose metabolism also have additional roles in immune cells. GAPDH is involved in glycolysis. It has also been shown to be part of the

GAIT (IFN-γ-activated inhibitor of translation) complex that in response to IFN-γ binds inflammatory mRNA, supressing their translation. The two functions are likely to be mutually exclusive, thus under conditions of high rates of glycolysis, the inflammatory mRNAs can be translated unchecked [36].

The close links between metabolism and immunity are highlighted by the very different metabolic phenotypes exhibited by M1 and M2 macrophages. Unlike their proinflammatory counterparts, M2 macrophages show increased oxidative phosphorylation and decreased production of NO and TNF-α. Furthermore, macrophage metabolism of arginine plays a key role in determining M1/M2 phenotypes: it may be metabolized via NOS to NO and citrulline or by arginase to generate ornithine and urea. The former is favored by M1 macrophages, while arginase activity is favored by M2 macrophages.

As we will see later, there are a multitude of cytokine factors that can also determine macrophage phenotype. This again demonstrates that no single environmental factor acts in isolation.

36.4 Impact of Host–Pathogen Interactions on Innate Immune Cell Function

Both host and pathogen have evolved strategies to emerge from their interaction with advantage. In tissue, an effective innate immune response must:

a) recognize pathogen and recruit other immune cells to the area, typically with an initial marked influx of neutrophils and a subsequent monocyte–macrophage recruitment important for clearing and resolution. This is triggered by both the pathogen itself and the tissue damage associated with infection via pathogen-associated molecular patterns (PAMPs) or "self-" damage-associated molecular patterns (DAMPs), respectively;
b) rapidly engulf and kill pathogen;
c) induce an adaptive immune response; and
d) clear infected and apoptotic cells to enable resolution of inflammation.

Pathogens may subvert innate immune function at any of these stages to persist and/or progress to systemic infection. A pathogen may avoid recognition, prevent recruitment of innate immune cells, avoid (or indeed enhance) phagocytosis, prevent antigen presentation, and subvert apoptosis of both neutrophils and macrophages to suit their needs.

36.4.1 Damage-associated Molecular Patterns Activate the Innate Immune System

Infective or sterile tissue injury will result in the generation of DAMPs, predominantly from necrotic cells. DAMPs include ATP, heat shock proteins (HSP), mitochondrial DNA and formylated peptides, and nucleic acids. The presence of DAMPs in the microenvironment has a significant impact on the innate immune response, creating microenvironmental "danger signals" that favor immune cell recruitment.

Extracellular ATP enhances neutrophil recruitment, but the mechanism is complex and not a simple chemotactic signal. Extracellular ATP may alter cell adhesion through an indirect mechanism: ATP can upregulate macrophage IL-1β via a different receptor ($P2X_7$ receptor) that in turn can increase expression of adhesion molecules on the endothelium. Chemotaxis is, however, amplified by neutrophil release of ATP in response to stimuli such as fMLP, which then acts in an autocrine fashion to ligate the G protein-coupled $P2Y_2$ receptor and control orientation. This signal is further amplified by signaling via the adenosine A3 receptor in neutrophils [37]. ATP secreted from apoptotic cells also induces monocyte recruitment, again in a $P2Y_2$ receptor-dependant manner.

Mitochondria-derived peptides also act as DAMPs. They are formylated peptides that resemble bacterial peptides and signal via the same cell surface receptors on neutrophils (FPRs) to mediate neutrophil chemotaxis. A hierarchy of processes is proposed to control neutrophil chemotaxis in response to DAMPs and mitochondrial formylated peptides are found to be crucial for the last stages of migration [38].

36.4.2 Pathogen Avoidance of Immune Cell Recognition

Tissue damage due to infection will also cause generation of DAMPs, but this will be augmented by danger signals from the pathogens themselves. Pathogens have developed a range of strategies to alter the function of innate immune cells and avoid eradication.

Pathogens may evade the innate immune system by avoiding recognition. A polysaccharide capsule "hides" PAMPs from receptors such as TLRs in *Haemophilus influenzae* and in Streptococci. Pathogens may also alter their PAMPs to avoid recognition; for instance, *Helicobacter pylori* can chemically alter its LPS and flagella to avoid TLR4 and TLR5 recognition, respectively.

Following recognition, key phagocytic receptors generate a cascade of intracellular signals often mediated by tyrosine kinase activity and polyphosphoinositol generation. *Yersinia* species and *Salmonella typhimurium* generate tyrosine phosphatases (YopH and SptP, respectively) to counteract these signals and avoid uptake [39]. Downstream signaling may also be influenced by the pathogen: enteropathic *E. coli* utilize a type III protein secretion system to translocate toxic effector proteins into host cells. One such effector, nleE, has been shown to stabilize IκB that inhibits NFκB signaling following TLR activation [40].

36.4.3 Preventing Innate Immune Cell Recruitment

Pathogens may also alter the infectious microenvironment to prevent the recruitment of innate immune cells. In response to pneumococcal infection in the lung, there is an influx of neutrophils that both engulf and kill the pneumococcus. This initial highly inflammatory response is found to be protective in mouse models of pneumonia. Neutrophil recruitment has been shown to be impaired by a zinc metalloproteinase (Zmp) produced by *S. pneumoniae*: ZmpC was found to inhibit PSGL-1 (p-selectin glycoprotein ligand-1), thus preventing selectin-mediated neutrophil rolling within blood vessels and resulting in reduced

recruitment and enhanced virulence of the pathogen [41]. *S. aureus*, an important pathogen in human disease, has evolved a number of strategies to evade the innate immune system including manipulating immune cell recruitment and survival (see below). For example, the chemotaxis inhibitory protein of staphylococci (CHIPS), through binding to C5a receptors, reduces the initial influx of neutrophils and macrophages to the sites of *S. aureus* infection [42].

36.4.4 Manipulating Phagocytosis and Antigen Presentation

Opsonisation is a process whereby a pathogen is "tagged" by immunoglobulin and complement proteins and this facilitates its uptake and destruction by phagocytes. This process may be targeted by a pathogen to prevent uptake. Both *S. pneumoniae* and *S. aureus* produce immunoglobulin proteases to avoid Fc receptor-mediated uptake. *S. aureus* and *S. pneumoniae* may also scavenge opsonins such as Factor H, a key activator of complement [39].

Mycobacterium tuberculosis is a hugely successful pathogen in humans. Its capacity to persist within the host is in part due to the subversion of innate immunity. It has been found to not only interfere with normal phagolysosome maturation in macrophages but also inhibit the assembly of the NADPH oxidase machinery by binding to the RAC1 component, thus reducing the generation of ROS that would otherwise facilitate mycobacterial killing [43]. *M. tuberculosis* is also able to interfere with antigen presentation in macrophages, thus impairing the activation of adaptive immune responses. Chronic TLR stimulation by mycobacterial lipoproteins results in downregulation of MHC class II transcription. *M. tuberculosis* infection also interferes with trafficking of MHC class II molecules to the cell surface, further reducing the capacity of the macrophage to present *M. tuberculosis* antigen and activate the adaptive response [44].

36.4.5 Subversion of Immune Cell Death

Intracellular pathogens may gain an advantage by slowing or reducing apoptosis and hijacking the immune cell as a "Trojan Horse." Other pathogens accelerate apoptosis of phagocytes in order to limit their capacity to phagocytose and kill.

Neutrophils achieve bacterial killing via extracellular release of granules during degranulation and through release of ROS and granule contents into the phagolysosome following phagocytosis of a pathogen. Phagocytosis ordinarily induces the neutrophil's apoptotic machinery (phagocytosis-induced cell death, PICD) [45] allowing subsequent efferocytosis by macrophages and clearance of the pathogen. This pathway may be hijacked by a number of intracellular pathogens that through prolonging neutrophil survival are able to perpetuate infection. *Chlamydia pneumoniae* is an obligate intracellular pathogen. It is effectively taken up by neutrophils through phagocytosis but not effectively killed [46]. It not only delays constitutive neutrophil apoptosis but also causes enhanced MIP-1β-mediated macrophage recruitment and increased phagocytosis of infected neutrophils by macrophages, thus allowing propagation of the infection [47].

S. aureus PAMPs include LTA that is recognized by TLR2 and peptidoglycan (PGN) that is an agonist for the intracellular pattern recognition receptor NOD 2. TLR 2 ligation leads to NFκB-mediated neutrophil survival that may initially favor the host [48], allowing further activation of the innate response by the neutrophil. However, if the pathogen is able to persist inside the cell (as *S. aureus* has been shown to do), the host advantage may be lost as the cell becomes a reservoir of infection. The effect of *S. aureus* on apoptosis is not straightforward; there is some evidence of neutrophils showing enhanced PICD in response to *S. aureus* exposure, possibly allowing safe removal of both neutrophil and pathogen and thus resolution. The *S. aureus* pore-forming toxin Panton–Valentine leucocidin (PVL) can induce neutrophil necrosis or apoptosis with higher concentrations of PVL favoring necrosis [49]. *S. aureus* certainly manipulates neutrophil cell death, but this is context dependent and also crucially dependent on multiplicity of infection (MOI) [48].

Conversely, if a pathogen is able to accelerate neutrophil apoptosis sufficiently, it can impair the immune response. Pyocyanin is a toxic metabolite released by *Pseudomonas aeruginosa* that has been found to induce rapid neutrophil apoptosis via lysosomal alkalinisation and subsequent destablization of Mcl-1 and caspase activation [50]. In murine models, pyocyanin production is associated with reduced bacterial clearance.

The examples above are only a small selection of the ways in which pathogens may alter the microenvironment and subvert innate immune cell function.

36.5 Inflammatory Mediators

Both neutrophils and macrophages have the capacity to modify the immune response through the production of a wide range of pro- and anti-inflammatory mediators. Their migration and activation are, in turn, guided by the expression of such mediators. There will, therefore, exist a complex interplay between pathogen, damaged host tissues, and neutrophils and monocytes/macrophages.

36.5.1 Oxidants

Neutrophils and macrophages generate ROS both by NOX2 activity and by mitochondria, that is, mROS. They also generate RNS by interaction of NO-produced inducible nitric oxide synthase (iNOS) with ROS (Figure 36.4).

While these oxidants have important antimicrobial actions, they are also important signaling molecules and can dramatically alter the innate immune response. ROS are predominantly active within the phagolysosome, but they may also diffuse into the cytosol and may cross the cell membrane into the extracellular environment. The more volatile the species, the more rapidly it will react with other target molecules. Conversely, less reactive species such as H_2O_2 may diffuse further and have both paracrine and autocrine effects.

RNS may have both pro- and anti-inflammatory effects and the outcome of the response will depend on location, duration, concentration, and interaction with

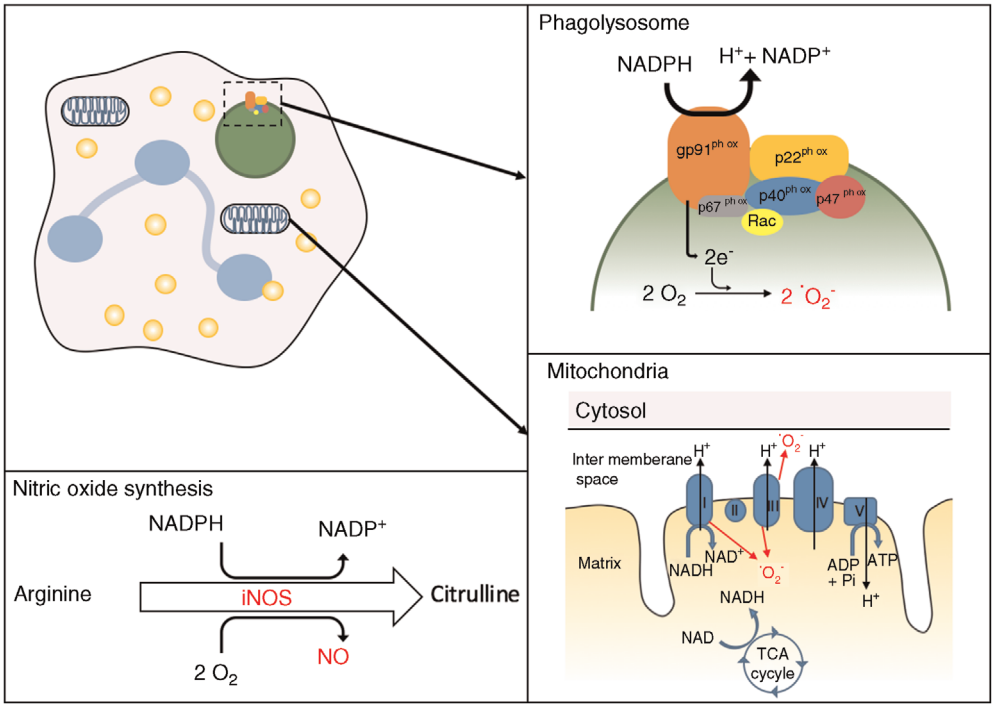


Figure 36.4 Sources of oxidants in neutrophils.

cofactors. The localization of iNOS within neutrophils and macrophages is not fully characterized, but it is seen within small vesicles in the cytosol [51].

As they are potent oxidizing agents, ROS and RNS have the capacity to alter the redox state of the cell that, in turn, has implications for signaling. ROS and RNS may also act directly as signaling molecules. Tyrosine phosphorylation is pivotal in cell proliferation, differentiation, and in cell to cell communication. It is involved in transducing signals from diverse mediators including fMLP, TNF-α, and GM-CSF. Oxidants may alter the balance of phosphatase versus kinase activity either through inhibition of phosphatase activity or through induction of kinase activity [52]. A further layer of complexity is added when we consider that the effect may be direct or indirect and that the alterations in phosphorylation may be activating or inhibitory. Examples of key enzymes subject to oxidant regulation include Syk (implicated in integrin signaling in neutrophils), MAP kinases such as ERK and protein kinase C (PKC).

Mitochondria are increasingly recognized as more than simply the energy generator of the cell especially in neutrophils that carry out little or no aerobic respiration. As discussed above, mitochondrial TCA cycle intermediates play a key role in signaling in immune cells. Mitochondrial ROS (mROS) are implicated in a wide range of immune cell functions. mROS are required for effective bactericidal activity in macrophages. TNF-α and ligation of TLR 1, 2, and 4 lead to an increase in mROS generation via TNF receptor-associated factors (TRAFs) [53], neatly linking "danger signals" with antimicrobial activity.

Increased levels of mROS are also an important signaling mechanism. mROS have been shown to stabilize HIF-1α and to upregulate NFκB activity downstream of TNF receptor signaling. mROS are also required for activation of inflammasome components such as NLRP3, a key player in recognizing and leading the response to various DAMPs and PAMPs. mROS are also implicated in a number of adaptive immune cell functions including T-cell activation. As they are able to alter the redox state of the cell, mROS may also affect other signaling pathways by analogy to NOX2-derived ROS.

Key neutrophil and macrophage functions are affected by ROS/RNS-mediated signaling changes. FcR signaling is blocked in the absence of ROS *in vitro* and the neutrophils of patients with CGD show a failure of amplification of FcR-mediated phagocytosis [54]. Macrophage phagocytosis is intricately linked to ROS and RNS generation. Redox signaling in dying cells modifies their uptake by macrophages; for example, the presence of oxidized phosphatidylserine (PS) on the surface of an apoptotic cell is a key "eat me" signal. In addition, redox reactions within the macrophage may alter phagocytic function. One example is the delay in fusion of the phagosome and lysosome caused by RNS. This is proposed to allow time for more proficient antigen processing and thus antigen presentation by the macrophage [55]. Oxidative stress (leading to excessive oxidation of proteins, lipids, and nucleic acids) can directly trigger apoptosis. CD45 and SHP-1 that modulate apoptosis have oxidant-dependent activity and peroxynitrate (a NO derivative) can also induce apoptosis in neutrophils. Oxidants can also alter gene expression. ROS can activate NFκB expression that in turn upregulates key cytokines including IL-1, IL-6, IL-8, and MIP2. ROS may also participate in LPS-mediated NFκB signaling in macrophages. Finally, TNF-induced NFκB activity is also ROS dependent.

Leukocyte ROS may also affect local cells including the vascular endothelium where it is shown to increase phospholipase D and P-selectin expression (and thus neutrophil binding).

The requirement for molecular oxygen for ROS and RNS generation means that there exist links between hypoxia and oxidant availability and activity. Bacterial killing by NADPH oxidase-derived ROS may be impeded in hypoxia and interestingly, iNOS is a HIF target gene.

36.5.2 Cytokines and Chemokines

Neutrophils are exquisitely sensitive to the cytokine environment and possess a wide range of cytokine receptors. Cytokines acting upon neutrophils can be divided into different classes with different structures, different receptors, and different signaling pathways.

Type I cytokines consist of four α-helices and include IL-4, IL-6, G-CSF, and GM-CSF. Type II cytokines contain six α-helices and include IFN-α, β, and γ and IL-10. Both Type I and type II cytokine signaling is mediated by JAK-STAT pathways.

The IL-1 superfamily includes the three known forms of IL-1 (IL-1α, IL-1β, and IL-1Ra) and IL-18. IL-1β is a critical regulator of the inflammatory response and is predominantly produced by macrophages and may also be produced by epithelial

cells and fibroblasts. In neutrophils, its main effect is to prolong survival. As well as being secreted by macrophages, IL-1β may also act as a transcription factor in macrophages in an autocrine fashion.

TNF-α is a key inflammatory cytokine. It is produced in large quantities by activated macrophages and can act upon both macrophages and neutrophils. It is important in priming and activating neutrophils and in macrophages can result in increased NFκB activity and sustained IFN-β production that acts in synergy with the TNF-α in orchestrating the inflammatory response [56].

Neutrophil functions are guided by the presence of cytokines and other inflammatory mediators. Neutrophil priming describes the phenomenon whereby exposure to a "priming" stimulus results in an amplified response from the neutrophil following subsequent activation. Neither the priming stimulus nor the activating stimulus in isolation is able to unleash the full antimicrobial arsenal of the neutrophil, but together they induce a potent effector cell function. Priming agents include pathogen-derived factors such as LPS and the influenza A virus and "self" inflammatory mediators such as TNF-α, IL-8, and GM-CSF [57]. These priming stimuli may be generated by other immune cells or by endothelial cells. The consequences of priming are manifold: classically, priming is measured by enhanced ROS production and increased phagocytic activity; however, primed neutrophils may also alter the makeup of their surface receptors, thus changing their capacity to respond to subsequent cytokine signals. Primed neutrophils also show enhanced degranulation and survival. The physiological importance of priming is clear: a fully activated neutrophil is potentially highly toxic to host tissue, but neutrophils must be activated quickly to be effective when required to fight infection. The intermediate primed stage avoids having high numbers of active neutrophils in the circulation, but it means that in response to a danger stimulus, there is a rapidly available population of potent effector cells available.

Interestingly, priming may be reversible in response to certain stimuli, including platelet activating factor (PAF). This "depriming" is also a safety mechanism – if after a period of priming, the neutrophil is not fully activated it can "stand down" again, retaining the capacity to be reprimed and fully activated at a later stage [58]. Recent studies in acute lung injury (ALI) suggest that a failure of this depriming may contribute to the neutrophil-mediated cell damage seen in this condition [59].

Chemokines are small molecules with characteristic conserved cysteine residues. They are subdivided based on the position of these residues (CXC and CC chemokines). Their receptors are seven-transmembrane G protein-coupled receptors whose downstream signaling includes activation of PI3-kinase required for chemotaxis. Chemokines are released by cytokine-activated endothelial and epithelial cells, resident tissue cells, and other leukocytes in injury or infection, leading to neutrophil chemotaxis. This response is then amplified as neutrophils themselves secrete further chemokines to recruit further effectors of the immune response.

Survival stimuli for the short-lived neutrophil include not only bacterial products such as LPS but also a number of cytokines, such as GM-CSF, G-CSF, IL-1β, IFN-γ, and TNF-α. Their downstream signaling converges on overlapping pathways including PI3 K, ERK, and NFκB to tip the balance of pro versus anti-apoptotic factors and delay apoptosis.

Cytokines have a myriad of roles in monocyte/macrophages. They not only govern recruitment and activation but also polarization of macrophages that is critical in determining the outcome of the immune response through both cytokine production and through polarization of the subsequent T helper cell response.

The concept of macrophage polarization is a much debated topic. Most cytokine-directed polarization is driven by signal transducer and activator of transcription (STAT) proteins: the balance between STAT1 activation and STAT3/6 activation determines the functional outcome for the macrophage. Other transcriptions factor families are also involved, though, including HIF and PPAR family members.

IFN-γ is the key cytokine in the polarization of M1 macrophages. IFN-γ and TLR4 ligation, triggers activation of transcription factors including NFκB, IRF-1, and STAT 1 that, in turn, leads to transcription of proinflammatory genes including TNF, IL-1β, IL-6, type I interferon, CXCL-10, and iNOS. IL-4 and IL-13 polarize macrophages to an M2 phenotype. They share the IL-4Ra receptor subunit and IL4R ligation leads to activation of Jak kinases and subsequent activation of STAT 6 that mediates transcription of M2 genes including IL-10, PGE2, ARG1, CCL-17, and CCL-22.

The different subsets of macrophages also exhibit differential chemotactic behavior. M1/M2 recruitment appears to be phase dependent with initial recruitment to a site of inflammation favoring M1 macrophages with later recruitment of M2 macrophages. The physiological benefits of this are clear: initial antimicrobial activity with subsequent anti-inflammatory and healing activity. The mechanisms by which this is controlled are not fully elucidated. The two populations show some differences in chemokine receptor expression and also subcellular localization of receptors, thus providing a mechanism for fine-tuning of migration in response to cytokines and chemokines [60].

The M1/M2 paradigm is likely to be an oversimplification and there may be more of a spectrum of macrophage phenotypes whose development depends on the complex interaction of many factors. Cytokines, oxygen levels, pathogens, and the tissue in which the cell is differentiating all affect polarization and contribute to the macrophage phenotype that develops. In addition, there is significant interplay between these polarization mechanisms with simultaneous activation and suppression of certain genes; for example, NFκB upregulates HIF-1α. An increased HIF-1α:HIF-2α ratio is known to favor M1 polarization and a predominance of HIF-2α favors M2 polarization. STAT6 works with Kruppel-like factor (KLF) 6 to inhibit NFκB, thus reducing HIF-1α and further skewing the cell toward M2 activation [61]. There is a fascinating link between Th2/M2-type responses, immunity against helminthes, and tissue repair. In helminth infection, the large size and migratory capacity of the pathogen means a highly inflammatory type 1 response is likely to be more damaging to host than to pathogen. An alternative response is, therefore, induced that encourages not only control of parasitic infection but also tissue repair. There is a clear overlap in the cytokine and signaling profiles of antihelminth immunity and tissue repair with STAT 6, IL-4, and IL-13 being crucial mediators of both. Alternatively activated macrophages are not only more effective than naïve macrophages at killing helminths but also contribute to tissue repair in helminth infection [62]. This is illustrated by

the nematode *Nippostrongylus brasiliensis* whose migration through the lung causes significant tissue damage. The repair of this damage is mediated by M2 macrophages via IL-4R signaling [63].

36.5.3 Lipid Mediators in the Innate Immune Response

Prostaglandins and leukotrienes are predominantly proinflammatory lipid mediators. Alternative breakdown of their precursors can, however, generate anti-inflammatory products such as the specialized proresolving mediators that include lipoxins, resolvins, and protectins (Figure 36.5).

Prostaglandin E2 (PGE2) is the predominant prostaglandin in innate immune modulation. Levels depend on the activity of COX2-driven synthesis and 15-hydroxyprostaglandin dehydrogenase (15-PGDH)-mediated degradation of PGE2. There are four different PGE2 receptors that mediate heterogeneous effects. The receptors have varying affinity, varying expression, and varying desensitization to PGE2 and so the nature of the PGE2 signal determines the outcome. Thus, PGE2 can have seemingly contradictory effects. It contributes to

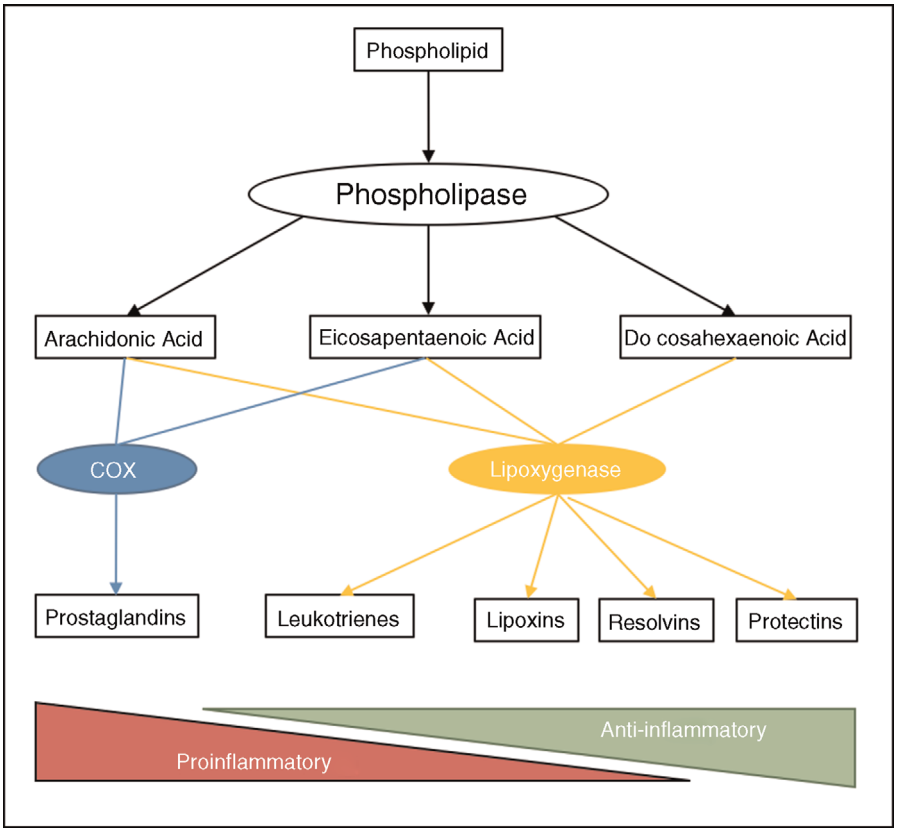


Figure 36.5 Generation of lipid mediators. Differential metabolism of arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid generate a range of mediators that can have pro- or anti-inflammatory effects.

an influx of neutrophils and macrophages and mediates vasodilation, but it also reduces phagocytosis and bacterial killing. PGE2 may also play a role in polarizing the adaptive immune response by reducing IL-12 production by macrophages and thus favoring a Th2-type response.

Leukotrienes are also products of arachidonic acid metabolism. Cysteinyl leukotrienes (CsyLT) are important in airway inflammation in asthma and CysLT antagonists are commonly used in asthma treatment. Leukotriene B4 (LTB4) is an important chemoattractant for neutrophils and, similar to the CXC and CC chemokines, ligates a G protein-coupled receptor. Interestingly, increased levels of LTB4 are found in the airways of patients with COPD, potentially contributing to the increased neutrophil- and macrophage-driven inflammation through increased recruitment of these cells [64].

Certain cytokines and chemokines lead to downregulation of the innate inflammatory response and myeloid cells can also have key anti-inflammatory roles. Interest is mounting in specialized proresolving mediators (SPMs), a group of proresolution lipid mediators including lipoxins, resolvins, protectins, and maresins. They share a common pathway of origin: phospholipase A2 enzymatically cleaves essential fatty acids to create arachidonic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) creating the precursors for SPMs. Lipoxins are the best characterized of this group and we discuss them in more detail next to illustrate these proresolution mediators.

Lipoxins are synthesized via two different pathways (via lipoxygenase action on LTA4 or arachidonic acid directly). Both pathways can generate lipoxin A4 and lipoxin B4. Lipoxins possess powerful anti-inflammatory effects: they reduce neutrophil chemotaxis and are highly effective at blocking leukotriene B4-mediated migration [65]. They also control neutrophil proliferation and reduce persistent neutrophilic inflammation, acting via a number of mechanisms. They prevent accumulation of nuclear NFκB and consequently reduce IL-8 levels [66]. Lipoxins bind to the ALX/FPR2 receptor and restore apoptosis pathways to aid resolution of inflammation. They competitively inhibit serum amyloid A (SAA) that is antiapoptotic (signaling via ERK and Akt pathways). SAA is also found in increased levels in COPD airways and this pathway has been mooted as a potential therapeutic target in COPD [64].

Lipoxin A4 and B4 increase monocyte migration and adherence [67] and lipoxin A4 augments macrophage nonphlogistic neutrophil phagocytosis [68]. In contrast to its restoration of apoptosis in neutrophils, lipoxin A4 prolongs the life span of macrophages, through the PI3K/Akt pathway [69]. Interestingly, macrophages pretreated with lipoxin A4 demonstrated upregulation of NrF2, the potent activator of antioxidant responses, when exposed to apoptotic stimuli [70].

36.5.4 Microvesicles

Microvesicles (MVs) are microparticles (diameter of 0.1–2 μm) formed from plasma membrane and shed by most cell types. The number of circulating microvesicles is increased in inflammatory and immune-mediated illness. Platelet-derived MV production begins with increased cytoplasmic calcium activation of calpain. Endothelial-derived MVs are triggered by oxidized LDL (among other

signals) and leukocyte MVs in response to NO-mediated signaling. Microvesicles shed by cells of the innate immune system are highly influenced by their microenvironment and the nature of their target cells. MVs may possess either pro- or anti-inflammatory functions.

Neutrophils treated with a broad spectrum NO inhibitor produce more microvesicles causing an increase in IL-8-mediated chemotaxis. Similar to platelet MV production, this is mediated via calpain activity [71]. Coincubation of neutrophil-derived microvesicles with human umbilical vein endothelial cells (HUVEC) induced release of the proinflammatory cytokine IL-6, while incubation with human macrophages induced the anti-inflammatory TGF-β [72,73]. These intriguing particles are under investigation as potential therapies for inflammatory disorders such as arthritis [74].

Conversely, the increase in microvesicle formation by stimulation of a murine macrophage cell line was abrogated by a NO inhibitor. Macrophage MV production was increased following TLR stimulation, particularly via TLR4 and TLR2. Increasing NO levels paralleled increasing MV production when cells were stimulated with LPS, and NO donors could independently increase murine macrophage MV production. Microvesicles derived from "resolving exudate" have been shown to improve macrophage efferocytosis of apoptotic cells, further demonstrating the varied roles these molecules play in the innate immune system [75].

36.6 Conclusions

The cells of the innate immune system are adapted to function in hostile inflammatory environments. They are able to sense a wide range of microenvironmental factors and integrate these signals to alter their phenotypes and respond appropriately to allow effective immunity with minimal damage to host tissues. However, these mechanisms can dysfunction – they may be hijacked by pathogens to subvert the immune system or become disordered in pathologies characterized by prolonged or excessive inflammation resulting in tissue damage. The overall outcome of the immune response is a highly regulated product. Cells of the innate immune system never act in isolation; they influence and are influenced by the microenvironment, and interactions between the innate compartments add further complexity. The outcome of all the extracellular factors depends on up- or downregulation of intracellular signaling pathways. Key pathways include HIF, NFκB, apoptosis pathways, ROS and RNS generation, MAPK and AKT signaling, and mTORc, and there is significant cross-talk between these pathways. The integration of all the different signals will thus alter the phenotype of the innate immune response.

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37

Epigenetics of Inflammation

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37.1 Epigenetics and Homeostasis Protection

Darwinian evolution principles designed a sensing, defending, and mending stress response to protect homeostasis and promote life. The first homeostasis survival process recorded by humans eventually was called inflammation, which a flame pictorial originally symbolized [1]. Later, physiologic properties of red color (rubor), heat (dolor), pain (dolor), swelling (tumor), and altered function (functio laesa) were added to the universal concept. It also became clear that inflammation departs from homeostasis in order to *defend and mend* an injury and return. Also appreciated was that inflammation could resolve or become chronic. Mounting data now support that epigenetic chromatin remodeling *sequentially* and *spatially* directs the course of inflammation.

Epigenetic control over inflammation involves both *innate* and *adaptive* immune cells and nonimmune tissues [2]. This chapter emphasizes well-characterized epigenetics of myeloid-derived circulating monocytes and tissue macrophages, as they become part of inflammation. However, depending on the spatial features of inflammation, epigenetics changes during inflammation reprogramming of dendritic cells, natural killer cells, eosinophils, basophils, and T lymphocytes, as well organ- and tissue-specific cells. This chapter also discusses the emerging and complex field of epigenetics in developmental epigenetics that program the many heterogeneities of yolk sac versus tissue-derived macrophages after they take residence tissues like lung, brain, and adipose tissue. Unfortunately, this field of immune epigenetics rarely looks at translation to clinical inflammatory diseases discussed in this chapter, and it is not known yet if or how these phenotype variances impact acute or chronic inflammation. Accordingly, epigenetic mapping of inflammation is immature, though growing. Figure 37.1 depicts the general homeostasis concept of inflammation epigenetics. Table 37.1 lists content sections. Table 37.2 provides a glossary.

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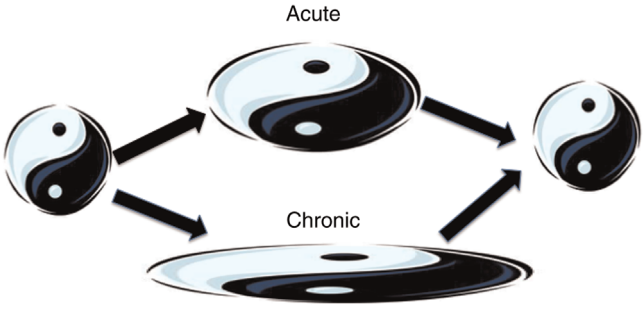


Figure 37.1 Inflammation and homeostasis: inflammation distorts homeostasis boundaries in acutely or chronically stressed cells and tissues. Homeostasis is retrieved when inflammation succeeds.

Table 37.1 Contents.

1. Epigenetics and homeostasis
2. Epigenetic principles
3. Epigenetics of successful inflammation
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5. Epigenetics and immunometabolism
6. Sirtuins as an inflammation homeostat
7. Epigenetics-based inflammation treatment
8. Summary

Table 37.2 Glossary of epigenetics.

DNA CpG methylation and hydroxyl-methylation: DNA epigenetic marks
Epigenetic code: chromatin functional marks on histone and DNA
Epigenome: genome-wide chromatin epigenetic code
Epimutations: stable epigenetic changes
Euchromatin: DNA accessible to transcription
Heterochromatin: reversibly silenced chromatin; also called facultative heterochromatin
Nucleosome: an orderly body of packaged histones and DNA
Immunometabolism: integrated immune and metabolic pathways
Inflammation initiation and adaptation: sequentially directed effector to repressor epigenetic reprogramming
Poised enhancer–promoter: rapidly transcribe monocyte sequences with predictable histone marks
Sirtuins: NAD+ redox sensors that critically regulate inflammation reprogramming as homeostasis guardians

37.2 Epigenetic Principles

The term “epigenetics” was introduced in 1942 [3]. Its classic definition emphasizes heritable changes in gene function not dependent on germ line DNA. This chapter uses a broader definition, in which nonreplicative chromatin histone proteins and DNA and transcription factors work together to temporally direct inflammation.

37.2.1.1 Epigenetic Language

The "epigenetic language or code" [4] – *sometimes divided into "readers, writers and erasers" – spatially and temporally maintains, activates, or silences gene expression by modifying histones and DNA*. Dynamic and flexible epigenetic reprogramming directs the development of immune cells and prepares them for defending and mending. During epigenetic reprogramming, mutations may permanently alter the epigenome by passing vertically as cells replicate (epimutations). Both histone and DNA epigenetic marks may be inherited [5].

Histones package nuclear but not mitochondrial DNA into orderly and repetitive nuclear chromatin structures called nucleosomes, which contain two tetrads of histones H2A, H2B, H3, and H4 with approximately two turns of 146 base pairs of DNA wrapped around the tetrad [6]. Linker histones (e.g., H1 linker histone) juxtapose DNA entering or leaving nucleosomes. Subtype histone variants (e.g., histone H2Z) "enter or leave" nucleosomes to modify their functions by remodeling or repositioning. Other important epigenetic regulators are histone protein 1 (HP-1) and high-mobility group (HMG) proteins, which also influence chromatin structure and function [7].

The term *euchromatin* signifies an "open" state with exposed DNA sequences that can support gene transcription. More compact chromatin called *heterochromatin* permanently or reversibly silences transcription [8]. Shifts between active euchromatin and reversibly silent heterochromatin – sometimes called *facultative heterochromatin* – dynamically direct gene expression reprogramming and inflammation phenotypes [9]. *Thus, flexible chromatin epigenetic coding and structural remodeling support the departure from and return to homeostasis during inflammation*.

Covalent post-translation chromatin modifications support histone epigenetic changes by adding or removing acetyl, methyl, phosphate, ubiquitin, and adenosine diphosphate (ADP) ribose to exposed side chains [10]. The best understood histone-coding marks are acetylation, methylation, and phosphorylation. Coding also adds or removes methyl to cytosine plus guanine (CpG) containing DNA. Chemical histone or DNA modifications generate epigenetic "coding" function by interacting with transcription factors, cofactors, and RNA polymerizes [8,11]. Epigenetics also controls micro-RNA or long coding RNA expression [12].

Acetylation of specific histone lysines (e.g., H3K9ac) often supports transcription, while methylation replacement of acetyl (e.g., H3K9me3) promotes silent heterochromatin formation, although this formula may vary. Histone H3K4me1 and H3K27ac commonly mark potentially active (poised) gene enhancer regions needed for rapid defense [13,14]. Switching between acetylation and methylation histone coding activates or represses gene transcription. DNA methylation, demethylation, and hydroxyl (OH) methylation controlled by specific epigenetic writers or erasers also dynamically increase or decrease gene transcription. Epigenetic reprogramming is also gene set-specific in that it programs physiologic pathways such as nutrient selection, protein synthesis, surface receptors, autophagy, and mitochondrial function, among others. Open access Epigenome Web-based sites provide access to epigenome information (www.blueprint-epigenome.eu; http://www.ncbi.nlm.nih.gov/epigenomics). Figure 37.2 conceptualizes epigenetic reprogramming of chromatin structure and function.

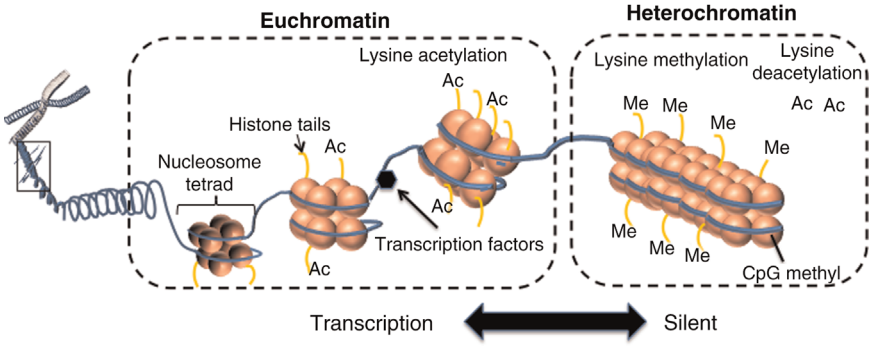


Figure 37.2 Chromatin structure and function: each nucleosome consists of an octamer of two copies of histones H2a, H2b, H3, and H4 encircled by about two turns of DNA (~146 base pairs). Reciprocal properties of lysine acetylation/deacetylation or methylation/demethylation combined with other post-translational changes such as phosphorylation, SUMOylation, and ubiquitination contribute along with DNA CpG methylation to dynamic shifts in euchromatin to reversibly silent heterochromatin. Interactions of chromatin and cofactors and transcription factors (e.g., NFκB) bound to accessible DNA direct transcription of genes encoding proteins and functional noncoding mRNAs.

37.3 Epigenetics of Successful Inflammation

37.3.1 Preepigenetic Sensing and Signaling

Preepigenetic sensors and signals transmit external and internal stress information to nuclear chromatin. First, external or internal cell sensing receptors detect danger signals (*alarmins*) and inform a signaling network [15]. Among external alarmins are pathogen-associated molecular patterns (PAMPs) from microbes and toxins sensed by Toll-like receptors (TLRs) [16], and other signals to complement receptors, immune globulin receptors, and receptors recognizing nutrients such as glucose, fatty acids, and amino acids. Internal alarmins include free nuclear or mitochondrial DNA, histones, and excessive oxygen and nitrogen-derived species (ROS, NOS, etc.), which are sensed by the *inflammasome* or *endoplasmic reticulum* (ER) stress response proteins [17]. Internal nutrient sensors (e.g., mTOR) inform epigenetics though sensing aminoacid levels. Sensing to signaling stress responses converge on common checkpoints such as the NFκB family, stress kinases p38, c-Jun N-terminal kinase (JNK), and extracellular signal-related kinase (ERK) [18]. Cyclic nucleotides and AMP sensor AMP kinases also inform nuclear epigenetics. Transcription factors binding to cognate DNA sequences with histone interactions make the definitive step to generating mRNA from RNA polymerase II.

The paradox that monocyte NFκB factor p65 could transfer into the nucleus but is unable to bind DNA and to transcribe proinflammatory genes pioneered the concept that epigenetics directs shifts between *initiation* and *adaptation* during acute inflammation [19]. To initiate a "defend and mend" response, preepigenetic stress sensors and signals converge their information on nuclear chromatin. First responder genes are quicker to initiate inflammation than others,

and epigenetically poised enhancer and promoter regions prompt the most rapid responses [14].

37.3.2 Epigenetically Poised Enhancer–Promoter Regions

A poised transcription state of DNA enhancer and promoter regions modifies chromatin structure, nucleosome positioning, and starts, but does not finish, mRNA transcription [14]. This first responder "language or epigenetic code" develops innate immune monocytes/macrophages to a differentiated and immune-poised state. Poised *enhancer* regions are marked by methylated histone H3K4me1, acetylated H3K27ac, demethylated DNA CpG, and constitutively bound PU.1 DNA-binding protein. Triple methylated H3K4me3 marks poised promoter regions, while closed promoters display repressor marks such as H3K9me2 and H3K27me3 polycomb complex. Poised epigenetic enhancer–promoter signatures await arrival of DNA binding of transcription activating factors such as nuclear factor kappa B p65 (NFκB p65), activator protein 1 (AP-1), and interferon regulatory factors (IRF) at their cognate DNA sequences. Then RNA polymerase II activity rapidly extends mRNA.

Poised enhancer–promoters rapidly trigger cytokine/chemokine gene expression to initiate inflammation after environmental stress receptors sense and signal the nucleus. First responders include tumor necrosis factor alpha (TNF-α) and interleukin 1 (IL-1). Intermediate responders transcribe their mRNAs after first responders, a process that often requires protein synthesis; examples include interleukin 6 and other proinflammatory cytokines and chemokines [13]. Memory T cells also are regulated by poised promoters to enable quick response to immune-specific antigens [20]. Also, very low intensity stress signals may "prime" or "train" innate immune memory by epigenetically generating poised enhancers [21].

Developmental processes epigenetically inform the identity of monocytes and macrophages, which are unique in their ubiquitous ability to become part of all organs. Tissue macrophages gain early identity in the fetal yolk sac and later from bone marrow origin [22,23]. An important emerging concept is that environment-specific cues dictate the location of macrophages among various tissues develops their enhancer identity [24]. For example, macrophage enhancers when in the blood differ from those in brain, lung, liver, spleen, and peritoneum. These differences are apparently environment-specific and plastic, since new macrophages coming into the region change and can change again if they migrate into another tissue. Unfortunately, most knowledge in epigenetics does not link to how inflammation – successful or unsuccessful – is affected by the developmental or acquired nuances, if at all. Such research might enlighten understanding disease such as Alzheimer's disease, alcoholic/nonalcoholic liver disease, acute/chronic kidney disease, and heart failure, among others.

37.3.3 Inflammation Initiation

During inflammation initiation, the poised repressor complex is replaced by an active euchromatin state of demethylated and acetylated H3K9, phosphorylated

H3S10, and hypomethylated CpG promoter DNA [25–27]. The precise mechanisms controlling enhancer–promoter derepression that launches initiation includes ubiquitination and proteasome degradation of basal repressor histone deacetylases and methyl-demethylase depressors [13]. Nuclear translocated and activated transcription factors then bind the "more" accessible euchromatin DNA transcription factor cognate sequences. *Initiation* reprogramming is gene-specific for several thousand protein-encoding genes and noncoding long and short regulatory mRNAs. Almost equal numbers of mRNAs increase and decrease during inflammation initiation, perhaps supporting the homeostasis yin/yang concept [28]. The dual activating and repressing function of many transcription factors interacting with cofactors (e.g., NFκB RelB) also underscore the importance of homeostasis. This yin/yang balance raises the possibility that both derepression and direct activation of transcription determines [29,30].

Most characteristics of gene-specific epigenetic *initiation* reprogramming are rapid responder proinflammatory/effector immunity cytokine and chemokine encoding genes and gene sets that regulate cell nutrition and cell survival. The repressed genes may include anti-immune mediators such as I kappa B family [31]. Epigenetic reprogramming also requires nucleosome repositioning, in which histone subtypes replace the canonical histone structure. Sequential epigenetic nucleosome repositioning occurs during inflammation *initiation* and *adaptation* [32].

Epigenetic inflammation reprogramming during *initiation* rapidly (minutes to hours) generates an *immune effector* state, in which microbicidal ROS form from different cell sources (e.g., xanthine oxidase, NADPH oxidase, and increased mitochondrial electron chain activity) and antimicrobial proteins become active as defense components. Initiation also activates *anabolic pathways* linked to the nonoxidative glucose-dependent pentose phosphate pathway in which nucleic acids are synthesized. Thus, post-transcriptional and post-translational processes also support homeostasis "defend and mend" distal to the epigenetic reprogramming chromatin network. Surprisingly, inflammation *initiation* in innate immune phagocytes after even mild or extreme stress is short-lived and reprogramming epigenetically switches to *adaptation* that begins in less than 12 h and is well established within a 24 h [27].

37.3.4 Inflammation Adaptation

A switch from "defend to mend" evolves as epigenetic reprogramming directing successful inflammation reprogramming toward its resolution. Epigenetic changes in chromatin and DNA direct *adaptation* reprogramming by switching euchromatin to heterochromatin [7]. Transcriptionally active NFκB p65 is replaced by NFκB factor RelB, which recruits a multi protein complex to generate silent heterochromatin, which is reversible. This repressor complex contains lysine transmethylase (G9a), heterochromatin protein 1 (HP-1), histone H1 nucleosome linker protein, DNA CpG transmethylases DNMT 3a/3b, and likely many other epigenetic modifiers [7,33].

Epigenetic-based adaptation is gene specific [34] and "silences" immune effector genes encoding cytokines that initiate inflammation and activates genes

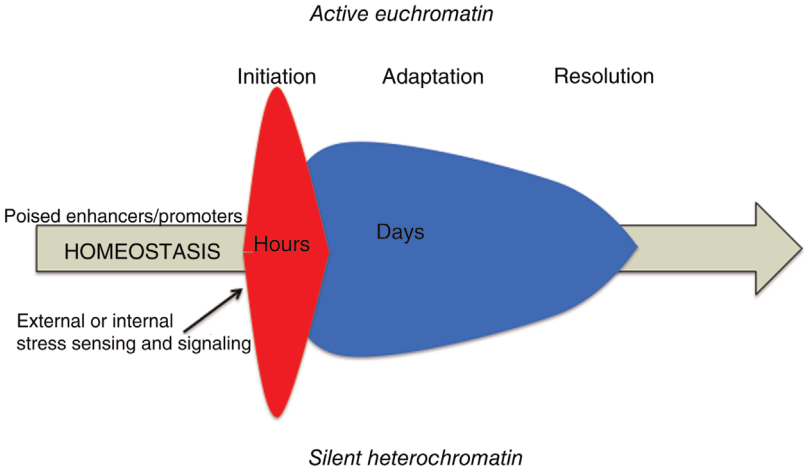


Figure 37.3 Epigenetics of successful inflammation: an orderly epigenetic and post-translational program informed by stress sensing and signaling temporally directs departure from and return to homeostasis. The *initiation* process shown in red instructs a rapidly mounted innate immunity program, which epigenetically reprograms genes responsible for immune effector and synthetic anabolic pathways. During this time, several thousand genes increase or decrease transcription, including those encoding proinflammatory mediators and glycolysis-dependent anabolic pathways. Within hours of initiation onset, homeostasis protective events launch an epigenetically controlled *adaptation* program shown in blue, which counters the effector immunity program and switches to reprogramming genes that limit inflammation and promote catabolic fatty acid oxidation to support a low-energy state. Successful inflammation then retrieves homeostasis by poorly understood mechanisms.

encoding antimicrobial peptides and IKB-α. RelB both represses chromatin at proinflammatory cytokines and chemokines and activates euchromatin at anti-inflammatory genes to promote a phenotypic shift in monocytes [25]. What directs RelB to perform in these two directions is unclear, but it is likely, at least in part, determined by its partnering with distinct cofactors, such as deacetylases and transacetylases as it concomitantly generates silent heterochromatin and activates euchromatin. Dual regulators also may change function based on post-translational processes such as phosphorylation or methylation. Of particular importance for inflammation resolution is disassembling the epigenetic repressor complex member (e.g., RelB, HP-1, and G9a), which is needed to restore homeostasis during successful inflammation [7], and the clinical shift is often evident within 2 or 3 days. Figure 37.3 conceptualizes epigenetic *adaptation* reprogramming during successful inflammation.

37.3.5 Inflammation Resolution and Epigenetic Memory

Successful inflammation resolution parallels epigenetic adaptation deprogramming. During resolution, NFκB p65 regains its capacity to bind and activate transcription of inflammation *initiation* genes (e.g., TNF-α) as RelB expression and function decrease [35]. Glucose and fatty acid metabolism, mitochondrial function (discussed subsequently), and microvascular leukocyte adherence

properties also return to homeostasis during inflammation resolution [36], as does tissue function. As adaptation resolves, monocytes are able to again defend and mend against new threats. Little is known, however, about how *adaptation* transitions to *resolution* at a more molecular level, or how often or how long epigenetic codes persist as epimutations. For example, studies in mice find that macrophage/dendritic cell epigenetic memory can persist in T cells for months after severe lung inflammation resolves [37]. Figure 37.3 conceptualizes the epigenetics of successful inflammation with homeostasis retrieval.

37.4 Epigenetics of Unsuccessful Inflammation

Unsuccessful inflammation occurs in billions of humans afflicted by chronic inflammatory diseases and the near 20 million who die each year of acute systemic inflammation from infection [38]. Many more suffer from unsuccessful inflammation in the form of chronic diseases such as diabetes, obesity, chronic lung failure, renal failure, liver failure, and dementias; aging is also accompanied by chronic inflammation. Important unknowns are what mechanisms arrest chronic inflammation in *initiation* and acute inflammation in *adaptation* state.

37.4.1 Acute Inflammation with Epigenetic Dysregulation

The initial environmental alarmin strength and its persistence, such as infection severity, determines the magnitude and duration of the acute inflammatory response and its eventual outcome. Above a presumed threshold of NFκB p65 DNA binding and transactivation, an extreme *initiation* response rapidly inflicts potentially lethal damage from reactive oxygen or nitrogen species (ROS or NOS), cell death pathways, and cardiovascular collapse. This epigenetically open and severe *initiation* state with its cytokine and ROS "storms" predicts early death or a sustained period of epigenetically controlled *adaptation* with immunometabolic failure [27,39]. During this postacute state, death rates remain high from severe immunosuppression and multiorgan dysfunction. Together, early and late deaths from uncontrolled acute systemic inflammation with sepsis may exceed 50% mortality globally and over 20% in more health-advanced regions [40]. Over 60% of the mortality during the acute systemic inflammation results during late/adaptation state [41]. The later effects of inflexible immune, metabolic, and bioenergy responses correlate with the high lethality. Organ dysfunction of liver, kidney, heart, and brain contributes to physiological collapse. Sustained original infections along with new viral/bacterial infections testify to the presence of an almost inert or hibernating state [42–44].

The fundamental driving force behind the failure of unsuccessful acute inflammation to epigenetically correct its inflexibility is not known, but maintaining the silent heterochromatin by RelB and its transcription repressor complex signify a resistance to restoring a flexible state of chromatin across thousands of genes [36]. It further suggests homeostat failure. During this time, ATP is low and cell death pathways may remain active in this catabolic state [45]. What prevents this epigenetic state from reversing is completely unknown.

Possibilities include alterations in redox balance between oxidized and reduced adenine nucleotides and repressed ability to generate ROS. Figure 37.4 conceptualizes the features of uncontrolled inflammation at the epigenetic level.

37.4.2 Chronic Inflammation with Epigenetic Dysregulation

Chronic inflammation reflects a low-grade and prolonged initiation or reduced adaptation; by definition it is unsuccessful epigenetic programming. In chronic inflammation, a persistent external or internal environmental stress stimulus resets the epigenetic homeostat in the proinflammatory state [46–48]. Known examples include elevated nutrients (glucose of proinflammatory fatty acids exterior to the immune cells during obesity with metabolic syndrome), abnormal microbial intestinal and immune cell interface in inflammatory bowel diseases, cholesterol and lipid deposits in inflammatory cells in atherosclerosis, amyloid plaques in Alzheimer's dementia, and chronic infections such tuberculosis. Possible epigenetic code inflexibility mechanisms include: (1) persistent acetylation activator histone codes and activated transcription factors (e.g.,

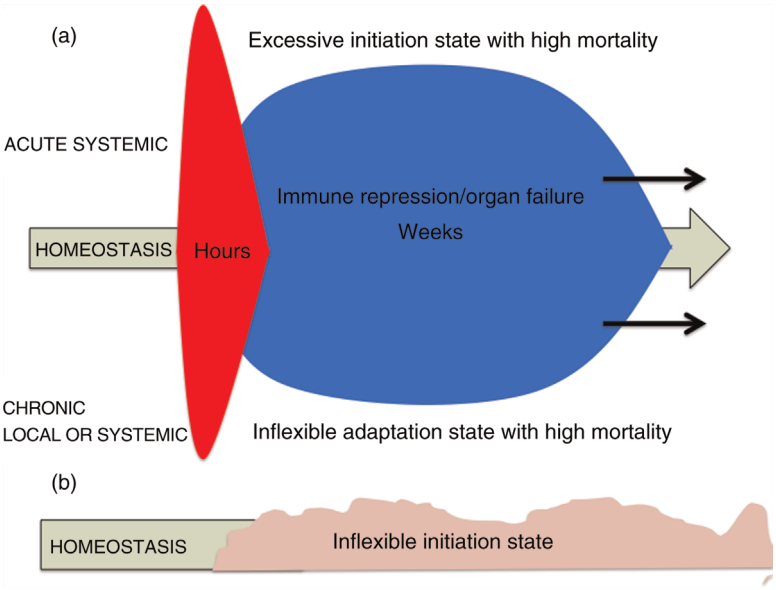


Figure 37.4 Epigenetics of unsuccessful inflammation. Evidence supports that the same sets of genes are involved in successful and unsuccessful inflammation, suggesting that dysregulated homeostasis protective reprogramming contributes to unsuccessful inflammation. This quantitative versus qualitative concept is depicted in (a) showing acute inflammation and (b) showing chronic inflammation. (a) If the inflammation homeostasis protector axis is set too low or the stress level is overwhelming, *the initiation state magnitude* reaches levels incompatible with cell and organ life, as depicted in the red zone. When the *adaptation state becomes inflexible* and persists in its catabolic and immune repressed state, infections cannot clear, organs cannot heal, and survival decreases as shown in the blue zone. (b) Chronic inflammation fails to temporally progress through initiation to adaptation to homestasis retrieval, suggesting that the inflammation homeostat is chronically set too low and/or is unable to progress through adaptation to resolution. However, the epigenetics of chronic inflammation is unclear.

NFκB p65) [49], (2) constitutively reduced *adaptation* [50], (3) inflexible nutrient overload with imbalanced mitochondria fueling [51], (4) persistently elevated ROS and NOS, which activate or inactivate inflammatory mediators [52]. Taken together conceptually, it is clear that chronic inflammation has a broken epigenetic homeostat with an abnormal epigenetic landscape not yet mapped. As depicted in Figure 37.4, chronically unsuccessful inflammation differs from acute in that it appears arrested in the *initiation* state. There is either too much *yin* or too little *yang*.

Two fundamentally important unknowns about unsuccessful acute and about chronic inflammation are as follows: (1) Why does lethal acute systemic inflammation get arrested in the adaptation state? (2) What prevents chronic inflammation from epigenetically directing adaptation and resolution?

Successful treatments depend on understanding the answers to these questions.

37.5 Epigenetics and Immunometabolism

Mounting evidence implicates cell nutrient selection as determinants of immune cell type and function. Glucose and amino acids fuel *initiation* (defend) and fatty acids fuel *adaptation* [25,53]*, and homeostasis retrieval rebalances the selection of cell nutrients*. The terms immunometabolism or meta-inflammation describe a duel partnership between metabolism and immunity, and this relationship is epigenetically coupled (see Figure 37.5).

37.5.1 Immunometabolism and Chronic Inflammation

A series of discoveries in obese human adipose tissue and later as a systemic response in obesity launched the immunometabolic and bioenergy concept. Proinflammatory macrophages (sometimes referred to as M1 classic monocytes and macrophages) that produce TNF-α become dominant over resident macrophages (sometimes called the M2 alternative type) in obesity with chronic inflammation [54,55]. This M2 to M1 macrophage polarity shift promotes chronically unsuccessful inflammation by misbalancing *initiation* and *adaptation* epigenetic polarity. This inflammatory process involves other cells: T effector proinflammatory lymphocytes also dominate over T regulatory lymphocytes in obese adipose tissue, and neutrophils and eosinophils contribute to immunometabolic imbalance [56]. Importantly, chronic obesity-induced inflammation promotes *systemic* metabolic dysregulation characterized by the diabetes phenotype, which is M1 macrophage *initiation* state dependent.

Fuel preferences by M1 effector and M2 regulatory/alternative macrophages and T effector CD4+ and CD8+ versus T repressor cells clearly differ [55,57]. Virtually all effector immune cells studies support a need for glycolysis to optimally mount their defense and anabolic properties, while repressor immune cells use catabolic fatty acid oxidation for a low-energy state. Cell nutrient selection between glucose and fatty acids becomes *inflexible* during obesity with chronic inflammation [51], but reversal or repair of the abnormal epigenetic

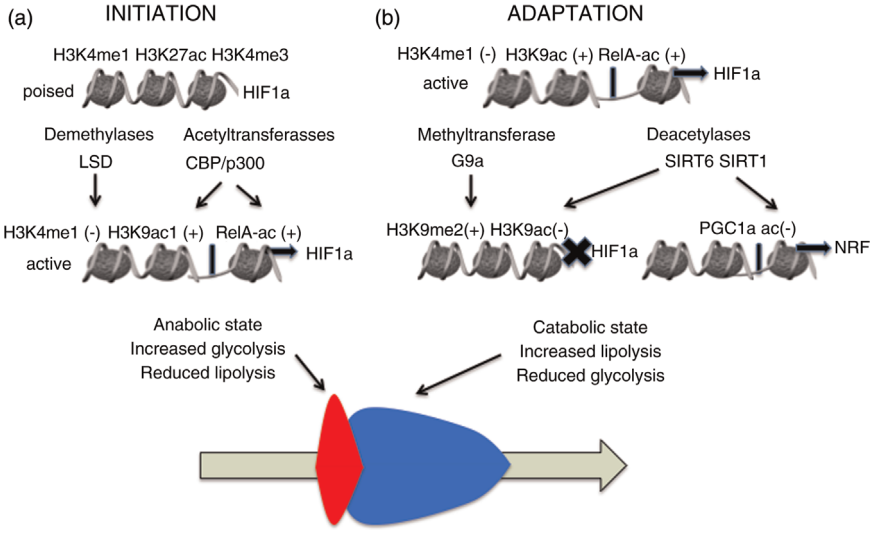


Figure 37.5 Epigenetics and immunometabolism: fueling the flame from poised promoters directly connects inflammation with cell nutrient selection. (a) Shows that NFκB p65 and HIF1a are epigenetically linked through histone acetylation and demethylation, which support increased glucose receptor expression and glucose uptake, promotes pentose shunt activity in monocytes, and directs aerobic or anaerobic glycolysis and glucose oxidation during inflammation *initiation*. This nutritional pathway is required for an optimal defense state – ROS generation, cell constituent synthesis, and ATP production – during which time lipolysis and fatty acid ß oxidation are decreased in favor of lipid synthetic processes. A critical switch from glycolysis to fatty acid oxidation as shown in (b) is a hallmark of inflammation adaptation and catabolic low-energy state of reduced pyruvate oxidation. Nuclear NAD+ generation and SIRT1 and SIRT6 deacetylase activation command this homeostasis switch, with SIRT6 repressing HIF1a and SIRT1 activating PGC1a and its control over lipolysis and mitochondrial biogenesis through nuclear respiratory transcription factors (NRFs). Histone H3K9 deacetylation followed by G9a histone methyl transferase activity promotes HIF1a gene silencing.

homeostat retrieves immunometabolic homeostasis and represses the chronic inflammation of obesity. For example, epigenetic dysregulation due to overnutrition is reversed by weight loss, supporting the notion that maintaining proper selection and flexibility of bioenergy fuel sources is essential to avoid chronic inflammation – also suggesting that pharmacologic approaches are tenable.

37.5.2 Immunometabolism and Acute Inflammation

Acute inflammation also couples immunity and metabolism through epigenetic programming [26,27]. This concept is becoming critically important for understanding acute inflammation. In naïve monocytes or macrophages, robust TLR4-induced inflammation *initiation* first increases glucose flux and generates a Warburg-like aerobic glycolysis state [21] controlled by hypoxia-inducible factor 1 alpha (HIF-1α) before switching to *adaptation* [21]. This stress recognition pathway concomitantly reprograms immunity and metabolism by gene-specific histone acetylation, nucleosome remodeling, NFκB p65 acetylation, HIF-1α

acetylation, and concomitant transactivation of immune effector/glycolysis controlling genes. Functionally, acetylated histone lysines and HIF-1α protein acetylation lead to a direct increase in glucose 1 transporter (GLUT1), hexokinases, glycolysis to lactate pathway, and pentose phosphate pathway (PPP) activity. When *initiation* epigenetically switches to *adaptation*, fueling by glycolysis switches toward increased fat uptake. This is accompanied by internal lipolysis, followed by fatty acid mitochondrial import, ß oxidation, and "feeding" of reducing NADH and FADH reducing equivalents to run the electron transport chain proton pump for ATP generation. During this nutritional and bioenergy switch, decreased ribosome expression and disrupted mTor pathways limit protein synthesis.

Bioenergy switching during initiation from an anabolic to a catabolic state resembles a starvation or hibernation state that seeks to conserve energy [58,59]. Nuclear receptors, such as peroxisome proliferative activated receptor (PPAR) and cofactor PGC 1-α [60], retinoic acid receptors, and estrogen-related receptors epigenetically promote the starvation or hibernation state, which depends on fat as primary fuel [61]. This "physiologic divorce" from using glucose carbons for energy compromises host defending and anabolic mending processes. Clinically, it is characterized by severe immunosuppression, persistent organ dysfunction, and high mortality rates.

37.5.3 Distinct Differences in Immunometabolic Coupling Occur During Acute versus Chronic Inflammation

In acute inflammation, immunometabolic epigenetic switching of monocytes from *initiation* to *adaptation* is rapid (within 6–12 h of *initiation*) [21]. In successful acute inflammation, the immunometabolic pathways both adapt and return to homeostasis. If unsuccessful inflammation, both the immune and metabolic responses concomitantly become inflexible; a state in which immunometabolic homeostat has failed.

In chronic inflammation, inflexibility differs by occurring in the initiation state, which through sustained system-wide dominance of the M1 monocyte and macrophage maintains the metabolic diabetic phenotype and the proinflammatory phenotype. Inflexible fueling of fat metabolism maintains a dominant proinflammatory response. Although there is evidence of concomitant anti-inflammatory M2 activity, chronic inflammation appears to unsuccessfully *adapt* and homeostasis *restoration* fails. Uncontrolled acute and chronic inflammations are similar in that they both are reversible, although they use different approaches.

Does metabolism inform or even direct nuclear epigenetic processes? Evidence supports that metabolism regulates histone acetylation/deacetylation states by modifying the availability of acetate and methyl for epigenetic use [10]. Acetyl CoA generated in mitochondria or cytosol from glucose and fat-derived carbons provide acetate for acetylation, and amino acid metabolism-derived *S*-adenosyl methionine (SAM) provides methyl for epigenetic coding. Reactive oxygen-derived and nitrogen-derived species from cytosol or mitochondria also can inform nuclear epigenetic processes through transfer to the nucleus and direct oxidation and reduction of nuclear proteins and DNA, which can change epigenetic functional pathways [62].

37.6 Sirtuins: An Epigenetic Homeostat

The spatial and temporal features of inflammation with its orderly and epigenetically controlled movement away from and back to homeostasis suggest that a common regulatory axis "guards" metabolic and immune homeostasis at epigenetic and post-translational levels. NAD+-dependent sirtuin (SIRT) family of redox-sensing deacylases and ribosylases are the leading homeostat checkpoint candidates [63]. First discovered in yeast, heterochromatin generator and metabolic sensor was *silent mating-type information regulator-2* (Sir2), an NAD+ -sensing protein with lysine deacetylase activity [64].

The sirtuin mammalian counterpart of yeast Sir2 founder protein, nuclear SIRT1, is an epigenetic checkpoint and homeostasis rheostat (homeostat), which reprograms chromatin structure of specific gene sets during inflammation [65]. Nuclear SIRT1 both represses NFκB p65-dependent euchromatin activation through deacetylation and concomitantly induces NFκB RelB-dependent silent heterochromatin formation, as inflammation *initiation* switches to *adaptation*. Accordingly, SIRT1 as a master inflammation rheostat proximally controls NFκB p65 and RelB participation in epigenetics by reciprocally decreasing *initiation* and promoting *adaptation*. In successful acute inflammation, the SIRT1 homeostat remains flexible, is turned down and immunometabolism returns to homeostasis with inflammation *resolution*. In an unsuccessful acute inflammation, sustained increases in NAD+ levels keep SIRT1 in an activated state [66], maintaining heterochromatin-based immune and metabolic inflexibility with its low-energy "hibernation" state – supporting the potentially lethal combination of immune repression and bioenergy failure [58,59].

As another example of the critically important SIRT1 homeostat, SIRT1 informs RelB to increase SIRT3 expression as a moderator of mitochondrial physiology [67]. SIRT1 through RelB, but not SIRT3, also supports mitochondrial biogenesis. The epigenetic functions of SIRT1 are directly controlled by redox, in which oxidized cysteines deactivate and reduced cysteines activate its function [68], supporting that redox control may direct epigenetic landscape reprogramming.

As the third example of the SIRT1 homeostat, SIRT1 proximally controls SIRT6 expression, the master regulator of glycolysis. Together, nuclear SIRT1 and 6 are reciprocal directors of glycolysis (SIRT6 reduces) and lipolysis and fatty acid oxidation (SIRT1 increases) [69]. SIRT1 and 6 distinctly deacetylate histones but similarly inactivate NFκB p65. SIRT6 by deacetylating histone H3K9 represses HIF-1α expression to limit GLUT1 expression, glucose flux, and glycolysis [70]. SIRT1 deacetylates peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1-α) to promote lipolysis and fatty acid oxidation [71]. Further supporting SIRT1 homeostat function are reports that reduced SIRT1 levels and activity amplifies inflammation *initiation*. Moreover, many chronic inflammatory diseases are associated with low SIRT1 levels [72]. Thus, SIRT1 is a master homeostasis checkpoint that informs the epigenetically controlled immunometabolic axis.

It is plausible that all SIRT family members cooperate to guard homeostasis [73]. They dominate in different cell compartments: nuclear SIRT1, 6, and 7; cytosolic SIRT2 with translocation to nucleus during acute inflammation; mitochondrial SIRT 4, 5, and 6. SIRT1 and 6 coordinate nuclear epigenetic pathways,

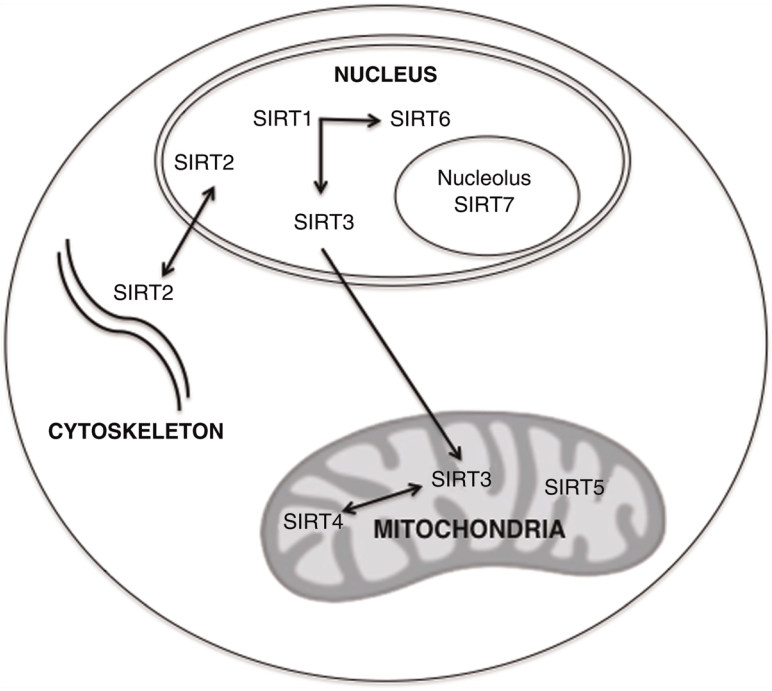


Figure 37.6 Sirtuins in inflammation: shown are locations of seven-family member SIRTs. Nuclear SIRT1 control of nuclear SIRT6 and mitochondrial SIRT3 expression and levels in mitochondria direct epigenetic immune, metabolic, and bioenergy reprogramming and its switching from initiation to adaptation. Low levels of SIRT1, SIRT2, and SIRT3 occur in chronic inflammation states such as obesity with diabetes. Other members of the SIRT family likely participate in homeostasis regulation. SIRT4 has reciprocal controls with SIRT3 and its expression may be separately regulated as a resolution contributor. Mitochondrial SIRT5 function as a desuccinylation agent may support energetics. SIRT2 is the major SIRT expressed in adipose tissue and shuttles between cytosol and nucleus. SIRT7 located in the nucleolus represses expression of ribosomes and may contribute to generating the catabolic state.

while SIRT3, 4, and 5 balance mitochondrial function with SIRT4 counter-regulating SIRT3 function by repressing fatty acid oxidation and glutamine metabolism [74]. SIRT2 is the dominant SIRT-expressed adipose tissue, regulates fat cell development, and supports obesity-associated immunometabolic reprogramming [75]. SIRT7, the least understood of sirtuins, is located in nuclear nucleoli and represses ribosomal DNA function and protein synthesis, which accompanies inflammation *adaptation* [76]. Figure 37.6 conceptualizes epigenetic homeostat properties of Sirt1 and its possible connections with other sirtuins family members.

37.7 Epigenetic-based Inflammation Treatment

37.7.1 Emerging Data Support that Targeting SIRT1 Homeostat Function may Treat Unsuccessful Inflammation

The *adaptation* inflammation state during severe systemic stress responses like sepsis persistently increases NAD+ and elevates SIRT1 levels and activity [66].

Mounting data support the potential of targeting SIRTs as novel treatments for inflammatory disease. For example, marked increases in survival occur in septic mice when a single dose of a SIRT1 inhibitor is administered after *initiation* switches to *adaptation* in septic mice with uncontrolled acute inflammation and high mortality [36]. This sepsis rescue rebalances immunity, metabolism of glucose and fatty acids, and mitochondrial function. Further supporting clinically applicable rheostat properties of SIRT1, studies show that increasing SIRT1 in low sepsis states like obesity substantially improves diabetes and decreases inflammation [77]. Although early results support that epigenetic therapeutic targeting of uncontrolled inflammation can be beneficial, indeed lifesaving, more research is needed to determine how effective the SIRT homeostasis axis is as a treatment target for unsuccessful acute or chronic inflammation and to identify what mechanisms dysregulate the SIRT homeostat. Critically important will be identifying epigenetic biomarkers, in order to identify which side of the epigenetic axis to target. Figure 37.7 depicts epigenetic-based prevention and rescue during acute systemic sepsis inflammation (upper panel) and chronic inflammation (lower panel). Table 37.3 lists immune, metabolic, and bioenergy processes improved through homeostasis retrieval.

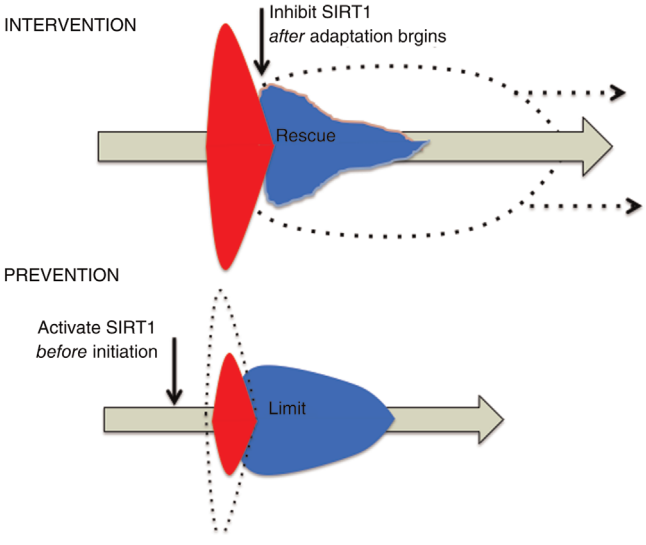


Figure 37.7 Epigenetics-based inflammation treatment: better understanding of homeostasis pathways is leading to new targets for inflammation *intervention* during sepsis or *prevention* in obesity with secondary stress. Based on the SIRT1 homeostat paradigm, administering a SIRT1 inhibitor to septic mice after initiation switches to adaptation rescues septic mice from death by reducing immune, metabolic, and bioenergy dysregulation (upper panel). In contrast to adjusting a high homeostat downward, preactivating SIRT1 in obese mice, whose SIRT1 levels are low, limits initiation magnitude, reduces sustained immune repression, and increases survival (lower panel).

Table 37.3 Targeting the Sirt1 epigenetic-based homeostat for sepsis inflammation treatment.

Sepsis	Immunity	Vascular	Metabolic	Bioenergy	Survival	Ref.
In vivo (mice): SIRT1 inhibition administered during early adaptation and immune repression	Rebalances effector/repressor Cytokine response Increases microbial clearance Restores NFκB p65 competence	Improves blood pressure and vascular flow Restores E-selectin and ICAM-dependent microvascular leukocyte endothelial adherence response	Rebalances glucose and fatty acid oxidation	Reduces mitochondrial stress	Markedly improves	[36]
Ex vivo (mice): SIRT1 inhibition of splenocytes or macrophages	Reverses silent heterochromatin Removes repressor complex	Not applicable	Rebalances glucose and fatty acid oxidation	Rebalances electron transport chain	Not applicable	[67] [69]
Ex vivo (humans): +/− SIRT1 inhibitor	Reverse silent heterochromatin Removes promoter repressor complex	Not applicable	Rebalances glucose and fatty acid oxidation	Rebalances oxidative phosphorylation and oxidoreductases	Not applicable	[35] [66] [67] [69]
In vitro (humans): Monocyte cell model Pharmacologic and genetic approaches	Reverses silent heterochromatin Controls epigenetic repressor complex on promoters. Regulated by NFκB p65 deacetylation	Not applicable	Rebalances glucose and fatty acid oxidation	Rebalances glucose/fatty acid oxidation and oxidoreductases		[7] [30–33] [35] [66] [67] [69]
In vivo (obesity in mice): Administering SIRT1 agonist before sepsis onset	Limits initiation magnitude	Reduces amplified microvascular leukocyte/platelet endothelial adherence	Not done	Not done	Markedly improves	[77]

37.8 Summary

Epigenetic reprogramming of immunity, metabolism, and mitochondrial bioenergetics plays a crucial role in directing the course of inflammation. In monocytes and macrophages, and likely in adaptive immune T cells, epigenetic processes first maintain and depart from a poised enhancer–promoter region after external or internal environmental alarmin signals reach nuclear chromatin. Feedforward processes reorganize active euchromatin into silent heterochromatin as inflammation *initiation* switches to *adaptation*. Successful inflammation restores homeostasis; unsuccessful inflammation does not.

Mounting evidence integrates inflammation with immunity and metabolism and supports that members of the NAD+-sensing sirtuin family, particularly SIRT1, control a homeostat that epigenetically controls immunometabolic and mitochondrial bioenergy reprogramming. When this homeostasis control axis becomes inflexible, acute and chronic inflammatory diseases occur. Applying treatment approaches based on SIRT-dependent epigenetic reprogramming of immunometabolic and mitochondrial bioenergy pathway is a promising new concept. Identifying critical checkpoint nodes connecting nuclear epigenetic processes with extranuclear processes could expand prevention and intervention treatment strategies for inflammatory diseases, thereby reducing the global health burden of unregulated inflammation.

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Part Five

Inflammation and Diseases

38

Allergy and Inflammation

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38.1 Introduction

In 1906, an Austrian pediatrician Clemens von Pirquet described cases of serum sickness in children receiving antibody preparations and used for the first time the term *allergy* to describe this phenomenon [1]. Since then, we have learned a lot about the initiation, development, and different types of allergic inflammation, as well as the immunological mechanisms involved in allergic reactions. There are four types of allergic reactions based on the main pathogenesis mechanism, historically defined as four types of hypersensitive reactions by Gell and Coombs [2], and later redesigned by Pichler [3]. Type I, Th2-dependent, IgE-mediated hypersensitivity includes mast cell degranulation upon surface IgE cross-linking and represents the main pathogenic mechanism of the most common allergic disorders such as allergic rhinitis, as well as skin prick test reactions. It is sometimes called "classical allergy" to differentiate with other less often allergic reactions such as drug allergies or contact dermatitis. Type II reactions, the antibody-dependent cellular cytotoxicity, are characterized by the cellular/tissue antigen–antibody (IgG/IgM) reactions that are able to activate complement and/or recruit cytotoxic cells, which finally lead to the tissue injury and function loss. The typical examples are the Goodpasture disease, thrombocytopenia, or autoimmune hemolytic anemia. Type III hypersensitivity is described by the formation of immune complexes, when antibody, most often IgG, binds to a soluble antigen. These immune complexes are further deposited within the vascular walls of various organs such as joints or kidney and lead to inflammation. Traditional examples are serum sickness, systemic lupus erythematosus, and several forms of glomerulonephritis. In classical Gell and Coombs description, type IV hypersensitivity, usually described as cell-mediated (or delayed-type)

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hypersensitivity, sensitized Th1 and Th17 cells, as well as cytotoxic lymphocytes release cytokines, activate macrophages and/or activate cell-mediate cytotoxicity programs that all lead to tissue injury and cell destruction. Examples of type IV reactions include contact dermatitis, diabetes type 1, multiple sclerosis, or some stages of tuberculosis. Another subclass of type IV hypersensitivity reactions play a major role in allergic diseases such as asthma and atopic dermatitis. In this subclass, Th2 cells, type 2 innate lymphoid cells (ILCs) play a major role in tissue inflammation, eosipnophilia, and IgE production. This group of type IV hypersensitivity usually coacts with type I hypersensitivity reactions in allergic diseases [3].

At present, it is known that the immune mechanisms underlying each allergic disorder are very complex and, in most cases, exchange or coexist at different stages of allergic disease. However, the modified Gell and Coombs classification is useful to grasp this complexity and put the clinical manifestations of the allergic diseases into a more pathomechanistic context.

38.1.1 Overview of Mechanisms of Allergic Inflammatory Response

The immune response in allergic diseases consists of sensitization/memory and effector/elicitation phase. Upon sensitization phase, innate immune cells such as airway cells, gut epithelial cells, or skin keratinocytes sense the allergens and their microenvironment via the pattern recognition receptors (PRRs) and particularly release thymic stromal lymphopoietin (TSLP), IL-33, IL-25, and GM-CSF, initiating the inflammatory response cascade [4–6]. Dendritic cells and monocytes/macrophages sample the allergen and travel to the draining lymph nodes to present the antigen to T cells inducing the differentiation and clonal expansion of allergen-specific $CD4^+$ Th2 cells. These cells produce IL-4 and IL-13 and induce class switch to the ϵ immunoglobulin heavy chain in B cells and the production of allergen-specific IgE antibodies (Abs). In addition, they support eosinophilia with IL-5. Allergen-specific IgE binds to the high-affinity FcϵRI on the surface of mast cells and basophils, which travel to different tissues, ready to react, degranulate, and release the wide range mediators upon the next encounter with the same allergen. At the same time, a memory pool of allergen-specific T and B cells is also generated. Type 2 ILC particularly release IL-5 and IL-13 and take an important part in the early phase of initiation of inflammation [7]. They react within a few hours with IL-33 released from epithelium, whereas Th2 cells require clonal expansion for at least 1 week to act. Although ILCs do not show any memory, Th2 cells display an antigen/allergen-specific memory with their increased frequency in the circulation and affected organs.

The effector phase usually happens as an immediate-phase response (IPR) and can be followed by the late-phase response (LPR) [8]. The new encounter with the causative allergen induces cross-linking of the IgE–FcϵRI complexes on the surface of sensitized effector cells, leading to the release of mediators including histamine, responsible for the classical symptoms of IPR such as increased vascular permeability, extravasation of fluid into the tissues, and smooth muscle contraction. Sneezing, watery discharge from eyes and nose, rash, or asthmatic bronchospasm are the classical symptoms of the IPR. The symptoms might be followed by the LPR, which occurs 6–12 h later, due to the other mediators

released from mast cells such as leukotrienes or upon the persistence of the allergen. Activated allergen-specific Th2 cells, accompanied by the innate lymphoid cell 2 (ILC2), produce IL-4, IL-5, IL-9, and IL-13, which play a key role in the maintenance of allergen-specific IgE levels, eosinophilia, recruitment of inflammatory cells to inflamed tissues, and persistence of the allergic inflammation into the chronic form [7,9,10].

In summary, various types of both innate and adaptive immune cells producing multiple mediators, including lipids, purines, cytokines, chemokines, soluble receptors, small molecules like histamine, adenosine, and reactive oxygen species take part in initiation, development, and maintenance of allergic inflammation.

38.2 Innate Immunity and Its Role in Allergic Inflammation

38.2.1 Microbial Pattern Recognition by the Innate Immune System

Both innate immunity cells and cells of adaptive immunity have a big repertoire of potent innate immunity receptors called PRRs to quickly recognize the most common and ubiquitous microbial, fungal, and viral threats. Through the evolution and natural selection, human organism adapted to survive and to recognize highly conserved molecular structures of these organisms called pathogen-associated molecular patterns (PAMPs) by PRRs [11]. These receptors also recognize other alarming situations in the body through sensing damage-associated molecular patterns (DAMPs), released by dying cells [12,13]. In many cases, activation of these receptors starts to initiate the cascade (Figure 38.1). There are two main types of PRRs: transmembrane or cytosolic signal-transducing receptors and secreted by the cells' soluble receptors.

Transmembrane and cytosolic PRRs such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), C-type lectin receptors (CLRs), and protease-activated receptors (PARs) are expressed constitutively in epithelial cells, dendritic cells, monocytes, and macrophages as well as T and B cells [14]. Their expression and activation often increases dramatically upon activation by the microbial PAMPs. Activation of these receptors leads to initiation of the immediate cell response through several mechanisms depending of the cell type, including NF-kB pathway triggering, activation of inflammasome and release of IL-1β in macrophages and DCs, release of interferons and various cytokines, chemokines, and lipid mediators in epithelial cells, aiming at the immediate and local threat elimination, and activation of the adaptive arm of immune response [14,15].

Secreted PRRs such as antimicrobial peptides (α- and β-defensins, cathelicidin (LL-37), dermcidin, and regIIIγ), collectins (mannose-binding lectin and surfactant protein A and D), and pentraxins (C-reactive protein) can directly kill invading pathogens or are involved in their opsonisation to facilitate phagocytosis and chemoattraction of innate and adaptive effector cells [16]. Some PRRs, such as CD14 and MD-2, can be both secreted or membrane-bound and serve as costimulators/adaptors for transmembrane PRRs, such as TLR4 in recognition of bacterial endotoxin.

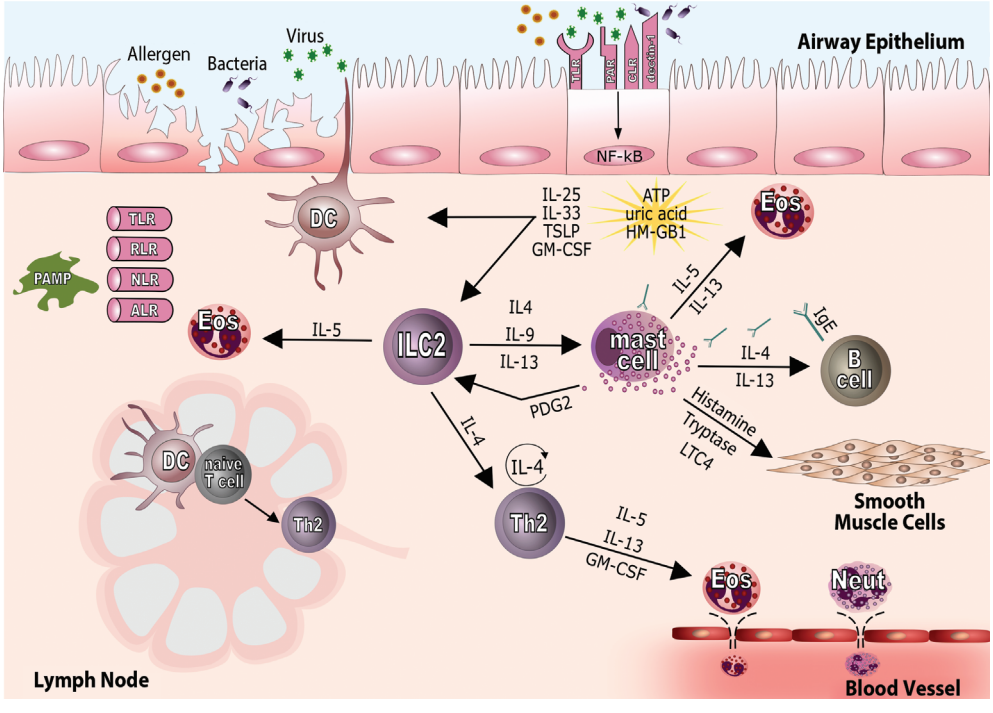


Figure 38.1 Airway epithelium response to allergens. Enzymatically active allergens invading airway epithelial cells may be recognized by PRRs such as TLRs, CLRs, PARs, and dectin-1, causing their activation and initiation of intracellular signaling pathways that result in production of various cytokines, chemokines, interferons, and lipid mediators. Activation of PRRs also leads to the release of endogenous danger signals such as ATP, uric acid, and HMGB1. Penetration of proteolytic allergens through the tissue may facilitate their access to submucosal dendritic cells. Upon activation, mature dendritic cells migrate to draining lymph nodes to present the antigen to the naive T cells through the MHC class II molecules that induce differentiation and clonal expansion of allergen-specific Th2 cells. Release of Th2-type cytokines (IL-4 and IL-13) by Th2 cell and ILC2 initiate class-switch recombination in B cells, leading to production of allergen-specific IgE antibodies that bind to the high-affinity receptor for IgE – FcεRI – on the surface of mast cells and basophils. Degranulation of these cells results in the release of wide range mediators responsible for smooth muscle contraction, vasodilation, goblet cell hyperplasia, and increased vascular permeability. TSLP, IL-25, and IL-33 released by epithelial cells potentiate Th2 responses. IL-5 and IL-13 produced by ILC2 contribute to early inflammation stage.
ALR: AIM2-like receptor, ATP: adenosine triphosphate, CLR: C-type lectin receptor, DC: dentritic cell, Eos: eosinophil, GM-CSF: granulocyte macrophage colony-stimulating factor, HM-GB1: high-mobility group box 1, IgE: immunoglobulin E, IL: interleukin, ILC: innate lymphoid cell, LTC4: leukotrien C4, Neut: neutrophil, NF-κB: nuclear factor κB, NLR: NOD-like receptor, PAR: protease-activated receptor, PDG2: prostaglandin D2, PAMP: pathogen-associated molecular pattern, RLR: RIG-I-like receptor, Th: T helper cell, TLR: toll-like receptor, TSLP: thymic stromal lymphopoietin

38.2.2 Epithelial Cells

Airway and gut epithelium, skin keratinocytes, and other epithelia are at the interface of the human body and the environment. Thus, they form a complex

physicochemical barrier and the first line of defense against environmental insults such as viruses, bacteria, fungi, parasites, allergens, and inorganic particles. Epithelial cells regulate both innate and adaptive immunity, among others, through the production of various costimulatory molecules, chemokines, cytokines, and lipid mediators in response to environmental stimuli, sensed by PRRs [17]. After sensing of allergens, epithelial cells can produce IL-1, IL-25, and IL-33, TSLP, and GM-CSF [17,18]. These cytokines start to orchestrate Th2 immunity. Upon allergen exposure, epithelial cells can also react by releasing danger signals such as adenosine triphosphate (ATP), uric acid, and high-mobility group box 1 (HMGB1), which increase local inflammation and might mount and further strengthen Th2 response [19]. When allergens have additional protease activities and/or they are accompanied by microbial components, such as endotoxin or inorganic particles, epithelial activation can lead to a mixed Th2 and Th17 or even Th1 immunity [20,21]. Different allergens activate several of these mechanisms and additional environmental factors such as viral infections, cigarette smoke, and diesel exhaust particle exposure might modify the final outcome in the direction of an IgE response against coexposed allergens. Infection with respiratory viruses or exposure to cigarette smoke increases the surface expression of TLR4 and MD-2, thus decreasing the threshold for allergen recognition [22]. Moreover, infection with several common respiratory viruses, such as human rhinovirus (HRV), the causative agents of the common cold, leads to impairment of nasal and bronchial epithelial barrier integrity [23] and as a consequence to facilitation of allergen uptake through the network of dendritic cells and monocytes [24,25]. Barrier function of epithelial cells in general plays an important role in allergy sensitization and further symptoms upon allergen exposure. Recent genome-wide association studies (GWAS) found novel links between genes involved in epithelial integrity such as cadherin-related family member 3 (CDHR3) and increased risk of atopy and asthma. CDHR3 and protocadherin (PCDH1) are highly expressed in epithelium [26,27]. They control epithelial polarity and integrity and might in this way facilitate allergic sensitization. It has been shown that epithelium of asthmatic patients has decreased barrier integrity and excess of Th2 cytokines such as IL-13 and IL-4 lead to further barrier impairment [28]. In summary, after recognition of allergen, epithelial cells response leads to activation of the network of DCs, ILC2, basophils, and monocytes that stimulate type 2 ILCs and polarize Th2 responses in naïve T cells [29].

38.2.3 Dendritic Cells

Dendritic cells are one of the most potent antigen-presenting cells (APCs), which play a crucial role in sensitization to allergens [30,31]. They reside beneath the epithelium in the skin and at the mucosal sites in the airways and gut. They can even extend dendrites between the epithelial cells in order to "patrol" and sample the external environment. Once they capture the allergen, they migrate to the draining lymph nodes and present the processed antigenic peptides to T cells. They present antigen within an antigen-binding cleft of major histocompatibility complex (MHC) molecules. These events start at the APC cell surface with the capture and endocytosis of antigens, followed by a complex sequence of

enzymatic activities leading to the association of antigenic peptides with MHC molecules and expression back to the cell surface. Intracellular molecules such as host self-antigens and intracellular invading pathogens' (e.g., viruses) proteins are processed and presented in the context of MHC class I to CD8+ T cells. This process plays a central role in recognition of the self-antigens and induction of immediate cytotoxic response in case of viral infection of the cell. In contrast, CD4+ T cells recognize antigenic peptides when presented by APC in the context of a class II MHC molecule together with the appropriate costimulatory signals. Allergen-specific Th2 subset is further activated and clonally expanded upon activation signals coming from combined actions of DCs, ILCs, and Th2 cells. In humans, there are two main groups of circulating DCs: (i) myeloid dendritic cells (mDCs) and (ii) plasmacytoid dendritic cells (pDCs) [31]. mDCs can be further divided into type 1 mDCs expressing BDCA1 and type 2 mDCs expressing BDCA3 [32]. Both mDCs and pDCs and their subsets express a different repertoire of PRRs and costimulatory molecules and thus display a diverse cytokine signature and further translate this cytokine profile to activation of specific subsets of CD4+ T cells [33]. In general, mDCs induce naive CD4+ T cells to produce IFN-γ, whereas pDCs stimulate naïve CD4+ cells to generate Th2 cytokines. However, in humans, there is also a special subset of fully mature pDCs that are also able to induce functional Treg cells [34,35], indicating that pDCs constitute a unique DC subset exhibiting intrinsic tolerogenic capacity [36–38].

38.2.4 Monocytes and Macrophages

Monocytes and macrophages show pleiotropic biological activities, including ability of antigen presentation, phagocytosis, cytotoxicity, and secretion of pro- and anti-inflammatory mediators (cytokines, chemokines, growth factor, etc.). The importance of monocytes in case of allergen sensitization has been shown in mice and humans [24,25].

Monocytes in general are the circulating precursors of tissue macrophages. However, recent fate-mapping experiments clearly showed that in many tissues there are resident macrophages, which develop during embryonic differentiation and play a pivotal role in tissue homeostasis in health and upon inflammation [39]. Macrophage function depends, to a large extent, on the monocyte activation process. Monocytes are heterogeneous population of cells, and on the basis of the expression level of CD14 (LPS receptor) and CD16 (FcγRIII), three functionally different subpopulations can be distinguished, namely, classical (CD14++CD16-), intermediate (CD14++CD16+), and nonclassical (CD14+CD16++) monocytes [40]. Nonclassical monocytes are referred to as proinflammatory cells because, in contrast to remaining two subpopulations, after stimulation, they release high amounts of tumor necrosis factor-alpha (TNF-α), but not IL-10 [41]. In fact, CD14+CD16++ monocytes are the major source of TNF-α on the periphery. These cells show an array of similarities to the M1-type macrophages, previously classified as classically activated macrophages. These cells are mainly involved in antimicrobial and antitumor responses. Therefore, they produce and release high amounts of proinflammatory cytokines, including IL-1, IL-6, IL-12, IL-23, and TNF-α. Furthermore, they posses high capacity for antigen

presentation to naïve T cells and polarize adaptive immune response toward Th1/Th17. On the other hand, CD14++CD16+ monocytes represent intermediate state of monocyte activation and maturation toward macrophages and show functional similarities to the M2-type macrophages (M2 a, b and c), previously referred to as alternatively activated macrophages. M2-type cells show an array of anti-inflammatory activities, including secretion of various anti-inflammatory mediators (e.g., IL-10, TGF-β, IL-1Ra, PGE_2 and others), induction of regulatory T cells, and lack of cytotoxic activity [42–44]. These populations are involved in suppression of adaptive immune response, inhibition of cytotoxic cell activity, rearrangement, reconstruction, and neovascularization of destroyed tissues.

38.2.5 Mast Cells and Basophils

Mast cells are one of the central effector cells in allergic inflammation. Upon first encounter with allergen, newly produced specific IgE binds the surface of mast cells through the FcεR1, the high affinity receptor for IgE. Upon next encounters with this allergen or cross-reacting allergen, tissue mast cells recognize it by at least two specific IgE molecules bound to the adjacent FcεR1 molecules [45]. This immediately activates mast cells and they respond by degranulation and release of granule contents that are preformed mediators such as histamine and several lipid mediators. Histamine, heparine, tryptase, chymase, prostaglandin D_2, cysteinyl leukotriens, and carboxypeptidase A3 are all preformed mediators released by activated mast cells [46]. At the same time, the production of many cytokines, chemokines, and growth factors is initiated by these mast cells. This rapid response of local mast cells leads to bronchoconstriction, vasodilation, increased vascular permeability, and increased mucus production in the bronchi of asthmatic patients or itching and eczema in case of skin manifestations. This response can be prolonged to the late phase, both due to the release of lipid mediators such as leukotrienes and due to infiltration of other effector cells such as eosinophils, monocytes, and neutrophils. Mast cells are distributed in all tissues, but they are found in high numbers especially near body surfaces, including the airway epithelium, the gut, and the skin [47,48]. Skin prick tests, widely used to document existence of specific IgE in the clinics, are based on the reaction of the resident skin mast cells in response to the applied allergen. Mature mast cells do not circulate in the blood; instead, hematopoietic stem cell-derived circulating mast cell precursors migrate to the peripheral tissues, where they complete their differentiation and maturation and become resident cells [49]. In contrast, basophils circulate in the blood in their mature form under the homeostatic conditions. They also express FcεRI, bind IgE and respond to allergen cross-linking in a similar way to mast cells. They release histamine and lipid mediators. They can also be a potent source of IL-4 and IL-13. Basophils are also used to document allergic sensitization in the basophil activation test, as the second-line test in allergy diagnosis [50].

38.2.6 Eosinophils

Eosinophils have been always associated with allergic disorders and helminthic parasite infection, but their roles in both of these states are not entirely clear [51].

They develop in the bone marrow from pluripotent progenitors, and in response to inflammatory stimuli they migrate into the blood and then accumulate in the peripheral tissues, such as lungs, skin, and gut in case of allergic disorders. They are important biomarkers of allergic disease [52]. Eosinophils contain many granules, contents of which are released upon activation. Human eosinophil-specific granules contain four major and proteins unique for eosinophils: eosinophil peroxidase, major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN). They also contain many preformed cytokines, enzymes, and growth factors. Eosinophils have also other granules storing Charcot–Leyden crystal protein (also known as galectin 10 and eosinophil lysophospholipase), as well as lipid bodies, which are the sites of synthesis of prostaglandins, cysteinyl leukotrienes, and thromboxane [51]. Eosinophils travel to the peripheral tissues in response to the several cytokines and chemokines, from which IL-5 and eotaxin (CCL11) are the most potent, respectively. Eosinophils have surface receptors for these agents, IL-5Rα and CCR3. Several Th2-inducing cytokines, such as TSLP, IL-25, IL-33, and other cytokines might indirectly promote eosinophilia by stimulating IL-5 production. HMGB1 and other danger-associated molecular patterns acting through TLRs and other PRRs on eosinophils are also able to induce recruitment and activation of these cells. Eosinophils communicate extensively with tissue-resident mast cells, which prolong their survival in the tissue [53]. They can induce DCs recruitment, as well as regulate specific adaptive responses among others by release of Th2-type chemoattractants such as CCL17 and CCL22, which promote the recruitment of Th2 cells and also through their interactions with DCs [54]. Eosinophils can also prime B cells for the production of antigen-specific IgM [55].

38.2.7 Innate Lymphoid Cells

Recent studies have identified a novel group of lymphoid cells, called innate lymphoid cells (ILCs), which lack rearranged antigen receptors. These cells can be divided into three majors groups, based on their phenotypic characteristic and functional properties [56,57]. ILC1s constitutively express T-bet and they can produce IFN-γ upon stimulation with IL-12 and IL-15. They are present in the intestines of patients with Crohn's disease and they might act in parallel with pathogenic CD8+ cells [58]. ILC2s were initially described in the gut, but they are also abundant in the lung and many mucosal barriers. ILC2s require the transcription factors RORα and GATA3, as well as Notch signaling and IL-7 and IL-33 for their development [56]. They produce IL-5, IL-9, and IL-13 after activation with epithelial cytokines IL-33 and IL-25, which promotes Th2 responses [57]. Thus, they control mucosal environment in helminthic infections and once dysregulated, they might induce and maintain proallergic inflammation [56]. Indeed, several studies confirmed their important role in the pathophysiology of allergic inflammation and asthma, as well as their involvement in the response to viral infections [59,60]. ILC2s express CD25 (IL-2Ra), CD90 (Thy1), variable amounts of CD117 (c-Kit), and CD127 (IL-7Ra). In addition, ILC2s express variable amounts of CD278 (ICOS), ST2 (IL-33R) and IL-17RB (IL-25 receptor), which makes them prone to respond and expand in the presence of IL-33, IL25, and IL-2 [57]. ILC3s require the transcription factors RORγt and

GATA3 for development, and in response to IL-1β and IL-23 they produce IL-17, IL-22, and IFN-γ [57]. Extensive research is going on for the investigation of further subsets and functions of ILCs.

38.2.8 NKT Cells

Natural killer T (NKT) cells express invariant T-cell receptor (TCR) and they are able to rapidly release many cytokines such as IL-4, IL-5, IL-13, IL-17, and IFN-γ after activation, thus exhibiting features of both T cells and NK cells, and they are able to amplify adaptive immune responses. Different subsets of NKT cells have been described in the pathogenesis of both allergic [61] and nonallergic asthma and especially bronchial hyperresponsivness of asthmatic bronchi [62–64].

38.2.9 Natural Killer Cells

Natural killer (NK) cells do not express CD3, CD4, or CD8 and they are especially important both in killing tumor and virus-infected cells, and in controlling certain microbial infections. They can produce high levels of cytokines including IFN-γ, TNF-α, TGF-β, IL-5, IL-10 and IL-22 after stimulation. However, they also contribute to allergic inflammation by increasing Th2 cytokines and eosinophilia as demonstrated after depletion experiments in a mouse model [65]. In addition, a tiny NK cell subset that secretes mainly Th2 cytokines but not IFN-γ has been shown to contribute to IgE production in humans [66]. On the other hand, TLR9-ligand-activated NK cells producing high levels of IFN-γ were shown to play a protective role in the development of asthma [67]. A tiny regulatory subset of NK cells that secretes IL-10 and suppresses T-cell responses has been demonstrated in humans [66].

38.2.10 Role of Microbiota in Allergic Inflammation

The enormously huge number of bacterial species inhabits human body, which constantly interacts with the innate and adaptive parts of immune system. All barrier tissues in the gut, lung, or skin are colonized by many bacterial species. It has been shown that the composition of microbiome, interactions of microbial products with other components of the diet and life period in which humans are colonized by the specific bacterial strains, they have a huge impact on the development of allergy, obesity, and cancer [68]. Several studies have suggested that risk factors associated with allergic sensitization such as lack of maternal exposure to pets or livestock during pregnancy, maternal consumption of antimicrobials during pregnancy, cesarean birth, formula feeding, and early-life antimicrobial exposure, all concentrated in the pre- and early postnatal phases of life, are influencing neonatal and maternal microbiome composition [68]. The infant gut microbiome accumulates bacterial diversity over the first years of life, although the source of this bacterial diversity remains unknown. In several allergic disorders, it has been found that the early aberrant microbial colonization

that lacks commensal species or has lower number and diversity of commensals takes place. Moreover, animal studies and epidemiological cohorts with interventions with the use of several bacterial species or bacteria-rich environment supplemented early in life or supplemented to mothers during pregnancy prevented allergic sensitization. For example, early-life introduction of a cocktail of Clostridium species or a diversity of bacteria significantly decreased circulating IgE concentrations in adulthood [69,70]. In terms of mechanism, it has been shown that several microbial-derived metabolites, especially short-chain fatty acids (SCFAs) such as butyrate and acetate, or propionate increased by consumption of yogurts or due to fermentation of complex dietary carbohydrates can prevent the development of allergy and asthma by influencing the regulatory T-cell compartment among several other mechanisms [30,71,72].

38.3 Adaptive Immunity and its Role in Allergic Inflammation

The most important and defining feature of the adaptive immune system is its memory. In response to antigen stimulation, specific T and B lymphocytes start to clonally expand. They express specific antigen receptors on their surface, the expression of which is shaped early in the fetal life by the sequences of recombination of genes encoding these receptors. Initially, T and B cells start to "learn" to be antigen specific in the thymus and in the bone marrow, where the process of recombination of the antigen receptor genes is initiated. This is the part of the process called central tolerance. Due to this process, they can differentiate self- from the nonself-antigens. Through the expression of antigen-recognizing T-cell receptor (TCR) and B-cell receptor (BCR), T and B cells can bind and become activated by a specific antigen. First encounter with the specific antigen activates lymphocytes and leads to the generation of long-lived, antigen-specific memory T and B cells by their clonal expansion and increased frequency in lymphatic organs. Upon the next encounter with the same antigen, there is a relatively faster recognition by these memory cells. Both cellular (mediated by T cells) and humoral (mediated by B cells) responses are generated more rapidly than during the first exposure to this antigen and more memory cells are produced.

38.3.1 Central and Peripheral Tolerance Mechanisms

Central tolerance occurs in the primary lymphoid organs, such as thymus in case of T cells and bone barrow in case of B cells, while peripheral tolerance is shaped in peripheral lymph nodes, spleen, tonsils, Peyer's patches, and other tissues [73]. The lack of effective central and peripheral tolerance mechanisms leads to autoimmune disorders and allergic diseases.

In the thymus medullary thymic epithelial cells, dendritic cells and macrophages present tissue-specific autoantigens to the immature T cells in the context of major histocompatibility complex I and II. Aire, the autoimmune regulator

gene in the medullary thymic epithelial cells and other antigen-presenting cells in the thymus, regulates this process. Aire is a transcription factor that promotes ectopic expression of tissue-specific antigens on medullary thymic epithelial cells [74]. When T cells react to the self-antigens with high affinity, they are depleted in the process called negative selection. Nonself-reacting T cells expressing CD4 or CD8 subsequently exit to the periphery. A small fraction of T cells that express low-affinity receptors for self-antigens that have escaped negative selection in the thymus are subjected to the peripheral tolerance mechanisms. In addition, some self-reactive CD4+ T cells that are not deleted by negative selection develop into central regulatory T (Treg) cells in the process also regulated by Aire. These central Treg cells circulate in the periphery as mature T cells and inhibit immune or inflammatory responses against self-antigens. B cells expressing immunoglobulin receptors with high affinity for self-components also undergo negative selection. They are eliminated in the bone marrow in the process of deletion and/or receptor editing. Similar to T cells, B-cell clones expressing receptors with low affinity might escape these central tolerance mechanisms and circulate to the periphery [73].

In the periphery, immune tolerance must be reinforced and maintained. There are several mechanisms of peripheral immune tolerance, including anergy, exhaustion, and immune regulation [75]. These mechanisms prevent overactivation of immune system to self-antigens and thus prevent autoimmunity, as well as control dysregulated immune responses to harmless antigens, which prevents allergic inflammation. Therefore, the fundamental strategy of allergen-specific immunotherapy for allergic diseases is to correct dysregulated immune responses by inducing peripheral allergen tolerance. Anergy happens in the periphery when CD4+ T cells receive signal through TCR only, without engagement of costimulatory receptors such as CD28 on antigen-presenting cells. On the other hand, on the surface of T cells, there are molecules, such as CTLA4, PD-1, and ICOS through which coinhibitory signals might be delivered [76]. Sustained expression of can also cause T-cell exhaustion. These terms need to be revisited, because in the nature there is no condition that T cells are not exposed to ligands for their costimulatory receptors, such as CD28, CD2, or ICOS. In addition, PD-1 and CTLA4 are strong tyrosine phosphatase-linked receptors, which have suppressive capacity by dephosphorylating tyrosines and acting as antagonists to tyrosine kinases. Another mechanism of peripheral tolerance is mediated by neighboring cells' death-inducing ligands, which activate processes leading to apoptosis of autoreacting cells. Moreover, effector T cells might be also suppressed by cytokines released by tissue cells and more effectively by regulatory T cells (Tregs).

38.3.2 Antigen Recognition by T Cell

Antigen-presenting cells (APCs) process intracellular or extracellular antigens and present on their surface in the context of MHC class I and II to CD8+ or CD4+ T cells, respectively. In general, APC present intracellular antigens to CD8+ T lymphocytes in the context of MHC class I, so as they are ready to quickly recognize the intracellular threats such as viruses and intracellular bacteria and

remove such cells in the series of cytotoxic events [77]. MHC class I molecules are expressed by all nucleated cells. Peptides originated from endogenous proteins, degraded in the cytosolic and nuclear proteasomes, are transported into the endoplasmic reticulum (ER) by trasporter associated with antigen presentation (TAP) proteins. In the ER, peptides are loaded onto the complex consisted with the polymorphic heavy chain of MHC class I and light chain-β2 microglobulin. Fully formed and stable complex is then transported to the plasma membrane and presented to CD8+ T cells (Figure 38.2). Naïve CD8+ T cells are able to directly recognize peptide–MHC class I complexes, but they require additional costimulation from APC to become potent effector CD8+ T cell with cytotoxic potential [78]. Extracellular antigens, including allergens, captured by the APCs, are engulfed in the process of endocytosis, phagocytosis, or macropinocytosis. The proteins are then degraded in the endosomal pathway. MHC class II molecules are primarily expressed by professional APCs such as DCs, macrophages, and B cells. α and β chains of MHC class II molecules are also assembled in the ER and stabilized by the invariant chain (Ii) and then transported to endosomes, where invariant chain is digested and only residual class II-associated Ii peptide (CLIP) stays in the MHC class II heterodimer. Next, there is an exchange of CLIP into the specifically recognized peptide, determined by the highly polymorphic sequences of MHC class II molecules. Fully assembled peptide–MHC class II complexes are then transported into plasma membrane to be presented to CD4+ T cells (Figure 38.3). Several costimulatory signals from APCs and cytokines such as IL-1 and autocrine IL-2 are required for CD4+ T cell to be activated after antigen recognition [78].

38.3.3 T Cell Subsets

As mentioned above, there are two classes of T lymphocytes carrying T-cell receptor (TCR) consisting of α and β chains and coreceptors CD4 and CD8 that determine their function in the adaptive immune response. CD8+ T cells are the highly specified, antigen-specific cytotoxic T cells (CTLs), whose function is to release the preformed mediators and/or to initiate cell death program by the interactions with the cell surface molecules. They are highly important in the immunity against viruses and intracellular bacteria. Activation of CD8+ cell arm of the adaptive immunity is an integral part of immunization by vaccination as well as in antitumor immunity. CD4+ T cells are traditionally seen and called T-helper cells (Th) cells, since the signals from them, after recognition of antigens, lead and direct other immune cell responses.

38.3.3.1 Th1/Th2 Paradigm

CD4+ T cells play an essential role in the development of fully mature, integrated cellular and humoral adaptive immune response. In the secondary lymphoid tissue after recognition of an antigen and signals from the costimulatory molecules from APCs, CD4+ T cells differentiate into the effector cells, with distinct functional properties defined by the pattern of cytokines they secrete. Compared with naive T cells, effector T cells do not require costimulation to be fully activated and they respond rapidly to antigen by producing high levels of

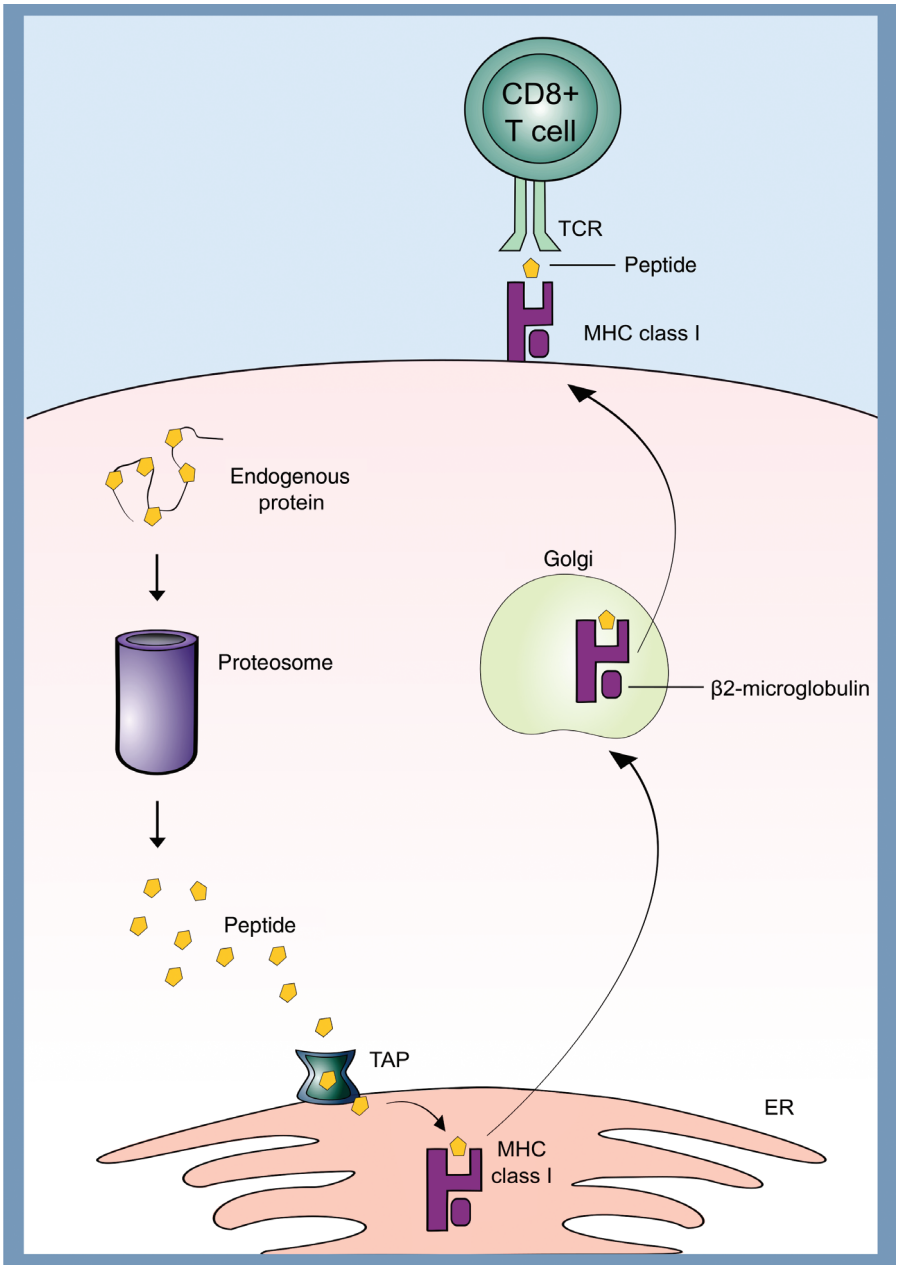


Figure 38.2 Antigen presentation in the context of MHC class I. MHC class I is involved in the presentation of endogenous antigens (viral antigens) to $CD8^+$ T cells. Endogenous proteins undergo degradation in the proteasome and subsequent transportation to the endoplasmic reticulum via transporter associated with antigen presentation (TAP) proteins. In the ER lumen, peptides are loaded onto the MHC class I composed of polymorphic heavy chain of MHC class I and β2-microglobulin. Then, peptide–MHC class I complexes are transported to the plasma membrane and presented to $CD8^+$ T cells.

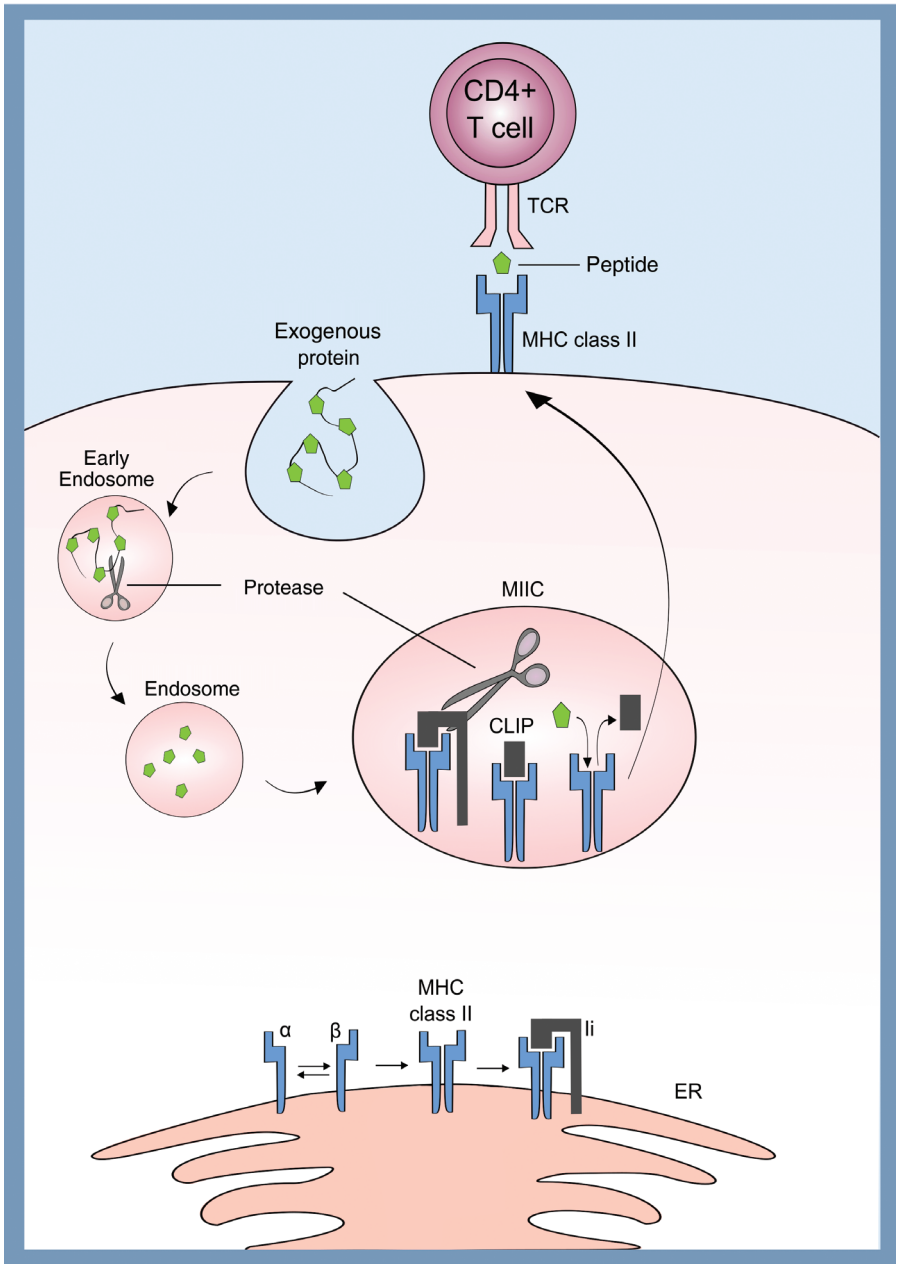


Figure 38.3 Antigen presentation in the context of MHC class II. MHC class II is involved in the presentation of exogenous antigens (i.e. bacterial antigens, allergens, etc.) to $CD4^+$ T cells. Assembly and stabilization of MHC class II via association with the invariant chain (Ii) take place in endoplasmic reticulum. Ii-MHC class II complexes are transported to the endosome where Ii chain is digested into CLIP that stays in the peptide-binding groove. Then, CLIP is replaced by the final peptide. Fully assembled peptide–MHC class II complexes are transported to the plasma membrane and presented to $CD4^+$ T cells.

cytokines and chemokines, which then direct the immune response. Many activated effector CD4+ T cells die following activation and an immune response through the process of activation-induced cell death, but a subset of CD4+ T cells will persist as memory cells for the life of the host. CD4+ memory T cells persist in lymphoid organs as central memory cells and in nonlymphoid tissues as effector memory cells. Th1 cells secrete mainly IFN-γ and differentiate with IL-12 secreted from DCs, while Th2 cells release IL-4, IL-5, and IL-13 in response to IL-4 secreted by basophils, mast cells, other Th2 cells, and ILC2. Th2 cells, after recognition of the antigen, presented by B cells, and engagement into CD40-CD40L costimulatory process, secrete IL-4 and IL-13, which leads to the immunoglobulin class switching and formation of IgE. The fate of CD4+ T cells into the development of Th2 responses depends upon master transcription factor GATA3 and master signal transducer STAT-6. Binding of IL-4 to IL-4R and IL-13 to IL-13R results in the phosphorylation of STAT-6. Phosphorylated STAT-6 translocates to the nucleus and binds to STAT-6 transcriptional elements and interacts with GATA-3. GATA3 promotes Th2 differentiation through instructing Th2 commitment, selectively stimulating the growth of Th2 cells, and suppressing Th1 differentiation [79]. On the contrary, T-bet is a major transcription factor for inducing IFN-γ production and Th1 cell differentiation [79]. Since the discovery of these two subsets of Th cells in 1986 [80], it was postulated that Th1 responses are predominant in infections and autoimmunity in parallel to CD8+ T cell cytotoxic responses, while unbalanced Th2-dependent responses underlies the pathogenesis of allergic diseases. The right balance between Th1 and Th2 responses was thought to be essential in prevention of IgE-dependent diseases. Indeed, infants with higher levels of IFN-γ in their cord blood less likely develop atopy, which is defined as a positive skin prick test to the allergens [81]. Moreover, expression of T-bet and IFN-γ production is decreased in the airways of patients with allergic asthma [82], similar to Th1-inducing cytokine IL-12 from DCs [83], and the expression of IL-12R on T cells [84]. Studies in the past two decades and accumulated knowledge on highly specialized novel subsets of Th cells such as Th17, Th9, Th22, and T follicular helper (T_{fh}) and the insights into the regulatory T-cell (T_{reg}) function have changed the simple dualistic view of Th1/Th2 imbalance in the pathogenesis of allergic diseases and other conditions. Proper immune regulation plays an essential role in autoimmunity, transplantation tolerance, tumor development, chronic infections, and successful pregnancy [7,85].

38.3.3.2 Other T-Helper Subsets

Th17 Cells

Th17 cells have been identified as a new T-cell lineage secreting mostly large amounts of IL-17 [33,34]. These cells are essential for the elimination of extracellular pathogens [86,87], and they are also involved in the development of psoriasis, Crohn's disease, and rheumatoid arthritis [88]. They might produce several cytokines such as IL-17A, IL-17F, IL-22, or IL-26 after activation [89–91]. The master transcription factor involved in Th17 cell fate is retinoic acid receptor-related orphan receptor γt/C2 (RORγt/RORC2) [92]. It has been shown that Th17 cells might play a pathogenic role in the development of asthma, yet probably in the

separate endotype from classical Th2-derived type of this disease [35,93]. Th17 cells contribute more to neutrophilic inflammation in acute airway inflammation models [94,95]. However, IL-17 is demonstrated to be the main cytokine driving the granulocyte influx observed into the lungs of allergic asthma models [96,97]. Also in humans, Th17 cells contribute to allergic airway disease by inducing airway smooth muscle cell migration as well as to neutrophilic asthma, chronic rhinosinusitis, and mixed eosinophilic and neutrophilic forms of these diseases [98].

Th9 Cells

TGF-β alone or in combination with IL-4 promotes the development of Th2 cells selectively secreting IL-9 [48,49]. The proviral integration 1 (PU.1) has been shown to be an important transcription factor for the Th9 cells' development and their association with allergic disease [99]. It has been shown that IL-9 released by Th9 acts both on other T-cell subsets and on B cells and mast cells. Moreover, it plays a role in promoting eosinophilic inflammation and other features of allergic asthma phenotype such as increased mucus secretion, elevation of IgE, and development of bronchial hyperresponsiveness [100–104]. The expression of IL-9 and IL-9 receptor is increased in bronchial tissue of atopic asthmatic subjects [105]. Moreover, mice that selectively overexpress IL-9 in the lung develop asthma-like symptoms [100,101].

Th22 Cells

Th22 cells are characterized by particularly high IL-22 production, which might be also produced by other T cells such as Th0 and Th17 cells [91,106–108]. In mice, there is no separate IL-22-producing T-cell subset; IL-22 is always coproduced with other cytokines, mainly IL-17, by Th17 cells [109]. In humans, though, separate Th22 cells have been recently characterized [110]. IL-22 acts on the mucosal sites, as mainly epithelial cells express IL-22R. However, the exact role of IL-22 in the development of allergy and asthma is not fully understood yet. It was reported that Th22 cells together with Th17 cells contribute to accumulation of airway smooth muscle cells, in asthma [88,98]. On the other hand, IL-22 could also inhibit allergic airway inflammation in the effector phase by altering the function of DCs and inhibiting IL-25 production from lung epithelial cells [111].

T Follicular Helper Cells (Thf)

T follicular helper cells are located in the germinal centers and can be identified by the surface expression of the CXC chemokine receptor 5 (CXCR5) and programmed cell death 1 molecules. They help B cells in clonal expansion and survival, as well as in the development of memory B-cell responses, and production of antibodies. Tfh cells might be a separate lineage, but this is still a matter of debate. Tfh cells generated *in vivo* in the course of Th1, Th2, or Th17 responses may secrete IFN-γ, IL-4, IL-13, or IL-17 signature cytokines in addition to IL-21 and CXCR5 [79,85].

38.3.3.3 Regulatory T (Treg) Cells

Regulatory T cells (Tregs) are essential in the maintenance of immune self-tolerance and homeostasis. They suppress abnormal or excessive immune

responses at several levels and by implementing various mechanisms. Treg cells comprise two main groups: (i) thymic-derived, naturally occurring CD4+CD25+, which constitutively express the transcription factor Foxp3, called natural Tregs (nTreg) or thymic Treg (tTreg), and (ii) the peripherally induced Tregs, called inducible Treg (iTreg) or more recently peripheral Treg (pTreg) [34,112]. nTreg cells are produced by the thymus as an antigen-primed and are functionally mature T-cell subpopulation specialized for immune suppression. Their main function is to reach the inflammation sites and suppress effector lymphocytes, especially helper T (Th)-cell subsets: Th1, Th2, Th17, and follicular Th (Tfh) cells [79,112]. nTreg cells constitutively express FOXP3, high levels of the alpha chain of the IL-2 receptor (CD25), and the suppressor costimulatory molecules CTLA4 and PD1 [113,114]. iTregs can arise from naïve CD4+ T cells in the periphery upon stimulation with TGF-β and IL-2. They also start to express Foxp3 and exhibit several regulatory functions, although in humans Foxp3 expression might be transient, can also happen in activated Th2 cells, and may not lead to acquiring regulatory properties [115,116]. Foxp3 is a master transcription factor in Treg cells' development and function as it was shown in patients with mutations in *FOXP3* gene that lead to the immune dysregulation polyendocrinopathy enteropathy-X-linked syndrome or the X-linked autoimmune and allergic dysregulation syndrome. These diseases are characterized by typical severe autoimmune and allergic phenotypes [117,118]. Similar phenotypes are observed in mice with *Foxp3* mutations (scrufy mice) that display comprehensive impairment in generating functional Treg cells [119]. nTreg and iTreg can be distinguished by several markers, including reported recently neuropilin 1 and helios [120]. There is a recent view that different Treg populations can be generated and specialized to control mainly the function of particular subsets of CD4 T cells. Upon IFN-γ stimulation, Foxp3+ Tregs upregulate T-bet and they are able to suppress T-bet expressing activated Th1 cells [121]. Similarly, it was noted that conditionally deleting Irf4 in Tregs selectively allowed the uncontrolled expansion of Th2 cells, suggesting that IFN regulatory factor 4 (IRF4) expression, important in Th2 differentiation, also played a role in differentiation of those Tregs that could control Th2 cells [122]. Moreover, to suppress pathologic Th17 cells, Tregs require STAT3 expression, which is essential in Th17 cell differentiation [123]. In humans, there is a population of iTreg cells generated in the periphery after antigenic stimulation, characterized by high levels of IL-10 production and playing a key role in the maintenance of healthy immune response to allergens [124,125]. They are called Tr1 cells. The suppressive capabilities of those cells are based on IL-10 and TGF-β [125–127]. In humans, Tr1 cells expand during the course of allergen-specific immunotherapy and inhibit the proliferation and cytokine responses of naïve CD4+ T cells as well as established Th1 and Th2 cells, including allergen-specific Th2 cell lines. Moreover, they directly or indirectly suppress effector cells such as mast cells, basophils, and eosinophils; suppress dendritic cells; suppress IgE production by B cells; and enhance IgG4 production [128–130]. It was shown that the homeostasis in healthy individuals and its disturbance in allergic patients depends on the imbalance between three different allergen-specific T-cell subtypes, Th1, Th2, and Tr1 [131,132]. What is even more important, during allergen-specific

immunotherapy, is that the success of the treatment depends on restoring the right proportion of those cells' populations. For example, during the exposure to venom allergen, venom-specific IL-10-secreting Tr1 cells are clonally differentiated from allergen-specific Th1 and Th2 cells [124]. Moreover, functional allergen-specific Treg cells in humans can be generated in the secondary lymphoid tissue, such as tonsils in the mechanism partially depending on plasmacytoid dendiritic cells [38]. Moreover, it has been shown that in case of chronic inflammatory conditions, represented by stimulations with IL-1β and IL-6, this allergen-specific T-cell tolerance in human tonsils and peripheral blood can be disrupted [133].

38.3.4 B Cells and Immunoglobulins

B cells and antibodies constitute the second essential part of the adaptive immunity, the humoral immune system. They form a potent weapon against viruses, bacteria, fungi, and parasites. Defects at the several stages of B-cell development, negative and positive selection, maturation, and antibody production may lead to autoimmunity, malignancy, immunodeficiencies, and allergy [134]. B lymphocytes develop in the bone marrow from hematopoietic precursor cells. At the early stage, B-cell precursors contact bone marrow stromal cells. During these processes, B-cell precursors undergo negative and positive selection steps, and their B-cell receptor is initially rearranged (Figure 38.4). Then, these immature B cells migrate to the peripheral lymphoid tissues, mainly spleen, where they differentiate into the mature naïve B cells, becoming either follicular IgM^+IgD^+ (majority of them) or marginal zone (MZ) B cells [135]. These mature naïve follicular IgM^+IgD^+ B cells recirculate through the secondary lymphoid organs (SLO) such as tonsil, lymph nodes, and also spleen until they encounter their cognate antigen and become fully activated, mostly in the presence of Th cells and further differentiate into either specific antibody-producing plasma cells or into long-lived memory B cells. Before antigen encounter, naive mature B cells express high levels of the chemokine receptor CXCR5 that allow them to respond to CXCL13 produced by follicular stromal cells of any secondary lymphoid tissue and lead them to the B-cell–rich follicles [135].

38.3.4.1 B-Cell Activation and Fate

As mentioned above, in the follicles of SLO mature B cell encounters its cognate antigen that is usually attached to the surface of neighboring APCs such as dendritic cells or macrophages [136]. Recognition of this antigen by the BCR, in a form of immunological synapse, is the first signal for B-cell activation [135]. BCR consists of a plasma membrane immunoglobulin (at this stage IgM or IgD) coupled to a signaling module Igα (CD79a)-Igβ (CD79b) dimer. LFA1 is an integrin also strongly involved in this process. Also, activation of the B-cell coreceptor complex, consisting of CD21, CD19, and CD81, enhances the BCR-dependent reaction. The first signal through BCR after antigen recognition leads to the several actin rearrangements in the B cells, which promotes antigen uptake by receptor endocytosis and starts the antigen processing into the MHC class II molecules, as well as induces CCR7 on B cells, stimulates them to traffic to the border of the T- and B-cell zones in

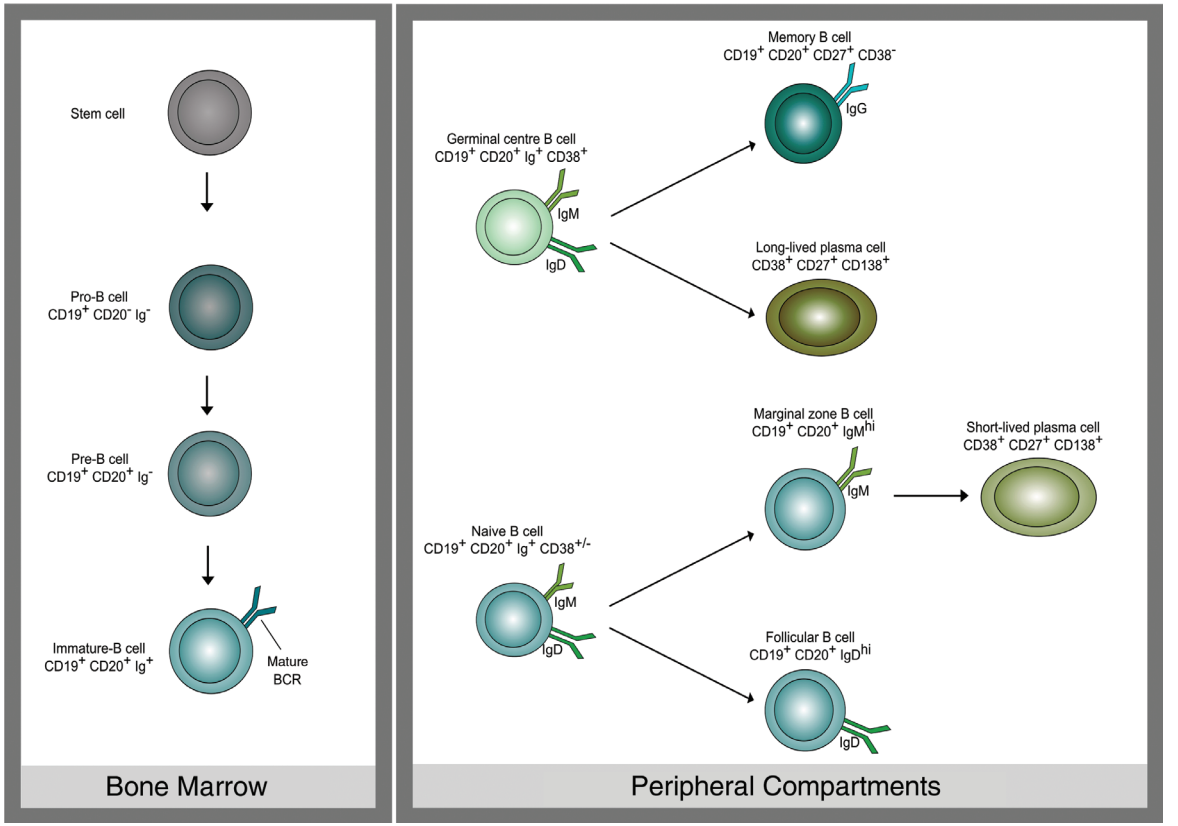


Figure 38.4 B-cell developmental stages. The B-cell development starts in bone marrow, where B cells develop from the hematopoetic precursor, and further progresses through the pro-B cells, pre-B cells, and immature B cells. Immature B cells migrate to peripheral compartments to differentiate into the mature naïve B cells. Depending on the specificity of their BCR, they might become follicular B cells or marginal zone B cells. Follicular B cells recirculate through the secondary lymphoid organs until they become fully activated by encountering their cognate antigen and in the germinal center develop into the memory B cells or long-lived plasma cells. Marginal zone B cells may develop into the short-lived plasma cells.

SLO and interact with Th cells. Interaction between Th cells and B cells consists of two essential processes: (i) antigen recognition through the TCR and MHC class II presented by B cell and (ii) the costimulatory signaling through CD40 on B cells and CD40 ligand (CD40L) on T cells [135]. Interaction between B and T cells is reciprocal. T cells provide help to B cells through CD40–CD40 ligand interaction and cytokine production, B cells provide peptide–MHC class II complexes in the context of costimulatory molecules to T cells, which facilitates their survival and cytokine production. After the initial interaction with Th cells, B cells can differentiate into extrafollicular plasma cells, germinal center (GC) B cells, or early memory B cells that have the capacity to recirculate [137]. BCR antigen recognition strength and differential expression of chemoattractant receptors are key factors in determining the fate of particular B cells [138]. The cytokine profile from interacting Th cells is the main determinant of the immunoglobulin class switch and final Ab production by plasma cells [137]. For example, classical Th2 cytokines IL-4 and IL-13 induce class switch recombination (CSR) to antigen specific IgG4 and IgE through activation of STAT6. IL-9 enhances IL-4–dependent IgE production in B cells through activation of STAT3 and STAT5, although IL-9 alone does not have this effect. TGF-β induces CSR to IgA through activation of Smad and Runx. IL-10 is known to induce CSR to IgG1, IgG3, IgG4, and IgA. IL-21 induces CSR to IgG1, IgG3, and IgA and also enhances IL-4-dependent CSR to IgE through activation of STAT3 [139]. The combined BCR, cytokine, and costimulatory activation signals in B cells induce activation-induced cytidine deaminase (AID) and other components of the somatic hypermutation (SHM)/class switch machinery, thus changing the affinity of the BCR and the isotype of immunoglobulin (IgM to IgG, IgA, or IgE) [140]. As a consequence of somatic hypermutation, BCR increases or decreases affinity for antigen. B cells with high-affinity BCR are preferentially activated, clonally expanded, and as a consequence high-affinity antibodies against this antigen are produced by plasma cells. Development of both short-lived and long-lived plasma cells depends on the expression of the transcription factor B lymphocyte-induced maturation protein 1 (Blimp-1), a master gene of plasma cell development [141]. Long-lived plasma cells are terminally differentiated and they do not divide. They travel to BM and reside there.

38.3.4.2 Immunoglobulins

Immunoglobulins consist of two heavy chains and two light chains. Each heavy chain or light chain has two major domains referred to as the constant region and the variable region. Heavy-chain variable regions are encoded by one V gene, which encodes most V-region amino acids, as well as one of the 23 diversity (D) and one of the 6 joining (J) gene segments. Only two types of genes encode the light-chain variable regions: V genes and J genes. Immunoglobulin remarkable diversity has four major sources: multiple V(D)J genes in the germline, random assembly of heavy chains and light chains, junctional nucleotide variability introduced during pre-B-cell immunoglobulin gene rearrangement, and somatic hypermutations (SHM) of immunoglobulin variable regions [142]. There are five classes of antibody molecules: IgM, IgD, IgG, IgA, and IgE. The IgG and IgA classes have more than one member. There are four IgG (γ) subclasses designated

as IgG1, IgG2, IgG3, and IgG4, and their constant regions exhibit 90% homology with each other. The two subclasses of IgA are similarly related to each other. There are two types of light chains: κ and λ. There are four λ subtypes but there is only one form of κ. These nine classes and subclasses of antibodies have different expression levels in serum and tissues, anatomic locations, and effector functions. IgA1-2, IgM, and IgG1-4 are involved in the antigen neutralization. IgM is most potent in complement fixation, although IgG1-3 and IgA1-2 are also involved in this process. IgG1-3 and IgE are also involved in antibody-dependent cellular cytotoxicity (ADCC). Finally, IgE is strongly involved in immediate hypersensitivity reactions [142].

38.3.4.3 Other B-cell Functions and B-cell Subsets

B cells at the different stage of their development can secrete various cytokines and thus they have additional functions. They can release a broad variety of cytokines, such as IL-2, IL-4, IL- 6, IL-10, IL-17, IFN-α, IFN-γ, TNF-α, TGF-β, and LT, and thus they can be classified into distinct subsets depending on the particular cytokine profile [143]. Due to the cytokine release, they can promote immune responses by regulating Th1/Th2/Th17 and neutrophil responses, induce macrophages, and DC and NK cell activation, as well as regulate their own differentiation and antibody-producing capabilities [144]. On the other hand, there are several lines of evidence that among them are also potent regulatory cells that can suppress proinflammatory responses of monocytes, DCs, CD4+, and CD8+ T cells [145]. In particular, human IL-10-secreting B regulatory cells (Br1) play an essential role in allergic inflammation [146]. They are able to suppress allergen-specific $CD4^+$ T-cell proliferation. In addition, the major bee venom allergen phospholipase A (PLA)-specific B cells from non-allergic beekeepers showed increased expression of IL-10 and IgG4 and the frequency of IL-10-secreting PLA-specific B cells increased in allergic patients receiving allergen-specific immunotherapy. Moreover, IL-10-overexpressing human B cells acquired a prominent immunoregulatory profile by upregulation of the suppressor of cytokine signaling-3 (SOCS3), glycoprotein A repetitions predominant (GARP), CD25, and PD-L1 [147]. They also secreted less IgE and potently suppressed proinflammatory cytokines in PBMCs, maturation of DCs, and antigen-specific proliferation *in vitro*.

38.4 Cytokines of Allergic Inflammation

Many cells including T cells, ILCs, epithelial cells, basophils, mast cells, eosinophils, monocytes, macrophages, and B cells produce cytokines that are involved in initiation and maintenance of type 2 immune responses. These cytokines, namely, IL-4, IL-5, IL-9, IL-13, IL-25, IL-33, and TSLP have a default role in the immunity against helminthes as well as a counterbalance to severe type 1 inflammation. However, in case of a dysregulation of immune responses, they might lead to initiation and maintenance of allergic responses. Therefore, several clinical trials in allergic diseases have targeted those cytokines or their receptors [7].

38.4.1 IL-4

IL-4 is produced primarily by Th2 cells, ILC2, basophils, mast cells, and eosinophils. It plays an important role in mounting protective immunity against helminthes and other extracellular parasites. It is a central cytokine for naïve CD4+T-cell development into Th2 cells after antigen stimulation [148–150]. In addition, IL-4 is critical to inducing class switch to IgE and IgG4 or IgG1 in human and mouse B cells, respectively. IL-4 stimulates the expression of MHC class II molecules, BCR, CD23, and IL-4R in B cells. IL-4- and IL-4Rα-deficient mice have compromised Th2 differentiation and the serum levels of IgG1 and IgE are reduced [151]. It also inhibits type 1 immune responses including Th1 cells and M1 macrophages. There have been several clinical trials targeting IL-4 and IL-13 or their receptors with more promising results on anti-IL-13 approaches for the treatment of allergic asthma and atopic dermatitis [152].

38.4.2 IL-5

IL-5, together with IL-4, plays a central role in allergic inflammation. It is predominantly produced by Th2 cells, ILC2, and also by mast cells, activated eosinophils, and NK and NKT cells. IL-5 receptor shares the β chain with IL-3 and GM-CSF, and together with these cytokines it leads to growth, activation, differentiation, survival, and mobilization of eosinophils to the inflammatory site. Therefore, eosinophils are one of the first cells influxing the site of inflammation in the lungs in asthma or gut in food allergy, and their number increases in the blood, which is used as a biomarker. IL-5 levels, Th2 cells, and eosinophils are increased in bronchoalveolar lavage and in biopsies of asthmatics and they correlate with severity of the disease [153]. IL-5-deficient mice are resistant to induction of experimental asthma [154]. Anti-IL-5 therapy has provided promising results in eosinophilic asthma, eosinophilic osephagitis, and chronic rhinosinusitis [152,155,156].

38.4.3 IL-13

IL-13 is mainly secreted by activated Th2 cells, ILC2, mast cells, basophils, eosinophils, and NKT cells [157]. One receptor chain, IL-4Rα is shared between IL-13 and IL-4; therefore, they have in many cases overlapping functions except an effect on Th2 differentiation because T cells do not express IL-13 receptor. IL-13 induces IgE class switch in B cells, recruits mast cells and eosinophils, and prolongs their survival [158]. IL-13 in addition to TGF-β has been linked to tissue remodeling and fibrosis [159,160]. Epidemiological data pointed out that several polymorphisms in IL-13 and IL-4 pathways significantly increase the risk of asthma, escalate asthma exacerbation frequency in childhood, and are correlated with elevated eosinophilia and total IgE in the blood [161,162]. IL-13 KO mice do not develop goblet cells hyperplasia in experiential asthma models [163,164].

38.4.4 IL-25 (IL-17E)

IL-25 is a member of the IL-17 family of cytokines. It is produced by Th2 cells, mast cells, and epithelial cells [18,165–167]. Eosinophils and basophils from

atopic individuals can also release IL-25. IL-25 stimulates the production of IL-4, IL-5, and IL-13 in non-B/non-T (NBNT) c-kit+FcεRI$^-$ cells in mesenteric lymph nodes, in a subset of natural killer T cells [168], and in ILC2 [169]. IL-25 also enhances IgE synthesis, induces mucus production and epithelial hypertrophy, and augments the numbers of eosinophils in blood in murine model of asthma [169]. Transgenic mice that express IL-25 only in lungs have increased numbers of eosinophils and CD4+ T cells upon allergen-specific challenge [167]. Anti-IL-25 treatment reduced several inflammatory outcomes in chronic rhinitis model in mice, suggesting a role for IL-25 also in the pathogenesis of chronic rhinosinusitis with nasal polyps [170]. IL-25 KO mice cannot get rid of parasite infection efficiently [171], because of the inefficient induction of Th2-type cytokine responses, and are very prone to Th1-dependent experimental autoimmune encephalomyelitis.

38.4.5 IL-33

IL-33 is a member of IL-1 family of cytokines. It plays a significant role in regulation of immune responses at the mucosal sites, together with TSLP [172]. It is predominantly produced by epithelial cells upon injury, but it can also be released by smooth muscle cells and fibroblasts. IL-33 acts through ST2 receptor, which is expressed in Th2 cells, mucosal ILC, mast cells, and NKT cells [173,174]. Soluble ST2, released by fibroblasts, monocytes, and macrophages upon activation by proinflammatory mediators, inhibits binding of IL-33 to its receptor and is a negative regulator of its activity. In mice, IL-33 induces airway inflammation through a process depending on IL-5-producing T cells but not IL-4 [175]. Administration of neutralizing antibodies or transfer of soluble ST2 impairs Th2-type inflammation in experimental induced asthma mice [176,177]. IL-33 is increased in serum of patients with anaphylaxis. It can also activate human eosinophils and mediate degranulation of mast cells in the absence of allergen [178,179].

38.4.6 TSLP

TSLP is produced among others by keratinocytes, airway epithelial cells, intestinal epithelial cells, thymic stromal cells, tonsilular crypt epithelial cells, mast cells, and basophils [180]. TSLP is released in response to viruses, bacteria, parasites, TLR engagement, and cytokines, such as IL-1β, TNF-α, IL-4, and IL-13 [180]. TSLP acts on DCs, monocytes, CD4+ T cells, mast cells, and B cells promoting building of the Th2 inflammatory response often in cooperation with IL-25 and IL-33 and other cytokines [180–182]. In antigen-presenting DCs, TSLP increases the expression of several costimulatory molecules, which further promotes the generation of IL-4-, IL-5-, and IL-13-producing T cells and inhibits IL-10 and IFN-γ production [183]. TSLP plays an essential role in allergic asthma. The expression of TSLP is increased in the asthmatic airways and in the skin of atopic dermatitis patients and correlates with disease severity [184,185]. Anti-TSLP treatment showed promising efficacy in reducing allergen-induced inflammation in clinical trials [186,187]. TSLP is also associated both with atopic dermatitis [188] and with chronic rhinosinusitis with nasal polyps [189].

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39

Sepsis

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39.1 Introduction

Sepsis is a syndrome of life-threatening organ dysfunction triggered by an excessive, dysregulated host response to infection. The immune system represents a complex interlinked system of activated cells, receptors, secreted proteins, enzymes, and structural and chemical defense elements such as epithelium and associated antimicrobial proteins, which protects the individual against infection. Pathogens access a normally sterile area following breach of epithelial or mucosal barriers, and this leads to a local infection. Rapid and highly effective innate immune responses usually result in local containment and clearance of the pathogens with limited systemic consequences. However, pathogens may overwhelm these early innate immune responses or, more commonly, can trigger an excessive systemic innate immune response in some individuals. This can lead to activation of multiple neural, cardiovascular, bioenergetic, metabolic, hormonal, endothelial, coagulation, and other pathways that can induce deleterious effects remote from the site of infection [1]. This is recognized clinically as organ dysfunction and conceptualized as "sepsis" [2].

39.2 Epidemiology

39.2.1 Incidence

Multiple epidemiology studies report the proportions of patients with severe sepsis, that is, infected patients with new-onset organ dysfunction – now termed "sepsis" by the new definitions (see later) from middle–high income countries [3]. The incidence of intensive care patients with infection-related organ function is approximately $10 \pm 4\%$ [4]. This figure is likely to be higher in developing countries but data are scanty. The population-treated incidence of "old-definition"

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sepsis (i.e., presumed infection and ≥2 of 4 systemic inflammatory response (SIRS) criteria) was estimated at 437 per 100 000 person-years and 270 per 100 000 person-years for infection plus organ dysfunction [3]. Extrapolating from these incidence estimates, 19.4 million patients are treated globally per annum for severe sepsis [3]. The incidence of sepsis is apparently increasing, especially in countries where national databases exist or where reporting is linked to remuneration [5–12]. Because of inconsistent use of definitions and coding for severe sepsis and septic shock, it is uncertain how real this increase is. Kumar *et al.* reported a 239% increase in discharge coding for severe sepsis from a nationally representative US hospital database over a 7-year period [13]. Using the same database, Rhee *et al.* reported a rise in cases of sepsis from 126 to 342 per 100 000 hospital discharges between 2003 and 2011, yet bacteremia rates fell (from 10 to 8 cases per 100 000) [8]. How much of the increase is due to previous underreporting and how much to reimbursement "gaming" is unknown [14]. Nevertheless, a steadily aging population, an increasing prevalence of comorbidities, an increasing complexity of surgical interventions, and widening use of immunosuppressive agents are likely to be increasing the risk of developing sepsis.

39.2.2 Acute Mortality

Depending on case-mix characteristics, cohort recruitment year and healthcare provision, acute mortality from sepsis varies widely from 11 to 80% [5–12]. Despite the lack of new therapies, alongside the increasing incidence of sepsis, mortality rates do appear to be reducing over time [9,15]. How much is due to improvements in the general processes of care and how much due to an altered denominator and incorporation of less sick patients is uncertain [15], although there does appear an improvement in outcome independent of changes in the generic case-mix characteristics of critically ill populations.

39.2.3 Implications of Improvements in Acute Mortality

Improving early outcomes in the context of an increasing incidence could potentially result in a higher prevalence of sepsis survivors with significant morbidity. This issue needs better characterization as sepsis survivors have ongoing inflammation for as long as 1 year posthospital survival and an increased risk of longer-term mortality [16]. Nearly, one in six sepsis patients who survive hospitalization die within the first year of discharge. If sepsis "causes" this increase in postacute mortality is uncertain, due to incomplete accounting of confounders during analysis and bias in study design [17].

39.3 Sepsis: Biology

39.3.1 Host Immune Response

The danger signals in sepsis could be either from the pathogen (pathogen-associated molecular patterns, PAMPs) or from intracellular constituents released by tissue damage (damage-associated molecular patterns, DAMPs) [18].

Perpetuation of inflammation by the innate immune system following bacterial clearance may be secondary to DAMPs [19].

39.3.2 PAMPs

Pathogen-associated molecular patterns are structures conserved among microbial species. The most common pathogens implicated in sepsis are bacteria, fungi, and, less commonly, viruses [20]. The lipoteichoic acid motif in Gram-positive bacteria and lipopolysaccharides present in the Gram-negative bacterial cell wall are examples of bacterial PAMPs. Common viral PAMPs include double-stranded or glycine/uracil-rich single-stranded ribonucleic acid (RNA). Fungal PAMPs include mannans, zymosans, mannosides, and beta-glucans [21].

39.3.3 DAMPs

Damage-associated molecular patterns are cell-derived endogenous molecules that have the potential to initiate and/or perpetuate the immune responses seen in sepsis. DAMPs include extracellular matrix products (such as hyaluronic acid, fibronectins, etc.), plasma components (such as fibrinogen, complement proteins, etc.), cytoplasmic proteins (such as heat shock proteins, high mobility group box protein 1, HMGB1, and cytokines), nonprotein molecules (e.g., adenosine triphosphate, uric acid, and heparin sulfate), Mitochondria, and nucleic acids (e.g., cellular and mitochondrial DNA, and RNA) [18,22].

39.3.4 Pattern Recognition Receptors Detect Danger Signals

PAMPs and DAMPs are sensed by pattern recognition receptors (PRRs), germ line-encoded receptors that act as transducers to initiate host immune responses. Innate immune cells (macrophages, dendritic cells, neutrophils, and monocytes) and nonimmune cells (such as fibroblasts, endothelial cells, and epithelial cells) express PRRs. Five PRRs subclasses have been described thus far – Toll-like receptors (TLRs), nucleotide oligomerization domain-like receptors (NODs), retinoic acid-inducible gene (RIG-1)-like receptors, C-type lectin receptors, and absence-in-melanoma 2 (AIM-2)-like receptors. PRRs could either be on the cell surface or intraellular. The sensing of PAMPs or DAMPs by PRRs triggers transcription of genes involved in inflammation. TLRs and NLRs are the key PRRs involved in bacterial recognition and signal transduction to immune cells [23].

39.3.5 Transduction of Danger Signals

Danger signals and PRR signaling alter gene expression. Subsequent transcription and translation is achieved using the signalosome and the inflammasome complexes. Inflammasomes are heterogeneous protein complexes assembled within the cytosol after sensing danger signals. These form a scaffold for procaspase-1 proteins and induce autoproteolysis and cleavage to generate active caspase-1. Active caspase-1-mediated proteolysis activates cytokines such as IL-1β and IL-18. Inflammasomes are also involved in proinflammatory cell death pathways

(pyroptosis) [24]. Most inflammasomes are of the NOD-like receptor (NLR) family, with relatively few non-NLR inflammasomes. The NLRP3 inflammasome is the best characterized to date and is activated by both DAMPs and PAMPs. When primed to a danger signal, there is an increase in cytosolic levels of the activated NLRP3 inflammasome. Aside from cytokine generation, caspase-1-mediated cell death is also an inflammasome-mediated effect. Active caspase-1-associated formation of cell transmembrane pores results in dissipation of cellular ionic gradients and cleavage of the chromosomal DNA without fragmentation, resulting in cell death [25]. The transcription of a plethora of pro- and anti-inflammatory cytokines are also induced by activation of the transcription factor NF-κB [26]. This, among other transcription factors, mediates many of the systemic responses seen in sepsis [27–32].

39.3.6 Immune Cell Abnormalities in Sepsis

Under physiological conditions, leukocytes travel in the higher-velocity, central laminar blood flow plane within blood vessels. Inflammation reduces vascular tone and increases adhesion molecule expression in endothelial cells via molecules such as P-selectin and E-selectin. Selectins interact with glycoproteins on neutrophil and monocyte surfaces, resulting in reversible adhesion and rolling. The next step is leukocyte adhesion to the inflamed endothelium. This is achieved by interaction between leukocyte integrin (CD11a:CD18; CD11b: CD18; and CD11c:Cd18) and endothelial adhesion molecules (ICAMs). Following this stage, leukocyte transmigration to the site of infection/inflammation occurs via the basement membrane, directed by a chemokine (CXCL8 and CCL2) gradient [33].

39.3.6.1 Neutrophils

Sepsis is usually characterized by neutrophilia with appearance of immature neutrophil subsets (such as band forms, meta-myelocytes, and myelocytes) in the peripheral circulation, a finding associated with poor prognosis [34–36]. In sepsis, neutrophils produce significant amounts of IL-10 [37,38], an anti-inflammatory cytokine that impairs monocyte–macrophage function [39]. Impaired neutrophil chemotaxis secondary to desensitization [40–42] and decreased respiratory burst capacity occur in sepsis [36,43,44]. Activated neutrophils impair T-cell function [45], while more immature neutrophil subsets are potentially cytotoxic to activated T cells [46]. In contrast to other immune cells, neutrophil life span is prolonged due to inhibition of apoptosis by reduced caspase-3, caspase-9 activity, and impaired release of mitochondrial cytochrome c [47–49].

39.3.6.2 Monocytes and Macrophages

In sepsis, monocytes are deactivated, impairing their antigen-processing ability [50,51]. This is associated with downregulation of HLA-DR expression in circulating monocytes [52,53], and a predisposition to secondary infections. Decreased HLA-DR expression is a risk factor for adverse outcomes from sepsis [53–56]. Monocyte deactivation is also associated with reduced inflammasome activity in patients with septic shock [57]. Macrophages have impaired

pathogen killing ability and altered cytokine production (reduced proinflammatory and increased anti-inflammatory cytokines) [54].

39.3.6.3 Dendritic Cells

Sepsis induces a greater expression of programmed death ligands (PD-L1 and PD-L2) on dendritic cells (DCs), resulting in impaired function [58]. Sepsis is also associated with DC loss secondary to apoptosis. Both these effects carry prognostic significance [59–62]. FDCs are antigen-trapping cells that reside in secondary lymphoid organs that are important for affinity maturation of the B-cell response. In sepsis, FDCs are depleted secondary to caspase-3-mediated apoptosis [63,64].

39.3.6.4 Natural Killer Cells

Aside from direct cytotoxicity toward microbe-infected cells, NK cells also contribute toward the cytokine responses seen in sepsis and impact other immune cell function [65,66]. In sepsis, NK cell numbers are diminished, while their cytotoxic function and ability to enhance cytokine secretion are both impaired [67–69]. The increase in NK cell apoptosis explains the reduced NK cell count observed in sepsis [54]. These findings may contribute toward the immunoparesis seen in sepsis.

39.3.6.5 Lymphocytes

Sepsis is associated with lymphopenia, the presence of which is associated with hypothermia, shock, bacteraemia, raised cytokine concentrations (TNFα, IL-6, IL-8, IL-10, and IL-15) and a higher risk of mortality [70–77]. Lymphopenia often persists beyond the acute illness [70] and can affect both B- and T-cell compartments [78]. In patients with sepsis, B- [68,76,79–83] and T-cell counts [68,69,76,81–85] are acutely decreased. Lymphopenia in sepsis is considered secondary to accelerated lymphocyte apoptosis via both intrinsic and extrinsic pathways [76,78,80–83,86–95]; implicated factors include elevated extrinsic pathway ligands and intrinsic pathway cellular stressors such as TNF, interleukins, and other cytokines and chemokines [18,19].

In patients with sepsis, upregulated inhibitory cell surface proteins such as PD1 [58,89,96] and BTLA (B- and T-lymphocyte associated; CD272) [97,98] are variably observed in B- and T-cell subsets. T cells also have impaired cytokine secretory ability (IL-6, IL-10, and interferon-gamma, TNFγ) when stimulated *ex vivo* [58]. However, cytokine secretion is largely preserved in an acute septic illness aside from interferon-gamma secretion [99]. There is also a decreased mRNA abundance of transcription factors that regulate CD4+ T cell plasticity, such as TGFβ1, T-bet, RORγ, and GATA3 [68,100]. The reduced mRNA abundance is associated with impaired generation of Th17 subsets of CD4+ T cells [68], which protect against bacterial and fungal infections [54]. Regulatory T cells (T_{reg}) impair proliferation and generation of other effector T-cell populations, resulting in immunosuppression [101]. Compared to other T-cell subsets, T_{reg} numbers and function are relatively preserved in sepsis [102–105]. Impaired TCR diversity is seen in sepsis and this recovers with clinical improvement [106].

CD28, a key costimulatory molecule for T-cell activation and proliferation, is downregulated [107].

B cells are activated, as suggested by increased CD69 expression [79,108,109]. BCR signaling is upregulated in trauma, which shares many gene expression patterns with sepsis [110]. Expression of CD86 and CD80 were increased in B cells taken from sepsis patients; of note, an increased proportion of CD80-positive B cells were associated with nonsurvival [79]. Using a murine influenza viral infection model, CD80 and CD86, coreceptors for T-cell activation by APCs and which are also involved in regulation of IgG secretion in activated B-cell subsets, were upregulated by an interferon-gamma mechanism [79,111–113]. In this context, IFN-1 administration to animal models resulted in improved outcomes, and this was associated with increased activation of B cells [108]. This study and others [108,114] imply that yet to be characterized B-cell functions have the potential to alter sepsis-related outcomes. When interpreted together, T-cell abnormalities in sepsis match the concept of T-cell exhaustion [115], while B-cell abnormalities imply impaired function over and above that which can be explained purely by T-cell abnormalities.

39.3.7 Metabolic Alterations in Sepsis

In additional to a greater metabolomic variance compared to matched healthy controls, sepsis-associated death is causally linked to alterations in the metabolomic profile [116]. Levels of 76 metabolites differed between septic survivors and nonsurvivors with alterations noted in the Krebs' cycle and fatty acid oxidation secondary to carnitine transport defects. A more recent study that included both transcriptomic and metabolomic profiling of septic patients highlighted mitochondrial dysfunction as a key defect in sepsis [117]. Other studies also report alterations in fatty acid oxidation [118,119].

39.3.8 Mitochondrial Function in Sepsis

Sepsis-associated mitochondrial dysfunction is multifactorial. It occurs secondary to impaired cellular oxygen delivery [120], increased production of endogenous mitochondrial respiration inhibitors (such as nitric oxide and other reactive species, carbon monoxide, and hydrogen sulfide) [121–123], and alterations in mitochondrial biogenesis [123]. Examining whole leukocytes taken from endotoxin-challenged volunteers, a decrease in mRNA abundance was seen in genes coding for mitochondrial respiratory chain complexes I–IV, ATP synthase, the Krebs' cycle, and the mitochondrial permeability transitional pore (MPTP) [124]. In circulating monocytes taken from septic patients [125,126], bioenergetic capacity was lower despite normal or raised levels of basal oxygen consumption [127]. This may contribute toward impaired immune function as it correlates with HLA-DR expression [125]. Although the mitochondrial dysfunction paradigm in sepsis is well accepted, the altered bioenergetic capacity in septic leukocytes is not universally observed [128]. In solid organs such as kidney, liver, and heart taken from patients who died of protracted sepsis [129] and in animal models [122], impaired mitochondrial function was seen alongside

hydropic changes in mitochondria and mitophagy, yet cell death was minimal. This has led to the hypothesis that mechanisms of organ dysfunction share similarities with hibernation physiology where cellular turnover is low and that organ dysfunction may even represent a late-stage adaptive process to a prolonged and severe insult [130].

39.3.9 Mechanisms of Organ Dysfunction in Sepsis

Organ dysfunction in sepsis is multifactorial and the precise underlying mechanisms remain unknown. Broadly, there are organ-specific pathways and more general shared systemic abnormalities [131]. These include systemic inflammation, impaired oxygen delivery due to macrocirculatory and microcirculatory abnormalities, alterations in cell cycle characteristics, and reprioritization of mitochondrial function toward cellular protection in the context of prolonged and excessive inflammation [19,130,132–134].

39.4 Clinical Management

The principles of clinical management of sepsis are enshrined within the Surviving Sepsis Campaign (SSC) guidelines [135]. These principles focus on prompt identification and treatment of infection (source control and antibiotics), alongside cardiovascular support to restore and maintain tissue perfusion using intravenous fluids ± vasoactive drugs. Organ support may be needed to support ventilator, renal, and cardiovascular function as a temporizing matter until the patient recovers enough function to cope unaided. Other interventions, such as correction of coagulopathy, rehabilitation, adequate nutrition, and infection control to prevent secondary episodes, are also necessary. These guidelines are based upon what evidence is available, though in many instances the quality of this evidence is poor and/or scanty.

39.5 Definitions

In 2016, the sepsis illness concepts provided by two previous International Consensus Conferences (1991 and 2001) [136,137] were revisited and new updated definitions and clinical criteria – "Sepsis-3" – published [138].

39.5.1 Limitations of Previous Definitions

Sepsis was previously defined as the presence of confirmed or suspected infection plus two or more systemic inflammatory response syndrome (SIRS) criteria [136,137]. These SIRS criteria (abnormalities in temperature, white count, respiratory rate, and/or heart rate) are, however, nonspecific [139] and very frequently seen in hospitalized patients, including many without infection [140]. Furthermore, 1-in-8 patients with infection-related organ failure admitted to

Australasian intensive care units did not have ≥2 SIRS criteria on ICU admission [139]. Finally, an evolving understanding of the underlying pathophysiology indicates that both pro- and anti-inflammatory activation occur early in the disease process, with both being of prognostic relevance [54,110]. The use of the SIRS criteria was thus dropped from the recent "Sepsis-3" definitions [138], though the individual components may still be clinically useful in the presumptive diagnosis of infection.

39.5.2 Sepsis-3 Definitions

Sepsis is now defined as *life-threatening organ dysfunction caused by a dysregulated host response to infection* [138]. Sepsis should be identified clinically using a change in Sepsis-related Organ Failure Assessment score (SOFA score) ≥2 temporally related to the infection. This system [141,142] consists of laboratory tests (platelet count for coagulation system, bilirubin for liver, and creatinine for kidneys), clinical assessment (Glasgow coma scale for neurology), and clinical interventions (vasoactive drugs for cardiovascular system and ventilation for respiratory system). A change in SOFA ≥2 from baseline accounts for patients with preexisting chronic organ dysfunction or those hospital inpatients who have developed organ dysfunction prior to the onset of the infection episode. In the absence of recent tests and any chronic ill health, a baseline score of 0 can be reasonably assumed. Patients with a SOFA score ≥2 around the time sepsis was diagnosed had a subsequent hospital mortality exceeding 10% [143].

Septic shock is now considered to be a subset of sepsis for patients who have a more severe illness with a higher likelihood of death than "sepsis" alone. Septic shock is now defined *as a subset of sepsis where underlying cardiovascular, cellular, and metabolic abnormalities are profound enough to substantially increase mortality* [144]. Septic shock is identified at the bedside using the following criteria: hypotension requiring vasopressors to maintain mean arterial pressure ≥65 mmHg (cardiovascular abnormality) plus a blood lactate >2 mmol/l (reflective of cellular/metabolic stress/abnormality) despite adequate fluid resuscitation. Using patients enrolled into the Surviving Sepsis Campaign database, hospital mortality was 42.3% for both hypotension and hyperlactataemia versus 30.1% (for hypotension alone), 25.7% for persisting hyperlactataemia despite fluid resuscitation, and 25.5% for patients with organ dysfunction but a normal lactate and not requiring vasopressors [144].

While SIRS represents components of the host systemic inflammatory response to an insult, it may be a completely appropriate host response and not necessarily a harbinger of clinical deterioration. On interrogating large US hospital population databases totaling over 5 million patients, approximately 850 000 patients were cultured and treated with antibiotics. Of 21 variables examined, three (respiratory rate ≥22 breaths/min, altered mentation, and a systolic blood pressure ≤100 mmHg) offered the greatest predictive validity of a poor outcome such as death or needing critical care. Having 0, 1, 2, or all 3 qSOFA criteria corresponded to an ultimate hospital mortality of <1, 2–3, 8, and >20%, respectively. Though needing to be prospectively validated, qSOFA offers a rapid, bedside tool for identifying patients at the risk of doing badly. Clearly, treatment

should not be delayed in the interim for patients with none or only one of the qSOFA criteria [143].

39.6 Conclusions

Sepsis is now considered to be a syndrome of life-threatening organ dysfunction triggered by an excessive, dysregulated host response to infection. The underlying pathophysiology is notable for its complexity, involving multiple systems and pathways and not just restricted to the immune and inflammatory systems. Mortality and morbidity remain high, thus maintaining the requirement for novel and effective therapeutics.

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40

Autoimmunity and Inflammation

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40.1 Introduction

Inflammation was once defined based on clinical signs such as rubor (redness), calor (heat), tumor (swelling), dolor (pain), and function laesa (dysfunction) [1]. Later inflammation was defined by the presence of immune cells in places where they usually do not occur. Nowadays, we define inflammation as induction or persistent expression of proinflammatory mediators causing these phenomena. It is obvious that inflammation can occur upon infectious and noninfectious triggers, the latter referred to as sterile inflammation. Triggers of sterile inflammation include trauma, toxic injury, malignancy, autoinflammation, alloimmunity, and autoimmunity [2]. Table 40.1 summarizes key differences between these three categories. Among these, autoimmunity is characterized by systemic or organ-specific inflammation of spontaneous onset, although autoimmunity can often be triggered by an episode of infection. Autoimmune disease becomes obvious mostly by organ injury and dysfunction, which focuses the attention of patients and doctors on this organ, which defines the patient's burden, complaints, and signs of the disease. However, the immunologist's perspective on autoimmunity is entirely different. Immunologists see autoimmunity as a problem of immune regulation in lymphoid organs such as thymus, spleen, and lymph nodes. The autoimmune attack of peripheral organs, organ inflammation, and organ dysfunction is already at the border of the immunologist's interest. Being aware of this entirely different perspective of researchers, clinicians, and patients on autoimmunity, we recommend dissecting the process into the pathogenesis of autoimmunity (mostly outside affected organs) and the pathogenesis of autoimmune tissue injury (mostly inflammation inside the affected organ). Hence, in this chapter, we approach the topic by first discussing the mechanisms of immune

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Table 40.1 Differences between autoinflammation, alloimmunity, and autoimmunity.

	Autoinflammation	Alloimmunity	Autoimmunity
Antigen	—	Foreign proteins of the same species	Proteins of the self
Genetic cause	Gene variants promoting spontaneous inflammation, often monogenic	—	Gene variants promoting loss of self-tolerance, often polygenic
Who is affected?	Children and young adults	Patients undergoing cell or organ transplantation	Adolescents and adults, women often more frequently affected
Central pathomechanism	Mutated pathway	MHC-II-driven alloantigen presentation via DC and B cells	MHC-II-driven autoantigen presentation via DC and B cells
Current treatment strategy	Cytokine antagonists targeting mutated pathway	Unselective immunosuppressants	Unselective immunosuppressants

tolerance, genetic and environmental factors that break immune tolerance, and the mechanisms of autoimmune tissue inflammation. Finally, we discuss systemic lupus erythematosus (SLE) as a paradigmatic example of systemic autoimmunity and tissue injury.

40.2 Immune Tolerance

The immune system's inherent property is to recognize, respond, and eliminate nonself-antigens, while not reacting harmfully to self-antigens, referred to as immune tolerance. The immune system must somehow avoid accidentally recognizing and destroying host tissues. Maintaining self-tolerance involves both the elimination of immune cells that can react with self-antigens and active inhibition of immune responses against self-antigens. In the first step of this process, a phenomenon termed "central tolerance" deletes T- or B-cell clones before the cells are allowed to mature if they possess receptors that recognize self-antigens with high affinity. Central tolerance occurs in the primary lymphoid organs: the bone marrow for B cells and the thymus for T cells. Because central tolerance is not perfect and some self-reactive lymphocytes enter into the periphery and secondary lymphoid tissues, additional safeguards limit their activity. These backup mechanisms called "peripheral tolerance," renders some self-reactive lymphocytes in secondary lymphoid tissues inactive and generates others that actively inhibit immune responses against self. The possibility of damage from self-reactive lymphocytes is further limited by the life span of activated lymphocytes, which is regulated by programs that induce cell death (apoptosis) following receipt of specific signals.

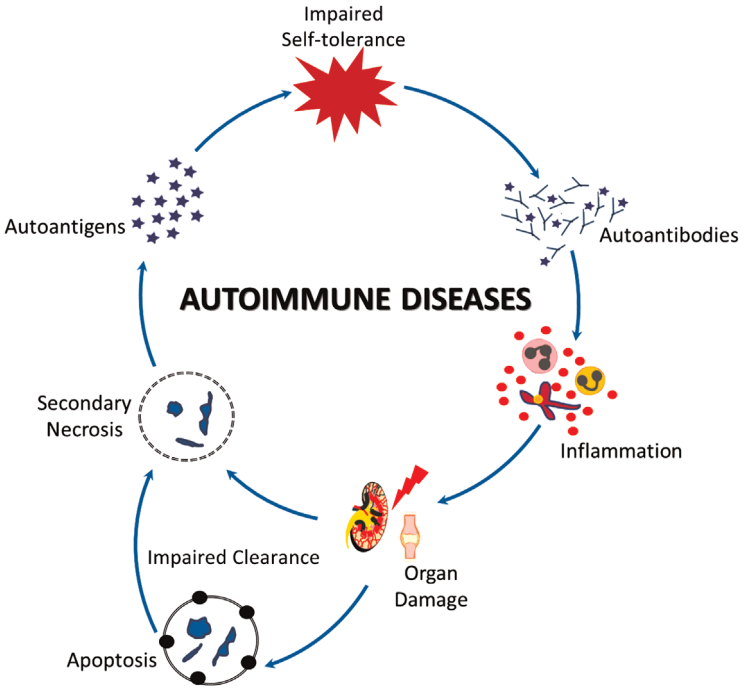


Figure 40.1 Progression of autoimmune diseases. Under healthy conditions, apoptotic cells are efficiently cleared by the immune system without an immune response. However, in situations of clearance deficiency, these cells progress to secondary necrosis causing accumulation of autoantigens. Not-ingested autoantigens are presented by the APCs to B cells, and thus break the tolerance to self. This subsequently leads to production of autoantibodies. Autoantibodies form complexes with autoantigens, called immune complexes that set up inflammation and tissue damage. This may lead to organ damage and, therefore, production of more autoantigens, setting up the loop leading to progression of autoimmune disease.

Apoptosis has an important role in establishing and maintaining immune tolerance (Figure 40.1). This is evidenced by the development of systemic autoimmune diseases in mice with naturally occurring mutations of death receptors like Fas or Fas ligand (FasL). Activated T cells express increased levels of Fas and FasL. In both B and T cells, engagement of Fas with FasL induces activation-induced cell death (AICD). Mice that carry inactivating mutations in Fas (lpr/lpr) or FasL (gld/gld) are able to develop autoimmune disease early in life [3]. Such a lack of self-tolerance can lead to autoimmune disease, whereas a host, displaying excessive tolerance may be more susceptible to pathogens. Ray Owen's experiments in cattle showed that a shared blood supply during development led to lifelong immune tolerance [4]. There are examples in which allografts, mismatched at a single HLA locus, are accepted with little or no immune suppression. In cases where the mismatched antigen is expressed by the mother, but not inherited by the offspring, there is the possibility that perinatal exposure induced subsequent tolerance to this antigen. Because human maternal cells do not normally cross the placental barrier, such specific tolerance to noninherited maternal antigens would be an exception rather than a common

place event. This self-tolerance is maintained by several mechanisms that prevent the maturation and activation of potentially self-reactive lymphocytes. The process of immune tolerance is often divided between central and peripheral mechanisms. Tolerance to self, acquired within the thymus, is called central tolerance, whereas tolerance acquired outside the thymus is called peripheral tolerance.

Central tolerance. Central tolerance refers to tolerance that is induced during development of T cells in the thymus and B cells in the bone marrow. The central tolerance mechanisms, clonal deletion, and receptor editing eliminate many autoreactive T cells in the thymus and autoreactive B cells in the bone marrow. Developing T cells with a high affinity for self-antigens are eliminated by clonal deletion. Strongly self-reactive immature B cells may be induced to make further immunoglobulin (Ig) gene rearrangements and thus evade self-reactivity. This phenomenon is called receptor editing. T cells also undergo T-cell receptor (TCR) editing in the thymus [5]. If editing fails, the self-reactive B cells are also eliminated by clonal deletion. Gene rearrangement to form the TCR is a random process that allows for the generation of T cells with an incredible repertoire of specificities. Invariably, some of these receptors will react against self-antigens. It is believed that lymphocytes specific for MHC plus self-peptides will encounter most of these self-antigens during development in the thymus. In contrast to mature T cells that respond to encounter with their specific antigen with a program of proliferation and differentiation, immature lymphocytes respond by undergoing clonal deletion. Although there are mechanisms to ensure a full repertoire of self is expressed in the thymus, it is invariable that some self-reactive cells will develop and migrate from the thymus to the peripheral lymphoid organs. Control of such "escaped" autoreactive cells is achieved through peripheral tolerance mechanisms.

Consider the mechanisms that generate diversity in T-cell or B-cell receptors. The genetic rearrangements that give rise to a functional T-cell receptor or Ig occur through a process called V(D)J recombination, commonly known as somatic recombination. During this process, generation of variable regions that react with self-antigens is almost inevitable. If this were allowed to occur frequently, such TCR or Ig receptors could produce mature functional T or B cells that recognize self-antigens and autoimmune disease would ensue. Clonal deletion eliminates developing lymphocytes whose antigen receptors bind strongly to self-antigens present in the generative lymphoid organs. In a classic experiment, Goodnow mated transgenic mice expressing hen egg white lysozyme (HEL) with mice expressing a transgenic immunoglobulin specific for HEL to demonstrate that self-reactive lymphocytes are removed or inactivated after encounter with self-antigen [6]. Clonal deletion of immature B and T lymphocytes is an important mechanism for maintaining tolerance to many self-antigens; this is also called central tolerance because it develops in the central (generative) lymphoid organs. Although both B and T cells participate in tolerance, T-cell tolerance plays the primary role.

T-cell Tolerance. The main process by which T lymphocytes acquire the ability to distinguish self from nonself occurs in the fetal thymus. This process, called clonal deletion, involves the killing of T cells that react against antigens present in

the fetus at that time. The self-reactive cells die by a process of programmed cell death called apoptosis. For negative selection to be efficient, the thymic epithelial cells must display a vast repertoire of "self-" proteins. A transcriptional factor called the autoimmune regulator (AIRE) enhances the synthesis of this array of self-proteins [7]. Mutations in the gene encoding the AIRE protein, which regulates protein expression in the thymus, result in the development of an autoimmune disease called autoimmune polyendocrinopathy. The AIRE also functions in the peripheral lymphoid organs such as the spleen and lymph nodes, where it contributes to peripheral tolerance.

Clonal deletion occurs at the double-positive (DP) to single-positive (SP) transition. The loss of over 95% of thymocytes reflects the stringent selection processes that shape the developing T-cell repertoire. Clonal deletion is a major mechanism of tolerance, in that T cells must be exposed to all self-antigens during thymocyte maturation. However, there are also self-antigens whose expression is restricted to a specific tissue or developmental time point. One possibility is that all proteins could be transported to the thymus by APCs. Recently, it was found that AIRE expression drives medullary thymic epithelial cells (mTECs) to express tissue-specific self-peptides in the thymus. Self-reactive developing T cells are thus exposed to tissue-specific self-peptides in the thymus and are deleted. AIRE-deficient humans and mice develop autoimmune polyendocrinopathy candidiais ectodermal dystrophy (APS-1 or APECED in humans), emphasizing the role of central tolerance in preventing T-cell-mediated autoimmune disease [8]. Negative selection ensures T cells displaying very high or very low receptor affinities for self-MHC undergo apoptosis and die in the cortex. Positive selection ensures T cells with TCRs that have intermediate affinities are rescued from apoptosis, survive, and continue along their pathway of maturation. T cells at this stage of maturation ($CD4 + CD8 + TCR^{low}$) go on to express TCR at high density and lose either CD4 or CD8 to become "single-positive" mature T cells. Less than 5% of thymocytes leave the thymus as mature T cells. Given the survival of some self-reacting T cells, a separate mechanism is required to prevent them from attacking the body.

B-cell Tolerance. B-cell precursors differentiate into immunocompetent B cells in the bone marrow; they do not pass through the thymus. Autoreactive B cells are eliminated by receptor editing and clonal deletion, which are recognized as mechanisms that lead to central tolerance. These processes reduce the occurrence of autoimmune diseases. On encountering antigens, the B cells form germinal centers and undergo affinity maturation to produce antibodies with higher antigen affinity and helps prevent antibody cross-reactivity with self-antigens. Immature B cells bearing an antigen receptor for a self-antigen can escape clonal deletion by changing their receptor in a process called receptor editing. David Nemazee and coworkers showed that some developing B cells could undergo receptor editing [9]. In this process, self-antigen recognition by immature B cells induces reactivation of the cell's V(D)J recombinase and the rearrangement and production of a new Ig light chain (edited), which changes the specificity of the receptor so that it no longer recognizes a self-antigen [9]. It is estimated that as many as 50% of self-reactive B cells undergo receptor editing.

However, tolerance in B cells is less complete than in T cells, an observation supported by the finding that antibodies mediate most autoimmune diseases. If receptor editing fails, the immature B cells that express high-affinity receptors for self-antigens and encounter these antigens in the bone marrow or the spleen may die by apoptosis. This process is also called clonal deletion. The antigens mediating clonal deletion – usually abundant or polyvalent self-antigens such as nucleic acids, membrane-bound lipids, and membrane proteins – deliver strong signals to IgM-expressing immature B lymphocytes that happen to express receptors specific for these self-antigens. Both receptor editing and deletion are responsible for maintaining B-cell tolerance to self-antigens that are present in the bone marrow. Negative selection of thymocytes removes autoreactive T cells; however, some autoreactive T cells do escape the thymus, because they have too low an affinity for self-antigens to induce clonal deletion. The body has evolved several other mechanisms to avoid autoimmunity, and one of such mechanisms is peripheral tolerance.

Peripheral Tolerance. Clonal deletion of either T cells or B cells during development is not perfect. Given the potential for autoimmune self-destruction, if the clonal deletion fails, there must be backup mechanisms to control autoreactivity for those few cells that manage to escape the thymus. Peripheral tolerance is necessary because some antigens are not expressed in the thymus and some tissue-restricted antigens are expressed only in adult life; therefore, some self-reactive T cells are not killed in the thymus. There are several mechanisms involved in peripheral tolerance; these mechanisms include immune privilege, anergy, and regulation. Clonal anergy is the term used to describe self-reactive T cells that are not activated because proper costimulation does not occur. The mechanism of clonal anergy involves the inappropriate presentation of antigen, leading to a failure of interleukin-2 (IL-2) production. Inappropriate presentation is due to a failure of "costimulatory signals" (e.g., CD28 on the T cell and B7 on the APC). For example, the inhibitory protein CTLA-4 on the surface of the T cells has a higher affinity than CD28 for B7 proteins, displaces CD28 and interacts with B7, resulting in a failure of T-cell activation. Thus, CTLA-4 is involved in the maintenance of unresponsiveness (tolerance) to self-antigens. Furthermore, B7 is an inducible protein, and failure to induce it in sufficient amounts can lead to anergy. In addition, the costimulatory proteins, CD40 on the APC and CD40L on the helper T cell, may fail to interact properly. The failure of costimulatory signals most often occurs when there is an insufficient inflammatory response at the site of infection.

The mechanisms mediating peripheral tolerance vary. Under normal conditions, an encounter of mature lymphocytes with antigen leads to induction of an immune response. However, presenting the antigen in some alternative form, time, or location may instead lead to tolerance. Antigens that induce tolerance are called tolerogens rather than immunogens. Here, context is important; the same chemical compound can be both an immunogen and a tolerogen, depending on how and where it is presented to the immune system. For instance, an antigen presented to T cells without appropriate costimulation results in a form of tolerance known as anergy, whereas the same antigen presented with costimulatory molecules can become a potent immunogen. One of the mechanisms of

B-cell tolerance is clonal exhaustion, in which the immunogen activates all of the B cells specific for it, leading to maturation of cells and transient antibody synthesis and thereby exhausting and diluting the B-cell response. It was found that T-cell tolerance is induced more rapidly and is longer lasting than B-cell tolerance. For example, T-cell tolerance may be induced in a single day, whereas B cells may require 10 days for induction. Maintenance of tolerance requires the continued presence of specific antigens. In immunocompetent adult animals, repeated administration of large doses of protein antigen or a massive single-dose administration of a polysaccharide antigen can induce a specific immunologic unresponsiveness termed high-dose tolerance.

T-cell Anergy. Cellular proliferation and/or potentiation of T-cell effector function are not inevitable consequences of TCR engagement. In fact, under some conditions, TCR ligation results in a state of T-cell unresponsiveness, known as T-cell anergy; this state appears to result either when the TCR is engaged without concomitant costimulation or when the ligand for the TCR is not of sufficient affinity to initiate the full spectrum of biochemical second messengers. This phenomenon was first noted by stimulating T cells in the presence of metabolically inactivated APCs. Subsequently, it was shown that CD28 costimulation could prevent the induction of anergy. Anergic cells are unable to proliferate or secrete cytokines upon TCR-mediated restimulation. Interestingly, supplementation of cultures with IL-2 can overcome the anergic state *in vitro*. Several unique experimental systems have recently provided insights into how TCR signaling events can lead to either anergy or full T-cell activation. Mice made transgenic for specific TCR complexes have proved to be useful models for studying differential responses to TCR engagement *in vivo* and *in vitro*. TCR transgenic mice produce T-cell repertoires that are largely restricted to a single specificity. T cells from such mice respond fully when presented with a specified peptide antigen, but exhibit an anergic response when that peptide is subtly altered. High doses of antigen are usually more tolerogenic, although repeated low doses can also induce tolerance in T cells. As a rule, T-cell tolerance is easier to induce and lasts longer than B-cell tolerance. Immune regulation includes regulatory T cells, which can actively suppress the immune system. By regulating the activation and proliferation of antigen-specific effectors, Tregs promote tolerance to self and suppress autoimmunity *in vivo*.

Regulatory T Cells (Tregs). Tregs play an important role in maintaining immune homeostasis. Tregs suppress the function of other T cells to limit the immune responses and allowing the establishment of immunological tolerance. Tregs are produced in the thymus as a functionally mature subpopulation of T cells and can be induced from naive T cells in the periphery. Alteration in the number and function of Tregs has been implicated in several autoimmune diseases including multiple sclerosis, active rheumatoid arthritis, and type 1 diabetes [10]. Tregs are vital for keeping the immune system in check, helping to avoid immune-mediated pathology. Treg cells can inhibit the proliferation of other T-cell populations *in vitro*, effectively suppressing autoreactive immune responses. They express on their surface CD4, CD25 (the chain of the IL-2 receptor), and a transcription factor, FoxP3, the expression of which is necessary and sufficient to induce differentiation to the Treg lineage. Foxp3 is a master regulator and expression of

the Foxp3 has been shown to correlate with regulatory activity in both humans and mice. The absence of Foxp3 either through spontaneous mutation or through targeted disruption of the gene leads to the absence of T cells with regulatory activity. Conversely, overexpression of Foxp3 leads to an excess of T cells with regulatory activity. Foxp3 genetic deficiency in humans and rodents leads to severe multiorgan autoimmunity [11].

Tregs manifest their functions through a myriad of mechanisms that include the secretion of immunosuppressive cytokines such as IL-9, IL-35, IL-10, and TGF-β, suppressing the activity of other nearby T cells and APCs. Cell contact-dependent suppression includes the high-affinity TCR and other costimulatory molecules such as CTLA-4 and GITR. Cytolytic activity-dependent mechanism kills T cells and APC through granzyme or perforin. Possible negative signals of Tregs include upregulation of intracellular cyclic AMP, which leads to inhibition of T-cell proliferation and IL-2 production. Activated Tregs may also down-regulate CD80/86 expression on APC. Depletion or inhibition of Treg cells before immunization may enhance immune responses to conventional vaccines and facilitate the development of antitumor immunity, leading to clearance tumours. Conversely, increasing the suppressive activity of Treg-cell populations could be useful in the treatment of allergic or autoimmune diseases and graft rejection. Tregs are broadly classified into natural or induced (adaptive) Tregs; natural Tregs (nTregs) are ($CD4^+CD25^+Foxp3^+$ T cells) generated in the thymus early in life to perform their key role in immune homeostasis (Table 40.2). Induced

Table 40.2 Regulatory T-cell subsets.

Property	Natural Treg (nTregs) nTregs	Induced Tregs (iTregs) Tr1	Th3
Development	Thymus	Periphery (MALT)	Periphery (MALT)
Phenotype	$CD4+CD25^+$ and $CD127^{low}$	$CD4^+CD25^-$	$CD4^+CD25^+$ from $CD25^-$ precursors
Other associated markers	$CTLA4^+$, $GITR^+$, and $FoxP3^+$	$CD25^{low\ variable}$, $CD45RB^{low}$, and $FoxP3^-$	$CD25^{low\ variable}$, $CD45RB^{low}$, and $FoxP3^+$
Suppression	Contact-dependent, granzyme B dependent, makes TGF-β	IL-10 mediated	TGF-β mediated
Target cells	APC and T effector cells	T effector cells	Not yet identified
CD28 Involvement	Thymic development and maintenance in periphery	Unnecessary for development or function	Unnecessary for development or function
In vivo role	Suppression of autoreactive T cells	Mucosal immunity and inflammatory response	Mucosal immunity and inflammatory response
In vitro expansion	Expandable using TCR/CD28 stimulation and IL-2	CD3, IL-10, and rehnoic acid	CD3 and TGF-β

$CD4^+Foxp3^+$ regulatory T (iTreg) cells develop outside the thymus in various tissues of the body including the mucosa-associated lymphoid tissue (MALT) throughout life and are typically induced by inflammation and disease processes, such as autoimmunity and cancer.

Natural Tregs. nTregs originate from the thymus as $CD4^+$ cells expressing high levels of CD25 together with the transcription factor FoxP3 [12]. The nTregs represent approximately 5–10% of the total $CD4^+$ T cell population. They are positively selected thymocytes with a relatively high avidity for self-antigens. The signal to develop into nTreg cells is thought to come from interactions between the T cell receptor and the complex of MHCII with self-peptide expressed on the thymic stroma. It seems that thymocytes that commit to the nTreg cell lineage are likely to receive unique stimulatory signals. nTregs are essentially cytokine independent and represent an alternative fate for autoreactive T cells. Most thymocytes that express receptors with high affinity for self-antigen die via clonal deletion. However, a small fraction appears to commit to the regulatory T-cell lineage and leave the thymus to patrol the body and prevent autoimmune reactions. These nTregs share the periphery with induced Tregs (iTrags) that can develop from mature T cells that are exposed to TGF-β and IL-10 cytokines. Suppression by these regulatory cells is antigen specific because it depends on activation through the T-cell receptor. nTregs regulate immune responses in a variety of means: directly inhibiting an antigen-presenting cell's ability to activate T cells, directly killing T cells, indirectly inhibiting T-cell activity by secreting inhibitory cytokines IL-10 and TGF-β, and depleting inflammatory cytokines such as IL-2. The existence of Treg cells that specifically suppress immune responses has clinical implications.

Induced Tregs. iTregs originate from the thymus as single-positive CD4 cells. They differentiate into CD25 and FoxP3 expressing Tregs (iTregs) following adequate antigenic stimulation in the presence of cognate antigen and specialized immunoregulatory cytokines such as TGF-β, IL-10, and IL-4. Under the influence of TGF-β, iTreg cells mature in peripheral sites, including MALT, from CD4+ Treg precursors, where they acquire the expression of markers typical of Tregs, including CD25, CTLA4, and GITR/AITR. Upon upregulation of the Foxp3, iTregs begin their suppressive effect. This includes the secretion of cytokines including IL-10 and TGF-β that may induce cell cycle arrest or apoptosis in effector T cells, and blocking costimulation and maturation of dendritic cells (DCs). iTreg cell populations include T regulatory 1 (Tr1) cells, which secrete IL-10, and T-helper 3 (Th3) cells, which secrete TGF-β. Both of them are involved in the suppression of autoimmunity. Tr1 cells do not express Foxp3, yet their properties *in vitro* are very similar to those of Foxp3+ Tregs [13].

Tolerogenic Signals of APC-presenting Autoantigen. DCs play a significant role in establishing self-tolerance through their ability to present self-antigens to developing T cells in the thymus. DCs are predominantly localized in the medullary region of the thymus. DCs in the circulation, skin, heart, and possibly other peripheral tissues, migrate to the thymus and present the peripheral tissue antigens. When antigen-presenting DCs make a high-affinity interaction with antigen-specific thymocytes, this interaction drives the interacting thymocytes to death, a process often referred to as clonal deletion, which fundamentally blocks

the self-reactive thymocytes from differentiating into mature T cells. Thymic DC presentation of self-antigens significantly contributes to the clonal deletion of $CD4^+$ thymocytes. In the absence of inflammation, classical DCs capture tissue antigens and migrate to the draining lymph nodes but do not produce cytokines and membrane molecules that are required to induce effective immune responses. The function of these DCs may be to present self-antigens to self-reactive T cells and thereby cause inactivation or death of the T cells or generate regulatory T cells. These mechanisms are important for maintaining self-tolerance and preventing autoimmunity.

Tolerogenic DCs are immature, maturation-resistant, or alternatively activated DCs that express low surface MHC molecules, and low costimulatory signals (e.g., CD80 and CD86), and an impaired ability to synthesize T-helper 1 cell-driving cytokines, such as IL-12p70, and high IL-10 production. Rapamycin is a potent immunosuppressive drug, impairs the maturation of DCs in response to "danger signals," such as high-mobility group box 1protein (HMGB1), Toll-like receptor (TLR) ligands, or CD40 ligation. Immunosuppressive drugs such as corticosteroids, cyclosporine, and so on have been used to target DC differentiation and function. All these molecules prevent DC maturation and/or activation or impair the capacity of DCs to produce IL-12p70. In addition, some of these agents prevent the nuclear translocation of nuclear factor-κB (NF-κB) family members, which are required for the differentiation of DCs [14].

Immune Privilege Sites. Immune-privilege sites are regions in the body that prevent the expression of inflammation within an immune-mediated environment. That is, when foreign antigens are introduced into the immune privilege site, the zone does not induce an autoimmune response, something that would usually occur in other areas of the body. Immune-privileged sites fail to induce an immune response because they are effectively sequestered from the cells of the immune system. The concept of "immune privilege" was first described by Medawar more than 60 years ago. Medawar believed that tolerance in these areas was due to limited lymphatic drainage [15]. In this model, lymphocytes cannot easily gain entrance to the privileged site and foreign antigens deposited there are not transported to secondary lymphoid organs, which prevents initiation of an immune response. The sites of immune privilege include the anterior chamber of the eye, cornea, fetus in pregnant females, uterus, testes, and brain. Each of these sites is characterized by an absence of lymphatic vessels and in some cases by an absence of blood vessels. Immune-privilege sites are thought to have evolved in order to protect sensitive areas of the body that are unable to regenerate their tissues and have vital functions. Both the eye and the brain have a limited capacity for regeneration, and immune-mediated inflammation in either organ could have devastating consequences.

Multiple mechanisms have been proposed to account for immune privilege. One mechanism that likely contributes to tolerance is a low level or complete absence of expression of classical MHC class Ia protein in cells of the eye and brain, especially cells with little or no capacity for regeneration [16]. The reduced expression of MHC class Ia molecules is required to protect some of these cells from cytotoxic T lymphocytes (CTL)-mediated cell lysis. In the case of the developing fetus, the cells of the villous trophoblast lack expression of class I

MHC. Cells in these areas also express cell surface and soluble factors that downregulate the immune response. For example, ocular cells express proapoptotic cell surface molecules such as Fas ligand (FasL) and TRAIL [17]. These TNF family members may contribute to apoptosis of infiltrating T and other inflammatory cells bearing "death receptors." Cytokines produced at sites of immune privilege are also likely to contribute to the inhibition of inflammation. The presence of high levels of the anti-inflammatory cytokines TNF-β and migration inhibitory factor (MIF) leads to inhibition of NK cell-mediated cytolytic activity. Consequently, the alloantigens of the graft are not able to sensitize the recipient's lymphocytes, and the graft has an increased likelihood of acceptance even when HLA antigens are not matched. The privileged location of the cornea has allowed corneal transplants to be highly successful. A potential solution for graft rejection would be triggering immune privilege mechanisms within a specific area, allowing the body to accept the transplanted tissue.

In addition to the various mechanisms of central and peripheral tolerance, an effective means to avoid self-reactivity is sequestration or compartmentalization of self-antigens. However, one possible consequence of sequestration is that the antigen is never encountered by developing lymphocytes, and thus active tolerance to the sequestered antigen is not established. If barriers between the immune cells and the sequestered antigens are breached, the newly exposed antigen may be seen as foreign because it was not previously encountered. Trauma to one eye that allows entry of immune cells can result in inflammation in that eye, leading to tissue destruction and impaired vision. Other immune privilege mechanisms involve the production of immunosuppressive and anti-inflammatory cytokines. This mechanism, observed in ocular macrophages and DCs, is thought to regulate immune response. The remarkably successful field of corneal transplantation in clinical practice is undoubtedly associated with corneal immune privilege. Antigens introduced into the anterior chamber elicit a unique form of immune regulation (anterior chamber-associated immune deviation) that inhibits T-cell-mediated inflammation and graft rejection [18]. Furthermore, the use of various transgenic mouse models has facilitated the identification of critical pathways, which upon disruption can modify the immune privileged status of the eye. Understanding these pathways has clinical implications for the transplantation field and for the treatment of autoimmunity.

40.3 Genetic Factors that Break Immune Tolerance

The broad antigen receptor diversity in lymphocytes is a result of two processes of somatic genome modification. At first, in the central compartments like bone marrow for B cells and thymus for T cells, lymphocytes undergo V(D)J recombination rearrangement of the B-cell receptor (BCR) and TCR genes during differentiation. Second, somatic hypermutation substitutes single nucleotides of BCR genes during the late phase of an immune response in peripheral lymphoid tissues. Because of these two processes, lymphocyte receptor diversity allows to react to a large variety of antigens [19–21]. Although, a significant fraction of these receptors can react to self-antigens, autoimmune diseases are

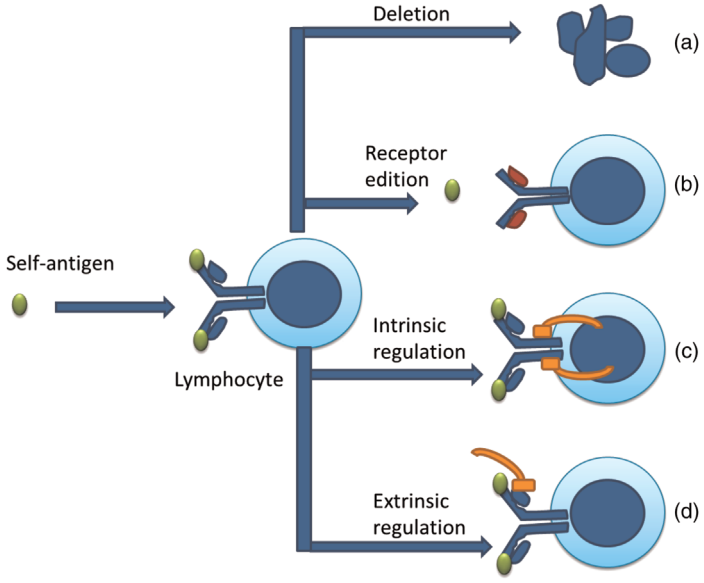


Figure 40.2 Major checkpoint mechanisms that regulate the development of B and T lymphocytes: (a) clonal deletion (apoptotic death of self-reacting lymphocytes, (b) receptor editing (V(D)J rearrangement of the self-reactive T-cell receptor or B-cell receptor, (c) intrinsic regulation of self-reactive receptor activation, and (d) extrinsic regulation of self-reactive lymphocyte activation.

relatively rare [22]. A series of checkpoints limit the reactivity of autoreactive lymphocytes to self-antigen (Figure 40.2): (1) deletion of self-reactive lymphocytes by inhibition of the Bcl-2-dependent survival pathway and/or activation of death receptor pathways. (2) Editing of self-reactive receptor by V(D)J recombination and somatic hypermutation [21]. (3) Intrinsic inhibition of BCR/TCR activation either by downregulation of BCR/TCR or by induction of inhibitory receptors (CD5 and CTLA4), or induction of phosphatase or ubiquitin ligases [23,24]. (4) Extrinsic control of lymphocyte activation either by downregulation of survival factors (BAFF and IL-7) or by inhibition of costimulation (anergy), or by limiting innate immunity.

Self-antigen ligating the BCR on immature B cells sets off a series of cellular events. Whenever the interaction with self-antigen exceeds a certain threshold, the immature B cells internalize the BCR and halt the maturation process [25]. This process downmodulates the expression of CD62 ligand (CD62L) [25], suppresses the receptors for BAFF (B-cell-activating factor) [26], and continues the expression of Rag1 and Rag2, which are required for V(D)J recombination for receptor editing [27,28]. B cells with unsuccessful editing of self-reactive BCR undergo cell death in the bone marrow or soon after arriving in the spleen [25]. B-cell deletion is a consequence of withdrawal of survival factors like BAFF or induction of proapoptotic factors like BIM. Vice versa, *Bim*-deficient mice are prone to spontaneous autoimmunity and produce anti-DNA autoantibodies [29].

In contrast to the BCR that can recognize antigen directly, the TCR is activated only upon antigen presentation by APCs. The self-peptide/MHC complex on the

surface of cortical thymic epithelial cells is presented to the TCR on naïve T cells. In case of TCR ligation by self-peptide, the respective T cell will start to proliferate, a process referred to as positive selection. Along this process, certain chemokine receptors are upregulated that promote the migration of such positively selected T cells into the thymic medulla where these are encountered with medullary thymic epithelial cells and dendritic cells, which express B7.1 (CD80) and other costimulatory molecules along with self-peptide/MHC [30]. The engagement of a costimulatory signal induces negative selection of autoreactive T cells. Deregulated self-tolerance is clearly observed in studies with mice lacking medullary MHC or B7 molecules or blocked by respective antibodies [20,31]. Deficiency of ZAP70 (ζ-chain-associated protein kinase of 70 kDa) allows autoreactive T cells to escape deletion [32]. GRB2 (growth factor receptor-bound protein 2) [33], MINK (misshapen–Nck interacting kinase-related kinase) [34], activation of ERK (extracellular signal-regulated kinase), p38, and JNK (Jun kinase) activation are also required for the efficient self-reactive T-cell deletion [30]. BIM, FAS, and Nur77 are required for the induction of apoptosis of self-reactive T cells [29,35]. Mice and humans with defects in FASL and FAS exhibit spontaneous lymphoproliferative autoimmune disease with similarities to human systemic lupus [36].

However, the mechanisms of central immune tolerance are not sufficient to control all self-reactive lymphocytes. Bone marrow and thymus do not express all self-antigens and autoreactive lymphocytes persist at low numbers in the secondary lymphoid organs. Hence, peripheral tolerance is required. The following lists the genes and factors that are required for peripheral tolerance:

- Recruitment of the tyrosine phosphatase SHP1 (SH2 domain-containing protein tyrosine phosphatase 1) and lipid phosphatase SHIP (SH2 domain-containing inositol-5-phosphatase) to the activated BCR and induction of CD5 receptor on the surface by self-reactive BCR cells is required for the inhibition of BCR signaling in self-reactive B cells (anergy) [24,37,38].
- Surface expression of CTLA4 (cytotoxic T-lymphocyte antigen 4) is induced at a high threshold of TCR self-reactivity and inhibits T-cell activation. Lack of CTLA4 causes accumulation of self-reactive T cells in peripheral lymphoid and nonlymphoid tissues. In addition, variants of the CTLA4 gene are associated with susceptibility to thyroid autoimmunity and type 1 diabetes in humans and mice [39–41]. Ubiquitin ligases CBL-B, GRAIL, and ITCH tag ubiquitin to TCR-CD28 or cytokine signaling molecules and inhibit the signaling and promote internalization of the TCR followed by proteolytic degradation [42,43]. cbl-b deficiency along with MHC haplotype causes type 1 diabetes in rats [44]. Mice lacking itch, cbl-b, or its close relative c-cbl develop large numbers of activated T cells and produce high titers of autoantibodies [45,46].
- In the periphery, survival of B cells depends upon BAFF that induces expression of antiapoptotic Bcl-2 [26], a prosurvival factor like PIM2 in B cells to promote survival [47,48]. Antagonism of BAFF or low expression of the BAFF receptor on self-reactive B cells eliminates these cells. Also, IL-7 plays an important role in the survival of mature T cells in the periphery [49,50]. IL-7 expression is usually low but in case like T lymphocytopenia IL-7 levels increase to

proliferate the naïve T cells. This could also provide an opportunity for self-reactive T cells to proliferate as well. This could explain why T lymphocytopenia predisposes people to autoimmune disease [51].

Moreover, the association of MHC gene variants with autoimmune disease is not surprising. Ability of T cells to respond to a particular antigen depends on genotype-dependent variants of MHC conformation. It is less likely that the susceptibility to an autoimmune disease is determined by differences in the ability of different allelic variants of MHC molecules to present autoantigenic peptides to autoreactive T cells [52,53]. Rather, certain MHC molecules may drive the positive selection of self-peptide-reactive T cells. Apart from MHC class molecules, there are numerous other genes that participate in the regulation of immune tolerance or checkpoints for the lymphocyte development. Mutations associated with genes like AIRE, BIM, ZAP70, CBLB, CTLA4, FAS, and ROQUIN have been shown to cause autoimmune diseases both in animal models and in humans.

Monogenic or Polygenic Autoimmune Diseases. Autoimmune diseases due to single-gene defects are rare, but studies with single-gene mutations in animal models have offered an important understanding how defects in the immune system can lead to autoimmunity. Some examples of monogenic autoimmune diseases and responsible genes are given in Table 40.3. In contrast to the monogenic autoimmune diseases, pathogenesis of polygenic autoimmune disease is much more complex. These diseases are the result of a combination of susceptibility alleles at multiple loci and environmental factors (such as smoking, pathogen exposure, and hormone levels). Recently, genome-wide association

Table 40.3 Examples of monogenic autoimmune diseases.

Gene	Human Disease	Mouse Mutant/ knockout	Mechanism of Autoimmunity
AIRE	APS-1	Knockout	Decreased expression of self-antigen in thymus
CTLA4	Graves's and T1D	Knockout	Failure of T-cell anergy and reduced threshold of autoreactive T-cell activation
FOXP3	IPEX	Knockout and mutation	Decreased generation of Tregs
FAS and FASL	ALPS	Mutation	Failure of apoptosis of autoreactive lymphocytes
Complement factor C4	SLE	Knockout	Defective clearance of immune complexes
Trex-1	AGS and chilblain lupus	Mutation	Increased-type IFN signaling

APS1: autoimmune polyendocrine syndrome-1; T1D: insulin-dependent diabetes;
IPEX: immunodysregulation polyendocrinopathy enteropathy X-linked syndrome;
ALPS: autoimmune lymphoproliferative syndrome; SLE: systemic lupus erythematosus;
AGS: Aicardi–Goutières syndrome

Table 40.4 Association of some genomic loci with autoimmune diseases.

Genes of Interest	Function	Diseases
PTPN22	T- and B-cell receptor signaling	RA, T1D, and CD
CTLA4	Transmits inhibitory signals to T cells	T1D and RA
MHC	Major histocompatibility complex	All autoimmune disorders
CD2/CD58	Activation of T lymphocytes	RA and MS
IL10	Downregulates immune responses, including cytokines, MHC class II, and costimulatory molecules	IBD, SLE, and T1D
IL2/IL21	IL2/IL21T cell trophic growth factors	CeD, IBD, RA, and T1D
IL12B	p40 subunit common to IL-12 and IL-23	IBD and PS
IL2RA	IL-2 receptor α chain	MS and T1D
TNFAIP3	Induced by TNF and pattern recognition receptor activation; inhibits NF-κB signaling	RA, SLE, and PS
TNIP1	Interacts with TNFAIP3	SLE and PS
PRDM1	Transcriptional repressor of IFN-β; induces B cell maturation	RA and SLE
BLK	B lymphoid tyrosine kinase	SLE and RA
PTPN2	T-cell protein tyrosine phosphatase	IBD and T1D

AS: ankylosing spondylitis; CeD: celiac disease; IBD: inflammatory bowel disease; MS: multiple sclerosis; PS: psoriasis; RA: rheumatoid arthritis; T1D: type 1 diabetes mellitus.

studies (GWAS) have identified a large number of major loci with many associations shared between various autoimmune diseases Table 40.4.

40.4 Environmental Factors that Break Immune Tolerance

Infection and Tissue Injury. In many clinical scenarios, genetically predisposed individuals require an exposure to a pathogen or tissue injury to trigger the onset of autoimmune disease, but how?

1) *Molecular mimicry by antigens*: Viruses and other microbes may eventually share epitopes with self-antigens causing cross-reacting immune responses that attack not only the pathogen but also host tissue; for example, anti-streptococcal immunity can cross-react with cardiac tissue [54].
2) *Molecular mimicry by adjuvants*: Nuclear material such as nucleosomes or U1sn ribonucleoprotein released by dying cells during infection and trauma has a particle structure similar to that of viruses. The degenerated nucleic acid component lacks protective methylation creating autoadjuvants that mimic viral adjuvants and rather promote autoimmunity [55].

3) *Activation of costimulation*: As discussed earlier, one of the major checkpoint mechanisms of controlling self-reactive lymphocytes is to suppress their activation. Effective antigen presentation requires costimulation. Peripheral tolerance mechanisms suppress costimulatory signals to induce anergy in T cells. Cell surface pattern recognition receptors activation by PAMPs and DAMPs during infection or tissue injury upregulate the surface expression of costimulatory molecules and, thus, promote self-reactive T cells overriding peripheral tolerance.
4) *Superantigens*: Superantigens are proteins produced by a variety of microorganisms, especially bacteria or mycoplasmae, or virus-infected cells. Superantigens can ligate TCRs and mount an aggressive T-cell response with different antigenic specificities. This activation can also lead to autoimmunity [56].
5) *Bystander activation*: An activation of T cells via TCR-independent mechanisms can enhance the processing and presentation of self-antigens. Bystander activation may induce the spreading of the immune response toward different self-antigens, referred to as epitope spreading [56].
6) *Dead cell clearance defects*: Infections and abrupt tissue injury may cause massive cell death and accumulation of necrotic debris in the extracellular space. If this is accompanied by lack of efficient clearing mechanism, the prolonged exposure of intracellular autoantigens can promote autovaccination and autoimmunity [57].
7) *Modification of self-antigen* is another factor associated with autoimmune diseases. Smoking can promote citrullination of proteins like fibrinogen, type II collagen, α-enolase, and vimentin. These citrullinated proteins act as self-antigens. For example, anti-CCP antibodies serve as a biomarker for rheumatoid arthritis.

40.5 Autoimmune Tissue Injury: Tissue Inflammation

Immune Complexes. Immune complexes are formed as a result of binding of autoantibodies to soluble autoantigens. Once formed, immune complexes cause several responses including phagocytosis, opsonization, and complement deposition. They are normally cleared efficiently either by mononuclear phagocytes that have complement and Fc receptors or by red blood cells bearing both complement and Fc receptors, while impairment in clearance of immune complexes leads to their deposition and subsequent tissue injury, which is a hallmark of autoimmune diseases. Classically, a complement cascade involves binding of complement component C1q to the Fc portion of the antibody, which triggers the activation of the anaphylatoxins C5a and C3a that attract effector cells such as neutrophils and NK cells and stimulate the release of proteolytic enzymes as well as inflammatory cytokines. The autoimmune responses that are result of immune complex formation and subsequent tissue injury are classified as type III autoimmune responses. They are usually systemic and are characterized by autoimmune vasculitis. More common examples are systemic lupus erythematosus (discussed in detail in the following section), cryoglobulinemic vasculitis, and all forms of immune complex glomerulonephritis.

Cryoglobulinemia refers to the presence of circulating immunoglobulins called cryoglobulins in serum. The disease mainly involves vasculitis due to cryoglobulin-containing immune complexes [58]. Conditions that lead to cryoglobulinemic vasculits include lymphoproliferative disease, collagen disease, and hepatitis C virus (HCV) infection. However, factors that cause cryoprecipitation are not yet well understood [58]. Manifestations of this disease include (a) skin: erythematous, infarction, hemorrhagic crusts, and ulcers [58]; (b) musculoskeletal: arthralgia and myalgia [58]; (c) neurological: peripheral neuropathy [59]; (d) pulomonary: dyspnea, cough, or pleurisy [58]; and (e) renal: membranoproliferative glomerulonephritis [60,61]. Moreover, deposition of immune complexes in the glomerulus induces glomerulonephritis, for example, circulating polymeric IgA1-containing immune complexes contributes in the pathogenesis of IgA nephropathy [62].

Lymphocytes. Lymphocytes play a crucial role in autoimmune diseases. Autoimmunity is rather defined by the presence of autoreactive T and/or B lymphocytes in the periphery. Lymphocytes with less affinity toward antigen can become activated when the stimulus is strong; for example, naive T cells are activated when encountered by activated dendritic cells presenting an autoantigen and expressing high levels of costimulatory signals.

T Lymphocytes. T lympocytes are present in the inflamed tissues, for example, kidney, skin, joints, and so on. They recognize antigens via the T-cell antigen receptor in conjugation with CD3-defined complex of transmembrane proteins to activate the signaling process [63]. During autoimmune diseases, this signaling is altered leading to heightened response to even low-affinity autoantigens, to which normal T lymphocytes do not respond [63]. T lymphocytes are also known to provide excessive help to B lymphocytes to produce autoantibodies, for example, anti-dsDNA antibodies in SLE. It has been observed that CD4+ and CD3+CD4-CD8- T cells are present in large amount in patients with systemic autoimmunity and help B cells. Therefore, inhibition of this interaction was thought to suppress systemic autoimmunity; for example, such costimulatory pair of molecules includes CD40 and CD40 ligand. The expression of CD40L is increased on the surface of T cells during systemic autoimmunity [64]. Another costimulatory molecule called inducible T cell costimulator (ICOS) is an essential costimulatory receptor for follicular Th (Tfh) cells that provide help for extrafollicular B-cell differentiation, IL-21 production, and CD4 helper function [65,66]. Circulating Tfh cell number is also increased during systemic autoimmunity [67,68]. Furthermore, CD4T cells undergo specific differentiation to become IL-17 producing Th17 cells [69–72], and IL-17 is considered an important contributor to inflammation and autoimmunity [73]. Mice deficient in IL-17 are protected from lupus-like systemic autoimmunity [74]. Moreover, IL-17 blockade has proven beneficial in other autoimmune diseases including psoriasis, rheumatoid arthritis, and uveitis [75,76].

B Lymphocytes. The reciprocal roles of T lymphocyte help for B lymphocytes during an adaptive immune response and B lymphocyte help in CD4+ T cell activation are recognized as important attributors in the pathogenesis of autoimmune diseases [77–79]. B cells are activated when the antigen binds to the B-cell receptor [80]. Different functions of B cells during the pathogenesis of

autoimmune diseases involves the following: (a) Secretion of autoantibodies, for example, autoantibodies directed to DNA, chromatin proteins, and ribonucleoproteins, and so on during systemic autoimmunity in lupus [81], against ribonucleoprotein antigens during Sjögren's syndrome [82], against DNA topoisomerase I, RNA polymerases, and fibrillin-1 during systemic sclerosis [79]. (b) Presentation of autoantigens and costimulation, for example, stimulation of antigen-specific CD4+ T-cell proliferation after naive T-cell priming by DCs [83]. B cells and T cells also interact physically involving costimulatory molecules such as B7/CD28, CD40/CD40 ligand (CD40L), and OX40 ligand/OX40 on the surface of B cells and T cells, respectively [84,85]. (c) Secretion of proinflammatory cytokines; for example, B cells produce multiple cytokines including IL-10, IL-4, IL-6, lymphotoxin-α, TGF-β, and IFN-γ [86,87]. (d) Modulation of antigen processing and presentation. (e) Generation of ectopic germinal centers, within inflamed tissues that can be observed during periods of chronic inflammation [88].

Other innate lymphoid cells that do not possess TCRs and mediate cytotoxicity and secretion of cytokines that activate adaptive immune response include NK cells, CD56+ T cells, natural killer T (NKT) cells, gamma/delta (γδ) T cells, and mucosal-associated invariant T (MAIT) cells [89].

Monocytes and Macrophages. Monocytes and macrophages are essential weapons of the innate immune system with a plethora of immunological functions that involves cytokine production, phagocytosis, and antigen presentation. Abnormalities in functions of monocytes and macrophages are associated with a variety of autoimmune diseases [90,91]. They express several surface markers in order to recognize the environment and respond to it, and therefore any aberrations in the surface markers lead to incomplete immune response (Figure 40.3). For example, the Fc gamma receptors (FcγRs), essential for phagocytosis, cytolysis, degranulation, and inflammatory cytokine production [92], are dysregulated during systemic autoimmunity [91]. In particular, these receptors are required for the clearance of immune complexes, which are produced abundantly in SLE, and therefore deletion of FcγRs on the monocytes protected mice from development of lupus nephritis [93].

Moreover, monocytes and macrophages from patients with systemic autoimmunity exhibit abnormalities in antigen presentation, for example, decreased MHC II expression that is associated with defective upregulation of CD80 on APCs [94,95]. They also express higher CD86 compared to the healthy individuals [96]. Overexpression of the adhesion molecules, for example, ICAM-1 and costimulators molecules, for example, CD40 and CD69 also leads to aberrant monocyte migration as well as lymphocyte activation during autoimmune diseases [90,97]. However, the mechanisms underlying these phenotypic changes of monocytes and macrophages during autoimmune diseases are largely unknown. Contributions of both genetic and environmental factors in this have been described.

Aberrant activation of the immune system also causes increased production of inflammatory cytokines from monocytes and macrophages [98–100]. Elevated levels of Th2 cytokines, IL-6 and IL-10, during SLE help to promote IgG production. IL-6, in cooperation with IFN-1, is also required for B-cell maturation [101], whereas TNFα, IL-1β, IL-6, and TGFβ also play pathogenic roles in RA [100].

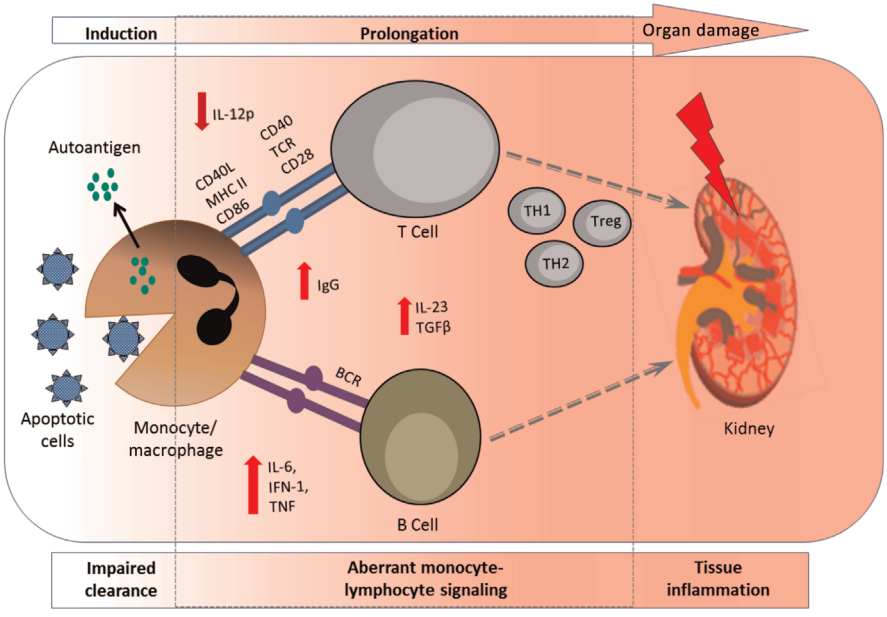


Figure 40.3 Role of monocytes and macrophages in pathogenesis of autoimmune diseases. Monocytes and macrophages participate at several levels during the pathogenesis of autoimmune diseases. They can misread apoptotic cells during their clearance and thus induce both inflammation and inappropriate presentation of autoantigens, initiating autoimmunity. Inappropriate antigen presentation leads to aberrant monocyte–lymphocyte signaling facilitating the prolongation of autoimmunity. At last, monocyte- and macrophage-mediated tissue inflammation may cause organ damage.

The phagocytosis of pathogens and homeostatic clearance of dying cells by monocytes and macrophages is essential for host defense. In systemic autoimmunity of the connective tissue diseases, the clearance of dying cells is impaired by monocytes and macrophages [102,103], which lead to increased availability of the intracellular autoantigens. These effects are partially because of reduced CD44 expression on the surface of monocytes [104], which is observed in SLE patients. Complement components also bind to apoptotic cells to promote phagocytosis by macrophages; however, antibodies against complement components, e.g. anti-C1q, impairs the clearance of apoptotic cells [105]. In addition to these mechanisms, autoantibodies may also directly act on macrophages [106].

Tertiary Lymphoid Organs. Tertiary lymphoid organs (TLOs) are formed when there is continued need for extravasation of leukocytes and a persistent source of antigen during an autoimmune attack [107] (Table 40.5). According to pathologists, the term TLO can be used only when all the following criteria are fulfilled [107]: (i) the organized infiltrate contains anatomically distinct yet adjacent T- and B-cell compartments; (ii) the T-cell area contains an extensive network of FRCs; (iii) PNAd+ or MECA79+ HEVs are present in the T-cell area; (iv) there is evidence of B-cell class switching and GC reactions in the B-cell

Table 40.5 Diseases in which TLOs have been found [107].

Disease	Location	Antigen Specific	References
Rheumatoid arthritis	Synovial space	Yes	[108,109]
	Lung interstitium	Yes	[110]
SLE	Tubulointerstitial nephritis	Yes (clonal restriction and mutated antibodies)	[111,112]
Diabetes	Periductally in pancreatic parenchyma	Yes, antiinsulin antibody responses	[113,114]
Primary biliary cirrhosis	Liver	No: absence of any clonal T- or B-cell proliferation	[115]
Secondary progressive multiple sclerosis	Meninges at deep cerebral sulci	Myelin and neuronal antigens: suggested	[116–118]
Hashimoto's thyroiditis	Thyroid gland	Thyroglobulin, thyroperoxidase	[119]
Autoimmune gastritis	Gastric lamina propria	Parietal cells	[120]
Myasthenia gravis	Thymus	Acetyl choline receptor: correlation of autoantibodies with infiltrates	[121]

follicles; (v) activation-induced cytidine deaminase (AID) enzyme is present; and (vi) FDCs are present. Often, it appears that not all criteria are fulfilled, but the organized structure can still function as a TLO [107].

The homeostatic chemokines and cytokines induce the formation of TLOs at the site of chronic inflammation (Table 40.5.). When LTα, CCL21 and CXCL13 are overexpressed from a tissue-specific promoter, ectopic lymphoid tissues are induced [107,122]. Similarly, either neutralization or genetic deletion of CXCL13, CXCR5, CCR7, or LTα inhibits TLO formation [108,123,124]. During this process, LTα signals the stromal cells to develop into follicular DCs and high endothelial venules, and CCL19 and CCL21 govern the organization of T-cell zone [107,123,125]. Moreover, deletion of IL7 causes absence of lymph nodes (LNs) in mice, whereas overexpression of IL7 causes development of additional LNs [107]. Also, RA patients show increased IL7 expression in the synovial tissue [126].

Similar to the secondary lymphoid organs, TLOs allow activation of naïve T cells and B cells by DCs within germinal centers [127]. The predominant APCs in TLOs are DCs [111]; however, their major constituents are the B cells [128]. Almost all TLOs show signs of B-cell class switching [128]. Plasma B cells found in the vicinity of TLOs secretes antibodies specific for the inducer of TLOs, whereas some B cells reach bone marrow and reside there as long-lived plasma cells [128].

TLOs are also considered as an inductive site for self-reactive T lymphocytes and antibodies. For example, the presence of T lymphocytes that destroy islets was detected in the pancreatic TLOs during the progression of diabetes in NOD mice [129]. Moreover, in lungs of RA patients, B cells in TLO produce rheumatoid factor and antibodies against citrullinated proteins [130]. TLOs have also been found in autoimmune thyroiditis or SLE [112,119].

Interindividual Variability of Organ Manifestations in Systemic Autoimmune Diseases. The confluence of remarkable advances in human genetics has furthered large amount of new information on genetic risk factors for human autoimmune diseases. For example, application of GWAS has revealed very important information on the common genetic risk variants, their frequencies in the population (risk allele frequency), and the risks on disease they confer (odds ratio) [131,132]. Most of the risk alleles identified by GWAS exert modest effect on the risk of disease (odds ratio >2), whereas only few risk alleles can exert large effect (odds ratio >3) [132,133]. In most of the autoimmune diseases, these important loci include HLA region [132,134,135]. However, other loci, for example, NOD2 and IL23R, contribute to Crohn's disease [136–138], and PTPN22 to type 1 diabetes and RA [139,140].

A recent large-scale GWAS of lupus nephritis provided strong evidence for multiple biologically relevant lupus nephritis susceptibility loci that are independent of HLA regions. For example, MHC locus rs9263871 located within *HCG27* had strongest association with lupus nephritis, independent of *HLA-DR2* and *HLA-DR3*, with an odds ratio of 1.7 [135]. Other loci outside the MHC region that are associated with lupus nephritis include rs1364989 on 4q11-q13, localized upstream of PDGF receptor-α [135]; rs274068 on 16p12.1, located within an intronic region of sodium-dependent glucose cotransporter *SCL5A11* [135]; rs7834765 on 8q24.12; and other two regions on 6p22 and 9p21 [135].

Some SLE susceptibility loci are also lupus nephritis susceptibility loci. For example, the most strongly associated SLE susceptibility locus rs2187668 within the HLA region is also associated with lupus nephritis [135]. Other such examples include rs2205960 within the 1q25.1 region near *TNFSF4* [135], and rs9888739 within ITGAM, located on chromosome 16p11.2 [135].

40.6 Lupus Nephritis, a Paradigmatic Example of Systemic Autoimmunity and Autoimmune Tissue Injury

In this chapter, the concept of systemic autoimmunity and autoimmune tissue injury is explained along the example of systemic lupus erythematosus. Systemic lupus erythematosus is a chronic autoimmune disease based on the loss of tolerance against nuclear autoantigens, which becomes evident by the presence of polyclonal antinuclear antibodies (ANA). The aberrant immunity of systemic lupus erythematosus can present as lymphoproliferation, immune complex disease, and inflammation in several organs [141,142]. Lupus nephritis is the most frequent organ manifestation. Genetic variants are fundamental for the development of lupus, but here the discussion is limited to the related disease mechanisms (Figure 40.4).

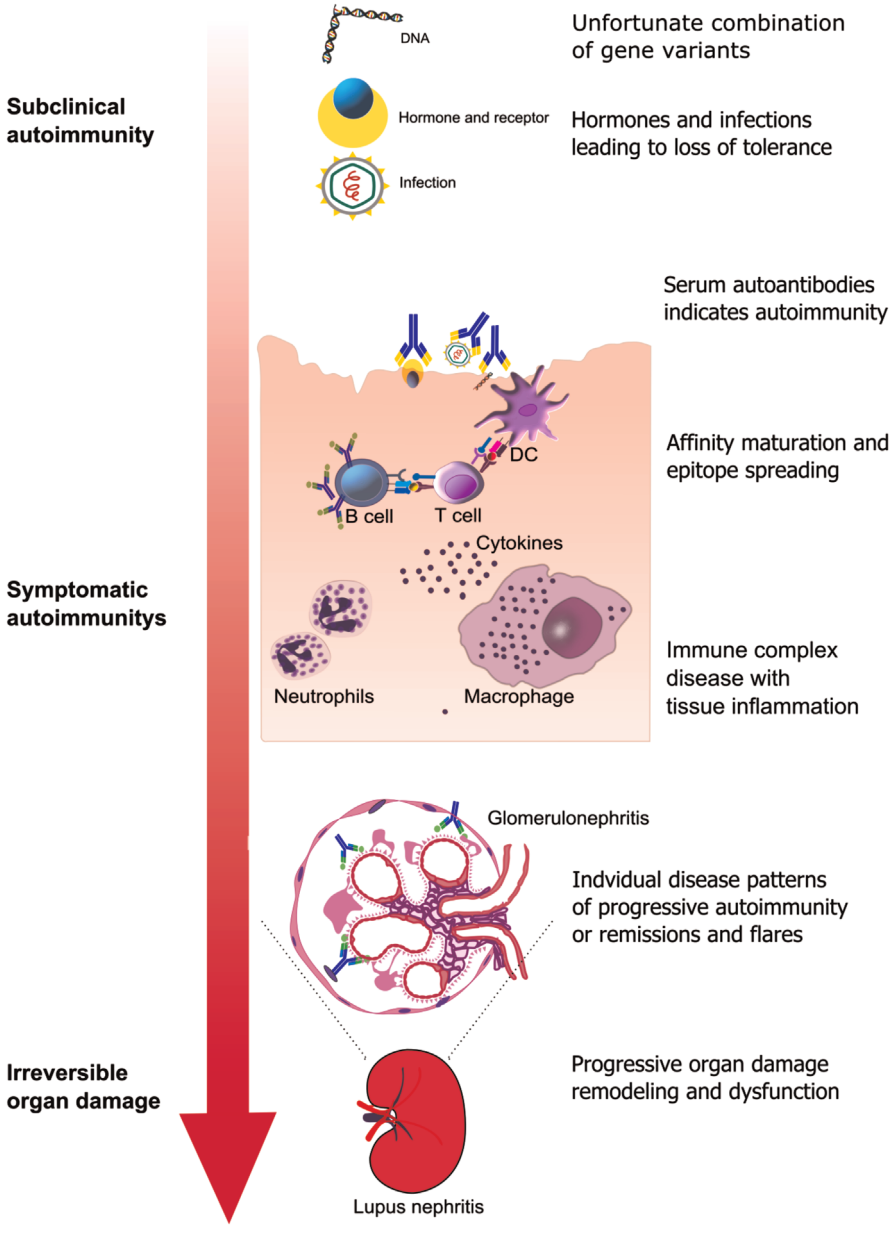


Figure 40.4 Evolution of autoimmune diseases. Autoimmunity is a very common phenomenon because everyone hosts some autoreactive lymphocyte clones that escaped negative selection in the thymus. Hormonal or environmental triggers such as infections promote an unspecific immune activation, which also drives a transient expansion of these clones that may cause a transient occurrence of autoantibodies in the circulation; for example, the occurrence of cryoglobulins after a mycoplasma infection does not develop without a distinct deposition. However, such phenomena do not necessarily lead to recurrent autoimmune disease unless other genetic variants compromise those elements that settle clonal expansion of autoreactive lymphocytes after disappearance of the infectious trigger. Gene variants in the autoantigen itself,

40.7 Pathomechanisms of Systemic Lupus Erythematosus

Aberrant Cell Death and Aberrant Handling of Dead Cells. Lupus, like any other autoimmune disease, is the result of autovaccination. The immunization process is imprinted into the immune memory via autoreactive long-lived plasma cells and memory T cells. These immune cells can hardly be eradicated by immunosuppressive drugs; hence, current drug options can only suppress disease activity but do not cure systemic lupus erythematosus [142,143]. Autovaccination and ANA formation imply that the nuclear autoantigens were accessible to antigen-presenting cells, a process normally prevented by rapid phagocytic clearance of dead cell corpses. Lupus patients are compromised in those mechanisms that normally assure low levels of chromatin in extracellular compartments. For example, certain gene variants modulate apoptotic removal of autoreactive lymphocytes [144,145] or the labeling of dead cells with opsonins such as complement factors or pentraxins. Also, the phagocytic clearance by macrophages can be impaired [81]. Recently, it was discovered that neutrophils undergoing NETosis releases nuclear material into the extracellular space [146–150], a process that drives autoimmunity on lupus [151].

Adjuvant mimicry Leading to Pseudoantiviral Immunity. Any persistence of dead cells and their components in the extracellular space holds the risk for autovaccination. This does not exclusively relate to autoantigens being exposed to the immune system, but it relates also to autoadjuvants that turn antigen presentation from tolerogenic to pathogenic. In this context, nucleic acids are important autoadjuvants. Delayed dead cell removal leads to degeneration of its components compromising elements that normally mask self-nucleic acids from being recognized as viral nucleic acids [152,153]. For example, the methylation of DNA and RNA inhibits their recognition by nucleic acid-specific Toll-like receptors that are important to translate viral nucleic acid recognition receptors into antiviral immunity [154]. Patients with systemic lupus erythematosus tend to process nuclear particles of dying cells as viral particles in which distinct nucleic acid structures activate these Toll-like receptors and thereby act as autoadjuvant. As the immune system mounts potent antiviral immunity against viruses, patients with systemic lupus erythematosus mount similar potent immune responses against those antigens that are presented in the context of these autoadjuvants. For example, ribonucleoprotein U1snRNP can be an agonist to Toll-like receptor TLR7 and trigger type I interferon release in plasmacytoid dendritic cells [155]. Immune complexes containing immunostimulatory RNA can activate B cells to produce antinuclear antibodies [156], a process regulated by SIGIRR [157,158].

its localization, and its antigen presentation, as well as in regulatory elements of the adaptive immune system, all can break the threshold of "loss of tolerance" and persistent autoantibody production. Autoimmune tissue injury then results from an ongoing attack of humoral and cellular effectors of the adaptive immune process directed against the autoantigen, which by nature persists and cannot be eliminated. Such antigens may be organ specific (e.g., autoimmune thyreoditis) or diffuse (e.g., in systemic lupus erythematosus). Autoimmune tissue inflammation then involves the same elements of innate and adaptive immunity that are involved in antimicrobial or antiviral host defense.

Similarly, DNA within immune complexes can activate TLR9 in plasmacytoid dendritic cells and promote B-cell proliferation [159]. Therapeutic inhibition of TLR7 and TLR9 can suppress type I IFN secretion, SLE, and lupus nephritis in mice [160–162]. The IFN receptor-dependent secondary gene expression ("signature") includes multiple antiviral genes, which should account for the unspecific symptoms shared by viral infections and SLE, such as fever, fatigue, arthralgia, and myalgia [163].

Expansion of Autoreactive Lymphocyte Clones. Dendritic cells and B cells are those antigen-presenting cells that regulate the behavior of the T-cell repertoire, that is, quiescence versus activation and proliferation [164]. Dendritic cells have a limited life span but upon persistent exposure to lupus autoantigens, activating TLR7 and TLR9 enhances their survival and renders them resistant to glucocorticoid-induced death [160]. Toll-like receptor activation of antigen-presenting cells changes their gene expression profile toward costimulatory molecules and proinflammatory cytokines, which shifts the interpretation of autoantigens from immune ignorance and lymphocyte anergy into lymphocyte activation and proliferation [165]. B lymphocyte stimulator (BlyS, also named B cell-activating factor, BAFF) and A proliferation-inducing ligand (APRIL) are only two lymphocyte mitogens secreted by activated dendritic cells and they also regulate B-cell differentiation and Ig class switching [166]. The two APRIL/BLyS receptors transmembrane activator and CAML interactor and B-cell maturation protein promote plasma cell survival [167]. B-cell maturation toward autoantibody-producing plasma cells involves interferon-related factor-4 [168]. T-cell activation involves MHC-II-mediated interaction with the clonal T-cell receptor (TCR), which implies that only T cells recognizing the presented antigen will get activated and undergo clonal proliferation.

Environmental Factors Promoting Systemic Lupus Erythematosus Activity. As viral infections activate the same kind of interferon-α-related immune response, viral infections can trigger disease activity of systemic lupus erythematosus [163]. In contrast, bacterial infections rather provide an unspecific immunostimulatory effect, in which the transient expansion of autoreactive lymphocyte clones is a bystander effect. Bacterial components also stimulate intrarenal immune cells and renal cells resulting in a transient aggravation of kidney injury. UV light causes keratinocyte death [169]. In case of a significant clearance defect, this process increases extracellular nuclear material and exposure of autoantigens and autoadjuvants to antigen-presenting cells [141]. Some drugs trigger drug-induced systemic lupus erythematosus by inhibiting methyltransferases, which unmasks endogenous nucleic acids for more potent activation of TLR7 and TLR9 [170,171].

40.8 Pathomechanisms of Lupus Nephritis Operating Inside the Kidney

Immune Complex Glomerulonephitis. The polyclonal autoantibody response in systemic lupus erythematosus accounts for the diagnostic hallmark of lupus nephritis, that is, the so-called "full house pattern" of IgM, IgA, and IgG deposits

inside the kidney's glomeruli [172]. However, antibody-deficient mice still develop lupus nephritis; hence, B cells are important for the disease pathogenesis beyond antibody secretion [173]. For example, B cells are important antigen-presenting cells that present autoantigens to autoreactive T cells and induce their clonal expansion [164]. Immune complexes can deposit in different compartments of the glomerulus, that is, the mesangium, the subendothelial, and the subepithelial space. In addition, they deposit along peritubular capillaries [174]. The histopathological classification of lupus nephritits acknowledges these distribution patterns; for example, immune complex formation in the mesangium is defined as class I and II, subendothelial immune complex formation as class III and IV, and subepithelial immune complexes as class V [175]. Initially, it was thought that circulating immune complexes passively deposit in the kidney, but this concept was disproved [176,177]. Glomerular immune complexes rather form *in situ* by binding to nuclear particles derived from glomerular cells [178]. Chromatin released from neutrophils undergoing neutrophil extracellular trap formation may be another source of intrarenal lupus autoantigens [146–151]. Heparin enhances DNA-I-dependent chromatin degradation and prevents chromatin binding to the GBM [179]. However, how do immune complexes cause glomerular injury? DNA antibodies can activate glomerular cells via different mechanisms. For example, DNA antibodies bind antigen also inside renal cells [177]. This process may involve cross-reactivity with α-actinin or annexin II on mesangial cells [180,181], but the role of this procs remains under debate [182,183]. Immune complex deposits also activate complement [172] demonstrating the dual role of complement in lupus nephritis [184]. On the one hand, complement deficiency impairs opsonization and removal of lupus autoantigens from the extracellular space; on the other hand, complement promotes immune complex-related inflammation inside the kidneys [185,186]. Immune complexes also activate glomerular cells via FcR receptors although data in this domain remains complex [187].

Toll-like Receptor Activation and IFN Signaling Inside the Kidney. Lupus immune complexes contain a nucleic acid component that activates not only TLRs in intrarenal macrophages and dendritic cells but also endothelial cells and mesangial cells to produce large amounts of proinflammatory cytokines and interferon-α and -β [188–195]. This intrarenal IFN signaling may contribute to lupus nephritis activity and triggers the formation of tubuloreticular structures or inclusions (TRI), that is, ultrastructural characteristic of IFN signaling [192,196]. Together, the activation of TLRs, complement receptors, and FcR activates renal cells to release proinflammatory mediators that further promote inflammation and cell injury inside the kidney [197,198].

Chemokines Recruit Leukocytes to the Kidney. All sorts of different leukocyte subsets infiltrate the kidney in lupus nephritis [199]. The different members of the chemokine family specifically recruit these different leukocyte subsets via distinct chemokine receptors and different chemokines direct leukocytes to different renal compartments [200]. For example, CCL2 recruits CCR2+ macrophages and T cells into both the glomerulus and the tubulointerstitium [201,202]. In contrast, CCR1+ cells only recruit to the interstitium and not to the glomerulus [203]. In addition to chemokines, renal cells secrete pentraxin-3, which inhibits leukocyte

recruitment by masking P-selectin on the luminal surface of activated endothelial cells [204–206]. In chronic forms of lupus nephritis, leukocytes can form perivascular tertiary lymphoid follicles inside the kidney, where clonal expansion and ongoing somatic hypermutation of B cells occurs in proximity to T cells, which contributes to local inflammation and tissue pathology [112,164,207–209]. These findings have encouraged physicians to test B-cell-targeted therapies in lupus nephritis [210,211]. Also, infiltrating T cells contribute to lupus nephritis, for example, CD3+/CD4+ or CD3+CD4/8 double-negative T cells that produce IL-17 [212]. Among the different macrophages phenotypes F4/80(hi)/CD11c(int) Gr1(lo)/Ly6C(lo)/VLA4(lo)/MHCII(hi)/CD43(lo)/CD62L(lo) macrophages were found to drive renal inflammation in lupus nephritis [90,213].

Aberrant Tissue Repair. Kidney injury triggers healing, which by itself can cause kidney dysfunction. Focal necrosis of the glomerular capillary loops induces an invasion of parietal epithelial cells that produce extracellular matrix and form focal scarring, referred to as "focal-segmental glomerulosclerosis." This process often progresses to global glomerulosclerosis and loss of function [214]. In addition, activated parietal epithelial cells can undergo hyperplasia and completely obstruct the urinary outfow [215,216]. This process can be triggered by GBM breaks that allow plasma leakage into Bowman's space, where mitogenic plasma components such as fibrinogen trigger parietal epithelial cell hyperplasia [217]. Subsequently, the parietal epithelial cells lose their polarity and produce matrix all around them, also leading to irreversible glomerular scarring, referred to as class VI lupus nephritis.

40.9 Summary

Autoimmunity is a form of sterile inflammation. In contrast to autoinflammation and alloimmunity, autoimmunity is driven by a spontaneous loss-of-tolerance against self-proteins and other structures of the self. The susceptibility to loss-of-tolerance is genetically determined by gene variants that break or weaken the checkpoints that ensure self-tolerance in the immune system. Infections often trigger loss-of-tolerance in susceptible patients, which then leads to autovaccination resulting in life-long persistence of immune memory. Therefore, cure of autoimmune disease is hardly possible unless immune memory that is laid down in long-lived plasma cells in bone marrow niches is eradicated. As such, therapy of autoimmune diseases focuses largely on the control of autoimmune tissue injury and tissue inflammation. Nevertheless, the driving force of autoimmune tissue injury is underlying autoimmune mechanisms taking place in lymphoid organs. MHC class II-mediated autoantigen presentation is at the heart of the pathogenesis and the critical interaction between antigen-presenting cells and autoreactive lymphocytes. Clonal expansion of the autoreactive lymphocyte clones leads to humoral and cellular immune responses. Immune complex disease, infiltration of autoantigen-specific T cells, and tertiary lymphoid organ formation all contribute to autoimmune tissue injury, inflammation, atrophy, and subsequently organ dysfunction. Interfering with the systemic and local pathomechanisms of autoimmune disease is the current strategy for induction of response,

maintaining a sufficient suppression of disease activity, and preventing relapse of a disease.

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Conflict of Interest Statement

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41

Psoriasis and Other Skin Inflammatory Diseases

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41.1 Introduction

Psoriasis vulgaris is a common inflammatory skin disease affecting 2–4% of the world population. As such, it has become one of the best understood T-cell-mediated disease for testing novel immune target treatments in humans. Since psoriasis immunopathogenesis represents amplifications of innate and adaptive immune systems in normal human skin, our understanding of psoriasis is now facilitating development of new treatments in other inflammatory skin diseases, such as atopic dermatitis and alopecia areata.

41.2 Normal Skin

41.2.1 Skin Structure and Its Function as an Immune Organ

Skin is composed of two layers, epidermis and dermis, which are distinct in architecture, but functionally interdependent. Epidermis is composed primarily of keratinocytes (>90%), with minority populations of Langerhans cells, melanocytes, and neuroendocrine (Merkel) cells [1]. The epidermal layer has downward invaginations termed rete ridges between dermal papillae, which form a honeycomb of interconnected ridges in three-dimensional structure. Dermis is a connective tissue composed of an extracellular matrix containing inflammatory cells such as T cells, dermal dendritic cells, macrophages, and mast cells. Among cellular components of skin, T cells, dendritic cells, and keratinocytes play key roles in skin function as an immune organ.

Healthy skin has the capacity to recruit recirculating T cells and also contains abundant resident T cells as a skin-associated lymphoid tissue (SALT) [2]. Skin-homing T cells are cutaneous lymphocyte antigen $(CLA)^+$ T cells, as they bind to dermal venules of the skin by interaction between CLA on the T cells and E-selectin on the endothelial cells [3]. Approximately 10% of T cells in the

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peripheral circulation of adults become differentiated for skin homing immunity through expression of CLA [4]. Beside those CLA^+ recirculating T cells, normal human skin also contains long-lived nonrecirculating resident memory T cells (T_{RM}) that persist in the absence of antigens and provide rapid on-site immune protection in the skin [5–7].

Dendritic cells play central roles in skin inflammation by taking up and processing antigens, activating antigen-specific T cells, and producing amplifying cytokines. The main dendritic cell populations in the skin are Langerhans cells in epidermis and myeloid dendritic cells and plasmacytoid dendritic cells in dermis. CD1a and langerin (CD207) are used as specific markers to distinguish Langerhans cells from other dendritic cell subsets, and the α_x integrin CD11c has been found to be the correct marker of dermal myeloid dendritic cells. Plasmacytoid dendritic cells are rare cell populations that can mount rapid and robust type 1 interferon (IFN) responses to viral/microbial infections [8,9].

Keratinocytes not only function as a barrier but also orchestrate innate and adaptive immune responses [4]. The outmost layer of epidermis (cornified layer) creates a physical barrier to inward bacterial penetration and outward water loss by a cross-linked protein membrane structure between which many layers of neutral lipids are deposited. Keratinocytes in granular layer, which is underneath the cornified layer, produce innate effector molecules such as antimicrobial peptides (AMPs), including cathelicidin LL37, defensins, and S100 proteins. Keratinocytes also participate in adaptive immune responses by directing migration of T-cell subsets into the skin through constitutive production of CCL27 (CTACK) and inducible production of chemokines such as CXCL9 and CCL20 [4,10].

41.2.2 Skin Response to Inflammation by Polarized T-Helper Cell Subsets

Keratinocytes in epidermis are highly responsive to cytokines made by polarized T-helper cell subsets, and the patterns of T-helper cell polarization determine characteristics of inflammatory skin diseases. For example, both psoriasis and atopic dermatitis are chronic inflammatory conditions with large infiltrates of T cells, but different T-helper cell polarization determines distinct clinical and histologic characteristics between psoriasis and atopic dermatitis: T_H17 polarization is predominant in psoriasis, while T_H2 polarization is predominant in atopic dermatitis [11]. Both diseases have T_H22 activation and T_H1 activation. Table 41.1 summarizes T-helper cell subsets with their main effector cytokines and functions in skin inflammation [12].

41.3 Psoriasis

41.3.1 Clinical, Histological, and Molecular Features of Psoriasis

Clinically, psoriasis vulgaris begins as a pinhead-sized papule on the skin that vertically grows to become raised, red, and scaly plaque, and radially expands to peripheral body surface area in progression to severe cutaneous disease

Table 41.1 Function of T-helper subtypes in skin inflammation.

T-helper Cell Subset	Effector Cytokines	Functions in Skin Inflammation
T_H1	IFN-γ	Induces hundreds of genes for innate and antiviral molecules in keratinocytes
T_H17	IL-17A	Highly induces antimicrobial peptides and psoriasin (S100A7)
	IL-17 F	Induces chemokine-recruiting neutrophils, dendritic cells, and T cells
T_H22	IL-22	Induces epidermal hyperplasia and inhibits terminal differentiation Induces S100A7, S100A8, and S100A9 (synergy with IL-17)
T_H2	IL-4	Inhibits epidermal cornification, but does not induce epidermal hyperplasia
	IL-5	Inhibits some AMPs, for example, human beta defensin 2 and 3
	IL-13	Involved in allergic responses and IgE switching

(Figure 41.1a). Psoriasis lesions are usually located on elbows, knees, scalp, and lumbosacral area, but it can be more extensive. Severe psoriasis is frequently associated with systemic inflammation and comorbidities, such as psoriatic arthritis, cardiovascular disease, diabetes, and depression [13–19]. About 80%

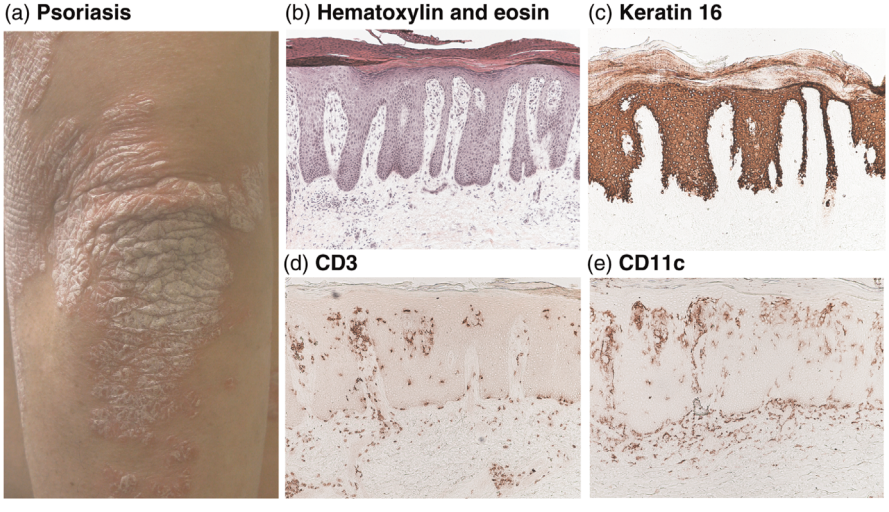


Figure 41.1 Clinical and histological features of psoriasis. (a) Psoriasis skin: raised, red, and scaly patches on the outside of elbow. (b) Histology of psoriasis skin biopsy with hematoxylin and eosin (H&E) stain: thickening of epidermis (acanthosis) with elongated downward projections (rete ridges). Thickening of stratum corneum (hyperkeratosis) with retention of nuclei (parakeratosis). (c) Keratin 16 stain: keratinocyte hyperproliferation of epidermis. (d) CD3 stain: $CD3^+$ T cell accumulation in epidermis and dermis. (e) CD11c stain: $CD11c^+$ myeloid dendritic cell accumulation in epidermis and dermis (all images 10× magnification).

of people with psoriasis have psoriasis vulgaris, but there are variant types of psoriasis – *small plaque psoriasis* in the Asian population, *guttate psoriasis* in childhood or young adulthood, *inverse psoriasis* in body folds, *pustular psoriasis* with white pustules surrounded by red skin, and *erythrodermic psoriasis* that leads to widespread redness over the whole body surface area.

Histologically, psoriatic plaque is caused by epidermal thickening (acanthosis) (Figure 41.1b), which represents keratinocyte hyperproliferation (Figure 41.1c). Keratinocytes in psoriatic plaques move through the epidermis over 4–5 days, a 10-fold acceleration over normal skin [20]. Because the normal process of differentiation cannot occur, there is a loss of the normal granular layer, thickened stratum corneum (hyperkeratosis), and retention of nuclei in the upper layers and stratum corneum (parakeratosis). There are often neutrophils collected in the epidermis and stratum corneum (spongiform pustule of Kogoj and Munro microabscess). In the dermis, abundant mononuclear cells, predominantly myeloid dendritic cells and T cells, are accumulated (Figure 41.1d and e).

Molecular changes in psoriasis skin are characterized by the immunologic interplays between T cells, dendritic cells, keratinocytes, and other inflammatory cells, which result in the production of psoriatic cytokines. Collectively, central IL-23/T_H17 pathway initiates immune response, and then polarized T-helper cell subsets (T_H17 cells, T_H1 cells, and T_H22 cells) amplify immune reactions (Figure 41.2). The cytokines IL-17, IFN-γ, and IL-22 from each T-helper cell subsets cause keratinocyte proliferation while producing cytokines, chemokines, and antimicrobial peptides [21–27]. This becomes a self-amplifying loop, where these products act back on the dendritic cells, T cells, and other inflammatory cells to perpetuate the cutaneous inflammatory process [4].

41.3.2 Cellular Players of Psoriasis Skin Inflammation

41.3.2.1 T Cells in Psoriasis Skin

Psoriasis lesions contain increased numbers of $CD3^+$ T cells (Figure 41.1d), and $CD4^+$ polarized T-helper cell subsets discretely produce IL-17, IFN-γ, and IL-22, with labeling of these cells as T_H17, T_H1, and T_H22, respectively (Figure 41.2) [4,28]. However, not only $CD4^+$ T-helper cell subsets but also other cell populations can produce IL-17. There are $CD8^+$ T cell populations that make the same range of cytokines (Tc17, Tc1, and Tc22). Innate lymphoid cells, which are immune cells that lack a specific antigen receptor yet can produce an array of effector cytokines, may be present in the skin and are potential source of IL-17 in psoriasis [29,30]. It was assumed that lesional IL-17-producing T cells expressed the αβ T-cell receptor only, but recent studies characterized many IL-17-producing T cells in human and murine psoriasis skin as γδ T cells [31].

Regulatory T cells (Tregs) are a heterogeneous group of cells that maintain antigen-specific self-tolerance and are one immune mechanism to prevent tissue damage due to inflammation [4]. When the above effector T cells are not being held in check by Tregs, unstrained effector T-cell effects lead to autoimmunity [32]. Some studies have shown Tregs to be dysfunctional in psoriasis suggesting that psoriasis may result from the inability to suppress autoinflammation [33,34]. The

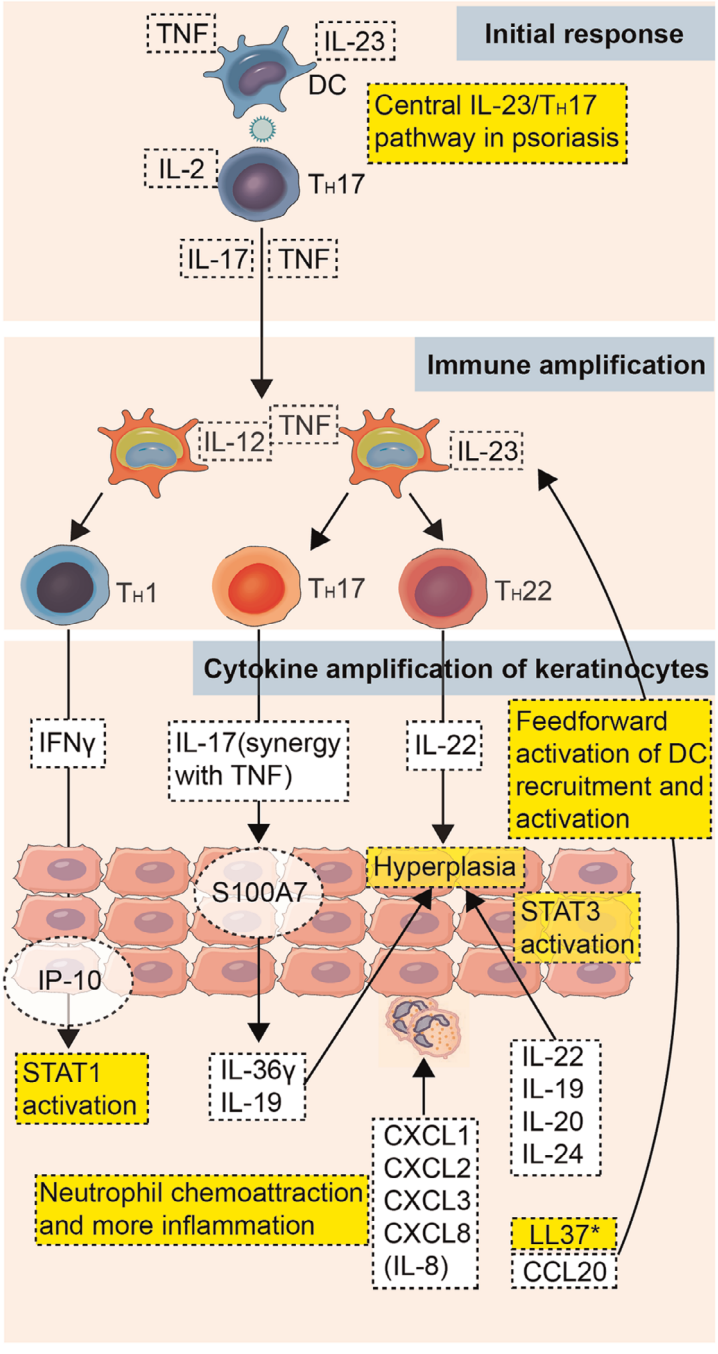


Figure 41.2 Psoriasis skin inflammation. Psoriasis immune pathways with cellular players and key cytokines in three different stages of skin inflammation: (1) initial response, (2) immune amplification, and (3) cytokine amplification of keratinocytes. As the immune circuits consist of a self-amplifying loop, psoriasis becomes an autoimmune skin disease. ∗LL37 is a possible autoantigen of psoriasis.

suppressor activity of $CD4^+$ $CD25^{high}$ Tregs was deficient in psoriasis patients' skin and blood in association with accelerated proliferation of $CD4^+$ responder T cells [35]. Also, $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs versus activated effector T cell ratio in progressive psoriatic skin was much lower than the ratio in normal human skin [36,37]. The reduced ratio in blood was associated with the severity and progressive course of psoriasis. However, further studies are needed to elucidate contribution of Tregs to psoriasis.

41.3.2.2 Dendritic Cells Interplaying with T Cells

Dendritic cells may play central pathogenic roles in psoriasis, both by activating T cells and by producing amplifying cytokines and chemokines during inflammation (Figure 41.2) [38]. There are two main subgroups of myeloid dendritic cells in psoriasis: blood dendritic cell antigen (BDCA)-1/$CD1c^+$ "resident" dendritic cells and BDCA-1/$CD1c^-$ "inflammatory" dendritic cells [39]. Inflammatory dendritic cells include "tumor necrosis factor (TNF) and inducible nitric oxide synthase-producing dendritic cells" or Tip-DCs, and produce IL-12p40, IL-23p19, and IL-20 [40,41].

Both resident and inflammatory dendritic cells stimulate T cells robustly in an allogeneic mixed lymphocyte reaction and similarly induce allogeneic T cells to produce IFN-γ and IL-17 [42]. However, while there is a 30-fold increase in myeloid dendritic cells in the dermis of psoriatic skin lesions compared to uninvolved psoriatic or normal skin [43], this increase is in inflammatory dendritic cells, not resident dendritic cells [44]. In addition, effective treatment of psoriasis was correlated with decreased numbers of inflammatory dendritic cells in psoriasis lesions [45]. Myeloid cells expressing 6-sulfo-LacNAc (slan) have been proposed to be inflammatory dendritic cell precursors in psoriasis, driving strong T_H17 and T_H1 responses [46].

Plasmacytoid dendritic cells have been studied as important upstream initiators of psoriasis development. Plasmacytoid dendritic cell-derived IFN-α may stimulate the activation and amplification of pathogenic T cells through the induction of myeloid dendritic cell activation/maturation [9]. In addition, psoriasis exacerbation reaction by TNF antagonist has been explained by unopposed IFN-α production by plasmacytoid dendritic cells in genetically predisposed individuals [47].

The role of Langerhans cells in psoriasis immunopathogenesis is still unclear, but recent attention has focused on the potential importance of Langerhans cells in uninvolved skin sites of psoriasis patients. It has been demonstrated that Langerhans cell migration is impaired in early-onset psoriasis, and treatment with biologics significantly restored epidermal Langerhans cell migration in uninvolved skin [48–50].

41.3.2.3 Keratinocytes Triggering Innate and Adaptive Immune Responses

In response to stimulation, keratinocytes trigger innate and adaptive immune responses by producing cytokines, such as TNF or the chemokine CCL20, and effector molecules, such as antimicrobial peptide LL37 (Figure 41.2). CCL20 functions to recruit both myeloid dendritic cells and T_H17 cells into active

psoriasis skin [4,51,52]. CCR6, a receptor for CCL20, is expressed in T cells and almost all the mature dermal myeloid dendritic cells in the dermal aggregates [53]. CCL20/CCR6 chemokine system may be crucial in self-maintaining the interaction between lesional mature dendritic cells and T cells where T cells are effectively activated *in situ*.

LL37 has a dual role: (1) nucleic acid-mediated maturation of dendritic cells and (2) possibly a T-cell autoantigen [54]. For dendritic cell maturation, LL37 can bind self-DNA and LL37/DNA complexes activate plasmacytoid dendritic cells by binding to intracellular Toll-like receptor (TLR) 9 [55–57]. LL37 also binds self-RNA, and LL37/RNA complexes activate plasmacytoid dendritic cells through TLR7. Activated plasmacytoid dendritic cells initiate psoriasis development by producing IFN-α, which activates myeloid dendritic cells. LL37/RNA complexes can directly activate myeloid dendritic cells through TLR8. In addition, LL37 is recognized as an autoantigen by circulating T cells in 46% of psoriasis patients and up to 75% of patients with moderate to severe psoriasis [54]. LL-37-specific T cells produce pathogenic cytokines, including IL-17, and can be tracked in patient blood by tetramer staining.

41.3.2.4 Neutrophils in Psoriasis

Neutrophils are a first line of defense against an immune attack, possessing many intracellular antimicrobial peptides. In psoriatic skin, neutrophils are predominantly located in the epidermis, and there are abundant neutrophil chemokines, such as CXCL1, CXCL2, CXCL3, and CXCL8/IL-8 (Figure 41.2). In addition, neutrophils express IL-17, suggesting another potential role in psoriasis [58]. However, neutrophils are inconsistently found in chronic psoriasis lesions and are absent from some mouse models of psoriasis [4].

41.3.3 Key Inflammatory Molecules for Targeted Psoriasis Treatment

41.3.3.1 IL-12p40

In the past, T_H1 axis (IL-12 and IFN-γ) was considered to be the dominant pathogenic model for psoriasis [59]. T_H1 cells, producing IFN-γ, are abundant in psoriasis lesions and blood, and approximately 400 of the genes upregulated in psoriasis lesions can be traced to signal transducer and activator of transcription (STAT)1 activation, a key IFN-γ transcription factor [60] (Figure 41.2). Because IL-12, a heterodimer of p35 and p40, drives T_H1 polarization, an antibody against IL-12p40 (ustekinumab) was created to target IL-12 and inhibit T_H1 cells. This therapy proved immensely effective, as 70–80% of patients attained a 75% reduction in the Psoriasis Area and Severity Index (PASI) score (PASI75) within 24 weeks. The improvement was initially considered to validate the pathogenic role of the IL-12/T_H1 axis in patients with psoriasis [61]. However, the paradigm changed with the discovery of T_H17 cells, which is regulated by IL-23 [62,63]. IL-23 is composed of two subunits, p19 and p40 (shared with IL-12), and it became apparent that ustekinumab could be targeting either IL-12 or IL-23 in patients with psoriasis [64].

41.3.3.2 IL-23/T_H17

Selective antagonists to IFN-γ, IL-23, IL-17, and IL-22 were used to dissect contributions of polarized T-cell subsets (T_H1, T_H17, and T_H22 cells) to psoriasis immunopathogenesis [64]. Studies have shown that blockade of IL-17A (secukinumab and ixekizumab), IL-17 receptor A subunit (brodalumab), or IL-23p19 (guselkumab and BI 655066) can improve psoriasis (PASI75 response) in more than 80% of patients [65–69]. In contrast, IFN-γ and IL-22 have lesser pathogenic roles, given that single antagonism of these cytokines with neutralizing antibodies did not induce high-grade improvements in the majority of patients [70]. Therefore, the predominant pathogenic axis in psoriasis is IL-23/T_H17. IL-17 induces IL-19 and IL- 36γ in psoriasis lesions, which lead to epidermal hyperplasia [4] (Figure 41.2).

41.3.3.3 TNF-α

TNF-α can be produced by many cell types including keratinocytes, T cells, and dendritic cells [38,41,71]. There are synergistic effects between IL-17 and TNF, as a combination of the two cytokines induces greater changes in gene expression than either alone [27,72]. Role of TNF-α in psoriasis has been evaluated in clinical trials of TNF-α inhibitors (etanercept, infliximab, and adalimumab), and a key effect of TNF-α is thought to be regulation of antigen presenting cells, impairing dendritic cell and T-cell interactions [73,74]. As TNF-α activates IL-23 synthesis in dendritic cells, TNF-α blockade is linked to suppression of the IL-23/T_H17 axis [75] (Figure 41.2). TNF-α inhibitors are also highly effective in reducing skin inflammation as well as joint inflammation in patients with psoriatic arthritis.

41.4 Other Inflammatory Skin Diseases

41.4.1 Atopic Dermatitis

Atopic dermatitis is clinically different from psoriasis (Table 41.2) and mediated by different subsets (T_H2 in atopic dermatitis versus T_H17 in psoriasis). However, other T-cell subsets (T_H22, T_H17, and T_H1 cells) also contribute to atopic dermatitis, and there are many histologic similarities in chronic stages of atopic dermatitis and psoriasis, such as increased epidermal hyperplasia and large numbers of T cell and dendritic cell infiltrations [11,76]. Considering those commonalities, immune-based strategies that correct epidermal defects in psoriasis may be also effective in atopic dermatitis.

To control T_H2 cell-mediated inflammation in atopic dermatitis, monoclonal antibody to IL-4RA (dupilumab) has been studied as a potential therapy [77]. IL-4 and IL-13 are key effector cytokines of T_H2 cells and IL-4RA is a common receptor of those cytokines. Therefore, dual antagonism of IL-4 and IL-13 with dupilumab potently inhibits T_H2 effector responses [77]. Dupilumab also potentially blocks IL-17, because IL-4 is a key regulator of T_H17 activity in atopic dermatitis skin [77]. In early-phase studies, patients with dupilumab had a marked and rapid improvement in all the evaluated measures of atopic dermatitis disease activity, and side-effects profiles were not dose limiting [78].

Table 41.2 Clinical differences between atopic dermatitis and psoriasis [11].

	Atopic Dermatitis	Psoriasis
Onset	85% before age 5 year old	Adolescence or early adulthood
Asthma/atopy	Commonly associated	Not associated
Wet/dry skin	Oozing, wet skin with crusting and exudate	Dry skin
Demarcation	Not well demarcated from uninvolved skin	Sharply demarcated
Flexor/extensor	Flexors usually affected	Extensors usually affected
Scale	Fine white scales	Thick, silvery-white scales
Itching	Prominent	Rare
Impetigo	Frequent	Not evident
Viral dissemination	Common	No risk

41.4.2 Alopecia Areata

Alopecia areata presents as patchy, nonscarring hair loss, and approximately 10% of patients progress to alopecia totalis or even whole body hair loss (alopecia universalis) [79]. Alopecia areata is a T-cell-mediated inflammatory disease, but molecular profiles of cytokine pathways have not been fully elucidated [80]. We recently performed microarray and RT-PCR profiling of lesional and nonlesional scalp samples from alopecia areata patients and established alopecia areata transcriptomes: T_H1 axis (IFN-γ, CXCL10/CXCL9), T_H2 axis (IL-13, CCL18, CCL26, TSLP, and periostin/POSTN), T_H9/IL-9, IL-23 (p40 and p19), and IL-16 mediators were upregulated, while there were no increases in T_H17 and T_H22 axis. Those data suggests consideration of anti-T_H2, T_H1, and IL-23 targeting strategies for alopecia areata treatment. Similar to psoriasis and atopic dermatitis, clinical trials with selective antagonists are required to dissect key pathogenic pathways in alopecia areata.

41.5 Summary

Clinical and translational research in human subjects has enabled a better understanding of the skin inflammation in psoriasis, and subsequently the development of novel immune-targeted therapeutics [81]. The translational research strategies have been successfully applied to other inflammatory skin diseases, such as atopic dermatitis and alopecia, to discover potential therapeutic targets. However, many basic questions about skin inflammation remain to be answered. Further studies must confirm safety of long-term cytokine antagonism and elucidate tolerance mechanisms. Ultimately, immune signatures of various

skin inflammatory diseases could be characterized to accomplish personalized immune-targeted treatments of individual patients.

Disclosures

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Rheumatoid Arthritis and Other Inflammatory Articular Diseases

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42.1 Inflammatory Articular Diseases

Inflammatory articular diseases encompass a spectrum of conditions that range from acute forms of septic or sterile arthritis to chronic, often polyarticular conditions. They may involve inflammation of synovial tissue of joints and other tissues, such as muscles and tendons, mucosal and epithelial tissues, and organs. With regard to etiology, the inciting inflammatory stimulus has been defined in some conditions, for instance, in septic arthritis (bacteria) and gout (uric acid crystals), while in others the etiopathogenesis is incompletely understood, for example, in spondyloarthritis (SpA) and rheumatoid arthritis (RA). The acute onset of some conditions suggests predominant involvement of innate immunity and autoinflammation, such as in gout and arthritis in adult-onset Still's disease. In other conditions, an often slower onset and the presence of autoantibodies suggest a more dominant role for adaptive and autoimmunity, like in RA and systemic lupus erythematosus. In a third group of conditions, the spondyloarthritides, an intermediate onset, absence of autoantibodies and alterations in microbiota, suggest that a combination of innate and adaptive immune responses is involved. Thus, a wide range of inflammatory cells and mediators is involved in different inflammatory rheumatic diseases.

Another layer of complexity is added by the fact that there is large variability in etiology and pathogenesis within the group of autoimmune arthritides. Furthermore, most of these conditions represent syndromes classified based on signs and

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symptoms rather than pathogenetic entities. In spite of these limitations, research in the past decades has revealed a number of key immunologic mechanisms that drive inflammation in the different patient groups. Here, we will focus on the two most prevalent forms of chronic immune-mediated inflammatory disease of the musculoskeletal system: RA and SpA.

42.2 Rheumatoid Arthritis

42.2.1 Clinical Phenotype

RA is a chronic, systemic inflammatory disorder affecting synovial tissue in multiple joints [1]. The clinical presentation typically manifests itself as symmetric polyarthritis of the small joints of hand and feet, but any joint can be involved. Patients present with tenderness, swelling, and impaired movement of their joints. If untreated, the disease leads to damage of cartilage and bone, resulting in functional impairment. Extra-articular manifestations can also be observed, such as subcutaneous or pulmonary nodules, pleuritis, periodontitis, vasculitis, and interstitial pneumonitis [2,3]. In some patients, there are overlapping features with other immune-mediated inflammatory rheumatic diseases, such as Sjögren's syndrome and systemic lupus erythematosus. Over the past decades, treatment has markedly improved, because of the development of targeted therapies, a more aggressive, early treatment approach, and the introduction of the treat to target principle (aiming for disease remission) [4]. It should be noted however that there is still a big unmet need, as a significant proportion of patients do not achieve disease remission in spite of currently available treatments. Chronic use of immunosuppressive treatment is also associated with side effects. Therefore, development of new treatments, individualized health care, and innovation in treatment algorithms aiming for disease remission, cure, and prevention of the disease should define the research agenda for the next decade [5,6].

42.2.2 Etiology

RA may develop over years by interplay of genetic, environmental, and stochastic factors. Twin studies estimated the relative genetic contribution to RA to be around 60% [7]. Since 2007, genome-wide association studies have allowed the identification of >100 genetic risk factors for RA [8]. The largest genetic risk factor lies within the human leukocyte antigen (HLA) class 2 region and encodes the HLA-DRB1 molecule [9]. The alleles within HLA-DRB1 that are associated with RA encode amino acids that are part of the antigen-binding groove of the major histocompatibility complex (MHC) class 2 protein and are referred to as "the shared epitope." It is thought that these alleles enhance binding of specific antigens to MHC class 2 or the T-cell receptor and thereby lower the threshold of activation of specific T-cell clones [10]. The second largest genetic risk factor lies within the protein phosphatase nonreceptor 22 (PTPN22) gene [11]. It encodes lymphoid tyrosine phosphatase that negatively regulates downstream signaling from the T-cell receptor. Mutations within *PTPN22* confer risk or protection to multiple autoimmune diseases [12,13]. The majority of other candidate genes are

part of major, predominantly T-helper and B-cell associated, immunological signaling pathways, and include *CD40*, *CTLA4*, *CD28*, *TRAF6*, and the autoimmune regulator (*AIRE*) as well as genes involved in NF-κB signaling [13–15]. Interestingly, a number of these genes overlap with other autoimmune diseases [13,16,17]; they confer small effect sizes. A substantial proportion of genetic risk remains unexplained, suggesting that epigenetic inheritance of gene–environment or gene–gene interaction plays a role in RA development [18].

Besides the patient-related factors, female sex, and age, several environmental risk factors have also been associated with RA. The best established environmental factor for RA is cigarette smoking. An association has been found between ever smoking, the number of pack years, and RA [19,20]. Furthermore, obesity, parity, and diabetes mellitus have been associated with RA, while alcohol consumption and breastfeeding were associated with lower risk in some cohorts [19–23]. We have recently established in a prospective study that smoking and overweight increase the risk of development of arthritis in a cohort of autoantibody-positive individuals at risk for developing RA [19,24]. Several studies have shown a strong interaction between smoking and the development of anticitrullinated antibodies (ACPA) in shared epitope-positive subjects [20,25]. These autoantibodies may develop in response to inflammation at sites other than the synovium in genetically susceptible subjects, in particular in the lung and periodontium, while alterations in the gut microbiome may also play a role [26–30].

Current research focuses on the interaction between genes and environmental factors in different phases of the disease. Increasing evidence indicates that RA may start years before the onset of clinical arthritis, with a central role for autoreactive T and B cells. Autoantibody-positive RA is characterized by the presence of rheumatoid factor (RF), anticitrullinated peptide antibodies (ACPA), and other autoantibodies; they are found in around 70–80% of RA patients [31]. The earliest identified autoantibodies were termed RF and react with epitopes on the constant domain of immunoglobulin G [32]. Later, a number of other, less prevalent autoantibodies were defined, such as against glycoprotein 39, glucose-6-phosphate isomerase, and collagen type 2 and more recently against peptides that have been posttranslationally modified by carbamylation and acetylation [33–36]. A major breakthrough was the discovery of ACPA that recognizes epitopes of proteins that are posttranslationally modified by citrullination [37]. Citrullination is a physiological process that among others occurs in the context of inflammation. ACPA are directed against a number of epitopes, such as citrullinated fibrinogen, vimentin, enolase, and histones [38]. These have not yet been fully characterized and differ between patients. ACPA are highly specific for RA and are found in 50% of patients with early disease and 70–80% of patients with late disease [31,39]. Both RF and ACPA predict disease severity and radiological damage [39,40]. Studies in ACPA-positive versus ACPA-negative subjects suggest that these are distinct pathophysiological disease subsets. Specific polymorphisms in HLA-DRB1 have been associated with ACPA-positive RA and confer risk from progression of ACPA positivity to ACPA-positive RA [41]. ACPA-negative RA is also associated with HLA-DRB1, but less strongly and with only one of the four HLA-DRB1 polymorphisms that associate with ACPA-positive RA [42,43]. Also, some non-HLA genetic factors associate specifically

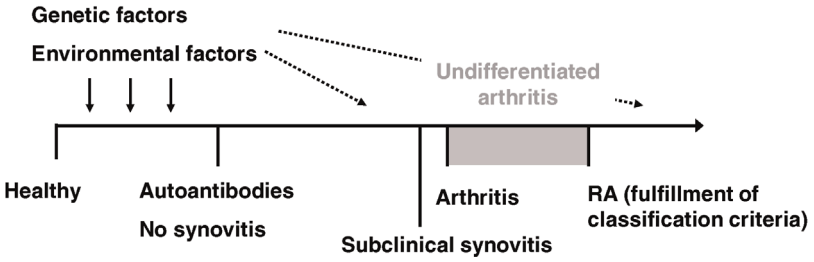


Figure 42.1 Timeline of autoantibody-positive rheumatoid arthritis (RA): the different stages of disease. Autoantibody formation may precede the development of clinical signs and symptoms of RA by several years. The presence of subclinical synovitis may probably last several weeks rather than months. (Reprinted with permission from Ref. [46].)

with ACPA-negative RA, such as CLEC16A and CLYBL. Of importance, RF and ACPA may be found years before onset of arthritis [44,45] (Figure 42.1).

In this phase, the ACPA response is not yet fully maturated. ACPA are of low avidity, have limited fine specificity, isotype distribution, and low titer up to a short interval before arthritis onset [47–50]. Their presence may concur with elevated levels of acute-phase proteins, chemokines, cytokines, and adipokines [51–53]. The recruitment of activated immune cells to the site of inflammation is generally preceded by a danger signal in a nearby lymph node followed by an immune reaction. Using ultrasound-guided core-needle biopsies of lymph nodes, we have recently demonstrated an increase in activated immune cells within lymph nodes of early arthritis patients as well as in autoantibody-positive individuals at risk for developing RA [24].

The presence of a type I interferon-activation status in peripheral blood mononuclear cells and the presence of increased numbers of T cells in peripheral lymph nodes appear associated with the development of arthritis in these individuals [24,54]). The synovial tissue of clinically and histologically uninflamed joints of patients with ACPA contain subtly increased numbers of CD8-positive T cells, most prominently in those individuals who subsequently develop arthritis after follow-up [55]. In some cohorts, smoking in subjects positive for the shared epitope is associated with ACPA positivity [56,57]. Given that smoking promotes citrullination of self-proteins, it might be directly linked to formation of ACPA in genetically susceptible subjects. Periodontitis, another possible risk factor for RA, has also been directly implicated in ACPA formation. Porphyroma gingivalis, one of the major bacteria involved in periodontitis, produces peptidyl-arginin deiminase (PAD), which mediates citrullination [58]. ACPA titers may be increased in the pulmonary and salivary secretions of some ACPA-positive individuals at risk for RA [59]. Taken together, the data suggest that autoantibody-positive RA may develop from a slow maturation of an aberrant autoimmune response in, for example, the lung through a second hit in the synovium, for instance, a viral infection or a trauma leading to citrullination in the joint, to autonomous disease progression in the joints [46]. Variation in the microbiome present in the gut has also been linked to RA, but clearly more research on individuals at risk of developing RA needs to be performed in a prospective manner to understand the importance of the relationship between the gut microbiome and the development

of RA [30]. Finally, recent research suggests that vagus nerve tone may modulate the immune response; reduced activity of the parasympathetic system is a predictor of arthritis onset in individuals at risk of developing RA [60].

42.2.3 Inflammatory Cells and Mediators Driving RA

Arthritis is the dominant clinical feature of RA, and is caused by inflammation of the synovial tissue that forms the inner layer of the joint capsule. The main function of the synovium is to provide nutrients to avascular structures such as the cartilage that lines the joint [61,62]. On a microscopic level synovial tissue consists of a number of cellular layers: the outermost layer is termed the intimal lining layer. It comprises one to three cell layers without a basal membrane. It consists of intimal macrophages and fibroblast-like synoviocytes (FLS). The synovial sublining consists of connective tissue with scattered FLS and contains relatively few cells and blood vessels. In patients with RA, the synovial tissue has become hypertrophic and is infiltrated with inflammatory cells. In the intimal lining layer, the number of intimal macrophages and FLS is increased. In the synovial sublining, FLS have proliferated and the amount of connective tissue is increased. Furthermore, it is infiltrated by variable numbers of T cells, plasma cells, macrophages, B cells, neutrophils, mast cells, natural killer cells, and dendritic cells [61–63]. This results in the formation of villous projections of synovial tissue into the joint space and overgrowth and invasion of synovial tissue at the junction with cartilage and bone. Proliferating synovial tissue in RA is referred to as pannus. The inflammatory response in RA involves both the innate and the adaptive immune system. Importantly, innate immunity and adaptive immunity are not two segregated systems, but these systems are largely intertwined and there is regulated cross-talk.

42.2.3.1 Innate Immunity and Stromal Cells

In RA, yet unknown factors activate innate immunity, thereby providing an adjuvant signal for the induction of adaptive immune responses that result in a breakdown of tolerance. The innate immune system involves many different cell types. In this section, we will discuss the most important players and their relative contribution to the inflammatory process in RA.

42.2.3.2 Fibroblast-Like Synoviocytes

FLS are mesenchymal cells that display many features of fibroblasts, including expression of collagen and CD90. However, they also express distinct molecules such as the adhesion molecule cadherin-11 that plays an important role in the formation of a defined intimal lining layer in diarthrodial joints [64,65]. Furthermore, FLS express uridine diphosphoglucose dehydrogenase, which is important to synthesize hyaluronic acid, a constituent of synovial fluid, and secrete lubricin, another critical protein for joint lubrication [66]. RA FLS create an abnormal stromal microenvironment that is thought to be crucial for the persistence of inflammation, not only by architecture, but also by secreting proinflammatory cytokines and chemokines, and by producing growth factors that stimulate neovascularization. These cells also secrete matrix metalloproteinases (MMPs)

that contribute to tissue destruction and receptor activator of nuclear factor-κB ligand (RANKL), which promotes osteoclast differentiation. In addition, RA FLS display unique features such as an invasive, tumor-like behavior that is normally not observed in fibroblasts [67,68]. This may be in part explained by epigenetic alterations, including changes in DNA methylation, histone methylation, or acetylation, and expression of microRNAs that affect gene expression [69]. FLS are able to interact with macrophages via the CD97/CD55 receptor–ligand system that may be involved in the spatial orchestration of the synovial lining layer and may contribute to maintenance and amplification of synovial inflammation [70], most likely by facilitating the retention of activated macrophages in the synovium [71]. Genetic deletion of CD55 or CD97 and targeting of CD97 with monoclonal antibodies in collagen-induced arthritis resulted in amelioration of disease [72,73].

42.2.3.3 Monocytes and Macrophages

Macrophages are derived from myelomonocytic cells and are key players of the innate immune system. In the inflamed RA synovial tissue, large numbers of macrophages are present, contributing to inflammation, which is substantiated by the observation that the number of CD68+ synovial sublining macrophages correlates with clinical signs and symptoms [63]. Clinical improvement after treatment is consistently associated with reduced macrophage numbers in the synovium [74]. The increased number of macrophages in RA synovial tissue can be explained by increased influx of circulating monocytes that differentiate locally in the inflamed synovial tissue, reduced efflux, enhanced local survival signals, reduced apoptosis [75], and local proliferation [76,77]. Using reinfusion of technetium-99m-labeled autologous monocytes in RA patients, we have demonstrated that monocytes migrate continuously into the inflamed synovial tissue, but at a relatively slow macrophage-replacement rate [78]. Consequently, blocking migration of monocytes to the inflamed synovial tissue may not be sufficient to induce clinical improvement unless cell egress is promoted as well. Macrophages are phenotypically heterogeneous, and are not only the main producers of proinflammatory cytokines like TNF, IL-1, IL-15, IL-18, IL-23, and IL-27 that play an important role in the persistence of inflammation, but they also generate inhibitory mediators such as IL-10, TGF-β, and soluble TNF-R (reviewed in Ref. [79]). Compared to other leukocytes, macrophages are more long-lived cells that also persist in inflammatory lesions during the resolution of inflammation and may actually contribute to this process [80]. Many macrophage subpopulations have been identified, mainly based on the expression of surface markers and/or production of certain cytokines [81]. For a long time macrophages have been characterized as either M1 (also known as classically activated/proinflammatory) or M2 (also known as alternatively activated/anti-inflammatory and involved in tissue repair). However, recently it has been recognized that macrophage populations are not fixed, but display plasticity and under certain circumstances can differentiate into one another [82]. Macrophages in RA synovial tissue can express a variety of markers, including those associated with M1 and M2 populations [79]. There is substantial heterogeneity in synovial macrophage populations and increased knowledge of the individual functions of these cells

may help to improve RA therapy by selective targeting of the proinflammatory macrophage subtype while leaving cells that contribute to resolution of inflammation unaffected.

42.2.3.4 Endothelial Cells

Endothelial cells (EC) play a crucial role in the pathogenesis of RA [83]. First, they express adhesion molecules and produce chemokines, thereby acting as the site of entry for immune cells into the synovial tissue [84]. In addition, EC proliferate and give rise to neovascularization, supplying oxygen and nutrients to activated stromal cells and immune cells, which contributes to the persistence of inflammation (reviewed in Ref. [85]). RA synovium contains a significant fraction of neoangiogenic, immature blood vessels [86]. In synovial tissue-containing lymphocyte aggregates, the so-called tertiary lymphoid structures (TLS), also highly differentiated EC, can be observed in high endothelial venules (HEV) [87]; these HEV may be central to the development of these structures [88].

42.2.3.5 Innate Lymphoid Cells

Innate lymphoid cells (ILC) are the most recently identified components of the innate immune system and have deserved a lot of attention over the last few years. ILC encompass both cytotoxic NK cells and lymphoid tissue inducer (LTi) cells, as well as more recently described noncytotoxic ILC populations. ILC are characterized by classic lymphoid cell morphology, but these cells lack the expression of cell-surface molecules that identify other immune cell types. The noncytotoxic ILC consist of three distinct groups: group 1 ILC (ILC1), group 2 ILC (ILC2), and group 3 ILC (ILC3), including LTi cells. Interestingly, the functional properties of ILC mirror to some extent those of CD4+ T cell subsets (Th1, Th2, and Th17) and depend on the same intracellular transcription factors: T-bet (ILC1), GATA-3 (ILC2) and ROR-γt (ILC3) [89]. ILC subsets have been identified in various inflammatory diseases, including RA, most notably ILC3 [88], but it is still early days for these cells and full characterization of the contribution of ILC subsets to the inflammatory response in RA remains to be determined.

42.2.3.6 Neutrophils and Mast Cells

In RA, neutrophils are mainly found in the synovial fluid [90]. Neutrophils possess great cytotoxic potential due to their ability to release both degrading enzymes and reactive oxygen species. In addition, neutrophils contribute to the production of cytokines and chemokines that drive inflammation, as well as to the regulation of immune responses via cell–cell interactions [91]. The notion that neutrophils are intimately involved in the clinical expression of the disease is supported by work showing that the development of clinical signs of RA is associated with increased synthesis of the chemokine CXCL8 (interleukin-8), which is a key driver of neutrophil migration [92].

Emerging evidence suggests that neutrophils may also contribute to autoimmune responses as they can release neutrophil extracellular traps (NETs)-containing chromatin associated with granule-derived enzymes. These NETs are not only capable of killing extracellular microorganisms but they also provide a source of autoantigens. This is relevant in the context of RA as citrullinated

proteins can be generated by neutrophil peptidylarginine deiminases, which replace arginine with citrulline residues [93]. The resulting citrullinated proteins can act as neoepitopes that may lead to loss of immune tolerance. As already discussed, ACPA can be detected before the onset of symptoms in patients with RA, and they are predictive of erosive disease. Furthermore, neutrophils from patients with RA have an increased propensity to form NETs containing citrullinated proteins, and sera from these patients contain autoantibodies recognizing these proteins [94,95]. Thus, in addition to their cytotoxic and proinflammatory role in RA, neutrophils may be a source of the autoantigens that drive the autoimmune processes underlying this disease [90]. Mast cells also contribute to synovial inflammation since they produce cytokines, chemokines, and proangiogenic factors, for instance, upon ligation of TLR [96].

42.2.4 Cross-Talk between Innate and Adaptive Immunity

Several cell types with predominantly innate immune functions may also induce adaptive immunity. These cells include the earlier discussed macrophages that are also capable of presenting antigen and inducing adaptive immune responses. Other cells that bridge innate and adaptive immunity are dendritic cells and γδT cells.

42.2.4.1 Dendritic Cells

Dendritic cells (DC) are professional antigen presenting cells (APC) and therefore not only have the capacity to stimulate innate immune responses (i.e., via TLRs) but they are also central for the induction of adaptive immune responses. DC may be categorized into various subtypes, including conventional myeloid DC (cDC) that are resident in lymphoid tissues and plasmacytoid DC (pDC). DC express MHC class II and costimulatory molecules such as CD80 and CD86 that interact with CD28 on T cells. This interaction usually takes place in secondary lymphoid organs (i.e., lymph nodes), but can also occur in local (inflamed) tissues [97]. DC can induce T-cell proliferation and differentiation into effector T cells (Th1, Th2, or Th17) or regulatory T cells (Treg). In RA synovial tissue, immature and mature DC subsets can be observed in close association with T- and B-cell follicles [98]. The number of pDC that are localized near perivascular lymphocytic infiltrates correlates with the presence of RF and ACPA. DC may contribute to ongoing inflammation through presentation of autoantigens and production of proinflammatory cytokines. DC can also have certain immunoregulatory functions in RA, such as the expression of the tryptophan depleting enzyme indoleamine-2,3-dioxygenase (IDO) or IL-10 [99], resulting in the capacity to induce tolerance (i.e., via the induction of Treg) [100]. The important role of DC–T-cell interaction in RA is substantiated by the beneficial clinical effect of CTLA4-Ig treatment, which blocks costimulation [101]. Interestingly, dendritic cells might also be used for immunotherapy in RA as a recent exploratory study demonstrated safety and biological activity after a single injection of autologous modified DCs exposed to citrullinated peptides in HLA risk genotype-positive RA patients [102].

42.2.4.2 γδ T Cells

γδ T cells are T cells that unlike most T cells do not express the TCRαβ chains, but γδ chains instead. They develop in the thymus and express a large diversity of antigen-binding TCRs. γδ T cells are mainly distributed in mucosal and epithelial tissues, and account for less than 5% of the total T cells in the peripheral blood. These cells respond more rapidly than αβ T cells. Upon activation, γδ T cells may express the IL-23R and IL-1R [103]. γδ T cells do not only produce proinflammatory cytokines such as IL-17 and IFNγ but they can also display antigen presenting functions through expression of antigen-presenting and costimulatory molecules, such as HLA-DR, CD80, CD86, and CD40, which is sufficient for strong induction of naïve αβ T-cell proliferation and differentiation [104]. Interestingly, DC and γδ T cells can also interact with one another, thereby mutually promoting each other's maturation and function through release of cytokines [105]. γδ T cells can also have immunoregulatory effects, for example, by suppressing the proliferation of CD4+ effector T cells under certain conditions [106]. In RA, γδ T cells have been demonstrated in the synovial tissue [107,108]. In the context of RA, it is important to note that γδ T cells have recently been demonstrated to inhibit human osteoclast formation and resorptive activity *in vitro* [109].

42.2.5 Adaptive Immunity

Despite the presence of autoantibodies from an early disease phase, genetic risk factors in adaptive immunity and the effect of B- and T-cell-directed therapies, it has not been formally proven that autoreactive B and T cells drive RA, nor which adaptive immune mechanisms prevail in each patient and disease phase.

42.2.5.1 T Cells

T cells and T cell-derived cytokines are present in RA synovial tissue from onset of arthritis [63,110]. These cytokines include TNF, IFNγ, GM-CSF, IL-22, IL-17A, and IL-17 F [111–115]. About half of synovial tissue T cells are expanded clones that are frequently shared between different joints [116]. Initially, it was thought that T helper 1 (Th1) cells were the main infiltrating subset, but after the discovery of Th17 cells and their detection in RA synovial tissue a larger role was attributed to these cells [117–119]. Th17 cells are increased in peripheral blood and synovial fluid of RA patients, also in early disease [120–122]. Th17 cells attract and activate monocytes and neutrophils and stimulate osteoclastogenesis [123]. *Ex vivo* Th17 cells, but not Th1 cells from RA patients, induce RA FLS to produce MMPs, IL-6, IL-8, and autocrine IL-17A production [121]. These effects are modulated by cyclo-oxygenase 2 and vitamin D [124,125]. Despite these data, IL-17A and IL-17 receptor A blocking agents were moderately effective at most in RA clinical trials [126–128]. Possible explanations might be found in mouse models of arthritis where inhibition of IL-17A was more effective early compared to later in the disease course [129,130]. In one model, IL-23-activated Th17 cells expanded together with B cells in germinal centers before arthritis onset and downregulated sialyltransferase in plasma cells in an IL-22- and IL-21-dependent manner. This resulted in proinflammatory glycosylation in autoantibodies and

autoantibody-mediated, but IL-23- and IL-17A- independent, arthritis in the effector phase [131]. Furthermore, Th17 cells have been demonstrated to exhibit plasticity and can transdifferentiate into regulatory T cells (Treg) during the resolution of inflammation. Consequently, IL-17 blockade may result in less Treg activity [132]. Another clue might lie in the recent identification of different Th17 subsets. These consist of classic Th17 cells, Th22 cells, and Th17.1 cells. The debate on the existence of Th22 cells as a separate subpopulation from classic Th17 cells in humans has not yet been resolved [133,134]. In RA, IL-22 is a growth factor for FLS and it plays a role in the communication with stromal cells to mediate terminal B–cell differentiation and production of autoantibodies that might be involved in osteoclastogenesis [135,136]. However, in an *ex vivo* coculture model of FLS with Th22 and Th17 cells, there was only a limited role for IL-22 in sustaining inflammation [136]. Th17.1 cells have both Th17 and Th1 characteristics. These cells express both IL17A and IFNγ and are found in relatively high numbers in peripheral blood and synovial tissue of RA patients [123,137,138]. They express high levels of GM-CSF and other proinflammatory mediators and are therefore hypothesized to be the most pathogenic Th17 subset. There is mounting evidence for conversion of classic IL17A+ cells into IL17+IFNγ+ and IFNγ+ T helper cells [139]. Of interest in this context, citrullinated peptide-specific T cells were identified in peripheral blood of RA patients that expressed a Th1 phenotype [140]. It remains to be determined what the phenotype of the dominant pathogenic T cells is, and whether this differs between patients, and between different phases of the disease.

42.2.5.2 B Cells

It is an open question to what extent RA is driven by RF, ACPA, or other autoantibodies and this might differ between tissues, such as cartilage, synovial tissue, and bone [141,142]. RA fluids and tissues contain increased numbers of B-cell products, such as free light chains and immune complexes that react with multiple autoantigens [141,143]. Immune complexes containing RF and ACPA have been shown to mediate increased proinflammatory cytokine production and enhance osteoclastogenesis *in vitro* [142,144]. The B-cell subset from which autoreactive B-cell clones originate is also under discussion. RA patients have been shown to have defects in early B-cell central tolerance checkpoints irrespective of disease activity [145,146]. Furthermore, RA patients have increased numbers of naturally occurring autoreactive B cells that show signs of diminished anergic tolerance [147]. Citrullin-specific B cells circulate at relatively high frequencies [148]. In the synovial tissue, B cells are only present in a proportion of patients, which suggests they may mainly reside in lymphoid tissue [149]. When present in synovial tissue, B cells are part of lymphocyte aggregates, together with T cells, surrounded by plasma cells in TLS [149]. A proportion of these B cells represent expanded clones that are highly enriched from blood [150]. In several studies a significant proportion (25–40%) of synovial tissue B cells from ACPA-positive patients were citrullin specific [151,152]. T-cell activation in TLS has been shown to be dependent on the presence of B cells [153]. It is controversial whether TLS amplify and refine local autoantibody

production [154], or occur secondary to the chronic inflammatory processes driving RA [149,155]. The B-cell depletive agent rituximab is only effective in a subset of patients, which might be explained by failure of rituximab to induce depletion of switched memory B cells in synovial and lymphoid tissue, together with the presence of inhibitory factors in clinical poor responders that remain to be identified [156].

Variability in clinical response to rituximab and other biological agents suggests the existence of RA subsets driven by qualitatively different autoimmune responses. New biomarkers predictive of the clinical response to treatments have been identified, which could provide further insight into the disease mechanisms in different subsets of RA. For instance, clinical response to rituximab is associated with high systemic levels of MRP 8/14 at baseline, whereas poor response has been linked to persistence of synovial tissue plasma cells, plasma cell products, and type 1 interferon signaling in peripheral blood mononuclear cells [143,156,157].

42.2.6 Structural Changes in RA: Cartilage Damage and Bone Erosions

The proliferating FLS and pannus are the major causes of cartilage damage in RA. FLS produce MMPs and other matrix degrading enzymes that can diminish cartilage integrity, particularly when endogenous inhibitors such as TIMPs fail to counterbalance MMP activity. Immune cells contribute to the destruction of bone and cartilage by the production of cytokines such as IL-17A and IL-1 together with reactive nitrogen species, causing apoptosis of chondrocytes resulting in destruction of cartilage and joint-space narrowing. Bone erosions occur rapidly in RA, are associated with inflammation, and are mainly caused by osteoclasts [158,159]. Osteoclasts, large multinucleated cells derived from the monocyte lineage, are the only cells capable of resorbing bone in the body. These cells are viewed as key innate immune cells in bone, as they express innate immune receptors, analogous to macrophages and dendritic cells, which regulate their ability to respond to inflammation in the joint, and direct their fate and function. In RA, development of osteoclasts occurs locally in the synovial tissue as a result of the expression of essential osteoclastogenic mediators, macrophage colony-stimulating factor 1 (M-CSF), and receptor activator of nuclear factor κB ligand (RANKL). In RA, osteoclasts are situated at the interface of inflamed synovial tissue and the periarticular bone surface, causing local bone destruction [160,161]. The main triggers of articular bone erosion are synovitis, including the production of proinflammatory cytokines and receptor activator of nuclear factor κB ligand (RANKL), as well as antibodies directed against citrullinated proteins [162]. Spontaneous repair of erosions rarely occurs and even potent anti-inflammatory therapeutic strategies, such as blockade of TNF or IL-6R, only results in limited signs of repair of bone erosions (reviewed in Refs [160,163]). Lack of repair is due, at least in part, to active suppression of bone formation by proinflammatory cytokines. More knowledge on the molecular pathways involved in bone loss and bone formation during inflammation is needed to facilitate the development of new targeted therapies to induce repair of bone erosions.

42.3 Spondyloarthritides

42.3.1 Clinical Phenotype

SpA are a group of related inflammatory arthritides that involve synovitis and enthesitis. They are related to and may involve psoriasis, inflammatory bowel disease (IBD), and uveitis. The main clinical syndromes are reactive arthritis that occurs after specific infections, psoriatic arthritis (PsA), arthritis associated with IBD, juvenile spondyloarthritis, undifferentiated spondyloarthritis, and ankylosing spondylitis (AS). AS primarily involves the axial tissues with inflammation of the ligamentous attachments and sacroiliac joints resulting in pain, stiffness, and immobility. It can be difficult to differentiate between these disorders because their clinical features may overlap. Different SpA subtypes may be found in different members of the same family and single patients may develop various clinical features over time. In contrast to RA, most types of SpA and especially AS are associated with new bone formation. PsA is not only associated with new bone formation but it can also involve bone erosions in a pattern different from RA. SpA treatment has markedly improved over the last decade, with the advent of novel targeted medicines. There is variability in the treatment response, irrespective of clinical phenotype, which suggests that the immunological mechanisms that drive SpA subtypes differ between patients.

42.3.2 Etiology

SpA is not associated with polymorphisms in the classical autoimmune genes, but is rather linked to alterations in genes involved in innate immune pathways, such as CARD9, and cytokine pathways including TNF, IL-1, and IL-23/IL-17 [164]. The lack of prototypic autoimmune features and the modest therapeutic efficacy of costimulation blockade (CTLA4-Ig) [165,166] and B-cell depletion (anti-CD20 therapy) [167,168] fuel the hypothesis that the pathogenesis of the disease is not primarily driven by altered adaptive immune responses, but rather by autoinflammatory pathways. The strong link with the genetic background and the importance of bacterial infections and mechanical stress in the development of the disease underscore this hypothesis. Twin studies have demonstrated that familial aggregation is more related to genetic than to environmental factors in AS [169,170]. Other SpA subtypes are also characterized by a strong genetic predisposition and susceptibility genes include HLA-B27 and IL-23R [164,171,172]. The major genetic risk factor is HLA-B27, an MHC class I molecule, and 80–90% of AS patients are positive for this HLA subtype. However, HLA-B27 only explains 20–40% of the genetic susceptibility to AS. In earlier times, it was hypothesized that HLA-B27 is involved in the presentation of specific bacterial peptides to cytotoxic T cells, resulting in activation of these cells and cross-reactivity with self-peptides [173]. However, it appears that HLA-B27 has a tendency to misfold inside the endoplasmatic reticulum, which may generate an unfolded protein response leading to the activation of NF-κB signaling, followed by increased production of proinflammatory mediators [174]. Furthermore, ER stress has been demonstrated to increase IL-23

production after TLR stimulation [175], which is also implicated in the pathogenesis of AS [176]. Therefore, HLA-B27 may have an intrinsic auto-inflammatory role. Tissue stress may contribute to the development of SpA. This is substantiated by the association between SpA and mechanical stress (development of inflammatory lesions at sites of high mechanical stress such as spine, entheses, and larger joints) and bacterial infections (in reactive arthritis after genitourethral or gastrointestinal infections). Consequently, it has been postulated that HLA-B27 in combination with mechanical stress or bacterial triggers leads to abnormal innate immune responses [177].

42.3.3 Cells and Mediators Driving Spondyloarthritides

Based on the susceptibility genes and the limited effect of targeted therapies that interfere with costimulation of B cells, the main driving force in SpA may be the innate immune system, including alterations in TNF and IL-23 signaling resulting in abnormal IL-17 responses [176,178]. This is substantiated by the overall beneficial effects of TNF blockade and IL-17 targeting therapies in SpA [179–184]. Detailed analysis of the inflamed synovial tissue of patients with peripheral SpA has provided further insight into the cell types and mediators of inflammation contributing to the disease. However, detailed analysis of the target tissue associated with remodeling, leading to osteoproliferation and ankylosis, has been hampered by the fact that it is difficult to obtain such tissues for in-depth analysis in humans.

42.3.4 Innate Immunity and Stromal Cells

42.3.4.1 Fibroblast-Like Synoviocytes

In many ways, FLS seem to be similar in RA compared to SpA, including surface expression of CD46, CD59, and the complement-regulatory protein CD55, as well as in cytokine production [185]. However, recently a novel and disease-specific myogene signature was identified in SpA synovial tissue and FLS, which includes genes involved in PDGF, TGFβ, and Wnt signaling [186]. These profibrotic pathways may contribute to osteoproliferation, which is a distinct feature of SpA.

42.3.4.2 Monocytes and Macrophages

SpA is characterized by a relative increase in the number of CD163+ macrophages compared to RA [187,188], although not in all studies [189]. The exact functional consequences of this possible difference remain to be determined, but M2 macrophages (associated with CD163 expression) have been demonstrated to correlate with disease activity and HLA-B27 positivity in SpA [187], as well as with subclinical ileal inflammation in AS, which is distinct from Crohn's disease [190]. In addition, CD163 expression was also associated with reduced lymphocyte activation as characterized by lower CD69 levels on synovial tissue T cells [187]. In line with this, synovial fluid from SpA patients contains less proinflammatory "M1" cytokines such as TNF, IL-1, and IL-12p40 compared to RA patients, whereas the "M2" cytokines IL-4, IL-13, and IL-10 are similar, which may result in a shift in the M1/M2 balance toward M2 polarization [191]. Besides these changes

in macrophage polarization, monocyte-derived macrophages from AS patients also exhibit differences in gene expression compared to healthy controls with a striking "reverse" IFN signature, which together with poor expression and responsiveness of the IFN gamma gene may suggest IFN gamma gene dysregulation as a contributor to disease pathogenesis [192].

42.3.4.3 Endothelial Cells

Compared to other inflammatory joint diseases, synovial tissue of SpA patients exhibits markedly increased neovascularization [193–195] and SpA patients have high serum levels of vascular endothelial growth factor (VEGF) [196], which is indicative of increased angiogenic activity in this disease. Angiogenesis not only contributes to inflammation by supplying oxygen and nutrients to activated stromal cells and immune cells, but activated EC are also involved in the active recruitment of new immune cells to the site of inflammation. In addition, two mechanisms have been described by which EC may contribute to new bone formation. First, EC can transform into mesenchymal stem cells that ultimately further differentiate into bone cells, a process called endothelial–mesenchymal transition (EndMT) [197]. Second, EC can recruit not only immune cells but also cells with broader differentiation ability, including osteoprogenitors [198]. Consequently, EC may not only contribute to the perpetuation of inflammation, but they may also be involved in pathological new bone formation in SpA.

42.3.4.4 Innate Lymphoid Cells

The contribution of ILC to the pathogenesis of SpA has as yet not been studied in great detail, since these cells have been described only recently. A recent study investigated ILC populations in the gut, peripheral blood, synovial fluid, and bone marrow of AS patients. There was expansion of ILC3, but not ILC1 and ILC2, in all of these compartments. Interestingly, these cells expressed IL-23R and produced both IL-17 and IL-22 [199]. Based on detailed analysis of gut biopsies, the authors hypothesized that ILC3 differentiate in the gut and may subsequently migrate to the inflamed joints due to the expression of specific integrins. More studies on ILC are required to draw firm conclusions on the role of these cells in AS and other SpA subtypes.

42.3.4.5 Neutrophils and Mast Cells

SpA synovitis is characterized by an increase in neutrophils and mast cells compared to RA [178,195,200]. Neutrophils could contribute to synovial inflammation, since accumulating evidence suggests that the majority of the IL-17 released in inflammatory arthritis is produced by innate immune cells, including neutrophils and mast cells, rather than T cells [201,202]. In SpA mast cells are not only increased in numbers but these cells also express more IL-17 than mast cells in RA. Targeting mast cells via a c-Kit inhibitor resulted in decreased levels of IL17 ex vivo [178].

42.3.5 Cross-Talk between Innate and Adaptive Immunity

The pathogenesis of SpA involves both arms of the immune response and is at the crossroads of immunity. Cells that bridge innate and adaptive immunity, such as γδT cells and dendritic cells, are thought to play an important role.

42.3.5.1 Dendritic Cells

Dendritic cells (DC) have been less studied in SpA compared to RA. These cells probably not only contribute to the pathogenesis of SpA via their direct effect on T cells, but also via proinflammatory cytokine production, including IL-12 and IL-23 [203]. *In situ* analysis of IL-23-positive cells in the spine of AS patients revealed that their numbers are significantly increased in the subchondral bone marrow of the facet joints compared to OA patients or normal control specimens obtained at autopsy. These IL-23-positive cells also comprised DC. Furthermore, the frequency of IL-23-positive cells was significantly higher than that of IL-12-positive cells in AS patients [204]. Monocyte-derived DC from HLA-B27+ axial SpA patients display altered functional capacity and deregulated gene expression. As a consequence, the capacity of these DC to stimulate CD4+ T cells was decreased [205]. Secretion of IL-12 and IL-23 by monocyte-derived DC from AS patients after TLR stimulation was however comparable to healthy controls [203]. Together, DCs are likely to contribute to the pathogenesis of SpA via IL-23 secretion, rather than via induction of effector T-cell responses.

42.3.5.2 γδT Cells, Invariant Natural Killer T Cells (iNKT), and Mucosal-Associated Invariant T (MAIT) Cells

There is increasing evidence that innate-like T cells, such as γδT, iNKT, and MAIT cells that function as a bridge between innate and adaptive immunity, may be involved in SpA pathogenesis [206]. MAIT cells express a genetically determined, restricted T-cell repertoire. They are more abundant in humans compared to mice and under physiological circumstances mainly reside in mucosal tissues and the liver. Their TCR reacts with nonpeptide antigens, such as bacterial vitamin metabolites, that are bound by the nonclassical class I antigen-presenting molecule MR1. They can also be activated by cytokines secreted by innate immune cells independent of TCR. IL-17-producing MAIT cells are relatively increased in blood and synovial fluid of patients with AS compared with healthy controls and patients with RA. Recently, it was demonstrated that circulating IL-17 secreting IL-23R expressing γδT cells are significantly increased in patients with active AS compared to healthy controls and RA patients [207]. In psoriasis patients, there is an increase in these cells in psoriatic skin with a reduction of the number of circulating γδT cells, suggestive of redistribution from the peripheral blood to the affected skin [208]. The dermal γδ T cells in psoriasis are poised to secrete IL-17 [209]. These data support the notion that γδT cells play a pathogenic role in psoriasis and they might also contribute to inflammation in PsA since these cells can also be found in synovial fluid of PsA patients [210].

42.3.6 Adaptive Immunity

It remains to be determined to what extent adaptive immunity is involved in SpA and whether this is based on a disturbed, overreactive alloimmune response or autoimmunity. SpA is often referred to as seronegative because of the absence of autoantibodies. However, recent work has suggested that autoantibodies against the bone remodeling molecules sclerostin and noggin as well as against the invariant chain peptide (CLIP/CD74), involved in MHC class II antigen loading,

may be involved [211,212]. The notion that humoral immunity is involved in some patients with SpA is supported by an open-label clinical trial in 20 patients with axial SpA using the anti-CD20 antibody rituximab, which was associated with improvement in 8 patients [167]. The association with the MHC class I allele HLA-B27 could suggest the involvement of CD8+ T cells. However, the search for CD8-restricted arthritogenic peptides did not yield any candidates. HLA-B27 may be involved in a different way, independent of CD8+ T cells, as HLA-B27 overexpressing rats develop disease independent of these cells [213]. Similarly, overexpression of TNF in mice is sufficient to generate a disease phenotype in the absence of B and T cells [214]. Besides HLA-B27, many candidate risk genes for SpA are involved in antigen presentation and T-cell activation. For instance, the risk genes ERAP1 and ERAP2 encode aminopeptidases that trim peptides to optimally fit in the antigen binding groove of MHC class 1 [215]. Polymorphisms in these genes may influence the capacity to present pathogenic peptides [216–218]. Alternatively, they may affect autophagy-associated HLA-B27 misfolding, endoplasmatic reticulum stress, or altered HLA-B27 surface expression that have also been implicated in SpA and that may induce activation of innate or innate-like immune cells [217–219].

Circumstantial human biologic evidence for a role of adaptive allo- or autoimmunity derives from studies of patient samples. Peripheral blood and synovial fluid of SpA patients contain elevated levels of IL–17A, IL-6, TGF–β, IFN–γ, and IL-22, and increased numbers of Th17 and Th22 cells [220–222]. Several studies suggest involvement of different T-cell subsets in different disease tissues and phenotypes. In a study in PsA and psoriasis patients Th17 and Th22 cells numbers were increased in peripheral blood in both conditions compared to healthy controls, but in synovial fluid of PsA patients only Th17 numbers were increased while Th22 numbers were lower compared to blood and IL-22 was absent in the synovial tissue [223]. In another study in PsA, there was proportionally more infiltration of CD4+ to CD8+ T cells in synovial tissue, while this ratio was reversed in synovial fluid [224]. In another study that compared PsA to RA, the number of CD4+ Th17 cells was increased in synovial fluid compared to blood in both conditions, but the number of synovial fluid IL-17A producing CD8+ T cells was only increased in PsA [225]. The latter correlated with disease activity and development of joint erosions. Th17 cells may contribute to development of bone erosions by stimulating osteoclastogenesis through RANKL, TNF, and/or IL–17 [123]. Of interest, a study that focused on IL-17 expression in inflamed facet joints of SpA patients found predominant expression of IL-17 by neutrophils and to a lesser extent by T cells and mast cells [201]. A recently developed mouse model supports the role of dysregulated adaptive immunity in SpA in the context of IL-23-driven innate immune activation [226]. This mouse model has a deficiency in the ZAP70 T-cell receptor-signaling protein, which leads to diminished central tolerance, alterations in the T-cell repertoire, and survival of autoreactive clones. After injection with the fungal component β-glucan, these mice develop a full SpA phenotype consisting of arthritis, enthesitis, spondylitis, uveitis, and Crohn's disease-like terminal ileitis. Arthritis and spondylitis were dependent on CD4+ T cells and IL-23 in this model, while enthesitis and ileitis depended on IL-23; enthesitis also required IL-17 and IL-22 [226,227]. Clinical

trials with IL-17A and IL-23 blocking agents have provided further experimental evidence for the role of these cytokines in the pathogenesis of SpA, although there was a variable response among different SpA-related conditions. Further investigations are expected to clarify the differences in the immune mechanisms that drive these interrelated conditions and whether adaptive immunity is involved through primary defects or whether it is secondary to defects in innate immunity. A picture emerges in which adaptive immunity modulates the disease process but it may not be necessary for the development of its signs and symptoms. This is illustrated by the fact that mice can develop a disease that resembles SpA in the absence of both T and B cells, as demonstrated in mice overexpressing TNF [214].

42.3.7 Bone Remodeling in SpA

In contrast to RA, SpA is characterized by osteoproliferation rather than bone degradation and joint erosions. Also, bone remodeling in SpA occurs to a large extent independent of (TNF-driven) inflammation as syndesmophytes and ankylosis may progress when inflammation is effectively blocked by anti-TNF therapies [228]. However, these processes may depend on other inflammatory pathways, involving cytokines such as IL-17, and clinical trials to formally study this notion are underway. Various hypotheses have been proposed with respect to the striking tissue remodeling that is observed in SpA, but research to further elucidate the mechanisms involved has been hampered by the difficulty to obtain tissues for detailed molecular analysis. Structural features of SpA may relate to important pathways of enchondral bone formation such as signaling by bone morphogenetic proteins (BMPs) and the Wnt pathways [229,230], but evidence for this is largely based on results obtained in animal experiments [231,232]. Stromal cells and mechanical stress appear to play a key role in the disease process. Recently, a novel and disease-specific myogene signature was identified in SpA synovitis, which included genes involved in TGFβ/BMP signaling. This signature was not modulated by TNF blockade [186]; the effects of IL-17-targeted therapies on this signature remain to be determined.

In conclusion, our knowledge of the mechanisms underlying bone remodeling in SpA is incomplete, but pathological bone formation is probably triggered by a combination of the inflammatory response and mechanical stress. Advanced understanding of the altered bone biology in SpA could facilitate development of novel targeted therapies to block osteoproliferation and prevent structural damage in patients.

42.4 Conclusions

In this chapter we have discussed the molecular and cellular mechanisms that contribute to synovial inflammation in inflammatory articular diseases with a focus on RA and SpA. We have highlighted both similarities and differences in the pathogenesis of these diseases, which may not only advance our understanding of the pathogenesis of these conditions but it may also help explain differential treatment responses: targeting IL-6R and B cells improves disease in RA, whereas

these treatments do not substantially ameliorate disease in SpA. For targeting IL-17 the case is reverse: excellent effects in SpA, but only modest effects in RA patients.

With the advent of new targeted therapies and a "reverse immunology" approach we may be able to further classify these diseases and build a new taxonomy [233,234]. This will enhance our understanding and simultaneously result in earlier appropriate treatment for patients. In addition, we notice a shift in the focus of research and treatment efforts to the early and preclinical phase of disease, with the objective to use the existing "window of opportunity" to either prevent disease or delay the onset of disease [6,235]. We expect that new treatment algorithms and preventive strategies will be developed in the coming years, which will be of great benefit for patients with inflammatory joint diseases or individuals at risk of developing these diseases.

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Missing Heritability of Crohn's Disease and Implications for Therapeutic Targeting and Improved Care

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43.1 Crohn's Disease: A Public Health Issue

Crohn's disease (CD) is a common relapsing–remitting intestinal illness that mainly affects young adults in industrialized countries. The CD patient's quality of life is profoundly compromised by severe diarrhea, abdominal pain, fever, weight loss, malnutrition, and bleeding. CD is associated with transmural inflammatory lesions that can potentially concern any part of the gastrointestinal tract. Some CD patients can also experience additional complications such as fistulae, abscesses, bowel strictures, and/or extraintestinal manifestations (mostly involving the eyes, skin, joints, and liver) [1]. While Crohn's disease can impair a patient's ability to work and his/her social interactions in various ways, its lifetime therapeutic management is far from optimal. In particular, invasive surgery and bowel resection is required in about two-thirds of Crohn's disease patients but it does not cure the disease. In addition, an overall 25–30% therapy refractoriness is seen, regardless of the biologics (most often antibodies against TNF-alpha) and/or immunosuppressive drugs, resulting in an accumulation of adverse events, including malignancies and serious infections [2]. Intriguingly, in Europe, the combined prevalence of CD is about 250–300 cases per 100 000 inhabitants, which results in as many as 2.2 million CD patients. The incidence is also on the rise in emerging countries, due to the influence of many genetic and environmental factors. Consequently, the annual economic burden of medical care of Crohn's disease patients ranges between €2.1 and 16.7 billion. In this context, there is an urgent need for more effective therapeutic interventions, which minimize complications and reduce the need for surgery in CD.

43.2 An Imperative Need for Understanding Missing Heritability in Crohn's Disease

A better understanding of the so-called missing heritability in CD remains a prerequisite for the overall disease understanding. Soon after its clinical

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description in the 1930s by Dr. BB. Crohn and his colleagues, epidemiological observations of patients with a family history of CD indicated that such an intestinal illness is a complex trait likely influenced by both environmental and genetic factors. Meanwhile, early-onset CD patients have more frequently a familial history of CD and experience more severe disease phenotypes than adults, suggesting a higher genetic load among familial cases of CD. Consistently, large European twin registries provided a valuable resource for estimating the heritability of CD. A greater concordance rate on disease location was revealed among identical twins when compared to fraternal twins, encouraging for the search of the causal genetic variations driving the association signal. Thanks to technological advances in genotyping, linkage studies provided some of the molecular basis for the involvement of genetic epistasis (also referred to as gene–gene interactions). Since the first genome-wide association study (GWAS) in 2005, over 160 risk alleles were identified by including additional cohorts and by performing meta-analysis. Besides patients with very-early-onset inflammation that have Mendelian-like form of CD [3], about 13.6% of the inherited risk of developing CD in adults is estimated to result from the combination of all risk alleles among which 16% are solely nonsynonymous variants [4]. Even if not all polymorphisms of the human genome have been studied so far, a bioinformatic compilation of GWAS has shown that nonsynonymous and synonymous coding single nucleotide polymorphisms have similar likelihoods and effect sizes for the association with human disease, as measured in the HapMap 3 project [5–7]. About 90% of alleles that are predisposing to such inflammatory condition are located in noncoding areas of the genome, as shown in the ENCODE project [8], indicating that the exome sequencing may not allow identifying the responsible susceptibility variants in most patients. These loci in noncoding regions presumably contribute to disease predisposition by regulating the expression of proteins that are relevant to intestinal homeostasis. Indeed, a unique, single synonymous variant may result in miRNA misuse, which predisposes to CD by altering the stability of the mRNA (rather than by deregulating the function) as what elegantly shown for the immunity-related GTPase M [9]. Meanwhile, whole-genome sequencing techniques revealed sequence differences that may explain some of the discordance between monozygotic twins. While copy number variants that predispose to CD are unlikely to explain all of the missing heritability of CD [10], more than ~36% of CD predisposing polymorphisms are expression quantitative trait loci (noted as eQTLs) [11] that may contribute to interindividual heterogeneity in gene expression. For example, the common risk allele C at SNP rs7746082 correlates with the reduced expression of the PR domain containing 1, with ZNF domain gene (encoding for the PRDM1 protein) in both ileal biopsy specimens and peripheral blood mononuclear cells of CD patients [12]. More recently, chromatin immunoprecipitation sequencing (ChIP-Seq) revealed an enrichment of several epigenetic marks in specific cell types of CD patients [13], including cell-specific active enhancer (e.g., H3K27Ac) or promoter (e.g., H3K4me1). Furthermore, a genome-wide methylome analysis revealed a link between a commonly observed complication of CD patients and differential DNA methylations that were primarily found within intergenic regions and introns [14]. Abnormal epigenetic reorganization of chromatin from CD patients may also

contribute to hyper/hypomethylation of promoters, modification of histone tails, and changes in miRNA expression of several genes that play a key role in intestinal homeostasis, such as immunity-related GTPase M [15]. While histone tail changes and methylation of promoters will allow transcriptional factors access to genes in an on–off mechanism, miRNA will optimally control gene expression as a remote control mechanism of cell function specificities, including cellular response to the CD-associated adherent-invasive *Escherichia coli* [9]. Furthermore, a loss of binding is predicted for hsa-miR-652 or hsa-miR-414 binding in the presence of the NOD2 risk haplotypes carrying the T allele at rs2066843 or the CARD9 risk haplotypes carrying the G allele at rs10781499, respectively, and the GTEx project [16]. In this context, studying the genetic of very-early-onset CD will provide information that promises a better understanding of the overall missing heritability of CD, while additional GWAS are unlikely to provide any additional valuable information on variants with larger effects.

43.3 Immunodeficiencies in Phagocytes as a Specific Cause of Crohn's Disease

Despite extensive efforts, only a few CD-predisposing genes have been clearly identified (including the genes encoding for the nucleotide-binding oligomerization domain 2, autophagy-related 16 like 1, and immunity-related GTPase M) [17–20]. In the gut, most of the aforementioned risk alleles are expressed by the mononuclear phagocyte system [21,22] that is mainly composed of monocytes, dendritic cells, and macrophages. The intestinal steady-state mononuclear phagocyte compartment comprises two types of conventional dendritic cells (CD11b− and CD11b+), both of which express the alpha E integrin CD103. In addition to these short-lived phagocytes, the steady-state lamina propria harbors a large population of resident, nonmigratory $CD11c^{lo}$ $CX3CR1^{hi}$ macrophages. Intriguingly, the latter phagocytic subset gain access to the gut lumen by forming transepithelial dendrites [23–26]. Our current understanding of the aforementioned genes suggests that a suppression of phagocyte function (e.g., defects in xenophagy) may contribute to disease development that may account for the greater colonization of patient's intestine by a large set of opportunistic microorganisms such as *Mycobacterium avium* subspecies *paratuberculosis* and adherent-invasive *E. coli* [27–29]. A fundamental attribute of such intracellular opportunistic microorganisms is the ability to avoid killing by macrophages by interfering with the endocytic pathway or by infiltrating the autophagic route for dissemination. Intriguingly, the development of another form of inflammatory bowel disease commonly referred to as ulcerative colitis is not influenced by these genes, distinguishing it from CD. The understanding of the contribution of the recently identified eQTLs in monocytes is now eagerly awaited for restoring phagocyte functions in CD [30]. It is indeed worth noting that bone marrow transplant has proved efficacy to resolve refractory CD that is caused by defects in interleukin-10 signaling [31].

43.4 Concluding Remarks

While the utility for diagnosis by genetic testing remains insignificant in the general population, opportunities for improved care may result from a better understanding of missing heritability. Indeed, it is a greater challenge for the research community to characterize noncoding variants that are predisposing to CD [32], as only underpowered studies have attempted to understand the synonymous allele contribution to the disease mechanism compared to non-synonymous polymorphisms. Furthermore, it is imperative for everyday clinical practice to evaluate the potential clinical value of cell-specific epigenetic markers in prospective studies that are currently being followed [33]. To this end, eQTLs studies with replicating cohorts are expected to be performed at the single-cell level by using specific cell types that are relevant for CD pathogenesis, such as monocytes. In this context, it is worth noting that the local microenvironment (including the gut microbiota) may determine the identity and functionality of intestinal phagocytes that are infiltrated into the tissue. This paradigm is essential as immunodeficiency affecting phagocytes (e.g., an abnormal bacterial handling) has been arguably reported within the mucosa of patients with CD.

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44

Inflammation and Transplantation

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44.1 Introduction

Many reviews consider organ transplantation primarily in the context of antigen-specific adaptive immune responses. This chapter will emphasize the innate immune responses to transplants. Antigen nonspecific inflammation and innate immune responses are unavoidable consequences of transplantation surgery. Excision of an organ from a donor results in ischemia followed by reperfusion after implantation into a recipient. Additionally, in clinical transplantation both the donor and recipient usually have preexisting conditions and undergo procedures before transplantation that can greatly augment innate immunity (Figure 44.1). These innate immune responses are superimposed on the exceptionally strong adaptive immune responses elicited by transplant antigens.

Innate and adaptive immune responses prevented successful organ transplantation until 1954 when a patient with renal failure received a kidney from his healthy twin brother [2]. Adaptive immunity was not elicited because the twins were genetically identical and therefore antigenically "matched." The facts that the kidney came from a healthy living donor and ischemic time was minimized were major factors contributing to the first success. Even with the greatly advanced immunosuppressive protocols used currently, unmatched kidneys from living donors – even living genetically unrelated donors – have significantly better outcomes than kidneys from deceased donors [3].

Inflammation has a significant effect on transplants because it can augment every step of the adaptive immune response, beginning with increased expression of antigens and progressing through more effective antigen presentation, greater expansion of responding cells, and augmented effector mechanisms.

In this chapter, we describe the modulatory effects of inflammation on successive steps of the adaptive immune responses to a transplant and then discuss therapeutic prospects to improve outcomes by modulating inflammatory responses in transplantation. We will concentrate on the available data from transplants in humans. Much of the discussion will be based on renal

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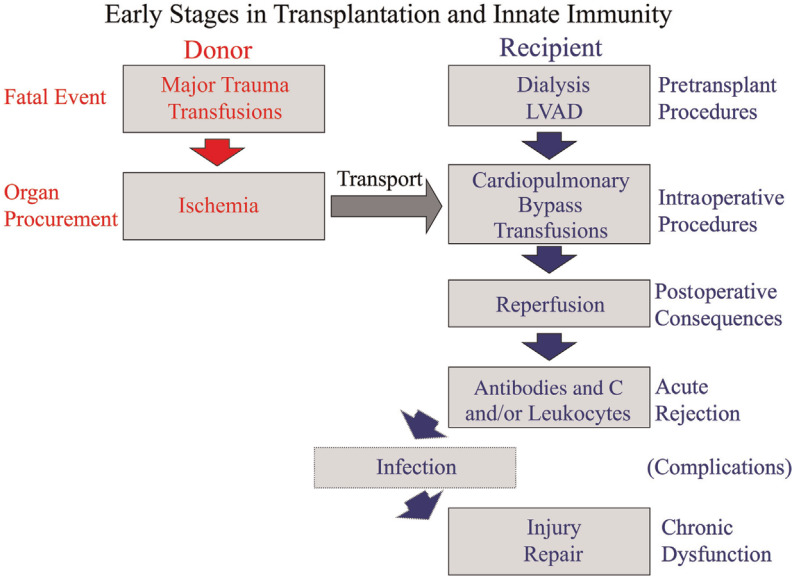


Figure 44.1 Sequence of events that provoke antigen nonspecific inflammation and innate immune responses in the donor and recipient before and after transplantation. (Adapted from Ref. [1].)

transplantation because of the large pool of data on kidneys from living and deceased donors and other variables that are informative about the interaction of innate and adaptive immunity.

44.2 Inflammation Increases the Expression of Transplant Antigens

Defining the antigens that elicit the adaptive immune response to transplanted tissue was a fundamental advance in immunology, for which George Snell, Jean Dausset, and Baruj Benacerraf shared the 1980 Nobel Prize in Physiology or Medicine [4]. Snell used transplantation experiments with tumors and skin in genetically inbred mice to define strong and weak (subsequently termed major and minor) histocompatibility antigens [5]. Genetic and serological evidence indicated that the major histocompatibility antigens are the products of multiple genes that are closely linked in a region now termed the major histocompatibility complex (MHC). Foreseeing the importance of antigenic matching for successful transplants in humans, Dausset identified the first histocompatibility antigens on leukocytes in man (subsequently termed human leukocyte antigens or HLA). Benacerraf found that genes in the MHC regulate the strength of an immunological reaction. The multiple functions of the MHC were resolved as the genetics and structure of the molecules were solved [6].

Enormous antigenic diversity results from multiple genetic loci in the MHC, an extensive number of different alleles for certain loci and codominant expression

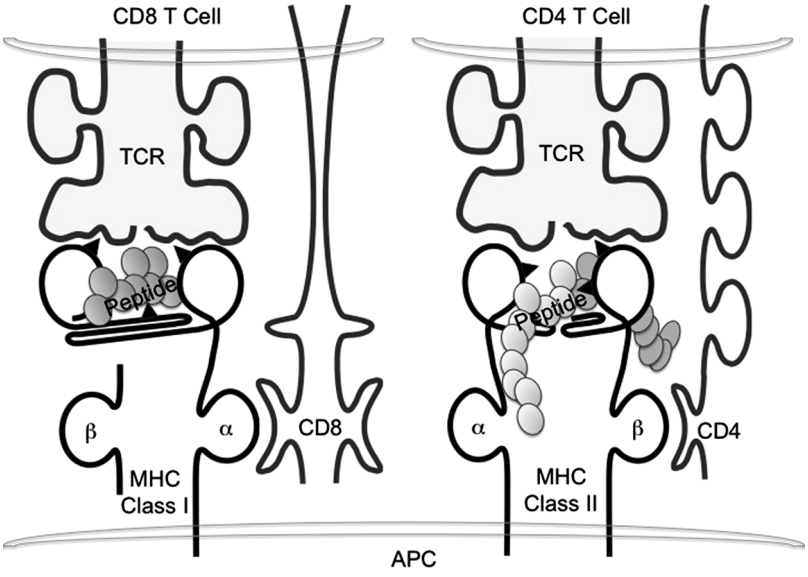


Figure 44.2 MHC molecules are grouped into two classes based on their structure and function. MHC class I antigens are heterodimers composed of a transmembrane alpha heavy chain (~45 kd) and a noncovalently associated beta chain (~12 kd). The alpha chain is extremely polymorphic. The beta chain is constant and imposes a tertiary structure on the alpha chain. MHC class II antigens are composed of two noncovalently associated transmembrane polypeptides: a less polymorphic alpha (~34 kd) and very polymorphic beta (~28 kd) chain. MHC classes I and II have binding grooves that contain short peptides (8–9 amino acids for class I and 13–24 amino acids for class II). The variability concentrated in the binding grooves is depicted by triangles. Antigen-specific receptors on T lymphocytes (TCR) bind to the peptide and the adjacent elements of the MHC molecule. The CD8 coreceptor on cytotoxic T cells and the CD4 coreceptor of helper T cells bind to nonpolymorphic domains of the MHC classes I and II molecules, respectively.

of the glycoprotein products on the cell surface. Antigens encoded by the MHC are grouped into two classes based on their structure and function (Figure 44.2). MHC class I antigens are composed of two polypetides: a transmembrane alpha heavy chain (~45 kd) encoded at the telomeric end of the MHC on chromosome 6 in humans and a noncovalently associated beta chain (~12 kd) that is encoded on chromosome 15. The beta chain is constant and imposes tertiary structure on the heterodimer. In contrast, the alpha chains of 3 MHC class I loci (HLA-A, B, and C) are extremely polymorphic with over 3000 genetic variants reported for the HLA-B locus [7]. In comparison, MHC class II antigens are composed of two noncovalently associated transmembrane polypeptides that are both encoded in the MHC: a less polymorphic alpha (~34 kd) and very polymorphic beta (~28 kd) chain. Three principal MHC class II antigens are expressed in human: HLA-DR, DP, and DQ. The extreme polymorphism of the MHC precludes complete antigen matching of unrelated donors with recipients on a genotypic level.

The evolutionary pressure for this diversity results from the protective function of these molecules as a surveillance system for potential pathogens. Crystallographic studies revealed that the three-dimensional structure of both MHC class I

and MHC class II forms a groove that binds short peptides (8–9 amino acids for class I and 13–24 amino acids for class II). When exposed to pathogens, peptide fragments are loaded into MHC for antigen presentation. In general, MHC class I is adapted to be loaded with peptides from intracellular organisms and MHC class II with peptides from extracellular organisms. The polymorphic variations of both MHC class I and MHC class II are concentrated in the binding groove and confer diversity in peptide presentation. This surveillance function underlies the association of some MHC polymorphisms with disease risks. When no exogenous peptides are present, the MHC molecules are loaded with endogenous peptides. Some of these endogenous peptides are recognized as minor histocompatibility antigens.

Antigen-specific receptors on T lymphocytes bind to the peptide and the adjacent elements of the MHC molecule (Figure 44.2). This interaction between T-cell receptors (TCR) and the peptides bound in the polymorphic domains of MHC limits the response to specific antigens. The low-affinity TCR interaction is strengthened by coreceptors on the T cells that bind to the nonpolymorphic domains of the MHC molecules. The CD8 coreceptor on cytotoxic T cells binds to conserved domains on MHC class I and the CD4 coreceptor of helper T cells binds to MHC class II.

The expression of both MHC class I and II antigens is dynamic and particularly responsive to inflammatory processes. This dynamic expression is an adaptive advantage for the surveillance function of MHC but has immunogenic consequences in transplants. Under normal quiescent conditions, MHC class I antigens are expressed at highest density on leukocytes, dendritic cells, and endothelial cells, but also at varying concentrations on most cells except erythrocytes. This allows any virally infected cell in the body to be eliminated by cytotoxic T cells. In contrast, MHC class II antigens normally are expressed by a more limited set of cells: dendritic cells, B cells, macrophages, and some endothelial cells.

The anatomic distribution and concentration of histocompatibility antigens shapes both the initiation of alloimmunity and the rejection of transplanted organs. Traumatic brain injury and ischemia–reperfusion upregulate the expression of MHC antigens on transplanted tissues [8,9]. Not only do individual cells increase expression of MHC antigens, but cells that normally only express MHC class I antigens also upregulate MHC class II antigens. As a result, all transplanted organs are more immunogenic immediately after transplantation, and organs from deceased donors are more immunogenic than organs from living donors [10].

44.3 Inflammation Increases the Presentation of Transplant Antigens

Following transplantation, the recipient is presented with histocompatibility antigens by multiple routes and different mechanisms. The three mechanisms of antigen presentation are termed direct, indirect, and semidirect. Direct presentation is unique to transplantation and results from recipient T cells, binding intact allogeneic MHC antigens on donor antigen-presenting cells.

Indirect presentation requires recipient antigen-presenting cells to take up donor antigens derived from damaged or dying cells or shed from living cells. These antigens undergo conventional processing and presentation as peptides in the groove of recipient MHC class II [11]. In semidirect presentation, portions of donor cell membrane with intact MHC molecules are integrated on the membrane of recipient antigen-presenting cells. Extracellular vesicles released from donor cells are one mechanism by which MHC molecules can be transferred to recipient antigen-presenting cells [12,13].

Each of these mechanisms of presentation differs qualitatively and quantitatively. Direct presentation to CD4 T cells requires donor antigen-presenting cells that express MHC class II antigen but recipient MHC class II molecules are not necessary. Therefore, the effectiveness of direct presentation was tested using mice that are genetically deficient for MHC class II molecules. These experiments demonstrated that CD4 T cells can cause acute rejection of allogeneic cardiac transplants in recipient mice that are MHC class II deficient [14]. Dendritic cells are the most potent agents of direct antigen presentation. The concept of dendritic cells has evolved from the first descriptions of "passenger leukocytes" that express MHC class II antigens [15] through very distinct short-lived cells that migrate out of transplanted organs [16] to the current view of a range of cells derived from a common precursor cell [17]. With the identification of increasing numbers of "markers" for subpopulations of dendritic cells and macrophages, more overlaps in function and transitional states have been discovered. Several important concepts have emerged. First, the different organs that are transplanted all have extensive networks of potential antigen-presenting cells that are diverse and responsive to the stresses of transplantation [17,18]. Second, subpopulations of dendritic cells function to present MHC class II antigens to CD4 T cells and MHC class I antigens to CD8 T cells. These cells are termed conventional or classical dendritic cells of myeloid origin (cDCs). Significant numbers of cDCs migrate out of transplants and interact with T cells in spleen, lymph nodes, and possibly bone marrow within days after transplantation [11,19]. Although many of these cells are destroyed by natural killer cells, it has been demonstrated that transfer of cDCs is an effective mechanism of generating vigorous T-cell responses to alloantigens [20]. Inflammatory dendritic cells are a third population of antigen-presenting cells in transplanted organs. These cells are derived from circulating monocytes that are recruited to sites of inflammation.

The direct response of T cells to intact allogeneic MHC antigens is exceptional in vigor and repertoire because the response is not confined to individual peptides in the binding groove. Instead, T cells with receptors that were selected in the thymus on templates of "self-MHC" molecules containing peptides cross-react with allogeneic MHC molecules. The binding affinity of individual receptors on a T cell for an allogeneic MHC may be low, but the cumulative avidity is increased by the density of allogeneic MHC molecules on the antigen-presenting cell. As a result, a polyclonal response is generated from 1 to 10% of the T-cell repertoire.

In contrast, T cells activated via indirect antigen presentation bind to peptides derived from donor antigens that are presented in the binding groove self-MHC molecules on recipient antigen-presenting cells. When allogeneic MHC antigens

are reduced to a few peptides, the response is narrowed to a few T-cell clones. The primary source of donor MHC antigens for indirect presentation is damaged or dying cells in the transplant. Therefore, inflammation associated with the logistics of transplantation process or subsequent infections or other complications can cause surges of donor antigen from the damaged tissue for indirect presentation [21].

Stresses such as ischemia–reperfusion also increase the basal release of microvesicles from cells [22]. Various forms of microvesicles are a source of intact MHC for semi-direct presentation [23]. Significant numbers of dendritic cells expressing both donor and recipient MHC molecules have been detected within 1 day after renal and cardiac allografts in experimental animals [24]. Although intact donor MHC is presented by the semi-direct mechanism, the density of antigen assimilated on individual recipient dendritic cells would likely be much less than on donor dendritic cells.

44.4 Inflammation Increases Antigen-Independent Activation Mediators

In addition to increasing the antigenic exposure, stresses associated with transplantation increase ancillary signals that modulate immune responses. Dendritic cells residing within healthy unstressed organs are immature and equipped with receptors to survey the milieu for pathogens. Pattern recognition receptors (PPR) such as toll-like receptors (TLRs) evolved with the dual function of binding signature molecules expressed by prokaryotes called pathogen-associated molecular patterns (PAMPs) and exposed on autologous tissues that are collaterally damaged by pathogens or other injury (damage-associated molecular patterns; DAMPs). The mechanisms connecting hypoxia and reperfusion to expression of DAMPs were detailed in previous chapters. Here we will concentrate on the known effects of DAMPs on transplants.

As illustrated in Figure 44.1, many steps in transplantation can release DAMPs and with the use of immunosuppression exposure to pathogens is also frequent. In the ideal situation of transplants from living donors, the period of ischemia followed by reperfusion is minimized. Moreover, fewer rejection episodes are incurred by organs from living than deceased donors. Consequently, recipients of organs from donors often are exposed to less immunosuppression and experience fewer infections. Exposure to PAMPs and DAMPs causes dendritic cells to mature. Mature dendritic cells express increased amounts of MHC class II molecules, upregulate costimulatory molecules (CD80 and 86), and produce various proinflammatory cytokines (Il-12, IL-6, TNFα and IL-1β). These cells also have increased migratory capabilities.

Increasing evidence implicates some DAMPs modulate the immune responses to transplants (Table 44.1). Experiments using genetically modified mice have established that TLR4 contributes to the inflammation caused by ischemia–reperfusion in kidneys [25]. TLR4 is expressed on intrarenal leukocytes within a day after ischemia–reperfusion and then these cells decline precipitously. TLR4

Table 44.1 DAMPs that have been demonstrated to modulate immune responses to transplants.

Source	Ligand	Receptor	References
Extracellular matrix			
	Hyaluronan fragments	TLR4, CD44, and RHAMM	[25]
	Biglycan	TLR2 and TLR4	[25,27]
Nucleus/cytoplasm			
	HMGB1	TLR2, TLR4, TLR9, RAGE, and CD24	[25–28]
	S100A8/A9	TLR4 and RAGE	[29]

expression is also increased on renal tubular epithelium on the first day after ischemia–reperfusion and this expression peaks at 3–5 days. The epithelial response is paralleled by increases in DAMPs that are known ligands for TLR4, including high-mobility group box 1 (HMGB1), hyaluronan, and biglycan. Mice constructed with a genetic ablation of TLR4 in their kidneys retained better renal function, incurred less tubular damage, and sustained fewer leukocyte infiltrates following ischemia–reperfusion. In complementary experiments, ischemia–reperfusion injury was effectively inhibited by blocking antibodies to HMGB1 [26]. Similarly, in humans TLR4 expression is upregulated on renal tubular epithelium following transplantation and kidneys that express genetic polymorphisms for nonfunctional TLR4 molecules have an increased incidence of immediate graft function [27]. As expected with longer ischemic times, renal tubular expressions of TLR4 and HMGB1 are higher in kidneys from deceased than living donors.

These findings support the concept that DAMPs that are ligands for TLR4 on renal epithelial cells promote inflammatory responses immediately after transplantation. TLR2 has also been implicated in experimental models of renal ischemia–reperfusion [30]. However, the effects of DAMPs on dendritic cells are more diverse and depend on the specific DAMP as well as the stage of maturation and type of dendritic cell.

HMGB1 has been linked to many steps in the maturation of dendritic cells for antigen presentation, including upregulation of MHC and costimulatory receptor expression on the cell surface, and increased production of proinflammatory cytokines [28]. In contrast, another DAMP that also is a ligand for TLR4, the S100A8/A9 heterodimer (calprotectin), protects the kidney from ischemia–reperfusion injury [29]. A third ligand for TLR4, hyaluronan, elicits a spectrum of effects. As an undegraded component of extracellular matrix, hyaluronan maintains quiescence and tissue integrity, but in sites of inflammation, hyaluronan is cleaved and the resulting fragments can exert pro- or anti-inflammatory effects depending on the fragment size [31]. The diversity of responses is related to the interaction of multiple receptors with hyaluronan. Depending on its size, hyaluronan can cross-link TLR4, CD44 (and its variants), Receptor for

Hyaluronan-Mediated Motility (RHAMM), and possibly TLR5 in various combinations.

Cold ischemic times of 24 h, which are not unusual for clinical renal transplants, induce dramatic changes in the dendritic cells of renal isografts between genetically identical rats [32]. Within 12 h after transplantation, about half of the donor dendritic cells migrate out of the kidney. Migration of dendritic cells is orchestrated by the downregulation or uncoupling of E-cadherin and chemokine receptors that retain immature cells in the tissue (CCR1, CCR2, and CCR5) and upregulation of other receptors (CXCR4 and CCR7) that direct the cells to T-cell compartments of the spleen and lymph nodes [33]. CCR7 is the receptor for CCL19 and CCL21, which are expressed on high endothelial cells and in the T-cell compartments of lymph nodes and spleens [34]. CXCL12 synergizes with the CCR7 ligands to direct dendritic cells to T-cell compartments where antigen presentation is most effective.

The surgically constructed vascular anastomoses allow dendritic cells to migrate from transplanted organs to the spleen. Migration of dendritic cells to lymph nodes has also been documented, even though the lymphatic vessels are not reconstructed [35–37]. The large numbers of leukocytes that emigrate from transplants via lymphatics are released into the peritoneal or thoracic cavity depending on the site of the transplant and reenter the lymphatic system of those cavities.

In addition to the efflux of antigen-presenting cells from the transplant, ischemia–reperfusion initiates an influx of leukocytes into the transplant. Both hypoxia and ligands for TLR2 and 4, such as uric acid, induce endothelial cells to exocytose contents of Weibel–Palade storage granules content, including von Willebrand factor and P-selectin [38]. These adhesion molecules initiate platelet accumulation and activation, which has been documented to occur within 20–40 min after the circulation is established to kidneys transplanted from deceased donors, but not from living donors [8]. This is accompanied by translocation of P-selectin from alpha-storage granules to the surface platelets. A more extended kinetic study of adhesion molecules demonstrated that expression of P-selectin was maximal between 2 and 6 h after transplantation in both renal isografts and allografts in rats [39]. Neutrophils that expressed both L-selectin and LFA-1 localized to the grafts by 2 h and maximized between 12 and 24 h. Expression of P-selectin glycoprotein ligand-1 (PSGL-1) is critical to neutrophil localization and subsequent immune responses to transplants because treatment with soluble PSGL inhibits not only neutrophil accumulation but also upregulation of E-selectin and MHC class II [40].

More recently, a subpopulation of resident macrophages in the kidney have been found to modulate the influx of neutrophils following ischemia–reperfusion [41]. The modulatory macrophages express the sialoadhesion molecule (CD169) and are located in close proximity to the capillary endothelium in the renal medulla. Depletion of this subpopulation of macrophages increases baseline ICAM-1 expression on endothelial cells.

Concomitant with upregulation of adhesion molecules, the microvascular endothelium and underlying epithelial cells produce chemokines and cytokines, including IL-1, IL-6, IL-8 (CXCL8), CCL2 (MCP-1), and TNFα that augment

migration of inflammatory cells into the transplant [42,43]. In human renal transplants, mRNA for IL-8, a potent neutrophil chemoattractant, increases in the reperfusion phase. The increase is greater in kidneys from deceased than living donors, and the increase correlates with duration of ischemia [44].

Besides releasing antigenic substrates, damaged and dying cells engage other components of the innate immune system [45]. The initial components of the complement cascade are essential for the clearance of damaged tissues. Both C1q and mannose binding lectin (MBL) bind to phospholipids and nuclear components that are exposed in the processes of regulated cell death and necrosis. With normal levels of cell turnover, C1q and MBL opsonize apoptotic bodies for phagocytosis by monocytes and macrophages without inducing inflammation [46].

These processes likely predominate when kidneys are transplanted from living donors. With deceased donors, however, brain death and longer ischemic times produce greater amounts of damaged and dying cells that bind C1q and MBL in quantities sufficient to activate more components of the complement cascade. Simultaneously, some of the regulators of complement activation that normally protect tissues from damage are downregulated following ischemia–reperfusion [47]. Under these conditions, the complement cascade progresses to activation of the terminal complement components that begins with the cleavage of C5. C5a, the small split product of C5, is a potent chemoattractant and activator of neutrophils and monocytes. These proinflammatory mechanisms may be favored in the kidney where local production of complement components and C5a receptors are upregulated by tubular epithelial cells in organs from deceased donors [48,49].

44.5 Immediate Adaptive Immune Responses to Allografts

The innate responses do not require any previous exposure to antigens and are immediately deployed in response to transplants because the receptors for DAMPs are constitutively expressed. In contrast, the preponderance of adaptive immune responses evolve after exposure to the allograft expands antigen-specific cells through proliferation and selection. However, previous exposure to transplant antigens or cross-reactive antigens can result in immediate adaptive immune responses to allografts.

Before 1969, about 20% of renal transplants from deceased donors were rejected within minutes to hours after circulation was reestablished [50]. This hyperacute rejection was characterized by extensive margination of neutrophils within 1 h, which progressed to endothelial damage, intracapillary fibrin microthrombi, and hemorrhage [51,52]. The first reports of hyperacute rejection implicated antibodies as the mediator because all of the patients had been exposed to transplant antigens through blood transfusions, pregnancies, or previous failed transplants. Consequently, most of the patients had measurable antibodies in their sera to donor tissue prior to transplantation. Moreover, IgG deposits were demonstrated in postoperative biopsies by immunofluorescence and antibody was eluted from the rejected transplant [52]. In 1969, Patel and Terasaki published convincing evidence that a serological cross-match test could

detect antibodies associated with hyperacute rejection [50]. Hyperacute rejection has been almost completely avoided since this test was introduced to screen potential recipients before transplantation.

Even in the absence of exposure to transplant antigens, some T cells do respond immediately to allografts. These immediate responders are memory T cells that accrue from a lifetime of exposure to pathogens and other environmental stimuli. The memory response of cross-reactive T cells is termed heterologous immunity [53]. Cross-reactivity occurs because the TCR interacts with only limited surfaces of the MHC molecule and the bound peptide. As a result, cross-reactivity is based on about five amino acids of the peptide that are projecting from the groove of the MHC and the adjacent surfaces of the MHC itself. After responding to an antigen, a population of memory T cells develops, which is primed for activation at a much lower threshold. This allows memory T cells to respond to cross-reactive peptide-MHC complexes with a much lower affinity for their TCR. Unequivocal evidence of cross-reactivity for transplant antigens has been demonstrated with clones of T cells produced to a virus that also respond to allogeneic cells [54].

In contrast to naïve T cells that are guided by CCR7 homing receptor to circulate through blood and lymphoid tissues, memory T cells upregulate adhesion receptors such as LFA-1 (CD11a) and CD44, which enhance emigration into transplants [55]. As a result, heterologous immunity manifests within hours after transplantation as an infiltrate memory T cell [56]. The influx of memory T cells is directed by the upregulation of adhesion molecules on endothelial cells. Extended ischemia times increases expression of adhesion molecules on vascular endothelium, which significantly increases the magnitude and effector functions of early memory T-cell infiltrates [57]. Infiltrating memory T cells are activated by direct antigen presentation on donor dendritic cells remaining in the graft and endothelial cells as well as by parenchymal cells [58,59].

44.6 *De Novo* Adaptive Responses to Transplants

In the absence of immunosuppression, *de novo* T-cell and B-cell responses can be detected in spleens, lymph nodes, and bone marrow within days after organ transplantation. The integrated structure and ordered movement of cells in spleen and lymph nodes promote effective interactions between antigen-presenting cells, T cells and B cells. Indirect presentation of alloantigens to naïve T and B cells is dependent on organized secondary lymphoid tissues. Acute rejection of cardiac allografts is abrogated in mice lacking spleen and lymph nodes [60]. T-cell responses to indirectly presented antigen were undetectable in these mice and the response to directly presented antigen is shifted from production of IL-2 and IFNγ to IL-4 and IL-10. These mice also did not produce detectable alloantibody responses. The absence of alloantibody responses is consistent with evidence that the spleen is the primary cite of alloantibody production to cardiac allografts in mice [61].

44.7 Acute Rejection Caused by T Lymphocytes

Extensive experimental data have established that thymus-derived lymphocytes can cause acute rejection of allografts. The experimental approaches include passive transfer of lymphocytes beginning with early work of Billingham, Brent, and Medawar [62], and extending through more refined transfers of subpopulations of T cells. T cells were further implicated by experiments demonstrating that transplants to neonatally thymectomized mice and mutant athymic mice were not rejected [63]. Finally, experiments with monoclonal antibodies and genetically modified mice have identified different functions for CD4 and CD8 T cells in acute rejection of organ allografts [14,64]. Cytotoxic CD8 that react to intact allogeneic MHC through direct presentation are capable of engaging MHC on allogeneic cells in grafts and causing cell death through the release of perforin and granzymes. In contrast, T cells generated in response to indirect antigen presentation cannot react to allogeneic MHC on the transplant except in cases where donor and recipients share MHC antigens. Otherwise, infiltrating dendritic cells or macrophages of recipient origin are required. Graft damage results from inflammation orchestrated by cytokines released from T cells. Although cytokines have very redundant effects, some have been found to correlate consistently with rejection. One of the most reproducibly detected is CXCL9 (Mig), which has been reported to correlate with acute rejection in biopsies of cardiac and renal transplants [65,66]. In a longitudinal study of endomyocardial biopsies, the expression of CXCL9 (Mig), CXCL10 (IP-10), CXCL11 (I-TAC), CCL5 (RANTES), CXCR3, and CCR5 were found to mirror the timing and grade of acute rejection episodes in cardiac allografts [65].

Improved immunosuppressive drugs have dramatically decreased acute T-cell-mediated rejection. From 1970 to 1977, only about 50% renal transplants from deceased donors survived 1 year [67]. Currently, 1-year renal graft survival is greater than 90% [68].

44.8 Antibody-Mediated Acute Rejection

Although hyperacute rejection provided proof of principle that antibodies can destroy allografts, antibodies were not considered a primary cause acute rejection until more sensitive markers were developed to detect antibody-mediated reactions in transplants. Routine immunofluorescent stains for IgM, IgG, and complement that were applied in hyperacute rejection did not provide evidence of antibody-mediated acute rejection [69]. The complement split product C4d became the first reliable diagnostic marker of acute antibody-mediated rejection [70,71]. C4d has several advantages for immunohistology. As a product of enzymatic cleavage, neoepitopes are exposed, which increase the specificity of monoclonals that react only with this activated complement component and not with the circulating native molecule. Sensitivity is increased because the enzymatic process cleaves many C4 molecules and C4d binds covalently with tissues. However, C4d was quickly recognized as an imperfect surrogate for complement-mediated injury to transplants [72]. Complement is

a multidimensional system that progresses from a largely anti-inflammatory series of mediators to extremely proinflammatory mediators [73]. The initiating components of the classical and lectin pathways of complement, C1q and MBL, are crucially important in clearing damaged and dying cells by macrophages without inciting inflammation [74]. Split products of the central complement component, C3, have a balance of modulatory effects on the immune system [75]. However, the progression of complement activation from C3 to C5 generates the soluble split product C5a, which is an extremely potent chemoattractant and activator for neutrophils and monocytes. Of relevance to antibody interactions, C5a stimulates macrophages to downregulate inhibitory FcgR and upregulate activating FcgR [76]. The potential harmful effects of the proinflammatory terminal complement components on endothelial cells are kept in check by multiple regulators of complement, many of which act on the enzymatic components of C1, MBL, C3 convertases, and C5 convertases. As a result, C5 and the subsequent complement components are only activated when the capacity for regulation is exceeded or the expression of regulators is decreased [48].

The vascular distribution of C4d reinforced the concept that endothelial cells are the primary targets of antibody action. Molecular assays on renal biopsies have delineated sets of gene transcripts that further implicate endothelial injury in association with circulating antibodies. These molecular signatures are frequently increased in the absence of C4d deposits [77] and are now incorporated into the diagnostic criteria for antibody-mediated rejection [71]. The recognition of a C4d negative category of antibody-mediated rejection is indicative of antibody functions that require either low levels of complement activation or no complement activation.

Several mechanisms of complement independent antibody functions have been defined in the context of transplants. Antibodies with sufficient avidity to cross-link MHC class I antigens stimulate endothelial cells to exocytose von Willebrand factor and P-selectin *in vitro* and *in vivo* [78,79]. These mediators rapidly bind and activate platelets and macrophages. Platelet storage granules contain a wide array of mediators that could potentially promote antibody-induced inflammation [80]. Of all these mediators, platelet factor 4 (PF4; CXCL4) is quantitatively most abundant [81]. In an experimental model of acute antibody-mediated rejection, PF4 and serotonin were recovered from renal allogarfts in much greater quantities than other cytokines stored in platelet alpha granules [78].

The expression of P-selectin by activated endothelial cells and platelets augments interactions with neutrophils and monocytes through P-selectin glycoprotein ligand-1 (PSGL-1). P-selectin expressing platelets attached to macrophages are evident by immunohistology in capillaries and venules of mouse and human renal allografts [78]. The effects of platelets on skewing macrophage function toward inflammation or wound healing functions in transplants is not known. However, PF4 has been demonstrated to elicit a distinct differentiation profile in macrophages *in vitro* that has been labeled M4 [82]. Clinically, antibody-mediated rejection has been associated with infiltrates of macrophages, and glomerular monocytes predict poorer graft outcomes [83].

In addition to regulating inflammatory responses by cross-linking MHC antigens on endothelial cells, antibodies can regulate neutrophil, monocyte, and NK cell function by binding with different types of Fc receptors [84]. Although the effects of activating (human FcγRI, FcγRIIA, and FcγRIIIA) and suppressing (human FcγRIIB) receptors for IgG have been appreciated for a long time [85], some single nucleotide polymorphisms and splice variants result in increased expression and function of specific Fc receptors on neutrophils and monocytes [86].

44.9 Chronic Rejection

Although advances in immunosuppressive drugs have steadily decreased the incidence and severity of acute rejection, progress in understanding and treating chronic deterioration of transplant function has been limited [87]. Many factors can be compounded over the course of years to result in chronic graft dysfunction. For example, with the persistent shortage of organs for transplantation, criteria for age and graft quality have been expanded, and these variables are associated with risk of chronic graft dysfunction. However, the dominant variable in clinical transplantation is immunosuppression. Excess immunosuppression can result in injury to the graft due to drug toxicity, opportunistic or recurrent infection, or cancer. Conversely, insufficient immunosuppression, either due to unrecognized subclinical rejection or nonadherence to medications, allows alloimmune responses to emerge. Surveillance biopsies of renal allografts with good function at 1 year confirm that about 75% of the transplants have no evidence of rejection, but the remainder have either subclinical cell-mediated rejection or subclinical antibody-mediated rejection [88]. Biopsies with subclinical antibody-mediated rejection were prognostic of a higher risk of eventual rejection over the subsequent 7 years. Antibody-mediated rejection is even more frequent in biopsies of renal transplants from patients with clinical evidence of rejection, and a large proportion of these patients are nonadherent [89]. The incidence of new antibody to donor HLA developing gradually increases following a first renal allograft to about 20% at 10 years, and is about three times higher in nonadherent patients [90]. Current diagnostic methods probably underestimate the contribution of antibody to chronic rejection. This relates to fluctuations in antibody levels over time and complement-independent effects of antibody [91]. Advances in molecular markers to identify tissue responses may begin to distinguish underlying mechanisms of graft injury.

44.10 Tolerance

The inherent risks of prolonged exposure to broad-spectrum immunosuppression have intensified the search for more specific regulation of the immune system. In 1953, Billingham, Brent, and Medawar demonstrated that inoculation of fetal mice (and rats, rabbits, and birds in later papers) with viable allogeneic tissue resulted not only in the acceptance of the initial tissue by the immature animal but also

tolerance of later transplants from the original or genetically identical donors [92]. Viable tissue introduced while the immune system is immature provides stem cells that can lodge in central lymphoid tissues (bone marrow and thymus) and participate in the negative selection of the lymphocytes. Negative selection is the normal process to eliminate lymphocytes that could react to autologous antigens. Deletion of lymphocytes by this mechanism is referred to as "central tolerance" [93]. Lymphocytes that escape deletion by central tolerance can be nullified by several mechanisms, including anergy and regulation, which are collectively termed "peripheral tolerance." Anergy and regulation occur when lymphocytes receive a signal through their antigen receptor but they either receive no second signal (anergy) or receive an inhibitory second signal (regulation). Tolerance is the ideal outcome for a transplant because it results in graft acceptance without compromising other immune functions.

In adults, tolerance can be attained when their immune system is ablated and then reconstitutes in the existence of allogeneic cells. The treatment protocols to ablate the adult immune system are complicated and intense. However, several strategies to achieve tolerance in adults are in clinical development [94–96]. When successful, these tolerance protocols will result in enduring graft function without immunosuppression. Again antibody-mediated rejection has been the most frequent cause of failure.

A minority of transplant recipients becomes tolerant to their transplants on conventional immunosuppression. Most often these patients independently decide to stop their medications and retain good graft function unlike the majority of nonadherent patients who experience acute or chronic rejection. Spontaneous tolerance has been most frequently reported for hepatic transplants (in the range of 20–40%) and much less frequently for renal transplants [97]. Extensive studies of these patients suggest that the mechanisms may be different for liver and kidney. Genes related to iron metabolism and natural killer cells have been found to be upregulated in peripheral blood of patients tolerant to hepatic but not in those tolerant to renal transplants [97,98]. In contrast, B cells and B-cell-related transcripts are increased in peripheral blood of patients tolerant to renal but not in those tolerant to hepatic transplants. However, these studies are based on small numbers of patients and there is not total agreement among different reports. Therefore, there are no tests that adequately predict patients who are prone to tolerance and can be safely allowed to discontinue immunosuppression. Attempts to purposefully wean patients off immunosuppression have been much more successful for hepatic than renal transplants. In one large study of 104 patients with hepatic transplants, the strongest predictor of successful withdrawal of immunosuppression was time after transplantation; the success rate was almost 80% for the patients undergoing withdrawal more than 10 years after transplantation compared to 12.5% success at less than 6 years after transplantation [99].

44.11 Tolerance and Accommodation of Blood Group Mismatched Transplants

A limited form of neonatal tolerance has been achieved for blood group incompatible cardiac transplants. Transplants mismatched for the major blood

group antigens are at risk for hyperacute rejection as the result of "natural" antibodies binding to the A and B antigens that are highly expressed on vascular endothelial cells. However, natural antibodies to A and B do not develop until after the first few months of life when the immune system is exposed to antigens on bacteria in the expanding gut flora that cross-react with blood group antigens. Blood group incompatible transplants performed before these cross-reactive antibodies are generated are not at risk for hyperacute rejection. Moreover, when newborns are transplanted with an A or a B antigen-incompatible heart, they are tolerized to the incompatible antigen and do not develop antibodies or B cells reactive to the blood group expressed on the graft [100]. Tolerance may be favored because these blood group antigens have distinctive properties [101]. The A and B antigens are carbohydrate groups that are expressed on a range of soluble and cell-bound proteins. This permits widespread exposure of the developing immune cells to these antigens.

After many years of avoiding blood group-incompatible renal transplants in adults because of the risk of hyperacute rejection, the increasing shortage of acceptable donor organs instigated reexamination of this source for living related donors who were well matched for HLA antigens. The major blood group antigens are encoded on a different chromosome than HLA (9 versus 6) and therefore inherited independently. As a result, about 40–50% of HLA-matched siblings are blood group incompatible. Hyperacute or acute rejection is avoided in these transplants by removal of antibodies through immunoadsorption or plasmapheresis before and during the first weeks after transplantation. Subsequent return of antibodies to the A or B blood group is often associated with the deposition of C4d on the vascular endothelium of the graft. However, these products of complement activation are usually not associated with evidence of inflammation or graft dysfunction [101]. This outcome is termed "accommodation." The mechanisms underlying accommodation are not fully understood, but upregulation of complement regulatory molecules on the vessels of the transplant may contribute.

44.12 Organ-Specific Inflammation

Since the introduction of cyclosporine, many organs, including liver, heart, lung pancreas, intestine, and various composite tissues, have been routinely transplanted. However, kidneys are the most frequently transplanted organs. In part this is due to greater number of donor organs because of the availability of living donors. Lobes of livers or lungs are also transplanted from living donors, but these operations have a higher risk and are usually limited to adult donors and pediatric recipients. There is more flexibility for kidneys from deceased donors as well because patients with delayed graft function can be supported on dialysis. Predictably, delayed graft function does increase the liability of tissue injury and decrease outcomes.

Although much of the experience with kidneys is applicable to other organ allografts, there are notable differences. The apparent immunogenicity of organs differs greatly in part due to the number and type of resident antigen-presenting cells, and also the content of lymphoid tissue. Most obviously, lungs and intestine

contain bronchial- and gut-associated lymphoid tissues (BALT and GALT). In addition, the lung and intestine are constantly exposed to microbial encounters. Lung transplants have also been found to be subject to inflammation from gastric reflux [102]. In contrast, the liver contains stellate cells that have been found to be tolerogenic [103].

This hierarchy of immunogenicity is evident in survival of experimental and clinical transplants. Clinically, transplants of kidney, liver, and heart have superior survival rates compared to lung or intestine. In mice, hepatic allografts are spontaneously accepted even between strains with major and minor histoincompatibilities [104].

The targets of rejection also vary among organs. Capillary endothelium is the primary target of acute rejection especially for antibody-mediated rejection, but endothelial cells are not uniform throughout the vascular system. For example, expression of adhesion molecules varies according to specific environmental and functional requirements [105,106]. In cell-mediated rejection, the infiltrating T cells encounter different parenchymal cells after emigrating through the endothelial layer.

The chronic changes induced in transplanted organs are also divergent. In hearts, the primary lesion is a proliferative neointima that impinges on blood flow in the epicardial coronary arteries and negligible pathological changes in capillaries [107]. In contrast, multilayering of glomerular and peritubular capillary basement membranes occurs in chronic rejection of renal allografts. This may reflect the different susceptibility of these organs to antibody-mediated injury. In lungs, the airways are sites of chronic rejection as would be predicted from constant exposure to inflammatory stimuli.

44.13 Treatment

The long-term success of the first renal allograft between genetically identical twins proved that transplanted organs could sustain life. However, transplants between genetically disparate living individuals before the development of immunosuppression were rejected acutely by antibody and cellular mechanisms [108]. Elion and Hitchings were awarded the Nobel Prize for developing a series of antiproliferative drugs including azathioprine that was the first effective immunosuppressive drug in transplants [109]. Azathioprine produced short-term renal allograft survival. Startzl added steroids to azathioprine and achieved the first long-term survival of renal transplants [110]. The major impact of steroids reflects the importance of modulating inflammatory responses to the transplant.

The introduction of cyclosporine A, a calcineurin inhibitor that decreases production of IL-2 and a set of inflammatory cytokines, including IL-4, IFNγ, and TNFα, controlled by the transcription factor nuclear factor of activated T cells (NFAT) elevated long-term survival of renal transplants and allowed the realization of transplants of other organs, including heart. Unfortunately, the benefits of calcineurin inhibitors are counterbalanced by serious adverse effects, including nephrotoxicity and new onset diabetes mellitus [111,112].

The adverse effects of chemically derived drugs has propelled development of biopharmaceutical or biologic agents, the most common of which are antibodies to immune competent cells, mediators, cell receptors, or adhesion molecules. Antibodies have the advantage of specificity and long half-life in the circulation. Monoclonal antibodies offer the advantage of standardized formulations and monoclonals produced in mice or other animals can be genetically "humanized" to diminish immune elimination. Soluble receptors or antagonists can be produced as fusion molecules with immunoglobulins to increase the half-life and to achieve higher affinity of dimeric forms. Examples of all of these types of biologics are in clinical application. One of the most commonly used biologic is antithymocyte globulin (ATG), polyclonal antibodies produced in rabbits to human thymocytes, that reacts primarily with T cells, but also with other leukocytes. Treatment with ATG at the time of transplantation and during the first few days afterward (induction therapy) causes leukopenia and decreases the incidence of acute rejection [113]. Monoclonal antibodies directed to the receptor for IL-2 are also used for induction therapy. More recently, CTLA4-Ig, a fusion protein between a soluble T cell costimulatory receptor (cytotoxic T-lymphocyte-associated protein 4) and immunoglobulin (Ig), has been demonstrated to provide effective immunosuppression and avoid nephrotoxicity [114].

Although these biologics have improved outcomes in transplantation, the resultant immune suppression is not antigen specific. Strategies to produce antigen-specific immune suppression have centered on enhancing the regulatory functions of immunity [115]. Regulation is a critical mechanism to modulate every phase of immune responses. Antigen-specific regulation is achieved by delivery of inhibitory signals in conjunction with the antigen. Inhibitory signals can be delivered through coreceptors or anti-inflammatory cytokines. Important inhibitory coreceptors on T cells include CTLA4, programmed cell death protein 1 (PD1), B- and T lymphocyte attenuator (BTLA), and lymphocyte-activation gene 3 (LAG3). IL-10 and TGFβ are anti-inflammatory cytokines that are produced by a variety of regulatory immune cells including T cells, B cells, dendritic cells, and myeloid-derived suppressor cells (MDSCs). Clinical studies are underway to expand regulatory cells *in vitro* for administration to patients [116].

44.14 Modulating Inflammation to Improve Transplantation in the Future

The recognition of inflammation as a fundamental component of transplantation has focused research on potential modulatory interventions. Transplants are particularly vulnerable to inflammation during procurement, transport, surgery, and reperfusion. These are all modifiable processes. Belzer pioneered the development of preservation solutions and perfusion pumps that counteracted organ deterioration during procurement and transportation [117]. Many of the advances were centered on metabolic and physiological considerations. More recently, immunological interventions have been introduced, including the use of

complement inhibitors [118]. The benefits of decreasing metabolic demands by cold storage is now challenged by evidence that normothermic perfusion can not only maintain viability but also reverse some injury [119,120]. Normothermic perfusion can decrease the antigenic content of organs by depleting emigrating donor leukocytes before transplantation [121]. Even more enticing is the potential to modify organs by the introduction of beneficial genetic alterations [122]. Application of these approaches will improve the currently acceptable organs and will greatly expand the available organs by allowing evaluation and repair of marginal organs.

Some existing interventions may need to be reevaluated for potential effects on the inflammatory component of transplantation. For example, new evidence suggests that cyclosporine A inhibits mitochondrial dysfunction during reperfusion [123]. Exploiting the effects of cyclosporine on reperfusion could enhance current protocols that focus only on its immunosuppressive functions [124].

In summary, inflammation is unavoidable in organ transplantation and if not modified can be a factor undermining graft outcomes.

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45

Inflammatory Mechanisms in Chronic Obstructive Pulmonary Disease

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45.1 Introduction

Chronic obstructive pulmonary disease (COPD) is a major global epidemic that is increasing throughout the world as populations age and survive previous causes of death [1]. COPD is now the fourth ranked cause of death worldwide and predicted to become the fifth ranked cause of disability, affecting approximately 10% of people over 45 years [2]. In developed countries, COPD now affects females and males equally, reflecting the equal prevalence of smoking. It is now recognized that there are different clinical phenotypes of COPD, with some patients having predominantly small airway disease, whereas others have mainly emphysema. Other differences include the age of onset, the frequency of exacerbations, and the association with other diseases such as chronic cardiovascular and metabolic diseases (comorbidities). Several attempts have been made to segregate COPD patients into different clusters based on clinical and radiological characteristics, but it has been difficult to identify these phenotypes in different populations and there has been no link to underlying disease mechanisms (endotypes) [3,4].

COPD is associated with chronic inflammation of the airways and lung parenchyma, which increases further during acute exacerbations and is also associated with systemic inflammation [5]. Although the nature of this inflammation in COPD lungs has been well described, it is still uncertain how this relates to clinical outcomes, disease progression, and response to different therapies, and more research is needed to understand this better. Longitudinal studies of COPD populations over many years have demonstrated that only about 50% of patients diagnosed with COPD have an accelerated decline in lung function, whereas the remainder has a normal age-related decline but start from a lower value, presumably due to impaired lung development [6]. This implies that only half pof "COPD" patients have inflammation and the normal decliners presumably do not have so, although in most studies no distinction is made as the trajectory of lung function is unknown.

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More research is urgently needed to understand different patterns of lung inflammation in COPD and how they relate to clinical outcomes, response to therapy, and prognosis. In particular, it is necessary to understand how inflammation changes in response to different environmental stimuli and how it changes over time in the same patient. COPD from different causes may differ, but the COPD associated with indoor air pollution appears to have a very pattern of inflammation similar to that seen in smoking-related COPD, suggesting that the respiratory tract may respond to different risk factors in the same way [7]. This review discusses the nature of inflammation in COPD and the underlying molecular mechanisms that may identify new targets for more effective anti-inflammatory therapy in the future [8].

45.2 Pathology of COPD

The progressive airflow limitation in COPD results from two major pathological processes: remodeling and narrowing of small airways and destruction of the lung parenchyma with consequent loss of the alveolar attachments of these airways as a result of emphysema. These pathological changes appear to be the consequence of chronic inflammation in the lung periphery, the intensity of which increases as the disease progresses [9]. Even in mild disease there is obstruction and loss of peripheral airways [10]. Analysis of serial computed tomography scans suggests that small airway obstruction usually precedes the development of emphysema [11], but the mechanisms for this are not clear. The small airway obstruction and loss of alveolar attachments result in airway closure and air trapping on expiration that is worsened by exercise and dynamic hyperinflation may account for exertional dyspnea, the major symptom of COPD patients, even in patients with mild disease [12,13]. It is presumed that the peripheral location of inflammation in COPD reflects the sites of deposition of inhaled irritant particles, such as cigarette and wood smoke. Indeed, in COPD associated with household air pollution (biomass smoke) in developing countries, small airway disease is predominant, whereas in cigarette smokers small airway disease and emphysema often coexist [14]. The pattern of inhalation of irritants may also determine localization, since wood smoke is usually inhaled tidally, where cigarette smoke is often inhaled deeply with a breath hold. This may be seen particularly in smokers of cannabis (marijuana) cigarettes, who may develop marked emphysema [15].

45.3 Characteristics of COPD Inflammation

In COPD, there is a characteristic pattern of inflammation with increased numbers of neutrophils in the airway lumen and increased numbers of macrophages, T lymphocytes, and B lymphocytes [9,16,17]. The inflammatory response in COPD involves both innate and adaptive immune responses, which are linked through the activation of dendritic cells [18]. A similar pattern of inflammation and mediator expression is found in smokers without airflow limitation, but in

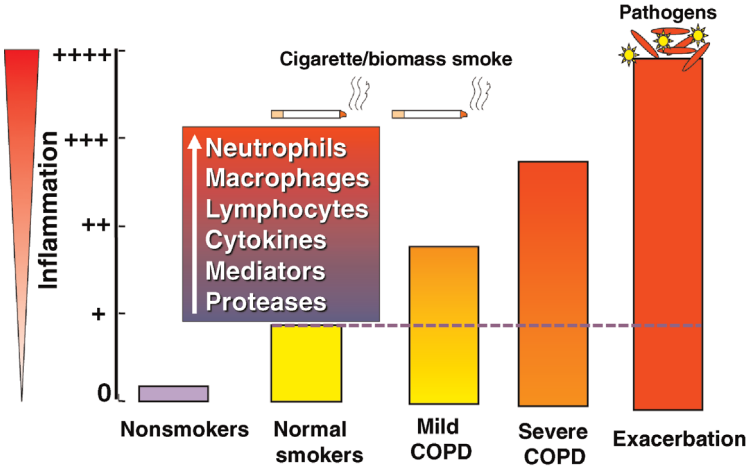


Figure 45.1 Amplification of inflammation in COPD compared to smokers who do not develop airway obstruction. Once established, this inflammation persists even after smoking cessation. Inflammation is further increased in acute exacerbations, triggered by bacteria or viruses.

COPD this inflammation is amplified and even further increased during acute exacerbations precipitated by bacterial or viral infection (Figure 45.1). The molecular basis for the amplification of inflammation is not yet fully understood but may be, at least in part, determined by genetic and epigenetic factors. The molecular mechanisms of amplification may determine which smokers are susceptible to the development of airway obstruction. Cigarette smoke and other irritants inhaled into the respiratory tract may activate surface macrophages and airway epithelial cells to release multiple chemotactic mediators, particularly chemokines, which attract circulating neutrophils, monocytes, and lymphocytes into the lungs. This inflammation persists even when smoking is stopped, suggesting that there are self-perpetuating mechanisms, although they have not yet been elucidated [19]. It is possible that long-lived memory T cells, bacterial colonization, or autoimmunity may drive the persistent inflammation in COPD patients.

45.4 Inflammatory Cells

The inflammation of COPD lungs involves both innate immunity (neutrophils, macrophages, eosinophils, mast cells, NK cells, γδ-T-cells, innate lymphoid cells, and dendritic cells) and adaptive immunity (T and B lymphocytes), but there is also activation of structural cells, including airway and alveolar epithelial cells, endothelial cells, and fibroblasts.

45.4.1 Epithelial Cells

Epithelial cells are activated by cigarette smoke and other inhaled irritants such as biomass fuel smoke to produce inflammatory mediators, including tumor

necrosis factor(TNF)-α, interleukin(IL)-1β, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and CXCL8 (IL-8) [20]. Epithelial cells in small airways express transforming growth factor (TGF)-β, which then induces local fibrosis. Vascular endothelial growth factor (VEGF) appears to be necessary to maintain alveolar cell integrity and blockade of VEGF receptors (VEGFR2) in rats induces apoptosis of alveolar cells and an emphysema-like pathology [14]. A reduction in peripheral lung concentration of VEGF is found in smokers and COPD patients, but another growth factor hepatocyte growth factor (HGF) is increased in smokers and may therefore protect against the reflect of reduced VE GF on alveolar integrity, whereas in COPD both VEGF and HGF are reduced, which may contribute to development of emphysema [21]. Airway epithelial cells are also important in defense of the airways, with mucus production from goblet cells, and secretion of antioxidants, antiproteases, and defensins/cathelicidins. Cigarette smoke and other noxious agents may impair these responses of the airway epithelium, increasing susceptibility to infection. The airway epithelium in chronic bronchitis and COPD often shows squamous metaplasia, which may result from increased proliferation of basal airway epithelial cells but the nature of the growth factors involved in epithelial cell proliferation, cell cycle, and differentiation in COPD are not yet certain. Epithelial growth factor receptors (EGFR) show increased expression in airway epithelial cells of COPD patients and may contribute to basal cell proliferation, resulting in squamous metaplasia and an increased risk of bronchial carcinoma [22]. Club (Clara) cells may act as progenitor cells in peripheral airways and may be susceptible to damage from inhaled irritants. Conditional depletion of club cells in mice leads to squamous cell metaplasia and peribronchiolar fibrosis, similar to the pathology of COPD [23]. Club cell secretory protein is deficient in patients with COPD [24].

Mucus hyperplasia is a feature of many patients with COPD and is a response to chronic airway irritation by cigarette smoke and other pollutants. EGFRs play an important role on mucus hyperplasia and secretion and may be activated by neutrophilic inflammation through neutrophil elastase secretion, which releases TGF-α (Figure 45.2) [25]. Oxidant stress is also able to activate EGFR and induce mucus hypersecretion [26]. An EGFR inhibitor is effective in blocking lipopolysaccharide and TNF-α-induced expression of MUC5AC in human airway epithelial cells and inhibiting LPS-induced mucus secretion and hyperplasia in rats exposed to cigarette smoke [27]. However, an EGFR inhibitor (BIBW2948) was ineffective in reducing mucon gene expression in COPD patients and was poorly tolerated [28].

45.4.2 Macrophages

Macrophages play a key role in orchestrating chronic inflammation in COPD (Figure 45.3) [29]. Macrophage numbers are markedly increased (5-10-fold) in airways, lung parenchyma, BAL fluid, and sputum of COPD patients. Macrophages are localized to sites of alveolar wall destruction in patients with emphysema and there is a correlation between macrophage numbers in the parenchyma and severity of emphysema. Macrophages may be activated by cigarette smoke extract to release inflammatory mediators, including TNF-α,

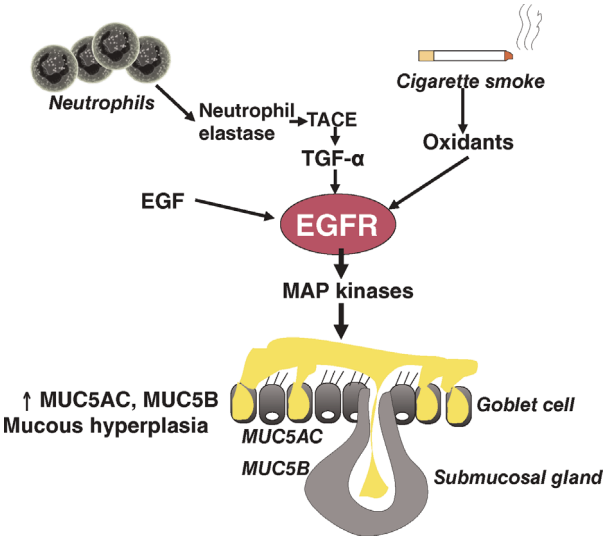


Figure 45.2 Mucus hypersecretion and hyperplasia in COPD. epithelial growth factor receptors (EGFR) may be activated by neutrophils through the effect of neutrophil elastase on TNF-α converting enzyme (TACE), which releases transforming growth factor-(TGF-α), a ligand for EGFR. EGFR may also be activated indirectly via oxidative stress. EGFR activates mitogen-activated protein (MAP) kinases, which increase expression of mucin genes MUC5AC and MUCB and also lead to hyperplasia of goblet cells and submucoisal glands.

CXCL1, CXCL8, CCL2 (MCP-1), LTB_4, and reactive oxygen species (ROS). Alveolar macrophages also secrete elastolytic enzymes, including MMP-2, MMP-9, MMP-12, cathepsins K, L, and S, and neutrophil elastase taken up from neutrophils [30]. Alveolar macrophages from COPD patients secrete more inflammatory proteins and have a greater elastolytic activity at baseline than those from normal smokers and this is further increased by exposure to cigarette smoke. Macrophages demonstrate this difference even when maintained in culture for 3 days and therefore appear to be intrinsically different from the macrophages of normal smokers and nonsmoking normal control subjects.

There are different phenotypes of macrophage that may be differentially activated and with different responses. The murine M1 of "classically activated" macrophages are proinflammatory, whereas M2 or "alternatively activated" macrophages are more anti-inflammatory and release IL-10 and show marked phagocytic activity [31]. However, these distinctions are less clear in human macrophages and the surface markers of these phenotypes less distinct. In general, it is likely that M1-like macrophages predominate in COPD but further studies are needed [32].

Matrix metalloproteinase-9 (MMP-9) is the predominant elastolytic enzyme secreted by alveolar macrophages from COPD patients. Most of the inflammatory proteins that are upregulated in COPD macrophages are regulated by the transcription factor nuclear factor-κB (NF-κB), which is activated in alveolar macrophages of COPD patients, particularly during exacerbations [33]. The

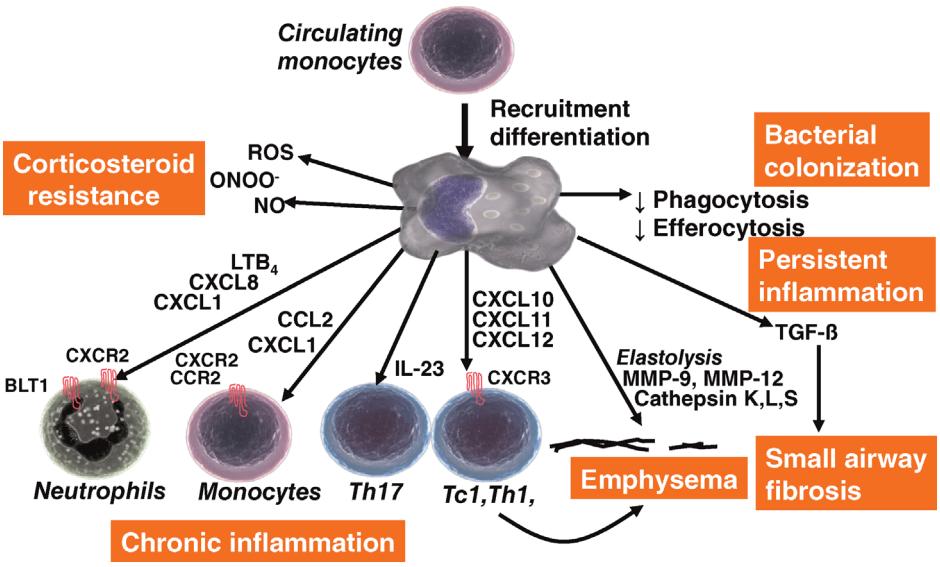


Figure 45.3 Central role of alveolar macrophages in COPD. Alveolar macrophages are derived from circulating monocytes that differentiate within the lung. They secrete many inflammatory proteins that may orchestrate the inflammatory process in COPD. Neutrophils may be attracted by CXCL8, CXCL1, and leukotriene B_4 (LTB_4), monocytes by CCL2, and Tc1 and Th1 lymphocytes by CXCL10, CXC11, and CXCL12. Release of elastolytic enzymes, including matrix metalloproteinases (MMP) and cathepsins, cause elastolysis contribute to emphysema together with cytotoxic T cells. Release of transforming growth factor (TGF)-β1 may induce fibrosis of small airways. Macrophages generate reactive oxygen species (ROS) and nitric oxide (NO), which together form peroxynitrite ($ONOO^-$) and may contribute to corticosteroid resistance. Defective bacterial phagocytosis may lead to bacterial colonization and defective efferocytosis.

increased number of macrophages in the lungs of smokers and COPD patients is due to increased recruitment of monocytes from the circulation in response to the monocyte-selective chemokines CCL2 and CXCL1, which are increased in sputum and BAL of patients with COPD [34]. Monocytes from patients with COPD show a greater chemotactic response to CXCL1 than cells from normal smokers and nonsmokers, but this is not explained by an increase in its receptor CXCR2 and appears to be due to greater receptor turnover [35]. Macrophages also release CXCL9, CXCL10, and CXCL11, which are chemotactic for $CD8^+$ Tc1 and $CD4^+$ Th1 cells, via interaction with the chemokine receptor CXCR3 expressed on these cells [36]. Macrophages from COPD patients release more inflammatory proteins than macrophages from normal smokers and nonsmokers, indicating increased activation [37].

Corticosteroids are ineffective in suppressing inflammation, including cytokines, chemokines, and proteases, in patients with COPD [38]. *In vitro* release of CXCL8, TNF-α, and MMP-9 from macrophages from normal subjects and normal smokers is inhibited by corticosteroids, whereas they are relatively ineffective in macrophages from patients with COPD [37]. The reasons for resistance to corticosteroids in COPD may be the marked reduction in activity

of histone deacetylase-2 (HDAC2), which is recruited to activate inflammatory genes by glucocorticoid receptors to switch off inflammatory genes [39]. The reduction in HDAC activity in macrophages is correlated with increased secretion of cytokines such as TNF-α and CXCL8 and reduced response to corticosteroids.

Both alveolar macrophages and monocyte-derived macrophages from COPD patients also show reduced phagocytic uptake of bacteria and this may be a factor in determining chronic colonization of the lower airways by bacteria such as *Haemophilus influenzae* or *Streptococcus pneumoniae* [40]. COPD macrophages are also defective in taking up apoptotic cells and this may contribute to the failure to resolve inflammation in COPD [41]. The nature of this defect in phagocytosis is not fully understood, but appears to be due to a defect in microtubular function that is required for phagocytosis rather than any abnormality of recognition of the phagocytosed particles [42]. Bacterial colonization of lower airways, found in at least 50% of COPD patients, may predispose to increased acute exacerbations and also to the increased risk of developing community acquired pneumonia in COPD patients [43].

45.4.3 Neutrophils

Increased numbers of activated neutrophils are found in sputum and BAL fluid of patients with COPD and correlate with disease severity, although few neutrophils are seen in airway wall and lung parenchyma, likely reflecting their rapid transit through these tissues. Smoking has a direct stimulatory effect on granulocyte production and release from the bone marrow and survival in the respiratory tract, possibly mediated by the hematopoietic growth factors GM-CSF and G-CSF released from lung macrophages. Neutrophil recruitment to the airways and parenchyma involves initial adhesion to endothelial cells via E-selectin, which is upregulated on endothelial cells in the airways of COPD patients [44]. Adherent neutrophils migrate into the respiratory tract under the direction of various neutrophil chemotactic factors, including LTB_4, CXCL1, CXCL5 (ENA-78), and CXCL8, which are increased in COPD airways. These chemotactic mediators may be derived from alveolar macrophages, T cells, and epithelial cells, but the neutrophil itself may be a major source of CXCL8. Neutrophils recruited to the airways of COPD patients are activated as there are increased concentrations of granule proteins, such as myeloperoxidase (MPO) and human neutrophil lipocalin, in the sputum supernatant [45]. Neutrophils secrete serine proteases, including neutrophil elastase (NE), cathepsin G, and proteinase-3, as well as MMP-8 and MMP-9, which may contribute to alveolar destruction. Airway neutrophilia is linked to mucus hypersecretion as neutrophil elastase, cathepsin G, and proteinase-3 are potent stimulants of mucus secretion from submucosal glands and goblet cells [46]. There is a marked increase in neutrophil numbers in the airways in acute exacerbations of COPD accounting for the increased purulence of sputum. This may reflect increased production of neutrophil chemotactic factors, including LTB_4 and CXCL8 [47]. Neutrophils from COPD patients show marked abnormalities in chemotactic response, with increased migration but reduced accuracy, reminiscent of the abnormal monocyte chemotactic responses [48].

45.4.4 Eosinophils

While eosinophils are the predominant leukocyte in asthma, their role in COPD is much less certain. Increased numbers of eosinophils have been described in the airways and BAL of patients with stable COPD, whereas others have not found increased numbers in airway biopsies, BAL, or induced sputum [39]. The presence of eosinophils in patients with COPD predicts a more favorable therapeutic response to bronchodilators and corticosteroids may indicate coexisting asthma or asthma–COPD overlap [49–51]. Approximately 15% of COPD patients appear to have clinical features of asthma [52]. The mechanisms for increased eosinophils in COPD patients are uncertain, but there has recently been considerable interest in the role of type 2 innate lymphoid cells (ILC2), which may be important in intrinsic asthma, but also in COPD [53,54]. ILC2 cells may be regulated by epithelial mediators such as IL-33, released as a result of epithelial cell injury (Figure 45.4). IL-33 expression is increased in basal epithelial progenitor cells in COPD and is associated with increased expression of IL-13 and mucin gene 5AC [55]. There appears to be considerable plasticity in ILCs and evidence that ILC2 cells may convert to ILC1 cells that release interferon(IFN)-γ after viral infection and exposure to IL-12 and IL-18 [56]. The presence of eosinophils in the airways may indicate a better response to corticosteroid therapy and therefore

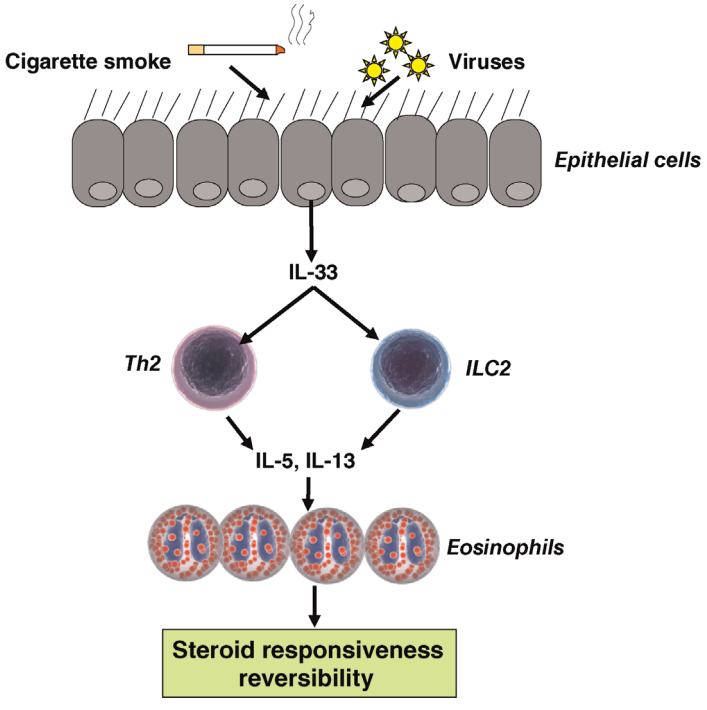


Figure 45.4 Eosinophilic inflammation in COPD. Both T helper 2 (Th2) and type 2 innate lymphoid cells (ILC2) may cause eosinophilic inflammation through the release of IL-5 and IL-13 and may result in a better response to corticosteroids and bronchodilators. Th2 and ILC2 cells may be activated by IL-33 from damaged epithelial cells.

may define a therapeutic phenotype of COPD patient. Sputum concentrations of IL-5 are correlated with sputum eosinophils and both are reduced by oral corticosteroids [57]. There is a relationship between bronchial wall eosinophils and blood eosinophils, so this may make it feasible to use blood eosinophil counts as a biomarker for steroid responsiveness in COPD patients [58]. In retrospective analyses of clinical trials, patients with increased blood eosinophils (>2% or >200/μl) show a greater reduction in exacerbations than in patients with lower eosinophils, although there was no difference in lung function or symptoms [59,60]. Increased numbers of eosinophils have been reported in bronchial biopsies and BAL fluid during acute exacerbations of chronic bronchitis [61].

45.4.5 Lymphocytes

There is an increase in the total numbers of T lymphocytes in lung parenchyma, peripheral, and central airways of patients with COPD, with the greater increase in $CD8^+$ than $CD4^+$ cells [9,36]. There is a correlation between the numbers of T cells and the amount of alveolar destruction and the severity of airflow obstruction. Furthermore, the only significant difference in the inflammatory cell infiltrate in asymptomatic smokers and smokers with COPD is an increase in T cells, mainly $CD8^+$ (Tc1), in patients with COPD [62]. There is also an increase in the absolute number of $CD4^+$ (Th1) T cells albeit in smaller numbers, in the airways of smokers with COPD and these cells express activated STAT-4, a transcription factor that is essential for activation and commitment of the Th1 lineage [63]. $CD4^+$ Th17 cells which secrete IL-17A and IL-22 are also increased in airways of COPD patients and may play a role in orchestrating neutrophilic inflammation [64,65]. Th17 cells may be regulated by IL-6 and IL-23 released from alveolar macrophages. $CD4^+$ and $CD8^+$ T cells in the lung of COPD patients show increased expression of CXCR3, a receptor activated by the chemokines CXCL9–CXCL11, all of which are increased in COPD [66]. There is increased expression of CXCL10 by bronchiolar epithelial cells and this could contribute to the accumulation of $CD4^+$ and $CD8^+$ T cells, which preferentially express CXCR3 (Figure 45.5) [67]. $CD8^+$ cells are typically increased in airway infections and it is possible that the chronic bacterial colonization of the lower respiratory tract of COPD patients is responsible for this inflammatory response.

As discussed earlier, ILCs are now thought to play an important role in regulation of lung immunity and may be regulated via danger signals and cell damage [68]. There is considerable plasticity between different subtypes of ILC in response to different triggers and cytokines. All three types of ILC have been identified in human lung [54]. In COPD, there is an increase in ILC3 cells, which secrete IL-17 and IL-22, and they may play a role in driving neutrophilic inflammation.

Autoimmune mechanisms may also be involved. Cigarette-induced lung injury may uncover previously sequestered autoantigens or cigarette smoke itself may damage lung interstitial and structural cells and make them antigenic. Oxidative stress may result in the formation of carbonylated proteins that are antigenic and several anti-carbonylated protein antibodies have been found in the circulation of COPD patients, particularly in severe disease [69]. Antiendothelial antibodies

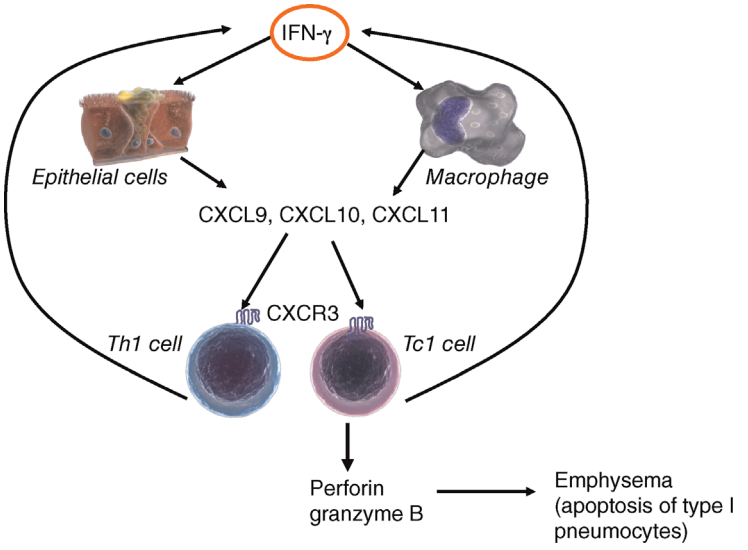


Figure 45.5 Lymphocytes in COPD. Epithelial cells and macrophages release the chemokines CXCL9, CXCL10, and CXCL12 that activate CXCR3 on Th1 and Tc1 cells, which release interferon (IFN)-γ, which in turn causes further release of these chemokines. Tc1 cells are cytotoxic because of the release of perforin and granzyme B, which may contribute to alveolar cell apoptosis and the development of emphysema.

have also been detected [70]. Autoantibodies may cause cell damage through the binding of complement, which is increased in the lungs of COPD patients [69]. Citrullinated proteins are also described in the lungs of COPD patients and may induce autoantibody formation, as in rheumatoid arthritis [71].

$CD8^+$ cells cause cytolysis and apoptosis of alveolar epithelial cells through release of perforins, granzyme-B, and TNF-α, and there is an association between $CD8^+$ cells and apoptosis of alveolar cells in emphysema [72]. There is evidence for immunological senescence in COPD with increased numbers of T cells with no expression of the costimulatory receptor CD28 ($CD4/CD28^{null}$ and $CD8/CD28^{null}$ cells) and these cells release increased amounts of perforins and granzyme B [73,74]. Senescence of T cells in COPD is associated with reduced expression of HDAC2 [75].

B lymphocytes are also increased in COPD lungs, particularly in patients with severe disease. B cells are organized into lymphoid follicles, which are located in peripheral airways and lung parenchyma [9]. B-cell activating factor is an important regulator of B-cell function and hyperplasia and in increase in lymphoid follicles of COPD patients [76].

45.4.6 Dendritic Cells

Dendritic cells are an important link between innate and adaptive immunity. The airways and lungs contain a rich network of dendritic cells that are localized near the surface, so that they are ideally located to signal the entry of foreign substances that are inhaled. Dendritic cells can activate a variety of other inflammatory and

immune cells, including macrophages, neutrophils, T lymphocytes, and B lymphocytes; so, dendritic cells may play an important role in the pulmonary response to cigarette smoke and other inhaled noxious agents. Dendritic cells appear to be activated in the lungs of COPD patients [77] and are linked to disease severity [78]. Numbers of dendritic cells are increased in the lungs of COPD patients and cigarette smoke increases their survival *in vitro* [79].

45.5 Inflammatory Mediators

Many inflammatory mediators have been implicated in COPD, including lipids, free radicals, cytokines, chemokines, and growth factors [80]. These mediators are derived from inflammatory and structural cells in the lung and interact with each other in a complex manner. Because so many mediators are involved, it makes it unlikely that blocking a single mediator will have significant clinical impact. Similar mediators that are found in the lungs of COPD patients may also be increased in the circulation and this systemic inflammation may underlie and potentiate comorbidities.

45.5.1 Lipid Mediators

The profile of lipid mediators in exhaled breath condensates of patients with COPD shows an increase in prostaglandins and leukotrienes. There is a significant increase in PGE_2 and $PGF_{2\alpha}$, and an increase in LTB_4 but not cysteinyl-leukotrienes [81]. This is a different pattern to that seen in asthma, which has increases in thromboxane and cysteinyl-leukotrienes. LTB_4 concentrations are increased in induced sputum and further increased in sputum and exhaled breath condensate during acute exacerbations [47,82]. LTB_4 is a potent chemoattractant of neutrophils, acting through high affinity BLT_1-receptors. A BLT_1-receptor antagonist reduces the neutrophil chemotactic activity of sputum by approximately 25% [82]. BLT_1 receptors have also been identified on T lymphocytes and there is evidence that LTB_4 is also involved in recruitment of T cells.

45.5.2 Cytokines

Cytokines are the mediators of chronic inflammation and several have been implicated in COPD [83]. There is an increase in concentration of TNF-α in induced sputum in stable COPD with a further increase during exacerbations. TNF-α production from peripheral blood monocytes is also increased in COPD patients and has been implicated in the cachexia and skeletal muscle apoptosis found in some patients with severe disease. TNF-α is a potent activator of NF-κB and this may amplify the inflammatory response. Unfortunately, anti-TNF therapies have not proved to be effective in COPD patients and may have serious adverse effects [84]. The reason for the failure of anti-TNF therapies in COPD is presumably because other proinflammatory cytokines druve the inflammatory process.

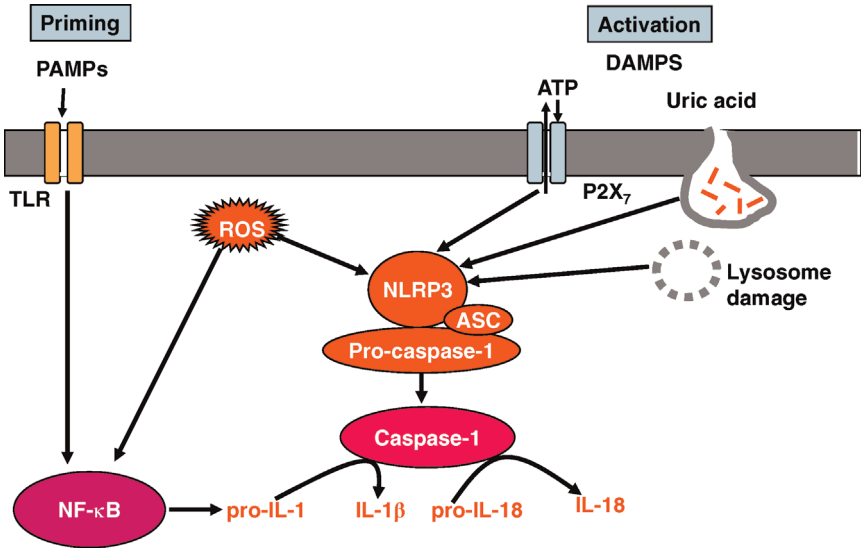


Figure 45.6 Inflammasome activation in COPD. The NLRP3 inflammasome is activated by two signals. The proming signal is activated by pathogens through pathogen-activated molecular patterns (PAMPs) with the generation of pro-IL-1β and pro-IL-8 by activation of nuclear factor-κB (NF-κB). The activation signal may include ATP (through $P2X_7$-receptors) and other damage-activated molecular patterns (DAMPS) such as uric acid. This causes the recruitment of the adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1 to generate caspase-1, which releases active IL-1β and IL-18.

45.5.3 Inflammasome

Inflammasomes are multiprotein signaling complexes that regulate the expression of the proinflammatory cytokines IL-1β and IL-18 in response to external and endogenous danger signals, resulting in neutrophilic inflammation [85]. Most attention has focused on NLRP3 inflammasomes, which are activated in several inflammatory lung diseases (Figure 45.6) [86]. The adaptor protein ASC is an important component of the NLRP3 inflammasome, which recruits pro-caspase-1 to the protein complex and is increased in lungs of COPD patients [87]. ASC accumulation is associated with the formation of extracellular "specks" that perpetuate the generation of IL-1β outside the cell. However, in patients with stable COPD, no increase in the NLRP3 inflammasome were found, apart from a small increase in patients with severe disease, possibly because an increase in inflammasome inhibitory molecules NALP7 and IL-37. It is more likely that NLRP3 inflammasome activation is linked to acute exacerbations, where pathogens, oxidative stress, and ATP, all of which activate NLRP3 inflammasome, are increased. The minimal role of the inflammasome in stable COPD is underlined by the failure of an IL-1β blocking antibody canakinumab to provide any clinical benefit in stable COPD [88].

IL-17, and other Th17 cytokines, are also increased in COPD sputum and airways and may play a role in orchestrating neutrophilic inflammation in the lungs [64,89]. These cytokines may also be released from ILC3 cells that are increased in COPD patients [54].

45.5.4 Chemokines

Several chemokines have been implicated in COPD and have been of particular interest since chemokine receptors are G-protein-coupled receptors, for which small molecule receptor antagonists have been developed [90]. CXCL8 concentrations are increased in induced sputum of COPD patients and increase further during exacerbations [33,36]. CXCL8 is secreted from macrophages, T cells, epithelial cells, and neutrophils, and is chemotactic for neutrophils via high affinity CXCR2, which are also activated by related CXC chemokines such as CXCL1. CXCL1 concentrations are markedly elevated in sputum and BAL fluid of COPD patients and this chemokine may be more important as a chemoattractant than CXCL8, acting via CXCR2, which are expressed on neutrophils and monocytes [34]. CXCL1 induces significantly more chemotaxis of monocytes of COPD patient compared to those of normal smokers and this may reflect increased turnover and recovery of CXCR2 in monocytes of COPD patients [35]. CXCL5 (ENA-78) shows a marked increase in expression in airway epithelial cells during exacerbations of COPD and this is accompanied by a marked upregulation of epithelial CXCR2.

CCL2 is increased in concentration in COPD sputum and BAL fluid and plays a role in monocyte chemotaxis via activation of CCR2 [34]. The chemokine CCL5 (RANTES) is also expressed in airways of COPD patients during exacerbations and activates CCR5 on T cells and CCR3 on eosinophils, which may account for the increased eosinophils and T cells in the wall of large airways that have been reported during exacerbations of chronic bronchitis. As discussed above, CXCR3 are upregulated on Tc1 and Th1 cells of COPD patients with increased expression of their ligands CXCL9, CXCL10, and CXCL11 [66]. CXCR3 ligands induce increased chemotaxis of monocytes and lymphocytes from COPD patients, which may reflect increased CXCR3 expression in COPD [91].

45.5.5 Proteases

The increase in elastase activity in COPD patients may contribute to the development of emphysema and to neutrophilic inflammation through the generation of chemotactic peptides such as acetylated Pro-Gly-Pro (matrikines), which are potent neutrophil chemoattractants that activate CXCR2 [92]. This may be self-perpetuating as neutrophils release MMP-9, which in turn generates more matrikine [93]. Human neutrophil elastase (HNE) not only has elastolytic activity but is also a potent stimulant of mucus secretion in the airways, mediated via EGFR [94]. MMP-9 is the predominant elastolytic enzyme in COPD and is secreted from macrophages, neutrophils, and epithelial cells.

45.6 Oxidative Stress as a Major Driving Mechanism

Oxidative stress occurs when ROS are produced in excess of the antioxidant defense mechanisms and result in harmful effects, including damage to lipids, proteins, and DNA. Oxidative stress is a critical feature in COPD [95].

Inflammatory and structural cells, including neutrophils, macrophages, and epithelial cells that are activated in the airways of patients with COPD, produce ROS. Superoxide anions ($O_2^{\bullet -}$) are generated by NADPH oxidase (NOX) and converted to hydrogen peroxide (H_2O_2) by superoxide dismutases (SOD). H_2O_2 is then converted to water by catalase. $O_2^{\bullet -}$ and H_2O_2 may interact in the presence of free iron to form the highly reactive hydroxyl radical ($^{\bullet}OH$). $O_2^{\bullet -}$ may also combine with NO to form peroxynitrite, which also generates $^{\bullet}OH$. Oxidative stress leads to the oxidation of arachidonic acid and the formation of a new series of prostanoid mediators called isoprostanes, which may exert significant functional effects, including bronchoconstriction and plasma exudation [96]. Peroxynitrite is also increased in the breath of COPD patients [97]. Nitric oxide may be increased in peripheral lung of COPD patients and appears to be linked to increased expression of inducible and neural NO synthases (NOS2, NOS1) [98].

The normal production of oxidants is counteracted by several antioxidant mechanisms in the human respiratory tract, including catalase, SOD, and glutathione, formed by the enzyme γ-glutamyl cysteine ligase, and glutathione synthetase. In the lung, intracellular antioxidants are expressed at relatively low levels and are not induced by oxidative stress, whereas the major antioxidants are extracellular. Extracellular antioxidants, particularly glutathione peroxidase, are markedly upregulated in response to cigarette smoke and oxidative stress. Extracellular antioxidants also include the dietary antioxidants vitamin C (ascorbic acid) and vitamin E (α-tocopherol), uric acid, lactoferrin, and extracellular superoxide dismutase (SOD3), which is highly expressed in human lung. Most antioxidants are regulated by the transcription factor nuclear erythroid-2-related factor-2 (Nrf2), which is activated by oxidative stress. However, in COPD lungs and cells, Nrf2 is not appropriately activated despite high levels of oxidative stress in the lungs [99] and this may be related to increased acetylation due to decreased HDAC2 [100].

ROS have wide-ranging effects on the airways and parenchyma and increase the inflammatory response. ROS activate NF-κB, which switches on multiple inflammatory genes resulting in amplification of the inflammatory response. Oxidative stress results in activation of histone acetyltransferase activity, which opens up the chromatin structure and is associated with increased transcription of multiple inflammatory genes [101]. Oxidative stress may also impair the function of antiproteases such as α_1-antitrypsin and SLPI, and thereby accelerates the breakdown of elastin in lung parenchyma. Oxidative stress markedly reduces HDAC2 activity and expression, through activation of phosphoinositide-3-kinase-δ (PI3 Kδ) and peroxynitrite-induced nitration of tyrosine resides [39]. This amplifies inflammation further and prevents corticosteroids from inactivating activated inflammatory genes. Through similar mechanisms, oxidative stress reduces the expression and activity of sirtuin-1, a key repair molecule that is implicated in aging. The reduction in sirtuin-1 in COPD lungs and cells may underlie the accelerated aging response seen in COPD [102,103]. Oxidative stress may also predispose to lung cancer, through the activation of growth factors and via DNA damage [104]. As discussed earlier, oxidative stress leads to formation of carbonylated proteins, which may be antigenic and stimulate the development of autoantibodies in COPD patients, which may cause persistence of inflammation [69].

45.7 Systemic Inflammation in COPD

Patients with COPD, particularly when the disease is severe and during exacerbations, have evidence of systemic inflammation, measured either as increased circulating cytokines, chemokines and acute phase proteins, or as abnormalities in circulating cells [105,106]. Persistent inflammation is associated with poorer clinical outcomes. Smoking itself may cause systemic inflammation, for example, an increased total leukocyte count, but in COPD patients the degree of systemic inflammation is greater. It is still uncertain whether these systemic markers of inflammation are a "spillover" from inflammation in the peripheral lung or are a parallel abnormality or related to some comorbid disease that then has effects on the lung. In any case, the systemic inflammation in COPD may contribute to its systemic manifestations and may worsen comorbid diseases. In a large population study, systemic inflammation (increased CRP, fibrinogen, and leukocytes) was associated with a two- and four-fold increased risk of cardiovascular disease, diabetes, lung cancer, and pneumonia but not with depression [107]. Using six inflammatory markers (CRP, IL-6, CXCL8, fibrinogen, TNF-α, and leukocytes), 70% of COPD patients had some components of systemic inflammation and 16% had persistent inflammation [106]. Patients with persistent systemic inflammation had increased mortality and more frequent exacerbations. Systemic inflammation appears to relate to accelerated decline in lung function and is increased further during exacerbations [108].

45.8 Defective Resolution of Inflammation and Repair

The reason why inflammation persists in COPD patients even after long-term smoking cessation is currently unknown, but if the molecular and cellular mechanisms for impaired resolution could be identified, this may provide a novel approach to COPD therapy. A major mechanism of airway obstruction in COPD is loss of elastic recoil due to proteolytic destruction of lung parenchyma, so it is unlikely that this could be reversible by drug therapy. However, it might be possible to reduce the rate of progression by preventing the inflammatory and enzymatic disease process. Similarly, the peribronchiolar fibrosis of small airways may not be reversible, but its progression may be halted by antifibrotic and anti-inflammatory therapies.

45.8.1 Proresolving Lipid Mediators

Resolution of inflammation is an active process that may be facilitated by several endogenous proresolving mediators, including lipoxins, E-series resolvins, D-series protectins, and maresins, all of which are derived from polyunsaturated fatty acids and act on distinct receptors [109]. These mediators promote the resolution of neutrophilic inflammation by preventing neutrophil recruitment and enhancing neutrophil removal by efferocytosis. Endogenous mediators or stable structural analogs that activate the same receptors may have therapeutic potential in promoting resolution of inflammation in COPD. Maresin-1 is the

most potent proresolving mediator that stimulates macrophage efferocytosis, so a stable analog of this mediator may be useful in COPD [110]. Defective efferocytosis by macrophages in COPD may prevent resolution of inflammation and may be the same defect that reduces bacteria phagocytosis [41,42].

45.8.2 Accelerated Aging

There is growing evidence that emphysema may be due to accelerated aging of the lungs and it hypothesized that accelerated aging may be due to defective function of endogenous antiaging molecules, such as sirtuins and FOXO proteins, as a result of increased oxidative stress in the lung [103,111]. SIRT1 is markedly reduced in the peripheral lung of patients with COPD and *in vitro* this is mimicked by oxidative stress, which reduces activity and expression of SIRT1, resulting in increased expression of MMP-9 [102]. The pathway linking oxidative stress to reduced SIRT1 expression involved PI3 K and activation of mammalian target of rapamycin (mTOR), which is activated in COPD and inhibited by rapamycin [112]. Since the same molecular pathways are shared by many of the comorbidities of COPD, which are also characterized by accelerated aging, it may be possible to identify novel targets and therapies to prevent disease progression and associated comorbidities [111]. Cellular senescence is found in COPD lungs [113] and senescent cells are known to secrete inflammatory proteins, including TNF-α, IL-1, IL-6, CXCL8, CCL2, and MMPs. This senescence-associated secretory profile (SASP) may account for the chronic low-grade inflammation that is found in COPD (Figure 45.7) [114].

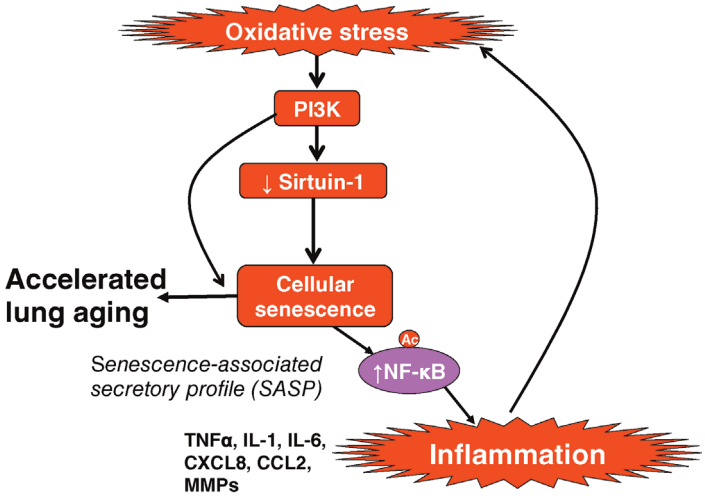


Figure 45.7 Accelerated aging and inflammation in COPD. Oxidative stress drives accelerated aging through activation of phosphoinositide-3-kinase (PI3 K) and reduction in sirtuin-1, which leads to cellular senescence and the release of inflammatory proteins (SASP), which further increase oxidative stress.

45.8.3 Airway Fibrosis

Increasing small airway fibrosis is an important mechanism of disease progression in COPD and is presumed to result from chronic inflammation, suggesting that effective anti-inflammatory treatments should prevent fibrosis. Fibrosis may be mediated via the activation of fibroblasts by fibrogenic mediators such as TGFβ, CTGF, and endothelin, which are secreted from epithelial cells and macrophages [115]. Endothelin-1 is also profibrotic and several endothelin receptor antagonists inhibit pulmonary fibrosis in animal models.

45.9 Future Implications

There is a need to identify phenotypes of COPD that respond to specific therapies and this will involve recognizing disease endotypes and biomarkers that predict response.

As already discussed, some patients with COPD have eosinophilic inflammation and this may be recognized by increased blood eosinophils, which may indicate patients who have a better therapeutic response to inhaled corticosteroids. Prospective studies are needed to define the cutoff point for blood eosinophils that indicate a good steroid response. Furthermore, COPD patients without elevated eosinophils would probably have inhaled corticosteroids withdrawn, which appears to be safe even in high-risk patients treated with high doses of inhaled corticosteroids [116].

Although inflammation is believed to be a key driving mechanism for COPD progression and exacerbations, it is apparent that in elderly COPD patients that cellular senescence may lead to low-grade inflammation, which may then drive further disease progression [114].

It is now recognized that there may be several coexisting mechanisms in COPD that interact in complex ways so that targeting a single pathway or mediator may not be effective in treating COPD, unless specific "responder phenotypes|" are identified. It is important to identify the molecular mechanisms that underlie susceptibility so that only a minority of smokers develop COPD. So far genome-wide association studies of COPD have been disappointing, but this may be because the studies include all types of COPD. It is also likely that epigenetic mechanisms such as DNA methylation play an important role in disease susceptibility and have yet to be explored.

Clinical studies to show effects of new drugs on disease progression are challenging since large numbers of patients studied over long periods (3 years) are necessary. It is important to identify biomarkers of disease "activity" or surrogate measurements that will predict the clinical efficacy of anti-inflammatory treatments in COPD patients. Analysis of sputum parameters (cells, mediators, and enzymes) or exhaled biomarkers (lipid mediators and reactive oxygen species) may be useful as biomarkers of inflammation [117].

COPD includes several different clinical and pathophysiological phenotypes, but so far it has been difficult to link any phenotype to a differential response to therapy, apart from the patients with eosinophilia discussed earlier. The PDE4

inhibitor roflumilast appears to work more effectively in a phenotype defined by disease severity, frequent exacerbations, and mucus hypersecretion, which may be a surrogate for neutrophilic inflammation [118]. Biomarkers that predict responsiveness to particular treatments are needed, particularly as treatment becomes more specific, such as targeting specific proteins. Patients with early disease who appear to decline more rapidly may show better responses to therapy, whereas currently the focus of treatment is on patients with late disease who have the most symptoms. If effective and safe anti-inflammatory therapies are developed for COPD, it may be more useful to introduce these early in the course of the disease to prevent disease progression and possibly to reduce the burden of concomitant comorbidities. This approach is akin to the therapy of systemic hypertension and hypercholesterolemia where treatment is given to prevent future risk.

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46

Obesity: A Complex Disease with Immune Components

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46.1 Introduction

Obesity has become a global epidemic and is one of the most significant public health challenges of the twenty-first century. Obesity contributes to a myriad of metabolic complications, including insulin resistance and diabetes, nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH), atherosclerosis, cardiovascular disease, and cancers. Among these complications, NAFLD/NASH is now seen as one of the most severe, as it also increases the risk of cirrhosis and liver carcinoma. NAFLD/NASH also further heightens cardiovascular risk.

Inflammation is currently regarded as one of the crucial pathophysiological processes associated with the progression of obesity and its related complications [1–3]. Additionally, adipose tissue (AT) now appears to be a key driver of complications; thus, this endocrine organ is not merely a simple fat reservoir. The observation that AT secretes the proinflammatory cytokine tumor necrosis factor alpha (TNF-α) marked the first milestone in studies investigating the immunological role of AT [4]. Subsequently, the innate and adaptive immune cells of AT depots have been found to secrete a diverse panel of proinflammatory cytokines/chemokines [5]. According to body composition, the adipose organ is distributed throughout several depots: visceral AT (VAT, including omental and mesenteric) and subcutaneous AT (scAT). The scAT is also composed of two main layers, the superficial and deep scAT that are separated by the fascia superficialis.

The relative importance of each depot in producing low-grade inflammation is due to adipocyte size (i.e., hypertrophic adipocytes secrete proinflammatory molecules) and differences in inflammatory cell infiltration. AT comprises two main fractions that can be isolated following collagenase digestion: adipocytes and the stromal vascular fraction (SVF) [6]. The number and phenotype of SVF cells can significantly change and adversely affect metabolism under obese conditions [7].

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AT is thus an active endocrine organ that controls energy homeostasis through the systemic release of adipokines (i.e., adipose-derived inflammatory molecules). Among a large panel of known molecules, leptin and adiponectin hormones are specifically secreted by adipocytes, but both hypertrophic adipocytes and SVF cells can also secrete various proinflammatory and anti-inflammatory cytokines (IL-1α, IL-6, IL-8, TNF-α, and IL-10) and chemokines (MCP-1/CCL2, RANTES/CCL5). These mediators may act not only locally by influencing adipocyte differentiation and/or survival but also systemically through the modulation of neuron activity in the central nervous system [8]. Here, we discuss the peripheral actors implicated in obesity-related inflammation, with a focus on AT.

46.2 Systemic inflammation: Circulating Cells as Contributors

This concept of "low-grade inflammation" in obesity was first based on the fact that obese people present a moderate but chronic increase in circulating inflammatory mediators, such as acute-phase proteins, proinflammatory cytokines, and adhesion molecules [8,9]. Over the last 20 years, the number of described molecules has continued to expand. In addition, changes in circulating immune cells also contribute to low-grade inflammation in obesity. Interestingly, this impaired immune profile in obese subjects is frequently reversible with weight loss following clinical intervention [10].

Monocytes ($CD14^+$ cells) exist in a proinflammatory state in obesity [11] and clinical studies indicate that the frequency of the $CD14^+CD16^{++}$ nonclassical monocyte subpopulation and the $CD14^{++}CD16^+$ intermediate monocyte population increases in obese subjects and decreases with weight loss [12,13]. In mice, the $Gr1^+Ly\text{-}6C^{high}$ monocytes, comparable to human $CD14^+CD16^+$ monocytes, could be precursors of proinflammatory macrophages accumulating in AT [14], but this has not yet been clearly demonstrated. In addition to monocytes [15], lymphocyte phenotypes are also modified. In a small study exploring 10 obese and 10 lean subjects, obese subjects displayed a decreased frequency of cytotoxic $CD3^+CD8^+$T cells (CTL), but an increased frequency of $CD3^+CD4^+$ and $CD3^+CD8^+CD95^+$ T cells. Increased CD95 (Fas) expression on T cells may indicate impaired regulation of $CD8^+$ T cell apoptosis [16]. T cells express receptors such as toll-like receptor 4 (TLR4) and IL-21 receptor (IL-21R) [17]. Subjects at high risk of developing diabetes demonstrate decreased numbers of $TLR4^+CD4^+$ T cells, which is associated with increased fasting plasma glucose [17]. The number of circulating $IL\text{-}21R^+CD4^+$ T cells was also negatively correlated with BMI in these subjects [17]. It has also been shown that obese subjects have fewer natural killer (NK) cells compared to lean individuals. Unhealthy obese subjects had even fewer NK and CTL cells compared to metabolically healthy obese people. Moreover, NK cells appear less activated in metabolically healthy obese subjects than unhealthy subjects [18]. These clinical studies indicate that higher levels of CTL and NK cells may protect against metabolic complications, but these findings require further exploration in

larger populations to clearly determine the relationship with the progression of obesity complications.

Circulating neutrophils from obese subjects also release high levels of proinflammatory mediators such as IL-8, chemokine motif ligand 2 (CCL2), matrix metalloproteinase (MMP9), and myeloperoxidase (MPO). Neutrophils are activated by the chemokine (C-X-C motif) ligand 2 (CXCL2) *ex vivo* and may stimulate adhesion and angiogenic capacities of neutrophils to the endothelial cells of visceral white AT [19]. In overweight children, cell counts of most circulating immune cells (i.e., total leukocytes, neutrophils, monocytes, total $CD3^+$ T lymphocytes, and $CD4^+$ T helper cells) are also modified, suggesting that this is an early phenomenon during obesity development.

As mentioned previously, this type of chronic low-grade inflammation can be reversible after weight loss. Dietary intervention and bariatric surgery significantly decrease the proinflammatory activation surface markers on circulating immune cells such as CD11b granulocytes, CD66b monocytes, and CD69 and CD25T cells. The number of proinflammatory Th1 cells and also the Th1-to-Th2 cell ratio are reduced. The specific reduction in the Th1-to-Th2 cell ratio is associated with weight loss and a reduced waist circumference [20]. In a study in obese individuals, the upregulated mitogen-induced blastogenic responses of peripheral blood T and B lymphocytes were significantly reduced following a very low calorie diet intervention [21].

46.3 Macrophages in Obese Adipose Tissue

In 2003, two research groups [22,23] revealed accumulation of AT macrophages (ATM) in genetically and diet-induced obese mice and in obese humans. Subsequently, there have been many discussions surrounding the specific phenotype of these cells, which are dissimilar in rodents and humans. Increased CD68 gene expression or macrophage infiltration in obese AT is associated with impaired insulin sensitivity and severe insulin resistance [24–27] in some but not all human studies. Nevertheless, at least in obese mice, ATM are implicated in inflammatory pathway activation [23] and contribute to obesity-induced insulin resistance [22] (Figure 46.1). ATM span a broad phenotypic spectrum, although two main populations have been described: the classically activated M1 and alternatively activated M2 cells. M1 cells express CD11c surface markers and secrete proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and inducible nitric oxide synthase (iNOS) in response to lipopolysaccharide (LPS) and Th1 cytokine IFN-γ treatment. M2 cells express CD206 and CD301, and classically secrete anti-inflammatory cytokines such as IL-10, IL-1 receptor antagonist (IL-1RA), and arginase 1 in response to Th2 cytokines IL-4 and IL-13 [28]. M2 cells in AT are located in the interstitial spaces surrounding adipocytes and are the dominant resident ATM in the lean state [29,30]. These cells are considered to be implicated in tissue remodeling and repair [30]. Obesity develops in rodents with a high-fat diet (HFD), along with a phenotypic switch from an M2-polarized to an M1 proinflammatory state [31]. Most ATM in obese mice, particularly M1 cells, primarily originate from circulating bone marrow-derived monocytes [23], for

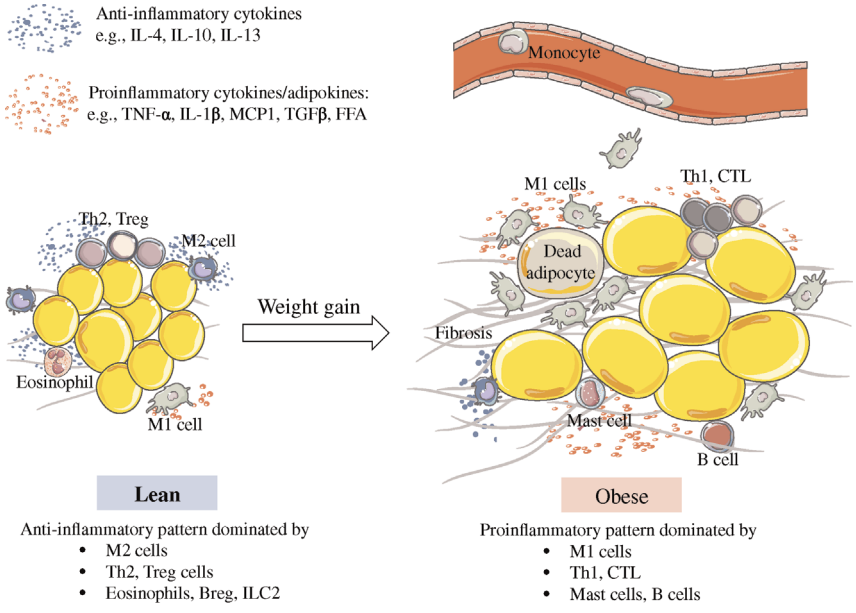


Figure 46.1 Adipose tissue inflammation in obesity. In the lean state, AT resident M2 cells, Treg cells, Th2 cells, group 2 innate lymphoid cells (ILC2s), eosinophils, and Breg cells predominate. The anti-inflammatory cytokines IL-4, IL-13 secreted by Th2 cells, eosinophils, and IL-10 secreted by Treg cells induce and maintain resident M2 cells, and improve insulin sensitivity. In the obese state, AT undergoes a switch from an anti-inflammatory cellular phenotypes to proinflammatory phenotypes (i.e., M1, Th1 cells, CTL, mast cells, and B cells), leading to adipose inflammation and insulin resistance. Adipose inflammation initiates tissue fibrosis that further aggravates inflammation. The proinflammatory cytokines and chemokines are released into circulation and subsequently enhance inflammation in other organs. A difficult issue is to examine whether this phenomenon exists during the progression of human obesity. Indeed, in human AT, macrophages express M2 markers and secrete a mixture of proinflammatory and anti-inflammatory molecules. The kinetics of macrophage accumulation and their phenotypic changes in human AT are not well understood.

example, circulating classical $Gr1^{+}Ly^{-}6C^{high}$ monocytes in diet-induced obese (DIO) mice [14]. ATM lipid content increases in obesity, and furthermore, leads to an accumulation of a small subpopulation of lipid-laden cells with foamy cytoplasm, named adipose foam cells. These adipose foam cells are implicated in local AT crosstalk that contributes to insulin resistance in obese rodents and humans [32].

During the transition from lean to obese states in humans, it is not clear if a switch from an M2-polarized state to an M1 proinflammatory state actually exists. Indeed, studies phenotyping these heterogeneous cells showed that macrophages from obese AT express M2 markers and secrete both proinflammatory and anti-inflammatory molecules [33]. The extravasation of monocytes into AT is not fully understood. Our team has demonstrated that monocytes express higher levels of the chemokine receptors CX3CR1, CCR2, and CCR5, and strongly respond to TLR4 and TLR8 stimulation in obesity, suggesting an increased cellular migratory capacity during obesity, which requires further investigation [13]. Monocyte

migration to inflamed AT probably occurs through many mechanisms, for example, an increased response to chemokines (e.g., monocyte chemoattractant protein-1 MCP-1/CCR2, CCL5/RANTES, osteopontin), and adhesion molecules (e.g., platelet endothelial cell adhesion molecule (PECAM)-1, intercellular adhesion molecule (ICAM)-1) [14]. Hypertrophic adipocytes initiate ICAM-1 and PECAM-1 adhesion molecule expression on endothelial cells, which concomitantly attracts monocytes to AT [34]. Adipocytes also produce increased IL-6, monocyte chemoattractant protein (MCP)-1, and inducible nitric oxide synthase (iNOS), which promote ATM infiltration and differentiation [6,34]. Cinti *et al.* showed that ATMs surrounding dead adipocytes form crown-like structures (CLS), which sequester and scavenge dead adipocytes. The release of cellular contents (e.g., toxic lipids) may also aggravate local inflammation [35]. In addition, a nonnegligible fraction of resident ATM was shown to undergo enhanced local proliferation in obesity. This process is induced by MCP-1 and specifically occurs in VAT where macrophage content and CLS density are enriched [36], emphasizing the involvement of resident ATM from alternative origins in obesity.

46.4 T, Treg, B, NK, and Mast Cells

Other innate and adaptive AT immune cells are also modulated in obesity. Similar to the M2 to M1 cell switch, obese AT undergoes a similar shift of T cells from protective Type 2 helper (Th2) cells to pathogenic Th1 and CTL [37–39] (Figure 46.1). Activated CTL produce IFN-γ, TNF-α, and IL-6 and participate in the formation of CLS, again amplifying AT inflammation [5,39,40]. Mice with genetic deletions of IL-7 receptor (IL-7R), a key regulator of T lymphocyte homeostasis, show attenuated ATM infiltration, improved glucose homeostasis, and insulin sensitivity [41]. Th2 cells regulate adipose M2 cells through secretion of IL-4 and IL-13 [37,42]. Overexpression of IL-13 in DIO mice prevents weight gain, insulin resistance, ATM infiltration, and AT inflammation [28]. Adipose resident $CD4^+$ $Foxp3^+$ Treg cells in obese mice are substantially decreased. These cells are considered to inhibit proinflammatory responses and ATM recruitment via the production of IL-10 and TGF-β. Modulation of Treg or Th2 cells could then protect against obesity-related insulin sensitivity, and also act as potential cellular targets to modulate inflammation and metabolic complications in obesity [38,43].

B cells accumulate in VAT and exacerbate adipose inflammation and insulin resistance through activating ATM, $CD4^+$, and $CD8^+$ T cells in DIO mice. This is likely driven by B-cell antigen presentation and the production of IgG antibodies [44–46]. As such, a particular profile of serum IgG autoantibodies was identified in obese individuals [44]. On the contrary, a subset of B cells – Breg regulatory B cells producing IL10 – negatively control AT inflammation and are reduced in obese mice. Transplantation of these cells into obese mice fat pads reduces the expression of CD44 and IFN-γ in $CD8^+$ T cells and TNF-α in macrophages [47].

Innate cells such as group 2 innate lymphoid cells (ILC2s) or eosinophils were also proposed as actors that maintain metabolic homeostasis. ILC2s are found in both mice and human AT, and are an innate source of IL-5 and IL-13. ILC2s can

regulate adaptive immunity (e.g., Treg cell accumulation) [48], eosinophil, and M2 responses, which are reduced in obesity [49,50]. ILC2s produce encephalin peptides, which induce the proliferation of PDGFRα^{+} adipocyte progenitors. Following activation by IL-33, these progenitors undergo differentiation toward beige cells that may improve AT metabolism [49,50]. Depletion of ILC2 in obese mice with a genetic background of impaired lymphoid development (Rag1$^{-/-}$ mice) exacerbates weight gain and glucose intolerance. Transferring ILC2s into obese mice leads to transient weight loss and improved glucose homeostasis [51]. Eosinophils produce IL-4 and IL-13, and further induce and maintain M2 cells. The absence of eosinophils aggravates weight gain, glucose intolerance, and insulin resistance in DIO mice [52]. Moreover, eosinophils in VAT can be activated by IL-33 via both direct pathways and an ILC2-mediated pathway [53], indicating close interactions between these adipose innate cells via key interleukins.

Finally, mast cells also contribute to this complex crosstalk and metabolic deteriorations. Obese humans and mice have increased WAT mast cells compared to lean [54], and these cells are preferentially located in AT fibrotic deposits [55]. The number of mast cells in scAT and oAT are positively correlated with fasting glucose and HbA1c in obese subjects [55]. A genetic deficiency of mast cells (*Kit*$^{W-sh/W-sh}$ mice) or their pharmacological stabilization in mice reduces weight gain, serum, and AT inflammation and improves glucose homeostasis and energy expenditure. Mast cells produce increased inflammatory cytokines (e.g., IL-6 and IFN-γ), angiogenic growth factors, and proteases (e.g., cathepsins), and probably enhance neovascularization and macrophage accumulation [54]. Invalidation of mast cell-secreted cathepsins, such as cathepsin L and K, protects mice from weight gain and insulin resistance [56,57]. However, one recent study attributed the protective effect against obesity and insulin resistance in mast-cell-deficient mice to the deficiency of hematopoietic c*Kit*, a growth factor required for mast cell differentiation and survival, rather than the mast cells *per se* [58]. Very recently, WAT mast cells from lean humans and mice were identified as being leptin-deficient and exhibiting anti-inflammatory properties, and transferring leptin-deficient mast cells into *ob/ob* mice improves glucose homeostasis [59]. All together, these studies show profound immune perturbations in obese fat. However, translating these observations from mice to humans has frequently proven difficult, in particular when exploring the links with obesity complications.

46.5 Many Other Inflammatory Cell Effectors

Nonimmune cells also contribute to AT inflammation, such as hypertrophic adipocytes that secrete a myriad of cytokines and chemokines. Mature adipocytes demonstrate increased expression of genes implicated in fibroinflammation, when cultured under proinflammatory conditions [60]. It was also shown that elevated adipocyte MHC II expression in obese subjects and mice enhances the dialog between adipocytes and resident T cells [61]. Our team has demonstrated that endothelial cells from obese VAT suppress lipolysis, adipokine secretion, and

insulin sensitivity of adipocytes, and increases the secretion of proinflammatory cytokines (e.g., IL-6, IL-1β), indicating that VAT endothelial cells contribute to AT inflammation [62].

Furthermore, preadipocytes and progenitors are also part of this complex interplay. Cocultured with macrophages, preadipocytes secrete elevated levels of proinflammatory cytokines [63]. A recent study showed that the fraction of adipose-derived stem cells (ADSCs) is reduced in obese mouse WAT. The delivery of lean mouse-derived ADSCs into obese mice attenuated adipocyte hypertrophy, reduced adipose CLS and TNF-α secretion, and sustained IL-10-secreting M2 cells, suggesting a crucial role of progenitors in adipose homeostasis. However, the relevance of a potential therapeutic approach targeting ADSCs in human obesity needs further investigation [64].

46.6 Impact of Adipose Inflammation on Local Biology: Numerous Molecular Effectors

Many inflammatory cells may contribute to obesity-induced insulin resistance via modification of local AT biology and enhanced lipolysis in mice. The role of molecular effectors and pathways has been highlighted as reviewed by Osborn and Olefsky [65]. Proinflammatory cytokines (e.g., TNF-α) are well known to impair insulin signaling, such as the IKKβ/JNK pathway that stimulates insulin receptor substrate (IRS-1) phosphorylation and inhibits the insulin receptor signaling cascade. Other kinases, for example, extracellular signal-regulated protein kinase (ERK), and the mammalian target of rapamycin (mTOR) are also implicated in IRS-1 phosphorylation. These proinflammatory kinases can regulate downstream transcriptional elements via transcription factor activator protein-1 (AP-1) and NF-κB. This results in amplified secretion of proinflammatory cytokines and perturbed metabolic signaling. *In vivo* studies demonstrate that genetic deletion of TNF-α, JNK, TLR2, or IKK improve metabolism in DIO or in *ob/ob* mice [8]. Proinflammatory cytokines (e.g., TNF-α, IL-1) enhance AT adipocyte lipolysis and increase circulating free fatty acids (FFA), which can alter the metabolism of other neighboring or distant organs (Figure 46.2). For example, FFA promotes the secretion of proinflammatory factors in AT, liver, and the pancreas through the TLR4 and JNK signaling pathways [66]. AT inflammation also disturbs normal adipogenesis via the downregulation of its key actor, peroxisome proliferator-activated receptor gamma (PPARγ) [8].

The inflammasome is yet another important effector. The inflammasome is a cytosolic multiprotein complex that is composed of an NLRP (NOD-LRR- and pyrin domain-containing) family member, the ASC inflammasome adaptor molecular, and the effector subunit pro-caspase 1. Obesity triggers inflammasome activation and the production of IL-1β, IL-18, TGF-β, and collagens [67]. Inflammasome-deficient mice demonstrate attenuated ATM activation, reduced effector-memory T cells, increased M2 cells, and improved glucose tolerance and insulin sensitivity upon HFD [68]. Saturated fatty acids and ceramides can activate the NLRP3 inflammasome through mechanisms involving defective autophagy and accumulated mitochondrial reactive oxygen species (ROS) [5]. However, the

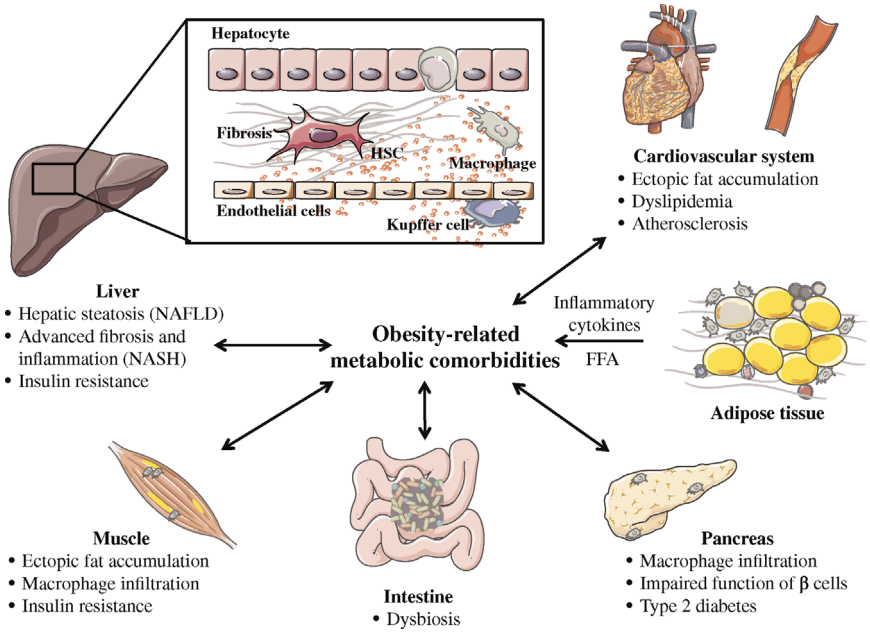


Figure 46.2 Obesity induces inflammation in diverse organs and leads to metabolic comorbidities. Inflamed AT in obesity results in increased lipolysis and free fatty acid (FFA) release, which play a crucial role in the mediation of insulin resistance and chronic low-grade inflammation throughout the body. In the liver, activated Kupffer cells and recruited macrophages produce proinflammatory cytokines, which further activate hepatic stellate cells (HSCs) to initiate fibrosis. Ectopic fat accumulation in the liver (steatosis) worsens insulin resistance. In skeletal muscle, elevated accumulation of lipids and proinflammatory macrophages impairs insulin signaling. In the intestine, the composition of the microbial population modifies, and lipopolysaccharide increases and aggravates systemic inflammation. In the pancreas, increased macrophage infiltration leads to impaired β-cell function and development of type 2 diabetes. In the cardiovascular system, lipid accumulation and proinflammatory mediators lead to dyslipidemia and atherosclerosis, and increases cardiovascular risk. Obesity-induced inflammation initiates pathological alterations in diverse organs and aggravates metabolic comorbidities.

role of the inflammasome complex in regulating AT leukocytosis in obesity has not yet been clearly deciphered.

46.7 Adipose Inflammation and Obesity Complications

The development of insulin resistance is crucial in the occurrence of obesity-linked comorbidities. In humans, it is unknown whether inflammation precedes insulin resistance or contributes to its maintenance once established. The accumulation of inflammatory cells such as macrophages and the secretion of proinflammatory cytokines in liver, skeletal muscle, and pancreas damage insulin signaling and aggravate whole-body insulin resistance as the disease progresses, regardless of the exact kinetic flow of events.

At least three major inflammatory signaling pathways are involved: (i) nuclear factor-κB-IκB kinase (NF-κB-IKK) signaling – a pathway activated by LPS, and

FFA via TLR4 activation in obesity. Inhibiting this pathway with anti-inflammatory agents such as salicylates, or by deletion of IKKβ or TLR4, improves insulin sensitivity [9,69]. (ii) The Jun N-terminal kinase (JNK)-activator protein-1 (AP-1) pathway, which is activated in AT, liver, and muscle also inhibits insulin signaling. Endoplasmic reticulum (ER) stress (for example, induced by palmitate) can activate JNK signaling and increase autophagy in adipocytes and pancreatic β cells that then aggravate insulin resistance. (iii) The inflammasome, as described above, activated by different molecules, primarily by proinflammatory cytokines, LPS, and secondary exogenous pathogen-associated and endogenous damage-associated molecular patterns. In all cases, the activation of these pathways leads to increased phosphorylaton of insulin receptors and insulin receptor substrates (IRS) at inhibitory sites and blocks downstream signals [9,69].

Insulin resistance develops particularly in those individuals with abdominal fat accumulations, a very well-known phenotype associated with increased cardiovascular risk [70,71]. The relationship between inflammatory cell accumulation in abdominal fat and cardiovascular complications is currently debated and needs extended investigation. For example, the presence of CLS in abdominal scAT from obese subjects is associated with worsened endothelium-dependent flow-mediated vasodilation [27]. However, the number of macrophages in scAT or VAT in severe obesity is not correlated with increased arterial intima-media thickness [72], a vascular phenotype associated with early cardiovascular risk. Nevertheless, relationships between AT-produced molecules and inflammatory cells from distant sites, and cardiometabolic phenotypes, have been described in overweight and obese subjects [73], although occasionally with inconsistent results. Among the key adipocyte-secreted hormones (i.e., adipokines), serum leptin is correlated with cardiovascular events, for example, myocardial infarction, stroke, and restenosis. Leptin stimulates the recruitment of intimal monocytes and the formation of foam cells, induces the secretion of proinflammatory and atherogenic cytokines, and the expression of C-reactive protein in human coronary artery endothelial cells [74,75]. Leptin is also prothrombotic as it enhances platelet aggregation and favors coagulation over fibrinolysis. Leptin also contributes to insulin resistance and diabetes through leptin–CRP interactions [75]. The adipokine resistin is regarded as a predictive inflammatory marker of coronary atherosclerosis [76]. It stimulates the expression and release of endothelin-1 by endothelial cells, and increases the expression of vascular cell adhesion molecule (VCAM-1) and MCP-1, and is therefore involved in cardiovascular disease. On the contrary, another key adipokine with anti-inflammatory properties, adiponectin, appears protective for the heart and vessels and is decreased with obesity and insulin resistance [77].

It is possible that liver alterations might generate a different picture (Figure 46.2). Liver Kupffer cells are regarded as hepatic resident macrophages and are activated toward an M1 phenotype in obesity. Activated Kupffer cells and recruited macrophages promote steatosis and produce proinflammatory cytokines [78]. TGF-β produced by Kupffer cells stimulates hepatic stellate cells to secrete extracellular matrix and induces fibrosis [79]. A “two-hit” hypothesis was initially proposed to describe liver injury in NAFLD/NASH: triglyceride accumulation in liver (steatosis) as the “first-hit,” followed by inflammation and

subsequent fibrosis (NASH) as the "second-hit" [80,81]. Adipose tissue inflammation is now considered as occurring concomitantly with liver damage. Van der Poorten *et al.* found that visceral fat volume is associated with hepatic inflammation and fibrosis, and is thus an independent predictor of advanced steatohepatitis [82]. In severe obesity, we showed that increased macrophage abundance in omental AT and deep scAT is associated with severe hepatic fibroinflammation. This association was independent of altered glycemic status [24,83,84]. These studies indicate that adipose-derived factors may contribute to NASH progression, notably via the release of adipose-derived molecules in the hepatic portal system.

Mechanistically, leptin promotes AMPK, activates lipid oxidation, inhibits lipogenesis by downregulating SREBP1-c, and upregulating PPARα [85,86]. In addition, leptin increases the expression of TGF-β1 in Kupffer cells, activates fibrogenetic hepatic stellate cells, and induces the production of key extracellular matrix components, such as α-smooth muscle actin, collagens, and tissue inhibitor of metalloproteinase 1 (TIMP1) [87,88]. Adiponectin is demonstrated to impede hepatic lipogenesis via the downregulation of SREBP1-c [89] and the stimulation of fatty acid oxidation and glucose utilization by activating AMPK [90]. In obese subjects, low circulating adiponectin is a predictive factor of steatosis and severe liver injury [91]. In contrast, some studies have shown that cirrhotic and hepatocellular carcinoma patients had elevated levels of serum adiponectin, which was associated with higher serum bile acids and decreased liver fat [92]. These data suggest that chronic hypoadiponectinemia in obesity may be an early trigger of cirrhotic and hepatocellular carcinoma formation. However, subsequent adiponectin increases might be induced by altered hepatic homeostasis [79,92]. Besides the above key proinflammatory and anti-inflammatory adipokines, AT contributes to the production of cytokines such as TNF-α and IL-6 that are markedly involved in NAFLD/NASH. [93,94]. For example, circulating IL-6 positively correlates with liver inflammation and fibrosis in NAFLD patients [82]. Glycoprotein 130 (gp130), a common signal transducer of IL-6, mediates AT lipolysis and stimulates hepatic steatosis and insulin resistance [95]. These observations led to the concept that the two-hit hypothesis might be too restrictive. Many other factors could be regarded together as "multihit" mediators involved in the progression from NAFLD to NASH, such as gut-derived bacterial toxins or dysregulated hepatocyte apoptosis, and among them, obesity-induced inflammation should be considered a pivotal player [81].

46.8 Obesity as a Profibrotic Disease?

A main consequence of long-term chronic tissue inflammation is the development of fibrosis defined as an excessive deposition of extracellular matrix (ECM) components (such as cross-linked collagens) and impaired degradation [96]. Obesity is associated with a high risk and prevalence of comorbidities with a strong fibrotic component, affecting different organs such as liver, kidney, heart, and lung. Fibrosis accumulation was discovered in obese rodent and human AT using picrosirius red staining. In obese subjects, scAT fibrosis is increased [97,98]

(Figure 46.1) and associates with adipocyte dysfunction and fibroinflammation [60]. Lysyl oxidase (LOX) is an important matrix fiber cross-linking enzyme that contributes to a tissue's mechanical properties [99]. LOX expression is upregulated in HFD or *ob/ob* mice and in obese subjects [60], whereas it is decreased after weight loss [100]. Inhibition of LOX activity leads to improved metabolism and reduced inflammation [101]. ScAT fibrosis in humans is associated with both altered stiffness and modified glucose homeostasis [102]. An important ECM component, collagen VI, which accumulates in obese AT, is also associated with insulin resistance [103,104]. Col6α1 expression in obese humans was found positively associated with ATM markers such as CD68 [104,105]. In mice, genetic ablation of membrane type 1-matrix metalloproteinase (MT1-MMP), a membrane-anchored metalloproteinase degrading collagen I, leads to increased periadipocyte fibrosis and severe metabolic complications such as hepatic steatosis [106]. In contrast, the absence of collagen VI in HFD or *ob/ob* mice results in uninhibited adipocyte expansion, reduced macrophage infiltration, and is associated with metabolic and inflammatory improvements [107]. Thus, the fact that the pericardial fat secretome (i.e., adipose tissue around the heart) promotes myocardial fibrosis [108] and that scAT pericellular fibrosis is associated with liver fibrosis [102] indicates that AT itself may promote profibrotic conditions at proximal or distant organ sites. The discovery of fibrosis in AT has added further complexity to the pathological alterations observed in obese AT.

Efforts are now being made to better understand fibrotic mechanisms associated with inflammatory events in obese AT. ATM and preadipocytes are considered to be important fibrotic effector cells. The profibrotic mediators (e.g., TGF-β, PDGF) produced by ATM directly attract and activate fibroblasts and control ECM deposition [96]. Vila *et al.* proposed that ATM TLR4 mediates the development of obesity- and endotoxemia (bacterial lipopolysaccharide-produced)-associated AT fibrosis through the TGF-β pathway [109], indicating the potential link between intestinal inflammation, the gut microbiome, and AT fibrosis. Human preadipocytes cultured with activated macrophage-conditioned medium overexpress a panel of ECM genes, along with increased collagen I and fibronectin proteins, and show increased migration and proliferation ability [63,98]. Endothelial cells (i.e., cells that could convert into myofibroblasts via epithelial mesenchymal transformation) and pericytes (i.e., main cellular component of blood vessels and potential adipocyte progenitors) both exhibit proinflammatory and profibrotic phenotypes and are capable of altering adipocyte function [62,110]. AT mast cells whose accumulation is preferentially located in fibrotic depots and positively associated with collagen, macrophage, and endothelial cell accumulations [55] are thought to be ECM producers, or at least contribute to ECM remodeling. Importantly, adipocytes themselves could also possibly produce fibrosis as significantly increased peri-adipocyte fibrosis is found in AT [97].

Alternative human and mouse studies proposed that ATM accumulation could be secondary to AT fibrosis [101,111], suggesting that other cellular or molecular factors might contribute to fibrosis initiation. For instance, the role of other immune cells accumulating in AT during obesity, for example, $CD4^+$ Th1 and

$CD8^+$ T cells [112], and their role in fibrosis needs to be further studied. As in many obesity-related fibrotic diseases, the complex pathophysiological mechanisms implicated in AT fibrosis remain to be elucidated, and may involve chronic low-grade inflammation, insulin resistance, lipotoxicity, aberrant secretion of many adipokines, oxidative stress, hypoxia, and gut microbiota alteration [1,113].

46.9 Toward New Obesity Phenotypes and New Therapeutics?

The discovery of AT inflammation and fibrosis led to a reassessment of the role of AT in obesity, and the generation of several different AT expansion models. Thus, two models of healthy or unhealthy AT expansion have been proposed [114]: healthy AT expansion comprises AT enlargement via effective recruitment of adipocyte precursor cells and increased numbers of small adipocytes, along with adequate angiogenic responses, appropriate ECM remodeling, and minimal inflammation; in contrast, unhealthy AT expansion consists of rapid growth of existing adipocytes, limited angiogenesis, high degrees of macrophage infiltration, and massive fibrosis [114]. The latter model supports the hypothesis that AT fibrosis and associated mechanical stress (e.g., stiffness) may limit AT expansion during obesity development. Fibrotic depots produce constraints on adipocytes that may lead to adipocyte dysfunction, ectopic fat storage, and metabolic disorders linked to ectopic fat accumulation in other organs (liver, muscle, heart, and pancreas) [115].

However, the picture might be more complex than initially thought. Asterholm *et al.* indeed showed in mouse models that adipocyte inflammation is necessary for healthy AT expansion and remodeling. The impairment of proinflammatory signals in AT leads to less efficient adipogenesis, affects VAT expansion, and impairs intestinal barrier function. In those mice, the absence of proinflammatory signals led to increased ectopic lipid accumulation, elevated hepatic steatosis, and impaired glucose tolerance [116].

46.10 If a Certain Degree of Inflammation Is Beneficial, Is It Really Relevant to Consider a Therapeutic Modulation of Low-Grade Inflammation in Humans?

Rodent studies have provided many potential drug targets. Manipulation of proinflammatory and anti-inflammatory cytokines has been tested with contrasting results, particularly when translating the findings in humans.

For most of the tested drugs, the objective was to improve insulin sensitivity and diabetes status. An illustration of this approach is the use of thiazolidinediones (TZDs), agonists for PPARγ, to control type 2 diabetes. TZDs activate PPARγ and subsequently suppress proinflammatory pathways, reduce ATM, enhance anti-inflammatory Treg cell stimulation, and increase circulating adiponectin and FGF21 [8,9]. Two main TZD drugs, rosiglitazone and pioglitazone,

were widely used to treat T2D with good efficacy. However, the use of these two drugs are now restricted or even suspended in some regions, such as Europe, due to safety concerns: rosiglitazone increases the risk of myocardial infarction and cardiovascular death [117] while pioglitazone increases the risk of bladder cancer [118]. Yet, the US Food and Drug Administration (FDA) recently lifted the rosiglitazone restriction based on newly reviewed data analysis. In addition, a recent meta-review with large, pooled, multipopulation analysis did not show any association between the incidence of bladder cancer and the cumulative use of pioglitazone [119], suggesting these two drugs might return to market after careful reevaluation.

Salsalate, an analog of salicylate, was also tested as an antidiabetic drug [120]. Clinical trials have confirmed that salsalate treatment improves insulin clearance and insulin resistance, reduces HbA1c and systemic triglycerides, and diminishes early glycation end products [9]. Similar effects have also been observed in patients treated with aspirin [121]. However, the beneficial effects remain quite modest.

Although anti-TNF-α antibodies do not significantly improve insulin sensitivity in obese or metabolically sick subjects, they decrease plasma glucose in obese subjects [122,123], improve insulin sensitivity and HbA1c, and decrease new-onset diabetes in subjects with severe immune diseases such as rheumatoid arthritis [124]. The antagonist of another key cytokine, IL-1, was also suggested as a favorable therapeutic molecule. The IL-1R antagonist enhances insulin secretion in β cells, decreases HbA1c, and reduces CRP and IL-6 circulating proinflammatory markers in overweight or obese diabetic subjects [125]. Neutralizing IL-1β antibody is found to decrease fasting glycemia, HbA1c, CRP, and IL-6 [126,127]. The treatment of the anti-inflammatory cytokine IL-10 in mice protects against IL-6-induced impairment of insulin action and signaling activity in liver and muscle [128]. Recently, a majority of infiltrating cells surrounding adipocytes were found to be CD44-positive macrophages. Administration of anti-CD44 monoclonal antibodies in HFD mice reduced AT inflammation and suppressed weight gain, fasting glycemia, insulin resistance, and liver steatosis [129]. These findings in rodents suggest the potential therapeutic possibilities of IL-10 or CD44 antibodies, which require further investigation in humans.

Administering the JNK-inhibitory peptide markedly ameliorates insulin resistance and improves glucose tolerance in diabetic mice [130]. Again, the efficacy of JNK inhibitors and its safety in humans remains to be evaluated. Two different chemical chaperones that reduce ER stress in cells and whole animals are able to effectively reduce glycemia, restore insulin sensitivity, improve fatty liver disease, and enhance insulin action in liver, muscle, and AT in obese and diabetic mice [131]. Furthermore, in a small clinical study, one of these chaperones was found to improve hepatic and muscle sensitivity and to increase liver insulin signaling [132], suggesting that the control of ER stress might have clinical benefit. Resveratrol (RES), an AMP-activated protein kinase activator, reduces hepatic steatosis, macrophage infiltration, insulin resistance, and improves memory alteration in HFD-fed mice, indicating that RES can be applied to treat obesity-related inflammation, metabolic deterioration, and memory deficiency [133].

Therapies that inhibit the chemotaxis or accumulation of immune cells into metabolic tissues can also improve insulin sensitivity, at least in rodents. Short-term treatment with CCR2 antagonists reduces ATM infiltration and ameliorates insulin resistance without significant improvement in body mass or hepatic steatosis [134]. Clinical studies showed similar but modest improvements in insulin resistance [135] and fasting glycemia [136] in diabetes. Leukotrinene B4 (LTB4) is a proinflammatory lipid mediator and promotes leukocyte chemotaxis through binding to its receptor LTB4R1. Administration of LTB4R1 inhibitor in HFD mice led to considerably improved glucose tolerance, decreased serum insulin and FFA levels, reduced inflammation, and ATM infiltration [137]. Treatment with CD3-specific antibody or its nonmitogenic $F(ab')_2$ fragment decreases the predominance of Th1 cells over $Foxp3^+$ Treg cells in VAT and improves insulin resistance and glucose hemostasis in mice existing on a HFD [138].

Another therapeutic example is linked to mast cells. A clinical study previously showed that administration of mast cell stabilizer with glimepiride had beneficial effects on systemic inflammation, and glucose and lipid homeostasis in obese diabetic individuals [139]. This suggests that development of molecules targeting mast cells could be promising, but this hypothesis requires exploration at a larger scale.

Nutraceutical or dietary methods have also been investigated. The fish oil ω-3 fatty acids, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) exert anti-inflammatory effects via binding to the G protein-coupled receptor (GPR) 120 in macrophages and adipocytes. The supplementation of ω-3 fatty acids in obese mice reduces ATM content and M1 proinflammatory genes, increases M2 anti-inflammatory genes, and improves systemic insulin sensitivity [140]. However, fish oil supplementation has had controversial results in human studies, and the amount of fish oil required to attain a GPR-120-mediated anti-inflammatory effect is too high [9]. Recently, a high-affinity GRP agonist was demonstrated to improve insulin resistance, chronic inflammation, and hepatic steatosis in obese mice, indicating that this GRP120 agonist molecule could be a promising new insulin-sensitizing drug for obese subjects [141]. Various spices have also been shown to exert antioxidant and anti-inflammatory activities in obesity. Among them, curcumin, a yellow pigment extracted from curry power, has been demonstrated to interact with adipocytes, macrophages, and hepatic stellate cells. Curcumin inhibits NF-κB and Wnt/β-catenin, activates PPARγ, and therefore results in downregulation of genes encoding TNF-α, IL-6, MCP1, leptin, and resistin and upregulation of genes encoding adiponectin. These curcumin-initiated modifications improve insulin resistance, hyperglycemia, hyperlipidemia, and other complications related to obesity in both animal and human studies. These results suggest that adding spices containing curcumin to a daily diet could be a favorable method to prevent obesity-associated insulin resistance [142].

Finally, intestinal microbiota is regarded as responsible for endotoxemia-induced inflammation and the onset of various metabolic complications in obesity. One animal study showed that antibiotic treatment decreased metabolic endotoxemia and cecal LPS content in DIO and *ob/ob* mice, and was associated with improved glucose tolerance, weight gain, inflammation, oxidative stress and macrophage infiltration [143]. Moreover, the administration of polymyxin B,

which eliminates Gram-negative bacteria, can improve hepatic steatosis [144,145]. Thus, these findings indicate that pharmacological drugs targeting gut microbiota inflammation could be useful therapeutic strategies for obesity and related metabolic complications.

In conclusion, major progress has been made regarding the understanding of immune-inflammatory and profibrotic processes occurring during obesity development. These novel findings reveal complex crosstalk between AT immune cells, impacting AT biology and the biology of distant organs. Although there are many studies proposing potential new targets, it is absolutely necessary to further explore whether, and in whom, these strategies will alleviate obesity and improve metabolic complications with acceptable side effects.

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47

Inflammation and Type 2 Diabetes

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47.1 Introduction

In 2014, the overall worldwide prevalence of diabetes was estimated to be 8–9%, according to the world Health Organization and the International Diabetes Federation. Currently, there are approximately 387 million people with diabetes, and this number is expected to increase to 590 million by the year 2035. Of these diabetes patients, 10% have type 1 diabetes and 90% have type 2 diabetes. Up to 50% of the people with type 2 diabetes are unaware of the fact that they have the disease.

In this chapter, we will discuss the current insights into how inflammation, particularly obesity-associated low-grade inflammation, may contribute to development of insulin resistance and β-cell failure, and hence comprise a major culprit for the development of type 2 diabetes. When available, we will focus on human data. We will approach this topic from the view of the obesity – inflammation – type 2 diabetes axis, since obesity comprises the most relevant risk factor for type 2 diabetes, worldwide.

47.2 Diabetes – Background and Epidemiology

In type 1 diabetes, the β-cells of the pancreas are destroyed by an autoimmune process. Pancreatic β-cells are the only insulin-producing cells in the body, and patients with type 1 diabetes therefore depend on insulin therapy for survival. Type 1 diabetes is often characterized by an acute onset, often at early age, that is, childhood, puberty, or early adulthood. In type 2 diabetes, various cells of the body show a decreased response toward the metabolic actions of the hormone insulin. This is also known as insulin resistance. Insulin resistance is a slowly developing condition and is usually counteracted by a compensatory increase in the

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production of insulin by the pancreatic β-cells. Type 2 diabetes will develop when insulin production of the β-cell is no longer sufficient to compensate for this increased demand for insulin. Because of the gradual onset of the disease, especially people with recent onset type 2 diabetes may be unaware of their condition. Type 2 diabetes used to be a disease that developed in adulthood but in recent years a steep increase in the incidence in youth, particularly in obese adolescents, has been seen [1,2]. Notably, already in the period when insulin resistance is compensated by increased insulin production, and full-blown type 2 diabetes cannot yet be formally diagnosed, chronic conditions such as cardiovascular disease are already developing. The exact underlying cause of type 2 diabetes is not known, but it is generally accepted that obesity and inflammation are important drivers of this disease.

The prevalence of type 1 and type 2 diabetes has been increasing over the past 20–30 years. Between 1980 and 2008, the worldwide number of people with diabetes has more than doubled [3]. The reasons for this increase are multiple. On the one hand, they are driven by population growth and aging. But on the other hand, environmental factors and lifestyle comprise relevant, and potentially modifiable, contributors. The worldwide prevalence of type 2 diabetes is projected to increase. Obesity is considered an important driver of the increase in diabetes prevalence. In the past three decades, both cardiovascular disease and diabetes, in particular obesity-induced type 2 diabetes, have been recognized as inflammatory diseases. The systemic low-grade inflammatory response that is often observed in obesity detrimentally affects both insulin signaling and β-cell function and may thus contribute to the development of type 2 diabetes and its complications [4]. Given the higher burden on the health care system and the community at large, the focus on increasing prevalence of diabetes is mainly on type 2 diabetes. Yet, there are also indications for an increasing prevalence of type 1 diabetes [5]. Several environmental risk factors have been identified for this increase. Some of these environmental risk factors such as viruses may initiate autoimmunity toward the β-cell, other exposures may put on overload on the already affected β-cell and thus accelerate the disease process [5]. Diabetes, especially type 2 diabetes, has long been regarded as a disease of affluence. However, currently more than 80% of deaths due to diabetes occur in low- and middle-income countries.

47.3 Obesity, Fat Distribution, and Inflammation

At the population level, the risk of developing type 2 diabetes rises with an increase in obesity. More specifically, it appears that particularly individuals with a larger amount of upper body fat are more often type 2 diabetes patients than those with less upper body fat, even with comparable body mass index (BMI) [6,7]. Upper body fat is also referred to as central obesity or abdominal obesity. Central obesity is generally associated with more visceral fat. This is the adipose tissue that surrounds the abdominal organs. Visceral fat may also be referred to as abdominal and/or omental fat. The visceral fat depot comprises 10–15% of total body fat [8]. It is generally considered an unhealthy fat depot and a relevant contributor to obesity-associated low-grade inflammation. The largest fat depot in the human

body, that is, subcutaneous fat, comprises 70–80% of total body fat and is often regarded as neutral or, when located around the hips and buttocks, even protective with respect to low-grade inflammation [9,10]. One exception may be made for deep subcutaneous fact in the abdominal area that may behave similar to visceral rather than to superficial subcutaneous fat [11]. There are also additional fat depots that are less pronounced in volume than subcutaneous and visceral fat, but appear highly relevant with respect to regulatory and metabolic functions. These additional fat depots are usually referred to as "ectopic" fat. Ectopic fat can occur as small but distinct fat depots that are located in close vicinity of specific organs, for example, the perirenal, perivascular, as well as epicardial and pericardial fat depots. Ectopic fat also can occur as accumulation of triglycerides in the parenchymal cells of tissues and organs that are usually not lipid-laden, such as hepatocytes and muscle cells. Both types of ectopic fact may contribute to (local) inflammation and to dysregulation of normal function and metabolism. There is accumulating evidence that a large capacity for storage of triglycerides in the superficial subcutaneous fat may actually result in less "overflow" of triglycerides into visceral and ectopic fat depots [12,13]. A high capacity for triglyceride storage in subcutaneous fat may contribute to the so-called (metabolically) healthy obese phenotype that has consistently been shown in approximately 25–30% of obese individuals [14]. Taken together, current data suggest that specific fat depots, including visceral and ectopic fat, are the culprit fat depots with respect to generation of an inflammatory response and insulin resistance. Prevention of overall adipose tissue dysfunction, of (visceral) fat inflammation, and of ectopic fat deposition may all contribute to preserving a metabolically healthy obese phenotype [14].

It is currently well accepted that the state of chronic low-grade inflammation that is induced by obesity [15–17] includes attraction and activation of an array of local and systemically derived immune cells in the adipose tissues, as well induction of systemic inflammation (see also Chapter 50). The adipogenic nuclear transcription factor peroxisome proliferator activated receptor (PPAR)-γ is a master regulator of adipocyte differentiation and promotes the storage of fatty acids in the large triglyceride droplet of the adipocyte [18]. PPAR-γ is also a key regulator of inflammatory and immune responses [19]. Adiponectin is the major anti-inflammatory adipokine. It is also one of the few adipokines for which the plasma concentration is lower in obese than in lean individuals [20]. Accumulation of abdominal fat can induce inflammation via several mechanisms. Expansion of adipose tissue and adipocyte hypertrophy may be associated with local hypoxia, which in turn generates signals to recruit macrophages [21]. In addition, the high rate of protein synthesis during adipose tissue expansion may lead to accumulation of unfolded or misfolded proteins. Hypoxia and intracellular presence of unfolded and misfolded proteins can both lead to endoplasmic reticulum (ER) stress [22,23]. This may then further contribute to the production of inflammatory and chemotactic signals.

Adipose tissue macrophages are mostly derived from bone marrow, although some are local, and their number is higher in obesity [24]. Macrophages that are located within the adipose tissue may be pro- or anti-inflammatory, depending on their activation status [25]. Classically activated macrophages (referred to as M1

macrophages) are considered proinflammatory, alternatively activated macrophages (referred to as M2 macrophages) on the other hand primarily function to resolve or dampen the M1-induced inflammatory response and are therefore considered anti-inflammatory. Although the M1 versus M2 status is a gradient rather than a black-and-white phenomenon, macrophages present in adipose tissue in obesity appear to be predominantly polarized toward the M1 phenotype [26]. Local production of chemo-attractants that enhance the homing of monocytes to adipose tissue depots may contribute to adipose tissue inflammation. Such signals can, for instance, be produced by adipocytes of the expanding adipose tissue. Hypertrophic adipocytes begin to secrete low levels of tumor necrosis factor (TNF)-γ, which stimulate preadipocytes and endothelial cells to produce monocyte chemotactic protein (MCP)-1 (also known as CCL2) [27]. Macrophages in adipose tissue are overrepresented around dead or dying adipocytes, thereby forming so-called crown-like structures [28,29]. Also in humans there is an increase in macrophages in obese adipose tissue. For instance, in a study of monozygotic twins acquired obesity was characterized by adipocyte hypertrophy and increased expression of the macrophage marker CD68 and TNF-α in subcutaneous abdominal adipose tissue [30]. These cellular and humoral proinflammatory changes in acquired obesity were associated with an increase in insulin resistance [30]. The exact signals from human adipose tissue that initiate macrophage infiltration still need to be identified.

In recent years, the role of other immune cells, including cells of the adaptive immune system such as proinflammatory T cells in obesity-induced type 2 diabetes, has gained significant interest. Human adipocytes and preadipocytes appear to possess the full machinery to prime inflammation and attract T cells independent of macrophages [31]. Moreover, subcutaneous adipose tissue of type 2 diabetes patients has increased presence of not only macrophages but also of proinflammatory T cells [32]; in mice fed a high-fat diet, infiltration of T cells actually preceded the infiltration of macrophages [32,33]. T cells derived from adipose tissue of obese mice produced more interferon (IFN)-γ than those from control mice [34] and hampered preadipocyte-to-adipocyte differentiation [35]. T cells that are infiltrated in adipose tissue may not only attract macrophages but also skew macrophage differentiation toward the M1 phenotype. In contrast, induction of T-regulatory cells was beneficial and reduced adipose tissue inflammation and insulin resistance [36,37]. Notably, the anti-inflammatory master switch in adipocyte differentiation, PPAR-γ, was recently identified as major driver of visceral adipose-tissue-resident regulatory T cells [38].

47.4 How Does Inflammation Cause Type 2 Diabetes?

For development of type 2 diabetes, at least two major events are necessary. First, the development of insulin resistance, and second the development of β-cell decay (see also Figure 47.1). Insulin resistance is a state in which the sensitivity (and hence the response) of target cells to the hormone insulin is reduced. In insulin resistance, particularly the metabolic actions of insulin are reduced (Section 47.4.1). Insulin resistance leads to an increased demand for insulin.

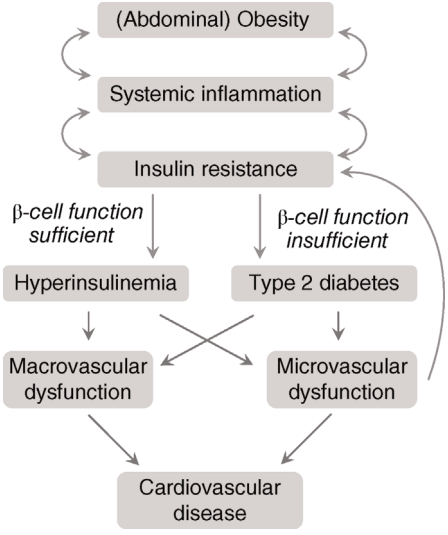


Figure 47.1 The main events that underlie the development of obesity-associated type 2 diabetes, that is, development of insulin resistance and the development of β-cell failure. When fat accumulates in the abdominal and ectopic depots, a persistent low-grade inflammatory response will develop. This low-grade inflammatory response will lead to cellular insulin resistance and also attract proinflammatory immune cells to adipose tissue, which can worsen the inflammatory response. Insulin resistance increases the demand for insulin. As long as the pancreatic β-cells can meet the increased demand for insulin with sufficient compensatory insulin production, this will lead to a state of normoglycaemia with hyperinsulinaemia. This is generally associated with dyslipidaemia and hypertension and is often associated with the metabolic syndrome. When, on the other hand, the secretion capacity of the β-cells is no longer sufficient, persistent hyperglycemia and hence type 2 diabetes will develop. The major vascular complications of type 2 diabetes, that is, macrovascular and microvascular disease are each presumed to start prior to the development of hyperglycemia. However, once diabetes develops, the progression of disease may be further accelerated.

The main driver for this is the physiological set-point of the body to maintain plasma glucose homeostasis. When insulin signaling is impaired, less glucose will be taken up by the cells and plasma glucose increases. Higher plasma levels of glucose stimulate the pancreatic β-cells to increase their insulin production. This will lead to a new balance in which normal plasma glucose is maintained but insulin concentrations are increased. For this reason, insulin resistance is in its early stages is characterized by normoglycemia but hyperinsulinaemia. The increased insulin production by the pancreatic β-cells to maintain normoglycaemia is referred to as compensatory β-cell function. This insulin resistant phase with higher circulating insulin concentrations but (relatively) normal glucose concentrations can be maintained for years. Notably, despite the normal glucose levels this is not a healthy condition. Hyperinsulinaemia is, among others, associated with dyslipidaemia and atherosclerosis [39]. Subsequently, in case of prolonged and/or worsening insulin resistance, β-cells may no longer be able to meet the persistent high demand for insulin. This will then lead to insufficient hepatic and peripheral glucose disposal, eventually to higher circulating concentrations of glucose and development of type 2 diabetes. The systemic low-grade

inflammatory response that is often observed in obesity detrimentally affects both insulin signaling and β-cell function and may thus contribute to the development of type 2 diabetes (Figure 47.1).

47.4.1 Inflammation Causes Insulin Resistance

Binding of insulin to its functional receptor induces autophosphorylation of tyrosine residues on the intracellular part of the receptor. In the metabolic pathway of insulin signaling, the insulin receptor substrate (IRS) then docks the insulin receptor and is trans-phosphorylated in its tyrosine residues via the kinase activity of the phosphorylated insulin receptor. These early events in insulin signaling comprise the key steps in metabolic insulin signaling that are particularly affected in case of insulin resistance. Subsequently, additional members of the metabolic insulin signal transduction pathway will dock onto the phosphorylated-IRS molecules. Most importantly, phosphatidylinositol-3-kinase (PI3 K) and Akt/protein kinase B (PKB) are recruited and activated in order to induce downstream effects [40]. Insulin signal transduction via PI3 kinase mainly affects metabolic pathways such as GLUT-4 translocation, inhibition of hormone-sensitive lipase, increased glycogen synthesis/decreased gluconeogenesis, and increased nitric oxide (NO) production, with the exact response depending on the metabolic machinery of the target cell. The other main pathway of insulin signal transduction involves signal transduction via the Ras G-protein/mitogen-activated protein kinase (Ras/MAPK) pathway instead of via IRS. Stimulation of Ras/MAPK signaling by insulin induces mitogenic, rather than metabolic, intracellular responses [40].

Several events can interrupt signaling via the insulin receptor. In general, these are physiological mechanisms that bring about a timely interruption of the insulin signal in order to prevent overshoot and maintain a beneficial physiological insulin response. First, protein phosphatases can dephosphorylate the insulin receptor and the IRS proteins. Second, there may be ligand-induced downregulation of the insulin receptor. Third, insulin receptor signaling inhibits its own signal via induction of pathways that inhibit insulin receptor-mediated signal transduction. This latter physiological negative feedback mechanism is induced when insulin activates mTOR and protein kinase C (PKC)-ζ. These intracellular serine/threonine kinases can then, either directly or indirectly (e.g., via IkappaB kinase beta (IKK-β)), phosphorylate the serine/threonine residues that are present in IRS. Phosphorylation of IRS on serine/threonine residues hampers the tyrosine phosphorylation of IRS via insulin receptor. Thus, serine/threonine phosphorylation of IRS interrupts, or at least reduces, insulin signal transduction via the IRS proteins [41]. In addition, serine/threonine phosphorylation can induce dissociation of IRS proteins from the insulin receptor [42,43], induce degradation of IRS proteins [44,45], remove IRS proteins from complexes that keep them in close proximity to the insulin receptor [46–48], and turn IRS proteins into inhibitors of insulin receptor kinases [49]. So taken together, serine/threonine phosphorylation is a key event in the control and inhibition of insulin signal transduction via the metabolic (IRS- PI3K-AKT/PKB) pathway (see Figure 47.2).

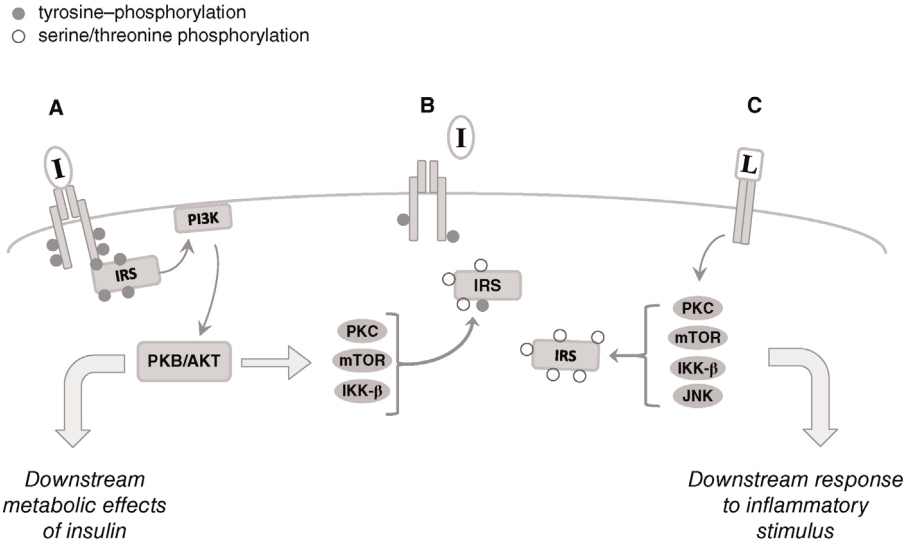


Figure 47.2 The central roles of tyrosine-phosphorylation and serine/threonine–phosphorylation in the control of insulin signal transduction. A: Binding of insulin (I) to its receptor leads to tyrosine autophosphorylation of the intracellular part of the receptor. The insulin receptor substrate (IRS) docks to the receptor and is transphoshorylated on tyrosine residues. Subsequently, the metabolic pathway of insulin signaling via phosphatidyl-inositol-3-kinase (PI3 K) and Akt/protein kinase B (PKB/AKT) is activated. B: activation of PKB/AKT also induces a response that aims to interrupt the insulin signal transduction in order to achieve a sufficient but proportionate insulin response. This includes the activation of factors that induce serine/threonine phosphorylation of IRS. This interrupts the signal transduction via IRS. C: Signaling of various inflammatory ligands (L) via their respective receptors can also induce serine/threonine phosphorylation of IRS. This subsequently hampers tyrosine phosphorylation of IRS in response to binding of insulin to its receptor and thus contributes to insulin resistance.

Notably, a state of chronic low-grade inflammation can directly hamper the efficiency of insulin signal transduction and induce insulin resistance via activation of these physiological negative feedback mechanisms of normal insulin signaling [50]. Various intracellular and extracellular substances that are increased in obesity-associated low-grade inflammation can actually induce serine/threonine phosphorylation of the IRS proteins. As such, they can hamper insulin signaling but in this case with pathophysiological consequences [50]. These substances include, for example, the proinflammatory cytokines TNF-α [51,52], interleukin (IL)-6 [53], IL-1α [54], and saturated fatty acids [55]. These factors employ various intracellular serine/threonine kinases such as IKK-β, Jun-NH2 -terminal kinase (JNK), PKC, and mTOR [50,55]. IKK-β is particularly interesting in this respect since it is a central effector protein in the inflammatory responses that are activated upon stimulation of the intracellular protein transcription factor nuclear factor (NF)-κB. The factors mentioned above primarily affect signal transduction via IRS. And indeed it has been shown that in obesity and in type 2 diabetes, particularly the PI3 K pathway was impaired, while insulin signaling via MAPK remained largely unaffected [56].

Saturated fatty acids, TNF-α, and IL-6 have all been demonstrated to induce insulin resistance in healthy humans [57,58]. This strongly suggests that the above-described induction of insulin resistance by inflammatory factors is indeed relevant in humans, even though most of the available information was obtained in cell and animal studies. Obese, hypertrophic, and/or insulin-resistant adipocytes were shown to have an increased release of fatty acids. Subsequently, saturated fatty acids could activate toll-like receptor (TLR)-4/NF-κB pathways in adipose tissue macrophages, in a paracrine fashion. This then induces release of TNF-α which, in turn, stimulates TNF-receptors on the adipocytes. TNF-induced release of fatty acids can then provoke a vicious cycle of worsening inflammation and insulin resistance [59,60]. JNK is not only activated upon exposure to cytokines and fatty acids but also in response to internal cues such as ER stress [23]. Given the high prevalence of NAFLD in obesity and type 2 diabetes, it is also of interest that experimental activation of JNK in the liver appeared to be sufficient to induce systemic insulin resistance [61]. These data corroborate the view that the liver may be a major contributor to the induction of obesity-associated systemic insulin resistance. The proinflammatory effects of fatty acids appeared to be mainly restricted to the saturated fatty acids, while unsaturated and in particularly ω-3 fatty acids, in contrast, appeared to be anti-inflammatory [62,63].

47.4.2 Inflammation Causes β-cell Dysfunction

The endocrine pancreas is a key player in the maintenance of normoglycaemia. It does so via an increase in β-cell mass and subsequent hyperinsulinaemia [64]. Hyperinsulineaemia may subsequently enhance β-cell mass since insulin may act as a "β-cell growth factor" [65]. However, once the demand for insulin exceeds the production of the pancreas, hyperglycemia will develop (Figure 47.1). β-cell failure can result on the one hand from an intrinsic insulin secretion defect in existing β-cells and on the other hand from insufficient β-cells mass [66]. β-cell failure may be partly due to genetic and partly due to acquired factors. It is probable that genetic disposition may render some individuals more sensitive to those acquired factors than others. This may explain why some (obese) individuals will remain normoglycemic for a long period of time, while others more rapidly progress to hyperglycemia and type 2 diabetes.

In addition to their interference with insulin signaling (as described in Section 47.4.1), inflammatory cytokines may also contribute to β-cell dysfunction and, as such, to development of type 2 diabetes [49]. In addition, prolonged exposure of pancreatic β-cells to high levels of glucose and lipids, also known as glucotoxicity and lipotoxicity, may contribute to oxidative stress – potentially via effects on mitochondrial function – and to high rates of β-cell apoptosis in type 2 diabetes [67–69]. Moreover, impaired signaling via IRS may add to β-cell dysfunction [70,71].

In a state of insulin resistance without diabetes, when fasting normoglycaemia is maintained, there will already be higher than normal increases in circulating glucose concentration after consumption of food. These enhanced postprandial glucose excursions are particularly present in those who have impaired glucose

tolerance [72]. Hyperglycemia can induce the production of IL-1β by β-cells [66,73] and this proinflammatory cytokine was shown to be involved in β-cell deterioration in both type 1 and type 2 diabetes [74,75]. IL-1β may, via induction of specific signal transduction pathways that include Fas (CD95), initially induce β-cell proliferation, but with prolonged hyperglycemia switch to increased β-cell apoptosis [76]. Notably, leptin, which is increased in obesity, was shown to enhance the release of IL-1β by β-cells [77]. In addition to its effects on β-cells, IL-1β may also induce insulin resistance via direct effects in insulin signaling. For example, IL-1β can down-regulate IRS mRNA expression in adipocytes [78]. Clearly, the effects of IL-1β are not the only way through which β-cell mass and function are affected in the development of type 2 diabetes, but the IL-1β pathway is a relevant representative of the many (inflammatory) pathways that are involved in the generation of β-cell failure in response to obesity-associated low-grade inflammation and the concomitant increased insulin demand. Pancreatic lipotoxicity partly results from dyslipidaemia (high small dense LDL cholesterol, low HDL cholesterol, high non-esterified fatty acids (NEFA) and partly from accumulation of fat (triglycerides) in the pancreas as an ectopic fat depot. Increased concentrations of circulating NEFA, particularly saturated fatty acids, are considered harmful for β-cells. First, because saturated fatty acids may induce IL-1β [79] and, as such, trigger an inflammatory response in pancreatic islets [80]. Second, because fatty acids can induce local production of other IL-1-dependent proinflammatory cytokines such as IL-6 and IL-8 [80]. In addition to those effects via interleukins, palmitic acid could also activate JNK in pancreatic β-cells, which was followed by a decrease in insulin gene transcription [81]. In addition, a reduction in the pancreatic triglyceride content led to an improvement in insulin secretion capacity of the β-cells [82]. The effects of lipotoxicity may be enhanced in case of hyperglycemia [83]. The data presented above indicate that both glucotoxicity and lipotoxicity can induce local production of cytokines and inflammation in pancreatic islets. The extent to which circulating cytokines can directly affect β-cell survival as their systemic concentrations remains to be established. *In vitro* studies suggest that externally added cytokines can affect the secretory function of β-cells [84].

Other mechanisms that may enhance β-cell failure in obesity-associated type 2 diabetes include ER stress, oxidative stress, amyloid deposition, and autophagy [85]. Most of these mechanisms have also been implicated in inflammation, either because they induce a (local) inflammatory response or because they result from inflammation [86,87]. The detrimental effects of inflammation on β-cell function may be particularly relevant in situations of a sustained inflammatory response. This is probably the case in obesity and associated glucose and lipid overload [86], but may also contribute to increased prevalence of type 2 diabetes in other conditions that are characterized by a chronic inflammatory state such as endotoxaemia [88], and, possibly periodontitis [89]. Increased numbers of macrophages have been shown in pancreatic islets of patients with type 2 diabetes [80,90], most likely in response to increased islet expression of IL-1β and chemokines [80,91]. Besides, angiotensin II, which is increased in insulin resistance and type 2 diabetes [92], can induce β-cell dysfunction, independent of vasoconstrictor effects [93].

47.5 How Does Inflammation Cause Vascular Disease in Type 2 Diabetes?

The vascular system can be divided into large (macrovasculature) and small vessels (microvasculature). The main inflammation-associated pathology of the macrovasculature is the development of atherosclerotic plaques in the arteries, which may advance to unstable plaques that can rupture and result in occluded vessels and ischemic events [94]. The microvasculature comprises arterioles, capillaries, and venules. The microcirculation serves to control whole body and tissue metabolism, and blood pressure [95,96]. It does so via three main mechanisms. First, the microcirculation regulates the exchange of nutrients, oxygen, and hormones, second, it drastically dampens large fluctuations in hydrostatic pressure at the capillary level, and third it is an important determinant of the overall peripheral resistance. The latter is because the quantitatively most substantial drop in hydrostatic pressure occurs at the level of the microcirculation [97]. Microvascular dysfunction is defined as impairment in one or more of these functions. In type 2 diabetes, either type of vascular bed can be affected leading to macrovascular or microvascular disease, respectively, or both.

47.5.1 Inflammation Contributes to Macrovascular Disease

Cardiovascular disease is a major long-term complication of insulin resistance and diabetes. Various aspects of (obesity associated) inflammation and macrovascular disease have been extensively reviewed before [98–101]. In short, atherosclerosis, the main process underlying macrovascular disease, starts with activation of the endothelial cells that line the intima. This activation can be induced by, for example, lipids (including NEFA and cholesterol) or inflammatory cytokines [98–101]. Notably, adipose tissue depots in close proximity to the vasculature and the heart have been implicated in the development of vascular dysfunction [98] production of mediators that contribute to a local inflammatory response [102,103] and local insulin resistance, which may directly affect vascular function [104,105].

Endothelial activation is characterized by, among others, increased expression of leukocyte adhesion molecules on the endothelial cells and enhanced binding of leucocytes to the inner lining of the large blood vessels. These leucocytes migrate through the endothelial cell layer to the intima, where they can attract monocytes that transform into macrophages and ultimately into lipid-laden foam cells. Foam cell formation comprises an early event in the generation of atherosclerotic plaques. All these processes may be enhanced in obesity-associated insulin resistance and type 2 diabetes, for instance, because of the ongoing systemic inflammation, the presence of an atherogenic lipoprotein profile, including small dense LDL particles, [106] and the presence of monocytosis [107]. Further progression of the atheroma and generation of rupture-prone atherosclerotic plaques involves a complex interplay of immune cells and inflammatory mediators. Inflammatory pathways are also involved in thrombosis, the late complication of atherosclerosis that is responsible for most of the complications of

macrovascular disease [94,101]. Macrovascular disease is thus perceived to be a major consequence of obesity-induced inflammation, insulin resistance, and type 2 diabetes. Moreover, individuals with insulin resistance or type 2 diabetes appear to be more sensitive to the detrimental effects of endothelial dysfunction than those with normal glucose metabolism [108]. This will render them more prone to develop macro- and possibly also micro-vascular disease in response to deteriorating endothelial dysfunction.

47.5.2 Inflammation Contributes to Microvascular Disease

Microvascular dysfunction may also contribute to the vicious cycle of adipose tissue dysfunction and inflammation. Capillary recruitment, which has been shown to be necessary for normal insulin-mediated glucose uptake by skeletal muscle, is impaired in obese insulin-resistant humans and rats [109]. Increased systemic concentrations of NEFA and inflammatory cytokines, and decreased concentrations of adiponectin, as is present in obesity-associated inflammation, can induce endothelial insulin resistance and reduce local NO production. This may lead to a reduction in insulin-mediated glucose uptake in muscle, by as much as 40% and, as such, contribute to whole-body insulin resistance. A small but metabolically relevant ectopic fat depot, that is, perivascular fat, is located around resistance arterioles of muscle. Perivascular fat may directly affect the function of the microvessels. Indeed, it appeared that in lean mice perivascular fat had a beneficial effect to stimulate insulin-induced vasodilation due to local adiponectin production, which was lessened in obese mice [110].

Functional capillaries in the expanding adipose tissue are necessary to provide optimal blood flow and delivery of nutrients and oxygen to adipocytes. Thus, insufficient adipose tissue angiogenesis and capillarisation may lead to hypoxia and induction of an inflammatory response [111]. A relative reduction in the density of the capillary network combined with microvascular dysfunction may further aggravate the hypoxic and inflammatory processes in expanding adipose tissue and lead to deterioration of insulin resistance and metabolic homeostasis [112]. Adipose tissue is capable of producing all the factors of the renin-angiotensin-system (RAS) that are needed to produce angiotensin II, and RAS activity is enhanced in obesity [109,113]. In the expanding adipose tissue, microvascular dysfunction may therefore be aggravated via increased (local) production of angiotensin II. Moreover, cross-talk between the signaling pathways of insulin and angiotensin II as well as a disturbed balance in relative contributions of AT1R and AT2R activation in response to antiotensin II in insulin resistance appear to modulate endothelial function and contribute to endothelials dysfunction [114].

Microvascular dysfunction may additionally contribute to the development of type 2 diabetes via effects on β-cell function. For example, transient periods of (mild) hyperglycemia that coincide with insulin resistance as well as low-grade inflammation – possibly in combination with increased NEFA and dysregulation of adipokines – may lead to reduced islet perfusion and (mild) islet ischemia [115] and control the recruitment of inflammatory cells to the islets [116].

47.6 Master Regulators and Potential Novel Targets to Counteract Obesity-Associated Inflammation in Type 2 Diabetes

As described in the previous sections, the various pathways via which obesity, inflammation, insulin resistance, and β-cell function are interconnected form a complex and intricate system. Together they determine whether an (obese) individual will develop chronic, systemic inflammation and insulin resistance, and also whether he or she will progress to develop hyperglycemia and type 2 diabetes. Several major contributors have been identified. These include cellular components such as the metabolic master switch 5′ AMP-activated protein kinase (AMPK), PPAR-γ, the inflammasome, and NF-κB, and also extend to metabolic or regulatory systems such as the incretin pathway, carbonyl and/or oxidative stress, the innate immune system, including complement, the microbiome, endotoxaemia, epigenetic signatures, and many more. Several of these have over the years been proposed – and partly also implemented – as potential targets for control of inflammation and prevention of insulin resistance and type 2 diabetes. In this section, we will discuss a selection of these potential targets for intervention, that is, PPAR-γ, the inflammasome, the gut microbiome, the complement system, dicarbonyl compounds/AGEs, and lifestyle.

47.6.1 PPAR-γ and PPAR-γ Agonists

PPAR-γ is a member of the nuclear receptor superfamily and acts as a ligand-inducible transcription factor. PPAR-γ plays a key role in the regulation of metabolic homeostasis and inflammation. PPAR-γ is strongly expressed in adipose tissue but also in cells of the immune system [117]. The natural ligand of PPAR-γ has not yet been identified. Most endogenous ligands identified thus far are dietary metabolites, mostly fatty acid metabolites that generally have low affinity [118]. Enhanced PPAR-γ signaling generally has beneficial effects on insulin resistance and type 2 diabetes and it remains to be determined to what extent these effects are primarily related to the metabolic or to the anti-inflammatory effects of PPAR-γ agonism.

Thiazolidinediones (TZDs) are synthetic PPAR-γ agonists that act as insulin sensitizers and are used in treatment of type 2 diabetes (reviewed in [119,120]). TZDs increase insulin sensitivity, lower postprandial glucose, and fatty acid concentrations and have anti-inflammatory effects [120]. Several lines of evidence, mainly derived from animal studies, show that the insulin sensitizing effect of TZDs is primarily conferred via effects in adipose tissue [119]. The adipose effects of TZDs include improved expandability of white adipocytes leading to a reduction in ectopic fat, increased production of adiponectin, and induction of the polarization of adipose tissue macrophages toward the anti-inflammatory M2 phenotype [119]. The first TZDs entered clinical practice in the late 1990s. These compounds were efficient with respect to improvement of glycaemic control, but had side effects that have hampered their full implementation and development [119]. For instance, all PPAR-γ agonists promote weight gain due to fat

accumulation (albeit in the subcutaneous, rather than abdominal of ectopic depots), fluid retention, and bone fractures [120]. Moreover, the first PPAR-γ agonist that was approved for glycaemic control, that is, troglitazone, was removed from the marked in 2000 due to severe hepatotoxic effects [121,122]. In contrast, another PPAR-γ agonist, pioglitazone, is actually implicated in improvement of nonalcoholic steatohepatitis [119,120]. This effect may partly be attributable to improvement in (adipose tissue) insulin resistance, and partly to anti-inflammatory of immunomodulatory effects [119].

For future development of novel PPAR gamma regulators, lessons learned over the past 15 years from first-generation drugs are currently used to develop more efficient drugs with less side effects. Directions that are currently explored include dual-action PPAR-α/γ agonists and selective PPAR-γ modulators (SPPARMs), which are partial PPAR-γ agonists [118–120]. SPPARMs are non-TZD compounds that separate the insulin sensitizing effects of PPARγ agonists from the transcriptional activation of genes that result in unwanted side effects. One SPPARM (INT131) has by now progressed into clinical trials [123]. Interestingly, one particular angiotensin II type 1 receptor blocker telmisartan has been identified to have partial PPARγ agonist properties [124]. Finally, modulatory rather than agonistic interference with PPAR-γ activation may be a viable future perspective. This approach is taken in the development of compounds that block cyclin-dependent kinase 5 (Cdk5)-mediated phosphorylation of PPAR-γ, preferably without transcriptional agonism [125].

47.6.2 Inflammasome

Recently, inflammasomes have been proposed as central regulators of early adipose tissue inflammation. The inflammasome is an important cellular danger-sensing system. It consists of pattern-recognition receptors (PRRs) that assemble into high-molecular weight platforms that control maturation and secretion of proinflammatory interleukins, primarily IL-1β, which has a central role in inflammatory processes [126]. The NOD-like receptor family pyrin domain containing 3 (NLRP3) is one of the best characterized members of the inflammasome-family [127]. NLRP3 releases bioactive caspase-1, which can cleave procytokines, for example, pro-IL-1β, into their mature active forms, that is, active IL-1β [128]. The expression of inflammasome NLRP3 components is increased in obesity, while whole-body knockout of components of this complex in mice resulted in protection from obesity (due to higher energy expenditure), and from inflammation and insulin resistance [26]. Several endogenous stress signals, including glucose, palmitate, cholesterol crystals, islet amyloid peptides, and reactive oxygen species, have been suggested as potential *in vivo* inflammasome inducers, but their relevance in the etiology of human obesity and insulin resistance remains to be elucidated. Notably, the NLRP3 inflammasome may be causally involved in induction of obesity and insulin resistance [129,130], at least in mouse models. Moreover, the NLRP3 inflammasome was shown to be upregulated in immune cells of human type 2 diabetes patients, *ex vivo* [131], although the trigger for this upregulation has not yet been identified. Controlling

obesity-associated chronic inflammation via modulation of the NLRP3 inflammasome and control of IL-1β activity is an area of active research.

A possible angle for control of NLRP3 activity in the future is to apply endogeneous or exogeneous miRNAs that are negative regulators of NLRP3 [132]. Also several known small-molecule inhibitors of inflammation have been shown to affect NLRP3 inflammasome activity [133,134]. However, at this point in time, the best option to control the effect of inflammasome activation is to target the main product of the NLRP3 inflammasome, IL-1β [133]. Already a decade ago it was shown that in islet of patients with type 2 diabetes there was an increased expression of IL-1, while expression of IL-1Ra, that is, the natural IL-1 receptor antagonist, was decreased [77], which makes IL-1β a target with good prospective. IL-1β activity can be blocked via for example, IL-1 receptor antagonists, or anti IL-1β antibodies [135]. The relevance of IL-1β blockade in the control of human type 2 diabetes, and in particular β-cell function, has been shown in a placebo-controlled proof-of-concept study with anakinra, an IL-1 receptor antagonist [136,137].

47.6.3 Gut Microbiome

Another target for the treatment of insulin resistance and diabetes that is still in the experimental stage is the gut microbiome [138,139]. The composition of the gut microbiome contributes to the inflammatory response that is seen in insulin resistance and type 2 diabetes. Fecal transplantation, an innovative approach to manipulate the gut microbiome, has been applied in humans and resulted in improved insulin resistance [140]. More indirect intervention in the gut microbiome such as bariatric surgery [141] or dietary interventions [142] may also induce beneficiary alterations of the microbiome composition and better insulin sensitivity. Moreover, higher concentrations of gut-derived endotoxins were seen in obese than in nonobese [143]. This has been observed both in humans and in animal models. Experimental endotoxaemia can induce adipose tissue inflammation and insulin resistance in lean human subjects [144]. Even at relatively mild levels, endotoxaemia may trigger TLRs, for instance, in adipose tissue or on pancreatic β-cells, and as such contribute to insulin resistance and β-cell failure [145]. Besides, gut-derived endotoxaemia may via the portal vein also induce inflammation in steatotic livers. As such, endotoxaemia may comprise a risk factor for progression of simple steatosis toward nonalcoholic steatohepatitis [146].

47.6.4 Complement

The complement system is a complex protein network that was initially identified as part of the innate immune system. Nowadays, the complement system is, in addition to its well-known role in immunity, increasingly recognized as an essential regulator of cell and tissue homeostasis [147,148]. Notably, the central component of the complement system (C3) has been identified as an independent risk factor for type 2 diabetes [149,150].

The complement system consists of soluble and membrane-bound proteins that function in cascades of stepwise protease activation. Complement can be

activated via several pathways – the classical, the lectin, and the alternative – all of which converge on complement C3. Activation of C3 initiates the activation of the terminal complement pathway. Compelling evidence from experimental animal studies indicates that the complement system – and more specifically the alternative and terminal pathways of complement activation – is causally involved in the development of insulin resistance as well as glucose and lipid metabolism. Most importantly, C3a-receptor knock-out mice are protected from high-fat-diet-induced insulin resistance and have less macrophage infiltration in adipose tissue [151] and similar data were obtained in C5a-receptor knock-out mice [152]. These observations on C3a and C5a and their receptors suggest that complement activation in obesity, and in particular in hypertrophic, metabolically stressed adipocytes, may contribute to recruitment of immune cells from the circulation to the adipose tissue. Also, recently the terminal pathway of complement activation was implicated in activation of the NLRP3 inflammasome [153,154]. Additionally, complement activation in humans is strongly associated with systemic (low grade) inflammation [155–157]. There is also a growing body of data that complement activation leads to endothelial dysfunction [156–158], especially when protection of the endothelial cells is compromised due to decreased expression of complement inhibitors [159]. Since the microvasculature comprises approximately 98% of the total vascular surface area [160], complement activation will inevitably affect microvascular function and may, as such, further contribute to development of insulin resistance and type 2 diabetes.

47.6.5 Dicarbonyl Compounds and AGEs

Advanced glycation end products (AGEs) form a heterogeneous family of unavoidable by-products that are formed by, among others, reaction of intracellular or extracellular proteins with reactive dicarbonyl compounds [161]. Reactive dicarbonyl compounds are generated during metabolism of glucose, lipid, and amino acids [162]. The most potent glycation product, the reactive dicarbonyl compound methylglyoxal (MGO), is mainly generated during glycolysis [163] and is increased in humans with insulin resistance and type 2 diabetes [164]. In obesity, the combined effects of enhanced food consumption, low energy expenditure, hyperglycemia, hyperlipidemia, and increased oxidative stress may augment the formation of AGEs. Indeed, obese individuals were characterized by accumulation of AGEs in adipose tissues [165] that may contribute to low-grade inflammation in obesity [166]. AGEs are involved in the development of vascular disease in diabetes [167,168]. Besides, AGEs have also been implicated in the development of obesity and diabetes [169] and have been found to be associated with insulin resistance [170]. In addition to the effects in insulin resistance, increased levels of dicarbonyl compounds and AGEs have been shown to also induce β-cell dysfunction and apoptosis, at least partly via the AGE-RAGE axis [171] and via effects of MGO [172,173]. There are indications in the literature that the effects of RAGE and IL-1β in obesity and type 2 diabetes are intertwined and have the potential to enhance one another [174] but the exact details for this remain to be elucidated.

47.6.6 Nonpharmacological Management of Inflammation in Type 2 Diabetes

Substantial progress in the management of inflammation in diabetes may be found in improvement of the quality of the diet and exercise. Dietary improvements may be achieved via two approaches; first, by consumption of a healthy diet with general anti-inflammatory effects [175], and second by addition of functional food and/or nutraceuticals with anti-inflammatory properties [176]. Medium and high energy physical exercise has long been known to improve low-grade inflammation and insulin resistance in obese individuals [177]. However, progress can also be gained by fine-tuning of interventions in physical activity [178,179]. Moreover, it becomes increasingly clear that, in addition to regular exercise, breaking sedentary patterns will also contribute to improved control of metabolism [180,181].

47.7 Concluding Remarks

Taken together, the interrelations between obesity, inflammation, and type 2 diabetes are becoming increasingly clear. The main points of this chapter are summarized in Box 47.1. The details on exactly where and how inflammation is induced, the temporal order of the events that contribute to insulin resistance and the development of β-cell function, and the role of vascular dysfunction therein remain to be fully elucidated. More detailed knowledge of these events will help to pinpoint optimal targets for prevention and management of type 2 diabetes. We

Box 47.1: Inflammation and Type 2 Diabetes: Key points

- Visceral and ectopic fat are implicated in the adverse metabolic phenotype that often coincides with central obesity.
- In obesity and type 2 diabetes, it is primarily the metabolic pathway of insulin signaling (via IRS and PI3 kinase) that is affected.
- Glucotoxicity, lipotoxicity, ER stress, oxidative stress, and amyloid deposition may all contribute to β-cell failure in obesity-associated type 2 diabetes.
- Endothelial dysfunction is a shared factor underlying both micro- and macrovascular dysfunction.
- PPAR-γ is a major target for anti-inflammatory, insulin sensitizing therapy but there is a need for more specific drugs.
- Inflammasomes, particularly the NLRP3-inflammasome, were proposed as central regulators of early adipose tissue inflammation and are a novel target for anti-inflammatory therapy in type 2 diabetes.
- Complement activation may contribute to insulin resistance and type 2 diabetes via (local) inflammation and attraction of immune cells, and may also induce endothelial dysfunction.
- Advanced glycation end products (AGEs) in obesity may contribute to dysregulation of adipokine secretion and the induction of insulin resistance and β-cell dysfunction.

also discussed the growing range of established and emerging targets for pharmaceutical management of chronic inflammation in obesity-associated insulin resistance and type 2 diabetes. In addition to pharmaceutical targets, substantial progress in the management of inflammation in diabetes can be reached by improvement of the quality of the diet and nonexercise movement patterns.

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Inflammation-Mediated Neurodegeneration: Models, Mechanisms, and Therapeutic Interventions for Neurodegenerative Diseases

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48.1 Introduction

Neuroinflammation is a protective response of the central nervous system (CNS) in an attempt to remove offending materials or harmful stimuli and to initiate a healing process. It is achieved mainly by the activation of microglia (the resident immune cells in the CNS) under tight spatial and temporal regulation by a complex regulating system. As the first line of defense, microglia become readily activated whenever injury or disease occurs and secrete a variety of proinflammatory and potentially cytotoxic soluble factors [1–3]. Excessive amount of these factors can make inflammation neurodestructive. Neuroinflammation is an important contributor to neurodegenerative process. Here we summarized recent progress in understanding inflammation-mediated neurodegeneration from creation of inflammation-related models to exploration of cellular and molecular mechanisms and development of therapeutic interventions for neurodegenerative diseases.

48.2 Neuroinflammation Is a Double-Edged Sword in Neurodegenerative Diseases

Neuroinflammation is a complex cascade of self-defense response of the CNS to injurious stimuli. It eliminates harmful agents (e.g., pathogens, damaged cells/tissues, and autoimmunogens) and initiates tissue repair. Being different from peripheral organs, the CNS only has microglia as the resident immune cell, and it lacks leukocytic infiltration resulting from restriction of blood–brain barrier (BBB), a membrane structure that restricts passage of cells and many substances

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from blood to brain. The CNS has been accepted to be immunologically specialized. We will review recent evidence showing that neuroinflammation plays critical roles in both maintaining brain function and participating in the pathogenesis of neurodegenerative diseases.

48.2.1 Neuroinflammation and Its Physiological Roles

Inflammation in the CNS (neuroinflammation) primarily involves the activity of two types of glial cells: microglia (the resident innate immune cells in the CNS) and astroglia. An acute insult to the CNS triggers rapid microglial activation, the principal component of neuroinflammation. During late embryonic and early postnatal brain remodeling and maturation, microglia participate in the programmed elimination of neural cells [2,4,5]. Microglia are distributed throughout the mature CNS and they constitute approximately 15% of the total CNS cells. In mature brains, resting microglia exhibit ramified morphology and conduct immune surveillance [4]. While the small cell bodies remain stationary, their long branches are constantly moving and surveying the surrounding area [5]. Microglia are very sensitive and sense a wide range of stimuli that disrupt physiological homeostasis, including trauma, ischemia, infection, toxic insult, and autoimmune injury in the CNS [3,4,6]. Activated microglia undergo dramatic morphologic changes (from a resting, ramified shape into an active, ameboid shape), upregulation of surface molecules (e.g., complement receptors and major histocompatibility complex molecules), phagocytosis, proliferation, and secretion of inflammatory/cytotoxic factors (e.g., eicosanoids, cytokines, chemokines, reactive free radicals, and proteases [1–3]. Phagocytic microglia (ameboid shape) migrate along a chemotactic gradient to reach the injury site, phagocytose cellular debris or foreign materials, release chemokines to attract more microglia, and secrete inflammatory factors to promote more microglia to proliferate [1–3]. These inflammatory mediators not only modulate immunological actions but also act on neurons to alter their function.

Astroglia also become activated in response to CNS injuries or immunologic challenges, but the proinflammatory function of astroglia is not as prominent as that of microglia [7,8]. Astroglia provide physical contact and nutrition to neurons, maintain ionic homeostasis, buffer excess neurotransmitters, modulate synaptic transmission, secrete neurotrophic factors, and serve as an important component of BBB [9,10]. The processes of astroglia enwrap synapses, the junctions between the axon terminals of a neuron and the receiving cell (a neuron, muscle cell, or gland cell). Astroglia express transporters (e.g., glutamate transporters) and participate in the reuptake and/or release of neurotransmitters including glutamate and GABA (gamma-aminobutyric acid), the principal excitatory and inhibitory neurotransmitter in the CNS, respectively [10]. When activated, astroglia upregulate the expression of cell-type-specific protein glial fibrillary acidic protein (GFAP) (GFAP), increase the secretion of neurotrophic factors, and release several proinflammatory cytokines [11,12]. Enlargement and increased proliferation of astroglia to fill up the left space after microglial phagocytosis of neural debris is thought to be a form of repair of CNS injuries [3]. Astrocytic scars (a dense glial scar formed by mature, hypertrophic astroglia)

prevent local infections/injuries from being widespread. Collectively, interactions among neurons, microglia, and astroglia are essential for maintaining the integrity and function of the CNS.

48.2.2 Association of Neuroinflammation and Neurodegenerative Diseases

Ample genetic, clinical, epidemiologic, and animal studies have demonstrated that inflammatory and immune responses play an important role in the pathogenesis of AD and PD, the two most prevalent neurodegenerative diseases. For instance, postmortem observation of brains of patients and animal models of AD revealed close association of amyloid plaques (the key pathological hallmark of AD) and activated microglia and astroglia [13]. Neuroradiological studies using positron emission tomography (PET) and the peripheral benzodiazepine ligand PK11195 detect activated microglia in the early stages of pathology of AD without cerebral atrophy [14]. Similarly, the substantia nigra of patients and animal models of PD revealed activated microglia and accumulation of inflammatory mediators [2,15]. These long-standing pathological observations uncover possible involvement of inflammation in neurodegenerative diseases but cannot distinguish whether inflammation initiates, exacerbates, or attenuates disease pathogenesis. Epidemiologic studies and case reports have suggested increased possibility of developing postencephalitic Parkinsonism several decades after early-life viral infections in people [16–20]. Experimental exposure of rodents to Japanese encephalitis virus, bacterial endotoxin lipopolysaccharide (LPS), and highly pathogenic H5N1 influenza virus can partially mimic PD-like neurodegeneration and gliosis in the substantia nigra as well as movement impairment [21–30]. Moreover, prospective studies suggest that higher plasma concentration of proinflammatory cytokine interleukin-6 correlates with an increased risk for PD [31]. The genetic studies from the past decade have identified several AD- or PD-associated genes functioning in inflammation and the immune system. DNA polymorphisms of several inflammatory cytokines might become risk factors for PD [32]. A recent genome-wide association study (GWAS) of 2000 PD patients and 1986 controls identifies an association of genetic variation in the HLA (human leukocyte antigen) with late-onset sporadic PD [33]. HLA contains a large number of genes related to immune function in humans. Additional GWASs have also implicated several AD susceptibility genes, such as *CR1*, *CD33*, *MS4A4A/MS4A4E/MS4A6E* cluster, *ABCA7*, *CD2AP,* and *EPHA1* [34–36]. Some evidence has indicated/implied that these genes are involved in the regulation of inflammation and the immune system [37]. Another GWAS of 59 AD candidate proteins has reported genetic associations with CSF levels of five proteins, of which chemokine (C–C motif) ligand 2 (CCL2), chemokine (C–C motif) ligand 4 (CCL4), and interleukin 6 receptor (IL6R) are important inflammatory mediators [38]. These hypothesis-free GWASs lend strong and independent support to an important role of inflammation in the pathogenesis of AD and PD, and more importantly, they pinpoint urgent investigations of potential causative involvement of these susceptibility genes in AD/PD etiology. Moreover, epidemiological studies and clinical trials suggest some beneficial effects of chronic use of nonsteroidal anti-inflammatory drugs

(NSAIDs) on the development of AD or PD, but there are some conflicting findings [39–46]. Altogether, the evidence summarized above has indicated that inflammation play an important role in neurodegenerative diseases.

48.2.3 Beneficial Effects of Neuroinflammation

A precisely controlled neuroinflammation will benefit the CNS by destructing pathogens (e.g., viruses or bacteria) or other foreign materials, removing cellular debris, eliminating toxic substances (e.g., aggregated amyloid and α-synuclein), preventing spread of infections and injuries, releasing neurotrophic factors, and promoting tissue repair and regrowth [12,47,48]. For example, in AD, microglia cluster around extracellular senile plaques in which they phagocytose and degrade amyloid-β (Aβ) deposits [49–51]. Activated microglia also secrete numerous cytotoxic factors, including reactive oxygen species (ROS), proteases, cytokines, and glutamate, to destroy infectious organisms and infected neurons [1–3].

Neuroinflammation also paves the way for repairing CNS-damaged tissue. During neuroinflammation, microglia and astroglia undergo several steps to promote regeneration of neural tissue. These include the secretion of anti-inflammatory cytokines, the recruitment of microglia and astroglia to the damaged site, and the release of neurotrophic factors. Indeed, glia-derived neurotrophic factor (GDNF) has been reported to promote neuronal survival and rescue injured dopamine neurons in several animal models of PD [52]. Microglia stimulate myelin repair and prevent neurodegeneration in chronic CNS diseases [47]. In addition, activated microglia physically remove the branches from neuronal perikarya near damaged tissue. This process is called synaptic stripping that facilitates regrowth and remapping of damaged neural circuitry [53]. Moreover, the localized production of chemoattractant molecules, the microglial chemotaxis, and the formation of astrocytic scars in the injury site prevent local lesions and infections from widely spreading.

Coordination among activated microglia, astroglia, and neurons plays a pivotal role in mediating tissue repair and fighting off the infection with minimal damage to the healthy CNS cells [5]. It seems that the beneficial effects of inflammation are apparently either inadequate or ineffective in neurodegenerative diseases. Thus, a reduction in generating these neuroprotective effects in response to CNS injuries might remove an important defense mechanism and consequently worsen the disease process.

48.2.4 Detrimental Effects of Neuroinflammation

As described earlier, a well-regulated neuroinflammatory process is essential for CNS homeostasis and proper function. However, excessive inflammatory response of microglia can become destructive. For example, although the initial aim of the secretion of various inflammatory factors from activated microglia is to destroy bacteria, viruses, and infected neurons, these inflammatory factors can also create considerable bystander damage to neighbor healthy tissue. In fact, ample evidence has demonstrated that in chronic inflammatory conditions,

microglia can be neurotoxic and play a major role in the pathogenesis of neurodegenerative diseases [1,54–56].

A restricted cell renewal and regenerative capacity of neural tissues renders the CNS extremely vulnerable to uncontrolled autodestructive immune and inflammatory processes. To date, a great amount of compelling data show inflammatory exacerbation of neurodegeneration in various neurodegenerative diseases. First, an excessive inflammatory response correlates with neuronal loss in various neurodegenerative diseases [13,55,57]. Second, epidemiological studies indicate a possible beneficial effect of long-term use of NSAIDs in both AD and PD [40–42]. Third, genetic studies reveal increased risk for AD and PD in individuals with DNA polymorphisms of several inflammatory cytokines [58,59]. Fourth and most important, experimental evidence shows that the suppression of inflammatory processes mitigates neuronal impairment in models of various neurodegenerative diseases [1,60–62]. Taken together, neuroinflammation displays neuroprotective and neurotoxic functions and is now increasingly accepted as a double-edged sword in neurodegenerative diseases [12]. The combined effects of individuals' vulnerability (e.g., genetic predisposition), level and period of insults (acute or persistent injurious stimuli), and concurrent events (e.g., stressors or existing diseases) with immune regulation eventually determine whether the immune reaction is a beneficial physiological one or a chronic neurodestructive one. With failed regulation of the immune reaction, uncontrolled sustained neuroinflammation, either as an initiator or as a secondary reaction to initial direct neuronal damages, could drive the chronic, progressive neurodegenerative process where detrimental effects of inflammation overcome its beneficial effects.

48.3 Inflammation-Related Models of Neurodegenerative Diseases

The most common features of various neurodegenerative diseases are the late onset, slow, progressive neurodegenerative process, and the prominent microglial activation in the diseased brain regions. The lack of animal models that recapitulate the delayed and progressive nature along with other key features of neurodegenerative diseases has impeded the progress in understanding the pathogenesis of these diseases and developing disease-modifying therapeutics. Several inflammation-associated models created in the last decade better mimicked the slow progression and other critical features of neurodegenerative diseases and provided an opportunity to explore the cellular and molecular mechanisms of the progressive nature of these diseases. Using PD as an example for a variety of neurodegenerative diseases, we summarize recently developed inflammation-associated models. PD, the second most common neurodegenerative diseases, is characterized by the progressive degeneration of the nigrostriatal dopaminergic pathway and consequent movement impairment. Intracranial or systemic administration of LPS or virus into rodents has been used to produce inflammation-associated PD model.

48.3.1 LPS-Induced PD Models

LPS, the active immunostimulant in the cell wall of Gram-negative bacteria, has been used as a representative inflammogen for direct immunological insults. Direct nigral injection of LPS causes robust microglial activation and acute loss of nigral dopamine neurons in rodents [22,24,63,64]. Because LPS has no direct effect on neurons, LPS-elicited neurotoxicity is entirely dependent on the activation of microglia. Chronic infusion of LPS for 2 weeks into the rat substantia nigra triggered a rapid activation of microglia, followed by a selective and gradual loss of nigral dopamine neurons that reached 70% by 10 weeks [25]. This is the first subacute PD model indicating that persistent microglial activation induced by chronic exposure to inflammogen was able to mimic the most fundamental pathological feature of PD, the delayed, progressive and selective degeneration of nigral dopamine neurons. Microglia-generated ROS play a critical role in dopaminergic neurotoxicity in this inflammation-mediated model of PD [25]. Next, a chronic inflammation-elicited PD was created by an intraperitoneal injection of LPS (5 mg/kg) in C57BL/6 mice. In this model, significant loss of nigral dopamine neurons was detected 7 months after the LPS injection and progressed further overtime [29]. Poor passage of peripheral LPS through the intact BBB [65] indicates the neuroinflammation and neurodegeneration induced by a systemic LPS injection is likely an indirect effect of LPS. Mice deficient in TNF receptors (TNF R1/R2$^{-/-}$) are protected from LPS-elicited chronic neuroinflammation and progressive dopaminergic neurotoxicity [29]. Thus, inflammatory cytokines such as TNFα played a critical role in transferring inflammation from the periphery to the CNS and mediating chronic progressive neurodegeneration in this chronic PD model [29]. Moreover, maternal exposure of developing rat fetuses to LPS was used to study how prenatal inflammation affects neurodevelopment of nigrostriatal dopaminergic system and subsequently alters the vulnerability of adults to inflammation-elicited neurotoxicity [26]. The neonates with *in utero* exposure to LPS displayed lesions of nigrostriatal dopaminergic system, which persists into adulthood [26]. Adult rats with prenatal exposure to LPS developed progressive dopaminergic neurodegeneration after chronic nigral LPS infusion in adulthood [27]. These findings together indicate that once reaching certain threshold, the inflammatory process is capable of initiating neurodegeneration. The higher density of microglia in the substantia nigra than other brain regions [23,66] may be partially responsible for the foremost loss of dopamine neurons in the inflammation-induced neurodegenerative process in PD models with systematic LPS exposure.

48.3.2 Virus-Associated Inflammatory PD Models

Epidemiologic studies and case reports have suggested an association between early-life viral infections and postencephalitic Parkinsonism [16–20]. People infected by highly pathogenic H5N1 influenza virus display acute neurological lesions ranging from mild encephalitis to motor disturbances to coma [67,68]. However, the long-term neurological manifestations of H5N1 infection in surviving hosts remain unknown. Whether viral infection can act as an initiating factor in human PD is unclear. Experimental administration of Japanese encephalitis virus or H5N1 influenza virus has been used to study whether virus-

associated inflammation can partially mimic PD-like pathological and behavior alteration in animals [21,30]. Exposure of neonatal Fisher 344 rats to Japanese encephalitis virus can induce degeneration of dopamine neurons and gliosis in the substantia nigra and movement impairments resembling human PD [21]. H5N1 virus administrated by intranasal inoculation in mice traveled into the CNS and induced microglial activation as well as α-synuclein phosphorylation and aggregation. Long after H5N1 virus became undetectable in the brain at 21 days after viral inoculation, microglial activation and α-synuclein pathological alterations persist and a significant loss of nigral dopamine neurons was observed [30]. This study provides important information on the role of virus-associated inflammation in potential long-term impairment to the CNS.

48.3.3 Inflammation-Associated Two-Hit PD Models

The etiology of various neurodegenerative diseases is unclear. Increasing evidence indicates that these diseases may be the final outcome of interactions, over decades of time, among multiple factors including genetic predisposition, exposure to environmental toxins, and the occurrence of neuroinflammation. In addition, most of the current animal models of various neurodegenerative diseases created by using a single environmental insult (e.g., toxin, heavy metals, physical injury, or stress) or a genetic lesion failed to reproduce the full spectrum of the diseases [69,70]. It suggests a combination of genetic vulnerability and an environmental trigger may be required for the development of neurodegenerative diseases. Thus, two-hit animal models are generated to test whether genetic deficiency increases vulnerability of neurons to potential environmental risk factors.

After the stereotaxic injection of LPS into the substantia nigra, transgenic mice overexpressing either wild-type or A53T mutant human α-synuclein, but not α-synuclein knockout mice, developed sustained microglial activation, accumulation of insoluble α-synuclein aggregates, and formation of Lewy body-like cytoplasmic inclusions in neurons, and death of dopamine neurons in the substantia nigra [71]. Furthermore, a two-hit, chronic, progressive model has been created by a single intraperitoneal injection of a relatively low dose of LPS (3×10^6 EU/kg) into 7-month-old transgenic mice overexpressing A53T mutant human α-synuclein [72]. This two-hit chronic PD model displays sustained low-grade neuroinflammation, α-synuclein accumulation and aggregation, Lewy body-like neuronal inclusions containing aggregated α-synuclein, and progressive dopaminergic neurodegeneration in the nigrostriatal pathway [72]. Compared with the previous LPS model [29], in which it took 7 months for LPS-injected mice to manifest PD-like dopaminergic neurodegeneration, this new two-hit model shows much earlier onset and more pronounced loss of nigral dopamine neurons [72]. The α-synuclein pathology, the pathological hallmark of PD, in the two-hit model has not been reproduced in the previous LPS model. This new two-hit model provides a better tool for studying mechanisms of interactions of chronic α-synuclein pathology and persistent neuroinflammation in chronic PD progression. Furthermore, loss-of-function mutations in the *parkin* gene cause early-onset familial PD. Deletion of *parkin* gene in mice failed to produce PD-like phenotypes. The intraperitoneal injection of low-dose of LPS (7.5×10^5 EU/kg)

twice a week into Parkin-deficient mice for 3–6 months resulted in persistent neuroinflammation, oxidative stress, and selective loss of dopamine neurons in the substantia nigra and subtle fine-motor deficits [73,74]. Thus, synergistic effects of low-grade neuroinflammation and dysfunction of α-synuclein or Parkin induce chronic PD neurodegeneration, while neither factor alone is sufficient to cause neuronal death.

Combination of LPS with neurotoxins was also used to mimic PD. Neurotoxin 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine (MPTP), a by-product of synthetic heroin, causes Parkinsonism in both human and nonhuman primates. MPTP trigger direct lesion to dopamine neurons after its uptake by dopamine transporter. Neither MPTP nor its toxic product 1-methyl-4-phenylpyridinium (MPP^+) can directly activate microglia [75]. Mice with systemic injection of both MPTP and LPS, but not with either toxin, showed striatal dopamine depletion and reduced stride length at 4 months after the injection. Systemic injection of MPTP alone triggered an acute reduction in striatal dopamine, but the reduction was transient and recovered to normal levels 4 months after the injection [76]. Earlier studies by our group reported synergistic dopaminergic neurotoxicity of a combination of individually nontoxic concentrations of MPTP and LPS or PD-related pesticide rotenone and LPS in midbrain neuron-glia cultures [77,78].

The two-hit models mimic PD multifactorial etiology and recapitulate the key features of PD. They underscore synergistic effects of genetic predisposition and environmental exposure or multiple exposures to environmental risk factors in the development of PD. More importantly, the chronic progressive nature of dopaminergic neurodegeneration makes the chronic two-hit models invaluable for the study of mechanisms of PD progression and for the screening of disease-modifying therapeutics.

48.4 Inflammatory Mechanisms of the Progressive Neurodegeneration

It is well accepted that neuroinflammation loses its defensive nature and becomes destructive to CNS homeostasis once it escapes from the strict regulation and exceeds the threshold of CNS tolerability. The two key question to understand whether and how neuroinflammation determines the slow, progressive nature of neurodegenerative diseases is how neuroinflammation converts initial neuronal damage into chronic, progressive neurodegeneration and how chronic neuroinflammation is formed and maintained.

48.4.1 Dysregulated Neuroinflammation and Injured Neurons Form a Self-Perpetuating Vicious Cycle Driving Neurodegeneration Progression

Various insults to the CNS (e.g., trauma, environmental neurotoxins, or genetic deficiency) can directly trigger neuronal injuries. As a result, microglia become activated. The microglial response secondary to direct neuronal lesions is usually

termed reactive microgliosis, a process involving increased proliferation, recruitment, and activation of microglia. Activated microglia, either as a direct result of immunologic stimuli or as reactive microgliosis, can release a variety of neurotoxic factors to induce or exacerbate neuronal damages. It is well documented that the inflammatory response has a self-amplifying nature. Once microglia escape from the strict regulation normally imposed on them, they lose their defensive features and switch to producing neurotoxic effects [3]. Consequently, a vicious cycle between injured neurons and uncontrolled inflammation occurs inevitably and further contributes to the ongoing disease progression. Both neuroinflammation and neurodegeneration continue even after the initial offending factors are overcome. Long after MPTP has been cleared from the brain, the substantia nigra of MPTP-exposed patients and monkeys displays an active and sustained neuroinflammation as well as active neurodegeneration [15,79]. Similarly, in AD, the extracellular environment remains hostile to neurons even after the removal of amyloid plaques [80]. Collectively, regardless of the type of initial lesions–inflammation by immune stimuli or neurodegeneration by traumatic brain injury, once one event starts, the other event joins in, forming a self-perpetuating vicious cycle that drives the chronic progressive neurodegeneration.

48.4.2 Interactions between Neuron-Derived Self-Compounds and Microglial Mac1 Bridges Microgliosis and Chronic Neurodegeneration

Release of a variety of soluble factors from injured neurons, along with loss of cell–cell contact between neurons and glial cells, can stimulate reactive microgliosis [81–83]. Neurons negatively regulate the reactivity of glial cells through cell–cell contact involving the neural cell adhesion molecules expressed on the surface of both cell types [83] and the CD200 receptor–ligand interaction [84]. A spectrum of noxious self-compounds released into the extracellular milieu following neuronal injuries, such as membrane breakdown products; released/leaked cytosolic compounds (e.g., α-synuclein and neuromelanin); abnormally processed, modified, or aggregated proteins (e.g., α-synuclein and Aβ); altered molecules (e.g., the active form of matrix metalloproteinase-3); and imbalanced neurotransmitters (e.g., elevated glutamate) can serve as stimuli to induce reactive microgliosis [85–89]. Microglia respond to these aberrant endogenous compounds and invading microbes similarly [90].

The pattern recognition receptors (PRRs) rapidly detect and eliminate invading microorganisms and also respond to host-derived ligands to activate inflammatory signaling cascades. Membrane-bound PRRs, such as toll-like receptors (TLRs) and C-type lectin receptors (CLR) are broadly expressed on the surface of innate immune cells, including macrophages and microglia. Mac1 (macrophage antigen complex 1; also known as CD11b/CD18, complement receptor 3, $\alpha_M\beta_2$) is an adhesion molecule of the leukocyte-specific β2 integrin family and also functions as a PRR [91–95]. In the CNS, Mac1 exclusively expresses on microglia and is involved in microglial adhesion, chemotaxis, and phagocytosis [96]. The expression of Mac1 is elevated in numerous neurodegenerative diseases including AD and PD [97,98]. Microglial Mac1 is an important mediator, bridging inflammation and neurodegeneration

in neurodegenerative diseases [85,91,92,99]. A binding study followed by a flow cytometry analysis using Mac1-deficient or wild-type microglia revealed Mac1 might bind with α-synuclein and/or affect microglial phagocytosis of α-synuclein [92]. Mac1 mediated α-synuclein-elicited microglia-dependent neurotoxicity in neuron-glia cultures [92]. Mac1-deficient mice were more resistant to MPTP neurotoxicity [100]. Additionally, deficiency in microglial Mac1 greatly mitigated neuronal death induced by *in vitro* exposure to MPP^+ [100]. Since MPTP and MPP^+ cannot directly activate microglia, Mac1 may react to noxious endogenous substances released from the damaged neurons, thereby enhancing MPTP/MPP^+ neurotoxicity. HMGB1 (high-mobility group box 1, a nonhistone DNA-binding protein), released from inflamed microglia and/or degenerating neurons, acted on microglial Mac1 to mediate chronic neuroinflammation and progressive neurodegeneration [101]. Aβ25–35 has been reported to bind to Mac1 and mediate microglial release of nitric oxide [2,92]. Mac1 might also participate in mediating microglial activation via the interaction with fibrinogen, a blood-clotting factor, which gains access to the CNS after BBB disruption in multiple sclerosis (MS) [99,102]. Interestingly, our recent studies identified Mac1 as a novel surface receptor for extracellular dsRNA in regulating macrophage immune responses. Extracellular polyinosinic:polycytidylic acid (poly I:C; a synthetic dsRNA) activates Mac1 to enhance TLR3-dependent signaling and to trigger TLR3-independent, but Mac1–dependent, inflammatory oxidative signaling. Altogether, Mac1 might play a critical role in mediating microglial activation, especially reactive microgliosis, and subsequent neurodegeneration in various neurodegenerative diseases.

48.4.3 The Coupling of Microglial Mac1 Receptor and NADPH Oxidase

Mac1 reacts to diverse stress stimuli to activate NADPH oxidase leading to production of ROS in microglia [103–107]. NADPH oxidase, an activity-dependent enzyme complex, is widely expressed in various immune cells including microglia. It is a main superoxide-producing enzyme during inflammation. Overexpression of mutant superoxide dismutase 1 (SOD1) enhances activation of microglial NADPH oxidase and subsequent secretion of TNFα elicited by TLR2 stimulation [107]. Regulation of NADPH oxidases activation by TLR2, TLR4, and other TLRs identified in peripheral immune cells and nonimmune cells have not been fully investigated in microglia. However, recent evidence has indicated a critical participation of Mac1 in the activation of NADPH oxidase during inflammation [101,108–111]. ADPH oxidase-generated ROS are required for Mac1-mediated phagocytosis [108]. Mac1 recognizes LPS and some endogenous ligands (e.g., α-synuclein, Aβ, or HMGB1) to induce NADPH oxidase activation [103–106]. The activation of NADPH oxidase and consequent ROS production elicited by these ligands are blunted in Mac1-null microglia, which resulted in less neuronal impairment [103–106]. Thus, the coupling between Mac1 and NADPH oxidase might be an important mechanism underlying microglia-mediated NADPH oxidase-dependent oxidative insults to neurons in neurodegenerative diseases.

48.5 Modulation of Neuroinflammation May Become a Disease-Modifying Therapy for Neurodegenerative Diseases

In neurodegenerative diseases, the role of inflammation can be beneficial and detrimental and can change dynamically over time. The dynamic modulation of inflammatory processes aimed at preventing or interrupting the vicious cycle between damaged neurons and dysregulated inflammation hold promise to halt or slow down the disease progression. When the inflammatory reaction is beneficial but not sufficient, immunization in a well-controlled way may become a choice for neurodegenerative disease treatment. When inflammation becomes destructive, therapeutic interventions to either suppress inflammation or boost endogenous anti-inflammatory signals will become an urgent need. In the meantime, exogenous administration or inducible release of neurotrophic factors to promote neuronal survival and/or regeneration could also become a disease-modifying therapy for neurodegenerative diseases. Most likely, the combination of these therapies could offer best treatment for neurodegenerative diseases.

48.5.1 Novel Anti-Inflammatory Therapy for Neurodegenerative Diseases

With increasing awareness of the detrimental effects of uncontrolled inflammation in the progression of neurodegenerative diseases, pharmacological targeting of progressive neurodegeneration mediated by the uncontrolled inflammation could become a potential disease-modifying therapeutic approach. In fact, various inflammatory pathways, receptors, or enzymes have been tested as prime therapeutic targets for neurodegenerative diseases *in vivo* and *in vitro* models. Correlation of pharmacological inhibition of inflammation with attenuated neurodegeneration as shown by a plethora of *in vivo* and *in vitro* studies [1,13,27,60,61,112] indicates dampening excessive inflammation to controllable levels could become beneficial for neuronal survival.

48.5.2 Therapeutic Targeting NADPH Oxidase

Neuroinflammation and oxidative stress are two intertwined major pathologic factors in neurodegenerative diseases. Among a myriad of inflammatory and cytotoxic factors released from activated microglia, NADPH oxidase-derived ROS are a crucial player in chronic neurodegeneration [50,113,114]. Pathological examinations reveal upregulated microglial NADPH oxidase in diseased CNS regions of patients with AD, PD, or ALS [115,116]. Peroxynitrite (a reactive product of superoxide and nitric oxide) and H_2O_2 (nonradical oxidants) derived from activated microglial NADPH oxidase can enter neurons. They are highly reactive and their excessive production can damage proteins, lipids, DNA, or RNA, leading to cell dysfunction and eventual death [117–119]. Pharmacological inhibition of NADPH oxidase activity with its well-known inhibitors (e.g., apocynin and diphenylene iodonium) and several structurally and functionally different compounds correlates with less neurodegeneration in models of neurodegenerative diseases including AD, PD, ALS, and MS [120–134]. These compounds include sinomenine

(a natural dextrorotatory morphinan analog), squamosamide derivative FLZ, pituitary adenylate cyclase-activating polypeptides, TGF-β1 (transforming growth factor-β1; a known endogenous immune modulator), verapamil, and resveratrol (a nonflavonoid polyphenol with antioxidant and anti-inflammatory properties). All these compounds suppress inflammation, inhibit superoxide release from wild-type microglia, and provide neuroprotection but fail to do so in the absence of NOX2, the catalytic subunit of NADPH oxidase [135–140]. Moreover, the genetic ablation of NOX2 attenuates the neurodegeneration induced by *in vivo* and *in vitro* exposure to diverse challenges, including neurotoxins (e.g., MPTP, rotenone and paraquat), inflammogen (e.g., LPS), aggregated proteins (e.g., Aβ and α-synuclein), extracellular HMGB1, nanometer size diesel particles, and genetic overexpression of mutant SOD and APP in mice as a model of ALS and AD, respectively [57,60,75,92,100,122,141–147]. These multiple lines of evidence strongly implicated overactivated NADPH oxidase as a major mediator of inflammatory oxidative neurodegeneration in various neurodegenerative diseases. Thus, NADPH oxidase, especially its NOX2 subunit, could become a promising therapeutic target for neurodegenerative diseases.

Although preclinical evidence provides a strong rationale for pharmacological modulation of NADPH oxidase activity for neurodegenerative diseases, long-term targeting NADPH oxidase has potential drawbacks. NADPH oxidase is indispensable for host defense. People with mutations in genes encoding subunits of NADPH oxidase are more susceptible to bacterial and fungal infections and chronic granulomatous disease (CGD) [148]. More than two-thirds of CGD cases are X-linked recessive due to NOX2 gene mutations, the remaining CGD cases are autosomal recessive due to gene mutations in $p47^{phox}$, $p67^{phox}$, or $p22^{phox}$. Importantly, one copy of causative NOX2 mutations in females reduces NOX2 activity but does not cause CGD or other ROS-related illness. Only CGD patients whose phagocytic ROS generation lowers to more than two orders of magnitude of healthy controls develop severe illness and poor long-term survival [149]. Thus, it appears feasible to develop therapeutic compounds for neurodegenerative diseases to decrease – but not to abolish – the excessive activity of the NADPH oxidase without compromise of immune function.

The limitations of available NADPH oxidase inhibitors, such as lack of selectivity of diphenylene iodonium for NADPH oxidases, the pro-oxidant potential of apocynin, the irreversible off-target effect on serine proteases and the very low efficacy of 4-(2-aminoethyl)benzenesulfonyl fluoride (IC50 >1 mM), and the nonoral administration routes of NOX2ds-tat peptide limit their clinical translation. Interesting, posttreatment with an ultralow dose of diphenylene iodonium inhibited microglial activation, reduced the expression of proinflammatory factors, attenuated α-synuclein aggregation, mitigated progressive dopaminergic degeneration, and improved rotarod activity in multiple PD models [150]. **T**he action mechanism and the selectivity of other putative NADPH oxidase inhibitors remain to be investigated thoroughly [151–155]. In view of the limitation of existing NOX2 inhibitors in clinical translation and the potential drawbacks to targeting NOX2, more efforts should be devoted to develop novel BBB permeable NADPH oxidase inhibitors with improved specificity, efficacy, and pharmacokinetic profiles. Taken together, with increasing awareness of the

prime role of NADPH oxidases, especially NOX2, in inflammation-mediated chronic neurodegeneration, proper modulation of NADPH oxidases activity might present a promising therapeutic strategy to retard the progression of neurodegenerative diseases.

48.5.3 Targeting Cyclooxygenase (COX) Using NSAIDs

Most NSAIDs act as nonselective inhibitors of the COX enzyme, inhibiting both the COX-1 and COX-2. COX catalyzes the formation of prostaglandins and thromboxane from arachidonic acid. Prostaglandins are produced by almost all nucleated cells and act on diverse receptors to exert a wide variety of effects including regulating inflammation. While COX-1 is constitutively expressed and regulates many physiological processes, COX-2 is expressed upon stimulation in scenarios of inflammation and growth. About 20 epidemiological studies reveal association of chronic use of nonselective COX-1/COX-2 inhibitors (e.g., indomethacin or ibuprofen) with reduced risk for AD [156,157]. The nonselective COX-1/COX-2 inhibitor diclofenac combined with misoprostol (a synthetic prostaglandin E1 analog) led to an insignificant marginal decrease in AD deterioration compared with placebo [158]. In much larger clinical trials, COX-2 inhibitors celecoxib [159,160], nimesulide [161], and naproxen [159,160] failed to show effectiveness in AD patients.

A variety of NSAIDs provide neuroprotection in animal models of PD [162,163]. Most of epidemiological studies suggest reduced risk for PD after chronic use of non-aspirin NSAIDs [39,40,44,45,164–166], but one study reported an increased risk [167]. Regular use of aspirin has been associated with a decreased risk of PD [39,45,165] and either no effect or increased risk [40,164]. A nested case-control analysis in a large cohort of 22 007 male physicians aged 40–84 years did not find evidence that NSAIDs use reduces PD risk [46].

At present, the number of clinic trials concerning the effects of anti-inflammatory drugs on the natural progression of neurodegenerative diseases is too limited to draw a firm conclusion, although there is encouraging experimental evidence [1,13,27,60,61,112]. The low success rate of translating anti-inflammatory drugs from animal models to the human scenario highlights the need for better approaches to anti-inflammatory drug design and more appropriate animal models for preclinical drug screening. In this regard, chronic models are better suited to assessing the progressive nature and to screening the putative disease-modifying therapies.

48.6 Conclusions

In neurodegenerative diseases, neuroinflammation can be neuroprotective and detrimental and its role can change dynamically over time. Dysregulated, prolonged, and self-amplifying inflammatory processes may be a common mechanism underlying the self-propelling nature of numerous neurodegenerative diseases. Owing to its vital role in pathogenesis, inflammation is a prime

therapeutic target for neurodegenerative diseases. Ample preclinical evidence has underscored a pivotal role of overactivated NADPH oxidase in microglia-mediated chronic neuroinflammation and progressive neurodegeneration. Given such an important role and the feasibility of pharmacological modulation of its activity, NADPH oxidase can become a promising target for retarding progression and improving outcome of neurodegenerative diseases. Because of the vicious cycle between uncontrolled neuroinflammation and damaged neurons and the present failure of single drug treatment (anti-inflammation or neuroprotection), drugs with multiple actions and multidrug approaches directed at both inflammatory and noninflammatory mechanisms might represent the most promising therapeutic strategies for neurodegenerative diseases. Although clinical trials have not yet produced encouraging results, conceivably this can change in the near future as we learn more about inflammatory mechanisms of progressive neurodegeneration, development of better disease models, and improvement of trial design.

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Conflict of Interest

The authors declare they have no actual or potential competing financial interests.

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49

Inflammation in Atherosclerosis

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49.1 Introduction

Atherosclerosis is a major cause of cardiovascular disease and its clinical manifestations such as stroke or myocardial infarction are among the leading causes of death worldwide. The pathology is characterized by a chronic inflammatory condition that is driven by an overload of lipids, particularly cholesterol, and formation of fibrous and lipid-laden plaques in arterial walls [1]. Animal, epidemiological, and clinical studies have all established that high levels of low-density lipoprotein (LDL) and its major constituent, cholesterol, promote atherosclerosis development. Plaque formation is initiated upon endothelial dysfunction at sites of disturbed blood flow. Increased vascular permeability and alterations in composition of the extracellular matrix allow for retention of LDL at these disrupted sites in the subendothelial space [2]. Concurrently, an increase in leukocyte adhesion molecule expression and chemokine release by vascular cells results in immune cell infiltration (e.g. monocytes and T cells) [3]. LDL trapped in the intima is then prone to oxidation, which enhances its uptake by scavenger receptors expressed on macrophages. Macrophages are unable to clear the large amounts of LDL cholesterol they ingest and store them as cholesterol esters in lipid droplets. This gives the macrophages a lipid-laden appearance, hence their description as "foam cells" [3]. In addition, oxidized LDL (oxLDL) is a potent danger-associated molecular pattern (DAMP) and elicits an inflammatory response. As these foam cells accumulate in arterial walls, they recruit more immune cells, including T cells that further drive plaque development. With progression of disease, smooth muscle cells migrate into the intima of affected arteries and start secreting extracellular matrix components resulting in

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formation of a fibrous cap. Generally, atherosclerosis manifests clinically when the fibrous cap impairs blood flow or ruptures, which may cause myocardial infarction or stroke as a result of arterial occlusion [1].

49.2 Cholesterol Transport and Metabolism in Atherosclerosis

Cholesterol is a major constituent of membranes and is essential for their stability and function. However, excess cholesterol is highly toxic to cells and is therefore rapidly converted to cholesterol esters for storage in lipid droplets or secreted from the cell [4]. Cholesterol can either be synthesized *de novo* from acetyl-CoA or taken up from dietary sources. In the bloodstream it is bound to lipoproteins; the major ones being LDL and high-density lipoprotein (HDL). LDL is secreted from the liver and transports cholesterol to peripheral organs, where it is taken up via the LDL receptor [4]. Additionally, foam cells can take up modified forms of LDL via the scavenger receptors, Scavenger Receptor Class A (SR-A), and cluster of differentiation 36 (CD36) [3]. HDL, in contrast, mediates cholesterol transport from peripheral organs to the liver, a process also referred to as reverse cholesterol transport (RCT). Unlike other lipids, cholesterol cannot be broken down by mammalian cells and as such the only way for foam cells to deplete excess cholesterol is through excretion and subsequent transfer to HDL [5]. RCT is mediated by two members of the ATP-binding cassette transporters: (ABC) ABCA1 and ABCG1, which efflux cholesterol from the cell and transfer it to HDL. Cholesterol loaded in HDL is in turn taken up by the liver, from where it is either secreted directly or as a conjugate form as bile acids [6]. The balance between cholesterol uptake by scavenger receptors and excretion by ABCA1 and ABCG1 is a major determinant of foam cell formation and disease progression. As cholesterol cannot be broken down, RCT and subsequent secretion provides the only way for the body to eliminate excess cholesterol. Thus, understanding and targeting RCT is subject of intense investigation and could have important clinical implications [7].

49.3 Mouse Models of Atherosclerosis

Many crucial insights into the development and progression of atherosclerosis were gained using mouse models. However, due to major differences in lipid metabolism, wild-type mice, unlike humans, do not spontaneously develop atherosclerosis when fed a high-fat or high-cholesterol diet. Therefore, transgenic mouse models are commonly used [8]. Mice deficient in apolipoprotein E (ApoE) or the LDL receptor (LDLR) are the most common transgenic mouse models for studying atherosclerosis. ApoE is the protein component of LDL and mediates the interaction with the LDLR. Absence of either ApoE or LDLR causes defects in cholesterol clearance from the bloodstream and results in hypercholesterolemia. LDLR-deficient animals require a high-fat or high-cholesterol diet to develop

atherosclerosis. ApoE$^{-/-}$ mice spontaneously develop atherosclerosis, but both onset and progression of the disease are accelerated by a high-fat diet [8]. A lack of consistency in diet composition represents a significant limitation of these murine models of atherosclerosis; indeed, a whole spectrum of both control- and high-fat diets, which differ vastly in nutrient composition, especially in regards to fatty acid and cholesterol content, are used for studying atherosclerosis. Therefore, care needs to be taken when comparing mouse studies, and especially when extrapolating implications for human disease from mouse data [3]. Nevertheless, mouse models represent a valuable tool in understanding the mechanisms and identifying mediators of atherosclerosis [8].

49.4 Triggers and Receptors of Inflammation in Atherosclerosis

Over the years, studies in both mice and humans have established that atherogenesis – the process of atherosclerosis progression – is driven by inflammation. Many pattern-recognition receptors (PRRs), which are predominantly expressed on innate immune cells such as macrophages and play key roles in host defense, have been implicated in atherogenesis, including scavenger receptors, toll-like receptors (TLRs), and inflammasomes, some of which will be discussed in the following section [1].

49.4.1 Scavenger Receptors

Scavenger receptors recognize a wide range of ligands and have broad functions in homeostasis and inflammation. The most intensively studied scavenger receptors are SR-A and CD36, which were initially identified as receptors for modified lipoproteins [9], but they also mediate clearance of apoptotic cell debris or cooperate with other receptors to sense pathogens. For example, CD36 is required for recognition of lipoteichoic acid, a component derived from the cell wall of gram-positive bacteria, as part of a complex with TLR2 and TLR6 [9]. *In vitro* studies identified Scavenger Receptors Class A-I/II and CD36 as the major receptors for oxLDL uptake [10]. By mediating this uptake, they not only have a crucial role in foam cell loading, but they also activate a multitude of receptors and signaling pathways.

As already described, free cholesterol is highly toxic to cells and thus rapidly esterified and stored in lipid droplets. In macrophages, oxLDL-derived free cholesterol can activate the unfolded protein response and induce apoptosis [11]. CD36 also mediates uptake of 9-hydroxyoctadecadienoic acid (HODE) and 13-HODE: oxidation products of linoleic acid that are abundant in oxLDL. These molecules are potent activators of peroxisome proliferator-activated receptor (PPAR) γ and promote foam cell formation by sustaining CD36 expression [12].

CD36 also facilitates the activation of other PRRs by oxLDL, including TLRs and inflammasomes.

49.4.2 Toll-like Receptors

TLRs are the best studies class of PRRs. They recognize a wide range of molecules of microbial origin, but also respond to exogenous and endogenous danger signals [13]. Receptor ligation upon ligand binding initiates a downstream signaling cascade that results in activation of transcription factors such as NFκB and expression of target genes, including cytokines, inerferons, costimulatory molecules, or antimicrobial prptides [14]. In the context of atherosclerosis, TLRs are expressed by a multitude of cells, including monocytes, macrophages, T cells and parenchymal cells [15]. TLR4 was originally discovered as the LPS receptor, but was also implicated in atherogenesis from early on. ApoE/TLR4-double-deficient mice fed a high-cholesterol diet exhibit a moderate phenotype with regards to inflammation and plaque formation compared to ApoE-deficient control animals [16]. However, while in a more recent study, LDLR/TLR4-deficient animals fed a high-fat, high-sucrose diet showed a marked reduction in disease severity, they showed no changes in inflammatory parameters. These LDLR/TLR4-deficient mice displayed improved plasma cholesterol levels and a drastic reduction in lesion size of 75% [17]. Interestingly, TLR4 engagement with TLR6 and CD36 upon stimulation with oxLDL induces the expression of proinflammatory cytokines *in vitro*. Mechanistically, CD36 facilitates oxLDL binding, internalization, and recruitment of TLR4 and TLR6. TLR4 and TLR6 activation in turn leads to downstream signaling and transcription of proinflammatory mediators [18]. TLR2 was initially discovered as a receptor for bacterial lipopeptides [14] and its expression is increased in atherosclerostic lesions [19]. Mouse studies further support the relevance of TLR2 in atherogenesis as administration of the TLR2/TLR1 agonist Pam3CysK4 to $LDLR^{-/-}$ mice fed a high-fat diet results in disease aggravation [20]. Interestingly, pharmacological inhibition of TLR2 in human atheroma explants diminishes the expression of inflammatory chemokines and matrix metalloproteases [21]. Together, these data suggest a role for TLR2 in the recruitment of immune cells by the activated endothelium and in plaque progression. Another study addressed the role of TLR2 in the ApoE model fed normal chow. ApoE/TLR2-double deficient animals exhibited reduced plaque formation, macrophage recruitment, and production of the chemokine macrophage chemotactic protein-1 (MCP-1), also known as C–C motif ligand 2 (CCL2) [22] 1. In addition to microbial ligands, TLR2 also recognizes a number of endogenous ligands, including oxidized phospholipids and serum amyloid A (SAA) [23]. SAA is an acute phase protein that is secreted from the liver during inflammation. It is also elevated in patients with coronary artery disease and was shown to induce factors that are involved in neutrophil activation in human coronary artery endothelial cells [24]. TLR2 also triggers apoptosis of plaque macrophages. As already mentioned, free cholesterol can induce ER stress and plaque macrophages express proteins of the UPR response. In concert with CD36, TLR2 activated by oxidized LDL or oxidized phospholipids drives apoptosis of macrophages undergoing ER stress [25].

49.4.3 Inflammasomes

Inflammasomes are another group of PRRs that sense a cholesterol imbalance. The NLRP3 (nucleotide-binding domain leucine-rich repeat containing (NLR) family, pyrin domain containing 3) inflammasome is a multimeric protein complex that consists of the receptor NLRP3, the adaptor molecule Asc (apoptosis-associated speck-like protein), and the effector molecule caspase-1. Inflammasome assembly results in cleavage of procaspase-1 to its active form and subsequent cleavage and maturation of the proinflammatory cytokines IL-1β and IL-18 [26]. NLRP3 inflammasome activation requires two steps: a priming step that drives NLRP3 expression and a second stimulus that leads to NLRP3 activation. Priming can be accomplished by a multitude of PPRs that activate NFκB [27]. NLRP3 itself is then activated by a multitude of molecules ranging from adenosine triphosphate (ATP) to pore-forming toxins and crystalline material. A direct ligand has not been identified so far, but in general NLRP3 activation is triggered in response to a deviation from cellular homeostasis [26].

Cholesterol, which accumulates in artery walls over the course of atherogenesis, is very poorly soluble in aqueous solutions and tends to crystallize at very low concentrations. Indeed, crystal deposition was reported in human atherosclerotic lesions as they advance from fatty streaks to more complex plaques [28]. *In vitro* studies also described intracellular cholesterol crystal formation in foam cells [29,30].

Cholesterol crystals are also activators of the NLRP3 inflammasome [31–33]. They are present in early stages of plaque formation and colocalize with macrophages in human plaque samples [31,32]. *In vitro,* cholesterol crystals that are engulfed by macrophages appear to activate NLRP3 by eliciting lysosomal damage [31]. OxLDL is also capable of activating NLRP3. When fed to macrophages, oxLDL-derived cholesterol accumulates in lysosomes, where it falls out of solution [31]. The uptake and subsequent crystallization of oxLDL-derived cholesterol is CD36 dependent [34]. As oxLDL can also activate NFκB via CD36, TLR4 and TLR6, it can therefore provide both signal 1 and signal 2 and treatment of macrophages with oxLDL indeed results in IL-1β mRNA induction and also secretion [18,34]. Interestingly, while priming requires the heterotrimeric complex of CD36, TLR4 and TLR6, cholesterol nucleation only requires CD36 and still occurs in TLR4- and TLR6-deficient animals *in vivo* [18,34].

49.5 Mediators of Inflammation in Atherosclerosis

49.5.1 Cytokines

IL1α and IL-1β are proinflammatory cytokines that exert their biological activities by binding to the same receptor: the IL-1 receptor (IL-1R) [35]. Both cytokines are synthesized as preforms and as they lack signal peptides, even after maturation do not enter the canonical secretory pathway. IL-1β is synthesized *de novo* upon PRR ligation and requires inflammasome activation and subsequent cleavage by

caspase-1 for secretion. IL-1α is constitutively expressed in a variety of cell types and can also be subject to cleavage by proteases [35]. In contrast to IL-1β, the IL-1α precursor as well as the processed mature molecule is capable of activating the IL-1R [36]. Both molecules however are released via distinct routes: dying cells that lost cellular integrity passively release the unprocessed precursor, while active secretion of Il-1α from intact cells requires processing by calpain-like proteases [37].

Both IL-1α and IL-1β play roles in atherogenesis, which was confirmed in studies using IL-1R-deficient mice [38,39]. Of the two cytokines however, Il-1β is the far more characterized and indeed models of IL-1β deficiency as well as administration of an Il-1β neutralizing antibody show decreased plaque formation [40,41]. However, in a comparative study, while both IL-1α- and Il-1β-deficient mice exhibited a phenotype, intriguingly Il-1α deficiency was more effective in reducing atherosclerosis [42].

The NLRP3 inflammasome is crucial for IL-1β release, however many established NLRP3 activators also elicit Il-1α secretion from macrophages [37] and crystalline activators including cholesterol crystals are able to trigger IL-1α release via an NLRP3-independent mechanism [32]. Reconstitution of LDLR-/- mice with NLRP3-deficient bone marrow nonetheless confers the same protection against atherosclerosis as Il-1α/IL-1β double-knockout bone marrow reconstitution, which suggests that Il-1α from hematopoetic cells is released in a NLRP3-dependent manner [31]. A more recent study highlighted a role for oleic acid in driving atherosclerosis via triggering Il-1α secretion in an ApoE model [43]. Using mass spectrometry, the authors found that after 6 months of high-fat diet, oleic acid was the most abundant fatty acid in plaque samples [43]. While unsaturated fatty acids, such as oleate, generally protect against the deleterious effects of saturated fatty acids [44], at high concentrations they can cause mitochondrial uncoupling [45]. Uncoupling of oxidative phosphorylation by unsaturated fatty acids is a conserved protective mechanism to deplete free fatty acids, reduce lipotoxicity, and protect from apoptosis [45]. In macrophages, this pathway in addition leads to secretion of pro Il-1α [43].

IL-18 is another member of the IL-1 cytokine family. It is synthesized as a preform, requires cleavage by inflammasomes, but in contrast to IL-1β is expressed constitutively [35]. Together with IL-12, IL-18 is a potent inducer of Th1 cells and interferon gamma. On its own it has proinflammatory properties and drives inflammation in many diseases [46]. The IL-18 receptor (IL-18R) is a heterodimer consisting of the IL-18Rα and IL-18Rβ chain [46]. IL-18 expression as well as expression of its receptor is increased in atheroma samples from patients. While the receptors are expressed by macrophages, endothelial cells, and smooth muscle cells, IL-18 expression is confined to macrophages [47,48]. IL-18- deficiency or overexpression of IL-18 binding protein, which sequesters IL-18 and thereby limits its activity, protect from atherosclerosis [49,50]. Conversely, administration of recombinant IL-18 results in a more severe disease phenotype, including increased lesion size and recruitment of proinflammatory cells into plaques [51,52]. Unexpectedly, it was found that while IL-18 deficiency has an effect on atherosclerosis, IL-18R deficiency alone does not. A recent study identified the sodium chloride (NaCl) cotransporter (NCC), also known as Slc12a3, as an IL-18 coreceptor, which

together with the IL-18 receptor complex is essential for IL-18-mediated effects in atherosclerosis. NCC is expressed by macrophages, smooth muscle cells, and endothelial cells and interacts with the IL-18 receptor. Strikingly, only ApoE/IL-18R/NCC triple-deficient mice exhibit a reduced disease phenotype, while ApoE/IL-18R- or ApoE/NCC double-deficient mice do not. IL-18 treatment triggers signaling through the IL-18R as well as NCC, although it remains to be established whether they signal in a complex or if they engage distinct downstream pathways to mediate the overall effects of IL-18 [53].

IL-6 is a pleiotropic cytokine also involved in atherosclerosis that initiates the acute phase response and activates the adaptive immune system during an infection [54]. It is a major mediator of autoimmune diseases such as rheumatoid arthritis and Crohn's disease and blocking of IL-6 signaling in these diseases with antibodies against the IL-6 receptor (IL-6R) is highly therapeutic [54]. The role of IL-6 in atherosclerosis progression is however more complex. In murine models, IL-6/ApoE-deficient mice showed increased diet-induced atherosclerosis, suggesting a protective role for IL-6 in disease progression [55,56]. However, administration of recombinant IL-6 into $ApoE^{-/-}$ mice increased atherosclerotic burden [57]. Interestingly, in two elegant Mendelian randomization studies, single nucleotide polymorphisms in the IL-6 receptor causing defective IL-6 signaling were associated with lower CRP levels and lower incidence of cardiovascular disease (CVD) events [58,59]. While these studies suggest a role for IL-6 in atherogenesis, whether this is protective, harmful or negligible remains unclear. Some of this apparent discrepancy within mouse models of atherosclerosis could be further investigated by interrogating the effect of IL-6R deficiency in an ApoE or LDLR background on atherosclerosis progression, and thereby determining if IL-6 or another cytokine of the family that signals through the IL-6R, contributes to disease.

IL-12 is a 70 kDa heterodimeric cytokine consisting of subunits encoded by two separate genes: *IL12a* (IL-12p35) and *IL12b* (IL-12p40), and is essential for driving a Th1 adaptive immune response [60]. IL-12p40 can also form a homodimer (IL-12p80) as well as another heterodimer with IL-23p19 to form the related cytokine IL-23 [60]. IL-12p40 deficiency is protective in mouse models of atherosclerosis, suggesting a role for IL-12 in atherosclerosis progression [61]. TNF, a 17-kDa cytokine, can further amplify inflammation via NFκB signaling upon activation of the TNF receptor. Furthermore, elevated TNF is a risk factor for CVD [62] and TNF-deficient mice are protected from atherosclerosis development [63], indicating a likely role for TNF in atherosclerosis [64].

49.5.2 Interferons

Interferon (IFN) α and β are members of the class of type I interferons. They are typically released in response to viral infection and are essential for inducing an antiviral state, but are also released during bacterial infection. Their role in atherosclerosis was only discovered quite recently. IFNα is detectable in plaque samples from patients and its levels correlate with plaque instability [65]. In mouse studies, type I IFNs alter monocyte recruitment into the plaque and foam cell lipid metabolism, while myeloid deficiency of their cognate receptor, IFNα/β

receptor (IFNAR), decreases macrophage recruitment and lesion size. Signaling through IFNAR leads to production of the chemokine CCL5 and subsequent monocyte infiltration into the plaque. In agreement with this, IFNβ injection results in increased CCL5 levels, plaque size, and macrophage infiltration [66]. IFNβ can also promote foam cell formation by affecting lipid metabolism. Foam cells treated with IFNβ increase SR-A expression and decrease ABCA1 expression, thereby fostering lipid accumulation [67]. A recent study further emphasized the role of type I IFNs in atherosclerosis: Myeloid cells deficient in the gene *N*-acetylglucosamine *N*-deacetylase-*N*-sulfotransferase 1 (Ndst1) display defects in heparan sulfate proteoglycan biosynthesis and are more sensitive to type I IFN due to decreased IFN sequestration. As a result, they exhibit a more inflammatory phenotype that accelerates atherogenesis [68].

49.5.3 Chemokines

Finally, chemokines also contribute to early-stage atherosclerosis by recruiting monocytes and other immune cells. Endothelial cells that have been activated upon lipoprotein deposition express adhesion molecules and immobilize chemokines, which initiates the leukocyte adhesion cascade. In particular, the capture and rolling stage of leukocyte adhesion appears to be dependent on C-C motif ligand (CCL)-5 and CXC motif ligand (CXCL)1 immobilization, as well as an increase in P-selectin expression on the luminal side of endothelial cells [69]. The interaction of chemokines with their receptors expressed on monocytes is also required for the transmigration of monocytes into the subendothelial layer. Different monocyte subsets with different inflammatory capabilities express different chemokine receptors, but the key chemokine–receptor interactions in atherosclerosis as determined in murine models are CCL2–CCR2, CXCL1–CXCR1, and CCL5–CCR5 [69,70]. Combined inhibition of this chemokine axis resulted in reduced monocyte recruitment and reduction of atherosclerosis progression in ApoE-deficient mice [71]. These chemokines also modulate total circulating monocyte numbers by controlling their release from the bone marrow and their growth and survival [72,73]. Reducing or inhibiting chemokine, chemokine receptor or adhesion molecule activity represents a potential strategy for treating inflammation in atherosclerosis, and indeed a P-selectin blocking inhibitor has shown some promise in initial clinical trials [74].

49.6 Targeting Inflammation Atherosclerosis

Atherosclerosis and its clinical manifestation cardiovascular disease (CVD) are still the leading cause of death worldwide [1] and as such there is intense interest in improving existing therapies, but also education to promote healthier lifestyles.

49.6.1 Potential of Dietary Changes

Epidemiological studies have identified several risk factors for CVD, including lifestyle factors such as dietary intake, exercise, smoking, and alcohol consumption [75,76].

Recent investigations have highlighted the potential of simple dietary adaptations in preventing and alleviating CVD. Two extreme dietary classifications are commonly made: the Western diet, which is rich in dairy products, meat, refined grains, and sugars and is classified as unhealthy [77] and the mediterranean diet, consisting of whole grains, vegetables, legumes, and olive oil [78].

Western diet increases CVD risk [77]. It is typically characterized by high amounts of saturated fats, cholesterol, and refined sugars. In contrast, a Mediterranean diet, which is high in unsaturated fatty acids, fiber, antioxidants, and vitamins protects against CVD development [78,79]. These diets differ not only in their cholesterol content but also in a multitude of other potent bioactive molecules that may modulate atherogenesis by either fostering or dampening inflammation.

Lipids taken up from different dietary sources can vary dramatically in their fatty acid composition. Animal fats are particularly rich in saturated fatty acids, the most prominent of these being palmitic and stearic acid. Saturated fatty acids are sensed by macrophages and have also been implicated in atherogenesis. At high concentrations they can be cytotoxic [44] and induce ER stress [80], which can drive macrophage apoptosis and necrotic core formation in plaques [11]. Strikingly, desaturation of fatty acids reduces ER stress in macrophages and mitigates atherosclerosis development [80]. Saturated fatty acids can also directly activate PRRs such as TLR4 and NLRP3. Palmitate conjugated to BSA activates NFκB signaling and induces cytokine expression in macrophages in a TLR4-dependent manner [81]. Furthermore, TLR4-deficient mice are protected against fatty acid-induced inflammation [81]. A more recent study addressed the molecular mechanism and found that stearate complexed with the serum protein fetuin A is a potent TLR4 activator [82]. Interestingly, serum fetuin A levels are increased in obese patients, which further highlights the relevance of this pathway in the context of metabolic and cardiovascular disease [82]. Palmitate was also shown to activate NLRP3 via inhibition of adenosine monophosphate (AMP)-activated protein kinase AMPK and generation of mitochondrial reactive oxygen species [83]. ApoE-deficient mice fed a diet rich in saturated fatty acids exhibit higher plasma cholesterol levels and accumulate higher amounts of cholesterol-esters in atherosclerotic plaques [84]. Notably, LDLR/TLR4- double deficient mice fed a diet high in saturated fatty acids are protected from atherosclerosis, which suggests that activation of PRRs by saturated fatty acids might be highly relevant in the context of atherosclerosis [17].

Unsaturated fatty acids, in particular omega-3 fatty acids, have been shown to exert protective effects in atherosclerosis. They can be subdivided into three groups: omega-3, omega-6, and omega-9. Mammals can produce omega-9 fatty acids themselves, but acquire omega-3 and omega-6 fatty acids from dietary sources. Plant fats are rich in unsaturated fatty acids, for example, olive oil is rich in oleic acid and linseed oil is rich in the omega-3 fatty acid linoleic acid [78]. Omega-3 fatty acids exert a multitude of beneficial physiological effects, in part by acting on macrophages. For instance, they bind to the G-protein-coupled receptor GPR120 [85], which has a multitude of functions, but notably is also expressed by macrophages. Several studies have established that omega-3 fatty acids can act as potent anti-inflammatory agents via macrophage GPR120.

Signaling through GPR120 inhibits TLR signaling and subsequent production of proinflammatory cytokines and chemokines via the downstream effector β-arrestin2 [86]. Mice fed an omega-3 fatty acid-rich diet are protected against high-fat diet-induced inflammation, while GPR120 deficiency has the opposite effect [86]. Another study addressed the effect of omega-3 fatty acids on inflammasome activation. Indeed, activation of GPR120 and GPR40, another fatty acid sensing GPR, by omega-3 fatty acids, interferes with NLRP3 inflammasome assembly and IL1-β secretion [87]. While this effect is also partially mediated by β-arrestin [87], the effects of omega-3 fatty acids on NFκB activation and in particular the priming step of inflammasome activation is still controversial, as these two studies showed contradictory results. Omega-3 fatty acids are also protective in mouse models of atherosclerosis: Replacement of saturated fatty acids with omega-3 fatty acids in high-fat diet reduces plasma cholesterol levels, aortic macrophage accumulation, and overall lesion size [88]. However, whether these effects are macrophage GPR120 dependent remains to be established.

The gut microbiome also metabolizes food-derived molecules to bioactive molecules that can affect macrophage function and atherosclerosis. Trimethylamine-*N*-oxide (TMAO) and protocatechuic acid (PCA) are two prominent examples of how metabolites of animal or plant origin can be turned into bioactive compounds by the microbiome. TMAO is synthesized from carnitine, which plays an essential role in fatty acid import into mitochondria. It can be synthesized *de novo* but is also taken up from dietary sources, in particular red meat. Recent studies have shown that carnitine metabolism to TMAO by the microbiome has atherogenic effects. TMAO affects macrophage recruitment into plaques and foam cell formation resulting in increased atherosclerotic burden [89,90].

In contrast, anthocyanins ameliorate atherosclerosis in a microbiome-dependent manner. These are plant-derived molecules with antioxidant and anti-inflammatory properties, which affect macrophage cholesterol metabolism. In mouse models of atherosclerosis, they have a protective role. Mice fed with a diet rich in anthocyanins exhibit reduced plaques and systemic cholesterol levels [91]. Cyanidin-3-*O*-β-glucoside, an anthocyanin that is found in pigments of fruits and vegetables has potent anti-inflammatory and atheroprotective effects. In fact, it is PCA, a metabolite of cyanidin-3-*O*-β-glucoside produced by the microbiome that is the active component with antiatherogenic effects. PCA inhibits expression of adhesion molecules in aortic endothelial cells and subsequent monocyte adhesion. It also affects foam cell formation and RCT by increasing macrophage ABCA1 expression and circulating HDL levels [92,93].

49.6.2 Pharmacological Intervention

In addition to preventative lifestyle changes such as improved diet or exercise programs, patients with CVD can also be treated medically to lower circulating LDL-cholesterol (LDL-C) levels. LDL-C, which is thought to be both a strong biomarker for CVD risk and a causative agent in disease progression, can be lowered by a number of therapies.

Key among these LDL-C-lowering therapies are the statins drugs, which, as competitive HMG-CoA reductase inhibitors, block *de novo* cholesterol synthesis and thereby reduce total circulating LDL-C. Statin-based therapies have been the cornerstone of atherosclerosis treatment for the past 25 years and are effective at lowering LDL-C and reducing CVD events [94]. However, although there is consistent reduction in CVD events and mortality with statin therapy, CVD-related deaths still account for 31% of all global deaths, with 80% of these deaths coming from low- or middle-income countries [95]. An alternative route to statin-mediated lowering of LDL-C levels is by inhibiting proprotein convertase subtilisin-kexin type 9 (PCSK9), which degrades the LDLR. PCSK9 inhibition therefore increases LDLR expression and LDL-C clearance from the bloodstream, thereby reducing total circulating LDL-C levels. The potential of PCSK9 inhibitors for treating CVD is boosted by evidence that people carrying inactivating mutations in PCSK9 have decreased risk of CVD, and indeed monoclonal antibodies against PCSK9 (alirocumab and evolocumab) are currently available to lower LDL-C, but their efficacy in reducing CVD outcomes in large-scale clinical trials has yet to be elucidated [96].

In addition to the *in vitro* and animal studies, a further indication that inflammation plays an important role in atherosclerosis is that the acute-phase C-reactive protein (CRP) is as strong a predictive biomarker of CVD risk as LDL-C or HDL-C levels [97]. CRP is a pentameric protein that binds to phosphocholine present on invading bacteria or dying cells. CRP binding activates the complement system and targets the cells or bacteria for opsonization [98]. CRP is produced in the liver in response to systemic cytokine (especially IL-6) production [98]. The initial observation that CRP and inflammation predict CVD risk came from the Physician's Health Study (PHS), where it was also observed that the common anti-inflammatory drug aspirin was more likely to prevent first time myocardial infarction in patients with elevated CRP levels [99]. Further evidence that systemic inflammation may contribute to disease risk arose from CRP level evaluation during the statin clinical trials. Intriguingly, even patients with low LDL-C starting values were also protected against CVD risk [100]. This finding was then validated directly in the Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) trial, which showed that in apparently healthy patients without hyperlipidemia but with elevated CRP, rosuvastatin treatment reduced the risk of a first myocardial infarction [101]. While the cholesterol-lowering effects of statins are well understood, the molecular mechanisms behind the apparent anti-inflammatory actions of statins are less well characterized, though are speculated to involve inhibition of prenylation of inflammatory signaling molecules, which is likely required for their effective activation [102]. While CRP is an excellent predictor or CVD risk, it does not appear to be causally involved in the inflammation driving atherosclerosis, as seen in both mouse and human studies (reviewed in Ref. [103]). It is therefore not considered a promising target for therapeutic intervention.

IL-6 levels also correlate with disease risk, though it is not used as a biomarker due to limitations of clinical assay sensitivity and reliability [103]. Blocking cytokine action, such as IL-6 or TNF, is an effective treatment method in inflammatory diseases such as arthritis, but whether this is an effective approach

in CVD has not yet been directly tested. However, the MEASURE study, a clinical study assessing vascular risk in rheumatoid arthritis patients treated with the IL-6 inhibitor tocilizumab, showed that IL-6 inhibition may adversely affect lipid profiles in CVD, as significant increases in total cholesterol, LDL-C, and triglyceride levels in tocilizumab-treated patients compared to placebo were observed, though the ApoB/ApoA1 ratio remained constant [104]. Anti-TNF therapy (such as with the TNF blocker Etanercept) has little or no benefit on cardiovascular outcomes in patients treated for rheumatic or psoriatic arthritis [105]. The ENTRACTE clinical trial, which is currently underway, will compare the effect of targeting TNF (e.g., with etanercept) to targeting IL-6 (e.g., with tocilizumab) on cardiovascular outcomes in patients with rheumatoid arthritis (ClinicalTrials.gov Identifier: NCT01331837). The clear drawback of the ENTRACTE study is that there is no placebo group, as all patients enrolled require anti-inflammatory therapy [97]. However, considering the potential adverse effects on lipid profiles, as well as the inherent increased risk of infection when blocking TNF or IL-6, the drugs targeting these cytokines have not yet been tested in large-scale clinical trials specifically investigating CVD.

Two large-scale clinical trials are however currently underway that are directly testing whether targeting inflammation in atherosclerosis can reduce adverse cardiovascular events. The first trial, the Cardiovascular Inflammation Reduction Trial (CIRT), has enrolled 7000 patients and will assess the efficacy of low-dose methotrexate on reducing CVD events [106]. Low-dose (15–20 mg/week) methotrexate is currently used as an effective treatment in rheumatic and psoriatic arthritis, and observational evidence suggests that there are reduced CVD events in methotrexate-treated arthritis patients [106]. Low-dose methotrexate is proposed to function at a cellular level by stimulating extracellular adenosine release [107]. This results in increased activation of the adenosine receptors, which can have direct anti-inflammatory effects [108], and importantly for atherosclerosis may enhance RCT by increasing the expression of ABCA1 and 27-hydroxylase in macrophages [109]. The results of the CIRT trial are expected in 2018 (Figure 49.1).

The second major clinical trial is the Canakinumab Anti-inflammatory Thrombus Outcomes Study (CANTOS), which was launched in 2011. Canakinumab is an anti-IL-1β antibody, which similar to its counterparts Anakinra (an IL-1R antagonist) and Rilonocept (an IL-1 trap) is currently used clinically to block excessive IL-1 signaling in the cryopyrin-associated auto-inflammatory syndromes (CAPS). Promisingly, Canakinumab reduced circulating IL-6 and hsCRP levels in patients with rheumatoid arthritis, type II diabetes, or CAPS without affecting lipid levels [110]. Further justification for targeting IL-1β with Canakinumab in a chronic disease setting is that it does not appear to reactivate tuberculosis or HIV infection unlike other blockers of cytokine signaling, possibly because IL-1α signaling remains intact [110]. The CANTOS study will particularly assess whether targeting IL-1β signaling in patients with elevated hsCRP levels will provide additional benefit to statin-based treatments in terms of CVD outcomes [110]. This study has enrolled over 10 000 patients worldwide and the results are expected in 2017.

An alternative approach to lowering LDL-C or targeting inflammatory mediators directly is to raise functional HDL levels. An inverse correlation

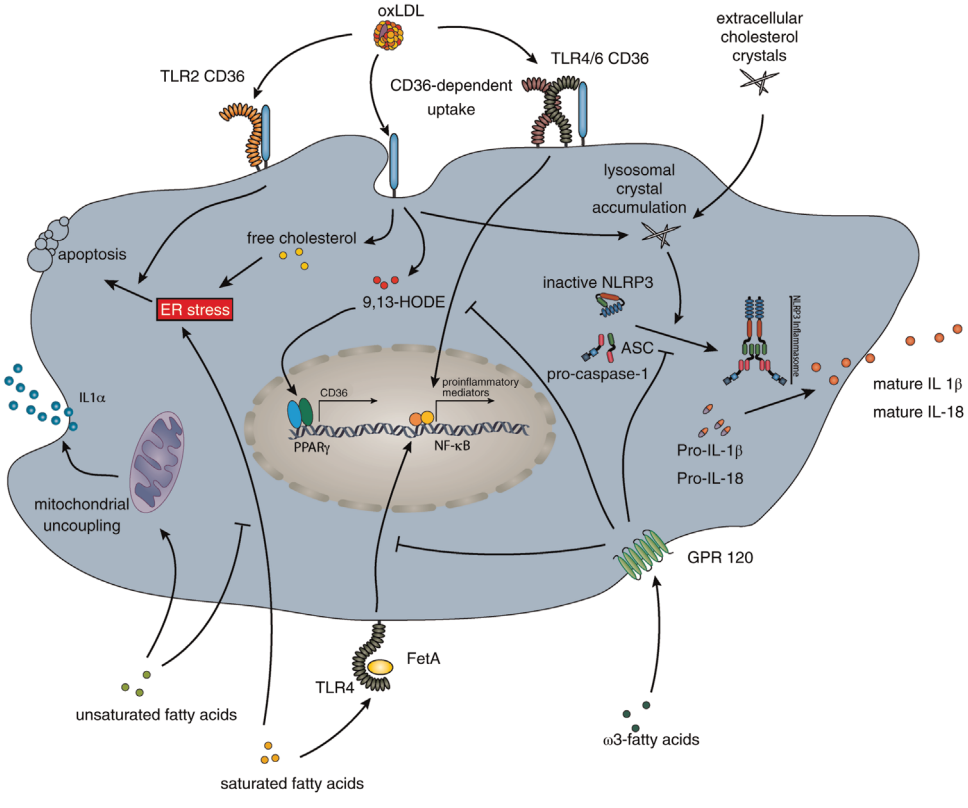


Figure 49.1 Macrophage inflammatory pathways in atherosclerosis. OxLDL engages multiple pathways. When recognized by a complex of TLR4 and TLR6, oxLDL triggers transcription of proinflammatory mediators [18]. oxLDL and/or oxidized phospholipids can trigger apoptosis in macrophages undergoing ER stress through TLR2 and CD36 [25]. CD36 also mediates macrophage oxLDL uptake [10] and subsequent particle breakdown and free cholesterol release, which can itself induce ER stress [11]. The linoleic acid-derived oxidation products, 9- and 13-HODE associated with oxLDL also activate PPARγ to sustain CD36 expression [12]. Cholesterol crystals are either taken up from the extracellular space via phagocytosis or precipitate from oxLDL-derived cholesterol in the lysosomal compartment [31]. Crystalline cholesterol activates the NLRP3 inflammasome by damaging the lysosome, which results in inflammasome formation and caspase-1-dependent maturation of IL-1β and IL-18 [31,32,33]. Saturated fatty acids alone induce ER stress but can also activate NFκB via TLR4 when complexed with fetuin A [44,82]. Unsaturated fatty acids protect from saturated fatty acid-induced ER stress [44], but cause mitochondrial uncoupling triggering IL-1α secretion [43]. Omega-3 fatty acids exert anti-inflammatory effects via GPR120 activation. GPR120 downstream signaling inhibits TLR-signaling [86] and interferes with NLRP3 inflammasome assembly [87]. oxLDL: oxidized low-density lipoprotein; TLR: toll-like receptor; CD: cluster of differentiation; HODE: hydroxyoctadecadienoic acid; PPAR: peroxisome proliferator-activated receptor; NLRP3: nucleotide-binding domain leucine-rich repeat containing (NLR) family, pyrin domain containing; ASC: apoptosis-associated speck-like protein; NFκB: nuclear factor "kappa-light-chain-enhancer" of activated B cells; GPR: G protein-coupled receptor; IL: interleukin; ER: endoplasmatic reticulum.

between HDL- associated cholesterol (HDL-C) levels and CVD risk was first reported over 50 years ago, and has been consistently reported since then in large-scale epidemiological studies such as the Norway Tromso study and the Framingham heart study [111,112]. However, to date, clinical trials

investigating the effect of raising HDL-C alone have shown no benefits in decreasing CVD risk, and indeed one trial showed significant harm [112]. Limitations to these clinical trials notwithstanding, these studies suggest that merely raising HDL-C levels does not lower CVD risk, and that HDL functionality may be a more pertinent factor in CVD protection. Accordingly, the HDL "flux" hypothesis, where HDL functionality is a better predictor of its protective effect in CVD than the amount of cholesterol associated with HDL, that is, HDL quantity, has also gained popularity [112]. A key study supporting this hypothesis showed in an *ex vivo* assay (measuring cholesterol efflux from lipid-laden macrophages to HDL isolated from patients) that HDL efflux capacity is indeed a better predictor of CVD risk than HDL-C levels alone [113]. This confirmed earlier studies in mice, where the rate of cholesterol efflux to HDL correlated with disease severity across a variety of genetic models and pharmacological interventions [5,112]. Besides, HDL is a complex lipoprotein with a myriad of functions beyond mediating RCT, including antioxidative, antithrombotic and anti-inflammatory functions [114–116]. Indeed, HDL can block cytokine, including IL-6, release from macrophages by modulating the activity of the transcriptional repressor activating transcription factor 3 (ATF3), which may contribute to its atheroprotective, anti-inflammatory effects [117]. Infusion of reconstituted HDL (i.e., purified human ApoA1 reconstituted *in vitro* with phosphatidylcholine) is currently being tested for efficacy in CVD: While one formulation (CSL-111) was discontinued due to liver toxicity, a second formulation (CSL-112) is currently in Phase IIa clinical trials (ClinicalTrials.gov Identifier: NCT02108262). Other formulations that are in development include ETC- 216, which is based on $ApoA1^{Milano}$, a naturally occurring antiatherogenic ApoA1 mutant, and CER-001, which contains sphingomyelin in its phospholipid shell [118]. The biology of HDL is however complex, and much remains to be discovered about HDL function and regulation before its full benefits can be clinically exploited.

49.7 Conclusion

Atherosclerosis can cause devastating pathologies leading to decreased lifespan. Evidence from population studies, intervention trials, and basic research has uncovered many different mechanisms that contribute to atherogenesis, yet two essential drivers have emerged: high LDL cholesterol levels and increased inflammation. Pharmacological interference to modulate cholesterol metabolism has proven to be a highly successful CVD therapy and now large studies testing anti-inflammatory therapeutic strategies are underway. Should the latter studies show therapeutic efficacy, further approaches targeting the individual innate receptors involved in the inflammatory response in the plaque could be developed. Finally, in recent years, numerous molecular insights have been gained that could explain how dietary factors can influence atherosclerosis in a positive or negative way. Life style modifications remain one of the most powerful strategies for preventing or halting atherosclerosis and therefore such approaches should be advertised in the general public.

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Acute Kidney Injury

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50.1 Introduction

The systemic inflammatory response to infection, trauma, and surgery is a major trigger of acute kidney injury (AKI) [1]. Such inflammation-associated AKI is typically seen as part of injury or dysfunction in multiple organs and is an independent contributor to excess mortality in critically ill patients [2]. In addition, long-term consequences of AKI include progression to chronic kidney disease [3] and supporting the notion of a sustained inflammatory response beyond the acute phase. Unfortunately, as no effective AKI treatment or prevention exists, supportive renal replacement therapy remains the only option when severe AKI leads to pronounced accumulation of circulating waste products and/or when inadequate diuresis leads to fluid overload and tissue edema.

AKI is defined as a rapid (within days) increase in serum creatinine and/or sustained oliguria [4]. Several mechanisms triggering the activation of kidney endothelial, glomerular, and tubular cells during the systemic inflammatory response syndrome (SIRS) have recently been identified. Since such mechanisms appear to precede the decline in kidney function, they are potential targets for future prevention and treatment of AKI. Additionally, unfolding the complex interactions between systemic and renal circulatory changes during SIRS will likely contribute to improved hemodynamic management of patients with or at risk of AKI.

This chapter gives a brief overview of the normal kidney anatomy and physiology. In addition, the consequences of systemic inflammation on kidney vascular, glomerular, and tubular structure and functions will be discussed in detail. Sepsis, one of the most severe triggers of SIRS and at the same time one of the most common AKI triggers, will act as a model for inflammation in this chapter.

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50.2 Normal Kidney Anatomy and Physiology

Under normal conditions, the kidneys receive approximately 1 l or 20% of cardiac output every minute. Through the afferent arteriole, renal blood flow (RBF) reaches the glomerulus where, in healthy kidneys, approximately 125 ml/min of plasma-water is filtered. The remaining blood volume leaves the glomerulus via the efferent arteriole to enter the peritubular capillaries in the renal cortex. In addition, approximately 10% of the RBF circulates through the vasa recta in the renal medulla. The unique design of the vasa recta allows circulating osmolytes (sodium chloride and urea) and water to equilibrate with the kidney interstitium and the tubular lumen – referred to as the countercurrent mechanism – creating the corticomedullary osmotic gradient necessary for water and electrolyte homeostasis in humans. In addition to osmolytes and water, oxygen also equilibrates between the descending and ascending vasa recta. Together with high oxygen consumption from ATPase-dependent sodium pumps in that part of the nephron, a hypoxic environment (pO_2 ~1.5 kPa) is often maintained in the inner medulla. Finally, after reabsorption of most of the filtered plasma-water, blood flows from cortex and medulla to the renal vein and the inferior vena cava.

The glomerular filtration rate (GFR) of plasma-water and molecules is dependent on RBF, on the net filtration pressure (NFP) across the filtration barrier, and on the specific features of the filtration barrier itself [5].

NFP is created by the hydraulic pressure in glomerular capillaries (P_{GC}) and is opposed by plasma oncotic pressure (π_{GC}), mainly determined on the plasma albumin concentration, and by the hydraulic pressure in Bowman's space (P_B) according to the following relationship:

$$\text{NFP} = P_{GC} - \pi_{GC} - P_B.$$

As water is filtered (and albumin retained), the plasma albumin concentration and hence the π_{GC} will increase along the glomerular capillary until it equals P_{GC} and, consequently, NFP ceases (Figure 50.1). Thus, higher RBF (and thereby higher plasma-water flow) leads to lower relative concentration of plasma proteins, delayed increase in π_{GC}, higher mean NFP, and greater GFR.

The relative resistance in the afferent and efferent arteriole is a major determinant of NFP and hence GFR; vasoconstriction of the afferent arteriole and/or vasodilation of the efferent arteriole decreases GFR, whereas afferent vasodilation and/or efferent vasoconstriction has the opposite effect. Renal autoregulation maintains RBF and GFR during fluctuations within a wide range of mean arterial blood pressures. Such autoregulation is achieved via two important mechanisms. When increased blood pressure stretches the afferent arteriolar wall it contracts, thereby lowering RBF and blunting NFP and GFR – the myogenic reflex. Additionally, higher blood pressure will increase the filtered NaCl load, which activates the "NaCl-detecting" macula densa cells in the distal nephron. In response to macula densa activation, the afferent arteriole contracts, which lowers P_{GC}, NFP, and GFR, thereby attenuating NaCl filtration.

The glomerular filtration barrier consists of three major components: the fenestrated glycocalyx-rich endothelium, the basement membrane, and the slit diaphragm between podocytes (Figure 50.2). The filtration barrier is size and

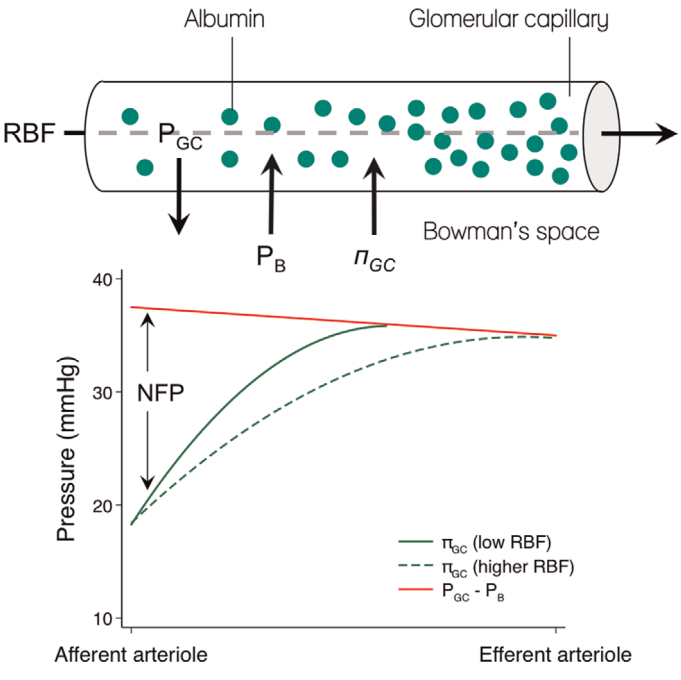


Figure 50.1 Hydraulic (red line) and oncotic pressure (green lines) changes along a glomerular capillary during lower (solid green line) and higher (dashed green line) renal blood flow (RBF).

charge selective [6]. Molecules up to 8 nm (80 Å) can theoretically pass the fenestrated endothelium. However, since the glycocalyx is negatively charged, filtration of negatively charged middle-sized molecules (7–60 kDa) is restricted. In fact, loss of negative charge of the filtration barrier is an important feature of

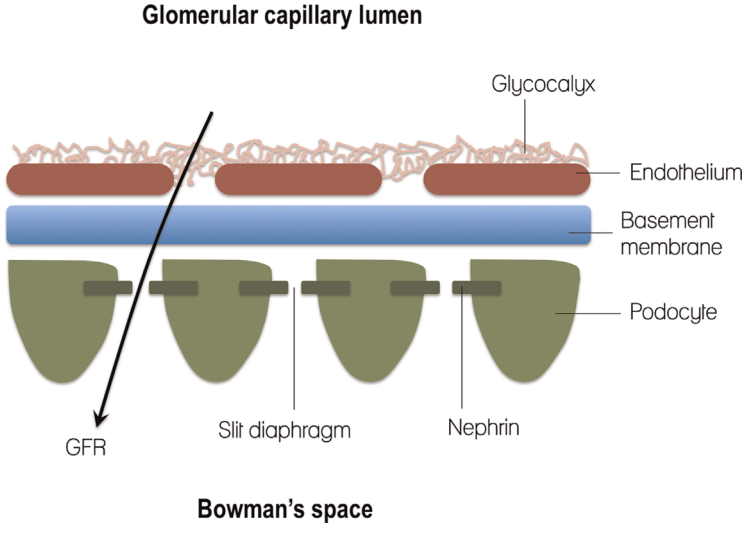


Figure 50.2 Glomerular filtration barrier.

some glomerular diseases and may partly explain the proteinuria that accompanies such conditions.

50.3 The Renal Response to Systemic Inflammation

Systemic inflammation causes stress or injury to kidney endothelial, tubular, and glomerular cells. The kidney tubular cells are particularly susceptible to circulating inflammatory mediators since they are continuously exposed to a filtrate derived from a large proportion of the circulating blood volume. Moreover, the majority of such mediators are amenable to filtration and can therefore injure cells facing the tubular lumen. In fact, several inflammatory mediators are being actively reabsorbed by the proximal tubule. An important clinical observation is that despite a sometimes complete shutdown of kidney function during severe inflammatory states such as sepsis, microscopic evidence of kidney parenchymal injury is scarce [7,8]. Recently, however, several cellular responses to inflammatory stimuli have been identified, which appear to have profound effects on kidney function (Figure 50.3).

50.3.1 Endothelial Activation

The vascular endothelial cells are part of the first line of defense during inflammation. However, systemic inflammation may activate endothelial cells distant from the primary injury, amplifying the systemic inflammatory response, and causing multiorgan injury and dysfunction. Endothelial activation is mediated by the recognition and binding of damage-associated molecular patterns (DAMPs) by so-called pattern recognition receptors (PRR), for example, toll-like

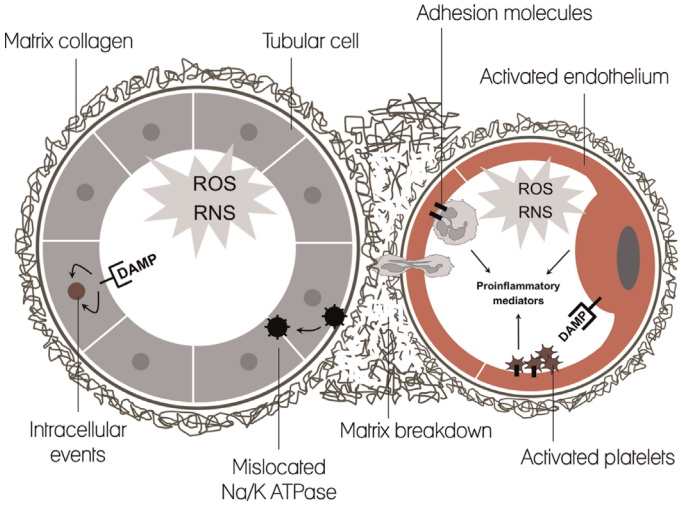


Figure 50.3 Inflammatory effects on kidney tubules and peritubular capillaries.

receptors, on the endothelial surface [9]. Such endothelial activation triggers the expression of adhesion molecules (e.g., P-selectin), which encourage recruitment, binding, and activation of platelets and neutrophils on the cell surface [10]. Upon their activation, platelets and neutrophils release hundreds of biologically active molecules, including cytokines, chemokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), and coagulation factors. Consequently, a vicious circle is created, maintaining or even aggravating endothelial damage and surrounding tissue injury [11].

In their aggregate, these alterations contribute to degradation of endothelial glycocalyx and intercellular tight junctions, ultimately increasing vascular leak of water, proteins, enzymes, and inflammatory mediators and migration of activated immune cells into the interstitial compartment [12]. Interstitial accumulation of protein degrading enzymes (proteinases) causes matrix breakdown, further impairing vascular stability [13]. Finally, outburst of platelet granular contents shift the balance toward a procoagulant state with increased risk of microthrombosis in renal capillaries [14].

50.3.2 Glomerular Filtration Barrier Changes

Glomerular capillaries are similarly activated during inflammation. Additional important alterations in the structure and function of other components of the glomerular filtration barrier are seen. Glycocalyx breakdown leads to loss of anionic charges, whereby the ability to reflect negatively charged molecules such as albumin is lost and albuminuria occurs [15]. In addition, loss of glycocalyx contributes to loss of vascular tone, microthrombosis (less adhesion to antithrombin III), and accelerated inflammation (increased adhesion of leucocytes). The important role of glycocalyx in inflammation was supported by Nelson *et al.* who found an association between circulating glycocalyx contents and mortality in patients with septic shock [16].

Increased glomerular capillary leakiness exposes podocytes to circulating cytokines. Such cytokine exposure suppresses nephrin expression by podocytes in *in vitro* studies. This, in turn, causes disruption of the slit diaphragm and further increased permeability to albumin over the glomerular barrier [17,18]. Indeed, proteinuria is commonly seen in critically ill patients with sepsis, even without obvious features of AKI such as elevated serum creatinine or oliguria [19].

Mesangial cells are important for the structure and function of the glomerular filtration barrier [20]. They produce several components of the extracellular matrix (e.g., collagens) and glycocalyx (e.g., proteoglycans), which provide support for glomerular capillaries. In addition, several growth factors important for cell proliferation are secreted by the mesangium. Moreover, they are actively involved in the removal of apoptotic cells by phagocytosis. Finally, mesangial cells share features with smooth muscle cells and contracts upon stimulation, thereby reducing the glomerular filtration area and GFR. Dysregulation of the mesangium appear to be involved in the progress of several chronic kidney diseases. Whether contraction of these cells contributes to reduced GFR in AKI is, however, yet to be determined.

50.3.3 Tubular Injury

During severe systemic inflammation, tubular cells are exposed to filtered (from the tubular lumen) and circulating (via capillary leakage and migration of immune cells) cytokines, DAMPs, ROS, and RNS (Figure 50.3). In addition to the degree and duration of such inflammatory stress, adaptive cellular mechanisms will determine the fate of injured tubular cells. Such adaptive responses appear to effectively reduce energy-consuming cellular activities until inflammation has subsided.

Triggered by the interaction of DAMPs with PRR on the kidney tubular cell surface, cellular turnover increases via complex intracellular signaling pathways [21] (Figure 50.3). However, episodes of cell cycle arrest have also been observed during AKI in animal models of ischemia–reperfusion injury [22] and sepsis [23]. In addition, urinary excretion of proteins involved in cell cycle regulation has been observed in critically ill patients with AKI [24], which supports the role of cell cycle disruptions in human AKI as well. The purpose of cell cycle arrest is uncertain. Yet, tubular cells are the major source for replacing injured cells by accelerated mitosis [25]. Therefore, the prevailing idea that cell cycle interruptions prevent injured cells from proliferating and dedifferentiating into dysfunctional cells – which would most certainly lead to permanent damage – appears logical.

In addition to cell cycle arrest, other mechanisms contribute to reduce renal oxygen consumption during inflammatory stress. Sodium chloride reabsorption via Na/K ATPase located at the outer cell surface (facing peritubular capillaries) is the major energy-consuming process in the kidney. During tubular stress, however, the sodium chloride pumps are relocated to the intercellular border or to the surface facing the tubular lumen [26]. Relocation inactivates these pumps and thereby effectively reduces energy consumption. Consequently, more sodium chloride is delivered to and detected by the "salt-detecting" macula densa cells, the tubuloglomerular feedback mechanism is activated, and ultimately GFR is reduced. Moreover, this reduces the filtered sodium chloride load, which further lowers oxygen demand.

Filtered proteins are mainly reabsorbed via megalin receptor-mediated endocytosis in the proximal tubule. Additionally, the megalin receptor mediates reabsorption of filtered toxins and cytokines and may therefore contribute to AKI [27]. However, downregulation of the megalin receptor has been observed during experimental endotoxemia and inhibition of the receptor appears to attenuate AKI. Collectively, these findings support the notion that megalin receptor downregulation is yet another mechanism by which kidney tubular cells protect themselves against further injury. In addition to glomerular filtration barrier changes (described in the previous section), megalin receptor downregulation likely contributes to proteinuria during systemic inflammation and AKI. In fact, accumulation of urinary biomarker proteins known to be reabsorbed by the megalin receptor, such as cystatin C and neutrophil gelatinase-associated lipocalin (NGAL), is typically seen in patients with SIRS and AKI [28].

Regulated cell death is associated with organ damage in many inflammatory diseases, including AKI. Experimentally, common pathways regulating both

inflammation and apoptosis have been identified. For example, upregulation of small intracellular noncoding RNA molecules, so-called miRNAs, was recently demonstrated in mice with septic AKI [29,30]. Furthermore, pharmacological stimulation of miRNA-21 production inhibited apoptosis, mitigated the systemic inflammatory response, and attenuated AKI. These findings suggest that miRNA synthesis is another mechanism by which kidney cells protect themselves during extreme inflammatory stress.

Tubular cell apoptosis has been detected in human AKI by terminal deoxynucleotidyl transferase–mediated digoxigenindeoxyuridine nick-end labeling (TUNEL) and activated caspase 3 staining [8]. Cellular stress, resulting in mitochondrial permeability, leakage of apoptogenic factors to the cytoplasm, and caspase 3 activation trigger apoptosis. Additionally, cytokine activation of membrane receptors can activate intracellular apoptotic pathways. Caspase activation is responsible for degradation of intracellular contents and fragmentation of the dying cell into apoptotic bodies, which are cleared by phagocytosis. The exact role of apoptosis in AKI and why it predominantly occurs in the distal nephron is uncertain. Autocrine and paracrine "cross-talk" between apoptotic epithelium and adjacent segments may trigger repair mechanisms after inflammatory insults [31]. Pharmacological modulation of apoptotic mechanisms may therefore be part of future AKI preventive or therapeutic interventions [32].

Recently, the importance of other regulated cell death mechanisms, such as necroptosis, was highlighted [33]. Unlike apoptosis, caspase-dependent phagocytotic clearance of cell debris is not a feature of necroptosis. Instead, necroptosis is caspase independent and involves cell membrane rupture with a massive outburst of cellular contents, including DAMPs into the extracellular space. The likely rationale for necroptosis is to attract immune cells to the site of injury/infection and thereby actively contribute to tissue homeostasis. However, the massive release of DAMPs from necroptotic cells further amplifies the inflammatory response and hence contributes to tissue injury in AKI. Since the discovery of some of its regulating pathways, pharmacological interference with necroptosis to prevent or treat inflammatory injuries such as AKI is currently being considered [33].

50.3.4 Changes in Renal Hemodynamics

The early systemic inflammatory response is characterized by increased cardiac output in the setting of systemic vasodilation and hypotension. In animal models of hyperdynamic septic shock, a proportional increase in RBF is a ubiquitous finding [34,35]. Similarly, in humans with early hyperdynamic sepsis, a relative increase in RBF (RBF/cardiac output) has been demonstrated [36]. Importantly, despite such increases in RBF, marked reductions in GFR are commonly seen. This apparent disconnection between RBF and GFR can physiologically be explained by excessive vasodilation of the efferent arteriole, causing NFP and GFR to drop. Indeed, under such circumstances, increased RBF will not be sufficient to compensate for reduced NFP (see Figure 50.1).

In addition to renal vasodilation, sepsis-induced systemic hypotension reduces the driving pressure needed for GFR. However, worsening kidney function is a

common clinical finding during sepsis despite normalization of MAP with vasopressors. Therefore, additional factors must contribute to sustained AKI in the context of systemic inflammation. Intravenous fluids are commonly used to treat hypotension in patients with hyperdynamic circulation during sepsis, after trauma or after major surgery. While such therapy may transiently stabilize cardiovascular function, it contributes to elevated central and renal venous pressure and kidney interstitial edema around leaky peritubular capillaries.

Venous congestion attenuates the difference between MAP and renal venous pressure, and hence the hydraulic transrenal pressure gradient needed for RBF [37]. Together with fluid accumulation in the kidney interstitium, venous congestion will increase subcapsular kidney pressure and P_B, further counteracting GFR. In fact, even in healthy volunteers with expected normal endothelial barrier, increased overall extravascular fluid volume and renal volume was observed together with reduced renal cortical tissue perfusion after a 2 l crystalloid infusion [38]. In addition, in septic patients with moderate to severe AKI after initial resuscitation with fluids and vasopressors, consistently decreased RBF, quantified by cine phase-contrast magnetic resonance imaging, was observed [39]. Finally, recent studies have confirmed a strong and independent association between elevated CVP and worsening kidney function in septic patients [40,41] and in patients with cardiac diseases [42,43].

Although physiological changes in intrarenal blood flow distribution can affect GFR [5], the importance of microcirculatory changes during AKI is not well understood. Preserved cortical RBF but decreased medullary RBF and tissue oxygenation was observed in sheep with hyperdynamic sepsis and AKI [44]. However, such findings have not been consistently demonstrated in previous studies. Moreover, attempts to enhance RBF with renal vasodilators in cardiac surgery patients did not prevent severe AKI [45]. In contrast, targeting a higher MAP with vasopressors attenuated AKI in septic patients with chronic hypertension [46]. Therefore, while the effects of renal microvascular alterations in AKI patients are being untangled, it appears critical to avoid fluid overload, venous congestion, and major deviations from premorbid blood pressure in such patients.

50.4 Summary

Systemic inflammation triggers adaptive responses in kidney endothelial, glomerular, and tubular cells. The clinical consequences of such responses depend on the degree of inflammatory stress and may vary from mild proteinuria to loss of GFR, need for renal replacement therapy and substantially elevated short- and long-term mortality risks. While novel therapies, targeting inflammatory pathways, are explored, hemodynamic management remain important in the management of patients with inflammation-induced AKI. Avoiding fluid overload and venous congestion appear particularly important since both are independently associated with worsening kidney function. In addition, maintaining systemic blood pressures close to premorbid levels appear to attenuate further deteriorations in kidney function during inflammatory stress.

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Ischemia–Reperfusion Syndrome

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51.1 Introduction

The events of ischemia–reperfusion injury trigger a local and/or a systemic inflammatory response that leads to cellular damage and even organ failure [1,2]. Ischemia induces cell death and inflammation through oxygen deprivation. However, restoration of blood flow (reperfusion), which is necessary for the recovery of cellular function, may first worsen the lesions present in ischemia, aggravating irreversible damage and cellular demise [1,3].

Although there are some similarities in the biological responses among ischemic episodes, there are important differences between a systemic reduction in perfusion (e.g., successfully resuscitated cardiac arrest) compared with regional ischemia and reperfusion of single organ (e.g., myocardial infarction, stroke, or organ transplantation) [3].

51.2 Pathophysiology

51.2.1 Ischemia

Prolonged ischemia results in a variety of cellular metabolic and ultrastructural changes such as altered membrane potential, cellular swelling, leakage of lysosome enzymes, cytoskeleton disorganization, altered ion distribution, and finally intracellular acidosis [2,4]. Initially, ischemia induces a large decrease in cellular oxidative phosphorylation due to a switch from aerobic to anaerobic metabolism, which results in a failure to resynthesize energy-rich phosphate such as adenosine 5′-triphosphate (ATP) and phosphocreatine. This leads to a dysfunction in the ATP-dependent ionic pumps, subsequently resulting in the entry of calcium, sodium, and water into the cells. Ischemia also promotes expression of some

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proinflammatory genes, which increases the activation of proinflammatory state [2]. As a result, during the hypoxia phase, only exiguous amounts of radical oxygen species are produced because of the redox reduction of cytochromes, nitric oxide (NO) synthases, xanthine oxidase, and NADPH oxidase activations [5,6].

51.2.2 Reperfusion

Paradoxically, reperfusion, either spontaneously or through a clinical intervention, does not restore initial homeostasis, but rather further enhances damage after normalization of oxygen levels and extracellular pH. As a consequence, this results in a normalization of the above-mentioned enzymes, and induces a large production of reactive oxygen species (ROS), which subsequently contributes to increase membrane and cytoskeleton structures. ROS also contribute in increasing mitochondrial calcium content, which causes opening of the mitochondrial transition pore (mPTP). Opening of mPTP represents one of the pathways that leads to cell death [7].

Hypoxia occurs in the boundary zone around the ischemic area after a reperfusion, and induces local and systemic inflammation response mainly through the hypoxia-inducible factor (HIF) [8], which is specifically dealt with in another chapter.

51.2.3 Ischemia–Reperfusion Pathophysiology

Although ischemia–reperfusion shares many similarities with infection responses, it usually occurs in a sterile environment except in the case of bacterial translocation after intestinal injury [9]. Various biological processes have been fully studied and many interact with each other (e.g., infection or noninfection).

51.2.4 Cell Death

Two main types of cell death have been described as apoptosis and necrosis. Autophagy has been more recently proposed as a process of cell death during which cells generate energy and metabolites by digesting their own organelles and macromolecules. Similar to apoptosis, autophagy is genetically controlled (e.g., programmed) and thus considered as a less immune-stimulating process as opposed to necrosis, which is considered to be a more brutal method of cell death. However, this dichotomic approach may be too simplistic and needs further investigation [7]. For instance, in some circumstances, necrosis could also be initiated by programmed control mechanisms [10].

51.2.5 Microvascular Dysfunction

Microvascular dysfunction is associated with endothelial cell dysfunction and increased vascular permeability, which might explain, at least in part, of tissue edema occurrences (e.g., lung edema). Abnormalities in vascular tone have been described to occur due to the imbalance of vasoconstricting and vasodilating factors that explain an intense vasoconstriction during the reperfusion phase such as

increased thromboxan A_2, prostaglandin H_2, leukotrienes, and adenosine production and decreased prostacyclin or nitric oxide production [11]. In addition, crawling neutrophils as well as increased adherence of circulating erythrocytes and platelets play a role in mechanical obstruction of microvascularization [11–14]. As a result, reperfusion can result in a "no-reflow phenomenon," which has been most commonly described in instances of myocardial infarction. This phenomenon is characterized by an increase in the impedance of microvascular blood flow and a decrease in intramyocardial blood flow, despite a reperfusion of the culprit coronary artery [15,16]. Similarly, ischemia was shown to induce a sustained contraction of pericytes on microvessels despite a successful reopening of the middle cerebral artery in a mouse model of stroke [17].

51.2.6 Innate Immune System

During ischemia and reperfusion, both innate and adaptive immune systems are activated and they interplay between each other. There is an imbalance among vasodilating and vasoconstricting factors in addition to activation of coagulation and complement systems.

Polymorphonuclear neutrophils are among the first cells that appear at the site of inflammation as both adherents and diapedes [18–20]. They are activated by inflammation and, in turn, release a variety of factors that further increase the inflammatory processes. In recent times, molecular targets (rather than cellular), such as complement receptors, Toll-like receptorss (TLRs), and proinflammatory cytokines, have been particularly emphasized for their role in this process [19–22]. Neutrophils seem to play a key role, as they are the first to be activated. Neutrophils aid in blocking capillaries to prevent reperfusion of the tissue and in secreting proinflammatory cytokines and chemokines to create a positive feedback loop with activation and recruitment of more neutrophils [19]. The role of natural killer (NK) cells and macrophages/monocytes relating to ischemia–reperfusion are unclear as studies have shown conflicting results [18].

Innate system activation can be summarized as follows: First, the system is activated through the recognition receptors, intra- and extracellular TLRs, the intracellular NOD-like receptors, and retinoic acid-inducible gene 1 receptors. TLRs play a major role in recognizing damage- or pathogen-associated molecular proteins (DAMPs and PAMPs) and work to activate various kinases (IRAK, TBK1, and IKK) that recruit cytoplasm adapter molecules. TLRs then amplify and transmit the signal to various transcriptional factors (NF-κB, MAP3, and the interferon IFR3) that encode the genes involved in the inflammation process [3,18,20].

Ischemia–reperfusion triggers excessive platelet aggregation and the release of platelet-derived mediators partly through integrin-mediated endothelial interactions [12]. They can also aggregate on leukocytes' surface through an integrin-dependent platelet activation and aggregation, and then migrate across the epithelial barriers to the sites of inflammation to further aggravate tissue damage [23]. Platelets can also release inorganic polyphosphate, polymers of 60–100 phosphate residues, that can activate factor VII, thereby acting as

proinflammatory and procoagulant factors [24]. However, inflammation observed after an ischemia and reperfusion can also trigger natural anticoagulation such as antithrombin, tissue factor inhibitors, and protein C [25–28].

The complement system plays a key role, and is the very first line of defense against pathogens and inflammatory reaction after an ischemia–reperfusion. It acts as an immune surveillance system that can discriminate cellular debris, apoptotic cells, and foreign intruders to vary its response accordingly [29]. DAMPs can also activate all three of the complement pathways (classic, alternative, and lectin) to bind either C1q, or C3 or mannose–lectin [30]. C3, C5 convertase, and the membrane attack complex (MAC) are well-known landmarks of the complement attack complex, which is a well-known landmark of the complement cascade. The activation of these proteins by the three overlapping pathways leads to cell opsonization and recruitment of many different inflammatory cells, such as neutrophils and B cells, which eventually result in phagocytosis and cell death [31,32]. Recent data have shown that some products of the coagulation pathway are able to activate the complement cascade. Serine proteases from the coagulation pathway can target C3 and C5 complement factors and produce fully active C3a and C5a complement factors that are known to play a major role in the inflammatory response [33].

51.2.6.1 Adaptive Immune Activation

Adaptive immune response involves T lymphocytes, along with other T-cell types. Pathways involved are not fully understood, but recent data suggest that there is a large contribution of both antigen-specific and antigen-independent mechanisms of activation [34,35]. Studies with mice deficient in specific populations suggest that $CD4^+$ and $CD8^+$ T cells play a detrimental role in ischemia–reperfusion tissue injuries [36–38], whereas T_{reg} cells seem to have a protective role [39,40]. Interaction between T and B cells through specific receptors generates an alloimmune response.

Autoimmunity is usually associated with the adaptive immune system. However, there are now evidences of innate recognition proteins that can be self-reactive and can initiate inflammation after ischemia–reperfusion as observed in response to pathogen; this phenomenon is also described as “innate autoimmunity” [41]. This process is related to the presence of autoantibodies produced by B cells in the absence of deliberate immunization, mainly IgM and more scarcely IgG [42]. However, the mere presence of these autoantibodies does not lead directly to tissue damage. Ischemia leads to cells injury and subsequent expression of new surface antigens, which represent a self-target of readily circulating autoantibodies [42–44]. The complex then activates a complement cascade, generating a positive feedback loop in the inflammation process.

These effects are not only observed within the same tissue area but can also affect remote tissue organ either in the same organ or even in a remotely different organ, such as kidneys and lungs, and are likely mediated through the release of circulating mediators [45,46].

Remote ischemic preconditioning has added more interesting insight as the protective effect was able to be observed in remote tissues and was initially

described as "reconditioning at distance" in an *in vivo* canine heart model of an ischemia–reperfusion [47]. The application of four 5 min occlusion–reflow cycles to the circumflex coronary artery before 60 min transient occlusion of left descending coronary artery reduced the infarct area by 60%, suggesting that protective agents are able to transfer from one cardiac site to another one. This concept was subsequently extended in the protection mechanisms that were induced by cycles of ischemia–reperfusion in remote organs such as kidney, liver, and brain [45]. The underlying mechanisms remain elusive and are likely related to release transferable mediators from the reperfused tissue and neuronal reflexes. For instance, after lower limb ischemia–reperfusion, remote effects on oxidative capacities have been variably observed in liver, lung, or kidney tissues [46].

51.3 Clinical Diseases

Most common clinical setting outcomes were particularly studied after instances of myocardial infraction, a neurologic stroke that can be induced spontaneously or through a thrombolysis agent [15,48–51]. However, this phenomenon has been largely in organ transplantation, particularly in kidney [4,11] and liver transplantation, to avoid any further damage when the organ is harvested after a necessary ischemia-reperfusion from cadaveric or living donors (kidney). The consequences of depriving an organ of its blood supply have also long been recognized as a critical factor in systemic hypoperfusion, as observed in hemorrhagic shock [52,53], aortic cross-clamping [54], cardiopulmonary bypass, or successful resuscitation of cardiac arrest [55,56].

Interestingly, even local ischemia–reperfusion phenomenon may induce important systemic inflammatory responses that could extend the damage beyond the ischemic area to include injury to remote nonischemic organs [3,57]. This has been observed, for instance, as remote effects of lower limb ischemia–reperfusion [46] after organ transplantation or after cardiac arrest that may have induced multiple organ failure, acute kidney injury, or intestinal injury [3,57]. In a particular model of systemic ischemia–reperfusion that is represented by a successful resuscitated cardiac arrest, severe hyperoxia was independently associated with decreased survival rates to hospital discharge, suggesting that hyperoxia may further increase systemic and brain tissue damage [58]. Nevertheless, exposure to high concentrations of inspired oxygen has shown not to worsen lung injury after cardiac arrest [59]. Moreover, a systematic review, meta-analysis, and metaregression of cohort studies have shown that an association between arterial hyperoxia and poor outcome is present in a subset of critically ill patients [60]. The same team [61] has suggested that oxygen retains its life-saving importance in critical care, accumulating evidence that further illustrated the role of hyperoxia and consequent formation of reactive oxygen species in the pathogenesis of several life-threatening diseases. Actually in sepsis and multiple organ failure, refractory shock can lead to a decrease in blood perfusion and supply of oxygen to cells, combined with arterial and uniform vasoconstriction; edema can explain in part of the resulting mitochondrial dysfunction. Furthermore, under conditions

of stress, cells can adapt to maintain viability through a decrease in the consumption of O_2 energy use, ATP demand, and basal cellular functions, as well as an increase in the consumption of energy reserves (e.g., cellular hibernation) and the activation of the damage repair mechanism. Restoration of oxygen delivery through therapeutic intervention (fluid resuscitation, for instance), spontaneous improvement, or, more commonly, both may lead to a better oxygen delivery that can at least temporarily aggravate tissue damage [62,63]. This situation strongly mimics what is observed in clinical situations more classically admitted for ischemia–reperfusion [3].

51.4 Therapeutic Perspectives

Ischemia–reperfusion is very often involved in various clinical diseases, as previously described, which can lead to numerous research teams who investigate how to limit the phenomenon by studying how to restore oxygenation either spontaneously or mechanically (angioplasty transluminal, for instance) or by using thrombolytic agents without inducing further damage related to the ischemia itself.

51.4.1 Ischemic Conditioning (Pre-, Post-, and Remote Conditioning)

The ischemic preconditioning might be effective in reducing kidney injury according to a recent systematic review on kidney animal models [64], although without wide translating therapies into clinical renal transplantation as of now. Ischemic preconditioning of a transplant graft before a liver transplantation or before a major liver resection has shown some benefit but without the expected scope from various animal studies [47,65]. Although these studies have shown to have some benefit, they have not been able to reproduce the profound tissue-protective effects of ischemic preconditioning observed in these animal studies, perhaps because of the difficulty in determining the most effective preconditioning protocol for a clinical study [3].

Similar to ischemic preconditioning, short episodes of ischemia following a reperfusion are associated with a reduction in myocardial infarct size, which can be described as postconditioning [16]. A prospective, randomized, controlled, multicenter study investigated whether postconditioning in 30 patients protected the human heart from a coronary angioplasty after an instance of myocardial infarction had any potential beneficial effects [66]. After a reperfusion into the occluded coronary artery using a stent, a protocol of 1 min inflation interspersed with 1 min deflation of the angioplasty balloon was used to induce postconditioning. The study then observed a persistent infarct size reduction and improvements in long-term (1 year) functional recovery in patients with acute myocardial infarction [66]. However, a more recent and a larger study did not observe any benefits in 1 year clinical outcomes such as a composite of death, myocardial infarction, severe heart failure, or stent thrombosis in patients with ST-segment elevation myocardial infarction who were undergoing primary percutaneous coronary intervention (PCI) [67].

As previously mentioned, ischemic preconditioning was suggested as a potentially effective protection on other remote tissues. The discovery of ischemia–reperfusion cycles in skeletal muscle, elicited by application of simple, noninvasive tourniquet, seemed to provide a similar ischemic preconditioning cardioprotection as well as protection of other vital organs and potentially for the whole body. This discovery fostered a rapid clinical evaluation of this concept that showed some promising results in smaller, underpowered, single center studies on the effects of remote ischemic preconditioning being administered to patients undergoing cardiac surgery [45]. However, two very recent large-scale, prospective, randomized, sham-controlled trials studying remote ischemic preconditioning in cardiac surgeries that required cardiopulmonary bypasses were negative [68,69]. A commonly used study regimen of four 5 min cycles of ischemia in the arm interspersed with 5 min cycles of reperfusion was used in both studies. These negative results may be partially explained through various factors that interfered with remote ischemic preconditioning such as less effective mechanisms in infarct-remodeled hearts, diabetes and, in older patients, redundant use of cardioprotective bypass technics, and the use of medications, specifically anesthetics, which may interfere with remote ischemic preconditioning [45].

Remote ischemic postconditioning induced by repeated cycles of limb ischemia (four cycles of 5 min inflation and 5 min deflation) before hospital admission was found to be effective as an adjunct of angioplasty in myocardial salvage of 333 patients with acute myocardial infarction [70]. The beneficial effect seemed to persist in the long-term clinical outcomes (median follow-up: 3.8 years) of the same population study [71].

51.4.2 Therapeutic Gases

Several therapeutic gases have been in use for the treatment of ischemia–reperfusion, among which are hydrogen, nitric oxide, hydrogen sulfide, and carbon monoxide. Of these gases, the most extensively studied is nitric oxide because it is endogenously produced by endothelial NO synthase, and also plays a role in regulating basal vascular tone, endothelial function, and maintenance of blood oxygenation through hypoxic pulmonary vasoconstriction. Multiple studies have indicated that the endogenous production of NO or its therapeutic delivery had effect in attenuating the damages of ischemia–reperfusion [72]. In a small, randomized, and placebo-controlled clinical trial ($N = 10$/arms) that used inhaled NO for the treatment of graft ischemia–reperfusion after a liver transplantation, NO gas was found to improve the restoration of liver function and to lower instances of hepatocyte apoptosis [73]. However, in contrast, inhaled NO was shown to be relatively inefficient in acute cell crisis time resolution. Inhaled NO and H_2S have both been shown to be helpful in rodent model of ischemia–reperfusion, such as in resuscitation of cardiac arrest [74,75].

51.4.3 Antioxidant Therapy

The enzyme superoxide dismutase (SOD) scavenges for superoxide anions on free radicals produced during tissue injury, and catalyzes the dismutation of

superoxide into oxygen and hydrogen peroxide. This function of SOD has been evaluated as a promising treatment approach to limit tissue injury after ischemia–reperfusion [76]. Numerous agents with antioxidant properties have been evaluated, and among those SOD and *N*-acetylcysteine are most extensively studied, particularly after a renal transplantation with some potential interests [77,78]. For instance, when SOD was administered intravenously during transplantation, it significantly reduced acute rejection and improved long-term outcome [78]. However, despite numerous trials with many different compounds in various organ targets, it showed that the aforementioned results do not seem to be as stringent as we might expect [79–81]. Several trials testing antioxidant agents are still under way.

51.4.4 Inhibition of the Innate Inflammatory Response

Inhibition of complement has been more specifically tested in clinical trials within renal transplantation [82]. Renal damage due to complement activation occurs in two phases: first phase during reperfusion after restoration of blood circulation, and second phase during an acute rejection when innate and adaptive immune systems recognize the donor antigens. Eculizumad, a recombinant C1 inhibitor (C1-INH), and soluble complement receptor 1 mainly inhibit C5, an alternative pathway, and C3/C5 convertase, respectively [11]. Several inhibitors are still under investigation in larger clinical trials. Experimental studies suggest that inhibiting TLR2 and TLR4 in ischemia reperfusion syndrome after myocardial infarction or renal transplantation could have potentially interesting results. Preliminary studies are under way to investigate the safety and efficacy of antibodies anti-TLR2 (OPN-305) and anti-TLR4 (eritoran). In these studies, TLR4 inhibitors are being studied in context of either myocardial infarction or renal transplantation [83–85]. Suppressing dendritic cells using antimyocytes globulins have been suggested as another potential interesting therapy in ischemia–reperfusion syndrome [86,87].

51.4.5 Improving Ischemia Tolerance

During ischemic phase, the energy metabolism pathway is shown to change from fatty acid oxidation to glycolysis to protect the body from tissue injury. This change is controlled by hypoxia-inducible factor, whose stability is regulated by three oxygen-sensing prolyl hydroxylase domain isoform (PHDs) enzymes. Deficiency or inhibition of PHD1 decreases oxygen consumption in ischemic skeletal muscle by reprogramming glucose metabolism to a more anaerobic route of ATP production, which is through a peroxisome proliferator-activated receptor-α pathway [88]. Incidentally, pharmacological doses of PHD inhibitors have shown to increase tolerance to kidney ischemia [89] and to cardio protection, resembling what was observed in heart ischemic preconditioning [90]. Moreover, heterogeneous deficiency of PHD2 has shown to be able to restore tumor oxygenation and to inhibit metastasis through endothelial normalization [91]. Therefore, inhibition of PHD2 may also offer alternative therapeutic opportunities for anticancer therapy.

Although HIF target genes mainly encode erythropoietin (EPO), a major regulator of red blood cell formation whose production and secretion are mainly regulated by tissue oxygen levels, promising preclinical studies have suggested that erythropoietin could have beneficial effects in metabolic response, apoptosis inhibition, and angiogenesis [3]. However, clinical trial evaluating erythropoietin treatment showed that the result from a successful reperfusion following a myocardial infarction through a percutaneous intervention of 4 h was disappointing, with no reduction in infarction size and with association in higher rates of adverse cardiovascular events [92].

Currently, other therapies using recombinant annexin 5, recombinant P-selectin glycoprotein ligand Ig fusion protein, anticomplement therapies, and induction of heat shock protein 70 have been recently reviewed [93].

51.5 Conclusion

Although rapid reperfusion is necessary after an instance of ischemia, the reoxygenation process can adversely contribute to further the tissue injury and destruction. Many clinical trials have studied this phenomenon, and many have tried to dampen the damage lesion in relation to reoxygenation. Despite procedure improvements and numerous investigations, it remains a serious challenge to circumvent the adverse impacts of ischemia–reperfusion, particularly after an organ transplantation, stroke, or revascularization of myocardial infarction. Despite promising results in basic research and several small clinical trials, many larger clinical trials have failed to provide clear evidence of protective effects of various approaches. Continuing research on the activation of innate and adaptive immune systems will play a key role in the pathogenesis of ischemia–reperfusion syndrome. Currently, numerous novel therapies are under investigation to evaluate whether they affect the reoxygenation of the products released after ischemia. To further improve tissue preservation, agents such as antioxidants and *N*-acetyl cysteine that interfere with pathways involved in ischemia–reperfusion mechanisms should continue to be investigated in the future to improve tissue preservation.

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Single-Nucleotide Polymorphisms and Inflammation

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52.1 The Principle

The concept that genetic predisposition may influence an inflammatory phenotype has been evolved by the clinical characteristics that many patients present with extreme phenotypes of an inflammatory disorder; for instance, some young and otherwise healthy patients develop severe sepsis, whereas other patients with several chronic comorbidities can survive severe sepsis despite their positive scoring for severity [1]. As such, most attention has been focused on how host DNA can encode the proteins involved in the inflammatory cascade. This was the driving force for conducting thousands of case–control studies with a common study design—to compare between cases suffering from the inflammatory conditions under investigation and controls well-matched for age, sex, and other probable confounding conditions for the frequency of one single-nucleotide polymorphism (SNP) of a gene encoding for one of these proteins. The end point of this comparison was the impact of the studied SNP on the susceptibility for the disease under investigation. Another end point was the impact of the SNP on the disease phenotype.

An SNP is the substitution of one nucleotide base for another, and this leads to functional change of the encoded protein (i.e., sense SNP) or it does not affect the function of the protein (i.e., missense SNP). The functional change may result by the amino acid change induced by the new nucleotide codon or by alternative splicing leading to different gene transcripts and altered protein production. People can be heterozygotes or homozygotes for the SNP. SNPs can be located at the exons or at the introns of a gene. For most genes, several SNPs have been described. This immediately generates the question: Which SNPs should be studied in one case–control study? In most studies this question remains unanswered. The selection of SNPs for a specific study design often relies on the presumed importance of the protein under study and on the frequency of the SNPs in the population. This is known as the candidate gene approach. For instance, some SNPs are so rare that the requested power of the study to

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document important associations should be extremely large. In some studies, patients are genotyped for SNPs located on the same chromosome, that is, for haplotypes.

With the advent of new technologies involving genome-wide association studies (GWAS) and next-generation sequencing (NXS), the need to select the candidate SNP is no longer required. In GWAS, chips with primers for a wide variety of SNPs are predesigned, whereas in NXS a full sequencing of the human exome is performed. The main limitations of these approaches are the huge cost and the need for bioinformatics analysis looking for associations at *p*-values of significance below 10^{-8}. At a next step, the elaborated association should be analyzed after adjusting for the clinical characteristics of the patients.

This analysis of the strengths and the limitations of the genetic workout of inflammatory conditions renders obvious why study results showing a genetic susceptibility by one SNP cannot have a universal value and why results may vary considerably from one publication to the next. This chapter provides an in-depth presentation of the most widely studied SNPs for which some association with either acute or chronic inflammatory disorders has been described in the last decade. To achieve this, a thorough MedLine search was conducted using the terms "SNPs," "genetic susceptibility," "GWAS," and "next-generation sequencing" for the disease entities that are analyzed in this chapter for the period 2005 until November 2015. Of the thousands of articles published, those who reported on GWAS, NXS, or meta-analysis of an SNP for a disease were finally included in the chapter.

52.2 SNPs and Functional Genomics

One of the major concerns is whether studied SNPs can modify the function of monocytes and lymphocytes. Some of the most broadly studied SNPs are those of *TLR4*; the first is rs4986790 leading to the substitution of aspartic acid by glycine at amino acid position 299 (Asp299Gly) and the second is rs4986791 leading to the substitution of threonine by isoleucine at amino acid position 399 (Thr399Ile). The emergence of these SNPs has evolved under natural pressure. People from Africa carry haplotypes with the rs4986790 SNP allele and with the rs4986791 wild-type allele. Their monocytes can overproduce tumor necrosis factor-alpha (TNFα) after stimulation with TLR4 ligands offering protection against *Plasmodium* spp. When people migrate from Africa to Europe, haplotypes with either both wild-type alleles or both SNP alleles emerge so that monocytes can no longer overproduce TNFα and be protected from sepsis [2]. In a similar way, the frequency of rs16910526 wild-type and SNP alleles of *Dectin-1* encoding for the receptor of β-D-glucan of *Candida albicans* does not differ between patients with candidemia and noninfected controls; however, carriage of minor frequency SNP alleles decreases the ability of TNFα and interleukin (IL)-1β production by circulating monocytes after stimulation with *C. albicans* [3].

Several studies have shown that carriage of the SNP rs1800629 A allele at the −308 position of the *TNF* gene modulates the capacity of circulating monocytes for the production of TNFα after *ex vivo* stimulation with bacterial endotoxin (LPS) compared with the carriage of only wild-type G alleles. This was shown in

Asian, Latin, and Caucasian populations [4–6]. Initial monocyte exposure to low LPS concentration impedes high cytokine production upon reexposure to high LPS concentration, a phenomenon known as LPS tolerance. One GWAS analysis indicated that the presence of rs522807, rs5745938, and rs625847 upstream of *TNFRSF1B* encoding for the receptor 2 of TNFα is probably associated with the expression of *TNFRSF1B* and mediates LPS tolerance [7].

52.3 Genetic Susceptibility for Acute Inflammatory Conditions

Severe sepsis is the prototype of acute inflammatory conditions. Available studies are characterized by great heterogeneity, and they have the limitations already analyzed in the first section of this chapter. However, these studies reflect the major limitation of the poor understanding of the pathogenesis of severe sepsis. More precisely, severe sepsis is the host reaction to an invading microorganism that is leading to acute organ dysfunction. This definition is very broad and introduces great heterogeneity because causative infections and responsible pathogens vary considerably from one patient to the other. This becomes far more complicated after considering that infection predisposition is severely affected by chronic coexisting illnesses and by the age and the ethnicity of the patient. As a consequence, how is it possible to identify specific SNPs responsible for predisposition to sepsis when adjusting for all this heterogeneity is mandatory? This is probably the reason why severe sepsis is considered the prototype of individualized medicine [8]. The following section provides some grouping of the available knowledge on the role of SNPs for severe sepsis. A synopsis is given in Table 52.1. The selection of SNPs to be included in this analysis was made by the following criteria: (a) studies conducted in the last decade; and (b) existing confirmation of the published evidence in other cohorts as this was coming from meta-analysis.

52.3.1 Genetic Susceptibility to Homogeneous Study Populations

Although homogeneous study populations in fact do not exist, some studies are characterized by relative homogeneity as this is expressed by the inclusion of patients with the same infection as the cause of sepsis or by the inclusion of patients with the same predisposing condition for sepsis acquisition.

52.3.1.1 Patients with the Same Underlying Infection

The only GWAS study in patients with sepsis was the multicenter GenOSept study of patients with community-acquired pneumonia (CAP) hospitalized in European Intensive Care Units (ICUs). To increase the power of analysis, patients of another three cohorts were analyzed together reaching a total of 3410 patients. Only one SNP was significantly below the set significance threshold of 5×10^{-7}; this was rs4957796 on chromosome 5 belonging to an intron region of *FER* that encodes for one Fps/Fes-related tyrosine kinase. Mortality after 28 days was 25.3% for patients with the TT genotype, 15.2% for patients with the TC genotype, and

Table 52.1 Synopsis of the most important single-nucleotide polymorphisms (SNPs) associated with susceptibility to the development of sepsis and with modulation of the physical course of sepsis.

SNP	Gene	Substitution and gene position	Encoded protein	Impact of minor frequency alleles
SNPs associated with susceptibility for sepsis				
rs1800629	*TNF*	−308 G/A	TNFα	Risk for severe sepsis in Asian populations; protection from leprosy in Brazilians
rs143634	*IL-1B*	+3594 C/T	IL-1β	TT genotype protects from sepsis
VNTR 86bp	*IL-1RN*	Intron 2 (1–6 VNTR alleles)	IL-1ra	Short sequence VNTRs increases susceptibility to septic shock
4G/4G genotype	*PAI-I*	−675 (4G/4G) homozygotes	Plasminogen activation inhibitor I	Increased risk for sepsis development
rs5743551	*TLR1*	−702 A/G	Promoter	OR = 2.56 for septic shock
rs6667605	*TNFRSF14*	Intron 4	Receptor for BTLA	Increased risk for infection by *Clostridium difficile* in UC patients
SNPs modulating the physical course of sepsis				
rs4957796	*FER*	Intron variant	Fps/Fes-related tyrosine kinase	OR = 0.56 for death with C allele carriage in CAP
rs1801274	*FCGR2A*	−167 C/T	Receptor for IgG2	OR = 2.90 for pneumococcal bacteremia in CAP
rs5743551, rs5743618, rs4833095	*TLR1*	−7202 A/G; −602 T/G; +743 A/G	TLR1	Increased risk for Gram-positive sepsis and sepsis-associated death after multiple injuries
rs1059703	*IRAK-1*	+532 C/T exon 12	IL-1 receptor associated kinase	Increased risk for MODS and death after multiple injuries
4G/5G genotype	*PAI-I*	−675 (4G/5G) heterozygotes	Plasminogen activation inhibitor I	Increased risk for death
SNPs modulating gene function				
rs1800629	*TNF*	−308 G/A	TNFα	Increased production of TNFα from circulating leukocytes after *ex vivo* stimulation

Ig, immunoglobulin; IL, interleukin; IRAK, IL-1 receptor associated kinase-1; MODS, multiple organ dysfunction syndrome; PAI, plasminogen activator inhibitor; UC, ulcerative colitis; TNFα, tumor necrosis factor alpha.

9.5% for patients with the CC genotype of this SNP. The combined protective odds ratio of the four cohorts was 0.56 with the carriage of C alleles [9].

Two studies are available in patients with bacteremia by *Staphylococcus aureus*: the first was a GWAS in 361 cases and 699 controls and failed to identify any SNP to be associated with susceptibility for bacteremia [10]; the second study in 127 cases compared SNPs of *MBL2* responsible for high, low, or absent production of mannose-binding lectin 2 (MBL2). Analysis revealed that SNPs responsible for low or absent production of MBL2 offered greater risk for persistent bacteremia (odds ratio 2.09, $p = 0.040$) [11].

The rs1801274 SNP of exon 4 of *FCGR2A* encodes for one arginine to histidine substitution at position 131 of the receptor FcγRIIA of the immunoglobulin IgG2. It is presumed that carriage of this SNP allele impedes adequate phagocytosis by neutrophils. The SNP was analyzed in 1262 patients with CAP and in 1244 controls. No differences in genotype distributions were found between patients and controls showing the lack of effect of the SNP on susceptibility for CAP. However, patients homozygous for this SNP had a greater risk for bacteremia by *Streptococcus pneumoniae* [12].

Using geographically different cohorts of patients with pneumonia from Germany and Greece, the rs2232613 SNP of LPS-binding protein (LBP) has been analyzed. The description of this SNP was largely based on differences in the crystallographic structure of LBP between patients. This SNP leads to the substitution of one cytosine to thymine at +988 position associated with 6.6 odds ratio for pneumonia by Gram-negative bacteria. Carriage of the SNP is also associated with lower production of TNFα by circulating monocytes compared with patients carrying only wild-type alleles [13].

52.3.1.2 Patients with the Same Predisposing Condition

Two large studies have recently been published exploring the role of candidate SNPs in patients with severe injuries. The first study enrolled 1498 patients of Caucasian origin analyzed for the carriage of three SNP alleles of *TLR1*: rs5743551 at the −720 promoter position, rs5743618 encoding one serine isoleucine transition at the 1804 position of the transmembrane domain of TLR1, and rs4833095 encoding one aspartate serine transition at the 742 position of the extracellular domain of TLR1. Patients homozygous for the SNP G allele rs5743551 and for the T allele rs5743618 had increased mortality due to sepsis contrary to patients who did not develop sepsis (odds ratios 3.16 and 2.48, respectively). Patients homozygous for the rs4833095 SNP allele had 4.13 odds ratio for sepsis by Gram-positive cocci ($p = 0.023$) [14]. These findings emerging in one homogeneous critically ill cohort of multiple injuries were confirmed in a nonhomogeneous cohort of 724 patients with sepsis admitted in the ICU. More precisely, patients homozygous for the G rs5743551 allele had 1.82 odds ratio for unfavorable outcome ($p = 0.028$); the odds ratio by carriage of this SNP allele was 2.56 for the development of septic shock. Furthermore, patients who were homozygotes for this SNP allele had increased prevalence of infections by Gram-positive cocci. This corroborates with the function of TLR1 that recognizes structures of the cell wall of the Gram-positive cocci. In the same study it was shown that

homozygosity of this SNP was associated with greater production of IL-6 after stimulation of leukocytes of healthy carriers with ligands of TLR1 [15].

The genetic variant rs1059703 of *IRAK-1* carried at the X chromosome, and encoding for the IL-1 receptor-associated kinase (IRAK-1) was genotyped in 321 patients admitted to one ICU for multiple injuries. Among patients homozygous for the wild-type haplotype, 4.7% developed multiple organ dysfunction (MODS) and 2.4% died; respective percentages were 11.1% and 10.3% for patients heterozygotes for the variant haplotype and 18.2% and 12.5% for patients homozygotes for the variant haplotype [16].

Although not always severe, it seems that the development of *Clostridium difficile* infection (CDI) is influenced by the genetic background of the host. As pathogenesis of CDI is characterized by marked heterogeneity regarding predisposing conditions, one recent publication focused only on the genetic make-up of patients with ulcerative colitis that is a common predisposing condition for CDI. Genetic analysis was done for the 163 SNPs that have been associated with susceptibility to ulcerative colitis. Seven of these SNPs (rs6667605, rs1363907, rs10051722, rs864745, rs4409764, rs1129555, rs17085007, and rs941823) were independently associated with the development of CDI. There was also a linear increase of the risk for CDI with the presence of each of these loci, so that each locus increased the risk for acquisition of CDI by threefold. Most susceptibility came from the TT genotype of rs6667605 influencing the activity of NF-κB [17].

52.3.2 Meta-Analysis Published

Since the beginning of case–control gene association studies, some SNPs have attracted much attention and they were thoroughly studied in multiple cohorts. The availability of studies allowed the publication of meta-analyses, although these cohorts were characterized by great heterogeneity. Available meta-analyses report on the role of SNPs of *TNF*, *IL-1*, and *PAI-I* for sepsis susceptibility and sepsis mortality.

Most case–control studies published so far involve the SNP rs1800629 of one guanine to adenine substitution at the −308 promoter region of *TNF*. One large meta-analysis published in 2010 [18] incorporated data from 25 studies conducted between 1996 and 2008; patients enrolled in these studies were of Caucasian origin, Asian origin, or mixed origin. The underlying condition predisposing to sepsis also varied considerably between these studies. Despite this heterogeneity, the meta-analysis indicated a pooled 2.15 odds ratio for sepsis development for carriers of the A SNP allele. This susceptibility was more pronounced in Asian populations (odds ratio 3.16 contrary to 1.94 in the Caucasians). Carriage of the A SNP allele was also associated with greater risk for death in Asian populations (odds ratio 10.75) but not in the Caucasian populations.

The results of this meta-analysis for the impact of the rs1800629 SNP in the Asiatic population were confirmed later in a cohort of 432 Chinese patients with severe sepsis who were compared with 624 Chinese healthy volunteers [4]. Carriage of the minor frequency A allele was associated with 1.92 odds ratio for the development of severe sepsis ($p = 0.00046$). We tried to follow up the

impact of carriage of SNP alleles of *TNF* in the physical course of Caucasian patients who were admitted in the ICU due to the need of ventilator support. These patients were uninfected on study enrollment, and the primary study end point was the time to the development of ventilator-associated pneumonia (VAP) as a result of exposure to intubation that is a well-known risk factor for VAP. Results revealed that wild-type haplotypes with major frequency G alleles for SNPs rs1800750 at −376 promoter region, rs1800629 at −308 promoter region, and rs361525 at −238 promoter region were associated with slower development of VAP compared with carriers of SNP haplotypes [5].

Meta-analyses for the role of the rs1800629 SNP involve not only sepsis risk but also the risk for leprosy. This later meta-analysis was conducted in case–control studies in Indian, Brazilian, Malawian, Thai, and Nepalese populations. Carriage of the minor frequency A allele was protective from leprosy, and this effect was more pronounced in the Brazilian populations. When whole blood of Brazilian patients was *ex vivo* stimulated, production of TNFα was greater than carriers of A alleles corroborating the findings coming from sepsis studies [6].

Meta-analysis has also been conducted on 18 case–control studies that explored the role of SNPs *IL-1A*, *IL1B*, and *IL-1RN* encoding for IL-1α, IL-1β, and IL1ra, respectively. Results showed that, among all analyzed SNPs, two conferred most susceptibility for sepsis. The first was the one SNP at the +3594 position of *IL-1B*; patients homozygous for the minor frequency T SNP allele were protected from sepsis (odds ratio 0.59, $p = 0.040$). The second was the presence of two alleles of tandem 86 bp repeats at intron 2 of *IL*-1RN that increased the risk of sepsis (odds ratio 1.85, $p = 0.020$); the effect on septic shock susceptibility was even more pronounced (odds ratio 10.00, $p = 0.040$) [19].

One of the meta-analyses involves genotyping for *PAI-I* encoding for the plasminogen activator inhibitor I that inhibits the activation of tissue plasminogen activator. Circulating concentrations of PAI-I are increased in severe sepsis. The published meta-analysis was done in 15 studies; 13 studies involved Caucasian populations and two studies involved Asiatic populations. Patients may carry one of 4G/4G, 4G/5G, or 5G/5G genotypes of *PAI-I* at the −675 position of the gene promoter. The 4G/4G genotype was associated with increased risk for sepsis acquisition (odds ratio 2.75, $p = 0.006$ for all the studies; odds ratio 1.23, $p = 0.03$ only for patients of Caucasian origin). However, higher risk for unfavorable outcome came from the 4G/5G genotype; the cumulative odds ratio for death for carriers of this genotype was 1.72 [20].

52.3.3 General Considerations

The above analysis generates doubts on which of the studied SNPs may really confer some genetic susceptibility to the host and modulate the physical course of severe infections or whether this is more or less confined to some ethnicities or even to individualized cases. One main reason for these doubts is the heterogeneity of the results. It may be argued that for a gene to affect the severe course of an infection, its implication should be shown in more than one disease states. One example is *CISH* encoding for cytokine-inducible Src homology-2 domain protein that belongs to the SOCS family. SOCS is the acronym for the suppressor of

cytokine signaling proteins that regulate cytokine production. Seven separate large cohorts of patients each suffering from one specific severe infection (bacteremia, tuberculosis, and malaria) were collected from different ethnicities and geographic regions from Gambia, Kenya, Malawi, and Hong Kong. Genetic analysis for the SNPs at positions −639, −292, −163, +1320, and +3415 of *CISH* was conducted, and results were compared with respective control cohorts of the same ethnicity and geographic region. Carriage of any of the five SNPs increased the likelihood for each of the studied infections, although most of the association signal came for the −292 SNP (pooled odds ratio 1.19 from the seven studied cohorts). The risk was more pronounced for patients carrying more than one SNP by at least 18%. Patients homozygous for the T allele of the −292 SNP had lower expression of *CISH* in their circulating mononuclear cells after *ex vivo* stimulation with IL-2 compared with patients homozygous for the A wild-type allele [21].

52.4 Genetic Predisposition for Chronic Inflammatory Disorders

Contrary to acute inflammatory conditions, evidence available for the role of genetic predisposition of the host for chronic inflammatory disorders is more robust due to the large number of available studies and to the nature of these studies. More precisely, the major study approach is the one of GWAS accompanied by confirmation of findings in independent cohorts. A summary of major findings is provided in this chapter.

52.4.1 Inflammatory Bowel Disease (IBD)

Large GWAS have been conducted for patients with both ulcerative colitis (UC) and Crohn's disease (CD). In most of these studies, findings are confirmed using a meta-analysis approach of published sequencing data or GWAS data (Table 52.2). One recent large GWAS involved 2693 patients and 6791 controls of European descent from the United States and Sweden; a replication cohort of 2009 patients and 1580 controls from Italy and The Netherlands was used for confirmation. Results identified 59 SNPs in 14 genetic loci that differed significantly between cases and controls. The most frequently implicated gene loci were *RNF186*, *OTUD3*, and *PLA2G2E* on chromosome 1 (rs3806308, rs1317209, and rs6426833); *IL23R* on chromosome 1 (rs2201841 and rs11209026); *FCGR2A* and *FCGR2C* on chromosome 1 (rs10800309); *IL10* and *IL19* on chromosome 1 (rs3024505); *REL*, *CCDC139*, and *PUS10* on chromosome 2 (rs6706689 and rs13003464); *MST1* on chromosome 3 (rs3197999); *CEP72* and *TPPP* on chromosome 5 (rs4957048); *C6orf10* and *BTNL2* on chromosome 6 (rs2395185); *DLD* and *LAMB1* on chromosome 7 (rs4598195); *CARD9* on chromosome 9 (rs4077515); *NKX2-3* on chromosome 10 (rs11190140); *IFNG* and *IL26* on chromosome12 (rs1558744 and rs971545); the *ORMDL3* region on chromosome 17 (rs2305480); and *PSMG1* on chromosome 21 (rs2836878) [22].

The implication of gene loci of *RNF186*, *IL23R*, and *CARD9* was confirmed in one GWAS of 200 UC patients of French Canadian ancestry [23]. One large

Table 52.2 Most common gene loci associated with susceptibility to ulcerative colitis as defined by genome-wide association studies [22–25].

SNP	Gene	Risk allele	Function	Odds ratio (95% CIs)
rs3806308	*RNF186*	G	E3 ubiquitin ligase for DSB repair	1.49 (1.17–1.90)
rs2858829	18 kb upstream of *FAM26F*	G	Increased *FAM26F* expression	1.12 (1.08–1.16)
rs11209026	*IL23R*	G	IL-23 receptor	1.74 (1.57–1.92)
rs1801274	*FCGR2A*	A	Immunoglobulin G Fc receptor II (low affinity receptor)	1.21 (1.16–1.26)
rs3024505	*IL10*, *IL19*	A	IL-10, IL-19	1.25 (1.19–1.32)
rs7608910	*PUS10*	G	Spliceosome biogenesis	1.19 (1.24-1.24)
rs7134599	*IFNG IL26*	A	IFNγ, IL-26	1.19 (1.14–1.24)
rs4077515	*CARD9*	C	Cardinal	0.39 (0.30–0.53)

CI, confidence interval; IL, interleukin.

meta-analysis of data collected from six UC GWAS confirmed the implication of these genes as well. This precise meta-analysis [24] comprised 6687 cases and 19 718 controls and added another new 29 gene loci associations with UC. This meta-analysis confirmed the importance of genetic variations of the previously reported genes such as *IL23R*, *FCGR2A*, *IL10*, *IL19*, *PUS10*, *IFNG*, *IL26*, and *CARD*9. Newly implicated genes were *TNFRSF8*, *TNFRSF9*, *TNFRSF14*, and *TNFRSF15* all encoding for receptors of the TNF superfamily; *IL1R2*, *IL7R*, and *IL8RA* encoding for receptors of IL-1, IL-7, and IL-8, respectively; *DAP* encoding for the death-associated protein of the apoptosis pathways; *PRDM1* encoding for a master transcriptional regulator in plasma cells and in T and B lymphocytes; and *IRF5* encoding for interferon regulatory factor 5 [24]. Another meta-analysis of 15 GWAS on UC and CD increased the number of implicated gene loci for predisposition to IBD in 193. This meta-analysis tried to provide the functional significance of the reported SNPs. It was found that SNPs associated with susceptibility to IBD are enriched either in the regulation of cytokine production pathways or in the activation of lymphocytes or in the JAK–STAT signaling pathways [26]. The enrichment of these pathways generated the hypothesis that selection of these gene SNPs is a process of natural pressure by the microbial gut flora.

Starting from new genotyping of 825 UC cases and 1527 controls from Spain and confirming the data with previous published GWAS data of the North-European ancestry, the rs2858829 located in chromosome 6q.22.1 was associated with significant risk for UC. This SNP is located 18 kb upstream of the *FAM26F* gene. Analyses of the microarrays from four studies in UC indicate that this SNP is associated with increased expression of *FAM26F* in the lamina propria of the colon mucosa [25].

GWAS for 19 799 coding SNPs was done in 735 German patients with CD. After bioanalysis, the G allele of rs2241880 of *ATG16L1* on chromosome 2 was associated with 1.45 odds ratio for susceptibility to CD. This was confirmed in sets of 498 German patients and 509 UK patients [27].

After capture and analysis of the whole exome from 42 CD patients from Germany, two missense SNPs of *PRDM1* on chromosome 6 were identified: rs106659789 as a risk variant for CD and rs106660076 as a protective variant for UC. Minor frequency C alleles of the rs106659789 risk variant were associated with greater potential of CD3 lymphocytes for replication and production of IFNγ. Moreover, carriage of C alleles of rs7746082 of *PRDM1* was associated with lower gene expression of *PRDM1* in T and B lymphocytes both in the gut lamina propria and in circulating lymphocytes [28].

52.4.2 Psoriasis Vulgaris and Psoriatic Arthritis

More than 30 studies have been published on the influence of carriage of A alleles of rs1800629 and rs361525 of the *TNF* promoter for susceptibility of psoriasis vulgaris and psoriatic arthritis. One meta-analysis of 26 studies has recently been published [29]. Results were homogeneous for patients of both Caucasian and Asiatic origin, and these showed an opposite effect of carriage of A alleles at −308 promoter region (rs1800629) and of A alleles at −238 promoter region (rs361525). More precisely, carriage of A alleles of rs1800629 was protective from psoriasis vulgaris (pooled odds ratio 0.574) but not from psoriatic arthritis, whereas carriage of A alleles of rs361525 increased susceptibility to both psoriasis vulgaris (pooled odds ratio 2.636) and psoriatic arthritis (pooled odds ratio 2.242).

A large genomic analysis of 9389 cases and 9477 controls pinpointed two important genetic elements predisposing to psoriasis vulgaris. The first is a 32.2 kb deletion of two *LCE* genes, known as *LCE3C_LCE3B-del* which is part of the epidermal differentiation complex. The second is one copy number variant offering the tag rs4112788 located 584 nucleotides downstream of *LCED*. The presence of the *LCE3C_LCE3B-del* offered overall odds ratio 1.21 for psoriasis at p-value 4.58×10^{-13}. This was also the case with the carriage of C alleles of rs4112788 associated with psoriasis with overall odds ratio 1.21 at p-value 1.42×10^{-12}. These associations were confirmed in populations of both European and Asiatic ancestry [30].

Most of the genetic susceptibility for psoriasis is coming from a region of 300 kb of *HLA-C* on chromosome 6p21 encoding in part for the major histocompatibility complex class I, also known as *PRORS1*. One large GWAS in 2622 cases and 5667 controls confirmed the implications of SNPs of *HLA-C* for predisposition to psoriasis with odds ratio 4.66. This chromosome encompasses four main genes, *TRAF3IP2*, *NFKBIA*, *REL*, and *TYK2*, involved in the activation of IκB, IL-17, and IFN signaling that are key molecules for the pathogenesis of psoriasis [31]. Another GWAS was conducted in 1831 cases and 2546 controls and a confirmation cohort of 4064 cases and 4685 controls. Authors validated three SNPs to be associated with psoriasis: rs4795067 of *NOS2* encoding for inducible nitric oxide synthase, rs10782001 involved in the activation of NF-κB, and rs12586317 also involved in the activation of NF-κB [32].

Table 52.3 Most common gene loci associated with susceptibility to systemic lupus erythematosus as defined by genome-wide association studies [33–36].

SNP	Gene	Risk allele	Function	Odds ratio (95% CIs)
rs7574865	*STAT4*	T	Intracellular signaling	1.55 (1.34–1.79)
rs13397	*TMEM187*	A	Yet unknown	1.41 (1.12–1.79)
rs4898475	TKTL1	A	Transketolase of pentose phosphate pathway (nonoxidative phase)	1.44 (1.15–1.82)
rs1059702	*IRAK1*	A	IL-1 receptor-associated kinase-1	1.18 (1.17–1.87)
rs2734647	*MECP2*	T	Repression of transcription from methylated gene promoters	1.42 (1.16–1.80)
rs2075596	*MECP2*	A	Repression of transcription from methylated gene promoters	1.45 (1.16–1.80)
rs1616369	*MECP2*	C	Repression of transcription from methylated gene promoters	1.50 (1.20–1.88)
rs7704116	*PPP2CA*	A	T lymphocyte proliferation	1.3 (1.1–1.3)
rs34933034	*CSK*	A	Expansion of late transitional B lymphocytes	1.32 (1.20–1.44)

CI, confidence interval; IL, interleukin.

52.4.3 Systemic Lupus Erythematosus (SLE)

SLE is the prototype of autoimmune disorders involving women in more than 90% of cases. Multiple large GWAS have been published for patients with SLE. Here, we report the results of studies published in the last decade with confirmation of the genetic evidence in European and several other ethnicities (Table 52.3). The first study highlighting the importance of individual SNPs used a candidate gene approach for SNPs of *STAT4* encoding for STAT4 that modulates intracellular signaling. Besides three independent cohorts, it was shown that carriers of the minor frequency T allele of rs7574865 have increased risk for SLE; the association was stronger for patients at a state of SNP homozygosity [33].

Often, presentation of SLE in women shed light on the importance of X chromosome for the genetic susceptibility for SLE. One large GWAS was performed for SNPs located in X chromosome in Americans of European Ancestry (3915 cases and 3462 controls). Identified SNPs below the threshold of significance of GWAS were confirmed in separate cohorts of African origin, Asian origin, and Hispanic origin. Data confirmed that 34 SNPs at the Xq28 locus are a risk factor for SLE. These SNPs are contained in the same haplotype, called haplotype H1, and they are located at the *IRAK1* and *MECP2* genes. The frequency of the H1 haplotype comprises 17.4% in cases and 13.3% in controls of European ancestry, 69.1% in cases and 64.2% in controls of Asian ancestry, 52.1% in cases and 40.1% in controls of Hispanic ancestry, and 63.3% in cases and 4.3% in controls of African ancestry. SNPs rs13397, rs4898375, rs1059702, rs2734647, rs2075596, and rs1616369 are the core of this haplotype. Of these

SNPs, carriage of rs1059702 was associated with lower expression of *MECP2* in circulating lymphocytes (Table 52.3). The significance of this biological association comes from the fact that IRAK-1 encoded by *IRAK-1* is implicated in the downstream activation of IκB and *MECP2* is implicated in the activation of the IFN signaling pathways [34]. Using the same cohorts of patients, it was found that the A allele of rs7704116 increases susceptibility for SLE in all ethnic groups and, in particular, for renal manifestations. This SNP is located in the *PP2CA* gene, and the A risk allele is associated with decreased gene expression of *PPC2CA* linked with defective proliferation of T lymphocytes. This is probably due to the significance of *PPP2CA* for the production of IL-2 [35].

Analysis of another three cohorts identified that patients homozygous for the minor frequency A rs34933034 allele of *CSK* have increased likelihood for SLE. This is associated with increased activation of gene expression of *CSK* accompanied by increased mobilization of calcium and expansion of late transitional B lymphocytes [36]. Other large genetic studies have shown that SNPs at the genetic region of *HLA* confer susceptibility for SLE and that they are associated with the phenotype of the disease. However, findings are limited in European populations and confirmation sets in other ethnic groups are not yet available [37,38].

52.4.4 Rheumatoid Arthritis (RA)

RA affects more than 1% of the general population worldwide, and genetic predisposition is a major component of pathogenesis in at least 20% of cases (Table 52.4). Using the candidate gene approach, SNPs located at *HLA-DRB14*

Table 52.4 Most common gene loci associated with susceptibility to rheumatoid arthritis as defined by genome-wide association studies [33,39–44].

SNP	Gene	Risk allele	Function	Odds ratio (95% CIs)
rs6457620	*HLA-DRB1*	C	HLA class II	2.55 (2.40–2.71)
rs6679677	*PTPN22*	A	Negative regulator of T-cell receptor (TCR)	1.79 (1.65–1.94)
rs7574865	*STAT4*	T	Intracellular signaling	1.14 (1.06–1.23)
rs3761847	*TRAF1-C5*	A	Negative regulator of TNF signaling pathway	1.32 (1.23–1.42)
rs44181354	*CD40*	T	CD40	0.91
rs6920220	*TNFAIP3*	A	Inhibition of NF-κB	1.32 (1.23–1.43)
rs10499194	*OLIG3*	—	Neuronal development	0.88 (0.83–0.93)
rs3890745	*TNFRSF14*	A	TNF receptor	1.13
rs2872507	*IKZF3*	A	B cell differentiation	0.90
rs10489629	*IL23R*	C	IL-23 receptor	1.15 (1.06–1.24)
rs7517847	*IL23R*	G	IL-23 receptor	1.18 (1.09–1.28)

CD, cluster of differentiation; CI, confidence interval; IL, interleukin; TNF, tumor necrosis factor.

and *PTPN22* were associated with increased likelihood for RA testing positive for the antibodies against cyclic citrullinated peptide (anti-CCP). The first large GWAS was conducted in 1522 RA cases testing positive for anti-CCP and 1850 well-matched controls. A confirmation of the results in 1053 cases and 1798 controls was done. Results identified gene loci at chromosome 6, where the *HLA-DRB1* is found, and at chromosome 9 to be associated with increased likelihood for RA. Loci of chromosome 9 referred mainly to nine SNPs of *TRAF1-C5*, of which rs3761847 was associated with an odds ratio of 1.32 for RA [39].

Another large GWAS was conducted by the Wellcome Trust Case Control Consortium (WTCCC) in 3714 cases and 5870 controls. Results were validated in another study of 9644 cases and 7390 controls, and indicated a profound role of rs6920220 located at chromosome 6p23 for susceptibility to RA. The effect was more pronounced for patients testing positive for anti-CCP (pooled odds ratio versus controls 1.32) and for patients testing positive for rheumatoid factor (pooled odds ratio versus controls 1.28) [40]. The significance of this SNP was also highlighted by another GWAS of 2680 cases and 4469 controls [41]. These results of the GWAS database of the WTCCC were confirmed in a new collection of 3929 RA patients testing positive for anti-CCP or for rheumatoid factor and 5807 matched controls. In this analysis, the SNP rs4810485 surpassed the level of significance; it is located in the gene encoding for CD40 and carriage of the minor frequency T allele is associated with protection from extensive joint involvement [42].

Trying to further validate the findings, genomic analysis in RA patients from five different countries used a new immunochip that was designed to comprise all known SNPs defined by the 1000 Genomes Project and all SNPs of the 186 loci defined to be involved in 12 autoimmune disorders. Results fully confirmed the implication of *HLA-DRB1*, *PTPN22*, *STAT4*, and *TNFAIP3* [43]. A multiethnic GWAS approach in 5505 RA cases and 22 603 controls followed by validation in 6768 cases and 8806 controls of European ancestry identified two more gene loci: rs3890745 of *TNFRSF14* located at chromosome 1 and rs2872507 of *IKZF3* located at chromosome 17q12; rs3890745 was associated with increased susceptibility for RA and rs2872507 with protection from RA [44].

In light of the conduct of the large GWAS, some meta-analysis of case–control studies of candidate genes managed to outscore the significance of some SNPs for RA acquisition and for the RA phenotype [45–47]. One meta-analysis was conducted for two SNPs of *IL23R* in populations of European origin coming from Australia, New Zealand, Norway, Spain, and the United Kingdom: rs10489629 and rs7518847. Analysis revealed that carriage of the C allele of rs10489629 and carriage of the G allele of rs7517847 were associated with increased likelihood for RA [45]. Another meta-analysis of 30 studies focused on the significance of two SNPs of *CTLA-4*: rs231775 and rs3087243. rs231775 is one adenine to guanine substitution at +49 position that increases the risk for RA in Asians (pooled odds ratio 1.27) and Americans (pooled odds ratio 1.87) but not in Europeans. rs3087243 is one guanine to adenine substitution at +60 position that decreases the risk of RA in Europeans (pooled odds ratio 0.89) without affecting other ethnicities [46]. Finally, one meta-analysis of 10 studies comprising 2053 patients revealed that the presence of A alleles of rs1800629 at −308 promoter of *TNF* was protective from erosions with 0.78 common odds ratio [47].

52.5 Conclusive Remarks

The current chapter rendered evident that genetic susceptibility for an acute or chronic inflammatory condition does not rely on single SNP but rather on a constellation of SNPs. This constellation may offer genetic susceptibility or modify disease phenotype. Existing evidence suggests that as genetic analysis goes on using the new high throughput techniques more susceptibility loci will be identified. The main question remains: Is this identification of clinical significance? The need for an answer becomes more demanding because some of these SNPs do not necessarily belong to genes directly implicated in the inflammatory process or to genes with known function. One recent example comes from an attempt to identify new genetic loci in patients with RA. To this end, a meta-analysis was conducted in data from 22 GWAS and then findings were replicated in two independent cohorts. The authors of this study followed such a thorough approach because they thought it was necessary to combine genetic data coming from patients with different ethnic backgrounds. Analysis managed to replicate the significance of already-reported SNPs in Table 52.4 associated with RA. Moreover, 42 new gene loci were identified to confer the susceptibility for RA. All identified SNPs, both the formerly acknowledged and the new described, were related with pathways of either immune regulation or hematologic cancer

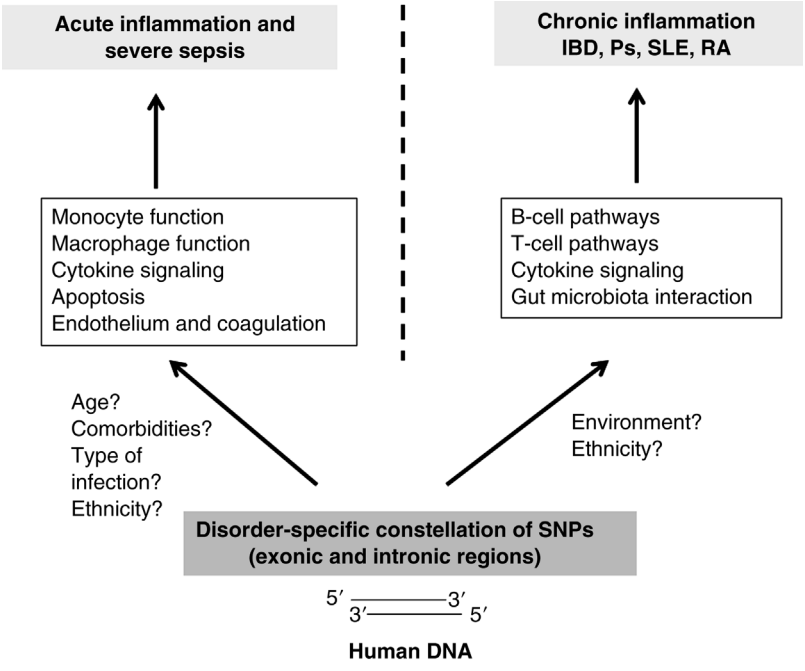


Figure 52.1 A simplistic representation of our current understanding of the impact of SNPs for acute and chronic inflammatory disorders. A specific for each disorder constellation of SNPs modulates pathways that dominate the development of acute and chronic inflammatory processes. Factors interfering as confounders to this modulation are marked with a question mark. IBD, inflammatory bowel disease; Ps, psoriasis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SNPs, single-nucleotide polymorphisms.

mutation. Those related to immune regulation were pathways of B-cells, T-cells, and cytokine signaling. One salient finding was that the described loci were related with functions targeted by many of the available biological disease-modifying agents for RA such as tocilizimab, tofacitinib, and abatacept [48].

Although the genetic predisposition provided by a constellation of SNPs for a chronic state of inflammation like in IBD, SLE, and RA seems clear-cut, the significance of a constellation of SNPs for susceptibility to severe sepsis seems far more complex and heterogeneous. This is probably a reflection of the disease process *per se* where both acquisition of severe infection and outcome are affected by a variety of factors. The intensity of the initial inflammatory reaction of the host to a microbial insult, the type of infection triggering septic phenomena, and the age and comorbidities of the host are the most important of these factors (Figure 52.1). It should also be underscored that part of the variability in findings is associated with the genetic background of different ethnicities. Heterogeneity of the genetic make-up between different ethnicities seems to largely affect predisposition for both acute and chronic inflammatory disorders.

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Part Six

Resolution of Inflammation and Tissue Repair

53

Pentraxins in the Orchestration of Defense and Tissue Repair during the Acute Phase Response

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53.1 The General Scenario

Microbial invasion and tissue injury trigger a complex response at the organism level commonly referred to as acute phase response [1,2]. Cytokines are key triggers of a complex set of changes centered on the liver, with IL-1 and IL-6 playing a key role in their orchestration. In general, the liver protein factory is reoriented from albumin synthesis to production of proteins relevant to defense and tissue repair (Table 53.1).

The general significance of this complex set of changes can be viewed in the perspective of defense and orchestration of tissue repair (Table 53.2). During an acute phase response, levels of components of the humoral arm of innate immunity increase (Table 53.2). These include the classic short pentraxins C-reactive protein (CRP) and serum amyloid P component (SAP), the long pentraxin PTX3, mannose-binding lectin (MBL), and C1q. These molecules act as fluid phase pattern recognition molecules (PRMs) with antibody-like properties [3]. They recognize microbial moieties, have opsonic activity, and activate and regulate the complement cascade. Moreover, as discussed later, they interact with matrix components [4]. Another set of acute phase reactants include components of the complement cascade (Table 53.2). Activation and regulation of the complement cascade parallels PRM production and can be viewed in the same general perspective of augmentation of antimicrobial resistance and regulation of inflammation.

It is important to note that CRP was originally identified by Tillet and Francis as an antibody-like molecule recognizing the C-type polysaccharides of pneumococcus in the blood of infected patients [3]. Thus, CRP, a component of the humoral arm of innate immunity, can be considered the first pattern recognition molecule to be identified [3].

Another set of acute phase proteins is related to the extracellular matrix (ECM) and tissue repair. These include fibronectin and fibrinogen which, by generating fibrin, provide a provisional matrix (Table 53.2). The production of protease

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Table 53.1 Main modifications of plasma proteins during an acute phase response.

50% increase in concentration
Caeruloplasmin
C1q, C3, and C4
Fibronectin
Serum amyloid P (SAP)
Two- to fourfold increase in concentration
α1 acid glycoprotein
α1 antitrypsin
α1 antichymotrypsin
Fibrinogen
Haptoglobin
Mannose-binding protein (MBP)
C4-binding protein (C4BP)
Hundredfold increase in concentration
C-reactive protein (CRP)
Serum amyloid A (SAA)
Reduction in concentration
Albumin, transferrin, apolipoprotein AI, apolipoprotein AII, alphafetoprotein (AFP), α2 HS glycoprotein

inhibitors is likely a mechanism to limit tissue damage and contribute to the orchestration of repair.

It is important to emphasize that the distinction between antimicrobial PRM and matrix components is a useful oversimplification. Indeed, PRMs interact with matrix components, and matrix components have opsonic activity for microbes and may contribute to defense, as discussed later [4,5].

Having presented a general scenario of acute phase proteins in the perspective of antimicrobial resistance and tissue repair, we will now focus on pentraxins and, in particular, on the long pentraxin PTX3 used as a paradigm for humoral innate immunity.

Table 53.2 General significance of acute phase proteins.

√	Amplification of humoral innate immunity (complement, pentraxins – CRP, PTX3, etc.)
√	Metabolic innate resistance (Fe)
√	Coagulation (fibrinogen)
√	Tissue repair (e.g., fibronectin, fibrinogen)
√	Inhibition of tissue damage (α1 antitrypsin)

53.2 Genes and Proteins

The human *CRP* gene has been mapped in the chromosome 1q23.2 locus and comprises two exons: the first coding for the leader peptide (1–18) and the initial two amino acids of the mature protein (19–20); the second for the remaining 21–224 sequence [6,7]. The expressed protein is made of five identical non-glycosylated subunits, whose tertiary structure is stabilized by an intrachain disulfide bond (Cys36—Cys97), highly conserved among pentraxins [8]. The human *SAP* gene has been identified as close paralog of *CRP* on chromosome 1, where the two genes share the same exon/intron architecture, and code for 51% identical protein sequences. SAP is a plasma glycoprotein that comprises 5 or 10 subunits, which again noncovalently associate into pentameric rings. As opposed to CRP, human SAP carries a single *N*-glycosylation site at Asn32 that is occupied by complex-type oligosaccharide [9]. X-ray crystallography allowed generating 3D atomic models of both short pentraxins in association with small ligands. Based on these, CRP and SAP protomers adopt a flattened jelly-roll topology, analogous to lectins, and expose composite binding sites on opposite sides of the pentamers [9–11]. Moreover, modeling of the SAP/FcγRIIa complex has provided a general structural frame for the interaction of pentraxins with FcRs [12], and highlighted similarities to antibody recognition by these receptors, thus bridging molecular functions of the innate and adaptive immune systems [13].

CRP and SAP are soluble PRMs in the serum. As such, they recognize pathogen-associated molecular patterns (PAMPs) as well as membrane phospholipids and nuclear components (collectively known as danger-associated molecular patterns, DAMPs) that appear on, and are released by, host cells at the sites of injury [14]. Indeed, CRP and SAP can opsonize microbial pathogens and apoptotic cells, and promote their complement-mediated clearance [15]. Their synthesis mainly occurs in the liver and is primarily induced by IL-6, typically as a reaction to microbial challenge or tissue damage. CRP expression has been reported in other tissues than liver; however, extrahepatic sites marginally contribute to the plasma levels of this protein [16]. CRP is a major acute phase protein in humans, with levels ranging from 5 mg/l at baseline up to 1000-fold higher in the acute response [17]. Therefore, since its first report in the 1930s, CRP has found extensive clinical applications as a sensitive yet nonspecific systemic marker of infection, inflammation, and tissue damage [18]. SAP is an acute phase protein in the mouse, whereas its levels in the human serum are constitutively found in the 30–50 mg/l range [19].

The human and murine *Ptx3* genes both localize on chromosome 3 and comprise three exons, which code for the leader peptide, N-terminal domain, and C-terminal domain of the protein, respectively. Proximal promoters of both genes share several regulatory elements, including Pu1, AP-1, NF-κB, SP1, and NF-IL-6. The NF-κB site is required for the transcriptional response of *Ptx3* to TNF-α and IL-1β, while AP-1 mediates its basal transcription [20–22]. In addition to inflammatory cytokines, Toll-like receptor (TLR) agonists, such as microbial moieties (e.g., lipopolysaccharide (LPS), OmpA, lipoarabinomannans) and intact microorganisms, can rapidly induce PTX3 expression in several cell types, including myeloid dendritic cells (DC) and macrophages (Figure 53.1).

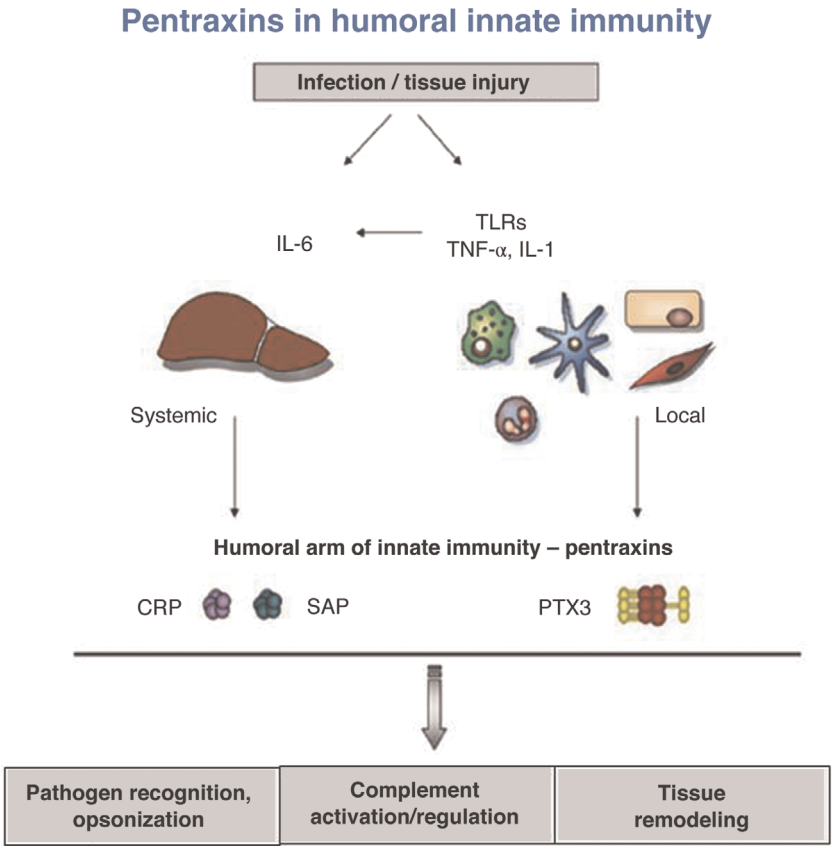


Figure 53.1 Complementary functions of CRP and SAP versus PTX3. The liver is the main source of CRP and SAP and these acute phase proteins are present in huge amounts in the circulation. PTX3 is made mainly in tissues and serves a local defense function.

PTX3 is a homomultimeric glycoprotein made of eight protomer subunits, each containing 381 amino acids, including a 17aa signal peptide [20]. Like other long pentraxins, the PTX3 protein comprises a unique N-terminal region (residues 18–178 in the preprotein) and a C-terminal domain (residues 179–381 in the preprotein) homologous to the short pentraxins (with up to 57% similarity) [23]. The N-terminal region is unrelated to any known protein structure; however, secondary structure predictions suggest that this domain contains coiled-coil elements, which likely drive the quaternary assembly of the protein [24]. Three-dimensional models of the C-terminal pentraxin domain have been generated instead, based on the crystal structures of CRP and SAP, which show this region to adopt a jelly-roll topology, typical of the family [25–27]. Interestingly, the calcium-binding pocket of CRP and SAP is missing in the pentraxin domain of PTX3, which might explain some differences in the binding properties across these pentraxins (i.e., calcium is not required for the interaction of PTX3 with C1q). A single *N*-glycosylation site has been identified in the C-terminal domain of PTX3 at Asn220, which is fully occupied by core-fucosylated and sialylated

complex-type oligosaccharides. This glycosidic moiety modulates the interaction of PTX3 with other soluble PRMs, including C1q [27], factor H [28], and ficolin-1 [29], and is required for recognition of influenza virus [30] and engagement of P-selectin [31]. Biophysical investigations indicate that the PTX3 octamer has an elongated conformation with a large and a small domain interconnected by a stalk region [24]. The rather unique quaternary structure and protein glycosylation status dictate ligands recognition by PTX3 and, ultimately, the biological functions of this prototypic long pentraxin [24,32].

53.3 Microbial Recognition

The first ligand described for CRP was the C-polysaccharide of *Streptococcus pneumoniae* to which CRP binds through phosphorylcholine (PC), a major constituent of C-type capsule polysaccharides. Moreover, CRP binds various microorganisms, including fungi, yeasts, bacteria (e.g., *S. pneumoniae*, *Hemophilus influenzae*, *Salmonella thyphimurium*), and parasites (*Plasmodium*, *Leishmania*) through PC and carbohydrate structures, promoting complement activation, opsonization, phagocytosis, and resistance to infections [33]. Like CRP, SAP binds various bacteria, such as *Streptococcus pyogenes* and *Neisseria meningitidis*, and to influenza virus, playing a protective role [33,34]. SAP binds to LPS, but controversial consequences have been described in SAP-deficient mice challenged in endotoxemia models [35]. SAP also interacts with and neutralizes Shiga toxin 2, the main mediator of severe hemorrhagic colitis and hemolytic uremic syndrome that occurs following ingestion of enterohemorrhagic *Escherichia coli* O157:H7 [36].

PTX3 was originally described as a humoral pattern recognition molecule exerting a protective role in resistance against selected fungal (*Aspergillus fumigatus*) and bacterial (*Pseudomonas aeruginosa*) pathogens [37]. Its nonredundant protective role in innate resistance to infections was extended to *Paracoccidoides brasiliensis* and *Klebsiella pneumoniae* [30,38]. PTX3 was shown to act as an opsonin for bacteria and fungi, facilitating recognition and phagocytosis by myeloid cells in a complement- and FcγR-dependent manner, and by neutralizing virus infectivity [14].

In addition, PTX3 emerged as an essential component of the innate resistance against uropathogenic *E. coli*, playing a nonredundant role in both animal models and humans in urinary tract infections [39]. In particular, PTX3, which is produced locally by the uroepithelium and by infiltrating myelomonocytic cells in a TLR4/MyD88-dependent manner, bound *E. coli* and facilitated the clearance of microbes by phagocytosis, and tuned detrimental local inflammatory responses and tissue damage [39]. PTX3 was also shown to reduce the bacterial load in an infant rat model of meningococcal meningitis by interacting with acapsular *N. meningitidis*, through outer membrane vesicles and selected meningococcal antigens, and acting as an amplifier of humoral responses to this bacterium [40].

Like for SAP, the interaction between PTX3 and certain influenza A viruses occurs through sialic acids expressed on the glycosidic moiety of PTX3 and the viral hemagglutinin (HA) glycoprotein, and results in virus neutralization and

antiviral activity. The specificity of this interaction determines the susceptibility of H1N1, H3N2, and H7N9 strains to the antiviral activities of PTX3 and SAP [30,38]. It has recently been shown that amino acid residues in the vicinity of the receptor-binding pocket of HA are critical determinants of sensitivity to PTX3, and that in a mouse model of infection, PTX3-resistant mutants were responsible of increased virus replication, pulmonary inflammation, and lung injury [41]. In contrast with the protective role in influenza virus infection, PTX3 was shown to interact with arthritogenic alphaviruses (chikungunya virus and Ross River virus) through the N-terminal domain, and to facilitate viral entry and replication during the acute phase of infection, leading to enhanced viral infectivity and prolonged disease [42].

PTX3 genetic variants have been recently described and associated with susceptibility to different infections, in particular pulmonary tuberculosis [43], *P. aeruginosa* infections in cystic fibrosis Caucasian patients [44], *A. fumigatus* infections in patients undergoing hematopoietic stem cell transplantation [45], fungal infections in solid organ-transplanted patients [46], and urinary tract infections [39], thus suggesting the relevance of functional evolutionary conservation of PTX3 in innate immunity. The protective haplotypes are associated with higher protein expression and circulating levels [45,47], although they are still poorly understood molecular mechanisms.

53.4 Complement and FcγR

Pentraxins recognize a number of complement components, and shape activation of this primordial system in a variety of physiopathological settings, including infection, inflammation, and cancer [48].

Both CRP and SAP, in an aggregated or ligand-bound form, interact with C1q, the recognition unit of the classical complement pathway (CCP), and lead to the activation of CCP on pathogens [49]. In addition, CRP can synergize with ficolins in amplifying the effector functions of the lectin pathway (LCP) on bacteria, such as *Salmonella enterica* [50]. The crosstalk between these two protein families might also contribute to the removal of cellular debris, thus subsidizing tissue homeostasis [49]. For instance, when bound to self-antigens (e.g., apoptotic cells, damaged tissue), CRP retains the ability to enhance CCP activation; however, this effect is restricted to the initial stages of the proteolytic cascade, with little deposition of the late components C5–C9 [51]. However, whether these short pentraxins exert protective or pathogenetic roles in Complement-mediated inflammation is still controversial [18,52,53]. Nonetheless, surface-bound CRP engages in a complex with factor H, the major soluble inhibitor of the alternative pathway (ACP), and inhibits ACP deposition [54,55]. In addition, both CRP and SAP bind C4b-binding protein (C4BP), the main soluble inhibitor of CCP and LCP [56,57]. These processes might restrain complement activation and prevent tissue injury, especially where the regulatory functions of factor H and C4BP are impaired, like in the case of factor H mutations and autoantibodies associated with atypical hemolytic uremic syndrome (aHUS) and age-related macular degeneration (AMD) [58].

C1q was the first PTX3 ligand identified [59,60]. This interaction has opposite functional outcomes, depending on settings; when immobilized on a surface, PTX3 induces CCP deposition, whereas it inhibits this cascade in solution, possibly due to competitive blocking of activating sites on C1q [59]. PTX3 also recognizes ficolin-1, ficolin-2, and MBL [29,61,62]. In particular, the association with ficolin-2 or MBL results in a functional cooperation that promotes recruitment of either molecules onto microbial pathogens (i.e., *A. fumigatus*, *Candida albicans*) and LCP amplification [61–63]. Furthermore, similar to the short pentraxins, PTX3 binds factor H and C4BP [28,64]. The interaction with factor H downmodulates ACP by promoting factor H recruitment onto PTX3-coated surfaces and preventing exaggerated complement activation [28]. It has been reported that aHUS-associated mutations within the PTX3-binding region of factor H and factor H autoantibodies inhibit the interaction of factor H with PTX3 [65]. In addition, PTX3 recruits C4BP onto apoptotic cells or ECM, increasing the rate of C4b inactivation and reducing deposition of the lytic C5b-9 terminal complex [64]. Most interestingly, we have recently described that PTX3 can act as an extrinsic oncosuppressor gene by regulating complement-dependent, macrophage-sustained, and tumor-promoting inflammation [66].

Other than complement, both PTX3 and short pentraxins share with antibodies the interaction with FcR. In this regard, SAP and IgGs bind FcγR with similar affinity, compete for common sites on FcγR, and inhibit immune complex-mediated phagocytosis [12]. Thus, pentraxins and antibodies have similar ancestral functions, and activate both complement and FcγR pathways [67].

53.5 Tissue Repair

SAP is a normal tissue protein as well as a plasma protein. SAP binds fibronectin, and is an abundant nonfibrillar component of all amyloid deposits of systemic amyloidosis because it avidly binds to and stabilizes all types of amyloid fibrils. It was recently shown that the administration of a small-molecule drug (CPHPC), which effectively depletes circulating SAP, and reduced SAP from systemic amyloid deposits, followed by a monoclonal anti-SAP antibody, which activates complement- and macrophage-dependent destruction of the SAP-containing amyloid deposits in tissues, safely cleared amyloid deposits and had therapeutic effects both in mice and humans [68,69].

SAP was shown to be involved in wound healing and regulation of fibrosis. In particular, SAP-deficient mice are more susceptible to bleomicin-induced lung fibrosis [70], and SAP was shown to prevent the differentiation of monocytes into fibroblast-like cells (fibrocytes) *in vitro* in part by interacting with Fc*γ*RI and delay normal murine dermal wound healing, through this mechanism [71]. Furthermore, animal studies and human phase 1 and phase 2 clinical trials showed that treatment with SAP decreases fibrosis in chronic fibrosing conditions, such as interstitial pulmonary fibrosis and myelofibrosis [72]. It has recently been shown that SAP plays antifibrotic effects independently from the interaction with Fc*γ*RI [73]. Indeed, in the absence of FcγR, SAP reduced but did not completely inhibit fibrocyte differentiation.

PTX3 plays a nonredundant role in tissue repair and remodeling, but through a different mode of action [5]. In different models of tissue damage (skin wound healing, chemically induced sterile liver and lung injury, arterial thrombosis) PTX3 deficiency was associated with increased clot formation, fibrin deposition, and persistence, followed by increased collagen deposition. In these conditions of damage, macrophages and mesenchymal cells produced PTX3 in response to TLR activation and amplification by IL-1, which localized in the pericellular matrix of macrophages and mesenchymal remodeling cells. PTX3-deficient remodeling cells showed defective pericellular fibrinolysis *in vitro* and defective directional collective migration in the provisional fibrin-rich inflammatory matrix *in vivo*.

The phenotype of PTX3-deficient mice in skin and liver damage was attributed to the interaction of PTX3 with fibrin and plasminogen at acidic pH, occurring *in vivo* in conditions of tissue damage-associated hypoperfusion and hypoxia, and to facilitated plasminogen-dependent fibrinolysis. Upon injury, fibrin acts as a provisional matrix protein, and its timely remodeling plays a major role for subsequent normal repair, as demonstrated by the phenotype of mice with genetic deficiency of components of the fibrinolytic pathway [74]. These data indicate that PTX3-dependent promotion of fibrin-rich inflammatory matrix remodeling ensures a normal tissue repair, providing a novel link between innate immunity, hemostasis, and tissue repair [75].

53.6 Concluding Remarks

Pentraxins are a phylogenetically conserved superfamily of proteins exerting common essential functions in defense from pathogens and in tissue repair. CRP is a widely used biomarker of inflammation in humans; however, lack of a strict evolutionary conservation between mouse and humans has precluded the use of straightforward genetic approaches to explore its functions *in vivo*. On the contrary, gene-targeted mice have allowed defining the functional role of PTX3 in innate immunity and inflammation as a functional ancestor of antibodies. Interaction with microbes and ECM components is a common characteristic for pentraxins, in particular SAP and PTX3 and collectins. In addition, several ECM components (e.g., fibronectin, mindin, osteopontin, and vitronectin) interact with microbes. Among humoral PRM, ficolines and collectins contain ancestral collagen and fibrinogen domains [14]. Altogether, these data suggest the evolutionary link between microbial and ECM recognition in the humoral arm of innate immunity.

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Anti-Inflammatory Cytokines, Soluble Receptors, and Natural Antagonists

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54.1 Introduction

For any successful host response to danger, a careful equilibrium between the inflammatory and anti-inflammatory response is vital to success. In this respect, it should be noted that, as has been suggested by Matzinger, our immune system is more concerned with entities that do damage than with those that are foreign [1]. Thus, a delicate balance is needed in the immune response, wherein pro- and anti-inflammatory processes in response to stress (e.g., trauma, burns, infection, germinating pollen grains, and so on) must be balanced to maintain homeostasis and prevent host damage. Lack of regulation will lead to inflammation and autoimmunity [2]. This chapter reviews recent insights in the anti-inflammatory response offered by the impressive amount of research that has been conducted in this field over the last two decades. The focus is on anti-inflammatory cytokines, soluble receptors, and natural antagonists and their regulation.

54.2 Anti-Inflammatory Cytokines and Soluble Receptors

54.2.1 Regulation of the Activity of IL-1 Family Cytokines

The interleukin (IL)-1 family of cytokines consists of 11 members, including 7 cytokines with agonist activity (IL-1α and β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ), 3 receptor antagonists (IL-1 receptor antagonist [IL-1Ra], IL-36Ra, and IL-38), and an anti-inflammatory cytokine (IL-37) (Table 54.1) [3,4]. Members of the IL-1 receptor (IL-1R) family include six receptor chains forming four signaling receptor complexes: two decoy receptors (IL-1R2 and IL-18 binding protein [IL-18BP]) and two negative regulators (Toll/IL-1R 8 [TIR8] or single-immunoglobulin IL-1R related [SIGIRR], and IL-1R accessory protein b [IL-1RAcPb]). Some of the family members are produced in proforms that need to be cleaved for

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Table 54.1 Interleukin-1 family of cytokines.

Cytokine	Receptor	Coreceptor
IL-1α (IL-1F1)	IL-1R1, IL-1R2	IL-1RAcp
IL-1β (IL-1F2)	IL-1R1, IL-1R2	IL-1RAcp
IL-1Ra (IL-1F3)	IL-1R1	
IL-18 (IL-1F4)	IL-18Rα	
IL-33 (IL-1F11)	ST2 (IL-33R)	IL-1RAcp
IL-36α (IL-1F6)	IL-36R	IL-1RAcp
IL-36β (IL-1F7)	IL-36R	IL-1RAcp
IL-36γ (IL-1F8)	IL-36R	IL-1RAcp
IL-36Ra (IL-1F5)	IL-36R	
IL-37 (IL-1F7)	IL-18Rα	
IL-38 (IL-1F10)	IL-36R	

Italicized family members indicate negative regulators. IL-1R1, type I IL-1 receptor; IL-1R2, type 2 IL-1 receptor; IL-1RAcP, IL-1 receptor accessory protein.

generation of active cytokines. Caspase-1 is important for the cleavage of pro-IL-1β and pro-IL-18 upon inflammasome activation. N-terminal processing is also required for the IL-36 cytokines to generate both agonist and antagonist activities. IL-1α or IL-1β binds first to the type I IL-1R, the ligand-binding chain, followed by recruitment of the coreceptor IL-1RAcP and signaling via the adapter protein MyD88 (myeloid differentiation primary response-88). Multiple mechanisms regulating the activity of IL-1 family members have been revealed, involving decoy receptors, receptor antagonists, and negative regulators. The presence of so many levels of inhibition reflects the physiological need to control the potential damaging proinflammatory effects of the IL-1 system.

IL-36α, IL-36β, and IL-36γ bind to IL-36R and use IL-1RAcP as a coreceptor [3,4]. IL-36 cytokines copy many IL-1 activities and likely contribute to amplification of innate immunity and inflammation in certain tissues, including lung and skin. IL-36Ra shares more than 50% homology with IL-1Ra and acts as a receptor antagonist.

The anti-inflammatory cytokine IL-37 does not have a murine homolog, making functional *in vivo* studies somewhat difficult. Transgenic mice overexpressing human IL-37 display attenuated inflammation during endotoxic shock, colitis, and ischemic heart and liver injury. In accordance, silencing of IL-37 in human monocytes resulted in enhanced lipopolysaccharide and IL-1β induced proinflammatory cytokine release. IL-37 is located in the nucleus where it inhibits gene transcription. Recombinant IL-37 binds with the IL-18Rα chain after which it can deliver an inhibitory signal through TIR8.

IL-38 shares 41% homology with IL-1Ra and 43% homology with IL-36Ra [3,4]. IL-38 likely serves an anti-inflammatory role considering that it binds to the extracellular domain of recombinant IL-36R. IL-38 acts as a partial receptor

antagonist of IL-36R, as illustrated by the finding that IL-38 can attenuate IL-36γ-induced IL-8 release by human peripheral blood mononuclear cells. Genetic association studies have implicated IL-38 in human inflammatory diseases, including the metabolic syndrome, psoriatic arthritis, and ankylosing spondylitis.

The IL-1R family entails two decoy receptors, IL-1R2 and IL-18BP [3,4]. IL-1R2 exists as a transmembrane receptor with a short cytoplasmic tail lacking signaling capacity and a soluble form released after proteolytic processing or alternative splicing, thereby acting as a molecular trap for IL-1. Soluble IL-1R2 exerts additional inhibitory activity by binding pro-IL-1β and blocking its processing by caspase-1. Similarly, cytoplasmatic IL-1R2 interacts with pro-IL-1α precluding its cleavage and activation by different enzymes such as calpain and elastase. Although IL-1R2 can also bind IL-1Ra, it does so at least 100 times less efficiently than IL-1. In addition, IL-1R2 can form a complex with IL-1 and IL-1RAcP, exerting a dominant-negative effect. Soluble IL-1RAcP, generated by alternative splicing, enhances the affinity of soluble IL-1R2 for IL-1, thereby exerting an IL-1 inhibitory effect. IL-1RAcPb is a different form of IL-1RAcP limited to the central nervous system. IL-1RAcPb deficiency results in neuronal damage, suggesting that it may dampen IL-1-induced neurotoxicity. IL-18BP is structurally and functionally similar to IL-1R2. IL-18BP binds mature IL-18 but not pro-IL-18. The human *il18bp* gene encodes four different isoforms (IL-18BPa–IL-18BPd), produced by alternative splicing. Physiologically, IL-18BP is present in blood at a 20-fold molar excess to IL-18, suggesting it is a circulatory safe keeper of systemic IL-18 activity. While IL-18 is an important cofactor for interferon (IFN)-γ production, IFN-γ induces IL-18BP expression, indicating that IL-18BP is part of a negative feedback loop limiting IFN-γ-elicited inflammation.

The IL-1 family contains two receptor antagonists, IL-1Ra and IL-36Ra [3,4]. IL-1Ra binds IL-1R1 with a higher affinity than IL-1, but does not induce cell signaling because it fails to engage IL-1RAcP. Four isoforms of IL-1Ra have been identified, of which one is secreted (soluble IL-1Ra) and three remain intracellularly (intracellular IL-1Ra1-3). Intracellular IL-1Ra isoforms can be released by dying cells or secreted by an unknown mechanism. The anti-inflammatory potency of IL-1Ra is illustrated by children born with IL-1Ra deficiency that suffer from severe inflammation involving the skin, blood vessels, and bone. In accordance, IL-1Ra-deficient mice display arteritis, arthritis, and psoriatic-like skin disease. IL-36Ra acts as a specific receptor antagonist for IL-36R, thereby inhibiting the IL-36-elicited pathway comprising IL-23, IL-17, and IL-22. Recombinant human IL-1Ra (Anakinra) is an approved drug for the treatment of rheumatoid arthritis and autoinflammatory diseases such as cryopyrin-associated periodic syndromes.

TIR8, which is expressed in several tissues, mostly in the kidney, digestive tract, liver, lung, and lymphoid organs, attenuates signaling in response to stimulation of IL-1R family members (IL-1R, IL-18R, and ST2) or Toll-like receptors (TLR1–TLR4, TLR7, and TLR9). The anti-inflammatory effects of TIR8 have been demonstrated in TIR8-deficient mice during experimental infections, kidney ischemia and transplantation, colitis, and autoimmune disorders. Intracellular negative regulators of IL-1R signaling include IRAK-M (IL-1R-associated kinase-M) and MyD88short.

54.2.2 IL-2

IL-2 controls inflammation via various mechanisms. First, IL-2 is essential for maintenance of regulatory T cells in the periphery, and elimination of IL-2 results in autoimmunity [5]. Second, IL-2 regulates inflammation by inhibiting T helper 17 differentiation through inhibition of IL-6-dependent signaling [6]. Third, IL-2 impairs the development of follicular helper T cells, a subset of T cells that regulate humoral immune responses; as an illustration of the functional consequences thereof, IL-2 reduced influenza virus-specific immunoglobulin G1 titers in mice infected with influenza virus [7]. The anti-inflammatory properties of IL-2 may have therapeutic implications, as indicated by two recent studies showing that the administration of low-dose IL-2 can reduce inflammation and improve disease in patients with chronic graft-versus-host disease [8] or hepatitis C virus-related vasculitis [9].

54.2.3 IL-6

IL-6 exerts wide-ranging effects on immune and nonimmune cells and often has hormone-like features that affect homeostatic processes [10]. IL-6 can have pro- and anti-inflammatory properties. Mice with a targeted deletion of the *il6* gene display impaired innate and adaptive immunity to viral, parasitic, and bacterial infection. On the contrary, enhanced IL-6 activity contributes to the maintenance of chronic inflammation such as in arthritis, autoimmune encephalomyelitis, lupus, and plasmacytomas. The IL-6R complex is composed of a ligand-binding portion IL-6R (CD126) and gp130, the signal-transducing β-receptor subunit (CD130). Gp130 also functions as the signaling receptor for several other mediators, including IL-11, IL-27, oncostatin-M, ciliary neurotrophic factor, cardiotrophin-1, leukemia inhibitory factor, and cardiotrophin-like cytokine. IL-6R expression is confined mainly to hepatocytes, leukocytes, and megakaryocytes. A variety of stimuli can induce the shedding of IL-6 from the cell surface, giving rise to soluble IL-6R, which can promote IL-6 signaling.

While IL-6 has been implicated in modulation of many components of the innate immune system, its inhibitory properties include inhibition of the expression of the chemokines CXCL1, CXCL8, and CX3CL1; inhibition of neutrophil accumulation upon TLR stimulation; reduction of influenza virus-induced inflammation; inhibition of nuclear factor (NF)-κB activation in dendritic cells; and induction of IL-1Ra and soluble TNFR type I release. In addition, IL-6 can inhibit the microbicidal activities of macrophages and the production of proinflammatory cytokines. Together, there is ample evidence that besides its clear proinflammatory effects (most notably during the acute innate immune response during infection), IL-6 also directs anti-inflammatory activities necessary for resolution of inflammation.

54.2.4 IL-10

IL-10 belongs to a family of cytokines that further includes IL-19, IL-20, IL-22, IL-24, and IL-26 [11].These cytokines are grouped in a family based on their

similarities with respect to the structure and location of their encoding genes, their primary and secondary protein structures, and the receptor complexes used. Nonetheless, in spite of their resemblances, IL-10 family members possess different biological functions. While IL-10 is a prototypic anti-inflammatory cytokine, IL-19, IL-20, IL-22, IL-24, and IL-26 primarily act on epithelial cells, protecting these from invasion by extracellular pathogens. Some authors also reckon the more distantly related IL28A, IL-28B, and IL-29 to the IL-10 family of cytokines; this latter group of cytokines elicits antiviral responses in especially epithelial cells. IL-10 is produced by almost every type of immune cell. The main cellular sources of IL-10 *in vivo* are monocytes, macrophages, and T cells. The IL-10 receptor is composed of IL-10R1 and IL-10R2. While IL-10R1 is specific for the IL-10 receptor complex, IL-10R2 is also part of the receptor complexes of other ligands (i.e., IL-22, IL-26, IL-28α, IL-28β, and IL-29). IL-10 influences immune responses at multiple levels resulting in efficient inhibition of inflammation. IL-10 confines the immune response during infection, thereby reducing inflammation-mediated injury to the host. IL-10 constrains the production of proinflammatory cytokines and chemokines by dendritic cells, macrophages, and monocytes; impairs antigen presentation by reducing major histocompatibility complex expression; and inhibits phagocytosis. IL-10 can also modulate T-cell functions, for example, IL-10 inhibits the proliferation of proinflammatory T helper 17 cells *in vivo* and also acts on so-called T helper 1 plus T helper 17 cells. The anti-inflammatory properties of IL-10 are particularly clear in the gut. Mice with targeted disruption of the *il10* gene develop colitis after colonization by particular microorganisms [12], and humans with mutations in the genes encoding IL-10 or IL-10 receptor components suffer from the early-onset severe inflammatory bowel disease [13], indicating an essential role for IL-10 in intestinal homeostasis. Treatment with recombinant IL-10 has been evaluated in patients with various inflammatory conditions. While early studies showed promising results in patients with psoriasis and Crohn's disease, larger studies have been unable to confirm these findings. Conceivably, targeted administration rather than systemic injections could be of value in limiting inflammation in patients. Notably, enhanced production of IL-10 has been implicated in the pathogenesis of a variety of diseases, including autoimmune diseases, such as systemic lupus erythematosus, malignancies like melanoma, and infectious diseases such as leishmaniasis.

54.2.5 IL-27 and IL-35

The IL-12 cytokine family consists of four heterodimers, that is, IL-12, IL-23, IL-27, and IL-35 (Figure 54.1) [14]. While IL-12 and IL-23 are considered proinflammatory cytokines, IL-27 and IL-35 have anti-inflammatory properties. IL-27 and IL-35 share the β-chain Epstein–Barr virus-induced gene 3 (EBI3); IL-35 shares its α-chain p35 with IL-12, while IL-27 (together with IL-23) has a unique α-chain (p28 or IL-30). IL-27 and IL-35 are secreted in small quantities by myeloid cells (mainly macrophages and dendritic cells) and regulatory T cells, respectively.

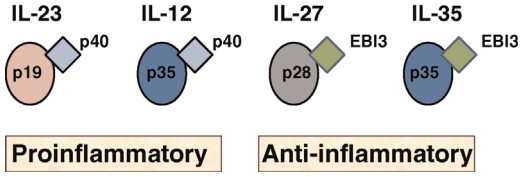


Figure 54.1 Interleukin-12 family of cytokines. The heterodimeric IL-12 family cytokines consist of an α chain (p19, p28, or p35) and a β chain (p40 or EBI3). The p40 chain can pair with p35 or p19 to form IL-12 or IL-23, respectively, while EBI3 can pair with p28 or p35 to form IL-27 or IL-35, respectively. While IL-23 and IL-23 are proinflammatory cytokines, IL-27 and IL-35 exert a variety of anti-inflammatory effects.

The anti-inflammatory function of IL-27 was clearly shown in IL-27R-deficient mice with leishmaniasis, which die from excessive immune responses [14]. Mechanistically, IL-27 converts activated, inflammatory $CD4^+$ T cells into IL-10-producing T cells. IL-27 also precludes the expansion of T helper 2 and T helper 17 cells in various inflammatory conditions. Notably, IL-27 also inhibits IL-2 production, which might hinder the growth of regulatory T cells, thereby contributing to the initiation of colitis in mice. The IL-27 subunit p28 can function as a gp130 antagonist, thereby inhibiting signaling mediated by IL-6 and IL-11. The anti-inflammatory properties of IL-30 are illustrated by its capacity to reduce hepatotoxicity mediated by IL-12, interferon-γ, and concanavalin A.

Knowledge of IL-35 is still limited [15]. IL-35 can mediate the transformation of $CD4^+$ effector T cells into so-called inducible IL-35-producing regulatory T cells ($iT_{reg}35$ cells), a subset of regulatory T cells that express IL-35 but lack expression of Foxp3, TGF-β, and IL-10. $iT_{reg}35$ cells can stop and revert the development of autoimmunity in various mouse models, including autoimmune encephalitis and inflammatory bowel disease. Moreover, ectopic IL-35 expression in pancreatic beta cells averts diabetes, and IL-35 protects against collagen-induced arthritis. IL-35 also controls T-cell-driven immune responses in allergic disease. The signaling pathway of IL-35 is not clear yet.

54.2.6 Transforming Growth Factor-β

Transforming growth factor (TGF)-β belongs to a family with more than 40 members, among which the TGF-β subfamily have six isoforms, of which three are expressed in mammals [16]. TGF-β1 is involved in embryogenesis and has the most important role in the immune system, controlling inflammatory responses, T-cell differentiation, B-cell isotype switching, and tolerance. Many different cell types can produce TGF-β, but it is always produced as an inactive complex that needs to be activated to become functional. As such, activation of latent TGF-β is an essential step in the regulation of TGF-β function. TGF-β triggers cellular signaling via the TGF-β receptor complex, a tetrameric structure composed of two TGF-βRI and two TGF-βRII.

Dependent on the inflammatory context and the presence of cell surface coreceptors, TGF-β can inhibit adaptive immune responses (through induction of regulatory T cells and by suppressing T helper 1 and $CD8^+$ T cells) or enhance

adaptive responses (e.g., through induction of T helper 17 cells). TGF-β1-deficient mice suffer from fatal inflammatory disease mediated by $CD4^+$ and $CD8^+$ T cells, possibly as consequence of autoreactivity. Unlike IL-10, TGF-β is constitutively expressed in many tissues, suggesting a role for this mediator in immune homeostasis. Alterations in TGF-β signaling may contribute to several pathologies, such as cardiovascular diseases, fibrosis, and cancer.

54.2.7 Soluble Cytokine Receptors

Most transmembrane receptors for cytokines also exist in a soluble form, which can be generated by either shedding (as a consequence of proteolytic cleavage) and/or alternative mRNA splicing [17]. Several of these soluble cytokine receptors have already been discussed. Most soluble cytokine receptors function as competitive inhibitors by virtue of their ability to prevent binding of a cytokine to its membrane-bound signaling receptors. Prominent "inhibitory" examples are the type I (p55) and type II (p75) soluble TNFRs. The extent to which a soluble cytokine receptor can inhibit biological activity of its ligand depends on multiple factors, including the concentrations of cytokine and soluble receptor, the ligand affinities of membrane-bound versus soluble receptors, the density of membrane-bound receptors on target cells, and the portion of membrane-bound receptors that need to be occupied to trigger a biological response. Of note, some soluble cytokine receptors have agonistic effects, including soluble IL-6Rs, which can interact with gp130, thereby inducing IL-6-mediated signal transduction and allowing cells that lack IL-6Rα but do express gp130 to respond to IL-6. For some soluble cytokine receptors, including soluble TNFRs, a carrier function has been suggested, which may result in enhanced ligand activity.

54.3 Negative Regulation of Toll-Like Receptor-Mediated Immune Responses

The innate immune system can sense danger via a limited number of pattern-recognition receptors (PRRs). PRRs recognize so-called pathogen-associated molecular patterns (PAMPs), which are conserved motifs expressed by micro-organisms [18,19], or danger-associated molecular patterns (DAMPs), which are endogenous molecules released by cells injured directly by, for instance, bacteria or indirectly as a consequence of, for example, ischemia or necrosis [20]. Prominent examples of PAMPs include lipopolysaccharide, peptidoglycan, lipopeptides (a component of many pathogens), lipoteichoic acid (a cell wall component of Gram-positive bacteria), flagellin (a mobility factor of bacteria), and bacterial DNA. Examples of DAMPs include extracellular matrix components (e.g., fibronectin, hyaluronan, and heparan sulfate), stress-response molecules (e.g., heat shock proteins, high mobility group box-1, histones, and nucleic acids [RNA, DNA]), and immunomodulatory proteins (e.g., calprotectin, β-defensins, surfactant proteins, and uric acid). The main classes of PRRs identified so far include TLRs, C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), and

NOD-like receptors (NLRs) [19]. NLRs are further subcategorized based on differences in the N-terminal domains: NODs are NLRs that recognize common fragments of bacterial peptidoglycan, while the largest NLR group, comprising 14 members, has an N-terminal pyrin domain (PYD) and is therefore called NLRP. In recent years, a growing number of new extracellular and intracellular PRRs have been characterized, including a rising number of cytoplasmic nucleic acid sensors [21].

Extensive crosstalk exists between PRRs in mounting an effective immune response toward eminent danger. Not surprisingly, this reaction has to be tightly regulated. Direct or indirect degradation or destabilization of signal transduction factors are the principal mechanisms that regulate TLR signaling [22,23] (Table 54.2). Transmembrane receptors that act as negative regulators of TLR-mediated inflammation include SIGIRR (TIR8) and IL-1R-like 1 (ST2) and radioprotective 105 (RP105). Cells that lack either one of these receptors respond more avidly to TLR stimulation. In addition, the adapter proteins involved in efficient signaling by TLRs (MyD88, TIRAP [TIR-containing adapter protein]/Mal, TRIF [TIR domain containing adapter inducing IFN-β], and TRAM [TRIF-related adapter molecule]) are target for negative control by several mechanisms. For example, TAG (TRAM adapter with Golgi dynamics domain) is a TRAM variant that competes with TRAM for TRIF binding, thereby inhibiting the TRIF-dependent pathway [24]. TAG also mediates internalization of TLR4 to the endosomes for subsequent degradation. Together with TMED7 (transmembrane emp24 protein transport domain containing 7) TAG disrupts the TRAM–TRIF complex, resulting in inhibition

Table 54.2 Negative regulators of the TLR signaling pathway [23].

Regulator	Affected TLR	Mechanism
sTLR2	TLR2	TLR2 antagonist
sTLR4	TLR4	Blocks interaction of TLR4 and MD2
MyD88s	TLR4	MyD88 antagonist
IRAK-M	TLR4, TLR9	Inhibits phosphorylation IRAK-1
SOCS1	TLR4, TLR9	Suppresses IRAK
NOD2	TLR2	Suppresses NF-κB
PI3K	TLR2, TLR4, TLR9	Inhibits p38, JNK, and NF-κB function
TOLLIP	TLR2, TLR4	Autophosphorylates IRAK1
A20	TLR2, TLR3, TLR4, TLR9	Deubiquitylates TRAF6
SIGIRR	TLR4, TLR9	Interacts with TRAF6 and IRAK
TRAILR	TLR2, TLR3, TLR4	Stabilizes IκBα

IRAK, interleukin-1 receptor-associated kinase; NF-κB, nuclear factor-κB; NOD2, nucleotide-binding oligomerization domain protein 2; PI3K, phosphatidylinositol 3-kinase; SIGIRR, single immunoglobulin interleukin-1-related receptor; SOCS1, suppressor of cytokine signaling 1; sTLR, soluble decoy TLR; TOLLIP, Toll-interacting protein; TRAILR, tumor-necrosis factor-related apoptosis-inducing ligand receptor. (Adapted from Ref. [23].)

of TLR4 signaling from the endosome [25]. SARM (sterile α- and armadillo-motif-containing protein) is another TIR domain containing protein that binds and inhibits TRIF [26]. Moreover, inflammatory signaling can be shut down by ubiquitination, a process during which proteins are "tagged" with ubiquitin for proteasome-mediated degradation. SHP (small heterodimer partner) inhibits TLR signaling by suppressing the ubiquitination of an essential downstream signaling molecule (TRAF6 [TNFR-associated factor 6]) [27]. Consequently, SHP-deficient cells release increased levels of proinflammatory cytokines upon lipolysaccharide stimulation. USP4 (ubiquitin-specific peptidase 4) also negatively regulates TRAF6, by a mechanism that involves removal of polyubiquitin chains. A20 is an essential intracellular negative regulator of TLR signaling, regulating NF-κB activation via multiple mechanisms. A20-deficient mice display spontaneous inflammation in various organs, which is caused by enhanced MyD88-dependent signaling as indicated by the fact that mice with combined A20 and MyD88 deficiencies do not show aberrant inflammation [28]. Other intracellular negative TLR regulators include MyD88 short, TOLLIP (toll-interacting protein), suppressor-of-cytokine signaling (SOCS), IRAK-M, TNF-related apoptosis-inducing ligand receptor (TRAILR), and phosphatidylinositol 3-kinase (PI3K) [23]. In addition, recent work has indicated a fundamental role for microRNAs (miRNAs) in regulating PRR-induced immune responses [29]. Some key soluble negative regulators of the TLR signaling pathway are discussed in the following sections.

54.3.1 Soluble Decoy Toll-Like Receptors

Soluble decoy TLRs are one of the mechanisms by which the biological activities of TLRs are under control. These soluble TLRs compete with TLR agonists and can directly diminish TLR signaling activation and therefore prevent further exacerbation of inflammatory responses [23]. Soluble forms of TLR2 have been described that attenuate the inflammatory response by direct binding to PAMPs; for instance, the extracellular TLR2 domain can directly bind peptidoglycan derived from *Staphylococcus aureus* [30]. Similarly, soluble TLR4 can inhibit TNFα release following lipopolysaccharide stimulation [31]. The mechanism involved in the inhibitory capacity of soluble TLR4 remains to be elucidated; it has been hypothesized that soluble TLR4 blocks the interaction between TLR4 and its coreceptors MD2 and CD14, leading to the termination of signaling [32,33].

54.3.2 Soluble ST2

Soluble ST2 is a decoy receptor that inhibits membrane-bound ST2 signaling. The transmembrane form of ST2L, which is expressed on macrophages, mast cells, and Th2 cells, serves an important negative regulatory function for TLR signaling [23,34,35]. This is illustrated by the fact that mice lacking ST2L are unable to develop endotoxin tolerance [23,34,35]. Of note, IL-33 mediates its biological effects via ST2L, which activates NF-κB and MAP kinases, and drives production of Th2-associated cytokines. Soluble ST2 can bind to IL-33, thereby inhibiting signaling by ST2L [36]; as such sST2 functions as a decoy receptor for

IL-33. Blood concentrations of sST2 are increased in inflammatory and heart disease, and are considered a valuable prognostic marker in both conditions [37]. For instance, in patients with sepsis, sustained elevation of serum soluble ST2 levels correlates with disease severity and mortality [38]. Functional studies showed that administration or overexpression of soluble ST2 attenuates inflammation in mouse models of ischemia/reperfusion injury and allergic lung inflammation [39,40].

54.3.3 Soluble TREM-2

Upon recognition of still unspecified ligands, TREM (triggering receptor expressed by myeloid cells)-1 and TREM-2 recruit and phosphorylate the adapter molecule DAP12 (DNAX adapter protein 12) after which the cellular response is initiated [41,42]. TREM-1 is expressed on neutrophils and subsets of monocytes [43], and has been demonstrated to act as an amplifier of the proinflammatory response *in vitro*, enhancing the TLR response to specific TLR ligands [44]. TREM-2 is primarily expressed on macrophages, dendritic cells, microglia, and osteoclasts [45–48]. In contrast to TREM-1, TREM-2 is thought to act as a negative regulator of inflammatory responses in macrophages and dendritic cells [45,47]. In addition, TREM-2 is involved in phagocytosis [49,50] and killing of bacteria by macrophages [51]. Blocking TREM-2 by a recombinant protein in a mouse model of polymicrobial sepsis showed that TREM-2 was not only required for bacterial clearance but also led to an improved survival [52]. Soluble TREM2 was found to be elevated in cerebrospinal fluid (CSF) of patients with multiple sclerosis and other inflammatory neurological conditions in comparison to patients without inflammatory neurologic disorders [53]. It has been suggested that these elevated levels of soluble TREM2 in patients may inhibit the anti-inflammatory function of the membrane-bound receptor [53].

54.4 Additional Anti-Inflammatory Mechanisms

Neuronal and hormonal mechanisms can also attenuate abundant proinflammatory reactions [54,55]. The so-called neuroinflammatory reflex can inhibit inflammation upon sensory input transmitted through the afferent vagus nerve to the brainstem; this results in stimulation of the efferent vagus nerve and subsequent activation of the splenic nerve in the celiac plexus, norepinephrine release in the spleen, and acetylcholine secretion by a subset of CD4+ T cells. The neurotransmitter acetylcholine diminishes proinflammatory cytokine release by macrophages. The *in vivo* relevance of the neuroinflammatory reflex is illustrated by animal studies, demonstrating that vagotomy renders animals more vulnerable to endotoxin shock, while stimulation of the efferent vagus nerve reduces systemic inflammation in experimental sepsis. Catecholamines can inhibit the production of proinflammatory cytokines primarily via an effect on β-adrenergic receptors. Inflammation can also be inhibited by other hormones (glucocorticoids, vasoactive intestinal peptide, α-melanocyte stimulating hormone) and lipid mediators (e.g., prostaglandin E2).

54.5 Concluding Remarks

Greater understanding of the balance between pro- and anti-inflammatory pathways will increase our understanding of infectious and inflammatory diseases and will pave the way for novel approaches of selective therapeutic manipulation of immune signaling pathways. The delivery of anti-inflammatory cytokines, soluble receptors, or their natural antagonists holds great potential. In this respect, the success story of recombinant IL-1Ra is a prime example. The commercialized version of this anti-inflammatory cytokines is now widely used to treat patients with rheumatoid arthritis, adult-onset Still's disease, and Behcet's disease. As research continues in this field, an improved understanding of these anti-inflammatory cytokine networks will bring renewed approaches to the treatment of a wide range of inflammatory diseases [2].

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Regulatory T Cells

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55.1 Introduction

The concept of immunosuppression mediated by T cells is a remarkable example in the history of science. Originally described in the early 1970s by Gershon and Kondo [1], so-called "T suppressor cells" turned into an area of intense research until the mid-1980s when molecular genetics failed to confirm proposed suppressor factors and receptors, and it became very unpopular to use the "S"-word [2]. One of the major difficulties in those early days of immunology was also the exact definition of T suppressor cells as a distinct cellular subset, and with the discovery of T helper (T_H) 1/2 cells, "suppression" was more and more thought to reflect the activities of counterregulatory cytokines [3]. The use of CD4 and CD25 as defining surface markers and the renaming of T suppressor cells into "regulatory T cells" (T_{REGS}) by one of the pioneers in the field, Shimon Sakaguchi [4], was a key step toward the rediscovery and rehabilitation of these fundamental negative regulators of the immune system. It was also the revival of one of the most significant immunological concepts since Paul Ehrlich coined the term "horror autotoxicus" over 100 years ago and postulated that counterregulatory mechanisms against autoimmunity must exist [5]. Since then, several milestone publications have corroborated the concept of T_{REGS} and introduced a paradigm shift away from central tolerance, highlighting the previously underestimated importance of peripheral tolerance mechanisms: (i) the observation that the absence of a transcription factor, Foxp3, leads to fatal autoimmunity [6–8]; (ii) the finding that Foxp3 expression is highly restricted to

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the T_{REG} compartment [9–12]; (iii) the proof that Foxp3 expression confers suppressive capacity [9,11,12]; and (iv) the direct demonstration in Foxp3-driven diphtheria toxin receptor (DTR) transgenic mice that $Foxp3^+$ T_{REGS} are indeed indispensable for protection from fatal autoimmunity [13–15]. Of note, although other subsets, such as IL-10-producing T_R1 and TGF-β-producing T_H3 cells (reviewed in Ref. [16]), have also been described to possess regulatory capacity, they will not be considered in this chapter as they are less well characterized on a molecular level. Instead, we will focus only on Foxp3-expressing $CD4^+CD25^+$ T_{REGS} as they are currently the best-defined T_{REG} population.

$Foxp3^+$ T_{REGS} can suppress immune responses on several levels, acting, for example, on effector T cells, NK cells, dendritic cells, and other proinflammatory effectors of the innate and adaptive immune system. This may be one of the reasons why there is no unique mechanism of suppression, and many different modes of action have been demonstrated experimentally. Some examples of T_{REG} suppressive functions include competition with effector T cells for IL-2, CTLA-4-dependent suppression of dendritic cell activation, secretion of anti-inflammatory cytokines (TGF-β, IL-10, IL-35), granzyme B-mediated killing, and galectin-1- and cAMP-dependent inhibition (reviewed in Ref. [17]). Although this is still an area of ongoing research, the use of these different mechanisms may depend not only on the target cell, the target organ, and/or the particular disease context but also on specific T_{REG} subpopulations. In fact, more and more subsets of $Foxp3^+$ T_{REGS} have been characterized in recent years which are discussed in Section 55.2. Most T_{REGS} positively selected in the thymus are skewed toward recognizing self (more precisely thymic self-ligands) and are referred to as "natural" or thymic-derived T_{REGS} (tT_{REGS}). T_{REGS} that recognize foreign antigens are induced in the periphery and are therefore termed "induced" or peripherally derived T_{REGS} (pT_{REGS}). These pT_{REGS} play important roles in the maintenance of immune homeostasis at mucosal surfaces and are most prominent in barrier tissues, such as the intestine, where particular mechanisms (e.g., the conversion of vitamin A to retinoic acid) promote the conversion of naïve T cells toward a Foxp3-driven T_{REG} phenotype. We will explicitly concentrate on the molecular events occurring during thymic and peripheral T_{REG} development and their differentiation into highly suppressive tissue-resident effector T_{REGS}, as these molecular checkpoints might have clinical relevance for the translation of basic science into immunotherapy. Apart from a better understanding of the mechanisms that govern the differentiation and function of T_{REGS}, some central questions still remain: Why did we evolve to have T_{REGS} and, most importantly, how can we exploit them therapeutically in the future to treat human diseases? Key gain-of-function events occurred during the evolution of Foxp3 in higher vertebrates, suggesting that T_{REG} development allowed placentation to occur and prevented rejection of the semiallogeneic offspring in mammals [18,19]. The importance of T_{REGS} for the recognition of self-antigens and prevention of autoimmunity is discussed in detail in Section 55.3. We will consider the clinical perspectives of interfering with T_{REG} numbers and function in autoimmunity and cancer. Some of the most interesting approaches include the transfer of T_{REGS}, for example, to prevent graft rejection in graft-versus-host disease (GvHD) or the precise ablation of T_{REGS} to trigger antitumor immunity. Many pathogens causing chronic

infections, on the other hand, may have evolved to trick the immune system by inducing immunosuppressive T_{REGS}, hence permitting immune evasion. Depletion of T_{REGS} could therefore be an interesting therapeutic option to boost cellular immunity against certain microbes, as discussed in Section 55.4. We will focus our overview mainly on recent work where $Foxp3^{DTR}$ transgenic models have been used, as other approaches targeting T_{REGS} less accurately have sometimes led to controversial results. While we are well aware of the differences between mice and men, we still believe that with regard to Foxp3 essential mechanisms and functions are sufficiently well preserved to guide ideas for novel clinical approaches harnessing T_{REGS}.

55.2 T_{REG} Subpopulations

55.2.1 Thymus-Derived versus Peripherally Derived T_{REGS}

T-cell precursors develop in the bone marrow and migrate as uncommitted progenitors to the thymus. Immature thymocytes then undergo differentiation passing through a series of stages from double-negative cells (DN1, DN2, DN3, and DN4) to double-positive (DP) cells and finally single-positive (SP) CD4 and CD8 T cells. SP CD4 or CD8 T cells that were not eliminated by negative and positive selection exit the thymus and populate the spleen, the lymph nodes, and also other organs as naïve T cells. Alternatively, SP CD4 T cells can give rise to "natural" T_{REGS}, more recently redefined as thymus-derived T_{REGS} (tT_{REGS}) [20,21]. While the processes of negative and positive selection are controlled by c-Kit, the SCF receptor, IL-7, Notch signaling, and the TCR itself [22], the developmental pathways that govern tT_{REG} development have only recently begun to be understood. In this sense, it has been proposed that tT_{REG} commitment occurs via a two-step process [23]. The first signaling events are dependent on the TCR and the costimulatory molecule CD28, and lead to the expression of the α and β chains of the IL-2 receptor (CD25 and CD122) as well as to epigenetic changes at the Foxp3 locus [24]. In the second step, expression of Foxp3 is induced in $CD25^{+}Foxp3^{-}$ precursors via an IL-2/STAT5-dependent, but TCR-independent, pathway [23,25]. Signaling via the IL-15R, which shares the β chain and the common γ chain (CD132) with the IL-2R, can also promote Foxp3 expression in $CD25^{+}Foxp3^{-}$ thymocyte precursors, suggesting that IL-15 contributes to tT_{REG} development or, at least, can act as a compensatory mechanism in the absence of IL-2 signaling [26–28].

Since the TCR repertoires of T_{REGS} and conventional CD4 T cells only show a small overlap and T_{REGS} are more resistant to TCR-induced clonal deletion, the current paradigm proposes that tT_{REGS} develop from thymocytes that carry TCRs with "intermediate" affinity between that driving positive and negative selection [29]. Recent evidence, however, suggests that besides the affinity of the interaction between the TCR and the self-peptide/MHC-class-II complex, other factors, including the amount of cognate antigen presented by thymic antigen-presenting cells (APCs) and the number of APCs presenting a specific antigenic complex, determine the tT_{REG} lineage [29]. Of note, tT_{REG} differentiation is, in

part, dependent on Aire-driven expression of self-antigens by medullary thymic epithelial cells (mTECs), suggesting that TCR activation plays a major role in commitment toward this subset [30].

Under defined experimental conditions, $CD4^+Foxp3^+$ T_{REGS} can also be generated in the periphery by antigen-driven conversion of naïve T cells. These *de novo*-induced T_{REGS} (formerly known as induced or adaptive T_{REGS} and now renamed as peripherally derived (p)T_{REGS}) usually recognize environmental antigens such as food- or commensal flora-derived antigens [31,32] and are thus believed to contribute to adaptive tolerance. *In vivo*-generated pT_{REGS} share many characteristics of tT_{REGS}, including the expression of T_{REG} canonical genes, stable Foxp3 expression, and similar epigenetic changes at the Foxp3 locus [33]. As for tT_{REGS}, signaling emanating from the TCR is crucial for pT_{REG} development, although it is believed that generation of pT_{REGS} occurs preferentially following weak and short TCR stimulation, mild costimulation, and in an anti-inflammatory environment [34]. Indeed, while costimulation via CD28 favors commitment toward tT_{REGS} [35,36], it impairs pT_{REG} development [37,38]. Yet once differentiated, T_{REGS} seem to require CD28 expression for proliferation and the maintenance of tolerance [39]. Similar to what is seen for tT_{REGS}, differentiation into pT_{REGS} also requires IL-2. IL-2 upregulates prosurvival signals that prevent apoptosis and maintain cellular fitness, and at the same time it directly acts on the Foxp3 locus via STAT5 [40]. TGF-β, in turn, promotes pT_{REG} formation by engaging the transcription factor Smad3, which acts in cooperation with NFAT to directly promote Foxp3 transcription. TGF-β alone, however, is not sufficient for the stable expression of Foxp3, which is a prerequisite for T_{REGS}' suppressive function and stability, especially when they encounter an inflammatory environment. In addition, the particular genetic signature of T_{REGS} is only partially dependent on Foxp3; other genes such as Eos, IRF4, SATB1, and GATA-3 cooperate with Foxp3 to induce the complete transcriptional program [41,42].

Induction of pT_{REGS} is also influenced by environmental cues [43]. In the gut, metabolites present in the intestinal microenvironment such as retinoic acid, a derivative from vitamin A, favor the TGF-β-dependent generation of pT_{REGS} by attenuating costimulatory signals and promoting histone acetylation at the Foxp3 promoter [44]. Similarly, short-chain fatty acids (SCFA), which are the product of fiber digestion by the gut microbiota, have also been shown to promote pT_{REG} conversion from naïve T cells [45]. Furthermore, defined commensals such as *Bacteroides fragilis* and *Clostridia* have been associated with pT_{REG} induction [46]. Thus, emerging data reveal that pT_{REG} development and function can be shaped by environmental signals, such as nutrients, bacterial products, and host-derived metabolites with the potential to be exploited as immunomodulators [47].

55.2.2 Molecular Mechanisms Underlying T_{REG} Differentiation

From a clinical point of view, understanding which events determine tT_{REG} lineage commitment in the thymus and how these cells retain a stable phenotype under inflammatory conditions will be critical to design strategies aiming to expand stable T_{REGS} for immunosuppression purposes, as in the case of autoimmunity. Furthermore, approaches tending to attenuate or increase T_{REG}

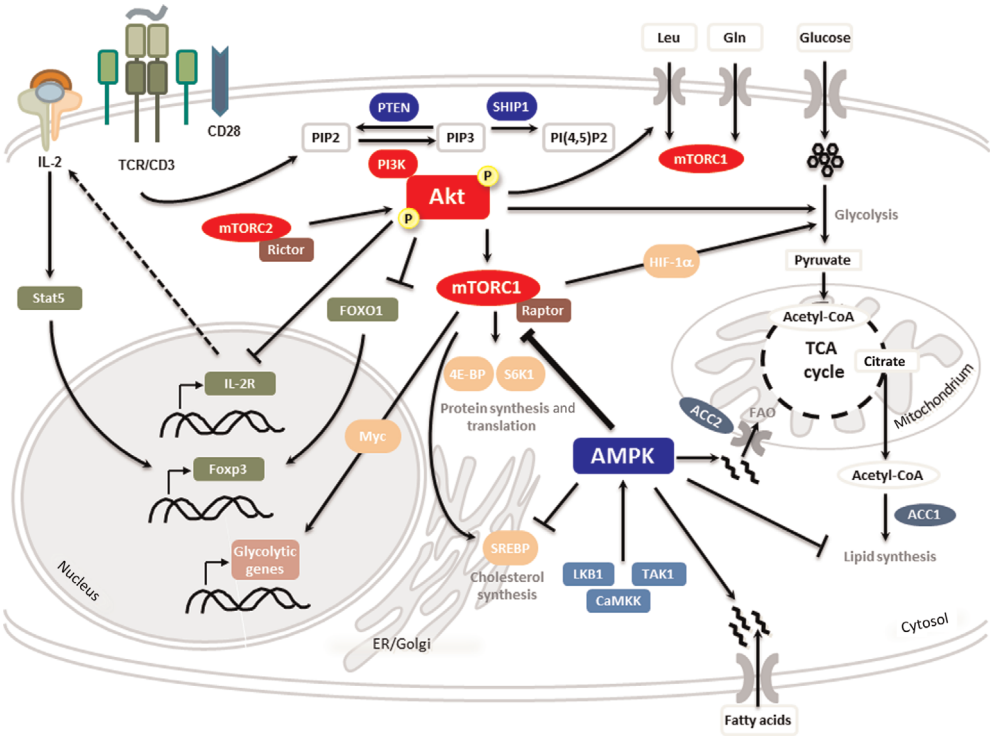


Figure 55.1 Role of activated mTORC1 in several key aspects of T-cell biology.

suppressive activity will profit from a better knowledge of how to discriminate pT_{REGS} from tT_{REGS} in terms of stability, specificity, localization, and function. In this sense, a comprehensive view of the molecular mechanisms required during T_{REG} development will help in designing new strategies and will also be beneficial when considering additional novel applications for drugs previously designed for other medical purposes.

Ligation of the TCR, CD28, or the IL-2R leads to the activation of class IA phosphoinositide 3-kinases (PI3K) and the consequent accumulation of the second messenger phosphatidylinositol-(3,4,5)-triphosphate (PIP3), which serves as a docking platform for proteins containing pleckstrin-homology domains, such as the phosphoinositide-dependent kinase 1 (PDK1) and the serine/threonine kinase protein kinase B (PKB/Akt). Phosphorylation of Thr308 in Akt by PDK1 initiates the mechanistic target of rapamycin complex 1 (mTORC1) cascade via the phosphorylation and consequent inactivation of tuberous sclerosis complex (TSC) (reviewed in Ref. [48]). Activated mTORC1 is then required for several key aspects of T-cell biology, including the integration of cellular and environmental cues for cell proliferation and differentiation [49] (Figure 55.1). How and whether these pathways are differentially regulated during tT_{REG} versus pT_{REG} differentiation is still not known; however, recent evidence suggests that the intensity and/or type of intracellular signals emanating from these receptors in thymocytes and $CD4^+$ naïve T cells might be crucial in determining T_{REG} fate.

Class IA PI3K are heterodimeric kinases composed of a 100 kDa catalytic subunit (p110α, p110β, or p110δ) and a regulatory subunit (p85α, p85β, and p55γ). Studies on the role of class IA PI3K have been hampered by the fact that, except for PI3Kδ, these kinases are ubiquitously expressed, and therefore germline deletion of the p110α or p110β subunit leads to embryonic lethality, while mice with a deletion of either p85α alone [50] or p85α and its alternative splicing variants p55α and p50α die soon after birth [51–53]. RAG-complementation analyses, however, suggest that these mice have impaired B-cell development and function, but normal T-cell compartments [54], similar to mice lacking the p85β subunit [55]. Unfortunately, direct proof for a role of these subunits in T_{REG} differentiation is still missing, as many of the studies mentioned above did not measure Foxp3 directly. More recent data, however, indicate that mice with deletions of both p85α and p85β in T cells only (known as r1ΔT/r2n mice) have reduced T_{REG} numbers in the periphery but not in the thymus and develope spontaneous autoimmunity as they age [56,57]. Similar studies have focused on PI3Kδ, which is mostly restricted to hematopoietic cells, and therefore p110δ knockout mice have a normal life span. While p110δ-deficient mice have no defects in thymocyte development, $p110\delta^{D910A\text{-}D910A}$ mice, which retain the expression of a catalytically inactive subunit, have more T_{REGS} in the thymus but less in peripheral lymphoid organs [58–60]. The differences in these two systems can therefore be attributed to the compensatory expression of other isoforms, as has previously been observed upon deletion of other individual subunits [53,61]. In addition, $p110\delta^{D910A\text{-}D910A}$ mice develop mild inflammatory disease in the intestine, probably due to an inability of T_{REGS} to secrete IL-10 and to suppress autoimmune reactions [60]. Thus, although these data propose that PI3Kδ activity restrains tT_{REG} differentiation, a direct proof for the intrinsic requirement of this kinase and the other class IA PI3Ks for T_{REG} lineage commitment is still missing.

Indirect evidence for a role of PI3K in T_{REG} differentiation and function comes from studies using mice with a genetic deletion of the phosphatases opposing PI3K activity. Phosphatase and tensin homolog (PTEN) catalyzes the conversion of PIP3 to PI(4,5)P2, whereas Src homology 2 domain-containing inositol-5-phosphatase (SHIP1) converts PIP3 into P(3,4)P2. $PTEN^{+/-}$ mice develop autoimmunity due to excessive T_H1 responses and B-cell proliferation, initially associated with resistance to FAS activation-induced cell death [62]. Besides, targeted ablation of PTEN in T cells (using either CD4-Cre or Lck-Cre) leads to fatal thymic lymphoma development, partly attributed to defective thymic negative selection [63,64]. Yet, after thymectomy, these mice are affected by a multiorgan autoimmune disorder, suggesting that PTEN is required to maintain self-tolerance. An interesting question is whether this phenotype is due to the overactivation of the PI3K pathway in conventional T cells or due to an intrinsic defect in the T_{REG} compartment. Interestingly, disruption of PTEN using OX40-cre, which is expressed in tT_{REGS} and also T_H cells upon activation, prevents autoimmunity despite exacerbated T-cell responses, possibly due to the presence of T_{REGS} with normal suppressive function [65]. Recently, mice carrying a specific deletion of PTEN in $Foxp3^+$ cells were generated [66,67]. Although initially having a normal thymic T_{REG} compartment, Foxp3ΔPTEN mice develop a

progressive lymphoproliferative disease due to the loss of $CD25^{+}Foxp3^{+}$ cells over time. Thus, PTEN seems to be required for the stability of Foxp3 because its disruption leads to altered T_{REG} homeostasis and function. Interestingly, a slightly different phenotype is observed in mice with unrestrained PI3K signaling as a consequence of absent SHIP1 expression. Using several mouse models, Collazo *et al.* demonstrated that germline or systemically induced disruption of SHIP1 expression leads to an increase in the proportion, but not the total number of T_{REGS} in the thymus [68]. Furthermore, SHIP-deficient mice show increased numbers of peripheral T_{REGS} that retain normal suppressive function [68,69]. As a consequence, SHIP1 knockout mice do not develop autoimmunity, but instead suffer from a myeloproliferative disorder and are protected from GvHD. In addition, SHIP1 is required for the differentiation of effector T_H cells [69]. Since SHIP1 was believed to terminate PI3K signaling similar to PTEN, it was surprising that deletion of the two phosphatases has differential effects. Evidence now suggests, however, that SHIP may not just act as a repressor of the PI3K pathway, but instead may redirect the signal, recruiting PH-domain containing proteins that bind PI(3,4)P2 [70].

PI3K is believed to restrict T_{REG} development via the Forkhead box family (Foxo) of transcription factors. Phosphorylation of Foxo proteins by fully activated Akt (via mTORC2) prevents translocation to the nucleus and binding to the Foxp3 locus [71,72]. Mice lacking Foxo1 and Foxo3a have reduced numbers of T_{REGS} in the thymus, suggesting a role during tT_{REG} development [72–74]. Similarly, mice with a constitutively active form of Akt develop signs of autoimmune disease [75]. Hyperactivation of Akt blocks canonical genes related to Foxp3 induction and T_{REG} lineage specification such as *Il2ra* and *Ctla-4* [76,77]. Furthermore, nuclear localization of Foxo1 is also pivotal to T_{REG} function [78]. Engagement of the IL-2R in T_{REGS}, but not on effector T cells, maintains PTEN upregulated and prevents activation of PI3K downstream targets such as Akt [69,79,80]. Thus, limited Akt activity is required for efficient T_{REG} induction and stability.

The PI3K/Akt axis has also a central role in the regulation of cell growth and metabolism. While naïve T cells use mostly catabolic processes to obtain energy, the proliferation burst occurring upon antigen encounter shifts T-cell metabolism toward increasing cell biomass. Indeed, quiescent naïve T cells rely on the oxidative phosphorylation (OXPHOS) of glucose, amino acids, and lipids in the mitochondria to generate ATP for their survival, whereas activated T cells obtain energy by the less efficient process of aerobic glycolysis (Warburg effect) that allows the synthesis of biomolecules such as proteins and lipids (reviewed in Refs [81–84]). Notably, T_{REGS} preferentially use fatty acid oxidation and external fatty acid uptake as a process to obtain energy [85,86], but the importance of these pathways for T_{REG} lineage decision is still not known. PI3K can coordinate metabolic changes at different levels. For example, Akt drives glucose metabolism and aerobic glycolysis both at the transcriptional and posttranscriptional levels, by increasing the translocation of glucose and amino acid transporters to the plasma membrane and by augmenting the enzymatic activity of glycolytic enzymes. Furthermore, activation of mTOR, an evolutionarily conserved serine/threonine kinase, is critical for the control of T-cell metabolism and lineage

commitment [49,87]. MTOR exists as two distinct complexes, mTORC1 and mTORC2, both of them being activated within minutes of TCR restimulation. MTORC1 is associated with the scaffolding protein Raptor, and acts via p70 ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor 4E-binding protein (4E-BP) to initiate ribosome formation and increase protein translation. Furthermore, mTORC1 activation sustains the glycolytic response via hypoxia-inducible factor 1 (HIF1-α) and MYC and augments the activity of sterol regulatory element-binding protein (SREBP) transcription factors that are responsible for *de novo* synthesis of cholesterol and fatty acids [88,89]. Although it is widely accepted that Akt is directly required for mTORC1 activation, Navarro and Cantrell have recently proposed that mTORC1 activation depends mostly on amino acid metabolism [90]. TCR activation controls the activation of the leucine transporter Slc7a5 and the glutamine transporter ACST2, thus regulating the availability of these amino acids inside the cells [91,92]. MTORC1 therefore acts by integrating signals from the environment such as nutrient availability with T-cell activation. The second complex, mTORC2, associates with Rictor and is required for full Akt activation in T cells via the phosphorylation of Ser473 in response to cytokines and costimulation. Deletion of mTOR at the DP stage of T-cell development (using CD4-cre), which abrogates the activity of both mTORC1 and mTORC2 complexes, did not disturb naïve or tT_{REG} differentiation [93]. However, engagement of the TCR in naïve cells from CD4ΔmTOR mice promotes spontaneous *in vitro* T_{REG} differentiation. Since repression of Foxp3 expression by Akt is partly counteracted by rapamycin treatment, it has been proposed that mTORC1 activation could prevent T_{REG} differentiation [76,94]. However, rapamycin can also affect mTORC2 under certain circumstances [95] and mice that lack mTORC1 signaling alone (CD4ΔRheb) did not have more T_{REGS} or spontaneous T_{REG} induction [93]. Furthermore, mice lacking Raptor in $Foxp3^+$ cells develop a fatal inflammatory disorder due to the loss of T_{REG} function accompanied by an increase in mTORC2 phosphorylation [96]. Thus, mTORC1 seems to be mainly involved in T_{REG} function by counteracting mTORC2 activity. Likewise, PTEN-deficient T_{REGS} upregulate the activity of mTORC2 and loss of this activity restores their function [67]. Further studies will be therefore required to reconcile these findings with the fact that optimal mTOR activation requires both the activation of the TCR and CD28 costimulation, as occurs during tT_{REG} commitment.

Activation of the TCR also leads to an increase in mitochondrial mass, membrane potential, and mitochondrial DNA (mtDNA) [97]. Part of these effects is mediated by AMP-activated protein kinase (AMPK), a highly conserved serine–threonine protein kinase, implicated in the regulation of mitochondrial function [98]. AMPK is a heterotrimeric complex composed of α-catalytic subunits and regulatory β and γ subunits. Upstream kinases such as the serine–threonine kinase liver kinase B1 (LKB1) phosphorylate Thr172 in the α-subunit in a constitutive manner, whereas upon TCR ligation, AMPK is activated via the Ca^{2+}-calmodulin-dependent protein kinase kinase 2 (CaMKK) [98]. In addition, AMPK activity is regulated by the levels of AMP and ATP found in the cells. Binding of AMP or ADP (signifying low energy) to the γ subunit of the enzyme causes a change in the conformation that allows for phosphorylation of Thr172

linking the activity of AMPK to the energy status of the cells. Activation of AMPK induces catabolic processes in the cells, and at the same time by preventing mTORC1 activation, it inhibits anabolic metabolism, including lipid synthesis (via SREBPs), polysaccharides, ribosomal RNA, and protein synthesis, as well as the induction of HIF-1α. AMPK also directly inhibits ACC1 and ACC2 to prevent lipid synthesis and promote fatty acid oxidation (FAO). We have previously demonstrated that inhibition of ACC1 particularly affects T_H17 development, although it promotes T_{REG} differentiation [85]. Similarly, Michalek *et al.* suggest that T_{REGS} are characterized by the activation of AMPK and the preferential use of FAO as a mechanism to obtain energy [86]. However, whether AMPK activation is a prerequisite for T_{REG} differentiation remains to be defined. Interestingly, AMPK also directly phosphorylates Foxo3 [99] and can modify the transcriptional activity of several genes by altering chromatin structure [100]. Mice deficient in AMPKα1, the main isoform expressed in lymphocytes, displayed normal thymocyte development and peripheral T-cell homeostasis, although primary immune responses are altered [101,102]. Likewise, LKB1-deficient T cells exhibit defects in T-cell activation, glycolytic and lipid metabolism, as well as excessive inflammatory cytokine production. However, ablation of LKB1 in T cells (using Lck-cre) led to severe defects in thymic cellularity and a reduction in peripheral T cells [103], suggesting that LKB1 exerts AMPK-independent functions essential for T-cell development. Although mTORC1 activity was elevated in LKB1- and AMPKα1-deficient T cells, and elevated cytokine production by these T cells could be reversed by rapamycin treatment, no data are yet available on the effect of AMPK or LKB1 deletion on T_{REGS}.

Besides LKB1 and CaMKK, transforming growth factor (TGF)-β-activating kinase 1 (TAK1) also leads to AMPK phosphorylation. TAK1 belongs to the MAP3Ks family and, in addition to the TCR, is involved in IL-2, IL-15, and TGF-β signaling [104]. T-cell-specific TAK1-deficient mice have markedly reduced SP populations in the thymus in both the CD4 and CD8 compartments, with fewer mature T cells in lymphoid organs [104,105]. Despite this, spleens of TAK1-deficient mice are enlarged compared with those of wild-type mice, with a 50% reduction in the number of T_{REGS} due to the lack of tT_{REG} development. TAK1 is required for the TCR-induced expression of members of the TNF superfamily (TNFSF) such as glucocorticoid-induced tumor necrosis factor receptor (GITR), tumor necrosis factor receptor superfamily member 4 (OX40), and member 1B (TNFR2) on tT_{REG} progenitors which participate in fine-tuning TCR signaling [106]. Yet, it remains to be determined which particular signals activate TAK1 and whether its effects are mediated, at least partially, via AMPK.

Despite the vast knowledge accumulated in the last decade of T_{REG} research, many questions are still open regarding the molecular pathways that can control their differentiation, function, and stability. The PI3K/Akt/mTOR pathway is generally considered to be detrimental for T_{REG} development, while PTEN activation and the AMPK axis seem to promote T_{REGS} and prevent excessive inflammation. Recent studies, however, suggest a higher level of complexity. For example, it is difficult to reconcile the idea that PI3K signaling inhibits T_{REG} development with the fact that tT_{REG} differentiation requires more potent TCR signaling than do naïve T cells undergoing positive selection. In addition, many of

the above-mentioned pathways are common to conventional T cells and T_{REGS}. Deletion of particular signaling molecules using conditional knockout mice crossed to Lck- or CD4-cre lines has narrowed the search, although in these mice deletion occurs in all mature thymocytes at the DP stage of development and thus new studies will be required to determine cell intrinsic versus cell extrinsic defects. Similarly, the use of Foxp3-cre lines does not allow any conclusions about these pathways in tT_{REG} differentiation because these events may be upstream of Foxp3 expression itself. Furthermore, more information is needed regarding tT_{REG} precursors as well as better markers to discriminate tT_{REGS} from pT_{REGS}. Helios and neuropilin have been proposed as such markers only recently, but they can be upregulated upon inflammation and their expression levels may therefore change in situations of autoimmune activation. Thymic T_{REGS} are believed to develop from $CD25^+Foxp3^-$ precursors. However, the existence of thymic IL-15-dependent $CD25^-Foxp3^+$ cells with the ability to differentiate into $CD25^+Foxp3^+$ tT_{REGS} in response to IL-2 has recently been described [107]. Moreover, the presence of recirculating T_{REGS} that arrive at the thymus from the periphery should be taken into account when analyzing the contribution of particular pathways to tT_{REG} differentiation [108]. Another important aspect is the role of enzymatic isoforms. For example, PI3K isoform-specific inhibitors have been designed as possible candidate drugs not only against autoimmunity, but also for cancer therapy, and many of them are already being tested in phase II and III clinical trials. The action of such inhibitors in T-cell and, particularly, T_{REG} function should be taken into consideration. Further studies clarifying these questions will help to safely target defined pathways for immune modulation.

55.2.3 Effector T_{REGS} (eT_{REGS})

Stable expression of Foxp3 is the key feature of T_{REGS} and distinguishes them from all other T-cell populations. Yet, the $Foxp3^+$ T_{REGS} that can be identified in the different tissues of the body do not represent a homogeneous group of cells. In addition to their different thymic or peripherally derived origins that have already been discussed, T_{REGS} can be further divided into several phenotypic subsets. The definite phenotype of a given T_{REG} subset is not only closely related to its activation status and suppressive function, but is also determined by its location and is thus a consequence of the environmental conditions that are present in the different lymphoid and nonlymphoid tissues.

In lymphoid organs such as the spleen or the peripheral lymph nodes, most T_{REGS} display naïve T-cell markers, including the homing receptors CD62L and chemokine CC receptor (CCR)7, which allow them to recirculate through peripheral lymphoid tissues [40]. However, a substantial fraction of T_{REGS} in lymphoid tissues shows a surface marker phenotype similar to that of "activated" or effector conventional T cells. Those effector (e)T_{REGS}, while maintaining high expression of Foxp3 and CD25, have downregulated CD62L. In addition, they display high expression of CD44, inducible T-cell costimulator (ICOS), GITR, killer lectin-like receptor subfamily G member 1-positive (KLRG1), and varying expression of markers such as CD69 and CD103 [40,109]. Although the molecular events that lead to the induction of the activated eT_{REG} phenotype are only poorly

defined, recent findings suggest that continuous TCR stimulation is an essential prerequisite to maintain the activated phenotype of T_{REGS} *in vivo* [110]. Transcriptional profiling of T_{REG} populations from different origins and anatomical sites demonstrated that *in vivo*-converted splenic $Foxp3^+CD103^+KLRG1^+$ T_{REGS} display substantial similarity to intestinal lamina propria (LP)-resident T_{REGS}, supporting the notion that the majority of T_{REGS} that reside in nonlymphoid tissues display an activated phenotype that is very similar to the population of eT_{REGS} that is found in lymphoid organs [111].

Besides displaying an activated surface marker phenotype, $Foxp3^+$ eT_{REGS} can co-opt the expression of key transcription factors that are associated with the differentiation and function of conventional effector $CD4^+$ T_H cell lineages. This adaptation leads to further phenotypic and functional heterogeneity that enables T_{REGS} to modulate different types of immune responses at both lymphoid and nonlymphoid tissue sites. $Foxp3^+$ T_{REGS} can selectively upregulate T-bet, considered to be the "master-regulator" of T_H1 differentiation. T-bet promotes the expression of the T_H1-associated chemokine receptor CXCR3 on T_{REGS}, leading to the accumulation of T-bet^+ T_{REGS} at the site of T_H1-mediated inflammation. Consequently, T-bet-deficient T_{REGS} display diminished expression of CXCR3 and fail to rescue Foxp3-deficient mice from T_H1-cell-mediated inflammatory disease [112]. Interestingly, it was demonstrated that IFN-γ produced by effector T cells can induce T-bet expression in T_{REGS} in a STAT1-dependent manner. Yet, IL-12-mediated activation of STAT4, another critical component of T_H1 differentiation, was not functional in T_{REGS}. Thus, by adapting only a part of the molecular T_H1 differentiation program, T-bet^+ T_{REGS} can be generated that effectively modulate T_H1-cell responses, without acquiring proinflammatory effector functions themselves [113]. In addition to T-bet, it was shown that T_{REGS} can also express the T_H2-associated transcription factor GATA-3 [114,115]. Intriguingly, GATA-3-deficient T_{REGS} displayed defects in their ability to suppress inflammatory responses, failed to accumulate at inflamed tissue sites, and even upregulated the expression of inflammatory cytokines. It appears that GATA-3 functions in a large multiprotein complex with Foxp3 to control a set of Foxp3 target genes as well as the expression of high levels of Foxp3 itself [116]. Consequently, GATA-3-deficient T_{REGS} display decreased levels of Foxp3 expression, which may account for the loss of suppressive function in these cells [114,115].

A special subset of eT_{REGS} has been identified in the germinal centers of mice and humans. Similar to their follicular helper $T_{(FH)}$ cell counterpart, these $Foxp3^+$ follicular regulatory $T_{(FR)}$ cells express Bcl-6, CXCR5, and PD-1 [114,117]. It was shown that expression of CXCR5 depends on Bcl-6, and cells lacking Bcl-6 expression did not give rise to T_{FR} cells despite the presence of germinal centers. Lack of T_{FR} cells resulted in enhanced germinal center reactions, including increasing numbers of germinal center B cells, enhanced affinity maturation of antibodies, and differentiation of plasma cells. Together, this indicates that T_{FR} cells play a defined and essential role in the regulation of germinal center reactions.

The expression of STAT3 also seems to have a critical function for the *in vivo* regulatory capacity of T_{REG} cells because Foxp3-specific deletion of STAT3 resulted in a dysregulated, exaggerated T_H17 response and severe intestinal

inflammation [118]. STAT3-deficient T_{REGS} displayed a normal suppressive capacity *in vitro*, but lacked CCR6 expression, resulting in an impaired recruitment of T_{REGS} to the intestine. Neutralization of IL-17 but not IFN-γ prevented colitis induction, indicating that STAT3 expression in T_{REGS} is indeed essential to specifically regulate the T_H17 lineage. Interestingly, it was shown that STAT3–Foxp3 complexes can recruit the enzyme histone acetyl transferase-1, which epigenetically modifies the *il10* promoter, making a space for docking STAT3–Foxp3 complexes to this locus [119]. Thus, STAT3 expression in $Foxp3^+$ T_{REGS} enhanced transcriptional activity of the *il10* locus, which lacks Foxp3-responsive elements and is therefore not directly regulated by Foxp3. In fact, IL-10 is an important immunosuppressive cytokine that is produced by a wide variety of innate and adaptive cells including T_{REGS}. Although not required to suppress systemic autoimmunity, IL-10 plays a critical role in limiting inflammation at epithelial and mucosal barrier sites [120]. Of note, $Foxp3^+IL\text{-}10^+$ cells display a cell surface phenotype characteristic of eT_{REG} cells and are highly enriched in the intestine [121]. Interestingly, T_H17 cells express the IL-10 receptor, and the expansion of this lineage is efficiently controlled by IL-10 [122]. It is thus possible that the colitis phenotype observed in mice with a T_{REG}-specific deletion of STAT3 can be attributed, at least in part, to suboptimal IL-10 production by T_{REGS} [123].

Blimp-1 and IRF4 are two other important factors required for the development and function of eT_{REGS}. IRF4 is a transcription factor with pleiotropic functions in the immune system, and is critically involved in the differentiation of $CD4^+$ T cells into the T_H2, T_H17, and T_{FH} lineages [124]. With respect to its function in T_{REGS}, it was shown that mice carrying a T_{REG}-specific IRF4 deletion develop a T_H2-mediated autoimmune phenotype with enhanced levels of T_H2 cytokines and IL-4-dependent IgG1 and IgE concentrations in the serum [125]. This was not due to reduced amounts of T_{REGS} or a general inability to suppress, as IRF4-deficient T_{REGS} were suppressive *in vitro*. However, IRF4-deficient T_{REGS} lacked ICOS expression and were unable to produce IL-10. Interestingly, a recent report indicated that IRF4-deficient T_{REG} cells also failed to upregulate T-bet, suggesting a broader role of IRF4 for the development of eT_{REG} cells [126]. In the same study, it was also shown that IRF4 interacts with Blimp-1 in the induction of ICOS and IL-10 expression, suggesting that IRF4 and Blimp-1 jointly govern the differentiation and function of eT_{REGS}. These data are in accordance with the notion that eT_{REGS} sorted from splenic $Foxp3^+$ cells on the basis of Blimp-1 expression showed a distinct transcriptional profile consistent with functional differentiation and active suppression [127]. This included the enhanced transcription of IL-10, CTLA-4, granzyme B, the coinhibitory molecule Tigit, and the ectonucleotidase CD39. Expression of T-bet, GATA-3, and RORγt, as well as KLRG1 and genes encoding peripheral tissue homing chemokine receptors and adhesion molecules, was also upregulated in $Blimp\text{-}1^+$ T_{REGS}, further supporting the idea that eT_{REG} cells constitute an activated, highly suppressive population of T_{REG} cells.

Importantly, T_{REG} populations that mirror the characteristics of murine eT_{REGS} have also been identified in humans. Based on the expression of chemokine receptors such as CCR6, CXCR3, and CCR4, several different T_{REG} populations have been found in peripheral blood and showed enhanced expression of

CTLA-4, ICOS, and IL10, as well as factors associated with effector T_H cell lineages, including T-bet, GATA-3, or RORγt [128].

55.2.4 Nonlymphoid Tissue-Resident T_{REG} Cells

In addition to their presence in secondary lymphoid organs, distinct T_{REG} populations have been described in various nonlymphoid tissues of humans and mice, including the skin, lung, liver, intestinal mucosa, adipose tissue, muscle, and placenta (reviewed in Ref. [129]). As mentioned earlier, nonlymphoid tissue-resident T_{REGS} display an activated phenotype and express factors that are characteristic of eT_{REGS}. Yet, the analysis of T_{REGS} in nonlymphoid tissues revealed additional features, such as the expression of certain transcription factors, homing molecules, and surface receptors, that indicate a substantial degree of adaptation to the tissue environment. It has been suggested that most lymphoid tissue-resident eT_{REGS} develop from tT_{REGS}. However, as will be discussed in this section, there is now good evidence that the majority of eT_{REGS} found at mucosal sites such as the intestine are derived from pT_{REGS}. Since the best described examples for nonlymphoid tissue-resident T_{REGS} to date are adipose tissue T_{REGS} and intestinal T_{REGS}, we will concentrate our description on those two populations.

55.2.4.1 Adipose Tissue T_{REGS}

The expansion of visceral adipose tissue (VAT) in obesity is associated with a proinflammatory milieu and enhanced amounts of proinflammatory immune cells, including $CD8^+$ T cells and M1-polarized macrophages. It is believed that this low-grade inflammation in obese VAT contributes to the development of insulin resistance, type-2 diabetes, and fatty-liver disease [130]. More recently, it has been reported that a unique subset of T_{REGS} exists in the VAT of mice. Interestingly, such VAT T_{REGS} were enriched in VAT of lean male mice, but their numbers were strikingly reduced in models of obesity [131]. VAT T_{REGS} were shown to express a range of eT_{REG} markers, including KLRG1, GATA-3, Blimp-1, GITR, CD69 (but not CD103), and high amounts of IL-10 [127,131]. In addition, VAT T_{REGS} expressed also a distinct chemokine receptor pattern that may account for the accumulation in lean VAT: CCR1, CCR3, CCR5, CCR9, and CXCR6, but not CCR6 or CXCR3 [131]. VAT T_{REGS} also displayed a TCR repertoire distinct from lymphoid organ T_{REGS} as well as the local conventional T cells, and the presence of repeated VAT T_{REG} cell TCR clones suggest that VAT T_{REGS} expand due to the recognition of local antigens. T_{REGS} found in VAT also express high levels of the IL-33 receptor ST2 [127]. IL-33 is a cytokine that promotes the development and function of multiple cell types, and is also considered to be an "alarmin" that is produced in response to inflammation and tissue damage [132]. It was shown that IL-33 is essential for the development of VAT T_{REGS} by maintaining their proliferative capacity and by increasing the expression of GATA-3 and Foxp3 [127]. Intriguingly, IRF4 together with BATF was found to cooperate at the *Il1rl1* locus (encoding for ST2) to induce the expression of the IL-33 receptor. Thus, in the absence of either IRF4 or BATF, ST2 expression was not detectable on T_{REGS} and, as a consequence, the

development of VAT T_{REGS} was abolished. Of note, IRF4 was also essential for the expression of another VAT T_{REG}-associated factor: peroxisome proliferator-activated receptor (PPAR)γ. PPARγ, the "master regulator" of adipocyte differentiation, is not only expressed by VAT T_{REGS} but also turned out to be crucial for VAT T_{REG} accumulation, phenotype, and function [133]. In mice carrying a T_{REG}-specific deletion of PPARγ, T_{REG} numbers were decreased in VAT. Furthermore, PPARγ-deficient T_{REG} cells failed to induce the VAT T_{REG}-defining gene expression profile. Interestingly, treatment of $Foxp3^+PPAR\gamma^+$ T cells with PPARγ agonists induced the expression of several factors associated with fatty acid metabolism, including upregulation of the fatty acid transporter CD36 as well as several key enzymes involved in fatty acid metabolism such as Lipe, Scd, Dgat1, and Cpt1a. These findings suggest that the co-option of local metabolic programs may be crucial for the adaptation of tissue T_{REGS}, and further underlines the general importance of the fatty acid metabolism in T cells (reviewed in Ref. [81]). From a therapeutic point of view it is important to mention that *in vivo* treatment with pioglitazone, an antidiabetic drug and agonist of PPARγ, resulted in a strong increase of VAT T_{REGS} in mice fed with either a standard or a high-fat diet. In the latter case, this positively correlated with an improvement of insulin resistance [133]. Thus, targeting the exact molecular and metabolic requirements of VAT T_{REGS} may offer novel ways to interfere with obesity-related pathologies.

55.2.4.2 Intestinal T_{REGS}

The gastrointestinal tract harbors the largest reservoir of tissue-resident T_{REGS} in the body. The gut and its associated lymphoid organs are also a major site for the development of peripheral-induced T_{REGS}, which are of functional importance for maintaining tolerance to food- and microbiota-derived antigens. Many factors contribute to the preferential induction and accumulation of T_{REGS} to the intestine, including food-derived vitamins and their derivatives, as well as the presence of certain commensal bacteria or commensal-produced metabolites such as SCFA, which are derived from the fermentation of dietary fibers (reviewed in Ref. [43]). Not surprisingly, the T_{REGS} found in this environment show several particular adaptations, for example, the expression of the SCFA receptor GPR43, which was shown to be essential for normal T_{REG} homeostasis in the colon [45]. A fraction of colonic T_{REGS} also expresses the IL-33 receptor ST2 together with GATA-3 [134]. Similar to what was observed in VAT T_{REG} cells, IL-33-mediated signaling enhanced Foxp3 expression by activation and recruitment of GATA-3 to the Foxp3 promoter. In addition, it was also shown that IL-33 provides a signal for the accumulation and proliferation of colonic T_{REGS} in response to epithelial damage. Nevertheless, the vast majority of T_{REGS} found in the intestine are negative for GATA-3, but instead express the T_H17 "master" transcription factor RORγt [135–138]. These $ROR\gamma t^+$ T_{REGS} express many eT_{REG}-associated factors, including IRF4, Blimp-1, ICOS, CTLA-4, CCR4, CCR6, CCR9, and IL-10 [135,137,138]. Importantly, the development of $ROR\gamma t^+$ T_{REGS} requires the presence of microbiota, as shown by the fact that germfree mice are completely devoid of this subset [136,137,139]. Indeed, several different bacterial species can induce the induction of $ROR\gamma t^+$ T_{REGS}. Also, feeding mice with a diet

rich in the SCFA butyrate increased the frequency of these cells [136,137]. Intriguingly, it seems that the expression of GATA-3 and RORγt by gut T_{REGS} is mutually exclusive. Since the majority of RORγt$^+$ T_{REGS} in the colon does not express Helios, and therefore is likely to be induced peripherally, it is tempting to speculate that the induction of GATA-3 in tT_{REGS} represents the default pathway, which is counterregulated in the gut by bacterial colonization and induction of RORγt$^+$ T_{REGS}. In line with these considerations, IL-23, a proinflammatory cytokine secreted by intestinal dendritic cells in response to activation by bacteria-derived pathogen-associated molecular patterns, restrained the expansion of GATA-3$^+$ T_{REGS} [134]. In contrast, IL-23 seems to be involved in the selective expansion of RORγt$^+$ T_{REGS} because mice deficient for the IL-23 p19 chain developed significantly fewer RORγt$^+$ T_{REGS} [136]. So far, the exact role of RORγt in T_{REG} function is not clear. In Ref. [136], it was demonstrated that mice with a T_{REG}-specific deletion of RORγt exhibited higher susceptibility toward T_H2-mediated oxazolone-induced colitis, but, on the other hand, were more resistant to infection by the helminth *Heligmosomoides polygyrus*, indicating that loss of RORγt leads to a dysregulation of type 2 responses. Yet, another study demonstrated that chemical blockade of RORγt results in dysregulation of T_H17/T_H1 responses, with mice showing enhanced susceptibility to disease in the T_H17/T_H1-mediated TNBS colitis model [137]. It is therefore possible that the exact role of RORγt$^+$ T_{REGS} is highly context dependent. In any case, RORγt$^+$ T_{REGS} display a very stable phenotype *in vivo* and were also shown to be highly suppressive in a transfer colitis model, suggesting that these cells indeed represent a potent eT_{REG} lineage, being highly specialized to regulate immune responses at intestinal sites [138].

55.3 Control of Immunity to Self-Tissues by Foxp3$^+$ T_{REGS}

55.3.1 Autoimmunity

Antigen receptors are generated through the random and imprecise assembly of V(D)J gene segments during lymphocyte development [140]. In addition, rearranged immunoglobulin genes are further modified by somatic hypermutation and class-switch recombination during antigen-driven B-cell selection in germinal centers [141]. The enormous diversity of antigen receptors that is produced ensures optimal protection from infections through adaptive immunity, but bears the risk of generating self-reactivity. Indeed, the majority of developing human B cells are self-reactive [142]. Moreover, immature T cells are positively selected to recognize self-peptides loaded onto MHC-class-II molecules [143]. Several checkpoints known as central tolerance usually eliminate highly self-reactive lymphocytes before they complete their development and are released into the circulation. This can, for example, be achieved by editing the antigen receptor specificity or by inducing cell death [143,144]. However, autoreactive T and B cells readily escape central tolerance and access the periphery [145,146]. In addition, somatic hypermutation can generate new autoreactive B cells in germinal centers [146,147]. Thus, self-reactive lymphocytes must be controlled

by additional mechanisms of peripheral tolerance to prevent autoimmune diseases [145,148].

The nonredundant function of Foxp3$^+$ T_{REGS} to prevent autoimmunity is best exemplified by rare *Foxp3* mutations that disrupt T_{REG} activity. In humans, those mutations lead to the IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome characterized by multiorgan autoimmunity in newborn boys [6,149,150]. Similarly, male *scurfy* mice harboring a *Foxp3* frame-shift mutation develop a fatal autoimmune disease closely resembling the human IPEX syndrome [7,151,152]. *Scurfy* mice played a historic role in identifying Foxp3, but have also been valuable for studying IPEX pathogenesis, experimental therapies and T_{REG} biology [153–155]. *Scurfy* T_{REGS} exhibit a complete loss of their immunosuppressive function, which can be measured by functional assays *in vitro* [156–159]. The *scurfy* phenotype could be linked to activated autoreactive CD4$^+$ T cells which are sufficient to transfer multiorgan inflammation to RAG-deficient mice in the absence of B cells [156,160,161]. Consistently, interfering with T_H1 CD4$^+$ T-cell effector functions reduces inflammation and prolongs the survival of *scurfy* mice [162]. Moreover, dampening the activation of effector CD4$^+$ T cells through blockade of the CD4 co-receptor ameliorates autoimmunity [161,163]. Although less is known about the cross talk of T_{REGS} with humoral immunity, it has recently been shown that autoantibodies contribute to *scurfy* disease pathology, and the transfer of *scurfy* CD4$^+$ T cells into B-cell competent *nude* mice is sufficient to trigger autoantibody production [164–167]. *Scurfy* mice and/or IPEX patients have been shown to develop antinuclear antibodies (ANA) (including anti-dsDNA, anti-histone, anti-Sm, and anti-RNP-A subtypes), rheumatoid factor, antimitochondrial-, anti-glucose-6-phosphate isomerase-, anti-bullous pemphigoid 180 (BP180)-, anti-keratin-14-, anti-tryptophan hydroxylase 1 (TPH-1)-, anti-autoimmune enteropathy-related 75 kDa antigen (AIE-75)- and anti-villin antibodies [161,164,165,167,168]. This diverse self-reactive response can be explained by increased autoreactive T-cell help in the absence of T_{REGS}. In addition, recent evidence suggests that T_{REGS} directly regulate humoral immunity. One study reported the expansion of autoreactive naïve B cells in IPEX patients, suggesting defective peripheral tolerance [169]. Furthermore, the CTLA-4-dependent regulation of germinal center reactions by a specialized population of T_{FR} was recently reported by several groups [114,117,170–172]. T_{REGS} also seem to inhibit the differentiation of long-lived plasma cells [173]. Thus, T_{REGS} regulate B-cell immunity on several levels. Frequent human autoimmune diseases, such as type I diabetes, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and multiple sclerosis (MS), are caused by an incompletely understood interplay of genetic and environmental factors. However, a partial T_{REG} dysfunction has been discussed to contribute to some of them [174,175]. Notably, expanded autoreactive naïve B cells and the presence of serum autoantibodies are hallmarks of RA and SLE in humans [146,176], similar to IPEX patients and *scurfy* mice. Interestingly, B-cell-deficient *scurfy* mice eventually succumb to death despite a greatly extended life span and reduced inflammation [164], underlining the important pathogenic role of CD4$^+$ T cells. Indeed, *scurfy* mice are protected from disease on the athymic *nude* background [177]. Seminal experiments showed that

autoimmunity is also induced in normal mice after neonatal thymectomy, which simulates transient T_{REG} depletion, as well as in athymic *nude* mice after the transfer of $CD4^+$ T cells depleted of $CD25^+$ T_{REGS} [178].

The identification of Foxp3 as a T_{REG}-specific marker and the development of Foxp3 reporter mice greatly extended our knowledge of this important cell population. $Foxp3^{DTR}$ mice, including bacterial artificial chromosome (BAC)-transgenic DEREG mice developed in our laboratory, proved that the selective depletion of Foxp3$^+$ T_{REGS} in neonates is sufficient to induce the *scurfy* phenotype [13,14]. However, a high degree of T_{REG} depletion or functional inactivation (>90%) is necessary to initiate the *scurfy*-like disease in adult $Foxp3^{DTR}$ BAC transgenic mice [15,179]. This is in good agreement with the finding that a modest wild-type T-cell chimerism of 3–6% is sufficient to rescue *scurfy* mice [180]. These findings could relate to the characteristics of T_{REGS} to sense their niche size and to rapidly compensate for a reduction in T_{REG} numbers and/or suppressive capacity through activation, proliferation, and reduced apoptosis [15,181,182]. This is a particular advantage of the DEREG mouse strain in studying T_{REG} biology [15,183], as adult $Foxp3^{DTR}$ knock-in mice allowing nearly complete T_{REG} depletion develop lethal autoimmunity, notably even after transient DT treatment on a Balb/c background [13,184]. The different mouse models thus allow valuable insights into how T_{REGS} prevent autoimmunity *in vivo*. In addition to the mentioned role of T_{REG} depletion efficiencies, genetic susceptibilities can lower the threshold at which autoimmune diseases occur [15,185]. Additionally, differences may also exist in the depleted T_{REG} repertoire or in environmental factors.

55.3.2 Cancer

Why is there such a high degree of self-reactivity in the lymphocyte repertoire despite the associated risk of developing autoimmunity? On the one hand, autoreactivity may be seen as an erroneous by-product of the adaptive immune system and the price for its highly diverse and protective repertoire. On the other hand, there is a high degree of flexibility in T-cell receptor specificities, and many human antibodies can bind various structurally unrelated antigens including self-antigens [146,186]. Autoreactivity may thus also reflect significant advantages such as the ability to broaden the lymphocyte repertoire or to increase antibody avidity through heteroligation [186,187]. Another intriguing role of autoreactive lymphocytes is the possibility to attack self-tissues that have transformed into tumors. Tumor development is usually prevented by several cell-intrinsic checkpoints. However, it is well accepted that the immune system makes an important contribution to the cancer cell-extrinsic control of tumor development and progression, also known as cancer immunosurveillance [188,189]. Consequently, the circumvention of cancer immunosurveillance has been suggested to be one hallmark of tumor development [189,190], and there is increasing evidence that T_{REGS} are involved in this process. T_{REGS} expand in the peripheral blood of tumor patients and have been shown to accumulate in murine and human tumor tissues [191,192]. Special care needs to be taken in the analysis of clinical data because Foxp3 can also be upregulated in human activated effector T cells, confounding some of the studies that may have to be reanalyzed focusing

exclusively on CD45RA$^-$CD25^{high}Foxp3^{high} T_{REGS} (reviewed in Ref. [193]). Importantly, however, the presence of tumor-infiltrating T_{REGS} often negatively correlates with patient survival [192,194,195] and the adoptive transfer of T_{REGS} inhibits tumor-specific immunity in animal models [196,197]. Thus, certain tumors may harness T_{REGS} to shield themselves from immunosurveillance through various mechanisms [148,192,198,199]. T_{REGS} likely also interfere with clinical attempts to induce tumor regression by vaccination [192,200]. Consequently, an effort has been made to deplete T_{REGS} through anti-CD25 antibodies or CD25-targeted diphtheria toxin (Denileukin diftitox). Yet, clinical responses were limited in animal models and patients with established tumors [201–205]. As effector T cells and many other immune cells express CD25, the true potential of targeting T_{REGS} in cancer remained unclear for many years. Using DEREG mice, we could for the first time demonstrate that the selective depletion of Foxp3$^+$ T_{REGS} has potent therapeutic effects on established B16 melanoma, MCA-induced fibrosarcoma, and B16F10 lung metastases, accompanied by CD8$^+$ T-cell activation and infiltration of tumors [206,207]. Transient T_{REG} elimination also enhanced the efficacy of vaccination against tumor antigens [206,208]. The detrimental role of T_{REGS} in cancer has been confirmed with two independent Foxp3^{DTR} strains and was extended to oncogene-driven cancer models [209–211]. Thus, the therapeutic potential of targeting T_{REGS} in cancer has previously been largely underestimated. Foxp3 is widely accepted as the most selective T_{REG} marker known to date, although some discussion arose concerning Foxp3 expression in other cell types. We, along with others, have found no evidence for Foxp3 expression outside the T-cell lineage, especially in naïve and B16 melanoma-associated macrophages, and in non-hematopoietic cells including epithelial cells [157,212–216]. Therefore, T_{REGS} seem to be a promising target for cancer therapy. Notably, anti-CTLA-4 checkpoint inhibition acts, in part, by depleting T_{REGS} in tumors but also directly on effector T cells [217]. In mice, T_{REG} depletion does not synergize with anti-CTLA-4 or anti-PD-1 therapy, but with tumor irradiation and vaccination highlighting potential new treatment strategies [206,209]. In summary, T_{REGS} clearly contribute to the escape of cancer cells from immunosurveillance and may be an interesting target for future immunotherapy.

55.4 T_{REGS} in the Context of Infections

While regulation of the immune response is necessary to protect the host from autoimmunity and tissue-destructive immunity directed toward commensal bacteria and innocuous antigens, T_{REG}-inducing mechanisms are also used by a large number of pathogens to promote immune evasion and their own survival within the host [218]. The role of T_{REGS} in infection is, however, a double-edged sword: While enhanced T_{REG} activity or T_{REG} numbers almost always correlate with higher pathogenic burden, immunopathology in the infected tissue is generally reduced. This observation led to the hypothesis that, from the host perspective, in certain chronic infections it may even be favorable to live with the pathogen (and high T_{REG} numbers) rather than inducing sterilizing immunity and

tissue damage. This view, however, has been challenged by studies showing that anti-inflammatory T_{REGS} can in certain cases also facilitate effective proinflammatory T_H17-mediated immunity [219,220] as well as the formation of long-term memory [221], suggesting additional levels of complexity on the biology of T_{REGS} during infection.

55.4.1 Viral Infections

The role of T_{REGS} during viral infections is probably the most ambiguous. Coevolution of mammals and viruses has led to an intertwined network with many different outcomes of T_{REG} induction in response to viral infection. Numbers of T_{REGS} and viral load often correlate, which suggests that T_{REGS} may be induced during chronic infection to allow viral immune evasion. One of the most prominent examples is human immunodeficiency virus [222]. Generally, unsuccessfully treated HIV patients present higher T_{REG} frequencies [223], while successfully treated patients show normalized T_{REG} numbers [224]. HIV patient-derived T_{REGS} can dampen both effector and bystander $CD8^+$ T-cell responses *in vitro* [225,226]. Conversely, T_{REGS} can also restrain the activation of effector T cells needed for efficient HIV replication [227], thereby inhibiting early viral spread. Yet, during later stages of the disease, T_{REGS} can block $CD8^+$ T-cell activation – this being one of the main causes of AIDS development [228]. A different controversial example is hepatitis C virus infection, another relevant human chronic disease, where spontaneous recovery correlates with a loss of T_{REG} activity [229], but excess pathology and liver inflammation [230,231].

From an evolutionary point of view, it seems likely that T_{REGS} play beneficial roles during acute infection when the immune pathology induced by the virus is most detrimental to the host. T_{REGS}, through dampening the immune response, might in fact be the major cause for the establishment of infections while, at the same time, protecting the host from immunopathology. This dual role of T_{REGS} has been addressed experimentally in various mouse models. T_{REG} depletion during lymphocytic choriomeningitis virus (LCMV) infection led to a reversal of $CD8^+$ T-cell exhaustion, but caused morbidity and increased weight loss in infected mice. Interestingly, the viral load was not affected [232]. Enhanced immunopathology as a consequence of T_{REG} depletion seems to be a common phenomenon during various viral infections: T_{REG} depletion during mouse MHV coronavirus infection can be fatal [233] possibly because T_{REGS} reduce infection-induced demyelination [234]. In a mouse model of RSV infection, T_{REGS} also dampen the immune response and limit the pathology, but similar to the findings in the LCMV model [232], depletion does not alter viral load [235,236]. Depletion of T_{REGS} during the initial immune response toward HSV-1 in a mouse model of stromal keratitis also causes massively enhanced ocular inflammatory lesions [237,238]. Here, T_{REGS} mainly seem to be protective during the early development of ocular lesions, but not during their progression at later stages [239]. While the aggravated pathology in response to T_{REG} depletion during HSV-1 infection clearly correlates with a stronger immune response, the opposite was observed in a mouse model of HSV-2. Here, surprisingly, T_{REG}

depletion results in an altered cytokine milieu and fewer effector cells at the site of infection resulting in a blockade of protective immunity [240].

T_{REG} depletion during Friend retrovirus (FV) infection has been shown to be exclusively advantageous for the host. T_{REGS} expand in response to both acute and chronic FV infection. Depletion of T_{REGS} using DEREG mice [14] led to reversal of T-cell exhaustion and strong FV-specific CD8 T-cell responses together with diminished viral load, without causing measurable immunopathology [241,242]. Transient depletion of T_{REGS} together with blocking of inhibitory ligands (PD-1 ligand and Tim-3) on $CD8^+$ T cells efficiently lowered the viral load in chronically infected mice beyond the level of any individual treatment strategy [243]. This suggests that inhibitory receptor expression on the effector T cells and suppression by T_{REGS} are, at least in part, nonredundant mechanisms. This is an encouraging finding in relation to the strategy of transient T_{REG} depletion as part of a combinatorial treatment strategy for viral chronic infections.

Importantly, T_{REG} depletion could also strongly enhance short-lived effector T-cell responses to modified vaccinia virus Ankara (MVA; used as live viral vector for vaccinations) and hence presumably also to natural infections with similar characteristics, such as vaccinia virus itself. In a mouse model of MVA infection, it could be demonstrated that T_{REG} depletion during the development of the immune response is absolutely crucial for this effect, which can mediate direct protective immunity. The CD8 memory T-cell pool was, however, unaffected, showing a time-dependent mode of action of the T_{REGS} and arguing for a use of T_{REG} depletion primarily for therapeutic (as opposed to prophylactic) vaccination regimen [244].

Together, the specificity, efficacy, timing, and duration of T_{REG}-depleting therapies seem to be the key factors for the successful treatment of chronic viral infections, while cases of virus-induced immunopathology may even require T_{REG}-expanding approaches to prevent overshooting immune responses from causing tissue damage.

55.4.2 Bacterial Infections

In contrast to the controversial role of T_{REGS} during viral infections, T_{REG} activity during bacterial infection has primarily been proven to be disadvantageous for the host. One possible explanation for the differing roles of T_{REGS} in viral and bacterial infections might be the evolutionarily different levels of host invasion by the two pathogenic classes: While viruses adapt more easily than bacteria, and the human genome, in part, even consists of viral components, nonmucosal tissues within the body are regarded as sterile with respect to bacterial colonization. As a consequence, immune-mediated clearance of bacteria seems a better defined goal, and the main action of T_{REGS} in this task is the termination of a protective immune response. As mentioned in Section 55.2, T_{REGS} can be efficiently induced in the intestine by members of the commensal microbiota and their metabolic products. In line with this, beneficial roles for T_{REGS} have been reported in models of inflammatory bowel disease, where pathology is triggered by immune recognition of commensal bacteria that breach the mucosal

barrier. This section, however, presents the role of T_{REGS} during infections driven by pathogenic bacteria and disregards commensal species that can become pathogenic upon barrier breach, immune suppression, or other extrinsic factors.

One example of pathogenic bacteria that are very efficient at inducing T_{REGS} is *Mycobacteria* spp. In humans, T_{REG} numbers were reportedly elevated in patients infected with *Mycobacterium leprae* [245] and *Mycobacterium tuberculosis* [246,247] and immunity to the latter inversely correlated with T_{REG} numbers. Increases in T_{REG} numbers and function after infection have also been modeled in mice, which showed an induced delay in priming and migration of effector T cells [248]. *Mycobacterium bovis* BCG infection in mice also caused an expansion of pulmonary T_{REGS} [249], and BCG vaccination of mice led to an initial increase in T_{REGS} [250], underlining the importance of studying the role of T_{REGS} in vaccination regimens [218,251]. Surprisingly, in the DEREG model only complete T_{REG} ablation coinciding with autoimmune side effects significantly improved mycobacterial clearance. This illustrates again that during chronic bacterial infection, the risk of autoimmunity seems to be the price we pay for sterilizing immunity [15,252].

On the other hand, in acute bacterial infections such as those caused by *Listeria monocytogenes*, the combination of vaccination and subsequent T_{REG} depletion (day 1 and 2 after vaccination) strongly enhanced protective $CD8^+$ T-cell immunity without obvious side effects [253]. Again, the timing of the T_{REG} depletion might be the critical difference because a decrease in specific $CD8^+$ T-cell memory was observed if T_{REG} depletion was carried out before infection with *L. monocytogenes* (day −1 and day 0) [221]. In this setting, T_{REGS} suppress low affinity responder T cells upon primary infection, and in turn promote the high affinity response, which is associated with the development of functional immune memory.

55.4.3 Fungal Infections

Besides few pathogenic species, fungi comprise many commensal organisms that can cause opportunistic infections in immune-compromised patients. Human T_{REGS} have been shown to expand antigen specifically in response to mucosal fungi (*Aspergillus* spp. and *Candida* spp.), potentially reflecting an important mechanism to suppress allergic asthma [254]. In contrast to their role during most other infections described above, T_{REGS} were exclusively beneficial in a model of *Cryptococcus neoformans* infection. Here, T_{REGS} were shown to suppress pathologic T_H2 responses while promoting T_H1-mediated pathogen clearance [255]. The role of T_{REGS} during *Candida* infection seems to be more complex. Depletion of T_{REGS} leads to improved resistance [256]. However, similar to what is seen in *Listeria* and *Leishmania* infections, T_{REGS} have also been shown to allow the establishment of immune memory, as T_{REG} depletion impaired protection from reinfection [257]. Interestingly, T_{REGS} also promote T_H17 responses against *Candida albicans* during acute infection (as also shown for *Citrobacter rodentium* infection [220]), while they prevent the establishment of T-cell-mediated transfer colitis (a model of inflammatory bowel disease) during later stages of infection [219]. The dynamics of T_{REGS} during the course of

infection are thus an important parameter to take into account when planning the manipulation of T_{REGS} during infection [258].

55.4.4 Parasitic Infections

One of the most important diseases worldwide is malaria, caused by *Plasmodium* spp. As already described above for viral infections, the debate as to whether T_{REGS} are beneficial or detrimental during parasitic infections is ongoing. Parasite load correlates with T_{REG} numbers in humans [259], although it is discussed that a certain threshold of parasitic load is needed to cause the observed increase in T_{REGS} (reviewed in Ref. [260]). Malaria patients were shown to be in a transiently immune suppressed state in response to many unrelated triggers, a so-called "bystander" effect of T_{REG} function [261,262]. Of note, although T_{REG} numbers correlate with infection, the appearance of effector T cells follows, leading to a stable T_{REG}/T effector ratio [260]. T_H1-prone C57Bl/6 and T_H2-prone Balb/c mice differ in their response to T_{REG} depletion during *Plasmodium* infection [263]. In C57Bl/6 mice, anti-CD25-mediated T_{REG} depletion greatly reduced parasitic burden and prevented features of cerebral malaria [264]. While Balb/c mice are generally resistant to the T_H1-driven cerebral malaria, the consequence of the same depletion strategy in these mice was likewise a reduction of the parasitic burden, but accompanied by enhanced features of the disease [265,266]. Interestingly, the T_H2-driving cytokine IL-33 could mediate protection from experimental cerebral malaria in C57Bl/6 mice and depletion of T_{REGS} using C57Bl/6-DEREG mice led to abrogation of this protection [267]. Mouse strain-dependent differences were also found in models of leishmaniosis: blocking IL-10 signaling (one of the major T_{REG}-driven suppressive mechanisms described in this model) in C57Bl/6 mice was shown beneficial due to the enhancement of a clearing T_H1 response [268]. Depletion of T_{REGS} in Balb/c mice could enhance pathogenesis by promoting the predominant T_H2-type immunity [269]. The absence of CD103 in Balb/c mice has, however, been shown to block susceptibility to *Leishmania major* infection, presumably due to inhibition of the T_{REGS} access to infected sites [270]. While enhanced pathogenicity in response to T_{REG} depletion does not seem predominant in *Leishmania* models, loss of long-term immunity to reinfection after initial clearance was observed [268]. It is tempting to speculate that mechanisms similar to those discussed earlier in this chapter for CD8 memory in response to *Listeria monocytogenes* are involved [221].

Schistosoma, such as the trematode helminths that cause Bilharzia, establish long-term chronic infections associated with immune suppression [271]. Studies showed that depletion of T_{REGS} in mice infected with *Schistosoma mansoni* leads to aggravated liver pathology and enhanced destruction of the worm eggs [272]. Infection with filarial nematodes also induces T_{REGS}, and defective T_{REG} activity was shown to correlate with the development of immunopathological symptoms, such as lymphedema and elephantiasis [273]. Using a mouse model of filarial nematode infection, it was established that T_{REG} induction takes place in two distinct waves, of which at least the first wave was necessary for the establishment of the parasite [274]. The third group of helminths is composed of intestinal

nematodes, which also trigger expansion of T_{REGS} at the site of infection, as shown in mouse models (reviewed in Ref. [271]). Systemic numeric expansion in humans could not be detected, but the general immunosuppressed state in infected individuals suggests a qualitative increase in T_{REG} activity [271]. Whether depletion of T_{REGS} during intestinal nematode infection can contribute to expelling of worms is not fully established. T_{REG} depletion in a *Heligmosomoides polygyrus* mouse infection model led to no differences in worm burden, but elevated T_H2 responses [275]. In line with these findings, *H. polygyrus*-induced T_{REGS} were shown to have a suppressive effect on the development of allergic airway inflammation [276]. While parasite burden was not affected by T_{REG} depletion in infections with *Trichinella spiralis* [277], T_{REG} manipulation led to enhanced immunopathology and lowered the overall worm burden in a model of *Trichuris muris* infection [278]. Depletion of T_{REGS} in a *Strongyloides ratti* model also led to enhanced worm expulsion in Balb/c mice (but not in C57Bl/6 mice) [279]. Taken together, T_{REGS} are important players in the host–parasite interaction, and breaking tolerance against the parasite generally leads to a decrease in parasitic burden.

55.5 Clinical Perspectives

With the tremendous amount of knowledge gained in the field of T_{REG} biology over the last decades, we could only review a few of the outstanding studies addressing T_{REG} manipulation in experimental settings. Future clinical perspectives might involve the inhibition of T_{REGS} during vaccination, chronic infection, or tumor therapy. Another example calling for more careful investigation is the role of T_{REGS} in coinfections such as with helminths and malaria or tuberculosis [280], as well as the impact of infection-induced T_{REGS} on vaccination or allergy [281]. More specific drugs will be needed to target these important cells directly *in vivo* – interference with the molecular pathways discussed in Section 55.2 could therefore lead to novel therapeutic avenues and will require intense research activities. The recently documented therapeutic effects of so-called checkpoint inhibition (e.g., anti-CTLA-4/ipilimumab, anti-PD-1/pembrolizumab/nivolumab/MK-3475, anti-PD-L1/MPDL3280A) and chimeric antigen receptor (CAR) strategies have led to a revolution of cancer immunotherapy [192,282–284]. Yet, tools to more selectively target $Foxp3^+$ T_{REGS} in humans are still of great interest with regard to their therapeutic potential and side effects in comparison to available therapies. One promising approach is targeting human effector T_{REGS} by anti-CCR4 antibodies or immunotoxins [285,286]. Various combinatorial approaches of checkpoint/T_{REG} inhibitors, conventional therapies, and/or vaccination strategies will also be an important future question [283,287]. Conversely, the opposite scenario of T_{REG} transfer, expansion, and stimulation could be envisioned when tolerance is defective, for example, during autoimmune diseases or when immunopathology is caused by excessive immune responses to foreign antigens. The first phase I and II clinical trials in GvHD treatment and prophylaxis, type 1 diabetes, MS, Crohn's disease, steroid-resistant asthma, and uveitis are to be initiated or are already ongoing and

some of the results are truly encouraging (reviewed in Ref. [288]). While cellular transfer of T_{REGS} will always be limited by the specific requirements of individualized medicine, including expensive and laborious GMP production steps, new approaches such as the manipulation of metabolic pathways to increase T_{REG} numbers and reduce effector cell responses might be of future importance [47,81].

To conclude, it is evident that T_{REG} manipulation is a double-edged sword: evolution has led to a fine-tuned balance of immunity, immunopathology, and immune tolerance, and these aspects need careful attention when designing putative treatment strategies. More research and careful evaluation of studies using T_{REG} depletion or stimulation as a means of immune engineering are thus necessary for every individual approach.

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56

Leukocyte Reprogramming

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56.1 Introduction

Inflammation is an extremely fast response of the host to any type of sterile or infectious insult. However, the proinflammatory response needs to be rapidly counteracted, even in the maintained presence of activating signals. Indeed, an overzealous inflammatory response, as illustrated by the cytokine storm seen during sepsis, can quickly become deleterious. Many mediators are produced soon after initiation of the inflammatory response to dampen it: anti-inflammatory cytokines, resolving lipid mediators, neuromediators, acute-phase proteins, glucocorticoids, and cross-talk with regulatory T cells (Treg) (Figure 56.1). Some inhibitors can specifically neutralize proinflammatory cytokines (e.g., soluble TNF receptor, IL-1 receptor antagonist (IL-1Ra), IL-36Ra, soluble IL-1 receptor, and IL-18 binding protein). Furthermore, a complex mechanism of cell reprogramming is initiated. This aims to limit the perpetuation of the inflammatory process, while maintaining the capacity of the host to fight the infection, and favoring the resolution of inflammation. First identified as endotoxin tolerance, the process is now recognized to not be specific to endotoxin [1] but to occur following any activation by ligands of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) or NOD-like receptors (NLR). Triggering factors can be pathogen-associated molecular patterns (PAMPs) released by microbes or damage-associated molecular patterns (DAMPs) released by injured or necrotic cells. This process is not a global switch off of all bioactivity but rather an adaptation. The term "reprogramming," first coined by David Morrison, perfectly describes the situation [2].

56.2 Endotoxin Tolerance

In 1946, Paul Beeson (1908–2006) injected rabbits with typhoid vaccine (1.25×10^8 heat-killed *Salmonella typhosa*) on a daily basis [3]. On the seventh

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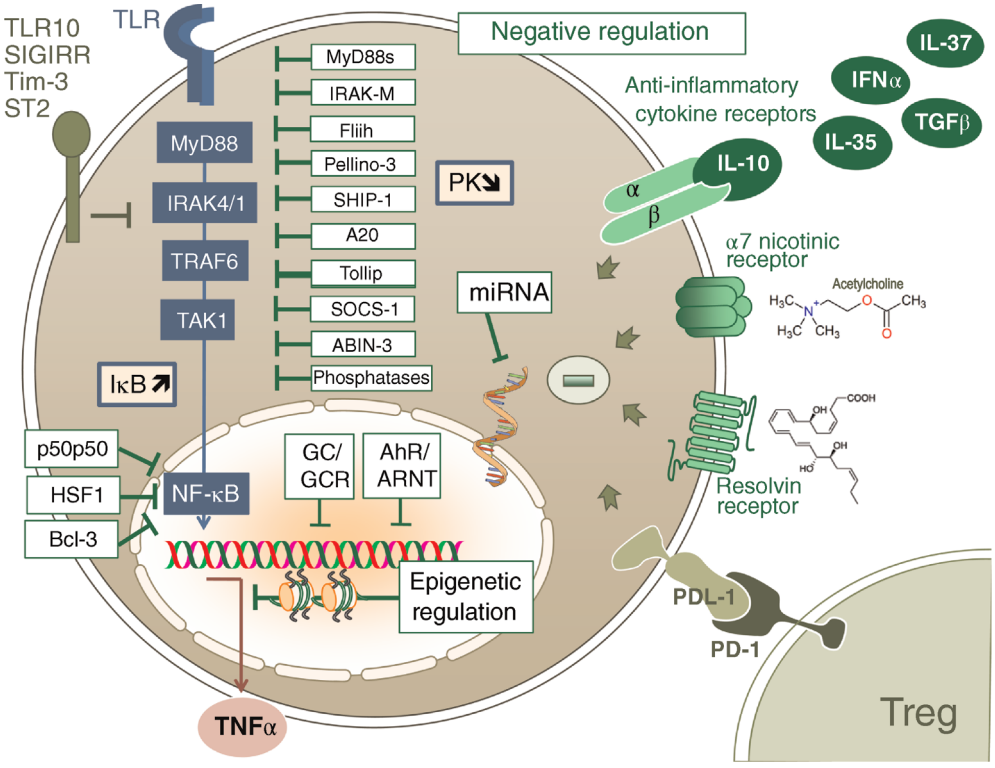


Figure 56.1 Exogenous signals, intracellular negative signaling molecules and intracellular mechanisms aimed to dampen the intracellular inflammatory cascade initiated by TLR ligands, ending to reprogrammed leukocyte.

day, he observed that the rabbits developed a markedly attenuated febrile response as compared to the first day during which they displayed a typical biphasic fever over 8 h following the injection. The next year, Beeson reported that tolerance to bacterial pyrogens was not bacterial strain-specific and could be abolished by blocking the reticuloendothelial system [4,5]. A similar observation on pyrogenicity was obtained using endotoxin instead of whole bacteria and could be conveyed by passive transfer of tolerant plasma [6]. In 1948, Morgan showed that human volunteers receiving repeated iv injection of somatic antigens from *S. typhosa*, *S. schottmuelleri*, and *Shigella dysenteriae* became tolerant as judged by their reduced febrile response [7]. He postulated that the tolerance was reciprocal for all three bacteria.

Pretreatment was soon associated with protection against a second lethal challenge. In 1955, Rowley reported that *Escherichia coli* cell walls (when given either ip or iv 48 h earlier) could protect mice against a lethal infection induced by 2×10^6 *E. coli* ip [8]. The following year, Landy and Pillemer showed that protection of mice could be achieved with the *S. typhosa* endotoxin given 1–3 days before different lethal bacterial infections (e.g., *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *S. typhosa*) [9]. The authors similarly showed that injection of a nonlethal dose of (lipopolysaccharide) (LPS) could protect

against a subsequent lethal dose. In rabbits, endotoxin tolerance was associated with protection against the lethal effect of LPS [10].

Numerous studies have been undertaken to decipher the phenomenon of endotoxin tolerance. Although the process can be fully mimicked *in vitro* with many cell types, macrophages were shown to be the main cells *in vivo* orchestrating endotoxin tolerance. In elegant transfer experiments performed in the LPS-nonresponding strain C3 H/HeJ rendered sensitive to LPS by the injection of normal C3 H/HeN macrophages, Freudenberg and Galanos [11] showed that prior transfer of tolerized macrophage from C3 H/HeN protected the mice against a lethal LPS injection. If one excludes the regulation of endogenous pyrogen, interferon was the first cytokine for which circulating levels were shown to be markedly depressed in LPS-pretreated mice challenged with a virus or LPS itself [12]. In rabbits, endotoxin tolerance was associated with a failure to detect circulating tumor necrosis factor (TNF) after the second challenge [13]. Of note, the diminished release of circulating cytokines is not a global defect: in mice, circulating levels of TNF, IL-6, and interferon-γ (IFNγ) were profoundly abolished by endotoxin pretreatment before an LPS challenge; however, reduction of IL-12, CXCL1, and CCL2 was less pronounced while levels of IL-1β and IL-18 were almost unchanged [14]. The abolished production of circulating TNF in tolerant mice was not observed if the mice were adrenalectomized [15], suggesting a role for glucocorticoids in this *in vivo* phenomenon.

The *in vitro* capacity of LPS to directly affect the capacity of macrophages to produce TNF was first established with cell lines [16,17], rabbit peritoneal macrophages [13], and human monocytes [18]. These observations indicate that even if circulating mediators present in plasma can contribute to the state of tolerance, the process can be intrinsic to the macrophage lineage. However, the phenomenon has been described with most cell types, including mast cells, endothelial cells, dendritic cells, epithelial cells, and fibroblasts.

56.3 Cross-tolerance

The cross-tolerance between endotoxins from different bacterial origins was rapidly recognized [9]. As early as 1957, Zweifach *et al.* [19] used the word cross-tolerance to characterize the finding that rats in whom endotoxin tolerance was induced by repeated doses of LPS became highly resistant to the lethal effects of both drum trauma and hemorrhagic shock. The following year, another cross-tolerance was reported between virus and endotoxin in terms of induction of endogenous pyrogen [20]. The concept of cross-tolerance was then forgotten; indeed, a review published in 1973 considered tolerance to be specific to endotoxin [21]. Rather surprisingly, Sheldon Greisman, the co-author of this review, had published the results of a very important experiment a few years earlier that he did not even dare to mention in the abstract of the paper! Rabbits treated with turpentine administered via different routes were rendered tolerant to the pyrogenic effects of LPS, with a 70% reduction of fever [22]. With the contemporary understanding that sterile inflammation induced by turpentine is associated with the generation of DAMPs, these findings displayed an undeniable

innovation, demonstrating indirectly for the first time the cross-tolerance between DAMPs and PAMPs. Whether the reverse would be possible, that is, an endotoxin injection protecting against a sterile inflammation was addressed in the late 1990s and renamed LPS preconditioning. Such pretreatment with LPS showed protection in various models of ischemia/reperfusion of the heart, brain, neurons, kidney, liver, or lungs, and after myocardial infarction and stroke.

Different stimuli can induce endotoxin tolerance, including alcohol, complete Freund adjuvant, sodium arsenite, phorbol-12-myristate-13-acetate (PMA), heat stress, and exercise. In addition, Gram-positive bacteria (*Corynebacterium parvum*, *Streptococcus pyogenes*, *S. aureus*), bacterial toxin (Shiga toxin-2, Cholera toxin), fungi (*Candida albicans*), mycobacterial lipoarabinomannan, and parasites (*Toxoplasma gondii*, filarial nematode) can also induce endotoxin tolerance. The reverse is also true: LPS can induce tolerance to heat-killed bacteria and zymosan, a fungal glucan [23]. Most importantly, the two main proinflammatory cytokines, IL-1 and TNF, can also induce endotoxin tolerance [24–26] in a synergistic manner [27].

56.4 From Endotoxin Tolerance to PRRs Ligands Tolerance

Most lipopolysaccharides activate cells via the TLR4/MD2 complex, with a contribution from CD14 and, eventually, LPS binding protein (LBP). TLR4 is the only TLR that can initiate two signaling pathways: the MyD88-dependent pathway shared with all TLRs except TLR3, and the TRIF-dependent pathway that is shared with TLR3. Of note, the MyD88 pathway is also shared with the IL-1 and IL-18 receptors. Accordingly, it is not surprising that cross-tolerance exists between most TLR ligands, IL-1 and IL-18.

The cross-tolerance between endotoxin and TLR2 ligands was established with the synthetic lipopeptide Pam3Cys–Ala–Gly even before this activator was recognized to be a TLR2 agonist [28]. Specific TLR2 agonists, such as the mycoplasmal "macrophage-activating lipopeptide-2" (MALP-2) [29], the lipoteichoic acid (LTA) of Gram-positive bacteria [30], *Yersinia* V-antigen [31], *Propionibacterium acnes* [32], fimbriae [33], or *Porphyromonas gingivalis* LPS, a rare endotoxin known to be a TLR2 ligand [34], were also demonstrated to cross-tolerize with LPS.

The cross-tolerance between poly I:C (polyinosinic:polycytidylic acid), a TLR3 ligand, and other TLR ligands led to controversial results. On the one hand, some authors reported that poly I:C pretreatment failed to induce tolerance to LPS [35,36], but could instead prime the response to TLR2 and TLR9 ligands [37]. Surprisingly, pretreatment with a TLR3 agonist tolerized cells when the Lipid A moiety of LPS was employed as the challenge [38]. Other reports showed that poly I:C could tolerize LPS responsiveness both *in vivo* and *in vitro* [39]. LPS and TLR2 agonists induce tolerance to flagellin, a TLR5 ligand, but the reverse seems only true with TLR2 ligands [37,40]. Stimulation via TLR7 tolerizes cells to TLR2 and TLR4 ligands [35,41]. Similarly a cross-tolerance exists between LPS and the oligodesoxynucleotide CpG, which is a TLR9 ligand [42,43]. CpG pretreatment protected against sepsis [44], and against

CpG + galactosamine-induced shock [45]. Of note, when human volunteers were injected with LPS, their peripheral blood cells tested a few hours after were tolerized to TLR2, TLR3, TLR4, TLR5, and TLR7 ligands [46].

Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2 are cytoplasmic PRRs. Muramyl tripeptide (MTP) and muramyl dipeptide (MDP), subunits of peptidoglycan, a constituent of both Gram-negative and Gram-positive bacteria, are NOD1 and NOD2 ligands. MTP pretreatment was protective against bacterial sepsis and LPS-induced shock [47,48], while MDP could induce self-tolerance and cross-tolerance to TLR2 and TLR4 ligands [49]. In contrast, LPS did not tolerize MDP either *in vivo* or *in vitro* [50,51]. Finally, LPS can also desensitize unrelated signaling pathways, as shown by its action prior to CD40 stimulation by CD40 ligand [52].

The demonstration of cross-tolerance between bacterial or synthetic ligands of TLRs is mimicked with DAMPs such as mitochondria [53], high mobility group box 1 (HMGB-1) protein [54], heat shock protein (HSP) [55], myeloid-related protein 8 (MRP8) (S100A8) and MRP14 (S100A9) [56], or fragments of hyaluronan [57].

56.5 Does Endotoxin Tolerance Affect Anti-Infectious Immunity?

As mentioned earlier, endotoxin tolerance was identified in the mid-1950s to protect against subsequent bacterial infection in mice [8,9], rats, and guinea pigs [58]. It was subsequently reported that endotoxin tolerance could induce protection against viral (influenza virus) [59] and fungal infection (*C. albicans*) [60]. More recent studies confirmed that mice pretreated with LPS had enhanced protection against bacterial [61], fungal [62], and viral [63] infections. However, other studies showed that the anti-infectious defenses could be altered [64,65]. These differences could reflect different protocols, timings, dosages, or routes of injection. Enhanced immunity following LPS pretreatment can be due to an increased killing capacity of macrophages [66], increased macrophage resistance to bacterial-induced apoptosis [63], improved bacterial clearance [67], increased formation of neutrophil extracellular traps (NETs) [68], redirection of neutrophils toward the site of infection [69], enhanced capacity of neutrophils to produce hydrogen peroxide [69], and limitation of the cytokine storm [70] that prevents the occurrence of multiple organ injury [71].

56.6 Clinical Relevance

The hyporesponsiveness of human leukocytes to LPS was first reported in 1949 in patients with malaria who displayed a lower febrile response to typhoid vaccine compared to healthy controls [72]. A similar reduced pyrogenic response was also observed in patients convalescing from typhoid fever [73]. The association of malaria with a reduced endotoxin-induced fever was directly demonstrated in

prisoner volunteers inoculated with *Plasmodium cynomolgi* or *P. vivax* before injection with endotoxin [74]. Still assessed in terms of fever, patients with infectious pyelonephritis were shown to be tolerant after a typhoid vaccine injection [75]. The tolerized status of circulating monocytes from sepsis patients was demonstrated *ex vivo* in terms of reduced production of TNF, IL-1α, IL-1β, and IL-6 in response to LPS [76]. Similar tolerant-like status was demonstrated with circulating leukocytes of patients with bacterial, parasitic, or viral infections, after severe surgery, particularly after cardio-pulmonary bypass, pancreatitis, burns, trauma, severe heart failure, acute coronary syndrome, chronic liver failure, liver cirrhosis, cystic fibrosis, and in patients resuscitated after cardiac arrest [77].

The altered immune status of these patients has been qualified in different ways: deactivation, adaptation, desensitization, anergy, refractoriness, immunodepression, immunosuppression, or immunoparalysis. The concept of leukocyte reprogramming most probably appears as the best expression to qualify these various situations after infectious or sterile insults [78]. Of importance, the systemic inflammatory response syndrome (SIRS) and the compensatory anti-inflammatory response syndrome (CARS) are occurring both concomitantly [79] and very soon after the insult [77,80]. The *ex vivo* hyporesponsiveness of the leukocyte to LPS is proportional to the severity of the insult [81] and the clinical severity of the patient [82]. In this study, the authors found an "endotoxin tolerance signature" generated by 99 genes uniquely differentially expressed in endotoxin-tolerant human peripheral blood mononuclear cells in patients with sepsis; this was associated with the severity of the disease in terms of the occurrence of organ dysfunction [82].

56.7 From Tolerance to Reprogramming

Zhang and Morrison [2] employed the word "reprogramming" when they found that release of nitric oxide (NO) and tumor necrosis factor was independently regulated in LPS-tolerized macrophages. Numerous authors have subsequently claimed that not all macrophage functions are extinguished in LPS-tolerant cells. For example, NO production [83], hydrogen peroxide release [82], lipocalin-2 expression [84], MRP8 & MRP14 expression [85], and phagocytosis [86,87] can be enhanced. The concept that endotoxin tolerance was not a global immunosuppression was convincingly demonstrated when Medzhitov's group studied chromatin modification [88]. They demonstrated that on repeated exposure to LPS, one class of gene ("tolerizeable" including inflammatory cytokines) was transiently silenced to prevent the pathology associated with excessive inflammation. The second class of genes (nontolerizeable) includes antimicrobial effectors, which remained inducible to protect the host from infection. That this process is not a global defect is illustrated in sepsis patients whose monocytes display enhanced phagocytosis, antimicrobial activity and capacity to produce matrix metalloproteinase 9 & 14, and vascular endothelial growth factor (VEGF) despite an altered response to LPS in terms of inflammatory cytokine production [89]. This process is not observed in all tissues; indeed, alveolar macrophages

and Kupffer cells of mice undergoing trauma-hemorrhage plus sepsis displayed an enhanced capacity to produce TNF in response to LPS [90]. This finding contrasted with that seen using blood or spleen macrophages. Neither murine alveolar macrophages [91] nor human alveolar macrophages [92,93] could be tolerized.

56.8 Reversal

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFNγ are well-known macrophage-activating factors. They are also the two main cytokines able to counteract the induction of tolerance. To exert their counter-regulatory activity, they can be added either during the first contact with LPS, or added subsequently to LPS preculture or before the second LPS challenge. Addition of IFNγ (but not IFNα or IFNβ) to Mono-Mac-6 cells during the LPS preculture period led to an inhibition of desensitization [94]. Using THP-1 cells, Shi *et al.* mitigated endotoxin-induced tolerance with interferon-α2 [95]. The capacity of IFNγ to prevent endotoxin tolerance was confirmed with human monocyte-derived macrophages [96] and *in vivo* in mice [97]. Not only could GM-CSF and IFNγ prevent the induction of tolerance, but also their pretreatment of human mononuclear cells could prime the responsiveness of the cells [98]. Both cytokines could also reverse the protective effects of endotoxin tolerance in a lethal LPS challenge [99]. These cytokines prevent endotoxin tolerance induced by low, but not high, doses of LPS by inhibiting IRAK degradation and also by promoting its association with MyD88 after a second LPS stimulation. This in turn leads to NF-κB activation and TNF production [100]. The properties of GM-CSF and IFNγ to reverse endotoxin tolerance were further demonstrated in sepsis and trauma patients by their capacity to reverse the acquired status of tolerization [101,102].

56.9 Mechanisms

56.9.1 Anti-Inflammatory Cytokines

As expected, pretreatment of human monocytes by anti-inflammatory cytokines such as IL-10 and TGFβ can mimic endotoxin tolerance [23]. Of note, *in vitro* pretreatment of monocytes by IL-10 requires cells to be adherent to the plastic wells [103]. If this adherence is prevented, either in cultures of whole blood samples or on Teflon, the IL-10 pretreatment can prime the subsequent LPS response. The involvement of IL-10 and TGFβ was fully established by Randow *et al.* [104] when they prevented the induction of LPS tolerance using anti-IL-10 and anti-TGFβ antibodies. In human sepsis, IL-10 was found to be a major functional deactivator of human monocytes [105]. However, IL-10-deficient mice can be rendered fully tolerant to LPS [106] illustrating that one anti-inflammatory cytokine alone cannot explain the whole reprogramming mechanism. Among anti-inflammatory cytokines, IL-1 receptor antagonist (IL-1Ra) behaves differently in its capacity to prevent the induction of LPS-induced tolerance [107]. This observation has to

be associated with the capacity of IL-1α or IL-1β to mimic LPS-induced tolerance [23], and their possible involvement within an autocrine loop after LPS activation.

56.9.2 Glucocorticoids

As previously mentioned, the observation of endotoxin tolerance requires the presence of intact adrenals; glucocorticoid administration restored the capacity of adrenalectomized mice to be rendered tolerant to LPS [15]. This early observation was confirmed by the use of the glucocorticoid receptor antagonist RU486 that prevented LPS tolerance [108]. Similarly, RU486 counteracted the LPS-induced protection against hypoxia-ischemia induced brain damage [109]. LPS activates the hypothalamic–pituitary–adrenal axis, ending in the production of adrenocorticotropic hormone (ACTH) and corticosterone [110]. Endotoxin tolerance also alters the responsiveness of the hypothalamic–pituitary–adrenal axis itself [111]. While glucocorticoids can modulate nearly 2800 genes [112], half of them are induced, including the glucocorticoid-induced leucine zipper (GILZ). Using GILZ KO mice, it was demonstrated that this molecule contributes to endotoxin tolerance [113]. Whether other glucocorticoid-induced molecules play a similar role is not known. Of note, annexin-1, which is known to dampen monocyte activation, was also shown to act through GILZ [114].

56.9.3 Regulatory Signaling Downstream TLRs

TLR dimerization following their interaction with their ligands results in the binding of their Toll-interleukin receptor (TIR) domain to TIR domain-containing signaling adapter proteins, that is, the myeloid differentiation primary-response protein 88 (MyD88) and, eventually, the MyD88–adapter-like (MAL) protein. Their engagement stimulates downstream signaling pathways that involve interactions between IL-1R-associated kinases (IRAKs), the adapter molecules TNF receptor-associated factors (TRAFs), and TGFβ-activated kinase (TAK). An activated IκB-kinase high-molecular-weight complex favors IκB phosphorylation, which is then followed by its ubiquitination and dissociation from nuclear factor-κB (NF-κB). NF-κB then translocates into the nucleus and binds to promoters of numerous genes, favoring their transcription.

Numerous intracellular signaling molecules are upregulated in tolerized cells and act at different levels on the positive signaling cascade to counteract the different players (Figure 56.1). For example, MyD88short (MyD88s), a splice variant of MyD88, termed MyD88 short, competes with MyD88. Once bound it prevents the binding of IRAK4 and the phosphorylation of IRAK1 [115], IRAK-M prevents the formation of IRAK/TRAF6 complex [116,117]. Suppressor of cytokine signaling (SOCS)-1 is a negative regulator molecule of the JAK-STAT signal cascade [118]. SHIP-1 (SH1-containing inositol-5′ phosphatase), which is produced in response to TGFβ, exerts its inhibitory effects primarily through the hydrolysis of phosphatidylinositol 3-kinase [117,119]. A20 accomplished its termination of the TLR response by directly removing ubiquitin moieties from the signaling molecule TRAF6 [117,120]. ABIN-3 (A20 binding

inhibitor of NF-κB activation) inhibits NF-κB activation, downstream of TRAF6 [121]. Pellino-3b deactivates IRAK-1, TBK1, MyD88, and TRIF-dependent signaling cascades [122]. Toll-interacting protein (Tollip), an adapter protein that associates with the cytoplasmic TIR domain of IL-1R, TLR2, and TLR4, potently suppresses the activity of IRAK after TLR activation [123]. Flightless I homolog (Fliih) directly interferes with the formation of the TLR4-MyD88 signaling complex, and acts as a negative regulator [124]. Of note, numerous phosphatases also contribute to leukocyte reprogramming [125] while different kinases are deactivated [126–128].

Some players prevent the binding of the active p50p65 NF-κB heterodimer such as Bcl-3, which is induced by IL-10 [129], p50p50 homodimer [130], and HSF1 [131]. Furthermore, IκB is more rapidly regenerated in endotoxin-tolerant cells [132], reflecting a failure of IκB kinase induction on second exposure to LPS [133]. Negative signals can also be delivered by cell surface receptors such as SIGIRR (single immunoglobulin IL-1R-related molecule) [134], TIM3 (T-cell immunoglobulin domain and mucin domain 3) [135], ST2 [136], and TLR10 [137]. Of interest, some of these negative regulators are upregulated in cells from sepsis or SIRS patients [89,138–143].

56.9.4 Epigenetics

During leukocyte reprogramming, chromatin remodeling, histone acetylation, and methylation occur at the promoter sites of many genes. For example, during LPS tolerance, phosphorylation at serine 10 and methylation at lysine 9 of histone H3 prevent the binding of NF-κB p65 onto the IL-1β promoter [144]. Depending on the nature of the modification, some genes will be tolerized and non responsive to a second stimulus whereas others will not be tolerized [88]. Accordingly, cells display a reduced capacity to produce proinflammatory genes while maintaining their capacity to produce anti-inflammatory, anti-microbial, and healing players. The group of Charles McCall further deciphered the epigenetic mechanism in tolerized cells (see Chapter 38). For example, they showed that H3K9 histone methyltransferase G9a and heterochromatin-binding protein-1 (HP1) cooperatively interact to silence TNF and IL-1β expression [145,146]. Of note, the H3K9 histone methyltransferase G9a is recruited by the stress-response transcription factor, ATF7 [147].

The relevance of the *in vitro* epigenetic analysis has been illustrated in a murine model of sepsis by the observation of a long-term altered histone methylation on the *il12* promoter [148]. In human sepsis, McCall's group found that the NAD^+-dependent sensor, sirtuin 1 (SIRT1), which is known to deactivate RelA/p65 through lysine 310 deacetylation during endotoxin tolerance, was increased in both promoters of TNF and IL-1 of patient leukocytes as compared to healthy controls [149]. Similarly, the expression of H3K4me3, H3K27me3 and H3K9Ac was either increased or decreased in leukocytes from septic patients as compared to healthy controls, depending on the nature of the genes [150].

56.9.5 miRNA

MicroRNAs (miRNA or miR) are regulators of gene expression acting at the posttranscriptional level via an mRNA interference mechanism. miR-146a is

critical for endotoxin tolerance [151]. miR-146a expression gradually increases by 8 h post-LPS exposure in a dose-dependent manner. Transfection of miRNA-146a mimics LPS priming in an LPS tolerance assay while its blockade abrogates LPS-induced tolerance. miR-146a promoted binding of the transcription repressor RelB to the TNF promoters [152]. Furthermore, overexpression of miRNA146 resulted in a marked reduction of IRAK-1, a key signaling molecule downstream of TLR, and attenuated IκBα phosphorylation [153]. miR-146a also represses TRAF6, another adapter kinase downstream of TLR [154]. miR-146a is not only induced by LPS but also by peptidoglycan, Pam3CSK4, flagellin, and IL-1β [154], suggesting an universal role for this miRNA independent of the agonist. Of note, morphine abrogated LPS tolerance and downregulated miRNA146a expression resulting in a persistent inflammation during sepsis [155]. Other miRNAs (miR-125b, miR221, and miR-579) are significantly increased in tolerant cells [156]. Their concurrent inhibition allows TNF mRNA and protein expression in LPS-tolerant cells. These three miRNAs are significantly induced in the macrophages of septic mice. The protein kinase Akt1 is required for the development of endotoxin tolerance. Akt1 positively regulates miRNA Let-7e, which represses TLR4, and negatively regulates miR155 that represses SOCS1, a negative regulator of cytokine signaling. Overexpression of let-7e and suppression of miRNA-155 in $Akt^{-/-}$ macrophages restored their capacity to undergo endotoxin tolerance [157]. Other miRNAs involved in the regulation of cytokines such as IL-10 which contribute to cell reprogramming can be indirectly involved in endotoxin tolerance. For example, miR-98 negatively regulated IL-10 and endotoxin tolerance [158].

56.10 Macrophage Plasticity

Charles Mills discovered that macrophages could be subdivided into two subsets: M1 and M2. M1 cells are involved in the fight against pathogens whereas M2 cells contribute to the healing phase of inflammation. M1 are characterized by the processing of arginine by inducible nitric oxide synthase (iNOS) to generate NO, while M2 are characterized by processing of arginine by arginase leading to the production of ornithine [159]. Others authors established that LPS or IFNγ was favoring M1, while M2 was induced by IL-4, IL-13, or IL-10 [160]. M1 and M2 produce different subsets of chemokines, and express different cell surface receptors [160]. This is an oversimplification since the gene transcription profile in response to IFNγ or IL-4 is greatly influenced by the compartment from which the macrophages are derived [161]. Endotoxin tolerance was associated with an M2 polarization [162,163]. Of note, for cells recovering from endotoxin tolerance, a unique hybrid status was reported [164]. Some gene expressions had returned to their initial status, some were expressed as in tolerized cells, while others were upregulated. Although the duration of this new profile was not addressed, this observation fits with the new concept of trained innate immunity, which associates a type of immunological memory for innate cells such as macrophages and NK cells [165]. In macrophages, changes in expression of PRRs and cytokines following molecular modification (epigenetic changes, different miRNA

expression . . .) contribute toward priming the cells during their encounter with a new pathogenic stimulus.

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57

Roles of Specialized Proresolving Lipid Mediators in Inflammation Resolution and Tissue Repair

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57.1 Resolution of Acute Inflammation: Identification of Specialized Proresolving Mediators

Acute inflammatory responses are necessary for survival because they protect against exogenous pathogens that disrupt host homeostasis. In contrast, inappropriate activation of the immune system by inert stimuli or endogenous neoepitopes can lead to persistent chronic inflammation and can promote tissue fibrosis and dysfunction (Figure 57.1a) [1]. Indeed, chronic unresolved inflammation is classically associated with many chronic diseases, including asthma and neurological degenerative disorders, metabolic diseases, arthritis, and periodontitis [2-5].

The acute inflammatory response is divided into an initiation phase and a resolution phase [6]. The initiation phase is accompanied by the cardinal signs of inflammation known to ancient civilizations as heat, swelling, and pain [1] with eventual loss of function that are controlled for the most part by local chemical autacoids. These include proteins (cytokines, chemokines) as well as lipid-derived mediators (e.g., eicosanoids) that form chemical gradients to regulate leukocyte trafficking into the injured tissue. Polymorphonuclear neutrophil (PMN) infiltration and monocyte/macrophage accumulation ensues as a characteristic sequence of events during the initiation of the acute inflammatory response (Figure 57.1a). Once in tissues, these phagocytes contain invading microbes and clear apoptotic cells. Without an appropriate termination and clearance of phagocytes from the injured tissue, their continued presence is associated with collateral tissue damage, as is observed in chronic inflammation [5]. Hence, controlling the infiltration of leukocytes, as well as promoting their clearance from tissues, could attenuate nonresolving chronic tissue inflammation and is a characteristic feature of active resolution of acute inflammation.

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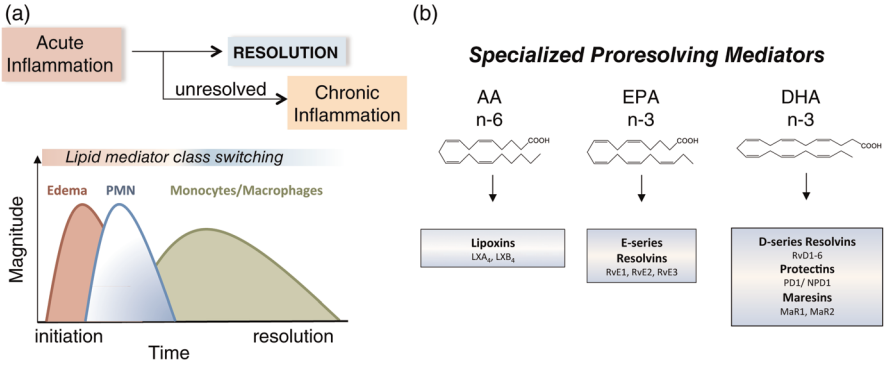


Figure 57.1 Role of specialized proresolving lipid mediators in resolution of acute inflammation. (a) Diagram of the acute inflammatory response, highlighting the initiation and resolution phases and that distinct lipid mediators are associated with each phase. (b) Polyunsaturated fatty acids serve as precursors to the biosynthesis of specialized proresolving mediators. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

The resolution of inflammation and return to homeostasis was previously thought to occur via dissipation of initiating chemotactic signals during the acute inflammatory response [1]. Indeed, several intrinsic negative feedback systems blunt production of inflammatory mediators, such as transcriptional repressors, including activating transcription factor 3 (Atf3) [7]. However, substantial evidence now shows that resolution of contained inflammatory exudates is regulated by temporal biosynthesis of novel chemical mediators during the resolution phase [6]. These proresolving mediators not only counter-regulate inflammatory gene transcription, but also directly limit excessive PMN migration (e.g., downregulate adhesion receptors) and *stimulate* distinct cellular processes, such as macrophage uptake of apoptotic PMN, microbes, and cellular debris, that are required for tissue homeostasis to be reestablished [8-11]. These findings raised the possibility that failed resolution of an inflammatory challenge could potentially lead to recurring bouts of persistent tissue inflammation and diseases associated with chronic inflammation, as well as that proresolving mediators could be a new class of targeted therapeutics [6,12]. Using a systems approach with LC-MS/MS-based analysis of self-limited inflammatory exudates (ones that resolve to homeostasis on their own) new families of specialized proresolving mediators (SPM) were identified. These SPM comprise four families, the lipoxins, resolvins, protectins, and maresins, that have unique structures and are biosynthesized in exudates within the resolution phase of acute inflammation [8,9,11,13]. The identification of these new locally produced mediators demonstrated that the resolution phase of acute inflammation is a biosynthetically active process [10]. Importantly, SPM are conserved from invertebrates to nonhuman primates to humans and several recent studies have documented the presence of SPM in human tissues (e.g., spleen, plasma, serum, urine, and breast milk) at levels commensurate with their biological actions [14-21].

57.2 Lipid Mediator Class Switching During Inflammation and Its Resolution

Lipid mediators play integral roles in both the initiation and resolution of the inflammatory response and lipid mediator class switching governs the duration of acute inflammation (Figure 57.1a) [11]. The eicosanoids, such as prostaglandins (PGs) and leukotrienes, are formed from arachidonic acid (AA; omega-6) and regulate blood flow, endothelial permeability, and PMN chemotaxis [22]. Transendothelial migration and chemotaxis of PMN toward injured tissue and/or pathogens are governed in part by leukotriene B_4 (LTB_4), which is itself a major product generated by PMN and regulates their activation in an autocrine manner [22,23]. After PMN enter tissues, there is a temporal switch in lipid mediators from the initiation phase to resolution in which AA is diverted away from LTB_4 biosynthesis and toward lipoxin biosynthesis [11,24]. Lipoxin A_4 (LXA_4) serves as an endogenous "stop" signal that attenuates PMN infiltration in part by downregulating the surface expression of adhesion receptors. The biosynthesis of lipoxins and related SPM can occur in a single cell or via transcellular biosynthesis (e.g., leukocyte:leukocyte or leukocyte:epithelial interactions) and their biosynthesis also increases when macrophages are actively clearing apoptotic PMN [25]. These SPM also act in an autocrine manner to facilitate phagocytosis. This mechanism of biosynthesis, coupled with potent regulation of inflammatory gene transcription, is similar to that of other "find me" and "eat me" signals (e.g., adenosine, ATP, and CX_3CL1) generated by phagocytes that play a key role in the immunologically silent process of apoptotic cell clearance [26,27]. Systematic identification of these endogenous SPM indicates that they are novel structures and that the precursors for resolvins, protectins, and maresins are omega-3 essential fatty acids EPA and DHA, while the lipoxins are generated from the ω-6 polyunsaturated fatty acid (PUFA), AA (Figure 57.1b). An update on the biosynthesis and biological actions of lipoxins and resolvins is given below and readers are referred to more extensive reviews covering protectins and maresins [28].

57.3 Lipoxins

The lipoxins were the first lipid mediators recognized to have both anti-inflammatory and proresolving actions [29] and are comprised of two distinct regioisomers denoted lipoxin A_4 (LXA_4) and LXB_4. They are biosynthesized from AA via lipoxygenase (LOX) enzymes, whereby 15-LOX generates 15-hydroperoxyeicosatetraenoic acid (15-HpETE), which is further transformed via 5-LOX to LXA_4 and LXB_4 during cell:cell interactions (i.e., transcellular biosynthesis). An alternate lipoxin biosynthetic route involves platelet:leukocyte interactions, in which the intermediate in leukotriene biosynthesis, leukotriene A_4 (LTA_4), is produced within leukocytes and converted to lipoxins by platelet 12-LOX [30]. This biosynthetic mechanism allows for direct shunting of the same intermediate from leukotrienes to lipoxins, thus regulating the duration of leukocyte recruitment. Lipoxins (and their aspirin-triggered epimers; see below)

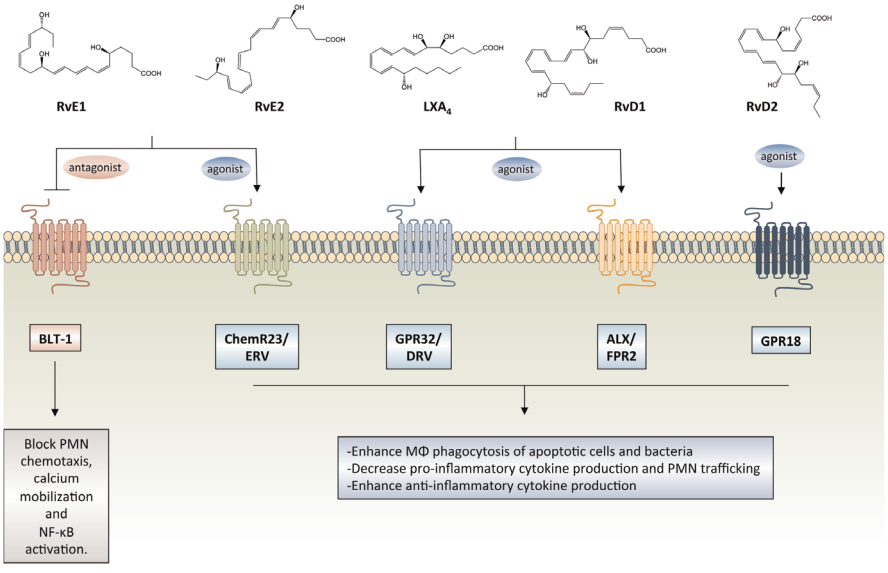


Figure 57.2 Specific receptors and key biological actions of SPM.

are formed in humans, with their levels inversely correlating with peripheral artery disease, local aggressive periodontitis, cystic fibrosis, heart failure, and Alzheimer's disease [31-35].

The biological actions of LXA_4 are mediated in part by a specific G-protein-coupled receptor (GPCR) termed ALX (also denoted formyl peptide receptor 2; FPR2) [30,36]. Specific high-affinity binding (low nanomolar range) of LXA_4 to ALX is stereoselective and ALX signaling mediates the protective actions of LXA_4 in myeloid cells, which include inhibition of NF-κB activation, blockade of leukotriene biosynthesis, attenuation of superoxide production, and regulation of leukocyte chemotaxis (Figure 57.2). Overexpression or genetic deletion of both human and murine homologs of ALX/FPR2 [37] has provided unequivocal evidence that ALX is a bona fide target for LXA_4 [38,39]. Interestingly, ALX also serves as the endogenous receptor for other SPM, including resolvin D1 (RvD1; see below) as well as the proresolving glucocorticoid-inducible protein, annexin A1 [40]. This agonist diversity positions ALX as a central mediator of the resolution of inflammation. In addition to ALX, which is a receptor for LXA_4 and RvD1 in mice and humans, these SPM also bind an additional receptor in humans, termed GPR32 [41].

The anti-inflammatory role of lipoxins has been demonstrated in both animal and human systems, in which administration of LXA_4 or LXB_4 reduces PMN transmigration, PMN-mediated vascular permeability, adhesion receptor expression, and proinflammatory cytokine generation in response to inflammatory stimuli [42-46]. Lipoxin biosynthesis initiated by platelet:leukocyte interactions during ischemia-reperfusion is associated with protection from vascular inflammation in an ALX-dependent manner [47]. In addition to this anti-inflammatory role, lipoxins also stimulate resolution. That is, lipoxins stimulate macrophage-mediated clearance of apoptotic PMN in a noninflammatory manner [48,49].

Interestingly, lipoxins also promote macrophage efflux to peripheral lymph nodes. Thus, lipoxins are dual acting mediators that not only reduce further leukocyte infiltration but also promote their removal from inflamed sites [49,50]. In addition to innate immune cells, lipoxins also have direct actions on adaptive immune cells (e.g., B cells, T cells, and natural killer cells), vascular endothelial and smooth muscle cells, fibroblasts, and epithelial cells. For an extensive chronicle of these cell-specific targets, the reader is referred to a recent review on lipoxins by Romano *et al.* [51].

The identification of the lipoxins as inflammation-resolving mediators raised the possibility that they could be used as a novel genus of agonist-based therapeutics for diseases in which inflammation plays a causal role. Indeed, lipoxins stimulate resolution of inflammation associated with cystic fibrosis, periodontitis, asthma, and sepsis [51]. More recently, it was demonstrated that both LXA_4 and LXB_4 resolve allergic and LPS-driven lung inflammation and that topical administration of LXA_4 promotes corneal wound healing [52-54]. The endogenous role of PMN-derived LXA_4 in mediating resolution of corneal injury was recently established [55]. Lipoxins stimulate the resolution of adipose tissue inflammation in obesity, which translates into improved systemic metabolism [56,57]. In addition to regulating inflammation in metabolic disease, a recent study assigned new biological roles to lipoxins by demonstrating that they promote reverse cholesterol transport in the context of hypercholesterolemia [58]. Lastly, a multicenter, double blinded, placebo-controlled randomized clinical trial recently reported that a stable lipoxin analog denoted 15 *R*/*S*-methyl LXA_4 (which resists metabolic inactivation) blunts the progression of infantile eczema, providing direct evidence that proresolution therapeutics could be a novel approach to treating maladaptive inflammation in humans [59].

57.4 E-series Resolvins

Like the ω-6 PUFA AA, ω-3 PUFA, including eicosapentaenoic acid (EPA), is also used as substrates for cyclooxygenase (COX) and LOX enzymes to generate novel families of SPM. The ω-3 PUFA have long been regarded to have anti-inflammatory actions and have been shown to reduce inflammatory cytokine production in humans [60], although the mechanisms underlying these actions remained unclear. Using murine models of self-resolving acute inflammation, initial studies identified that EPA is converted a novel trihydroxy-containing mediator, which was denoted resolvin E1 (RvE1) [61]. In these studies, mice were administered both EPA and aspirin, because the combination of ω-3 PUFA and aspirin was shown to be protective in cardiovascular disease patients enrolled in the GISSI Prevenzione trial [62]. In addition, it had previously been shown that aspirin acetylation of COX-2 increases the formation of epimeric lipoxins by converting AA to 15*R*-HETE [63]. Like the lipoxin biosynthetic pathway, monohydroxy fatty acids derived from EPA, including 18-hydroxyeicosapentaenoic acid (18-HEPE), were also present in exudates of mice given EPA and it was postulated that this product could potentially be a biosynthetic intermediate derived from acetylated COX-2. Further studies demonstrated that this is indeed the case, whereby

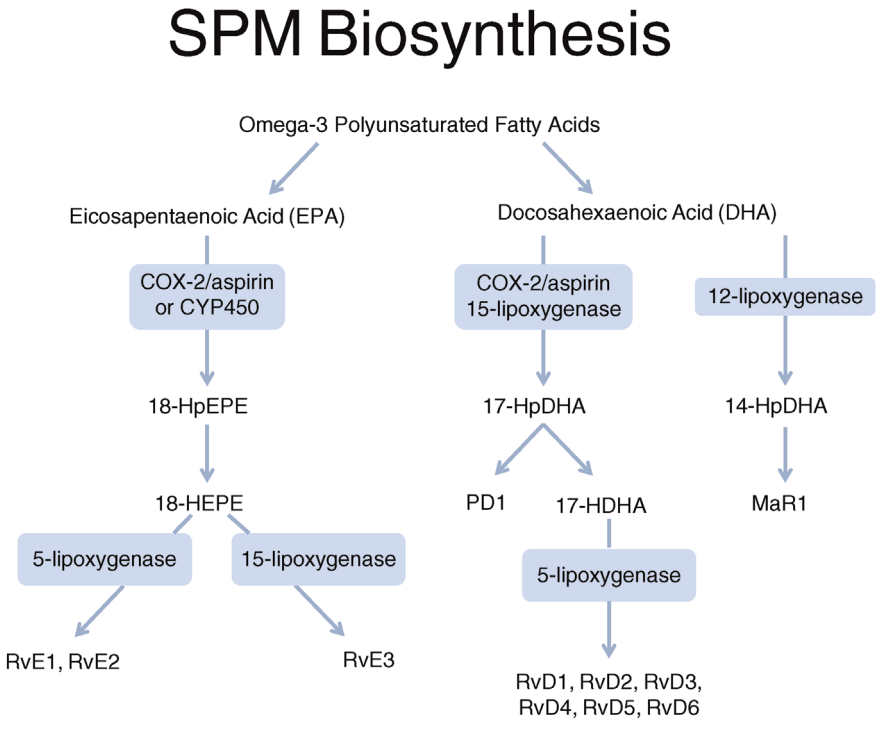


Figure 57.3 Biosynthesis of specialized proresolving mediators generated from omega-3 polyunsaturated fatty acids. Omega-3 polyunsaturated fatty acids serve as substrate precursors for specialized proresolving mediator (SPM) biosynthesis, in which EPA is converted to the E-series resolvins (RvE1, RvE2, RvE3), and DHA is converted to D-series resolvins (RvD1-6), protectins (PD1), and maresins (MaR1).

acetylated COX-2 generates 18-hydroperoxy(Hp)EPE, which is converted by 5-LOX to RvE1. The RvE1 biosynthetic pathway was recapitulated in isolated human cells, which elucidated that 5-LOX and LTB_4 hydrolase act sequentially to form RvE1 from 18-HpEPE [18]. The complete structural elucidation and stereochemical assignment of endogenous RvE1 was subsequently completed by matching the chromatographic and physical properties with synthetic stereoisomers [64]. Importantly, several studies have documented RvE1 formation in humans [18,64-66]. A summary of the E-series resolvin biosynthetic pathway is shown in Figure 57.3.

In animal models of acute inflammation, RvE1 reduces PMN infiltration in response to an inflammatory challenge, which is partially related to its ability to block LTB_4-dependent actions on PMN [64]. Indeed, RvE1 signals as a partial agonist/antagonist of the LTB_4 receptor, BLT1, and attenuates LTB_4-induced proinflammatory signaling in PMN [67] (Figure 57.2). Thus, both the biosynthesis and biological actions of RvE1 compete with proinflammatory LTB_4. In addition to regulating the proinflammatory functions of PMN, RvE1 also stimulates macrophage phagocytosis. Unbiased receptor screening approaches revealed that in addition to BLT1, RvE1 binds and activates ChemR23 (also

denoted ERV) as an agonist. RvE1 inhibits TNF-α-stimulated NF-κB activation in cells transfected with ChemR23, consistent with *in vivo* actions of RvE1 in blocking TNF-α-stimulated leukocyte trafficking and the endogenous role of ChemR23 in counterregulating inflammation was recently demonstrated [68]. Mechanistically, RvE1 stimulates the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, leading to activation of the translational regulator, ribosomal protein S6 [69]. This pathway is involved in RvE1 stimulation of macrophage phagocytosis. Pretreatment of ChemR23-expressing cells with pertussis toxin blocks the effects of RvE1; suggesting downstream signaling through $G\alpha_{i/o}$. ChemR23 is highly expressed on dendritic cells and monocytes, although its expression is low on PMN [64,67]. RvE1 regulates leukocyte trafficking and proinflammatory signaling to promote resolution of peritonitis, colitis, periodontitis (a chronic infectious inflammation), and retinopathy [49,70-72]. Industrial scale-up synthesis has been achieved and RvE1 is currently in clinical development for the treatment of inflammatory pathologies of the eye under the International Nonproprietary name, *Navamepent* [73,74]. More recently, RvE1 was found to blunt the progression of atherosclerotic plaques in a rabbit model of periodontitis and atherosclerosis [75].

The stereochemistry and downstream metabolism are important determinants of the biological actions of RvE1. Aspirin acetylation of COX-2 produces more 18*S*-HEPE (and therefore more 18*S*-RvE1) than 18*R*-HEPE [18]. The 18*S* isomer binds to RvE1 receptors with a higher affinity, but is also inactivated more rapidly. Like eicosanoids, RvE1 can be enzymatically converted into further downstream metabolites. Indeed, RvE1 is a substrate for 15-hydroxyprostaglandin dehydrogenase (15-PGDH; an eicosanoid oxidoreductase; EOR [76]), which converts RvE1 to 18-oxo-RvE1 [77]. This metabolite is essentially inactive compared with RvE1 and is a major metabolic route for RvE1 inactivation in the lung [77]. Other RvE1 metabolites include the ω-oxidation product, 20-hydroxy-RvE1, and 20-carboxy-RvE1 and 10,11-dihydro-RvE1, the latter two of which are essentially inactive [78]. Therefore the structure-function relationship of resolvins is critical to their targeted actions on multiple cell types and the stereochemistry of each resolvin is a unique determinant of their endogenous effects. Importantly, these studies also suggest that a failure of resolution could potentially be related to both impaired biosynthesis of resolvins, as well as accelerated metabolic inactivation. Along these lines, a stable analog that resists metabolic inactivation, denoted 19-(*p*-fluorophenoxy)-RvE1 methyl ester, shows similar biological activity as RvE1 *in vivo* [77], suggesting that this and related stable analogs may be important new candidates for resolution pharmacology approaches.

Two additional members of the E-series resolvin family have been characterized, namely RvE2 and RvE3 (Figure 57.3). The 5*S*-hydroperoxide formed prior to epoxidation can undergo reduction to 5*S*,18(*R/S*)-dihydroxy-eicosapentaenoic acid, denoted RvE2 [79]. Like RvE1, RvE2 blunts PMN infiltration in murine models of acute inflammation, stimulates macrophage phagocytosis, and promotes the generation of anti-inflammatory cytokines (Figure 57.2) [80]. The newest member of the E-series resolvin family is RvE3, which unlike RvE1 and RvE2, does not require 5-LOX for its biosynthesis, but rather only depends on

12/15-LOX to form the 17, 18 dihydroxyEPE product [81]. Like RvE1, both 18*R* and 18*S* isomers of RvE3 are biologically active and reduce PMN chemotaxis [81,82].

57.5 D-series Resolvins

Like EPA, DHA is also enzymatically biotransformed into structurally diverse families of mediators that comprise D-series resolvins, protectins, and maresins (Figure 57.3) [28]. Similar to the biosynthetic route elucidated for RvE1, aspirin acetylation of COX-2 leads to the formation of 17*R*-hydroperoxy(Hp)DHA when DHA is available as a substrate. In the absence of aspirin, 15-LOX converts DHA primarily to 17*S*-HpDHA [83]. Subsequently, both epimers of 17-HpDHA are converted to a 7,8 epoxide intermediate by 5-LOX and then hydrolyzed enzymatically into 7*S*,8*R*,17*R*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-DHA and 7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-DHA [9,83]. These two mediators were given the names aspirin-triggered-resolvin D1 (AT-RvD1) and RvD1, respectively. Both RvD1 and AT-RvD1 attenuate trans-endothelial migration of human neutrophils and reduce excessive PMN accumulation during acute inflammation [83]. As noted above, RvD1 elicits its biological actions by binding to specific GPCRs. Two human receptors have shown to transmit RvD1 signals, the LXA_4 receptor (ALX/FPR2), and the previous orphan receptor GPR32 (Figure 57.2) [41]. This overlap in biological function may exist to ensure adequate resolution of inflammation in the presence of different nutritional fatty acid sources (i.e., AA vs. DHA). Importantly, RvD1 does not activate ChemR23, a receptor for RvE1, illustrating selectivity among SPM receptors. That these two resolvins have some overlapping but also other unique actions (*vida infra*), emphasizes the potential that each individual resolvin possesses in mediating resolution-phase processes.

In PMN, RvD1 reduces actin polymerization, actions that are abolished by pertussis toxin, and not cholera toxin, suggesting downstream signaling through $G\alpha_{i/o}$. Additionally, RvD1 stimulates macrophage phagocytosis of both apoptotic neutrophils and opsonized-zymosan. This enhancement of phagocytosis in macrophages is increased when ALX/FPR2 or GPR32 are overexpressed, while short-hairpin (sh) RNA knockdown of each receptor decreases rates of phagocytosis by RvD1, implicating direct receptor signaling in this biological actions of RvD1 [41]. Interestingly, a positive feed-forward loop was recently identified wherein RvD1 stimulates LXA_4 production in macrophages by promoting the redistribution of 5-LOX [24]. Specifically, RvD1 blunts the activation of p38 and MK2 kinases, thereby preventing the phosphorylation and subsequent nuclear localization of 5-LOX [24]. This mechanism promotes a sustained shift in the production of SPM (e.g., LXA_4) instead of proinflammatory LTB_4 in macrophages. Along these lines, RvE1 also stimulates endogenous LXA_4 production in the lung [84]. Interestingly, 5-lipoxygenase activating protein, which is classically associated with leukotriene production, has also been implicated in both lipoxin and resolvin biosynthesis [85]. In addition to regulating proinflammatory lipid mediators, RvD1 also downregulates the transcriptional co-activator, CARM1,

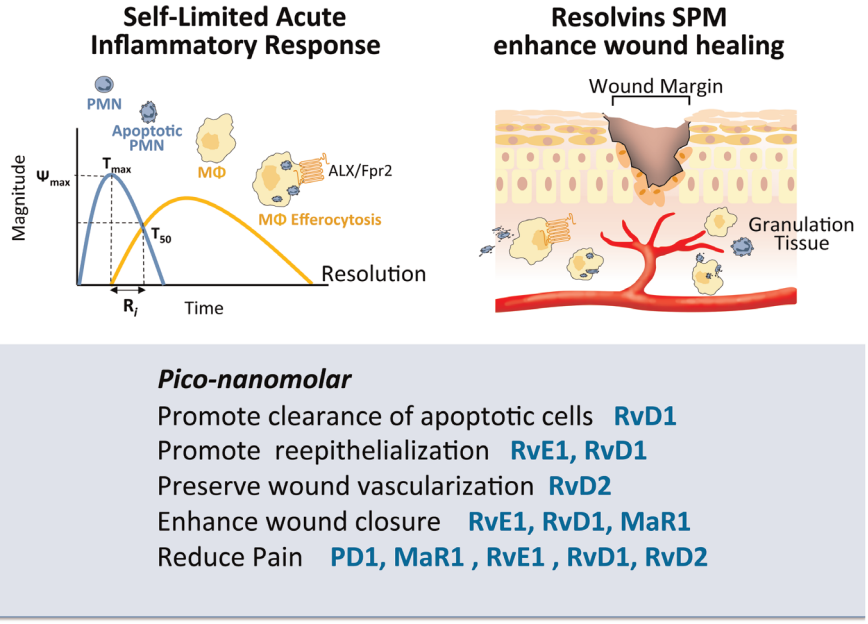


Figure 57.4 Resolution of inflammation and wound healing. The key cellular events during an acute inflammatory response are depicted, in which infiltrating polymorphonuclear neutrophils (PMN) undergo apoptosis and are cleared by macrophages during resolution. Resolution indices are shown, in which T_{max} represents the time at which maximum PMN infiltration occurs, T_{50} represents the time point at which PMN have declined to half of their maximum levels, and R_i represents the resolution interval, the time interval between T_{max} and T_{50}. SPM receptors, including ALX/Fpr2, mediate the biological actions of SPM, which include stimulation of macrophage clearance of apoptotic cells during resolution. Similar events take place during tissue injury and wound healing, whereby SPM resolve inflammation while promoting wound reepithelialization and reducing pain.

which in turn reduces the expression of mediators that regulate PMN activation and chemotaxis (e.g., GM-CSF and CXCL2) [86].

Protective actions of RvD1 have been demonstrated in several animal models of inflammatory diseases [87]. In adipose tissue from obese rodents and humans, RvD1 and its receptors are present and exogenous administration of RvD1 improves insulin sensitivity in obese mice by resolving adipose tissue inflammation, effects that are associated with improved cutaneous wound healing (see Figure 57.4) [15,88-90]. The resolution of adipose tissue inflammation and promotion of wound healing are in part related to actions on monocytes and macrophages, as RvD1 reduces monocyte transmigration in adipose tissue and restores defective efferocytosis [91]. Along these lines, apoptotic cells are a stimulus for RvD1 production in macrophages, which is in part related to the delivery of SPM precursors from microparticles shed by apoptotic cells [25]. Using this system as a form of biomimicry, humanized nanoparticles loaded with RvD1 reduce inflammation in the temporomandibular joint and accelerate keratinocyte wound closure *in vitro* [92]. In the context of cardiovascular disease, RvD1 reduces neointimal hyperplasia during vascular injury and accelerates

resolution of inflammation and tissue repair during myocardial ischemia [93,94]. A similar protection against ischemia-reperfusion injury by RvD1 was observed in the liver [95].

Additional members of the D-series resolvin family have been subsequently identified and their structures have been systematically deduced [96]. The complete structure of RvD2 was recently determined as 7*S*,16*R*,17*S*-trihydroxy-4*Z*,8*E*,10*Z*,12*E*,14*E*,19*Z*-DHA [97]. Both RvD1 and RvD2 are generated from a common precursor, namely 7,8-epoxy-17-HpDHA and, analogous to LXA_4 and LXB_4, are regioisomers of each other. RvD2 was originally identified in resolving inflammatory exudates and its structure was elucidated using total organic synthesis and matching with endogenous RvD2 to confirm its structure and stereochemistry by LC-MS/MS and GC/MS (i.e., functional group derivatization). In murine models of acute peritoneal inflammation, RvD2 reduces PMN infiltration at low picogram levels and its actions are stereospecific, as a Δ10-*trans*-RvD2 isomer is essentially inactive. Using intravital microscopy, it was determined that RvD2 reduces leukocyte adhesion to postcapillary venules, as well as transmigration. While like other resolvins RvD2 reduces the expression of PMN adhesion receptors, RvD2 also has direct endothelial actions, promoting the production of anti-adhesive mediators such as nitric oxide and prostacyclin [97,98].

In the context of polymicrobial sepsis, RvD2 reduces the amount of live bacteria in both the peritoneum, as well as the blood, which translates into markedly increased survival rates [97]. Interestingly, this increased survival and reduced infection occurs concomitantly with a reduction in peritoneal PMN recruitment. At the cellular level, RvD2 stimulation of neutrophils promotes their phagocytosis of live *E.coli*, providing evidence that SPM can combat infection. Several subsequent studies have determined that other resolvins (i.e., RvD5; 7*S*,17*S*-dihydroxydocosa-4*Z*,8*E*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid) stimulate bacterial clearance by phagocytes and thus reduce the amount of antibiotics needed to control infection [99,100]. In addition to infection, RvD2 (nanogram doses) also reduces PMN-mediated tissue damage in cutaneous burn wounds [101,102]. Recently, a specific GPCR was found to mediate the biological actions of RvD2 during acute inflammation. Using an unbiased GPCR screen, RvD2 was found to activate GPR18 in both PMN and macrophages, and specific binding was demonstrated using radiolabeled RvD2 and both heteroligand and homoligand competition in cells overexpressing GPR18 (Figure 57.2) [103]. Knockdown of GPR18 abolishes the stimulatory effects of RvD2 on macrophage phagocytosis and mice deficient in *Gpr18* fail to respond to exogenous RvD2 [103]. Importantly, RvD1 does not compete for RvD2 binding to GPR18, indicating that this RvD2 receptor is unique and specific. Given that GPR18 is abundantly expressed in the bone marrow, lymph nodes, spleen, and several individual leukocyte subsets, it is likely that RvD2 has additional biological roles that remain to be revealed.

The complete stereochemistry of an additional D-series resolvin member, RvD3, was recently elucidated as 4*S*, 11*R*, 17*S*-trihydroxydocosa-5*Z*, 7*E*, 9*E*, 13*Z*, 15*E*, 19*Z*-hexaenoic acid [104]. Like RvD1 and RvD2, an aspirin-triggered epimer (17*R*) of RvD3 is also formed in the presence of acetylated COX-2. Both epimers of RvD3 were identified in human plasma, spleen, and breast milk [14,16]. Unlike other D-series resolvins, RvD3 appears much later during

the resolution phase of acute inflammation, indicating distinct controlled biosynthesis that may be related to different leukocyte subsets during resolution [104]. Given that RvD3 binds GPR32, which is a proresolving receptor that is shared with RvD1 and LXA_4, RvD3 biosynthesis may serve to preserve SPM receptor ligands during all phases of resolution. Both epimers of RvD3 are biologically active in murine models of acute inflammation, reducing PMN trafficking to inflammatory loci, proinflammatory cytokine production and enhancing macrophage efferocytosis [104].

57.6 Novel Statin-Induced 13-Series Resolvins

Analogous to aspirin-mediated acetylation of COX-2 that drives the biosynthesis of AT-resolvins, it was recently found that *S*-nitrosylation of COX-2 by statins (e.g., atorvastatin) leads to the production of a novel series of resolvins generated from omega-3 fatty acid, docosapentaenoic acid (DPA) [105]. These newly identified resolvins were denoted 13-series resolvins (RvTs) because they carry an alcohol group at carbon 13 in the DPA backbone. The RvTs are generated during PMN:endothelial cell interactions via transcellular biosynthesis, as occurs during acute inflammation, and involves the initial formation of a 13-HpDPA intermediate in endothelial cells in a COX-2-dependent manner. This intermediate is converted to RvTs by 5-LOX in PMN [105]. The biosynthesis of RvTs is increased by atorvastatin and inhibited by nitric oxide synthase inhibitors, suggesting that nitrosylated COX-2 plays a role in their biosynthesis. Along these lines, nitrosylation of recombinant COX-2 by *S*-nitrosoglutathione increases the formation of 13-HDPA. The biological actions of these novel RvTs (i.e., RvT1, RvT2, RvT3, and RvT4) include promoting the resolution of acute inflammation and phagocyte-mediated containment of bacterial infection, leading to increased survival in *E. coli* peritonitis. The elucidation of these novel mediators may help to explain in part the anti-inflammatory actions of statins and may overcome therapeutic limitations to prolonged statin use.

57.7 Role of Novel Sulfido Lipid Mediator Conjugates in Tissue Regeneration

During tissue injury, a temporal program of events (i.e., inflammation, proliferation, and remodeling) ensures a restoration of homeostasis and function. While the inflammation phase is required to combat infection, prolonged activation of immune cells can cause tissue damage and impede its repair. As such, timely resolution of inflammation could be considered integral to this "wound healing" program. In addition to the aforementioned SPM that play a role in resolving inflammation and combating infection, recent studies have identified that novel families of sulfido-conjugated lipid mediators are generated during the transition from inflammation to tissue repair [106,107]. Specifically, glutathione conjugates of SPM are formed from DHA precursor during resolution, as well as by human

phagocytes, and they actively promote tissue regeneration. As such, they were given the names "conjugates in tissue regeneration" (CTR) and consist of maresin conjugates (MCTR), resolvin conjugates (RCTR) and protectin conjugates (PCTR) [106,107]. They are novel structures and their biosynthesis involves enzymatic conjugation of glutathione to the epoxides formed during the production of each respective family. Further downstream products of γ-glutamyl transferase, including cysteinylglycinyl and cysteinyl conjugates, are also formed and have biological activity. These novel conjugates were identified in regenerating plenaria during head resection, as well as in human and murine cells and tissues (e.g., spleen, breast milk, and inflammatory exudates) [106,107]. Overall, the identification of these novel CTRs provides evidence that the resolution of inflammation is directly linked to tissue regeneration in an active manner.

57.8 Summary

The resolution of inflammation is an active process characterized by the generation of SPM that potently resolve inflammation, promote clearance of infection, and stimulate tissue regeneration. These mediators are evolutionarily conserved and their identification has helped to open up a new era of "resolution pharmacology" based on using agonists of resolution, rather than inhibitors of the initiation events in inflammation [28]. In contrast to traditional pharmacologic agents that suppress inflammation, SPM enhance host defense and tissue repair. Given that inflammation is emerging as a central causative factor in numerous chronic diseases, the implications of SPM as proresolving therapeutics has considerable promise for the future of medicine, as well as potential for diagnosis and predicting clinical outcomes [108,109].

Acknowledgments

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58

Glucocorticoids

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58.1 Introduction

58.1.1 Historical Introduction

The fatal consequences of adrenal gland damage were first described in 1855 by Addison, in a series of autopsy cases [1]. Of note, Addison's findings were based on anatomical observations, preceding experimental studies. Experimental confirmation was provided by Brown-Séquard who demonstrated that bilateral adrenalectomy in laboratory animals invariably proved fatal [2]. Subsequent observations demonstrated that the adrenal cortex, producing specific hormones named corticosteroids, was in fact essential for survival. Two distinct families of corticosteroids were isolated, regulating on the one hand the metabolism of electrolytes and fluids (thereby earning the name mineralocorticoids) and on the other the metabolism of carbohydrates (named glucocorticoids). While mineralocorticoids, through their capacity to retain sodium and water, were found to be responsible of prolonging life in adrenalectomized animals, glucocorticoids were found to possess powerful anti-inflammatory properties. The synthesis of artificial corticosteroids, the first of which being cortisone, enabled physicians to use corticosteroids therapeutically [3]. Cortisone was administered for the first time to patients suffering from rheumatoid arthritis by Hench [4]. The 1950 Nobel Prize in physiology and medicine was awarded for findings pertaining to the structure and biological effects of adrenal hormones. It soon became apparent that glucocorticoid administration was associated with significant side effects. In order to reduce these side effects, numerous synthetic glucocorticoids, as well as novel ways of administrating these drugs, were developed making glucocorticoids one of the most frequently prescribed classes of drugs, available for a range of inflammatory or immune pathologies.

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58.1.2 Corticosteroid Biosynthesis

The adrenal glands lie at the superior pole of the kidneys and are divided into two distinct parts: the adrenal medulla and the adrenal cortex. The adrenal cortex synthesizes a group of hormones called corticosteroids. Natural steroids derive from cholesterol and possess four carbon rings. Cholesterol is either produced *in situ*, or else transported to the adrenal gland by cholesterol-transporting low-density lipoproteins (LDL). Circulating LDL recognize specific receptors on the adrenal cells, leading to the internalization of the LDL–LDL receptor complex in intracellular vesicles that fuse with lysosomes, leading to the degradation of LDL and the release of cholesterol. Steroid precursors are then shuttled between the mitochondria and the endoplasmic reticulum, where corticosteroid synthesis occurs. Each step of biosynthesis is controlled by a specific enzyme (Figure 58.1). Corticosteroids are preferentially produced in distinct regions of the adrenal cortex. Indeed, the adrenal cortex is anatomically and functionally composed of three different layers. These layers are, from the outermost layer beneath the

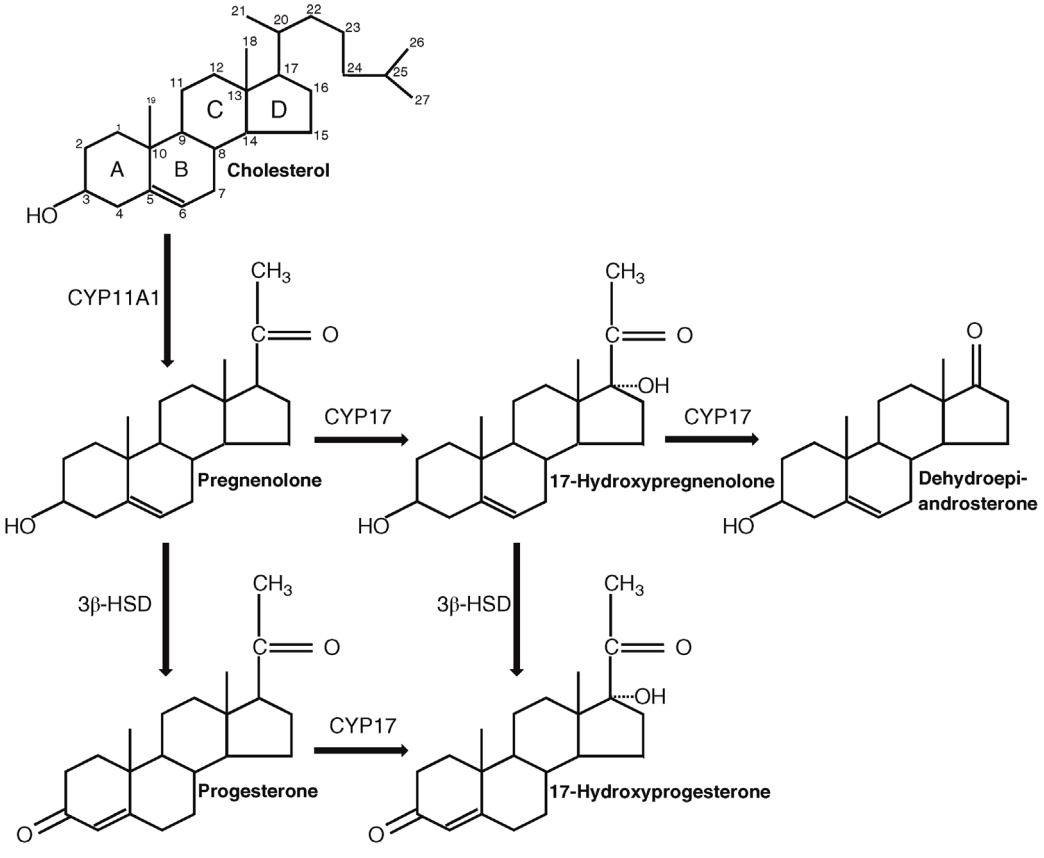


Figure 58.1 Pathways for corticosteroids biosynthesis. CYP11A1: cholesterol desmolase; 3β-HSD: 3β hydroxysteroid dehydrogenase; CYP17: steroid 17α-hydroxylase; CYP21: steroid 21-hydroxylase; CYP11B2: aldosterone synthase; CYP11B1: steroid 11β-hydroxylase.

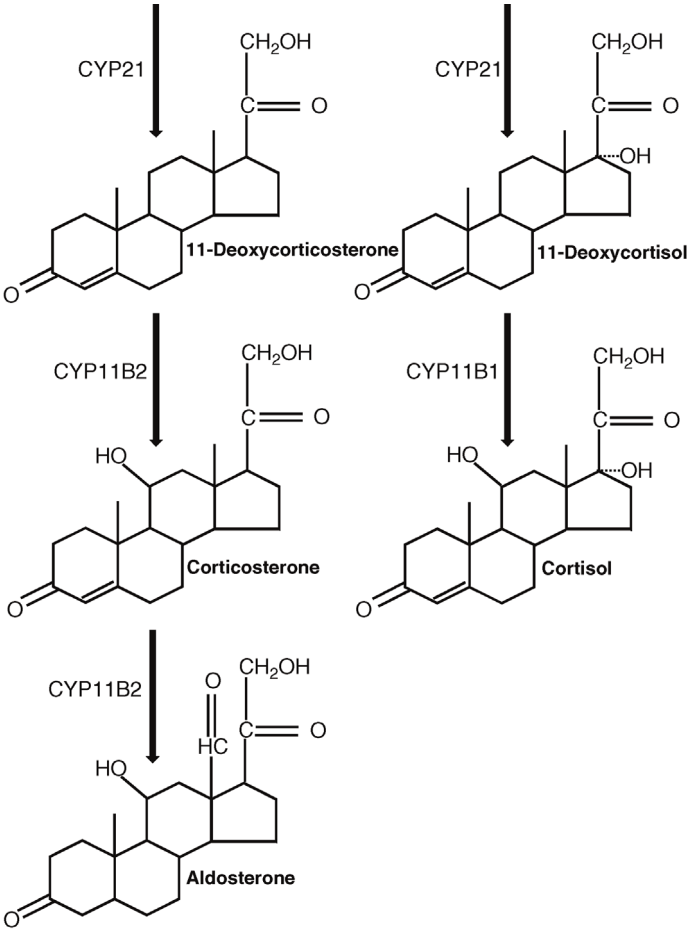


Figure 58.1 (*Continued*)

capsule, the zona glomerulosa, which secretes the mineralocorticoid aldosterone, the zona fasciculata, which mainly secretes the glucocorticoids cortisol and corticosterone, while the innermost layer, the zona reticularis secrets the adrenal androgen dehydroepiandrosterone (DHEA).

58.1.3 Hypothalamic–Pituitary–Adrenal Axis

Glucocorticoid production is regulated by the hypothalamic–pituitary–adrenal (HPA) axis. Indeed, steroids do not accumulate in the adrenal gland, their secretion and release is almost entirely controlled by the adrenocorticotropic hormone (ACTH), produced in the anterior pituitary gland. ACTH is synthesized as part of a large precursor protein, proopiomelanocortin (POMC). Other biologically relevant peptides, including the melanocyte-stimulating hormone, lipotrophin, and endorphins derive from the same precursor. ACTH is a 39 amino

acids polypeptide. The receptors for ACTH belong to the melanocortin receptor family, which are G-protein-coupled receptors. When ACTH binds its receptor, the adenylyl cyclase is activated, which induces a rapid rise in intracellular cAMP concentration, followed by the activation of the protein kinase A and the conversion of cholesterol to pregnenolone. In turn, ACTH production is controlled by the corticotropin-releasing hormone (CRH), a 41 amino acid peptide produced by the parvocellular region of the periventricular nucleus of the hypothalamus. CRH is secreted in response to proinflammatory stimuli (such as bacterial LPS as well as physical or psychological insults). Secreted CRH enters the hypophyseal portal system then reaches the pituitary gland where it binds to the CRH receptor, another G protein receptor that interacts with the adenylyl cyclase, leading to the biosynthesis and release of ACTH. Overall, the HPA axis is tightly regulated by several factors including (1) The circadian rhythm in basal steroidogenesis; plasma cortisol concentrations reach a peak in the morning and a trough in the evening/at night. (2) Negative feedback mechanisms control the level of circulating glucocorticoids. Circulating glucocorticoids bind to specific glucocorticoid receptors (GR) in the hypothalamus and pituitary gland, inhibiting the production and release of CRH and ACTH. (3) Inflammation; cytokines including interleukin 1 (IL-1), IL-6, and TNF affect the brain, which responds by regulating the immune system through the HPA axis and the production of glucocorticoids [5].

58.2 Pharmacokinetics

Most steroids exhibit both a glucocorticoid and a mineralocorticoid activity. The relative glucocorticoid and mineralocorticoid activity of steroids is presented in Table 58.1. In order to manipulate the relative mineralocorticoid or glucocorticoid action of glucocorticoids, thereby limiting their side effects, a number of synthetic drugs have been produced. The pharmacokinetics of glucocorticoids depends on their physicochemical properties. Glucocorticoids are usually administered as prodrugs. Glucocorticoids have a very good bioavailability [6]. Corticosteroids circulate bound to proteins. For instance 80%–90% of plasma cortisol binds to cortisol binding globulin (CBG, also known as transcortin), a protein produced by the liver, and to a lesser extent to albumin [7]. CBG may also bind synthetic glucocorticoids as well as steroids albeit with reduced affinity [8,9]. In basal conditions (in the absence of stress), approximately 5% of circulating cortisol is unbound. The free hormone hypothesis postulates that only the free fraction of cortisol is biologically active [10]. Free cortisol is converted in peripheral tissues into the inactive compound cortisone by the 11 β-hydroxysteroid dehydrogenase [11]. Since glucocorticoids derive from cholesterol, they are easily soluble in lipids and may easily diffuse through the cell membrane into the cytoplasm, where glucocorticoids bind their specific receptor. Glucocorticoids modulate gene transcription through modifications of the production of mRNA. Most metabolic effects are therefore delayed, requiring 60 minutes for proteins to be synthesized and several days for their full effect to be developed. Cortisol half-life is between 60 and 90 min. Corticosteroids are mainly metabolized by the liver,

Table 58.1 Relative potencies of natural and synthetic steroids.

Compound	Glucocorticoid activity	Mineralocorticoid activity
Natural steroids		
Cortisol	1	1
Corticosterone	0.3	15
Aldosterone	0.3	3000
Deoxycorticosterone	0.2	100
Synthetic steroids		
Cortisone	0.8	0.8
Fludrocortisone	10	125
Prednisone	4	0.8
Prednisolone	4	0.8
Methylprednisolone	5	0.5
Bethametasone	25	0
Dexamethasone	25	0

Glucocorticoid and mineralocorticoid activity of natural and synthetic steroids, relative to cortisol.

where they are transformed into inactive glucuronic acid. These metabolites are then excreted into the bile or by the kidneys. Topically administered glucocorticoids may exhibit systemic effects such as growth retardation in asthmatic children, or diabetes following intra-articular injections of glucocorticoids.

58.3 Mechanisms of Action (Figure 58.2)

58.3.1 Genomic Effect of Glucocorticoids

Glucocorticoids exhibit potent anti-inflammatory actions, both through increased synthesis of anti-inflammatory and repressed synthesis of pro-inflammatory proteins. Glucocorticoids mediate their activity through their interaction with a specific intracellular receptor, the GR. The GR belongs to the nuclear receptor family of transcription factors. The gene for the human glucocorticoid receptor is located on chromosome 5q31-31 [12]. Glucocorticoid-regulated transcription factors are 94 kD proteins, exhibiting specific regions; a ligand binding region composed of 12 α-helices, dedicated to the recognition and binding of glucocorticoids, a region specializing in the interaction of the hormone–receptor complex with specific DNA sequences, composed of two zinc fingers and a region dedicated to the binding of other transcriptional factors. Posttranscriptional modifications of the GR, such as phosphorylation, modulate the activity of the GR [13]. Nonbound glucocorticoid receptors reside in the cytoplasm and are stabilized by small proteins, including heat-shock proteins 70 (Hsp70) and Hsp90 as well as immunophilin. On binding with glucocorticoids, the GR is activated, leading to

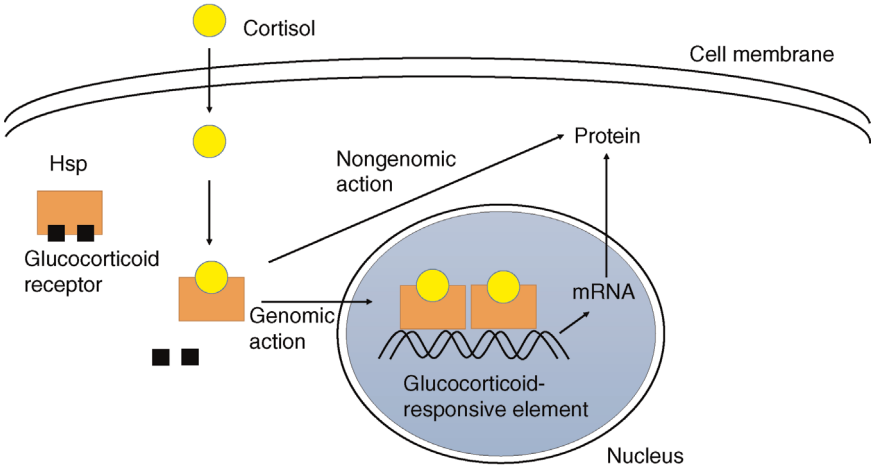


Figure 58.2 Schematic mechanism of action of glucocorticoids.

dissociation with chaperone proteins and nuclear translocation. In the nucleus, homodimers of the hormone–GR complex interact with specific DNA sequences within the regulatory region of target genes. DNA sequences recognized by the hormone–GR complex are named glucocorticoid-responsive elements, characterized by a consensus sequence of 15 base pairs GGTACAnnnTGTTCT, where n is any nucleotide. The expression of target genes for the hormone–GR complex is regulated through chromatin remodeling through the facilitation or inhibition of the assembly of key elements of the transcriptional machinery [14,15]. Chromatin consists of nucleosomes; DNA associated with core histone proteins. Resting state genes are characterized by DNA tightly wound around histone proteins, which hamper the capacity of the RNA polymerase to bind to the DNA and to produce mRNA. Core histones possessing Leu-rich N-terminal tails may be acetylated, leading to a modification of the nucleosome structure and an opening up of the chromatin, ultimately leading to gene expression. Indeed proinflammatory transcription factors such as NF-κB activate histone acetyltransferases (HAT) leading to acetylation of core histones, loosening of the chromatin and activation of the target gene. By opposition, histone deacetylases (HDACs) induce a tightening of the structure of the chromatin, repressing the expression of target genes. Activated glucocorticoid receptors inhibit HATs while activating HDACs, repressing the expression of proinflammatory genes.

For instance, glucocorticoids induce the synthesis of annexin 1, an anti-inflammatory protein inhibiting the expression of the phospholipase A2 and the subsequent production of proinflammatory elements derived from arachidonic acid, including prostaglandins and leukotrienes [16]. Glucocorticoids also repress the expression of proinflammatory genes. The expression of the IRF3 transcription factor, which is implicated in interferon production and viral protection, is downregulated by glucocorticoids [17,18].

Second, the GR–glucocorticoid complex inhibits the expression of proinflammatory proteins by directly interfering with other transcription factors, such as NF-κB [19]. NF-κB is particularly implicated in the transcription of proinflammatory cytokines genes [16,20]. In a resting state, inactive NF-κB is bound to IκBα. On cellular activation, NF-κB and IκBα dissociate and NF-κB translocates into the cellular nucleus. Glucocorticoids interfere with NF-κB by increasing the expression of the inhibitory protein IκBα that sequesters and therefore inhibits NF-κB in lymphocytes [21]. Glucocorticoids also induce the expression of glucocorticoid-induced leucine zipper (GILZ) that in turn inhibits NF-κB [22] as well as the anti-inflammatory protein MAP kinase (MAPK-1) phosphatase 1, which inhibits the nuclear translocation of GATA-3 implicated in Th2-type cytokine expression [23,24].

58.3.2 Nongenomic Effect of Glucocorticoids

The genomic action of glucocorticoids occurs over several minutes, with clinically relevant effects observed after several hours, following nuclear translocation of activated GR and gene regulation. This latency is related to the time needed for mRNA to be produced and modified, proteins to be synthesized, transported and reach a significant concentration threshold in order to exert a meaningful cellular effect [25]. Following glucocorticoid administration, rapid effects have been observed, which occur in a time frame too short for classic, genomic mechanisms to be implicated. These actions are described as nongenomic and they occur *in vitro* in the presence of inhibitors of DNA transcription and of protein synthesis. Nongenomic actions may also be triggered by macromolecule-bound glucocorticoids, unable to penetrate the cellular membrane. Nongenomic actions of glucocorticoids are either specific (i.e., mediated by the interaction of the hormone with a steroid receptor) or nonspecific (i.e., no receptor is involved, the effect is probably mediated by interactions with the cellular membrane and subsequent modification of ion transport) [26]. For instance, glucocorticoids, through the activated GR, may inhibit NF-κB by direct protein–protein interactions, such as with p65, one of the subunits of NF-κB as well as with other proinflammatory transcription factors such as activator protein 1 (AP-1) [27–29]. Glucocorticoids rapidly activate the endothelial nitric oxide synthetase (eNOS) through the protein kinase Akt pathway [30]. Glucocorticoids also induce posttranscriptional changes of mRNA coding for inflammatory proteins [31]. Early glucocorticoid action on neurons has been described [32] as well as rapid behavioral changes in response to corticosterone, the main glucocorticoid in amphibians and in rodents [33]. It has also been suggested that glucocorticoids might induce cellular signals through their interaction with membrane-bound receptors. Indeed, a glucocorticoid receptor-like protein has been detected in the membrane of human lymphoma cells [34]. Overall, these nongenomic actions although not completely elucidated, appear to be linked to the administration of higher doses of systemic glucocorticoids.

58.4 Pharmacological Effects

58.4.1 Immunomudulation

Cortisol reduces capillary permeability and fluid loss into the interstitium. Cortisol, through reduced production of leukotrienes and prostaglandins, reduces the migration of leucocytes into inflamed areas and inhibits phagocytosis of damaged cells. Glucocorticoids suppress the expression of endothelial adhesion molecules implicated in cell trafficking, including endothelial-leukocyte adhesion molecule 1 (ELAM-1), intracellular adhesion molecule 1 (ICAM-1), and vascular adhesion molecule 1 (VCAM-1) thereby interfering with the capacity of inflammatory cells to adhere and migrate through the endothelium [35–37]. Glucocorticoids inhibit the production of chemokines implicated in leucocyte chemoattraction [38–40]. Glucocorticoids also suppress the production of acute-phase reactants. Glucocorticoids exert powerful anti-inflammatory and immunomodulatory actions affecting cells from both the innate and adaptive arms of the immune system. Broadly speaking, glucocorticoids repress the maturation, differentiation, and proliferation of leucocytes of all subtypes.

58.4.1.1 Innate Immunity

Glucocorticoids reduce the number of monocytes/macrophages and of dendritic cells. The number of circulating neutrophil granulocytes increases through increased release by the bone marrow associated with increased demargination, while the number of eosinophil and basophil granulocytes is reduced. Glucocorticoids reduce the membrane expression of MHC class II receptors and Fc receptors and their production of proinflammatory cytokines [41–44]. Crosstalk between the Toll-like receptor 2 (TLR2) and the glucocorticoid receptor pathways accounts for the surprising observation that TLR2 KO mice exhibit impaired corticosterone production as well as alterations of adrenal tissues [45]. Corticosteroids stabilize the lysosomal membranes thereby causing the amount of proteolytic enzymes released by lysosomes to be greatly reduced. For instance, reduced IgE-dependent release of histamine and leukotriene is observed in steroid-treated allergy. The release of arachidonic acid and its metabolites (e.g., prostaglandins and leukotrienes) is also inhibited through inhibition of cyclooxygenase-2 and of phospholipase A2 and the expression of annexin 1. Cortisol attenuates fever by reducing the production of IL-1, TNF, IL-8, and MCP-1 by monocytes and macrophages. By contrast dendritic cells treated by glucocorticoids produce increased levels of the anti-inflammatory cytokines IL-10 and TGF-beta [46]. Glucocorticoids also promote the expression of another anti-inflammatory protein, MAPK phosphatase 1 [29]. Activating signaling induces a kinase cascade that phosphorylates and activates the transcription factor c-Jun a subunit of AP-1, implicated in the transcription of proinflammatory signals [47]. MAPK phosphatase 1 inhibits c-Jun-mediated transcription. Additionally, the GR binds directly and inhibits through protein–protein interaction the activity of AP-1.

58.4.1.2 Adaptive Immunity

Activation, proliferation, and the production of immunoglobulins by B cell lymphocytes are depressed in the presence of glucocorticoids [48,49].

Corticosteroids deplete thymic stroma cells and T cells by apoptosis [50,51]. Circulating T cell numbers are also reduced. Additionally, glucocorticoids induce a shift from a T helper 1 (Th1) to a T helper 2 (Th2) phenotype, both directly through the inhibition of the transcription factor T-bet and indirectly by effecting antigen presenting cells [52–55]. Additionally, glucocorticoids also inhibit the expression of Il-23, which promotes the differentiation of Th17 cells and the production thereof [56,57]. Interestingly, it has been proposed that glucocorticoids may expand the number of Foxp3(+)CD4(+)CD25(+) regulatory T cells that are functionally suppressive, these findings are however discrepant between different teams [58–61]. Glucocorticoids suppress the production by lymphocytes of the proinflammatory cytokines IL-2, IL-4, IL-5, IL-13, and INF [24,62].

58.4.2 Other Effects

Glucocorticoids are usually administered for their anti-inflammatory and immunomodulatory effects. Their administration, especially at higher doses and over a longer period of time is associated with multiple side effects. Some of these adverse effects include atherosclerosis and arterial hypertension, through sodium retention and the enhancement of vasopressor responses to angiotensin II and catecholamines [63]. Inhaled glucocorticoids have been reported to impede growth in children through systemic absorption [64,65]. In children, longitudinal growth depends on the proliferation and differentiation of chondrocytes, which is mediated in part by insulin-like growth factor I. Glucocorticoids inhibit insulin-like growth factor I and promote chondrocyte apoptosis through the Akt pathway [65,66]. Reduced bone density and osteoporosis occur in adults treated by glucocorticoids, through decreased activity of osteoblasts while increasing the activity of osteoblasts [67,68]. Wound healing is altered during glucocorticoid treatment through the inhibition of the synthesis of collagen and matrix metalloproteinase, implicated in wound repair [69]. Glucocorticoids promote liver gluconeogenesis, degradation of muscle proteins to free amino acids leading to muscle atrophy and lipolysis leading to hyperglycemia [70]. These modifications of the metabolism of carbohydrates, fatty acids, and proteins lead to the development of diabetes mellitus, increase of body weight, redistribution of body fat, and myopathy. Other unwanted effects include eye cataract, electrolyte imbalance, increased risk of infection, and mood changes, including euphoria or depression [71,72].

58.5 Therapeutic Use of Glucocorticoids

58.5.1 General Principles

With the exception of opotherapy for adrenal insufficiency, the therapeutically use of glucocorticoids is largely empirical. Both the risk and benefits of glucocorticoid administration must be balanced for all patients. Since side effects are both dose and time dependent, immunosuppressive treatments may be associated with corticosteroids in order to spare steroid usage. When treating potential life

threatening conditions, high doses of glucocorticoids must be administered until the condition is controlled. The smallest effective dose of glucocorticoids will thereafter be sought. Tapering should be progressive in order to avoid a flare of the disease or adrenal insufficiency. Glucocorticoids are usually administered in the morning, in order to preserve the HPA axis and to limit sleep disorders. Outlined below are important uses of glucocorticoids in a series of conditions. The reviewed indications for corticosteroid therapy are not inclusive. Additionally, we indicate through a grading system the level of evidence supporting the use of glucocorticoids in specific conditions.

58.5.2 Allergy and Asthma

In anaphylaxis and asthma, symptoms are mediated by IgE binding to antigens. In the case of anaphylaxis, symptoms are systemic, while in asthma the lung is preferentially affected. Anaphylaxis, which is life-threatening, must be immediately treated by epinephrine. Since the onset of action of glucocorticoids is delayed; they are used as a second-line treatment during anaphylaxis (very low quality of evidence) [73]. Corticosteroids should be administered intravenously for severe allergic conditions. Topical applications are appropriate for milder conditions. Asthma is a chronic inflammatory disorder of the airway characterized by bronchial hyperresponsiveness and the occurrence of transient symptoms of bronchial obstruction. Inhaled glucocorticoids are recommended as maintenance treatment for adults with symptoms more than twice a month (moderate quality of evidence) [74]. Exacerbation that are severe or do not respond to 48 h of treatment should receive oral glucocorticoids (high quality of evidence) [74]. Usual doses for adults are 1mg/kg/day of prednisone over a period of 5–7 days.

58.5.3 Rheumatic Disorders

Glucocorticoids are a cornerstone of the treatment of most severe inflammatory rheumatic diseases such as systemic lupus erythematosus (SLE) and most vasculitic disorders. Severe SLE flares affecting approximately a third of all patients at one time of their disease, typically mandate the use of high doses of glucocorticoids and/or initiation or intensification of immunosuppressive treatment [75]. In rheumatoid arthritis, because of the serious side effects associated with chronic use, glucocorticoids are administered temporarily if the disease remains active despite usual antirheumatic drugs or if the disease flares [76].

58.5.4 Renal Diseases

Glucocorticoids are recommended as the first-line treatment for nephrotic syndrome secondary to minimal change disease both for children (moderate quality of evidence) and for adults (low quality of evidence) [77]. Diminished proteinuria is usually observed within a month and most patients achieve remission within 3 months. Additionally, renal diseases secondary to SLE are treated by pulse IV followed by oral glucocorticoids (low quality of evidence) [78].

58.5.5 Preventing Graft Rejection

High doses of glucocorticoids in conjunction with other immunosuppressive agents are given at the time of transplant surgery. Most patients are thereafter kept on low-dose glucocorticoid maintenance regimen. Of note, the outcome of glucocorticoid-free immunosuppressive regimens following islet transplantation in type I diabetes mellitus seems to be improved [79].

58.5.6 Infectious Diseases

Preliminary data indicate that the daily administration of prednisone hastens recovery and hospital discharge in adults with community-acquired pneumonia [80,81]. Glucocorticoids (prednisone 40 mg twice a day for 5 days followed by progressive tapering over 3 weeks) for documented *Pneumocystis jirovecii* pneumonia in the acquired immunodeficiency syndrome causing hypoxemia (room air $PaO_2 < 70$ mmHg) reduces both the occurrence of respiratory failure (moderate quality of evidence) and mortality (high quality of evidence) [82,83]. Dexamethasone (0.15 mg/kg to a maximum dose of 10 mg, four times daily for 4 days) administered before or immediately after antibiotics in bacterial meningitis was found to reduce the incidence of hearing loss and long-term sequelae in both children and adults (high quality of evidence) [84]. Additionally, glucocorticoid administration also reduces the risk of death in HIV negative subjects affected by tuberculous meningitis (moderate quality of evidence) [85]. Steroid infusion (hydrocortisone; 200 mg/day) is currently the only adjunctive treatment available in septic shock. International guidelines suggest its use in case of persistent shock despite fluid resuscitation and vasopressor therapy (low quality of evidence) [86].

58.5.7 Adrenal Insufficiency

Adrenal insufficiency results from direct insults to the adrenal gland (primary adrenal insufficiency) or from insults affecting the anterior pituitary or the hypothalamus (secondary adrenal insufficiency). Acute adrenal insufficiency is a life threatening condition associating nausea, vomiting, and abdominal pain with dehydration hyponatremia, hyperkalemia, weakness, and hypotension. Management is based on intravenous vascular expansion, supplementation by hydrocortisone, 200 mg/day (moderate quality of evidence), and treatment of the precipitating factor [87].

58.5.8 Miscellaneous

Topical glucocorticoids are used in a variety of inflammatory dermatoses such as eczema. The risk of systemic absorption increases when the drug is applied on inflamed skin. Systemic glucocorticoids may be administered for severe episodes or for exacerbations of chronic disorders. Life threatening autoimmune dermatological conditions such as pemphigus are treated by systemic corticosteroids [88].

Mild forms of Crohn's disease confined to the ileum and/or right colon are effectively treated by controlled-ileal-release budesonide, a synthetic glucocorticoid that is inactivated by first pass hepatic metabolism and thus theoretically exhibits decreased levels of systemic toxicity (high quality of evidence). Moderate-to-severe Crohn's disease benefits from a treatment by corticosteroids until resolution of symptoms and weight gain (high quality of evidence) [89].

During severe liver disease, prednisolone should be used instead of prednisone, which requires hepatic conversion to be active. The first line of treatment for severe forms of autoimmune hepatitis is glucocorticoids (high quality of evidence) [90]. Patients with severe alcoholic hepatitis – defined as a Maddrey score of > 32 – should receive prednisolone (40 mg/day for 28 days, typically followed by discontinuation or a 2-week taper) (high quality of evidence) [91].

Glucocorticoids, which possess anti-lymphocytic effect, are used at high doses as a component in the chemotherapy of both lymphocytic leukemia and lymphoma. Corticosteroids are also commonly used in neurological conditions where inflammation is thought to be responsible of clinical symptoms such as brain metastases (low quality of evidence) [92] and spinal cord compression (moderate quality of evidence) [93]. In pulmonary sarcoidosis, glucocorticoids improve patients symptoms and spirometry over 3–24 months and may be of benefit in moderate-to-severe forms of the disease (low quality of evidence) [94].

Glucocorticoids are potent anti-inflammatory and immunosuppressive agents that are administered in a variety of conditions. However, except for adrenal insufficiency, glucocorticoids are nonspecific, symptomatic agents. Given the amount of serious undesirable side effects linked to these agents, both the risks and benefits must be carefully assessed before initiating glucocorticoid treatment.

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The Neuroimmune Communicatome in Inflammation

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59.1 Introduction

Numerous studies have shown that neurons, immune cells, and other cells with immune function communicate with one another. Neuroimmune communications occur throughout the entire body, involve many cell types, mediators, and pathways, and serve to maintain health in the face of environmental challenges. Neuroimmune communication in the central nervous system (CNS), in the periphery, and between the immune system and the brain serves to protect the host from damage and promotes repair. By contrast, dysregulated neuroimmune communication potentially contributes to numerous pathophysiological conditions, which are characterized by excessive and unresolved inflammation. In this chapter, we present an integrative view of neuroimmune communications during inflammation, which we term the neuroimmune communicatome. We also discuss new and emerging therapeutic approaches in disease treatment based on this neuroimmune communicatome.

59.2 What is Inflammation?

Classical inflammation is a critical component of the body's innate immune response to tissue injury caused by infectious or sterile stimuli. Inflammation involves a series of highly regulated, complex, and overlapping protective processes that require communication between different cell types. It is characterized by recruitment of immune cells to the site(s) of tissue injury/infection following specific interactions between pattern recognition receptors (PRRs) expressed by host immune cells, which recognize damage-associated molecular patterns (DAMPs), released by injured, metabolically deranged, or dead cells,

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and/or pathogen-associated molecular patterns (PAMPs) – which are highly conserved structures associated with pathogenic organisms [1,2]. Ligation of most PRRs with PAMPs and DAMPs results in activation of intracellular signaling and production of cytokines, chemokines, and other bioactive molecules. These molecules activate immune cells and the endothelium initiating a series of complex processes to recruit activated immune cells to the sites of infection or injury. Initially, this process is protective because it delivers critical host defense cells and molecules to affected sites. If the resultant inflammation is not proportional to the threat and subsequently resolved in a timely manner, it can lead to chronic inflammation and continual tissue damage. Inflammation resolution is an active, complex, and precisely orchestrated process [3]. Inflammation is mediated through both proinflammatory mediators (e.g., cytokines and chemokines) and inflammatory cells, as well as production of numerous biochemical factors, including anti-inflammatory factors (e.g., chemokines/chemokine receptors) [4] and proresolving mediators. These proresolving factors include lipids (e.g., lipoxins, maresins, and resolvins), proteins/peptides (e.g., annexin A1) [5], neurotransmitters such as acetylcholine (ACh) [6], and neuropeptides and axon guidance molecules, including substance P (SP) and netrin [7]. Chronic, nonresolving inflammation is a hallmark feature of numerous diseases (e.g., arthritis, inflammatory bowel disease, asthma, cardiovascular disease, diabetes, and cancer) [8].

Until recently, understanding of the neural involvement in inflammation was limited, but it has become clear that communication between immune cells and neurons plays an important role in the regulation of inflammation and restoring homeostasis [6,9–11].

59.3 Neuroimmune Dialogue in Inflammation and the Concept of the Neuroimmune Communicatome

The nervous system regulates a myriad of physiological functions and integrates cardiovascular, gastrointestinal, respiratory, and other vital functions to maintain homeostasis. Numerous scientific observations have challenged the concept that immune function is devoid of nervous system regulation and demonstrated that neural signaling regulates immune activation and inflammation. In addition, immune cell activation with the release of proinflammatory mediators affects neuronal activity and brain function. Neuronal and neurohumoral mechanisms, including catecholaminergic pathways, the hypothalamic–pituitary–adrenal (HPA) axis, and the vagus nerve-based inflammatory reflex regulate inflammation and are currently being explored in clinical studies for the treatment of inflammatory diseases with promising results. Ongoing research on neuroimmune interactions is expanding the range of neuronal circuits that regulate immune function and this novel insight holds promise for the development of innovative therapies for inflammatory disease. Herein, we highlight pathways and mechanisms of neuroimmune communications in inflammation and the concept of the neuroimmune communicatome (Figure 59.1).

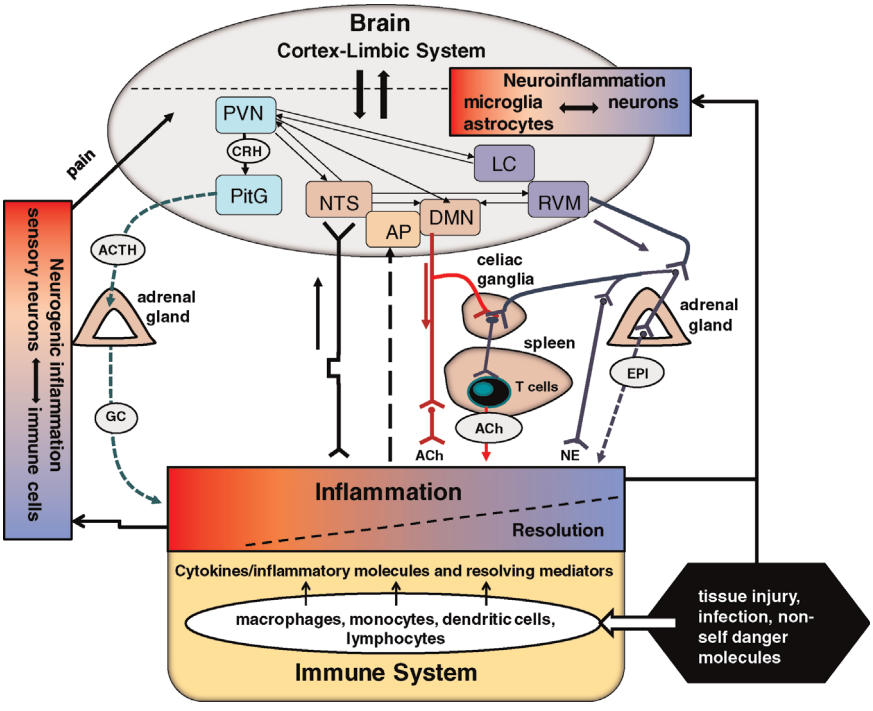


Figure 59.1 The neuroimmune communicatome.
Infection, tissue injury, and nonself danger molecules trigger complex inflammatory responses mediated by the activation of host macrophages and other immune cells of the host, which release cytokines and other inflammatory mediators. Inflammation is protective when it is localized and resolved in a timely manner, but also can be disease promoting and lethal when it is excessive, systemic, unresolved, and/or chronic. Multiple forms of bidirectional neuron – immune cell and immune system – CNS/brain communication occur during inflammation; these interactions govern its progression and resolution. We refer to these multifactorial interactions and communication in inflammation as the neuroimmune communicatome. Extensively studied specific loci of the neuroimmune communicatome are neuroinflammation in the CNS/brain and neurogenic inflammation, achieved within a sensory neuron axon–axon reflex manner in the periphery. The peripheral release of cytokines and other inflammatory products also trigger active communication between the immune system and the brain. Peripheral inflammatory signaling reaches the brain via afferent (sensory) vagus nerve fibers, circumventricular brain areas such as area postrema (AP), and other immune – to – brain pathways. Inflammatory molecules released in the periphery can promote or cause neuroinflammation and alter brain function ranging from temporary sickness behavior to chronic and severe neuropsychiatric manifestations. Peripheral inflammatory responses also trigger brain-derived immunomodulatory output. Afferent vagus nerve fibers terminate in the nucleus tractus solitarius (NTS) of the brainstem, which is interconnected with the dorsal motor nucleus of the vagus (DMN), where efferent (motor) vagus nerve fibers originate. These interactions mediate the brainstem integration of the vagus nerve-based inflammatory reflex, which suppresses peripheral proinflammatory cytokine release and controls inflammation. Interactions between the vagus nerve and the splenic nerve in the celiac ganglia mediate the release of acetylcholine (ACh) by T lymphocytes in the spleen. ACh exerts its anti-inflammatory effects by interacting with α7nAChRs expressed on macrophages and other immune cells. NTS and DMN are connected in a reciprocal manner with the hypothalamic paraventricular nucleus (PVN), a key component of the hypothalamic–pituitary–adrenal (HPA) axis, the rostroventrolateral medulla (RVM), and the locus coeruleus (LC), major brain components of the sympathetic

59.3.1 Major Components of the Neuroimmune Communicatome

A typical scenario of inflammation is triggered via activation of Toll-like receptors (TLRs), NOD-like receptors (NLRs), or other PRRs in macrophages and other innate immune cells. Subsequent activation of NF-κB and other transcription factors stimulates production of cytokines (e.g., TNF, IL-1β, IL-6, and IL-10), immune cell recruitment, eradication of invading pathogens and subsequent tissue repair. In parallel, an active cellular program to resolve the inflammatory response is initiated (Figure 59.1). The resolution of inflammation is a necessary step for restoration of homeostasis. Specialized proresolving mediators (SPMs) such as resolvins and protectins, limit leukocyte infiltration, cytokine production, and promote macrophage uptake of cellular debris and microbes. Failure of resolution results in a prolonged inflammatory response, a key factor in the pathogenesis of many inflammatory and autoimmune disorders, including sepsis, rheumatoid arthritis, inflammatory bowel disease, obesity, and diabetes. Aberrant inflammatory responses significantly add to the spectrum of pathological features of these disorders, which also involves metabolic, cardiovascular, and other homeostatic derangements [6,12–14]. Preventing and counteracting excessive and sustained inflammation are critical for homeostasis and present possibilities for therapeutic interventions.

Multiple mechanisms control the inflammatory response. In addition to the regulatory mechanisms that are inherent in the immune system, such as the release of anti-inflammatory cytokines and soluble cytokine receptors, neural signaling plays an important role in balancing defense and repair mechanisms in inflammation. Several scenarios of neuroimmune communication have been described. One example is neurogenic inflammation, which involves release of neuropeptides from sensory neurons that promotes fluid leakage, leukocyte activation, and pain (Figure 59.1) [15], as described in detail in Section 59.4.1.2. Neurogenic inflammation can be purposeful and protective, but excessive and sustained manifestations can contribute to autoimmune diseases and allergic symptoms [15]. Another example is the intricate interplay between neurons and resident cells with immune function in the CNS. Under normal conditions, microglia and astrocytes interact with neurons and modulate neurogenesis, synaptic functionality, and neurotransmission [16–19] and safeguard the integrity of the blood–brain barrier [17] (Figure 59.1). Importantly, microglia and astrocytes, major brain cell types with immune functions [17,18], actively participate and interact with neurons also in the so-called "neuroinflammation."

catecholaminergic outflow. The RVLM and LC also participate in the brain processing of pain (not shown). PVN neurons release corticotropin-releasing hormone (CRH), which in turn activates the release of adrenocorticotropin hormone (ACTH) by the pituitary gland (PitG). ACTH stimulates the secretion of anti-inflammatory glucocorticoids (GC) from the adrenal cortex. The release of norepinephrine (NE) and epinephrine (EPI) from catecholaminergic axon terminals and the adrenal medulla, respectively mediates proinflammatory and antiinflammatory effects via interactions with α- and β-adrenoreceptors expressed on immune cells. The role of forebrain regions and the limbic system in the brain integration of the neuroimmune communicatome and the involvement of the cholinergic and other neurostransmitter systems are areas of active research. See text for details.

Presence of PAMPs and DAMPs in the CNS can activate local TLRs and other PRRs and trigger production of proinflammatory cytokines, chemokines, and reactive oxygen species. Neuroinflammation is a necessary defensive event aimed at neuronal protection and preservation of brain function in response to threats. However, persistent neuroinflammation contributes to tissue injury and neurodegeneration and has been implicated in the pathology of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis [18,20]. In addition, there is active communication between the immune system and the brain in response to inflammation outside the CNS [21–25]. This is easily appreciated by considering how brain function is altered during inflammation manifested by typical "sickness behavior," including fever, fatigue, and anorexia [21,23]. In addition to these well-known symptoms, inflammation in the periphery also triggers immunomodulatory CNS output, including activation of the HPA axis, catecholaminergic signaling, the cholinergic anti-inflammatory pathway, and other neuronal mechanisms that regulate immune cell behavior [26] (Figure 59.1). Several sensory mechanisms play a role in "informing the brain about peripheral inflammation." For example, afferent vagus nerve fibers that terminate in the brainstem nucleus tractus solitarius (NTS) respond to increased levels of IL-1β and other proinflammatory cytokines in the periphery [25] (Figure 59.1). The signals are then communicated to other brain areas [25]. Blood-borne cytokines signal the brain through area postrema (AP) and other circumventricular organs [21,26]. Secondary mediation through the release of prostaglandins or activation of perivascular macrophages has also been documented [26]. In addition, infiltration of neutrophils, T cells, and other immune cells into the brain during inflammatory conditions has been described [22].

The HPA axis is an important neurohumoral immunomodulatory pathway. Major brain structures in the HPA axis are the hypothalamic paraventricular nucleus (PVN) and the pituitary gland [26–29] (Figure 59.1). The PVN contains neurons, specialized in synthesizing corticotrophin-releasing hormone (CRH) [27,29]. PVN neurons project to the pituitary gland and release CRH in its portal system, which leads to subsequent production of adrenocorticotropin hormone (ACTH) from the anterior pituitary [27,29]. The resultant ACTH action in the adrenal cortex generates the release of glucocorticoids. Glucocorticoids suppress proinflammatory cytokine production and promote production of anti-inflammatory mediators, suppress adhesion molecule expression, and inhibit neutrophil and monocyte infiltration in inflammation [29] (Figure 59.1). Based on these insights, synthetic glucocorticoids have long been used clinically to treat inflammatory disease and these mechanisms have been extensively reviewed elsewhere. [27,29].

Catecholamines, including the predominant postganglionic neurotransmitter in the sympathetic division of the autonomic nervous system, norepinephrine (NE), and epinephrine (Epi) from the innervated adrenal medulla, regulate leukocyte function by interacting with cognate receptors on immune cells [26,30] (Figure 59.1). Depending on context, the involved receptor and cell type, catecholamines can exert both pro inflammatory and anti-inflammatory effects [26,30]. For instance, NE acts on α_2-adrenoceptors on monocytes and macrophages to promote the production of TNF, and other cytokines [31]. NE

can also stimulate neutrophil accumulation and aggravate inflammation [30]. Conversely, NE and Epi inhibit proinflammatory cytokine production and stimulate anti-inflammatory cytokine production through activation of β2-adrenoceptors [30]. Brain structures, including the locus coeruleus (LC) and the rostroventrolateral medulla (RVM), regulate sympathetic neuronal activity through interactions with spinal cord preganglionic neurons (Figure 59.1) [24,30]. The specific interactions between these neural networks, afferent neural signals, inflammatory cytokines, and leukocytes remain to be characterized and understood in detail. The effects of local and systemic catecholamine release in inflammation are quite complex, have been extensively reviewed elsewhere, and are an area of ongoing research [30].

Cholinergic signals also play a key role in the control of inflammation. In this context, the vagus nerve-based inflammatory reflex has been well studied [11]. In this reflex circuit, cytokines and other inflammatory molecules activate afferent vagus neurons, which transmit signals to the nucleus tractus solitarius (NTS). Neuronal projections from the NTS to the dorsal motor nucleus (DMN) of the vagus make functional connections with efferent vagus nerve fibers, which provide a multistep anti-inflammatory signal that suppresses proinflammatory cytokine release (Figure 59.1) (see Section 59.3.2. for details) [11,26,32–36].

The brain integration of the inflammatory reflex in the brainstem medulla oblongata occurs through reciprocal neural projections between the NTS and the DMN, which also interact with the AP in a functional unit, termed the dorsal vagal complex (Figure 59.1) [26]. Neural projections among the constituents of the dorsal vagal complex, the main brain components of the HPA axis, and regions involved in the regulation of the catecholaminergic immunomodulatory outflow, including locus coeruleus and rostroventrolateral medulla, provide a neuroanatomical substrate for further brain integration of the inflammatory reflex with other brain-derived immunomodulatory pathways (Figure 59.1) [26]. In addition, the cortex and other brain components of the limbic system, including the hippocampus and amygdala, regulate peripheral immune responses [37]. These forebrain areas interact with the dorsal vagal complex and regions involved in the sympathetic and neuroendocrine regulation of immune function directly or through multisynaptic pathways [38].

59.3.2 Reflex Principles: The Inflammatory Reflex and Beyond

Nerves are uniquely capable transmitters of information. In contrast to endocrine factors, neurons can instantaneously deliver precisely targeted signals. Reflex circuits consist of sensory (afferent) neurons sending signals from the periphery to the CNS and motor (efferent) neurons that project from the CNS to peripheral target tissues. Sensory and motor neurons provide for information exchange between the CNS and practically the entire organism. The complex arrangement of the brain with sophisticated layers of multiple types of highly organized neuronal systems constantly process the numerous external and internal inputs and integrate set points and sensory signals to fine-tune motor responses. In this way, autonomic homeostatic reflexes provide rapid and precise control of a wide variety of physiological functions. In particular, reflexes based on autonomic

neuronal circuits help protect from a wide range of harmful events by regulating key physiological processes, including body temperature, heart rate, blood pressure, and metabolism. This system of balanced integration of input signals and set points provides for sophisticated optimization. It is easy to appreciate that dysfunction of the homeostatic reflexes can contribute to organ damage and even death.

Given the insights that the nervous system has evolved to provide control of key physiological functions for health and survival, it is reasonable that antimicrobial defense and wound healing – functions where the immune system plays a central role – are monitored and regulated by the brain and autonomic reflexes [39]. In this system, information on alterations in the immune function and inflammation are sent to the brain (Figure 59.1). Peripheral nerves are equipped with receptors for cytokines as well as PAMPs and DAMPs. Activation of these sensory mechanisms trigger brain-derived immunoregulatory mechanisms that may result in systemic release of anti-inflammatory factors, including corticosteroids, activation of specific motor neurons, and release of immune-regulatory neurotransmitters (Figure 59.1). The inflammatory reflex, in which the vagus nerve plays a key role, is a well-described circuit that regulates cytokine release. In this reflex, sensory signals are elicited by proinflammatory factors in tissues innervated by the sensory vagus nerve, and give rise to brain-derived motor activity in the vagus nerve (Figure 59.1). The efferent vagus nerve travels to the celiac ganglia where the splenic nerve arises. Electrical activity in the splenic nerve eventually triggers neuronal NE release that activates a particular subset of ACh-producing T cells in spleen. These choline acetyltransferase (ChAT)-expressing T cells release ACh and relay the neural signal to α7nAChRs on immune cells in the spleen, which ultimately reduces their release of proinflammatory cytokines. Intracellular mechanisms triggered by this interaction involve suppression of NF-κB nuclear translocation to mediate cholinergic suppression of TNF and other proinflammatory cytokine production [6]. The efferent arm of the inflammatory reflex, the cholinergic anti-inflammatory pathway, can be activated by electrical vagus nerve stimulation (VNS) and conversely, disrupted by surgical transection of the vagus nerve – vagotomy or splenic nerve transection [10,40]. The role of vagus nerve–splenic nerve functional integration regulating ACh release in the spleen is reflected by the increased splenic ACh levels in animals following VNS [35,41]. An immunomodulatory role of efferent cholinergic fibers innervating the liver, heart, gastrointestinal tract, and other visceral organs has also been demonstrated. Cholinergic anti-inflammatory output from these fibers is inversely correlated with proinflammatory cytokine levels and disease severity in animal models of hemorrhagic shock, ileus, pancreatitis, and inflammatory bowel disease [6,10,42,43]. Moreover, VNS decreases TNF and other proinflammatory cytokine levels and alleviates disease severity in various inflammatory conditions [39]. The study of the cholinergic regulation of inflammation activity, including the role of the α7nAChR on macrophages, has spawned investigation of the anti-inflammatory efficacy of selective α7nAChR agonists [44,45]. Inhibition of inflammation by GTS-21, choline, and other selective α7nAChR agonists has been observed in preclinical models of endotoxemia, sepsis, rheumatoid arthritis, type 2 diabetes, and other inflammatory and autoimmune disorders [10,46–48].

Hence, the detailed understanding of the inflammatory reflex has opened a number of novel avenues in the search for potential efficacious treatments of excessive inflammation.

Interestingly, in *Caenorhabditis elegans*, which is one of the least complex animals with a nervous system, the innate immune response to bacterial invasion of non-neural cells is regulated by neural signals [49]. In higher animals, neural control of inflammation has been reported in peripheral nerves other than the vagus nerve. For example, TRPV1 and Nav1.8-expressing neurons interact with dermal dendritic cells to regulate skin inflammation in a model of psoriasis [50]; however, the neurophysiology and molecular biology of these pathways are not completely understood. Neuronal activity also regulates the movement and homing of leukocytes with important effects on disease progression in experimental models. For example, alteration of the functional neural signals to hepatic invariant natural killer T (iNKT) cells can significantly decrease this post-stroke pneumonia-induced mortality in mice [51]. In another study, elimination of specific proprioceptive signals entering the lumbar spinal cord prevented CNS entry of pathogenic T cells in a murine model of multiple sclerosis [52]. Furthermore, electrical stimulation of the sciatic nerve can regulate production of catecholamines via vagus-nerve activation, and significantly reduces the mortality in sepsis [53]. Recent insights into these mechanisms have fundamentally changed our understanding of immune system regulation, which now includes neural reflex circuits. These discoveries are paving the way for the application of revolutionary ideas for support of tissue healing and treatment of chronic inflammatory diseases using electrical nerve stimulation [39,54–56]. Further research in the emerging field of bioelectronic medicine is likely to significantly expand our understanding of immune-regulatory neural reflexes in the years to come.

59.4 Points of Interaction in the Neuroimmune Communicatome

Inflammation affects neuronal activity and brain function, and neuronal circuitry has a major involvement in the regulation of inflammation. Specific features of this reciprocal communication and impact, and the loci of the neuroimmune communicatome in inflammatory conditions are described next.

59.4.1 How the Nervous System Is Affected in Peripheral Inflammation

The impact of peripheral inflammation on the nervous system is multifaceted. An example of this impact is neurogenic inflammation. Of note, neurogenic inflammation also implicates an active neural output modulating immune function. In classical inflammation, peripheral inflammatory products can be sensed by afferent vagus nerve fibers and the signal is transmitted to the brain. Moreover, brain neuronal function is altered during peripheral inflammation and these alterations can be protective. However, they can be exacerbated to the

level of life-threatening neurological complications in the context of inflammatory diseases.

59.4.1.1 Neurogenic Inflammation

The term "neurogenic inflammation" was introduced by Jansco and colleagues to describe the plasma leakage and edema following the application of capsaicin to the skin and/or mucus membranes of experimental animals [57]. The effect was abolished by surgically transecting the peripheral nerves or by desensitizing via repeat applications, thus, the authors concluded that sensory nerve stimulation contributes to inflammation in the periphery via the local release of biologically potent mediators that promote vascular permeability [57]. Today, the term "neurogenic inflammation" describes a complex and orchestrated response in the periphery mediated through a series of interactions between ligands and receptors on sensory neurons and immune cells. Sensory neurons serving as nociceptors signal afferent communication about noxious, and/or damaging stimuli leading to the perception of pain (Figure 59.1). Upon activation, these neurons release, in an antidromic fashion (i.e., in the opposite direction to normal conduction), neuropeptides and other mediators (e.g., SP, calcitonin gene-related peptide [CGRP], neuropeptide Y, and glutamate. These mediators trigger local release of histamine and other bioactive substances (primarily by mast cells), including 5-HT, adenosine, elastase, matrix metalloproteinases, chemotatactic factors for eosinophils and neutrophils, prostaglandins, thromboxanes, NO, and TNF and other cytokines and neuropeptides. Collectively, these substances increase vasodilation, vascular permeability, plasma extravasation, and recruitment of immune cells to the site [58–60]. Triggers for neurogenic inflammation include classical, infectious microbes (e.g., bacteria, viruses, and fungi), autoimmune processes, DAMPs, and other factors that increase neuronal activity (e.g., noxious stimuli). Similar to the classical inflammatory response of the innate immune system, neurogenic inflammation includes anti-inflammatory and resolving "regulatory components," including anti-inflammatory cytokines, neuroprotectins, SPMs, neurotransmitters, and neurotrophic factors [60]. However, prolonged stimulation and positive feedback release of proinflammatory factors from the tissue and immune cells can cause a sustained increase in vascular permeability, a disproportionately strong recruitment of immune cells, further tissue damage, and ultimately result in a state of persistent pain [59]. Hence, neurogenic inflammation can be beneficial, but also dangerous and destructive.

59.4.1.2 Neural Sensing of Inflammation

A sensory link between inflamed tissue and the nervous system was illustrated when Aulus Cornelius Celsus included "pain" among the four hallmarks of inflammation some two millennia ago. However, the molecular interactions between the nervous and immune systems long remained unexplored and are yet incompletely understood. Clearly, infection, injury, and inflammation can provoke wide-ranging and purposeful modifications of CNS-controlled behavior and physiological set points within the scope of sickness behavior, including

elevated body temperature, hyperalgesia, reduced food intake, and increased sleep [61]. Experimental evidence indicates that the nervous system and the immune system are hardwired in monitoring damage, microbial invasion and inflammation.

Signals for inflammation reaches the brain through a number of specific mechanisms, for example, via neural pathways, circumventricular organs, and receptors in CNS [25,26,62]. Interestingly, the systemic levels of TNF significantly influence the perception of pain in experimental models of inflammatory disease [63]. Furthermore, signals in peripheral sensory nerves are directly activated by inflammation-related events in the body. Body surfaces contain a dense network of sensory nerves equipped with similar molecular sensors as leukocytes, including Fc receptors [15,64,65]. The sensory vagus nerve and the carotid body express receptors for cytokines, TLRs, and other inflammation-associated molecules. Ligation of these receptors can elicit electrical activity in the vagus nerve [50,66,67] and may constitute a mechanism to inform the brain about peripheral inflammatory events. In peripheral tissues, immune cells commonly reside in close proximity of nerve fibers. Leukocytes are found near the vagus nerve in viscera, which provides an anatomical basis for a rapid neural response to factors released by these immune cells. In addition, invading bacteria can directly activate sensory nerves and elicit axonal signals [68]. Abdominal surgery provokes vagus nerve signals that regulate intestinal leukocyte recruitment [69]. Endotoxin and proinflammatory cytokines elicit sensory signals that result in a motor nerve response [32,67]. For instance, IL-1β injection in the portal vein increases splenic nerve activity [66]. Together, these and other sensory mechanisms enable a supply to the CNS of afferent information on dynamic changes in tissue integrity, microbial invasion and inflammation. Although important aspects of the neural sensing of immune-related signals have been delineated, understanding of the complete stepwise process is incomplete.

59.4.1.3 Brain Function in Peripheral Inflammation

Brain function can be altered in conditions characterized by peripheral inflammation. These brain alterations range from sickness behavior to severe neurological complications. Sickness behavior encompasses fever, changes in sleep, reduced physical activity and social interaction, appetite suppression, and other events [21,70]. A major contributor to and a mediator of sickness behavior is proinflammatory signaling within the brain, which is triggered or activated by cytokines and other inflammatory products released in the periphery (Figure 59.1) [21,36,71]. Peripheral inflammatory signals reach the brain via afferent nerve signaling, circumventricular organs, and other mechanisms described in Section 59.3. Local proinflammatory signaling in the brain is associated with microglial and astrocyte activation [36]. In classical scenarios of neuroinflammation following brain injury, infection and neurodegeneration, microglia, and astrocytes produce proinflammatory and anti-inflammatory cytokines, chemotactic factors, and neuropeptides and cytotoxic factors. These mediators of neuroinflammation interact with neurons to promote neuronal repair and restore neuronal function. However, chronic, unresolved neuroinflammation can lead to neuronal dysfunction. At least

some aspects of this pathophysiology are observed when the trigger is a peripheral inflammatory insult. Sickness behavior can be considered an important temporary protective mechanism. However, multiple transformations of sickness behavior can result in neuropsychiatric manifestations, described in septic patients and sepsis survivors, as well as patients with liver failure and cirrhosis, obesity and diabetes, systemic lupus erythematosus, and other inflammatory and autoimmune diseases [36,72,73]. The symptoms of such neurological complications range from relatively mild cognitive deterioration to delirium and coma within characteristic encephalopathies with debilitating and life-threatening consequences [36,74]. The clinical importance of these neuropsychiatric manifestations dictates the urgent need for early diagnoses and adequate treatment options. Despite increasing research efforts, the underlying pathogenesis of these neurological complications remains poorly understood. Proinflammatory signaling in the brain, derangements in cerebral blood flow and microcirculation, and alterations in brain metabolic activity have been described.

Dysfunctions of brain neurotransmitter systems have also been reported. For instance, brain cholinergic hypofunction has been observed in delirium and in sepsis encephalopathy [36,75]. This is an important finding for several reasons. The brain cholinergic system has a critical role in cognition, sleep, movement control, and peripheral/visceral function [76–78]. Brain cholinergic muscarinic acetylcholine receptor (mAChR)-mediated signaling has been implicated in the regulation of peripheral inflammation [6,41,79], and brain cholinergic mechanisms also regulate local brain inflammatory responses [80–83]. Therefore, brain cholinergic insufficiency may potentially be causally linked to brain neurological complications and may thus be targeted therapeutically. Centrally acting drugs such as acetylcholinesterase (AChE) inhibitors are clinically approved for treating cognitive impairment in Alzheimer's disease. Centrally acting AChE inhibitors also have powerful anti-inflammatory properties [36], and it is therefore conceivable that these modalities may provide therapeutic benefits in the context of neurological complications of inflammatory disease. Alterations in catecholaminergic, dopaminergic, GABA-ergic glutamatergic, and orexinergic systems have also been described in the context of sepsis [36]. Hepatic encephalopathy, manifested by a spectrum of symptoms such as cognitive and sleep deterioration, impairments in motor activity and coordination, lethargy, confusion, and sometimes delirium and coma, occurs during acute and chronic liver failure in cirrhosis [84,85]. Metabolic and immune derangements, including elevated ammonia levels, astrocyte enlargement and dysfunction, microglia activation, increased proinflammatory signaling, and oxidative stress, have been implicated in the pathogenesis of this neurological condition [84,85]. Increased AChE activity and alterations in brain cholinergic neurotransmission have been described in hepatic encephalopathy in the context of acute liver failure [86]. Interestingly, these brain alterations may occur in parallel with autonomic nervous system alterations and decreased vagus nerve activity as determined by heart rate variability analysis [14,87,88]. These observations suggest new therapeutic options for neuropsychiatric complications in diseases with inflammatory and metabolic etiology [36].

59.4.2 How Immune Functions and Inflammation Are Regulated by the Nervous System

The nervous system regulates and integrates a spectrum of physiological defense mechanisms. The bidirectional communication between the nervous system and the immune system facilitates the neural regulation of immune functions and various forms and aspects of inflammation.

59.4.2.1 Neural Regulation of Immune Cell Trafficking and Infiltration

Inflammation involves an orchestrated collaboration between immune/effector, vascular, and neural components to achieve immune cell infiltration to the site of inflammation or injury.

In neurogenic inflammation, sensory neurons residing in the periphery release neuropeptides and other bioactive agents upon stimulation [89]. Through ligand–receptor interactions, these neuronally derived substances communicate with a variety of cells (endothelium, immune cells, neurons found in the periphery) to enhance vasodilation, vascular permeability, plasma extravasation, and leukocyte infiltration [89]. For example, neuron-derived vasoactive intestinal peptide (VIP), a neuropeptide produced and released in the gut, enters circulation to promote vasodilation and subsequent immune infiltration in surrounding tissues [90]. Similarly, substance P (SP), produced by unmyelinated nerve fibers, induces vasodilation in the periphery, as well as plasma extravasation, leukocyte activation, and leukocyte migration and thus regulates immune cell trafficking [91,92]. Mast cells, which express specialized adhesion molecules for nerve fibers, are found within close proximity to the nerve fibers in the periphery and are thus proposed to be the dominant immune cell involved in neurogenic inflammation [93]. Furthermore, mast cells have receptors for numerous neuropeptides and neural factors implicated in promoting neurogenic inflammation [60]. Mast cells, as well as nerve fibers, produce and release several mediators that activate the vasculature and attract additional immune cells (e.g., macrophages, T cells, and dendritic cells) to the site [60]. These responses during neurogenic inflammation primarily serve to protect the host and maintain health; however, when these responses are continuous or amplified, they can lead to disease.

Neural regulation of immune cell trafficking can occur through controlling the production and/or the release of neuron-derived substances, including VIP, SP, CGRP, neuropeptide Y, adrenomedullin, neurokinins A and B, glutamate, and cytokines/chemokines (e.g., eotaxin) that promote: (1) vasodilation and/or vascular permeability, (2) immune cell chemotaxis, and/or (3) activation of immune/effector cells and/or endothelial cells, as well as immune cell-endothelial cell interactions required for immune cell trafficking during inflammation [15].

Neural regulation of immune cell trafficking can be achieved by actively suppressing the production of mediators that increase vasodilation and vascular permeability or activate immune/effector cells and endothelial cells that promote leukocyte or immune cell infiltration in the periphery. For example, neurons can produce mediators (e.g., ACh) that suppress endothelial activation, immune cell activation, and leukocyte trafficking. Consistent with the known functions of the cholinergic anti-inflammatory pathway, as the efferent or motor arm of the

inflammatory reflex and the neural circuit that responds to and regulates innate immune responses, including inflammation [34], endothelial cell activation, and subsequent leukocyte trafficking, have been reported to be regulated by ACh and nAChR agonists [94]. Suppression of endothelial cell activation by neural mediators could in this way help resolve inflammation. Thus, neural regulation of immune cell trafficking can involve production of neural factors that promote the resolution of inflammation and tissue repair processes.

59.4.2.2 Neurotransmitters in Neuronal Circuits Controlling Inflammation

Immune cells express receptors for neurotransmitters and respond to neuronal outflow. In the nervous system, neurotransmitter release commonly occurs in synapses, that is, anatomically defined structures between neurons or a neuron and a non-neuronal target cell. In addition, neurotransmitters released outside synapses (e.g., volume transmission) propagate by diffusion, and form tissue gradients with effects on multiple cells and regions. Immune cells may be targets of both types of signal transmission and a variety of neurotransmitters exert significant effects on immune cell function. Recent progress has provided important insights as outlined below.

NE and other catecholamines mediate interactions of sympathetic neurons with immune cells. These interactions can occur in a synapse-like fashion. Alternatively, NE diffuses through the parenchyma of the innervated tissue before interacting with these receptors [24,30]. This communication regulates immune responses and inflammation (Figure 59.1). Catecholamines interact with different types of G-protein-coupled adrenergic receptors (ARs) expressed on immune cells [30]. For example, NE activates β2 adrenergic receptors (β2ARs) on T cells, B cells, macrophages, dendritic cells, mast cells, and neutrophils [95]. This activation triggers subsequent activation of a traditional or canonical pathway via G-protein-dependent cAMP-protein kinase A (PKA)-mediated signaling, which results in suppression of immune responses and inflammation. These findings suggest that β2AR signaling pathways in immune cells could be targeted to reduce chronic inflammation, promote resolution of inflammation, and improve tissue homeostasis. However, a shift toward noncanonical signaling via β2AR-induced ERK1/2 activation has also been described in some inflammatory conditions [95]. This signaling pathway is G-protein-independent and occurs in a cell-type-dependent manner that augments and sustains cell-mediated immune and inflammatory responses [95]. In addition, ligation of NE with αARs on immune cells is associated with proinflammatory signaling [26,30]. Dopamine activates a multitude of dopamine receptors expressed on T cells with specific effects on cell migration and cytokine release [96,97]. Therefore, catecholamines, and specifically NE, exert multiple cell- and receptor-dependent effects on immunity and inflammation.

ACh, released from cholinergic neurons, T cells, or B cells inhibits macrophage release of proinflammatory cytokines by activating α7nAChRs through a mechanism that involves NF-κB and JAK2/STAT3 [35,40,98,99]. Glutamate has been reported to regulate the activation of modified T cells, possibly by controlling cAMP-mediated signaling and the MAPK pathway [100]. Serotonin can promote T cell activation and modulate function at different phases of the immune response [101].

Much remains to be learned about the role of neurotransmitters in the regulation of immune cell function and inflammation. Improved understanding may provide valuable tools in therapeutic regulation of immune responses.

59.4.2.3 Neural Regulation of Inflammation Resolution

As described above, signaling through the vagus nerve controls inflammation through suppression of proinflammatory cytokine responses and prevents excessive, damaging inflammatory reactions [12,40]. This modulation can be explored in treating acute and chronic inflammatory conditions [12,102]. Another critically important aspect of a "protective" inflammatory process is the resolution of inflammation [103,104], as described in Section 59.2. Resolution is actively mediated through numerous proresolving mediators, including SPMs such as resolvins, lipoxins, protectins, and maresins [104]. Inflammation resolution occurs through limiting leukocyte infiltration and increasing the phagocytosis of dead/dying cells by neutrophils and macrophages – a process termed efferocytosis [103,104]. Serhan and colleagues have recently reported that vagus nerve signaling is an important neural regulator of inflammation resolution, thus bridging the anti-inflammatory effects of the vagus nerve with the active immune processes mediating resolution [7]. By utilizing unilateral right side cervical vagotomy in mice, they discovered a pivotal role for the vagus nerve in controlling the axon guidance molecule netrin-1, a suppressor of local inflammation and endogenous proresolving mediator [7]. Leukocyte subsets – including granulocytes, monocytes, macrophages, T lymphocytes, and B lymphocytes – found in the lungs following zymosan-induced peritonitis produced netrin-1 in zymosan-induced peritonitis [7]. Vagotomy significantly reduced *Nnt1* gene and netrin-1 protein expression in the lungs and peritoneum, as well as netrin-1 localization in lung neurofilaments [7]. Lower netrin-1 levels found in vagotomized mice correlated with lower ACh and increased localized inflammation. In addition, leukocyte infiltration was increased in netrin-$1^{+/-}$ mice and inflammation resolution was significantly delayed when compared to wild-type netrin-$1^{+/+}$ mice [7]. Vagotomy also reduced the levels of resolvin D1 (RvD1) and other proresolving mediators [7], while RvD1 administration counteracted the effects of vagotomy and decreased proinflammatory cytokine levels [7]. Likewise, administration of RvD1 shortened the time to resolution of inflammation in netrin-$1^{+/+}$ mice, which was abolished in netrin-$1^{+/-}$ mice [7]. A bidirectional functional relationship between netrin-1 and specialized proresolving mediators was further established by the finding that netrin-1 enhanced the biosynthesis of proresolving lipid mediators, including protectin D1 (PD1) and RvD5 in mice [7]. Netrin-1 also decreased leukocyte migration and stimulated the uptake of apoptotic leukocytes by human macrophages, and efferocytosis was synergistically enhanced when netrin-1 and RvD1 were coincubated with macrophages [7]. Collectively, these findings reveal that vagus nerve signaling regulates inflammation resolution via modulation of netrin-1 and proresolving mediators. These findings strongly implicate a potential role of neural circuitry and specifically the vagus nerve in the resolution of inflammation. Further experimental studies of the effects of vagus nerve stimulation and manipulation of signals in other neural reflex

circuits, on proresolution pathways will provide additional insight into these intriguing possibilities.

59.4.2.4 Immune Cells and Cells with Immune Function in the Periphery Implement the Neuronal Repertoire

As discussed above, immune cells and other cells associated with immune/inflammatory responses in the periphery (e.g., endothelial cells) express receptors for neurotransmitters and these receptors mediate neural regulation of immune responses, including inflammation. Many of these same cells synthesize and release substances classically identified as neurotransmitters, which have been implicated in the regulation of immune processes through paracrine and autocrine mechanisms. These substances and neurotransmitter receptors are important components of the communication between the nervous system and the immune system. Below, we will focus on the functional role of non-neuronal ACh and catecholamines, including dopamine and NE.

ACh, synthesized from acetyl CoA and choline by choline acetyltransferase (ChAT), is a major modulator of immune cell responses. Although commonly designated as a neurotransmitter, ACh is synthesized and released by many cells in the periphery, including T cells, as well as other immune cells (e.g., monocytes, macrophages, B cells, dendritic cells, and endothelial cells) [105]. Lymphocyte-derived ACh participates in local innate immunity [99] through its interactions with both mAChRs and nAChRs, which are widely expressed on immune cells and nonimmune cells in the periphery [105,106]. ACh-producing T cells in the periphery suppress cytokine production following vagus nerve stimulation [35]. Fujii and coworkers have recently reported that ACh found in the blood was primarily produced by $CD4^+$ T cells [106]. The factors controlling the release of ACh are not completely understood [106]. ACh is rapidly degraded by ubiquitously expressed AChE and thus, ACh must act on its receptors in proximity of its release. ACh neuronal receptors are relatively well characterized. The nAChRs form ion channels comprised of five homologous subunit structures surrounding a pore [107]. The mAChRs are G protein-coupled receptors, with five main subtypes (M1-M5) [107]. In contrast to the neuronal ACh receptors, non-neuronal mAChRs and nAChRs (including those on immune cells) are not fully characterized. ACh, produced by T cells is known to exert immunomodulatory effects through binding to α7nAChRs present on macrophages and suppresses TNF production/release (Figure 59.1) [35]. Interestingly, a recent study has reported that interaction of ACh with α7nAChRs expressed on mitochondria results in stabilization of mitochondrial membrane permeability after ROS exposure, prevention on inflammasome activation and reduction IL-1β, IL-18, and HMGB1 release [108]. Endothelial cells also express α7nAChRs and cholinergic agonists have been shown to regulate endothelial cell activation and subsequent leukocyte trafficking via nAChRs *in vivo* [94]. Therefore, the α7nAChR is a possible molecular target for anti-inflammatory treatments. Interestingly, a recent study showed that peritoneal B cell-derived ACh can suppress neutrophil trafficking; this action is not the result of reduced cytokine/chemokine expression, but is regulated via mAChR-dependent modulation of adhesion molecule expression on endothelial cells [99]. As described above, there

is a potential role for ACh in regulating the development of inflammation as well as in the active resolution of inflammation [7]. However, much remains to be learned about detailed mechanisms and the functional implications of ACh released from immune and other non-neuronal cells in the regulation of inflammation.

Catecholamines, including dopamine, Epi, and NE, are produced and released within the CNS, and by peripheral neurons, where they function as neurotransmitters. Epi, produced mainly in the adrenal medulla, functions as a hormone (Figure 59.1) [109]. Sympathetic postganglionic fibers produce and release NE in the periphery (Figure 59.1) where it can bind immediately to neighboring immune cells or diffuse through the parenchyma of innervated tissue before interacting with immune cells [24,30]. Like ACh, NE can be found in numerous immune cells, including peritoneal macrophages, mast cells, and numerous lymphocyte subsets [110]. β2-adrenergic receptors (β2ARs), a common ARs on immune cells [110], are found on lymphocytes, monocytes, macrophages, dendritic cells, mast cells, NK cells, and neutrophils [95]. Binding of NE to β2ARs on immune cells can lead to cAMP production and functional changes that inhibit immune responses, including inflammation (Figure 59.1). For example, NE activation of β2AR on T lymphocytes reduces the production of proinflammatory cytokines, including IFNγ, IL-4, and IL-5 [111]. Similarly, NE-mediated signaling impairs NK cell cytotoxic potential [112] and adhesion to the endothelium [113]. In most cases, activation of β2ARs on LPS-stimulated monocytes typically produces anti-inflammatory responses, characterized by decreased cytokine (TNF, IL-12, and IL-18) and chemokine (CCL3/MIP-1α) production, as well as suppression of ICAM-1 adhesion molecule expression, which together limit leukocyte accumulation [110]. These findings suggest that modulation of AR signaling in immune cells may serve as a therapeutic target to reduce chronic inflammation, promote resolution of inflammation. It should be noted, however, that under certain conditions, βAR signaling can be proinflammatory [26,30] and significant differences have been reported in rodent vs. human immune cell responses following NE treatment [110].

Finally, to the best of our knowledge, other than its ability to suppress proinflammatory mediator production, the role of NE in resolution of inflammation has not yet been explored. Thus, further studies are warranted to better characterize the role of AR-mediated signaling in immune cells during inflammation.

Dopamine acts in the periphery as a paracrine or autocrine factor by binding to and activating either dopaminergic receptors or α- and β-AR. Several immune cells, including T and B lymphocytes, macrophages, bone marrow-derived mast cells, and granulocytes synthesize dopamine [110], and measurable amounts of dopamine are found in the circulation. Dopamine signals through five different dopamine receptors (D1-D5), which are categorized as either D1-like or D2-like dopamine receptors based on their activation or inhibition of adenylate cyclase, respectively [114]. Various immune cells (e.g., T and B lymphocytes, monocytes, neutrophils, and NK cells) express dopamine receptors [114,115]. Similar to NE, dopamine regulates immune cell activation and cytokine production, as well as cell proliferation [114,116]. For example, dopamine promotes T lymphocyte

proliferation and cytokine production following LPS stimulation [117]. A very recent study highlights the modulation of the NLRP3 inflammasome activation by dopamine [118], which leads to the release of mature/active IL-1β and IL-18 [119]. Excessive NLRP3 activation and subsequent overproduction of proinflammatory IL-1β and IL-18 are implicated in several chronic inflammatory conditions (e.g. diabetes, metabolic syndrome, and atherosclerosis) [119]. Thus, targeting the dopamine/dopamine receptor pathway may have therapeutic potential for treating inflammatory conditions.

59.4.2.5 The Brain as an Active Player in the Regulation of Peripheral Inflammation: Focusing on Cholinergic Mechanisms

The brain is not merely a passive recipient or a "victim" of peripheral inflammatory insults, but rather an active participant in the regulation of peripheral immune functions and inflammation. The role of the vagus nerve as a conduit of bidirectional immune system – brain communication has triggered interest in elucidating brain mechanisms regulating the efferent arm of the inflammatory reflex. We focus below on research that highlights a pivotal role for brain cholinergic mechanisms and mAChRs in this regulation. The brain cholinergic system, with somata localized in the basal forebrain and mesopontine tegmentum provides innervations to the cortex, hippocampus, amygdala, hypothalamic nuclei, thalamus, and other brain regions [76,120]. Postsynaptically located mAChRs, which predominantly belong to the M1 subtype, play a major role in processing ACh neurotransmission in the cholinergic synapses in these and other brain regions [76,120]. Brain areas innervated by the cholinergic system interact directly or via multisynaptic pathways with the dorsal vagal complex, the brainstem integrative center of vagus nerve activity, and the inflammatory reflex. These neuroanatomical substrates provide a means of brain cholinergic regulation of anti-inflammatory output via the efferent vagus nerve. The role of brain mAChR signaling in controlling peripheral inflammatory responses has been indicated by the observation that intracerebroventricular (icv) administration of an M1 mAChR agonist significantly suppresses serum TNF levels in endotoxemic rats [121]. In addition, icv injection of a mAChR ligand activates efferent vagus nerve activity, determined by heart rate variability analysis based on ECG recording [121]. M1 mAChR agonist delivery via icv infusion also reduces colonic cytokine production and disease severity in two models of colitis [41,122]. An intact vagus nerve and a splenic nerve are required to mediate these effects [41,122]. Another recently published study evaluates the anti-inflammatory potency of peripheral administration of xanomeline – a centrally acting M1 mAChR agonist studied for the treatment of Alzheimer's disease and schizophrenia [123]. Brain mAChRs and an intact vagus nerve-splenic nerve axis are required for xanomeline-mediated suppression of TNF in a mouse model of endotoxemia [123]. An anti-inflammatory role of brain mAChRs has also been shown in experimental hemorrhagic shock [124,125] and other preclinical settings of inflammatory disorders [36]. Intriguingly, anticholinergic drugs (and specifically centrally acting anticholinergics), which are frequently prescribed to the elderly, have been found to exacerbate inflammatory responses to endotoxemia in a murine model of neurodegenerative tauopathy [126,127].

In addition to using M1 mAChR agonists and other centrally acting mAChR ligands, brain cholinergic activation can be achieved by using AChE inhibitors, which inhibit ACh breakdown in the synaptic cleft and potentiate brain cholinergic signaling. Some of these agents are in clinical use for the treatment of Alzheimer's disease. The anti-inflammatory properties of these drugs have been demonstrated in murine endotoxemia, inflammatory bowel disease, and other inflammatory conditions [36]. For instance, peripheral administration of the centrally acting AChE inhibitor, galantamine, significantly reduces serum TNF and improves survival in mice with endotoxemia [79]. Brain mAChRs and vagus nerve α7nAChR-mediated signaling are required for the anti-inflammatory effects of galantamine in endotoxemia [79]. Galantamine also stimulates the efferent vagus nerve in rodents [128]. Treatment with galantamine alleviates inflammation and disease severity in a model of inflammatory bowel disease through brain mAChRs and activation of vagus nerve – splenic nerve neuronal axis [41]. Together these findings indicate that brain mAChR-mediated cholinergic activation by galantamine is functionally linked to activation of the efferent arm of the inflammatory reflex. Anti-inflammatory and disease alleviating properties of galantamine and other centrally acting AChE inhibitors have also been demonstrated in adjuvant-induced arthritis [129], spontaneous hypertension [130], obesity and the metabolic syndrome [131], type 2 diabetes [132], type 1 diabetes [133], and other preclinical models of diseases [36]. Of note, peripheral inflammation has been reported in patients with Alzheimer's disease, which is characterized by neurodegeneration affecting basal forebrain cholinergic neurons. Treatment of Alzheimer's patients with AChE inhibitors, which may provide a temporary boost in brain cholinergic signaling, results in suppression of peripheral inflammatory responses [134–136]. Together, these findings suggest an interesting connection between brain cholinergic signaling and peripheral inflammation.

59.5 Translational Exploration Based on the Neuroimmune Communicatome

The available therapy for treating excessive inflammation remains limited. Steroidal and nonsteroidal anti-inflammatory drugs, small molecule compounds, and specific anti-cytokine drugs in clinical use are not selective to particular tissues and often produce serious undesirable side effects. For example, systemic anti-TNF therapy, which has revolutionized the treatment of several chronic inflammatory diseases, may increase the risk of serious opportunistic infections [137,138]. The need for new, selective, and adaptable treatment options in inflammation is, therefore, pressing [8].

Providing insight into neuroimmune communication has gone hand in hand with evaluating new therapeutics for treating inflammatory and autoimmune conditions. This avenue of clinically oriented research has already identified several pharmacological agents that can be used in the context of human disease. In addition, harnessing neuroimmunomodulation by utilizing electrical stimulation and other types of device-generated output has identified new therapeutic strategies

for disease treatment in the growing field of bioelectronic medicine. Below we focus on the emerging clinical development of some of these new approaches.

59.5.1 Pharmacological Anti-Inflammatory Approaches Based on the Inflammatory Reflex

Active preclinical research on the inflammatory reflex in the context of septic shock, sepsis, hemorrhagic shock, arthritis, inflammatory bowel disease, obesity, type 2 diabetes, and other disorders [6,12,102] has identified new pharmacological approaches that can be further studied in clinical settings. Some of these approaches, utilizing clinically approved α7nAChR agonists and centrally acting AChE inhibitors are being tested in human inflammatory conditions. For instance, the α7nAChR agonist, GTS-21, at doses previously reported to be safe in humans [139] has been recently tested in human endotoxemia [140]. Although no significant anti-inflammatory effects were found, a correlation between higher plasma GTS-21 levels and lower plasma levels of TNF, IL-6, and IL-1 receptor agonist were observed [140], raising the question of whether higher GTS-21 doses would be effective. Treatment with another α7nAChR agonist, choline, has been reported to significantly lower TNF and other inflammatory molecules, reduce oxidative stress, and alleviate disease scores in patients with asthma [141], further supporting the potential benefit of targeting α7nAChR.

The pandemic proportions of obesity and obesity-driven disorders coupled with the health risks they present identify a large unmet medical need [6]. The preclinical efficacy of α7nAChR agonists and galantamine in alleviating inflammation and insulin resistance in preclinical settings has led to recent exploration of these drugs in clinical scenarios of obesity-driven disorders. The effects of the selective α7nAChR agonist, TC-6987, are being evaluated in type 2 diabetes (https://clinicaltrials.gov/ct2/show/NCT01293669?term=Targacept&rank=11). A recent trial is evaluating the effects of clinically approved (for the treatment of Alzheimer's disease) galantamine doses in individuals with metabolic syndrome (https://clinicaltrials.gov/ct2/show/NCT02283242?term=cholinergic+obesity&rank=2).

59.5.2 Bioelectronic Medicine

The inflammatory response must be fine-tuned to purge invading pathogens and promote resolution without causing excessive tissue damage and development of inflammatory diseases. The onset and resolution of inflammation are complex processes involving phenotypic changes in immune cells, switching effects of inflammation-related molecules, secretion of proresolving mediators, and more. Current pharmacological treatment carries a significant risk for serious adverse effects, partly because the treatment is commonly not sufficiently specific in terms of anatomical location and targeted mechanism. The complexity of involved mechanisms makes development of useful treatments difficult, and conceptual innovations in inflammatory and autoimmune disease therapy are urgently needed [8].

The emerging understanding of the neuroimmune communicatome described in this chapter suggests that interventions in neural signaling can be used for

therapeutic regulation of inflammation. By interfacing with the physiological autonomic reflexes that are already in place, more specific treatment in terms of anatomical and molecular targets coupled with immediate evaluation of effect of interventions might be possible, although an intense multidisciplinary experimental effort will be required to bring this concept from vision to reality. This effort brings neuroscience, immunology, molecular biology, engineering, and clinical medicine together in developing new technology to discover, define, and therapeutically modulate neural circuits and molecular mechanisms of the neuroimmune communicatome, and has spawned the field of bioelectronic medicine [36,142,143].

Importantly, bioelectronic medicine relies on detailed insights into the neurophysiological and molecular mechanisms that constitute the homeostatic neural reflexes that regulate immune activity. The experimental observations thus far have identified potential targets for nerve stimulation therapy in the peripheral nervous system and in the CNS. We anticipate that basic science studies of the neuroimmune communicatome will continue to identify a multitude of interesting potential therapeutic targets. These advances should provide insights for optimization of clinical trial design and a more detailed prediction of effects and potential side effects. Current experimental findings defining the inflammatory reflex have enabled the design of clinical trials for treatment of inflammatory disease. Studies of the effect of implanted vagus nerve stimulators on symptoms in rheumatoid arthritis (https://clinicaltrials.gov/ct2/show/NCT01552941) and Crohn's disease (https://clinicaltrials.gov/ct2/show/NCT02311660) have been performed. Results from these trials, demonstrating the efficacy of these treatments were recently published [144,145].

The prospect of using electrical stimulation of specific nerves to regulate inflammation and treat disease is attractive. The emerging concept of using nerve recording and stimulation for therapy tentatively provides a novel approach in personalized medicine: It may be possible to integrate sensory information on inflammation and other clinical data, and to use it to continuously guide disease diagnosis and treatment. It is tempting to speculate that perhaps even a fully implantable closed-loop device might be developed to provide uninterrupted access for clinicians and patients to data on disease development to optimize diagnosis and treatment, as well as monitor responses to treatment. However, much remains to be learned about the anatomy and physiology of the neuroimmune communicatome before the feasibility of such prospects can be determined.

59.6 Conclusions

Herein we have described important aspects of the current understanding of neuroimmune communication in inflammatory conditions by introducing the unifying concept of the neuroimmune communicatome. Inflammation has many facets and multiple forms. It represents a native defense mechanism when localized and resolved in a timely fashion. However, inflammation can be disease promoting and lethal when unbalanced and unresolved. Immune cells and neurons interact and communicate with each other during inflammation and

inflammation resolution in order to maintain and restore biological homeostasis. Specific forms of active neuroimmune communication occur during neurogenic inflammation in the periphery and neuroinflammation in the CNS and in the brain respectively (Figure 59.1). Peripheral inflammatory conditions also trigger active communication between the immune system and the brain, mediated by vagus nerve neurons and other mechanisms (Figure 59.1), which provide a reflex control of peripheral inflammation. Other brain-integrated and coordinated reflex pathways have also been described. Introducing the concept of the neuroimmune communicatome is aimed at providing an integrative view of the involvement of the nerves in inflammation. It is evident that various forms of neuroimmune communication do not occur in isolation. For instance, sensory neurons, which, in an axon–axon reflex fashion mediate and regulate inflammation, also serve as nociceptors that transmit pain sensation to the brain and potentially alter brain function. Moreover, in addition to triggering brain-derived anti-inflammatory output, peripheral forms of inflammation can exacerbate neuroinflammation and even generate brain proinflammatory signaling that contributes to aberrant brain function, even to a life-threatening extent. Neuroimmune communication takes place through multiple mechanisms. Neurotransmitters such as ACh and NE along with cytokines and other immune cell-derived inflammatory molecules are important mediators. Expression of neurotransmitter receptors on immune cells and cytokine receptors on neurons facilitate this communication. Release by immune cells of ACh and other substances classically perceived as neurotransmitters is also implicated. Preclinical findings in neuroimmune communication have provided a rationale for clinical studies using pharmacological modalities and electrical nerve stimulation to treat inflammatory disease and spawned the field of bioelectronic medicine. Clearly, the basic science underlying the neuroimmune communicatome is in its infancy and new pathways and mechanistic insights will be elucidated in time. Implementing optogenetics, Cre-lox, and CRISPR/Cas9 genetic modifications and other state-of-the-art methodologies should facilitate preclinical research in this area. The knowledge generated holds the potential of implementing new strategies in disease treatment.

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Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
ACTH	adrenocorticotropic hormone

AP	area postrema
AR	adrenergic receptor
AVN	afferent vagus nerve
CAP	cholinergic anti-inflammatory pathway
ChAT	choline acetyltransferase
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CRH	cortotropin-releasing hormone
DAMP	danger/damage-associated molecular patterns
DMN	dorsal motor nucleus of the vagus
ECG	electrocardiogram
Epi	epinephrine
HPA	hypothalamic–pituitary–adrenal
icv	intracerebroventricular
iNKT	invariant natural killer T cells
LC	locus coeruleus
LPS	lipopolysaccharide
mAChR	muscarinic acetylcholine receptor
nAChR	nicotinic acetylcholine receptor
NF-κB	nuclear factor kappa B
NK	natural killer
NLRP3	NACHT, LRR, and PYD domains-containing protein 3
NOD	nucleotide-binding oligomerization domain
NE	norepinephrine
NTS	nucleus tractus solitarius
PAMP	pathogen-associated molecular pattern
PD1	protectin D1
PitG	pituitary gland
PRR	pattern recognition receptor
PVN	periventricular nucleus
RvD1	resolvin D1
RVM	rostroventrolateral medulla
SNS	sympathetic nervous system
SP	substance P
SPM	specialized proresolving mediator
TLR	Toll-like receptor
VIP	vasoactive intestinal peptide
VNS	vagus nerve stimulation

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60

The Inflammatory Response in Tissue Repair

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60.1 Introduction

In multicellular organisms, millions of years of evolutionary pressures led to the emergence of sophisticated systems for recognition of injury and repair of damaged tissues. The effectiveness of the reparative response following injury is dependent on the severity and type of injury, the organ affected, and species-specific characteristics. The ability for regeneration of certain tissues by some species, but not others cannot always be explained by species-specific differences in the level of complexity of the injured structure. Amphibians, but not reptiles, birds, or mammals can regenerate limbs [1]. Fish can regenerate myocardium following injury [2,3]; in contrast, adult mammalian hearts do not regenerate and heal through formation of a collagen-based scar [4,5]. Despite a wide range of functional outcomes, reparative responses following injury share common characteristics. Healing of injured tissues is dependent on timely activation and suppression of an inflammatory cascade that serves to eliminate the noxious stimulus, and clear the injured area from dead cells and matrix debris, while setting the stage for activation of reparative cells. Perturbations of the inflammatory reaction following injury may result in defective repair, prolonged injury, fibrosis, and development of organ dysfunction. This chapter discusses the cellular effectors and molecular signals involved in regulation of the inflammatory and reparative reaction following tissue injury.

60.2 Initiation of the Inflammatory Response Following Injury: The Alarmins

Necrotic cells release a wide range of mediators that serve as "danger signals" and trigger an intense inflammatory reaction. These endogenous molecules, termed alarmins [6,7], can also be secreted by activated immune cells and are capable of

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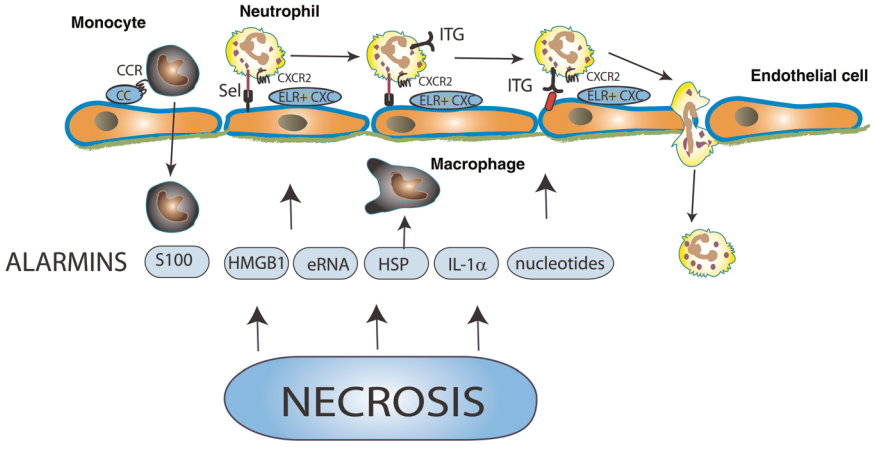


Figure 60.1 Adhesive interactions between leukocytes and endothelial cells mediate recruitment of inflammatory cells at the sites of injury. Dying cells (and some immune cell populations) secrete alarmins that activate Toll-like receptor (TLR)-dependent cascades, initiating the innate immune response. Activates endothelial cells express selectins (Sel) on their surface; these proteins interact with selectin ligands on leukocytes initiating rolling. Firm adhesion between leukocytes and endothelial cells require interactions between chemokines bound to endothelial glycosaminoglycans and their leukocyte receptors, and subsequent integrin (ITG) activation. Transmigration of the leukocytes predominantly occurs through endothelial junctions.

stimulating cellular responses that clear the injured tissue and promote a reparative program. The nuclear DNA-binding protein high-mobility group box-1 (HMGB1) is the prototypical member of the family. HMGB1 is passively released by necrotic cells, but may also be secreted by myeloid and NK cells without the need for *de novo* synthesis [8,9]. In contrast, cells dying through apoptosis do not release HMGB1. Members of the S100 family of proteins (such as S100A8/9, S100A12, and S100B), heat shock proteins, interleukin (IL)-1α, extracellular RNA or DNA, and uric acid can also act as alarmins, activating an immune response upon their release by necrotic cells (Figure 60.1) [10–12]. Alarmins target all cells implicated in tissue inflammation and repair, by activating pattern recognition receptors, such as Toll-like Receptors (TLRs) and nucleotide-binding oligomerization domain receptors (NLRs), and by binding to the promiscuous receptor for advanced glycation end-products (RAGE) [13]. Activation of TLR and RAGE signaling induces inflammatory mediator synthesis by innate immune cells, recruits antigen-presenting cells, and may be implicated in proliferation and differentiation of reparative cells, such as angiogenic endothelial cells and resident stem cells [14,15]. Moreover, prolonged secretion of alarmins may activate fibroblasts, promoting a fibrotic response [16]. Thus, alarmins have been implicated in both beneficial and detrimental actions following tissue injury. Tight spatial and temporal regulation of alarmin-mediated actions may be critical to activate a reparative program, while preventing inflammatory injury and progressive tissue fibrosis.

60.3 The Complement Cascade

The complement system is comprised by a large family of proteins, which are activated in a cascade-like fashion in response to host immunoglobulins, specific pathogens, or danger signals via three distinct pathways: (1) the classical pathway, (2) the lectin pathway, and (3) the alternative pathway. Regardless of the underlying mechanism, activation of the complement cascade ultimately leads to phagocytosis of pathogens, and ends with assembly and deposition of a membrane attack complex that kills bacteria and infected or damaged cells by disrupting their membrane integrity [17]. In addition to their cytolytic effects, components of the complement cascade also exert leukocyte chemoattractant actions [18], and trigger cytokine and growth factor release by a wide range of cell types [19–21]. Complement activation has been consistently demonstrated in healing wounds [22]. Experimental studies have suggested that activation of innate immune pathways by the complement cascade is crucial for recruitment of leukocytes [23] and may promote fibroblast proliferation and activation [24,25] in injured tissues. Moreover, evidence implicates components of the complement system in tissue regeneration. Mice lacking C3a and C5a exhibited a marked impairment in regeneration in models of hepatic resection and CCl4-induced parenchymal hepatic necrosis [26,27]. Thus, the complement cascade activates both cytotoxic and reparative processes following tissue injury.

60.4 Induction and Release of Proinflammatory Cytokines

Activation of innate immune pathways following tissue necrosis triggers the nuclear factor (NF)-κB cascade, leading to marked induction and release of proinflammatory cytokines and chemokines. Most cell types, including immune cells, vascular cells, fibroblasts, and parenchymal cells, residing in various organs are capable of producing large amounts of interleukin (IL)-1, tumor necrosis factor (TNF)-α, and IL-6 when stimulated by danger signals. Influx of abundant leukocytes with a proinflammatory profile, in response to the first wave of inflammatory mediators, further accentuates the cytokine response. Cytokines are highly pleiotropic, exerting a wide range of actions to all cell types involved in injury and repair. IL-1, the prototypical proinflammatory cytokine [28,29], is the classic endogenous pyrogen induced following inflammation, and critically modulates cellular behavior in injured tissues. Two major forms of IL-1, IL-1α and IL-1β, encoded by distinct genes have been identified and extensively characterized. Both act through the same receptor (IL-1RI) and have similar biological properties. In recent years, the IL-1 family has expanded with the discovery of several new ligands and receptor proteins.

IL-1α is constitutively expressed, and when released by necrotic cells acts as an alarmin, activating an inflammatory cascade [30]. IL-1β, on the other hand, is upregulated in immune cells following injury, and requires activation by caspase-1. Caspase-1 activation and subsequent release of active IL-1β requires assembly of a multiprotein complex, called the “inflammasome” [31]. When released in the area

of injury, IL-1 induces proinflammatory cytokine and chemokine synthesis and increases endothelial expression of adhesion molecules, mediating recruitment of leukocytes [32]. IL-1 also activates a proinflammatory program in fibroblasts, delaying myofibroblast transdifferentiation to prevent premature deposition of extracellular matrix proteins in injured areas, until the wound has been cleared from dead cells and matrix debris [33].

TNF-α synthesis is also upregulated in injured tissues and is expressed by many types of immune cells. Moreover, tissue resident mast cells may degranulate in response to complement activation, releasing significant amounts of preformed TNF-α from their granular stores [34,35]. TNF-α plays an important role in recruitment of leukocytes following injury by stimulating chemokine synthesis, by activating leukocyte integrins, and by inducing adhesion molecule expression by endothelial cells [36,37]. It should be emphasized that cytokines are highly pleiotropic and multifunctional, and have also been reported to exert prosurvival actions on resident cells in injured tissues [38].

60.5 Chemokines Regulate Leukocyte Recruitment

Chemokines are a family of small (8–12 kDa) chemoattractant proteins that plays a critical role in regulating trafficking of immune cells, both in homeostasis (during lymphoid organ development), and following tissue injury [39,40]. From a structural perspective, the chemokine family is subdivided into CC, CXC, CX3C, and XC subfamilies; this classification has important functional implications. CC and CXC chemokines are the largest subfamilies and exhibit distinct properties. Most CC chemokines function as potent chemoattractants for mononuclear cell subsets. On the other hand, CXC chemokines that contain the tripeptide motif glutamic acid–leucine–arginine (ELR) in the NH_2-terminal region (such as CXCL8/IL-8) are implicated in granulocyte recruitment [41,42]. Although chemokines predominantly target immune cells, several members of the family have been reported to function as modulators of many other cell types (such as endothelial cells, vascular smooth muscle cells, fibroblasts, neurons, and cardiomyocytes) [43,44], regulating cellular phenotype, proliferation, and survival.

On the basis of their mechanisms of regulation, chemokines can be divided into "homeostatic," which are constitutively expressed in certain tissues and may be involved in basal trafficking of leukocytes, and "inducible" chemokines that are upregulated through activation of innate immune pathways and mediate recruitment of leukocytes following tissue injury [45–47]. Clearly, this concept represents an oversimplification, as homeostatic chemokines (such as CXCL12/Stromal Cell Derived Factor (SDF-1) can be induced following immune activation [48], and inducible chemokines may also regulate trafficking of leukocyte subsets in normal tissues. However, the notion that some members of the family are predominantly upregulated in pathologic conditions and may participate in inflammatory and reparative responses following injury remains useful [43,46,49–51]. Chemokines act by binding to glycosaminoglycans on endothelial cells and on the extracellular matrix [52]. When immobilized into the microvascular endothelial surface, chemokines are presented to circulating

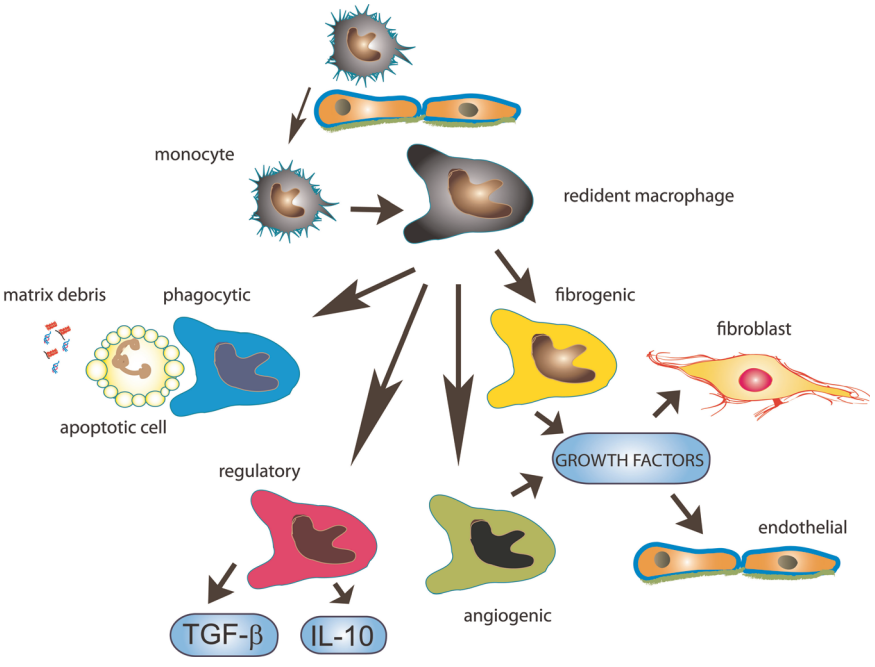


Figure 60.2 Macrophages in tissue repair. In injured tissues, macrophages do not exhibit M1/M2 polarization, but acquire nuanced phenotypes and critically regulate inflammatory, fibrogenic, angiogenic, and regenerative responses.

leukocytes expressing the corresponding chemokine receptors and can trigger an adhesive cascade that ultimately leads to extravasation of leukocytes into the area of injury (Figure 60.2).

60.6 Leukocyte Recruitment in Sites of Injury

Induction of adhesion molecules on the endothelial surface and release of chemokines and cytokines activate a multistep adhesive interaction between leukocytes and endothelial cells, ultimately leading to extravasation of leukocytes into the area of injury (Figure 60.2). First, circulating leukocytes are captured from the bloodstream through interactions that involve endothelial selectins and their corresponding ligands. Subsequently, leukocytes roll along the endothelial layer and sense chemokines bound to endothelial glycosaminoglycans. Selectin-mediated inside-out signaling and interactions between chemokines and the corresponding leukocyte ligands (chemokine receptors) induce conformational changes of leukocyte integrins [53,54], a family of adhesion proteins responsible for strong adhesive interactions and arrest of the leukocytes [55]. Leukocytes then crawl towards endothelial junctions, and migrate across the microvasculature. Experimental evidence suggests that leukocytes preferentially migrate through "matrix-poor" regions of the basement membrane [56] that often overlap with

gaps between pericytes [57]. In addition to the well-established paracellular route, leukocytes may also transmigrate via a transcellular route (through endothelial cells) [58]. Recruitment of various leukocyte subpopulations requires activation of distinct chemokine:chemokine receptor pairs and may involve a different set of integrins. In healing wounds, early release of neutrophil chemoattractants results in a first "wave" of infiltrating neutrophils, followed by upregulation of mononuclear cell-specific chemokines and recruitment of monocytes and lymphocytes [59].

60.7 The Role of Neutrophils in Injured Tissues

At the site of injury, extravasated neutrophils are activated by alarmins, cytokines, and chemokines and acquire a phagocytotic phenotype [60]. In the presence of infection, pathogen-associated molecular patterns potently activate neutrophils. Activated neutrophils generate large amounts of reactive oxygen species (ROS), and rapidly phagocytose pathogens and cellular debris. In addition, neutrophils degranulate releasing proteinases (both serine proteases and matrix metalloproteinases) and antimicrobial peptides. In order to effectively kill pathogens, neutrophils extrude networks comprised of chromatin fibers, granule-derived antimicrobial peptides, and enzymes (such as elastase and myeloperoxidase). These networks, termed neutrophil extracellular traps (NETs) play an important role in killing of pathogens [61], but may also accentuate tissue injury in sterile inflammation. A growing body of evidence suggests that neutrophils may also exert immunomodulatory actions, regulating phenotype, and function of monocytes/macrophages and lymphocytes [62].

60.8 Repression and Resolution of Inflammation: The Stop Signals

Acute inflammation triggers suppressive signals that downmodulate proinflammatory molecules and promote resolution of the inflammatory infiltrate. Suppression of inflammation following injury is accompanied by activation of reparative cells that restore tissue structure and function, or (if regeneration is impossible) repair the tissue through formation of a collagenous scar to prevent organ failure. Considering the catastrophic consequences of defects in suppression and resolution of inflammation, it is not surprising that many distinct pathways have evolved for negative regulation of inflammatory responses following injury [63]. As the wound is cleared from dead cells and debris, all cell types involved in tissue repair contribute to suppression and resolution of inflammation. Both secreted mediators (such as IL-10, and members of the TGF-β family) and intracellular cascades that inhibit innate immune signaling have been implicated in negative regulation of the inflammatory response following injury [64].

60.8.1 Negative Regulation of Cytokine Signaling

Several distinct mechanisms have been implicated in negative regulation of cytokine signaling following tissue injury. First, endogenous cytokine inhibitors may be induced to suppress the inflammatory response [65]. IL-1 receptor antagonist (IL-1Ra) is the prototypical endogenous competitive inhibitor of cytokine-driven inflammation. Second, expression of decoy receptors by inflammatory cell types may serve as a molecular sink for cytokines. Binding of IL-1 to the type II IL-1 receptor, a typical decoy receptor, does not transduce a signaling response, but serves to terminate IL-1-mediated responses [66]. Third, proteasomal-mediated degradation of signaling molecules may restrain cytokine-induced actions. Fourth, induction of inhibitory signals that terminate the JAK–STAT cascade, such as phosphatases that dephosphorylate JAKs [67] and members of the suppressor of cytokine signaling (SOCS) family [68] may play an important role in negative regulation of cytokine responses.

60.8.2 Anti-Inflammatory Cytokines

Secreted anti-inflammatory cytokines are critically implicated in suppression of the inflammatory response in injured tissues. Extensive evidence has documented the role of the interleukins IL-4, IL-13, IL-10, IL-35, IL-37, and of members of the TGF-β family in negative regulation of the immune response [69,70]. TGF-β1 restrains T-cell-mediated inflammation [71]; IL-10, on the other hand, limits immune responses to foreign antigens and suppresses inflammatory cytokine and chemokine synthesis by mononuclear cells [72]. The relative role of each anti-inflammatory cytokine in regulating inflammation following injury is dependent on the tissue studied, and on the specific type of injury. Several secreted mediators may cooperate to suppress inflammatory activation of all cell types involved in tissue repair.

60.8.3 Lipid Mediators in Resolution of Postinfarction Inflammation

A growing body of evidence suggests that resolution of inflammation may be mediated by release of specialized proresolving lipid mediators [73,74]. Four distinct families of anti-inflammatory mediators have been described: the resolvins, the lipoxins, the protectins, and the maresins. Endogenous release of proresolving lipids is associated with resolution of inflammation in a wide range of experimental models of tissue injury [75–78]. Moreover, treatment with resolvins and protectins accelerated resolution of inflammation in conditions associated with persistent and prolonged inflammatory activation, such as type 2 diabetes [79].

60.8.4 Inhibition of the Leukocyte Adhesion Cascade

Effective suppression of the inflammatory response requires inhibition of the adhesive interactions between leukocytes and endothelial cells, in order to restrain proinflammatory leukocyte influx in the tissue. Several distinct pathways may be responsible for downmodulation of leukocyte adhesion. First, induction of

anti-inflammatory cytokines (such as IL-10 and members of the TGF-β family) may repress adhesion molecule synthesis by endothelial cells. Second, chemokine-binding proteins (such as TNF-stimulated gene/protein-6 (TSG-6)) may perturb chemokine–glycosaminoglycan interactions, leading to inhibition of chemokine-driven leukocyte recruitment [80]. Third, inhibitory proteins may interfere with the early selectin-mediated steps of leukocyte rolling, decreasing the pool of leukocytes available to form strong adhesive links with the activated endothelium [81]. Fourth, endogenous endothelial cell-derived integrin ligands may be released during resolution of inflammation, serving as potent leukocyte adhesion inhibitors [82]. The integrin antagonist developmental endothelial locus (Del)-1 is an example of this mechanism: Del-1 is secreted by endothelial cells, binds to the β2 integrin CD11a/CD18, and inhibits leukocyte/endothelial cell adhesion *in vitro* and *in vivo* [83].

60.9 The Cellular Effectors of Resolution of Inflammation

Neutrophils, monocytes/macrophages, lymphocytes, vascular cells, and fibroblasts participate in suppression and resolution of inflammation following injury. Moreover, surviving parenchymal cells that reside in the injured tissue may also contribute to repression of inflammatory signaling, protecting the organ from prolonged injury.

Neutrophils are short-lived cells, programmed to undergo apoptosis. In injured tissues, ingestion of apoptotic neutrophils by macrophages triggers release of anti-inflammatory mediators (such as IL-10, TGF-β, and proresolving lipid mediators), suppressing inflammation [84,85]. Moreover, neutrophil-derived mediators, such as lactoferrin and neutrophil gelatinase-associated lipocalin (NGAL) may directly modulate macrophage phenotype, suppressing proinflammatory signaling, and stimulating a reparative program [86,87].

Recruitment of anti-inflammatory monocyte subsets may play a critical role in negative regulation of inflammation in injured tissues [88,89]. The mechanisms responsible for selective recruitment of anti-inflammatory monocytes during the transition from the inflammatory to the proliferative phase of repair remain unclear. Specific chemokine:chemokine receptor pairs may be involved. The fractalkine receptor CX3CR1 may selectively mediate recruitment of monocytes with reparative properties [90]. Moreover, Sphingosine 1-phosphate receptor 3 (S1P3) signaling has been suggested to play an important role in recruitment of anti-inflammatory monocytes in injured and remodeling vessels [91]. Several other subpopulations of immune cells have been implicated in negative regulation of inflammation following tissue injury. Dendritic cells release anti-inflammatory cytokines (such as IL-10) and may drive macrophages toward a regulatory phenotype [92]. Lymphocyte subpopulations, such as invariant natural killer T cells (iNK T cells) and regulatory T cells (Tregs), have also been implicated in negative regulation of inflammation in various models of tissue injury [93–95]. Specific chemokine:chemokine receptor interactions may be responsible for recruitment of these important lymphocyte subpopulations into sites of injury [96,97].

Macrophages are critically involved in negative regulation of the inflammatory response following tissue injury. Traditional concepts suggest that, in response to changes in their microenvironment, macrophages may acquire polarized M1 and M2 phenotypes; M1 macrophages are proinflammatory, whereas M2 macrophages exhibit anti-inflammatory and reparative properties [98]. A large body of evidence suggests that resolution of inflammation and tissue repair following injury are associated with an abundance of M2 cells [99]. Although the crucial role of macrophages in negative regulation of inflammation following injury cannot be overemphasized, the M1/M2 paradigm is oversimplified and of limited use in understanding the cell biological response responsible for tissue repair. In healing tissues, macrophages exhibit a wide range of "nuanced" phenotypes. Macrophage subpopulations with distinct properties and cytokine expression profiles may be involved in regulation of the regenerative, angiogenic, anti-inflammatory, and fibrogenic responses that cooperate in tissue repair (Figure 60.2).

60.10 The Extracellular Matrix

In injured and remodeling tissues, the extracellular matrix does not simply serve as a structural scaffold, but also plays a critical role in regulating cell phenotype. In all tissues, cells are enmeshed in a dynamic network of extracellular matrix proteins. Through interactions with the matrix, the cells sense microenvironmental changes, and respond by alteration their phenotype and gene expression profile. In injured tissues, the extracellular matrix proteins may also transduce key signals that regulate survival, proliferation, and migration of the cells. During the early inflammatory phase of tissue repair, activation of proteases generates matrix fragments with proinflammatory properties [100,101]. Ingestion of matrix fragments by professional phagocytes plays an important role in resolution of the inflammatory reaction. Moreover, the damaged matrix at the site of injury is replaced by a provisional matrix, comprised primarily of fibrin and fibronectin [102,103]. The provisional matrix serves as a conduit for migrating inflammatory and reparative cells, facilitating remodeling of the injured tissue. Alarmins, cytokines, and growth factors also induce expression of a family of structurally unrelated macromolecules, termed matricellular proteins [104,105]. These proteins are generally not part of the normal matrix network, but enrich the matrix following injury, and do not serve a structural role, but modulate cell:cell and cell:matrix interactions. Prototypical members of the matricellular family, such as thrombospondins-1 and thrombospondins-2, tenascins-C and tenascins-X, SPARC, osteopontin, periostin, and members of the CCN family, participate in the reparative response by activating growth factor and cytokine responses, by modulating protease activity, and by transducing signaling cascades that regulate cell proliferation and survival [104,106,107].

60.11 Tissue-Specific Events Leading to Regeneration

The regenerative potential of an organ following injury is dependent on the intrinsic proliferative capacity of the cellular constituents of the tissue.

Continuously dividing tissues (such as epithelia of the skin and gastrointestinal tract) exhibit continuous loss and replacement of cells, triggered by activation of progenitor cell populations that reside in the organ, or through growth factor-mediated stimulation of proliferative activity of mature cells. In these tissues, injury triggers activation of progenitor cells that restore structure and function.

In other tissues, the cells are quiescent at baseline, but maintain the ability to proliferate following injury. Parenchymal cells in the liver or kidney, fibroblasts, and vascular cells are capable of proliferation following injury. Thus, tissues comprised of these cells have the capacity to regenerate; however, the outcome is dependent on the severity of injury. Very extensive injury may overwhelm the regenerative capacity of the organ, resulting in permanent loss of function. Traditionally, certain cell types, such as cardiomyocytes and neurons, are considered terminally differentiated and nonproliferative in adult mammals. However, recent evidence suggests that cardiomyocytes in adult humans may have a (very limited) capacity for self-renewal [108]. Moreover, brain plasticity following injury suggests that neurons may also exhibit regenerative capacity, and neurogenesis has been documented in certain regions of the brain in humans [109,110]. The extremely limited regenerative capacity of these cell types is not sufficient to restore structure and function following injury in the heart or the nervous system. Thus, in these organs, injured areas heal through formation of a scar, driven by activation and proliferation of fibroblasts. In the heart, reparative fibroblasts may not be capable of performing the specialized contractile function of cardiomyocytes, but maintain the structural integrity and geometry of the organ, preventing catastrophic rupture [111].

60.12 Fibroblast Activation and Formation of a Scar

Most tissues contain resident fibroblast populations that can be activated following injury, undergoing transdifferentiation into matrix-synthetic myofibroblasts [112]. Moreover, in many cases, the local fibroblast population is enriched by cells of various origins that may acquire characteristics of fibroblasts. Thus, blood-derived progenitors, endothelial cells, epithelial cells, and pericytes have been demonstrated to contribute to the population of activated fibroblasts following injury [113].

In tissues with negligible regenerative capacity (such as the myocardium and the nervous system), fibroblasts become essential reparative cells, that deposit extracellular matrix to preserve the structural integrity of the organ. Even in regenerative tissues, fibroblasts may significantly contribute to repair, when injury overwhelms the regenerative process, or depletes the population of resident progenitor cells. Transition from the inflammatory to the proliferative phase of repair is associated with activation of abundant myofibroblasts, phenotypically modulated fibroblasts that express contractile proteins, such as α-smooth muscle actin (α-SMA). At the early stage of myofibroblast transdifferentiation, the cells may lack α-SMA expression, but have stress fibers rich in cytoplasmic actins, and exhibit mature focal adhesions comprised of β- and γ-actin microfilaments, associated with nonmuscle myosin. These cells are called “proto-myofibroblasts” [113]. Activated

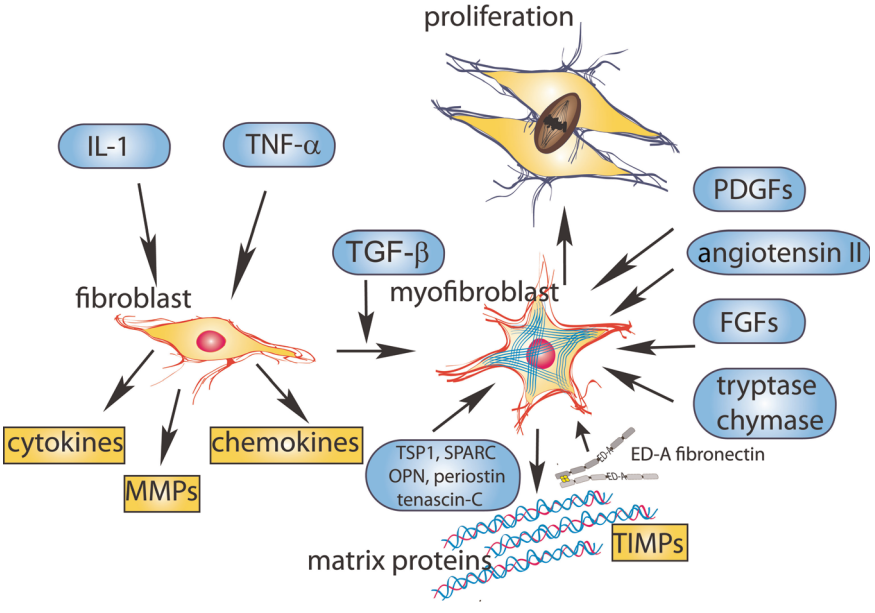


Figure 60.3 In injured tissues, fibroblasts transdifferentiate into myofibroblasts, proliferate and synthesize extracellular matrix proteins. In tissues with limited regenerative capacity, fibroblasts form a scar that serves to preserve the geometry of the organ. In the early stages of repair, fibroblasts acquire a proinflammatory and matrix-degrading phenotype, secreting cytokines, chemokines, and proteases. TGF-β activation promotes myofibroblast transdifferentiation and stimulates extracellular matrix protein synthesis. A wide range of fibrogenic mediators cooperate to stimulate fibroblast proliferation and matrix-synthetic activity. Specialized matrix proteins (such as ED-A fibronectin and matricellular proteins) contribute to fibroblast activation by transducing growth factor-mediated signals.

myofibroblasts have an extensive endoplasmic reticulum, synthesizing and secreting large amounts of extracellular matrix proteins [114–116]. Several distinct pathways cooperate to activate fibroblasts at the sites of injury (Figure 60.3):

1) TGF-β is critically implicated in myofibroblast transdifferentiation [117]. Most tissues contain significant amounts of TGF-β in a latent form that can be activated following injury. ROS, proteases, integrins, and matricellular proteins (such as TSP-1) have been implicated in activation of TGF-β in injured tissues [118,119]. Following activation, active TGF-β binds and sequentially transphosphorylates type I and type II receptors on the fibroblast surface, inducing α-SMA transcription [120]. Both Smad-dependent [121] and Smad-independent pathways have been implicated in myofibroblast activation *in vitro* and *in vivo* [122,123].
2) Alterations in the matrix environment plays critically regulate fibroblast activity. Specialized matrix proteins, such as the ED-A fibronectin variant, are upregulated in injured and healing tissues and promote myofibroblast conversion [124].
3) Release of fibrogenic growth factors (such as fibroblast growth factors, platelet-derived growth factors, and the mast cell proteases chymase and tryptase) and

activation of the angiotensin cascade may promote fibroblast activation, triggering cell proliferation, and stimulating matrix synthesis [125,126].
4) Expression of syndecans on the fibroblast surface may participate in transduction of growth factor-mediated signals that activate myofibroblasts [127].
5) Mechanosensitive signaling activates Rho/Rho kinase pathways, directly stimulates α-SMA transcription in fibroblasts [128].

Once activated, fibroblasts secrete large amounts of extracellular matrix proteins. In nonregenerating tissues (such as the heart), fibroblast-derived extracellular matrix proteins become cross-linked and form a scar that serves to prevent catastrophic failure of the organ. In both regenerating and nonregenerating tissues, fibroblast activity is restrained through poorly understood anti-fibrotic cascades.

60.13 Concluding Remarks

Tissue injury activates a superbly orchestrated inflammatory reaction that clears the wound from dead cells and matrix debris, while stimulating a reparative program. Although the cell biological characteristics of the reparative response are tissue- specific, in all cases immune cells play a critical role in wound healing [129]. In organs with regenerative capacity, macrophages may activate progenitor cells, while stimulating angiogenesis to provide blood supply, necessary for growth of new tissue. In organs with limited regenerative potential, lymphocytes orchestrate macrophage-mediated responses to activate fibroblast-derived matrix synthesis and formation of a collagen-based scar. Understanding the signaling pathways that regulate reparative responses in various tissues is critical for development of new treatment strategies for a wide range of pathologic conditions.

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Part Seven

Detection and Treatments

61

Biomarkers in Inflammation

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A biomarker is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [1]. Biomarkers may be used as diagnostic tools, tools for staging of disease, indicators of prognosis, or for prediction or monitoring of clinical response to therapeutic interventions [1]. They should be amenable to accurate and reproducible measurements.

Causes for inflammatory responses are numerous. However, it has become evident that more or less intraindividual response patterns are often similar particularly in acute inflammation irrespective of the inflammatory stimulus as the immune system seems to be more concerned about circumstances that do harm than those are just foreign. For instance, in infection microbial "pathogen-associated molecular patterns" (PAMPs), such as bacterial cell membrane components or bacterial or viral nucleic acids, are recognized by the innate immune system via "pattern recognition receptors" (PRRs), for example, toll-like receptors (TLRs) or retinoic acid-inducible gene(RIG)-1-like receptors (RLRs). Recognition of PAMPs by PRRs typically leads to induction of signaling cascades promoting pro- and anti-inflammatory host responses. On the other hand, disease entities of noninfectious origin, such as pancreatitis, burns, or trauma, leading to severe damages in the organism, for example, cellular necrosis, may cause release of endogenous substances functioning as danger signals or alarmins ("danger-associated molecular patterns" (DAMPs)), inducing similar pro- and anti-inflammatory cascades. However, differentiating systemic inflammation with infection from inflammatory disease entities without infection is one of the most important issues in daily intensive care allowing starting specific treatments timely to improve prognosis in these critically ill patients. Still, according to the above-mentioned similarities of inflammatory responses this differentiation is sometimes very difficult, particularly as clinical signs are typically similar, too, and microbial diagnostics often either fail to recognize causative pathogens or results are too delayed.

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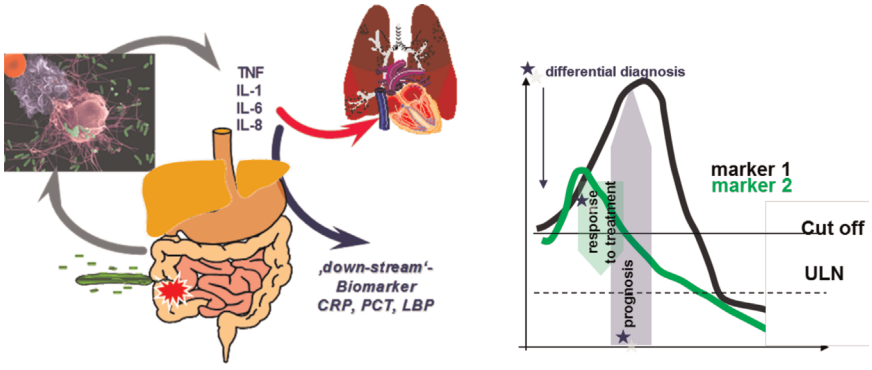


Figure 61.1 Biomarkers reflecting the host response to harmful stimuli. A perforated bowel serves as an example: Local or systemic activation of cells of the immune system will release cytokines that might contribute to remote organ injury and to induction of downstream proteins, such as CRP, PCT, or LBP, that indicate with different kinetics the underlying host response to the pathogen. Changes in expression of cytokines or downstream mediators may inform about severity and prognosis but also potentially reflect eradication or propagation of pathogens and response to treatment.

Due to the complex pathophysiology of sepsis involving a multitude of mediators of inflammation many biomarkers have been proposed. Consequently, in a review 178 different sepsis biomarkers were found in the literature [2] and the search is still ongoing. A selection of these biomarkers is presented in this chapter.

61.1 Acute-Phase Reactants

A variety of diseases and tissue damages, such as infections, trauma, burns, surgeries, or autoimmune diseases, induce inflammatory reactions mediated, for example, by interleukin-6 leading to a reprogramming most notably in liver parenchymal cells with profound changes in the plasma proteome (Figure 61.1). This highly regulated process is called acute-phase response. Changes on transcriptional and—to a lesser extent—on posttranscriptional levels lead either to reduced protein levels (negative acute-phase reactants, e.g., albumin) or higher levels of so-called positive acute-phase reactants, such as C-reactive protein (CRP), procalcitonin (PCT), serum amyloid A, or fibrinogen. While some acute-phase reactants play a distinct role in damage control, functions of other acute-phase reactants are uncertain.

61.2 Pentraxins

Pentraxins, a superfamily of evolutionary conserved proteins, act as pattern recognition molecules and play a crucial role in innate immunity. Members of this group of molecules are, for example, C-reactive protein, serum amyloid P, and pentraxin 3 [3].

61.3 C-Reactive Protein (CRP)

C-reactive protein is primarily released by the liver in response to cytokines, prototypically to Interleukin-6 (IL-6), and has pro- and anti-inflammatory effects. Pathophysiologic roles in inflammatory processes seem to be various. As a component of the innate immunity it binds to pathogens and damaged cells on the one hand and to phagocytic cells on the other hand and activates the complement system when bound to one of its ligands promoting their complement-mediated clearance [4]. Anti-inflammatory effects may be due to reduced endothelial adhesion of neutrophils, stimulation of IL-1ra synthesis, and inhibition of neutrophilic superoxide generation [5].

CRP values in healthy individuals are usually<2 mg/L. However, levels of up to 10 mg/L can be found, too. Sometimes, these patients have trivial injuries or minimally inflammatory processes but these values can also be found in patients, for example, with osteoarthritis [5,6]. After an inflammatory stimulus plasma CRP values begin to rise after (6)-12–24 h and usually peak after a single stimulus after 36–72 h. Plasma half-life is about 19 h [5,7]. In the critical care setting, CRP is often used to differentiate between presence and absence of infection [8]. It helps to differentiate patients with bacterial from viral infections or patients with exacerbated COPD and infection from those without exacerbation [9,10] and can help to predict infectious complications after gastrointestinal surgery [11,12].

In critically ill patients, CRP levels correlated in studies with multiorgan failure and mortality [13–15]. However, specificity and sensitivity of single CRP values were sometimes rather low and other studies could not demonstrate differences between survivors and nonsurvivors at onset of sepsis [15–17]. On the opposite, taking the course of CRP levels into account, decreasing CRP values over treatment time are associated with better prognosis than persistently high levels [4,18]. In the postoperative setting, absolute CRP levels and persisting high levels after the third postoperative day were associated with inflammatory complications and higher mortality [19,20], while low CRP levels on the fourth postoperative day reliably ruled out infectious postoperative complications after various gastrointestinal operations [21]. CRP levels decline when effective antimicrobial treatment is initiated [18,22,23]. However, prospective studies on CRP-guided treatment algorithms and their impact on outcome are missing [24].

61.4 Pentraxin 3

Pentraxin 3 is produced in response to TNF-α, IL-1β, and TLR signaling in several cell types, for example, macrophages, endothelial and epithelial cells, and fibroblasts, but not in hepatocytes as most other acute-phase reactants [3,25]. IL-6 does not induce pentraxin 3 hinting at a rather local than systemic function [25]. Pentraxin 3 acts as a fluid-phase pattern recognition molecule with a protective role in resistance against fungal, bacterial, and viral pathogens activating various antimicrobial effector mechanisms [26,27]. However, for alphavirus infection, a promotion of viral entry and replication was also described [26]. Pentraxin 3 contributes to opsonization of pathogens,

complement activation, and phagocytosis [3,26]. It has been proposed that pentraxin 3 exhibits a negative feedback mechanism in early neutrophil accumulation, thereby potentially limiting excessive inflammation-induced tissue damage [3]. In addition, it plays a role in tissue repair in acidic environment of tissue damage [28].

Pentraxin 3 was found to correlate with severity of disease in sepsis [29,30] and with sepsis-associated coagulation/fibrinolysis dysfunction [29]. In a study with febrile patients presenting at the emergency department, patients transferred to intensive/intermediate care had higher levels than those transferred to the ward [31]. Another study found higher level in emergency room patients with suspected infection in sepsis and organ failure than in other patients and an association with mortality. Again high levels predicted need for intensive care treatment [32]. In bacteremia both, initial pentraxin 3 values as well as high levels until day 4, were associated with mortality [33], and in patients with necrotizing soft tissue infection baseline levels were associated with need for amputation and mortality [34]. However, in another study in severe sepsis only persisting high levels were associated with mortality but not initial values [29].

61.5 Procalcitonin

The polypeptide procalcitonin (PCT) is the precursor of the hormone calcitonin. However, in inflammation and in sepsis, regulation of induction is different and independent from hormone regulation of calcitonin. PAMPs as well as DAMPs can stimulate cells in various tissues to produce and secrete PCT [35]. Peripheral blood mononuclear cells have also been shown to produce PCT *in vitro* [36]. PCT potentially augments the inflammatory response triggered, for example, by LPS, TNFα, or IFNγ and modulates gene expression of inducible nitric oxide synthase [37].

PCT exhibits faster kinetics than CRP. It becomes detectable within 3–4 h with peak serum levels usually after 24–48 h and declines by 50% within 24–36 h. Normal values are below 0.05 ng/mL. PCT appears in blood in response to bacterial infection, but also after other insults causing severe systemic inflammation, for example, after major surgery or trauma. Several studies demonstrated significant correlations between PCT levels and the diagnosis of sepsis [38]. PCT as a sepsis marker was first described in 1993 [39]. High PCT levels have a high positive predictive value for sepsis and normal or low PCT values likely rule out sepsis or severe systemic inflammation [40]. In a meta-analysis investigating PCT as a marker for separating sepsis patients from patients presenting signs of systemic inflammation due to noninfectious disease entities that included 30 studies, accounting for 3244 patients, a sensitivity of 0.77 (95% CI 0.72–0.81) and specificity of 0.79 (95% CI 0.74–0.84) and an area under the receiver operating characteristic curve (AUC–ROC) of 0.85 (95% CI 0.81-0.88) was found [41]. While bacterial infections resulting in a systemic host response seem to be a strong trigger for PCT production, in viral infections PCT induction is rather low [42–45,152]. An explanation for lower PCT values in viral infections might be that interferon γ typically released in viral infections attenuates its gene expression in response to IL1-β [46,153]. Thus, PCT

seems to be a good tool to rule out bacterial coinfection in influenza infection [47]. Nevertheless, the link between elevated PCT levels in bacterial infection is the severity of the host response [40]. Consequently, in trauma patients Wanner *et al.* found not only high PCT levels in patients with sepsis but also a correlation with severity of trauma and multiorgan dysfunction. Patients with sepsis exhibited the highest levels in the study cohort [48]. A systematic review of the diagnostic value of PCT after cardiac surgery demonstrated a correlation of PCT values not only with infection but also with the presence of organ failure and postoperative complications. Rather the course than single postoperative PCT values were informative with respect to infectious complications [49]. Nevertheless, in community-acquired low-to-moderate risk respiratory tract infections cutoff values between 0.1 and 0.25 to rule out bacterial infections seem feasible, while in critically ill patients higher cutoff values between 1 and 2 μg/l seem helpful for discrimination of patients with sepsis from other inflammatory condition [41] when the above-mentioned limitations in distinct disease entities are taken into account. In both settings repeated measurements increase diagnostic accuracy.

PCT has also been proposed for guiding antibiotic treatment with respect to both initiating as well as terminating antibiotic treatment. Such an antibiotic stewardship could be of high importance in the light of increasing antimicrobial resistance triggered at least in part by an overuse of antibiotics on the one hand and increasing mortality rates in sepsis patients with delay of appropriate antibiotic treatment on the other hand.

Several studies evaluated protocol-based decision making of initiating or withholding antibiotic treatment in patients with diagnosis of respiratory tract infection in dependence of PCT values in the primary care, emergency department, and hospital setting [38,50–58,153]. Chosen cutoff value of PCT to recommend withhold of antibiotic treatment was usually 0.25 μg/L, sometimes complemented by a strong recommendation to withhold antibiotics, if PCT value was below 0.1 μg/l. Overall, these studies demonstrated reduced antibiotic prescription rates and shorter duration of therapy without increase of adverse events.

In addition, in critically ill patients, various prospective studies have been performed evaluating PCT-guided algorithms to reduce length of antibiotic treatment. Five studies investigated a PCT-based protocol to stop antibiotic treatment in mixed ICU populations with various sites of infection [59–63], two studies in surgical ICU patients [64,65], and one in patients with ventilator-associated pneumonia [66]. The protocols used were similar, recommending discontinuing antibiotic treatment when PCT levels dropped below an absolute cutoff value (0.1–0.5 μg/L) and/or showed a relative decrease (80%–90%) from peak values. All but one study led to a decrease of initial antibiotic treatment duration of at least 2 days in the PCT-guided groups. Only one study could not demonstrate a significant reduction in antibiotic treatment duration, most likely due to a too low threshold of 0.1 μg/l as stopping rule [63]. In contrast, in the largest study including about 1500 patients using a threshold of 0.5 μg/L or a decrease of 80% demonstrated not only a decrease in antibiotic consumption, but a decrease in 28-day and 1-year mortality was also observed in the study group [60]. Thereby, in a high-risk ICU setting, a drop in PCT levels after initiation of antibiotic therapy to levels less than 0.5 or by at least 80%–90% from

baseline accompanied by a clinical improvement seems reasonable to stop antibiotic treatment [67].

61.6 Interleukin 6

The cytokine interleukin-6 (IL6), a peptide consisting of 184 amino acids, exhibits pro- and anti-inflammatory properties and has numerous biological functions in inflammatory processes, immune reactivity, oncogenesis, and hematopoiesis [68]. It is a crucial mediator in acute-phase reactions but plays also a role in chronic inflammatory processes, such as arthritis or chronic bowel disease, and in regenerative processes, such as liver regeneration after partial hepatectomy [69]. Downstream cellular reactions are induced after building a complex with cellular IL6 receptor or soluble IL6 receptor and binding of this complex to glycoprotein-130 receptor.

IL6 release is directly induced by the primary cytokines TNFα and IL1. Thereby, it appears early, that is, within 2 hours in the course of inflammatory reactions associated with infection, injury, or trauma [68]. In infection, it is mainly produced by activated macrophages and monocytes. Serum levels can increase 1000-fold. The rapid appearance of IL6 in infection seems to be an interesting characteristic.

Although in several studies IL6 levels were significantly higher in patients with infection, diagnostic accuracy was rather moderate [70–73]. In a study including 327 patients admitted to ICU, IL6 had higher AUC–ROC (0.76) than PCT, CRP and LBP to differentiate patients with infection and systemic inflammation from those without. However, it was inferior to PCT in discriminating between infected patients with or without organ dysfunction [71]. Two studies investigated patients presenting in an emergency department. In one IL6 was inferior to PCT and CRP in differentiating patients with and without infection [72], while another study found similar AUC–ROCs to differentiate patients with systemic inflammation and infection from patients neither with signs of infection nor systemic inflammation or only one of these two items [73].

Increased IL6 levels were also found in noninfectious conditions, for example, after major surgery, trauma, transplant rejection, and autoimmune disorders [24].

IL6 levels were related with severity and outcome in sepsis in several studies particularly when high levels were found in the course of disease [16,74–76]. For example, one study found that, while IL6 levels were significantly different between survivors and nonsurvivors on days 1, 3, and 7, the best discrimination was on day 3 with an AUC–ROC of 0.86 [75]. Similarly, in another study, values were different on admission, but AUC–ROC was higher on day 2 (0.799) [76]. When infection is successfully treated, IL6 decreased [16].

61.7 Lipopolysaccharide-Binding Protein (LBP)

Lipopolysaccharide(LPS)-binding protein (LBP) is 58 kD acute-phase protein synthesized in the liver. LBP transports phospholipids. It also binds to LPS, a cell

membrane component of Gram-negative bacteria, forming a complex mediating its binding to soluble CD14 and CD14 receptors on monocytes and macrophages. CD14 presents LPS to TLR4–MD2 complex leading to activation of several signaling cascades ultimately resulting in secretion of proinflammatory cytokines IL-1 and TNFα [77]. Normal values of LBP are between 1 and 20 μg/mL and can rise rapidly by severalfold in acute-phase response. High levels were demonstrated in patients with SIRS and sepsis [78,79]. In patients with suspected community-acquired infections, LBP distinguished between infected and non-infected patients [80]. However, other studies could not confirm a good discrimination between septic and nonseptic patients [71,81]. Furthermore, LBP had no prognostic significance in critically ill patients [71,81]. Interestingly LBP levels were similar in Gram-positive and Gram-negative sepsis [71].

61.8 Presepsin (Soluble CD14 Subtype)

CD 14, a glycoprotein expressed on monocytes and macrophages, functions as LPS receptor together with LBP serving as an activator of inflammatory signaling via TLR 4. Furthermore, CD14 can bind peptidoglycan and other microbial products with similar structural features and other ligands such as human heat shock protein 60 or ceramide [82]. Presepsin is a soluble *N*-terminal fragment of CD14, which is released from cell surface during monocyte activation after LPS recognition [83,84]. It can also bind LPS thereby either competing with membrane-bound CD14 for LPS binding or mediating LPS-induced activation of non-CD14-expressing cells [82]).

In a study of patients presenting at emergency department with systemic inflammation and suspected infection presepsin levels were higher in patients with sepsis with and without organ dysfunction than in patients without infection. Values tended to be higher in septic patients with organ dysfunction than in those without. However, AUC–ROC for presepsin with respect to infection was 0.7 and thereby significantly lower than for PCT (0.87) [85]. Another study in a similar setting including 859 patients found increasing presepsin levels from healthy controls over patients with systemic inflammation without infection to patients with infection and a correlation with sepsis severity with a higher AUC–ROC for predicting sepsis with organ dysfunction in septic patients for presepsin than for PCT (0.840 vs. 0.741) [86]. Other studies, however, found similar AUC–ROCs for presepsin and PCT for differentiating SIRS with and without infection (presepsin: 0.89, PCT: 0.82, CRP: 0.82) and bacteremia versus no bacteremia (presepsin: 0.750, PCT: 0.787, CRP: 0.602) [87,88].

A meta-analysis that included eight studies with a total of 1815 patients found a pooled sensitivity and specificity of 0.86 (95% CI: 0.79–0.91) and 0.78 (95% CI: 0.68–0.85, respectively. The AUC–ROC was 0.89 (95% CI: 0.86–0.92) demonstrating good diagnostic accuracy for the diagnosis of sepsis [89].

Initial high presepsin levels in patients with sepsis with and without organ failure were associated with mortality [85,90] and the number of failed organs and development of further organ failures [90]. Levels were higher within the first week in nonsurvivors [83]. In patients with appropriate antibiotic treatment and

in those with negative blood cultures presepsin levels on day 7 were moderately lower than on day 1, while they were higher in patients with inappropriate antibiotic treatment [90].

61.9 Soluble Triggering Receptor Expressed on Myeloid Cells-1 (sTREM-1)

Triggering receptor expressed on myeloid cells-1 (TREM-1), a receptor of the immunoglobulin superfamily, is expressed on the surface of neutrophils, monocytes, and macrophages [91]. It is an activating receptor of downstream signaling pathways stimulating inflammatory responses and has been demonstrated to be upregulated on cells that infiltrate human tissues infected with bacteria and on peritoneal neutrophils in microbial sepsis [92,93]. While Gram-positive and Gram-negative bacteria as well as fungi caused a strong expression of TREM-1, this was not the case in infection with mycobacteria and in noninfectious inflammatory response [92]. Upregulated TREM-1 expression is associated with increased levels of soluble TREM-1 (sTREM-1) [91].

The presence of sTREM-1 in bronchoalveolar lavage fluid strongly predicted presence of both community-acquired and ventilator-associated pneumonia (sensitivity 98%; specificity 90%) [94]. In adult patients with suspected sepsis, plasma sTREM-1 levels on admission had a sensitivity of 96% and specificity of 89% for indicating infection on a medical ICU [95] and a sensitivity and specificity of 79% each in postoperative ICU patients [96]. A meta-analysis including 13 studies with 980 patients from ICU, emergency department, and hospital ward found a sensitivity of 0.82 (95% CI: 0.68–0.90) and a specificity of 0.86 (95% CI, 0.77–0.91) for the diagnosis of infection, for example, in pneumonia, meningitis and urinary tract infection, or suspected bacterial infections, with samples drawn from blood, urine, pleural, and cerebrospinal fluid. The AUC–ROC was 0.86 (95% CI, 0.77–0.91). In urinary tract infection, diagnostic value was low [97]. However, in two later published studies either no differences were found between surgical ICU patients with inflammation of infectious and noninfectious origin and healthy controls [98] or diagnostic value for the differentiation of infectious and noninfectious origin of inflammatory response in critically ill patients was poor on the day of admission (AUC–ROC 0.62) due to a huge overlap although sTREM-1 levels were significantly different [99]. STREM-1 was associated with multiorgan dysfunction [99] and mortality in this study [99,100]. Conversely, other studies found lower serum values in nonsurvivors than in survivors in critically ill patients with infection with and without organ dysfunction [101] or no differences in surgical ICU patients [98]. While serum sTREM-1 levels decreased in survivors, there was rather an increase in nonsurvivors in a 5-day- and 14-day-period, respectively [100,101].

STREM-1 levels were also elevated in other disease entities accompanied by states of inflammation like in patients with chronic kidney disease on hemodialysis, in ulcerative colitis, and in myocardial infarction [102–105]. In the latter patient group sTREM-1 levels were an independent predictor of death [102].

61.10 Soluble Urokinase-Type Plasminogen Activator Receptor (suPAR)

Urokinase-type plasminogen activator is involved in the migration of inflammatory cells from bloodstream into tissues and can be found as a glycosyl-phosphatidylinositol(GPI)-linked protein on the cell membrane of various immune cells, for example, on monocytes and macrophages [106]. During inflammatory states it is cleaved from cell surface forming its soluble form, that is, suPAR, that plays a role in immunological functions such as cell adhesion and migration, chemotaxis, signal transduction, immune activation, proteolysis, angiogenesis, fibrinolysis, and tissue remodeling [107].

Serum/plasma levels of suPAR seem to reflect the activation of the immune system, for example, in response to infection. Increased levels can be found in various pathological conditions, including sepsis, streptococcus pneumonia bacteraemia, bacterial and viral CNS infection, active tuberculosis (TB), human immuno-deficiency virus type 1 (HIV-1)-infection, malaria, and but also in solid tumors [106]. Thus, suPAR is a nonspecific biomarker of inflammation. It has no diagnostic value with respect to the cause of inflammation. In a study evaluating patients with at least two SIRS criteria and suspected community-acquired infection, including patients with bacterial, viral, and parasitic infections, as well as noninfected patient, the AUC–ROC of suPAR for bacterial infection was 0.50 and for infection versus no infection 0.51 [108]. In another study, suPAR levels were similar in patients with and without infection, when no organ dysfunction was present but significantly higher in patients with infection and organ dysfunction, thus supporting the notion that suPAR is a marker of disease severity. Levels were higher in nonsurvivors [109]. Correlation with disease severity and prognostic value of suPAR with respect to outcome could be demonstrated in various critically ill patient cohorts including children [33,110–117].

Even in states of low-grade inflammation increased suPAR levels can be found. Higher levels were found in smokers than in nonsmokers, and in smokers higher levels were associated with increased risk to develop lung cancer [107] and elevated levels of suPAR were associated with incident chronic kidney disease and an accelerated decline in the eGFR [118].

While in tuberculosis patients suPAR levels decreased in response to therapy over an 8 months period, a similar effect could not be demonstrated in septic ICU patients during a 10- or 14-days period, respectively [112,113,119].

61.11 Adrenomedullin

Adrenomedullin (ADM) is a 52-amino acid peptide with vasodilatory effects that is secreted by the adrenal medulla and various other organs, including heart, lung, and kidney [120]. Vascular endothelial and smooth muscle cells produce ADM [121,122]. Apart from vasodilation, it has endocrine effects, affects renal function, and has antimicrobial properties [123]. In inflammation production of

ADM seems to be stimulated by TNFα, where it plays a major role in the hyperdynamic response during early sepsis [122,124]. ADM is derived from preproADM, a larger precursor peptide, from which different biologically active peptides are generated [120,125]. A reliable measurement is difficult due to short half-life, existence of a binding protein, and its physical properties [125,126]. Therefore, midregional proadrenomedullin (MR-proADM, proADM 45–92) a stable, nonfunctional peptide that is generated in stoichiometric amounts, is often measured instead [8,125].

Increased values of ADM and MR-proADm, respectively, have been described in patients with pheochromocytoma, sepsis, chronic renal failure, congestive heart failure, acute myocardial infarction, and acute cerebral hemorrhage [125–127]. In patients with suspected sepsis, MR-proADM levels showed an increase from inflammation of noninfectious origin to infectious origin with a further stepwise increase with presence of organ dysfunction and shock [128]. Higher levels in patients with organ dysfunction and/or shock were also seen in another study [124].

Elevated levels of ADM or MR-proADM seem to have a prognostic significance in a variety of disease entities with inflammatory states. In two studies including about 2000 patients with community-acquired pneumonia, initial MR-proADM levels were associated with severity of disease according to the pneumonia severity index and mortality [53,129]. Prognostic significance was also demonstrated in septic patients [124,128], but also in inflammation of noninfectious origin. For example, elevated levels of MRproADM after cardiac surgery were associated with increased mortality (AUC–ROC 0.940 after 6 h) [130]. Higher levels of MR-proADM as a predictor of complicated course of disease and mortality have also been reported in acute heart failure, myocardial infarction, and cerebral hemorrhage [127,131,132].

61.12 CD64

CD64 is a high-affinity immunoglobulin FC receptor for IgG (FC γ receptor I, FCγRI) expressed on monocytes. CD64 contains three Ig-like domains in its extracellular region, which bind both monomeric and aggregated IgG. It plays a role in antibody-dependent cellular cytotoxicity, clearance of immune complexes and phagocytosis [133].

Usually, CD64 is present on the surface of only a few circulating polymorphonuclear cells in low levels [133]. Upon activation of neutrophils it is strongly upregulated in response to IFN-γ and G-CSF [134]. CD 64 is considered to be a reliable indicator of acute inflammation. An increase in blood is seen in patients with bacterial infection but also with active autoimmune disease [134].

In a small cohort, 47 patients with infection had higher values than healthy controls with increasing values from patients without organ dysfunction over patients with organ dysfunction to those presenting with shock [135]. A meta-analysis including 13 studies with about 1900 patients demonstrated a sensitivity of 79% (95% CI: 70%–86%) and a specificity of 91% (95% CI: 85%–95%) for the diagnosis of bacterial infection. Values were even higher when only studies with

adult patients or with flow cytometry for CD64 assessment were included [136]. While one later study confirmed these results in patients where a blood culture was indicated [137], two others, however, presented conflicting results with either low sensitivity or low specificity in critically ill medical ICU patients (sensitivity: 87%; specificity: 41%) and patients presenting with fever at the emergency department (sensitivity: 63%; specificity: 89%). AUC–ROCs for the detection of infection were 0.8 or 0.66, respectively [138,139]. Only patients with microbiologically confirmed infection were included in the infection group. Patients with bacteremia had higher values than those without and values increased with severity of disease [139]. Neutrophil expression of CD64 was increased in influenza A with a higher proportion of cells expressing CD64 in complicated course of infection. It was, however, still lower than in bacterial infection [140].

After cardiac surgery with cardiopulmonary bypass, CD64 expression was enhanced. While values decreased in uncomplicated postoperative course, they remained high in patients with suspected or proven infection in the postoperative course [141].

CD64 values predicted mortality in several studies. Unfortunately, sometimes higher and sometimes lower values were associated with survival [135,138,139].

61.13 Panels of Combined of Biomarkers

As described in the previous paragraphs, single biomarkers of inflammation, albeit promising in some aspects, bear many limitations particularly in the light of distinguishing inflammatory states with infection from those without infection in critically ill patients. Furthermore, the complex pathophysiology of sepsis and the above-mentioned similarities of the host response to various infectious and noninfectious inflammatory stimuli lend support to the notion that successful diagnosis is more likely achieved by use of combinations of biomarkers and perhaps including also clinical features. The different aspects of diagnosis, prognosis, and monitoring of therapeutic interventions in different inflammatory disease entities or in different pathogens in diseases of infectious origin might obviously require different biomarker panels.

In this light, a study evaluated the combination of three biomarkers, that is, PCT, sTREM-1, and CD64 index of polymorphonuclear cells, in a two-step approach for diagnosing sepsis. In an inception cohort of 300 ICU patients, a bioscore was determined ranging from 0 to 3 with one point for each biomarker above the determined optimal cutoff value on admission. The bioscore performed better than each individual biomarker in diagnosing sepsis resulting in an AUC–ROC of 0.97. The bioscore was validated in an independent cohort of ICU patients revealing similar results (AUC–ROC 0.95) [142]. Another study evaluated neutrophil count, CRP, PCT, suPAR, sTREM-1, and macrophage migration inhibitory factor (MIF) to differentiate bacterial infection from nonbacterial causes of inflammation. Apart from sterile inflammation, the study cohort also comprises patients with viral, mycobacterial, and parasitic infections [108].

With respect to prognosis, a multibiomarker-based risk stratification model for 28-day mortality in septic shock was developed and tested in a multistep approach

in 881 patients. A decision tree was constructed that includes C–C chemokine ligand (CCL) 3 and 4, neutrophil elastase 2, granzyme B, heat shock protein 70 kDa 1B and interleukin-8. These 5 biomarkers provided maximum accuracy out of a panel 12 candidate biomarkers. They were complemented by serum lactate, age, and presence of chronic disease, as these parameters further improved predictive accuracy. When tested in the validation cohort, the decision tree resulted in a sensitivity of 85% and a specificity of 60% and an AUC–ROC of 0.726 [143]. In a similar approach, a decision tree was developed specifically for pediatric sepsis [144].

61.14 Multiplexed Molecular Markers Derived from "Omics" Technologies

There is growing evidence to suggest simultaneous activation of pro- and antiinflammatory signaling events in the host response to stress events, for example, associated with infection. As outlined above, this response is currently primarily monitored in the clinical context using single protein biomarkers restricted to the proinflammatory component of innate immunity. The advent of high dimensional biology and the ability to monitor genome-wide changes in gene expression on various levels from genome to metabolome allows for a broader assessment of both pro- and antiinflammatory signaling events and a rational selection of biomarker candidates on the different regulatory levels [8]. Applying tools of systems biology and bioinformatics, limited sets of transcripts, proteins, and/or metabolites can be selected to generate a compound biomarker that can be used for classification and diagnostics in a clinical setting. Essentially, these studies have paved the way to question the monopolar concept of SIRS proposed in the early 1990s and shed light on failure of central immune functions [145,146] that is further modulated by metabolic adaptations [147].

Due to feasibility, studies applying such an approach are currently available primarily for transcriptomic, that is, RNA-derived markers. Quantifying the systemic inflammatory response including assessment of both pro- and antiinflammatory innate and adaptive immune responses would hold great promise to identify patients at risk and to facilitate immune interventions in sepsis. Consistently, novel compound biomarkers based on genome-wide selection of candidates allow to cover the spectrum of the host response including antiinflammatory, immunosuppressive, and metabolic facets of the host response to stress.

The available data are currently restricted primarily to the transcriptome and introduce the need to corroborate them in the context of proteomics and metabolomics. Furthermore, most of the available evidence so far reflects elegant proof-of-concept while the studies typically do not fulfill rigorous criteria for diagnostic trials with independent training and confirmation cohorts under "real life" conditions across health care systems. However, the few studies available so far that fulfill criteria for validation of a molecular marker suggest that multiplexed transcriptome-based biomarkers perform superior to currently available single protein biomarkers of the host response (Figure 61.2) [148,149]. A

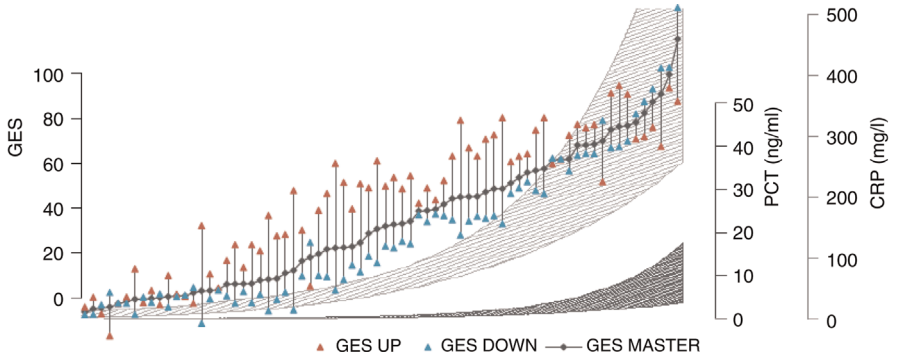


Figure 61.2 Potential of a gene expression score (GES, gray diamonds) computed based on seven transcripts that predicted the position of a given patient́s transcriptomic response in the continuum as reflected by a pangenomic assessment of the blood transcriptome. GES and its components GES UP (red triangles), that is, reflecting upregulated innate immune functions, and GES DOWN (blue triangles), that is, reflecting downregulated specific immune functions, compared to CRP (light gray shaded area) and PCT (dark gray shaded area; both represented by the 95% CI of the exponential regression fit). CRP, PCT, and downregulated adaptive immune functions (GES DOWN) are impacted significantly by disease severity. In contrast, upregulated transcripts encoding innate immune functions (GES UP) reached a plateau and were exceeded by GES DOWN in the most severe cases. The single protein biomarkers displayed broad scattering in particular with states of high-grade inflammation [148].

prerequisite to validate these compound biomarkers and assess their clinical utility requires a change of platform from research tools, such as microarrays to a feasible assay system. This can be easily achieved for both, RNA and protein markers with PCR and, for example, lateral diffusion assays respectively, while metabolites derived from metabolomic screening typically require mass spectrometry or high-resolution NMR spectroscopy [150]. The use of strategies of high-dimensional biology followed by a change in platform to a routine test system is by far the most promising avenue to develop biomarkers suitable for point-of-care testing that can classify states of severe systemic inflammation of infectious and noninfectious origin.

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62

In Vivo Imaging of Inflammation

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62.1 Introduction

Inflammation is a significant component of several chronic diseases involving the cardiovascular, metabolic, respiratory, gastrointestinal, musculoskeletal, and nervous systems. Many of these disorders are slowly disabling or fatal and account for a majority of deaths among adults in Western industrialized countries. Timely identification and localization of inflammatory foci are critical for adequate treatment of patients.

The main indications for *in vivo* imaging of inflammation are musculoskeletal disorders (e.g., painful prosthetic hip, diabetic feet and Charcot's joints, osteomyelitis, infected trauma), fever of unknown origin (FUO), abdominal infection and inflammation (e.g., sepsis, abscesses, chronic inflammatory bowel disease), vascular infection and inflammation (e.g., prosthetic valve endocarditis, vasculitis), and pulmonary infection and inflammation (particularly in immunocompromised patients).

Conventional imaging techniques (computed tomography (CT), magnetic resonance imaging (MRI), ultrasound (US)) may detect such pathology, but they only rely on anatomical changes and are not capable of discriminating active inflammatory pathology from the anatomical changes resulting from prior successful therapy or surgery. In addition, inflammatory foci cannot be detected in the early phase of development because of the lack of substantial anatomical changes at this time. Nuclear imaging with radiotracers that accumulate at the site of inflammation has become an established tool in the evaluation of several inflammatory and infectious conditions. They can reveal molecular and cellular changes associated with inflammation and provide sensitive detection of even small inflammatory foci at an early stage of disease. During the past two decades several new techniques and imaging agents have been developed for inflammation

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imaging, but as yet no imaging agent has been found with optimal characteristics. Some characteristics of inflammation make the development of imaging agents demanding due to unspecific uptake. Locally enhanced blood flow, increased vascular permeability, and influx of white blood cells (WBCs) promote the accumulation of any imaging agents to the site of inflammation. While acute inflammation is characterized by vasodilation and changes in blood flow, increased vascular permeability with exudation of plasma (protein rich, edema), and infiltration of neutrophilic leukocytes, the chronic inflammation is characterized by proliferative response with mononuclear cell infiltration (macrophages, lymphocytes and plasma cells), increased tissue destruction (extracellular matrix degraded by matrix metalloproteinases), and attempts to heal (angiogenesis) [1].

This chapter describes the principles of different techniques used for imaging inflammation; provides an outline and examples of their clinical use in evaluation of inflammatory conditions; and discusses some of the emerging techniques that are currently being developed for imaging of inflammation.

62.2 Methods for Inflammation Imaging

In vivo imaging plays an important role in medicine facilitating detection of diseases in early stages, stratification of patients for specific treatments for example targeted therapy (personalized medicine), and monitoring treatment efficacy or progression and extent of disease. Currently, most of the clinical *in vivo* imaging protocols are based on nuclear imaging modalities, that is, positron emission tomography (PET) or single photon-emission computed tomography (SPECT). In ongoing dynamic preclinical research, new potential target molecules for different diseases are identified, and selective imaging agents are developed along with state-of-art devices, hardware, and software for sensitive and quantitative *in vivo* imaging. Beyond radionuclide-based imaging, non-radioactive techniques such as contrast-enhanced US with targeted microbubbles or optical imaging with fluorescent probes are explored as alternative strategies as they provide inexpensive, real-time imaging with high resolution. Although, several targeted-US and -optical imaging approaches are used for preclinical animal studies, we are now seeing the first applications of these methods in patients with inflammatory diseases such as arthritis.

Properties of different *in vivo* imaging modalities are summarized in Table 62.1. CT, US, and MRI methods have excellent spatial resolution, even in micrometer range, and they are best suited for imaging the structure of tissues and organs. Nuclear and optical imaging has the advantage of a high sensitivity, even up to femtomole range, and thus they are best suited to measure the function of tissues and organs.

62.2.1 Nuclear Imaging

62.2.1.1 Positron Emission Tomography

PET is noninvasive, quantitative, and repetitive imaging modality using molecules labeled with positron-emitting radionuclides to visualize and measure rates of

Table 62.1 Comparison between different imaging modalities.

	Modality	Spatial resolution	Depth	Temporal resolution	Sensitivity	Molecular probe	Common radionuclides/ contrast agents	Advantages	Disadvantage
Nuclelar imaging	PET	1–5 mm	No limit	10 s-min	pmol-fmol	ng	• ^{15}O ($t_{1/2}$ 2.04 min) • ^{11}C ($t_{1/2}$ 20.4 min) • ^{13}N ($t_{1/2}$ 9.97 min) • ^{68}Ga ($t_{1/2}$ 68 min) • ^{18}F ($t_{1/2}$ 109.8 min) • ^{64}Cu ($t_{1/2}$ 12.7 h) • ^{76}Br ($t_{1/2}$ 16.2 h) • ^{89}Zr ($t_{1/2}$ 3.27 days) • ^{124}I ($t_{1/2}$ 4.18 days)	• Unlimited depth penetration • Whole-body imaging possible • Quantitative molecular imaging • Can be fused with CT or MRI for anatomical information	• Radiation exposure • Expensive • Low spatial resolution • Long acquisition time (min to hour)
	SPECT	0.5–5 mm	No limit	min	pmol	ng	• ^{99m}Tc ($t_{1/2}$ 6.0 h) • ^{123}I ($t_{1/2}$ 13.22 h) • ^{111}In ($t_{1/2}$ 2.80 days) • ^{67}Ga ($t_{1/2}$ 3.26 days) • ^{177}Lu ($t_{1/2}$ 6.65 days)	• Unlimited depth penetration • Whole-body imaging possible • Quantitative molecular imaging • Theranostic: can combine imaging and radiotherapy • Can be fused with CT or MRI for anatomical imaging	• Radiation exposure • Low spatial resolution • Long acquisition time
Optical imaging	BLI FOI	3–5 mm 2–3 mm	1–2 mm <1 mm	sec-min sec-min	fmol pmol-fmol	μg-mg μg-mg	• Light absorbing nanoparticles • Fluorescent molecules and dyes	• No ionizing irradiation • Real-time imaging/short acquisition time (sec-min) • Relatively high spatial resolution • Can be applied externally or internally (endoscopy) • Inexpensive • Highly quantitative and sensitive • Multiplexing	• Limited depth penetration • Whole body imaging is not possible, except for small animals

(*continued*)

Table 62.1 (*Continued*)

	Modality	Spatial resolution	Depth	Temporal resolution	Sensitivity	Molecular probe	Common radionuclides/ contrast agents	Advantages	Disadvantage
Anatomical imaging	MRI	25–100 µm	No limit	min-hrs	mmol	µg-mg	• Gadolinium (Gd^{3+}) • Iron oxide particles (PSPIO, USPIO) • Manganese oxide • ^{19}F	• Unlimited depth penetration • Whole-body imaging possible • No ionizing irradiation • Excellent soft-tissue contrast • High spatial resolution	• Expensive • Long acquisition time • Limited sensitivity for detection of molecular contrast agents
	CT	50–200 µm	No limit	min	—	N/A	• Barium • Iodine • Krypton • Xenon	• Unlimited depth • High spatial resolution • Whole body imaging possible • Short acquisition time • Moderately expensive • Anatomical imaging	• Radiation exposure • Poor soft-tissue contrast
	US	50–500 µm	mm-cm	sec-min	—	µg-mg	• Contrast microbubbles	• No ionizing irradiation • Real time imaging/short acquisition time (min) • High spatial resolution • Can be applied externally or internally (endoscopy) • Inexpensive • Highly sensitive	• Whole-body imaging not possible • Contrast agents currently limited to vasculature • Operator dependency

Modified from Pysz MA1 [2].

biochemical processes in tissues of living patients. PET is based on coincidence detection of annihilation radiation. The radionuclide in the radiotracer decays and the resulting positrons subsequently annihilate on contact with electrons after traveling a short distance (~1–10 mm) within the body. Each annihilation produces two 511-keV photons traveling in opposite directions (180°), which are detected by the detectors surrounding the patient and reconstructed onto three-dimensional tomographic images as a function of time (dynamic 3D images). PET requires several persons from different disciplines to be involved in, which can make method expensive. One of the major disadvantages of PET is that most of the positron-emitting radionuclides must be produced on cyclotrons, which due to short physical half-life ($t_{1/2}$) of the PET radionuclides measured in hours must be on site. Only few PET radionuclides are made by low-cost generator systems. Typical radionuclides used in PET include ^{15}O (oxygen-15 $t_{1/2}$ 2.04 min), ^{11}C (carbon-11 $t_{1/2}$ 20.4 min), ^{13}N (nitrogen-13 $t_{1/2}$ 9.97 min), ^{68}Ga (gallium-68 $t_{1/2}$ 68 min), and ^{18}F (fluorine-18 $t_{1/2}$ 109.8 min). Some nonstandard radionuclides with longer half-lives may also be used such as ^{64}Cu (copper-64 $t_{1/2}$ 12.7 h), ^{76}Br (bromine-78 $t_{1/2}$ 16.2 h), ^{89}Zr (zirconium-89 $t_{1/2}$ 78.5 h), and ^{124}I (iodine-124 $t_{1/2}$ 4.18 days). Beyond different physical half-life, the radionuclides also differ in their β^+ decay amount and energy, which has influence on PET image quality. Radionuclides with short half-lives, such as ^{18}F and ^{11}C, are useful for rapid signal acquisition, and their use will minimize patient exposure to ionizing radiation. However, PET imaging has many advantages. Most importantly it has very high sensitivity; a typical PET scanner can detect between nmol/L to fmol/L concentrations. Beside, in principle, there are unlimited number of compounds that can be labeled with PET radionuclides as tracers. Second is its quantitative feature, which makes it attractive tool *inter alia* for drug development purposes, such as for monitoring treatment efficacy of new anti-inflammatory drugs.

A glucose analog, 2-[^{18}F]fluoro-2-deoxy-*D*-glucose ([^{18}F]FDG) facilitates *in vivo* PET imaging of inflammation with high sensitivity [3]. This is based on the fact that during inflammation, glucose consumption is elevated due to the presence of activated inflammatory cells, such as macrophages, and rapidly proliferating healing tissue. Intravenously injected [^{18}F]FDG is rapidly transported through the cell membrane to the cytosol by glucose transporters (GLUT-1, GLUT-3), phosphorylated by hexokinase enzyme, and trapped inside the cell as [^{18}F]FDG-6-phosphate. Besides *in vivo* detection of inflammatory foci, dynamic [^{18}F]FDG PET imaging with the use of graphical method, that is, Gjedde-Patlak plot [4], allows quantification of the regional glucose metabolic rate. For the analysis, plasma radioactivity concentration, plasma glucose level, and the lumped constant (LC) are required. The net influx rate ($K_i = K_1 * k_3/(k_2 + k_3)$) incorporates both net inward transport and trapping of the tracer in tissue. Although [^{18}F]FDG PET imaging is useful for inflammation imaging, the uptake of [^{18}F]FDG is not specific for inflammatory conditions. High [^{18}F]FDG uptake is also observed at site of rapidly proliferating tumor cells. [^{18}F]FDG PET cannot differentiate between cancerous growth and inflammation or between sterile inflammation and infection. New more specific imaging agents are thus needed (see paragraph 4).

62.2.1.2 Single-Photon Emission Computed Tomography

SPECT is also a functional nuclear imaging technique. The radionuclides used in SPECT have relatively long half-lives (from few hours to few days) making them easy and rather cheap to produce. Most of the SPECT radiopharmaceuticals are based on commercially available kits and a generator-produced ^{99m}Tc (technetium-99 m $t_{1/2}$ 6 h). This represents the major advantage of SPECT as an imaging technique, since it is significantly cheaper than PET. However, it lacks good spatial and temporal resolution. Additionally, due to the radiation exposure related to long half-life of radiopharmaceuticals, there are safety aspects concerning the administration of the imaging agents to the patient, especially for serial studies.

^{67}Ga-citrate (^{67}Ga $t_{1/2}$ 3.26 days), radiolabeled WBCs and ^{111}In- (^{111}In $t_{1/2}$ 2.80 days) or ^{99m}Tc-labeled anti-granulocyte antibodies or antibody fragments, chemotactic peptides, liposomes, and certain cytokines are used for SPECT imaging of inflammation, and infection [5]. At this point, it should be noted that the migration of leukocytes to sites of inflammation and infection are practically taken the same as infection always has inflammatory components. The advantage of using radiolabeled WBCs is their specificity for infection imaging. Unfortunately, their use is limited for the detection of acute inflammation. The reason is that, during acute inflammation, WBCs (mainly neutrophils) and some macrophages extravasate at the injured site, but when the inflammation becomes chronic, the amount of neutrophils reduces. The radiolabeled WBCs consist mainly of neutrophils because lymphocytes are usually damaged by radiation during the quite demanding preparation procedure, which includes the isolation of WBCs from the patient's own blood (normally comprises 59% neutrophils, 34% lymphocytes, and 2% monocytes) and *in vitro* radiolabeling of the cells. In general, the SPECT imaging with radiolabeled WBCs should be acquired at three time points ("early image" at 30 min–1 h, "delayed image" at 3–4 h, and "late image" at 20–24 h post-injection) and radionuclide decay-corrected to the time of injection. When images are displayed with the same intensity scale, any focal increase in radioactivity with time should be considered as an infection, whereas if accumulation detected at 3–4 h would decrease at 20–24 h, it is a sign of a sterile inflammation.

Intravenously administered ^{67}Ga-citrate binds to plasma transferrin. This complex extravasates at the site of inflammation due to locally enhanced vascular permeability, and in the inflammatory lesion it may transchelate to lactoferrin in activated leukocytes and bacterial siderophores. While ^{67}Ga-citrate and [^{18}F]FDG are useful for the identification of both acute and chronic inflammation, neither of them is an inflammation-specific imaging agent. ^{67}Ga-citrate SPECT imaging has largely been replaced by [^{18}F]FDG PET, as ^{67}Ga-citrate offers no advantages for imaging of inflammatory diseases.

62.2.2 Computed Tomography

CT is based on the absorption of x-rays as they pass through the different parts of a patient's body. For example, iodine-based agents enhance soft tissue or vascular contrast and facilitate anatomical delineation of numerous structures. Method is

highly reproducible, has an excellent spatial resolution, and, although more expensive than US, is still relatively inexpensive. The disadvantages include exposure of the patient to radiation and the lack of functional information. Furthermore, the use of contrast medium to enhance image contrast may be limited or impossible in patients with impaired renal function or previous allergic reactions. CT plays an important role in the evaluation of patients with a pulmonary inflammation and variety of inflammatory bowel diseases.

62.2.3 Magnetic Resonance Imaging

MRI is based on strong magnetic field and resulting nuclear magnetic resonance in tissues. The method provides very high spatial resolution, but the sensitivity is limited. To achieve molecular imaging with MRI, contrast agent-linked peptides, antibodies/antibody fragments, and small ligands have been applied. Given the imaging is relatively long and noisy, MRI may be discomfort for the patient. Furthermore, there are limitations to the scanning of patients with claustrophobia, pacemakers, implants, and other devices, and the procedure is relatively expensive. However, MRI in combination with gadolinium (Gd) enhancement is useful for evaluation of, for example, synovial inflammation, bone erosion, and edema associated with arthritis.

62.2.4 Ultrasound

US imaging is cost-effective and widely available imaging modality that can reveal anatomical changes associated with inflammation, including tissue swelling, abscess formation, and synovial effusion. Contrast-enhanced US is a promising technique for imaging of inflammation. US contrast media are composed of small (usually 1–4 μm in diameter) gas bubbles (microbubbles) that oscillate in response to US and provide strong contrast in US images. After intravenous injection, microbubbles remain mainly in the intravascular space, provide enhancement of the interface between blood and tissues, and permit assessment of tissue perfusion and vascularity. While nontargeted US contrast agents are available for clinical use, modified microbubbles covered with ligands that bind specific molecular markers (targeted microbubbles) are developed in preclinical studies. Contrast-enhanced US with microbubbles targeted to bind endothelial leukocyte adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), or E-selectin, has been studied in some inflammatory conditions as a novel approach for early detection of inflammation.

62.2.5 Multimodal and Hybrid Imaging

Hybrid imaging is defined as combination of information from two imaging modalities into one image. It has become clinical routine in inflammation imaging since most of the PET scanners and an increasing number of SPECT scanners are currently hybrid devices. It enables combination of detailed anatomical information in CT or MRI images with functional information on inflammation, such as PET or SPECT scans.

62.3 Clinical Inflammation Imaging

Nuclear medicine techniques have a well-established role or have shown promise in the evaluation of several infectious and inflammatory conditions, including systemic infections, FUO, vasculitis, sarcoidosis, rheumatoid arthritis, inflammatory bowel disease, and vascular inflammation associated with atherosclerosis [6–8].

In addition to the presence or absence of an inflammatory focus, important clinical questions related to inflammation imaging are to assess the exact localization, extent, and degree of inflammatory activity; differentiate between infection and sterile inflammation; differentiate between acute and chronic inflammation; and to assess response of inflammation to therapy. Addressing these issues requires careful planning of the test and good knowledge of the limitations of different techniques [8].

The use of [^{18}F]FDG PET has increased in the assessment of inflammatory conditions. The strengths of [^{18}F]FDG PET include high sensitivity, high resolution of images, high reproducibility of results, and relatively short duration of imaging protocols (<2 h). The main limitation is that the signal is not specific for inflammation, but it can also be related to infection, malignant tumors, and even physiological glucose metabolism in some tissues, such as the myocardium, bone marrow, and brown fat [9]. Prolonged, increased glucose uptake associated with regeneration and healing process can be a problem after surgical procedures. Furthermore, anti-inflammatory therapy can rapidly decrease the [^{18}F]FDG PET signal resulting in nondiagnostic scan. As discussed earlier, labeled WBC are more specific for infection than [^{18}F]FDG. The risks related to ionizing radiation need always to be weighed against the expected benefits of imaging, particularly in young individuals and when repeated testing is considered.

62.3.1 Suspected Systemic and Local Infections

It has been shown that in systemic bacteremia, [^{18}F]FDG PET can find clinically relevant foci of infection in a large proportion of patients [7].

FUO is a diagnostic challenge due to unspecific symptoms and wide, heterogeneous group of underlying diseases. Whole-body [^{18}F]FDG-PET/CT can often provide findings that guide to more specific investigations. A recent meta-analysis summarized the results of 15 available studies and reported that [^{18}F]FDG PET has a sensitivity of 85% for establishing the cause of FUO. A particularly challenging group of patients are immunocompromised patients in whom conventional diagnostic tests often fail to find the cause for febrile neutropenia. In such patients, many studies have shown that [^{18}F]FDG PET has high sensitivity for identification of focal pathology requiring treatment, such as opportunistic infections caused by toxoplasmosis or tuberculosis.

Infectious endocarditis is a serious infection where early diagnosis and detection of complications are important for successful management. Recent studies have shown that [^{18}F]FDG can detect paravalvular inflammation associated with prosthetic valve endocarditis (Figure 62.1). The use of [^{18}F]FDG PET significantly increases the number of definite or rejected endocarditis diagnoses as well as reduces the number of cases classified as having possible endocarditis. Recent

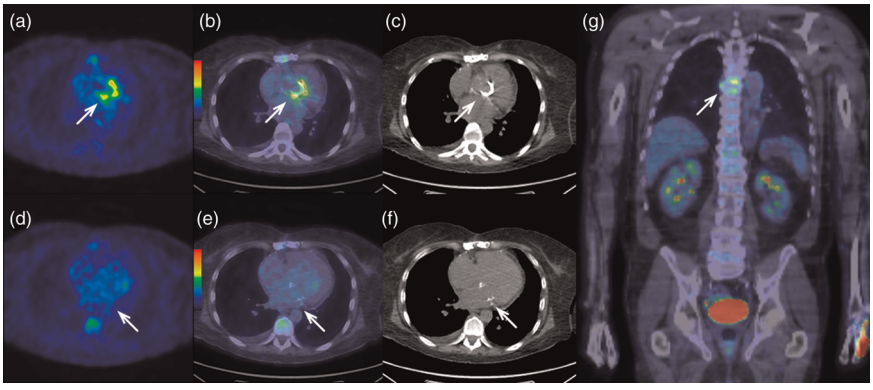


Figure 62.1 Imaging of systemic infections with 2-[^{18}F]fluoro-2-deoxy-*D*-glucose positron emission tomography/computed tomography ([^{18}F]FDG PET/CT). The patient is 67-year-old woman with aortic and mitral bioprostheses implanted several years earlier. She presented with fever, elevated C-reactive protein level (143 ng/mL), and positive blood cultures for *Staphylococcus epidermidis*. Initial transesophageal echocardiography showed no sign of endocarditis. Cardiac [^{18}F]FDG PET/CT study showed strong tracer uptake (arrow in PET image in panel (a), maximum standardized uptake value 8.4) that co-localizes with the aortic valve prosthesis (arrows in PET/CT fusion image in panel (b) and CT noncontrast CT image in panel (c). No uptake was visible in the region of mitral valve prosthesis (maximum SUV 3.6, arrows in panel (d, e and f). Myocardial uptake had been suppressed by overnight fasting and low-carbohydrate diet on the day preceding imaging. Findings were consistent with prosthetic aortic valve endocarditis that was confirmed later on and treated successfully with antibiotics. Whole-body PET images show increased [^{18}F]FDG uptake in the spine consistent with spondylodiscitis associated with endocarditis (Panel g).

European guidelines on the treatment of bacterial endocarditis indicate that the use of [^{18}F]FDG PET should be considered if the diagnosis of prosthetic valve endocarditis remains uncertain after the results of blood cultures and US examination. If the prosthesis has been implanted less than 3 months earlier, false-positive findings related to wound healing are concern.

In high-risk patients with bacteremia, such as patients with prosthetic and native valve endocarditis, [^{18}F]FDG PET and anatomical imaging tests (MRI and CT) are also important in identification of metastatic inflammatory foci, such as spondylodiscitis (Figure 62.1).

Other major clinical indications for [^{18}F]FDG PET in suspected infectious diseases include infections of joint prosthesis, spinal infections (spondylodiscitis, vertebral osteomyelitis), and peripheral bone osteomyelitis that have been reviewed in detail elsewhere [6,7].

62.3.2 Vasculitis

Vasculitis is an inflammatory disease characterized by inflammation and damage to the vessel wall. Large vessel vasculitis including giant cell arteritis and Takayasu's arteritis mainly affect the aorta and its branches. The diagnosis of vasculitis is mainly based on tissue biopsy, but the sensitivity of a single biopsy is relatively low due to the focal nature of inflammation. Several studies have shown

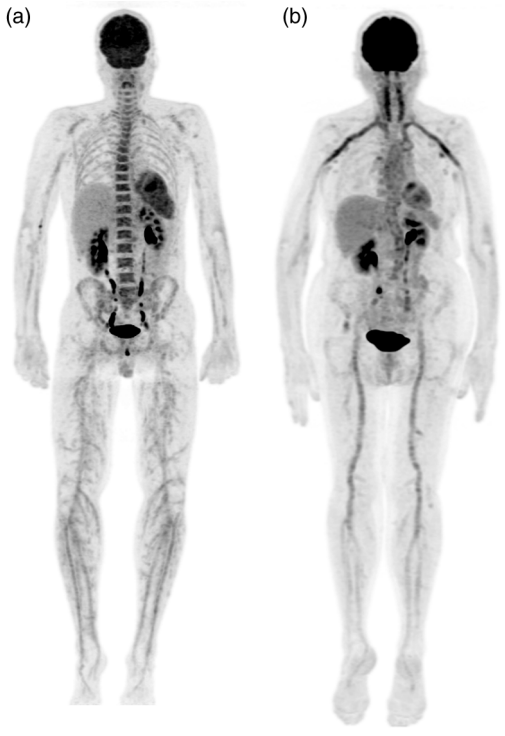


Figure 62.2 Vasculitis detected by 2-[^{18}F]fluoro-2-deoxy-*D*-glucose positron emission tomography/computed tomography ([^{18}F]FDG PET/CT). (a) A 47-year-old man had fever, fatigue, abdominal pain, and claudication due to antineutrophil cytoplasmic antibody-associated vasculitis. Whole-body PET/CT scan revealed abnormal [^{18}F]FDG accumulation in small- and medium-sized vessels of the upper extremities and in the lower extremities appearing as a tree-root-like uptake pattern. Increased [^{18}F]FDG uptake in vertebral bone marrow and the spleen was observed due to hematopoietic stimulation. (b) A 76-year-old woman had an atypical [^{18}F]FDG uptake pattern in the larger arteries, which indicated giant cell vasculitis. (Modified from Reference 9.)

that [^{18}F]FDG PET can detect inflammation in the aorta and its branches in large vessel vasculitis that can help identify a suitable biopsy site (Figure 62.2) [10]. A recent systematic review and meta-analysis concluded that [^{18}F]FDG PET appears to be a useful imaging test in the initial diagnosis and evaluation of the extent of disease in patients with large vessel vasculitis. It demonstrated a good performance (pooled sensitivity of 90% and specificity of 98%) in the detection of large vessel inflammation in giant cell arteritis patients. A potential caveat is rapid reduction in signal under immunosuppressive therapy [11]. It has been noted that standardization of image analysis and imaging protocols is still needed to optimize the use of [^{18}F]FDG PET in vasculitis [7].

62.3.3 Sarcoidosis

Sarcoidosis is a systemic granulomatous inflammatory disorder of unknown etiology. Lungs and mediastinal lymph nodes are most commonly affected, but the disease can manifest elsewhere with various presentations. Diagnosis of sarcoidosis is based on histological findings in conjunction with typical clinical and imaging findings [12]. In the absence of easily accessible tissue for biopsy, diagnosis can be very challenging.

Sarcoid lesions can be well identified by [^{18}F]FDG PET due to strong tracer uptake. [^{18}F]FDG PET is emerging as a useful diagnostic tool for sarcoidosis, especially in identifying extrapulmonic disease, such as cardiac sarcoidosis

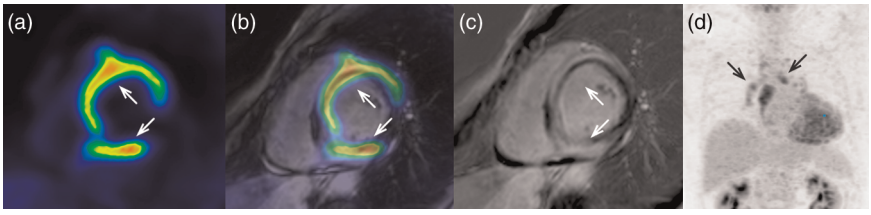


Figure 62.3 Imaging of myocardial inflammation with 2-[^{18}F]fluoro-2-deoxy-*D*-glucose ([^{18}F]FDG) positron emission tomography (PET) in cardiac sarcoidosis. Cardiac [^{18}F]FDG PET/magnetic resonance imaging (MRI) study was performed with a hybrid scanner after overnight fast to suppress physiological myocardial [^{18}F]FDG uptake. Images show cardiac PET (panel a), late-enhancement MRI approximately 5 min after intravenous injection of 0.15 mmol/kg of Gd-DTPA (panel c), and PET/MRI fusion image (panel b). There is strong [^{18}F]FDG uptake consistent with inflammation that co-localizes with late enhancement in the interventricular septum and inferior walls (arrows). Granulomatous inflammation consistent with cardiac sarcoidosis was confirmed in histological analysis of an endomyocardial biopsy obtained from the interventricular septum. In addition to myocardium, uptake of [^{18}F]FDG in mediastinal lymph nodes is typical of sarcoidosis (arrows in panel d). (Images are courtesy of Jussi Pärkkä, MD, Turku University Hospital.)

(Figure 62.3). Cardiac sarcoidosis is a serious form of sarcoidosis that is often associated with conduction abnormalities, heart failure, and ventricular arrhythmias that can even result in sudden cardiac death. To detect [^{18}F]FDG uptake associated with myocardial inflammation, a patient preparation to suppress physiological myocardial glucose utilization by fasting and avoiding carbohydrates is necessary. A typical finding consistent with cardiac sarcoidosis is focal uptake of [^{18}F]FDG co-localizing superimposed on a perfusion defect. With the recent PET/MRI hybrid scanners, [^{18}F]FDG PET findings can also be superimposed on delayed Gd enhancement, which indicates the presence of ongoing inflammation or scar tissue (Figure 62.3). [^{18}F]FDG PET can also identify extracardiac uptake in mediastinal lymph nodes that present a good target for tissue biopsy.

62.3.4 Other Applications

The use of [^{18}F]FDG PET imaging has been studied in inflammatory bowel disease showing increased [^{18}F]FDG uptake at the sites of histologically verified bowel inflammation.

Rheumatoid arthritis (RA) and psoriatic arthritis are diseases associated with synovial inflammation in often multiple joints. Functional imaging of inflammation with [^{18}F]FDG PET or ^{67}Ga-citrate SPECT has shown potential in evaluation of the degree and extent of inflammation in RA. Early detection and efficient therapy are important to prevent complications of RA and other arthritis. Some studies have indicated that [^{18}F]FDG PET may be useful to predict treatment response in RA (Figure 62.4) [13].

Inflammation plays an important role on the development of atherosclerosis and its complications. Several studies have shown visible [^{18}F]FDG uptake in atherosclerotic lesions. This may complicate detection of primary inflammatory

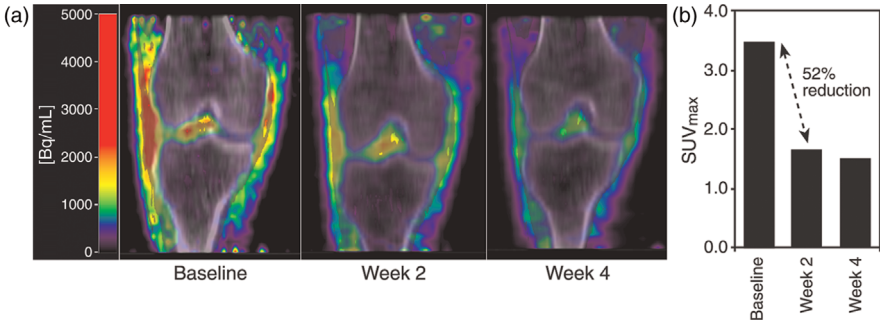


Figure 62.4 A study evaluated the potential of functional imaging to monitor disease activity and response to treatment with disease-modifying antirheumatic drugs (DMARD) in DMARD-naive patients with early rheumatoid arthritis (RA). (a) Representative [^{18}F]FDG PET/CT images of a knee in a patient with recent onset of RA presented on the same color scale, and (b) the corresponding [^{18}F]FDG maximum standardized uptake values (SUV_{max}) at baseline and 2 and 4 weeks after initiation of combination therapy. (From Reference 16.)

vasculitis but also provide significant information on the atherosclerosis risk. Some studies have shown that [^{18}F]FDG is associated with the risk of future incident cardiovascular disease that could be reduced by effective prevention strategies.

[^{18}F]FDG PET has been tested in many other conditions involving inflammation, such chronic obstructive pulmonary disease, venous thromboembolic disease, and inflammatory diseases of the central nervous system that have been discussed in detail elsewhere [7,8].

62.4 Emerging Protocols for Inflammation Imaging

In consideration of the limits of the various imaging agents currently used for inflammation imaging, the latest research has produced a series of new compounds that could increase the accuracy of functional imaging. Differentiation between bacterial and nonbacterial inflammation remains a diagnostic challenge. Although PET using [^{18}F]FDG has been introduced as a promising imaging modality for the evaluation of various infectious conditions, the diagnosis of infection remains a challenge. In general, development of new imaging agents resembles that of drugs (Figure 62.5). In general, the true value of new imaging protocols cannot be accomplished without translational studies, that is, by transforming sophisticated imaging approaches developed in experimental disease models into practical human application such as inflammation imaging for the detection of the vulnerable atherosclerotic plaques. New imaging agents that show promise after the preclinical phase will be validated in large-scale human studies.

An ideal target for *in vivo* imaging of inflammation would be a molecule that is normally absent from the endothelium of healthy tissues but is induced at the onset of inflammation. Vascular adhesion protein 1 (VAP-1) is an inflammation-

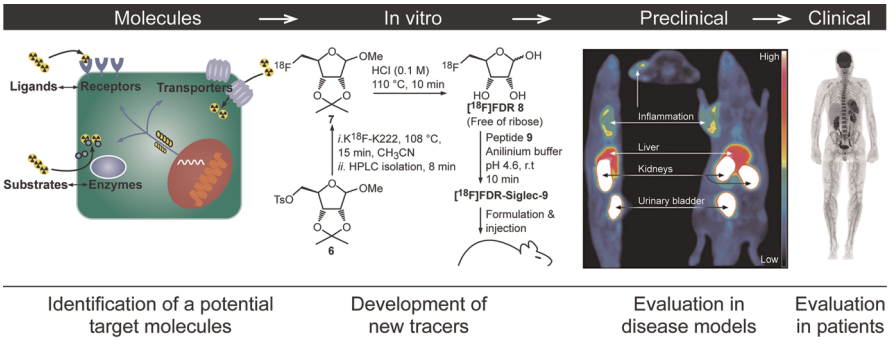


Figure 62.5 Developmental steps of the new imaging agents.

inducible endothelial cell molecule mediating leukocyte interactions with the lining of blood vessels [14]. It contributes to several steps in the extravasation cascade and controls the trafficking of lymphocytes, granulocytes, and monocytes to sites of inflammation. At the onset of inflammation, VAP-1 is rapidly translocated from intracellular storage granules to the endothelial cell surface. Besides being an adhesion molecule, VAP-1 is also a semicarbazide-sensitive amine oxidase (SSAO) enzyme (currently known as copper-containing amine oxidase AOC3, EC 1.4.3.6). The end products of the enzymatic activity are highly potent inflammatory mediators and can upregulate other adhesion molecules, such as E- and P-selectin, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1. The proof of concept regarding the use of VAP-1 as an inflammation imaging target was obtained with iodinated monoclonal antibodies against VAP-1. They were used to detect skin and joint inflammation in the pig by SPECT [15]. Currently, researchers are investigating VAP-1 as a potential target for *in vivo* imaging of inflammation by means of PET. ^{68}Ga-peptides; ^{68}Ga-, ^{124}I-, and ^{18}F-labeled antibodies; and ^{11}C-labeled small molecule inhibitors of VAP-1 are being evaluated as PET tracers. Furthermore, it is assumed that the developed imaging agents may be modified for MRI (Gd-DOTA conjugation) and SPECT (^{99m}Tc-labeling), in addition to PET. Based on a phage display approach, it has been discovered that sialic acid-binding immunoglobulin-like lectin 9 (Siglec-9) is a leukocyte ligand for VAP-1 and that PET with a ^{68}Ga-labeled peptide of Siglec-9 specifically detects VAP-1 in vasculature at sites of inflammation and certain cancer [16,17]. The endothelial molecules guiding the migration of leukocytes to sites of inflammation and infection are practically taken the same as infection always has inflammatory components. Therefore, it is highly unlikely that imaging endothelial molecules one can easily discriminate between infection and inflammation.

Integrin $\alpha_v\beta_3$, a cell adhesion molecule, is overexpressed not only in various cancer cells and sprouting tumor neovasculature but also in chronic inflammatory conditions, including inflammatory bowel disease and the inflamed synovial tissue of patients with RA. The $\alpha_v\beta_3$ integrin is expressed in angiogenic vessels and mediates intercellular adhesion of all proteins in which the arginine-glycine-aspartic acid (RGD) tripeptide sequence is exposed. In recent years, numerous

RGD peptides have been developed and tested for *in vivo* imaging of various inflammatory disorders and cancers [18].

Translocator protein (18 kDa; TSPO), formerly called peripheral benzodiazepine receptor, is a ubiquitously expressed protein localizing primarily in the outer mitochondrial membrane. It is involved in numerous cellular functions, such as synthesis of steroid hormones and transport of cholesterol. The expression of TSPO is high in activated macrophages, and therefore it has been targeted for imaging of inflammatory conditions. TSPO targeting prototypic tracer, *N*-methyl-[^{11}C]-(*R*)-1-(2-chlorophenyl)-*N*-(1-methyl-propyl)-3-isoquinoline carboxamide ([^{11}C]PK11195) has been studied for PET imaging of various inflammatory disorders, but the pharmacokinetic properties and target-to-background ratio have not been optimal. Therefore, several new TSPO ligands are currently under investigation [19].

Beyond nuclear imaging, indocyanine green (ICG)-enhanced fluorescence optical imaging (FOI) has shown good results for imaging of inflammation, for example in experimental animal models of arthritis. Recently, this emerging new technology has revealed its potential for sensitive imaging of inflammatory changes in humans with arthritis (Figure 62.6) [20]. However, further development of image interpretation and quantitative analysis are still required until the technology would be useful as a clinical imaging tool.

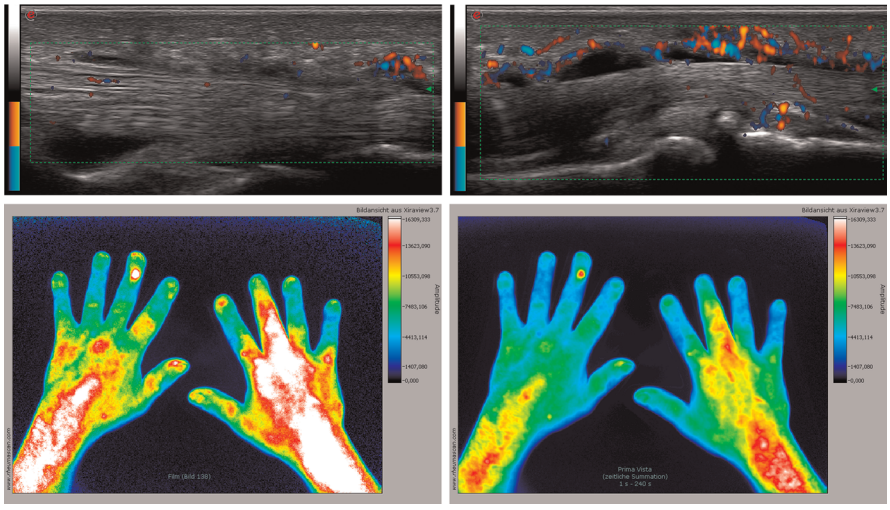


Figure 62.6 Comparison of power Doppler ultrasound (PDUS) imaging with fluorescence optical imaging (FOI) in a patient with the new diagnosis of psoriatic arthritis and clinical dactylitis of the third right finger. By PDUS (upper panels) above the third metacarpophalangeal and the third proximal interphalangeal joints (each from palmar), active tenosynovitis of the flexor tendon (and slight elevation of the MCP 3 joint capsule) is presented with the corresponding FOI images (lower panels; in phase 1 and PrimaVista mode) showing increased signal intensities especially in the third finger. (Copyrights by Charité. Permission to present the images were given by Sarah Ohrndorf, MD, and Anne-Marie Glimm, MD.)

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63

Novel Targets for Drug Development

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63.1 Introduction

Advances in our understanding of molecular components that activate and regulate the innate and adaptive immune systems have provided us with a plethora of novel targets for intervention to control the inflammatory response. Translating laboratory findings at the cellular level into effective clinical therapeutics has proven to be a major challenge. Many of these immune molecular targets have redundant pathophysiologic functions that can differ in magnitude depending upon the inciting inflammatory event, the duration of acute inflammation, and the status of immune responsiveness within the host when treatment is indicated. The capacity to do harm with immune regulators in the presence of a latent or an overt infectious process is substantial and should always be considered before initiating treatment.

The highly conserved, evolutionary balance between the advantageous survival benefits of intact host defenses and functional antimicrobial clearance mechanisms must be weighed against the potential deleterious effects of unrestrained collateral tissue damage induced by systemic inflammatory responses. This balance is highly variable in individual patients depending on their age, genetics, comorbid illnesses and medications, degree and duration of inflammatory response, and nature of injury and infectious challenge [1]. A selected group of immunomodulatory agents that are in development will be reviewed in this chapter with a focus on those drug candidates thought to be most likely to succeed as therapies for acute inflammatory disease processes. We will emphasize therapies targeting PAMPs (pathogen-associated molecular pattern molecules), DAMPs (damage-associated molecular pathogens), endothelial and epithelial barrier protection therapies, and regulators of cell apoptosis, autophagy, gene regulation, and survival. We will also highlight attempts to limit systemic inflammation with selected agents designed to terminate persistent, nonresolving

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inflammation, and in some circumstances, provide immune reconstitution by bolstering host defenses against microbial toxins and virulence.

63.1.1 Targeting the Pathogen PAMPs, Their Toxins and Virulence Factors

Since the first discovery of microorganisms by Antonie van Leuwenhoek and Robert Hooke in the late seventeenth century and the establishment of the germ theory of disease by Pasteur and Koch in the late nineteenth century, therapies have been sought to prevent or eradicate microbial pathogens. Vaccines, serum therapy, and later antimicrobial chemotherapy culminated in the dawn of the antimicrobial era in which we now still live today [2]. This remarkable achievement seemed to place microbes inevitably and permanently under the dominion of humankind with our ever expanding access to new potent antibiotics. Regrettably, this favored status over microorganisms might be short-lived as we now find ourselves stumbling to come up with adequate antimicrobial regimens to defend against an onslaught of multidrug-resistant (MDR) bacterial pathogens [3]. Efforts to develop nonantibiotic solutions to limit the inflammatory response and to defend against the emergence of MDR pathogens are now underway and are listed on Table 63.1.

63.1.1.1 Opsonic Antibodies and Antivirulence Factor Therapies

Clearing pathogens or their toxins and removal of pathogen-associated molecular pattern molecules without antibiotics is increasingly feasible with advances in polyclonal or monoclonal antibodies, microfluidic hemoperfusion devices, or even with the return to phage therapies against bacterial pathogens. Antivirulence therapies designed to disarm bacteria and render them harmless to the host is also a future possibility [13].

63.1.1.2 Bacteriophage Therapy

Lytic bacteriophages have been used therapeutically against bacterial infections for almost 100 years and still remain an active area of research in Eastern Europe and in the Republic of Georgia, a former state of the Soviet Union. These highly lethal phage particles are highly species- and strain-specific and are therefore not well suited for early empiric treatment for septic shock. Rapid and specific diagnosis with genomic methodology might help solve this limitation but other practical issues remain such as dosing of phage, distribution to sequestered sites, biofilm penetration, development of phage resistance, and possible immunogenicity. The need to maintain ready stocks of large populations of lytic phage against every conceivable bacterial pathogen is another significant drawback. Nonetheless, phage therapy might provide an alternative to antibiotics for selected, pan-resistant, health care-associated bacterial pathogens [13]. The lytic enzyme that is utilized by phage to dissolve bacterial peptidoglycan has been identified and is undergoing preclinical studies as an alternative to standard antimicrobial chemotherapy [14].

63.1.1.3 Hemoperfusion Systems to Clear Pathogens and PAMPs

An increasing number of hemofiltration and heme absorption columns and devices are now available in clinical trials. The basic approach is to remove

Table 63.1 Agents targeting the pathogen, virulence factors, or pathogen-associated molecular patterns.

Target	Potential therapy	Mechanism(s) of action	Development status
Pathogens	Hemoperfusion columns including PMX, heparin, or MBL-bound columns	Attach and filter out pathogens and their toxins and PAMPs	Late-stage clinical trials [4–7]
Pathogens	Immunoglobulins, new vaccines and opsonic mAb	Promote host clearance of the pathogen	Late-stage clinical trials [8]
Exotoxins, LPS, and virulence factors	Monoclonal antibodies	Inhibit toxins from binding to target; WN1 222–5 binds to conserved regions on LPS	Preclinical, clinical trials [9,10]
Microbial communications	Quorum sensing inhibitors	Disrupt population sensing and coordinated virulence	Preclinical studies [11]
Pore-forming exotoxins	Engineered liposomes	Decoy membrane binders to protect host cell lysis	Preclinical studies [12]
Pathogens	Bacteriophage therapy, or lytic enzymes from phage	Lyse specific bacteria with phage injections or local delivery of phage enzymes	Ongoing preclinical and clinical studies [13–15]
MDR pathogens	Repurposed drugs, Tyrosine kinase inhibitors, combination agents	Coadministration with or without standard antibiotics	Preclinical studies [16–19]
MDR pathogens	Defensins, BPI	Antibacterial peptides that can be active against MDR pathogens	Preclinical, clinical studies [20–26]

PMX: polymyxin B; mAb: monoclonal antibody; LPS: lipopolysaccharide; PAMPs: pathogen-associated molecular patterns; MBL: mannose binding lectin; BPI: bactericidal permeability-increasing protein.

circulating microbial pathogens and/or their toxins or PAMPs and thereby reduce microbial burden and rescue patients from disseminated infection. Advances in bioengineering have provided investigators with a number of ingenious hemoperfusion systems to study in clinical trials [4–7]. These devices could prove to be a valuable adjuvant therapy in treating MDR pathogens.

63.1.1.4 Engineered Liposomes to Clear Exotoxins

In addition to hemoperfusion systems and antibody-based therapies to eliminate pathogens, their toxins, or other virulence factors [4–10], a novel strategy to adsorb circulating cytolytic toxins is currently under investigation. Empty, specially engineered liposomes can be fashioned to serve as decoys to membrane toxins and thereby preventing host membrane pore formation and cytolysis [12]. Many Gram-positive and Gram-negative pathogens produce cell membrane, pore-forming exotoxins. Administration of engineered liposomes proved to be highly effective in experimental sepsis and clinical trials is now contemplated in the near future [12].

63.1.1.5 Antivirulence Factor Therapy or Quorum-Sensing Inhibitors

Another appealing approach to controlling bacterial infections without relying upon antibiotics is to disrupt the capacity of pathogens to communicate with each other and coordinate microbial invasion of the host [11]. This is accomplished by quorum-sensing signaling systems that regulate virulence genes in a population of pathogens based upon their population density. Soluble chemical signals are generated when bacterial pathogens are gathered in high concentrations. These chemical sensors are linked to intracellular kinase systems that de-repress virulence gene expression and promote lethal infection. A large number of inhibitory molecules are under investigation that might be able to block communication systems in bacteria and limit pathogenicity [11,15]. Targeting virulence has another advantage of limiting selection pressures on bacterial populations to evolve resistance to virulence traits, as opposed to selective pressures to resist bactericidal antibiotics [8,13].

63.1.1.6 Tyrosine Kinase Inhibitors and Other Novel Antibacterial Agents

Following the success of the Abl-family tyrosine kinase inhibitor, imatinib mesylate (GLEEVEC®) in the treatment of chronic myelogenous leukemia, and other cancers (gastrointestinal stromal tumors), it was discovered that the Abl-family of tyrosine kinases is also important in the pathogenesis of diverse microbes. Kalman and colleagues show that imatinib was effective in reducing bacterial load and associated pathology in mice infected with *Mycobacterium tuberculosis*, including drug-resistant strains [16]. These antibacterial effects were detectable at doses below those used for treating cancer [17]. Unexpectedly, it induced myelopoiesis, but not lymphopoiesis, and increased the numbers of myeloid cells in blood and spleen [16].

63.1.1.7 New Antibiotic Combinations

While inhibitors of serine-beta lactamases have been employed as co-drugs to overcome beta-lactamases that inactivate carbapenems (e.g., clavulanic acid,

tazobactam, sulbactam, and avibactam), there are no equivalent inhibitors of metallo-beta-lactamases (MBL) such as NDM-1 (New Delhi metallo-beta lactamase). King *et al.* identified a natural fungal product from *Aspergillus versicolor* aspergillomarasmine that inhibits MBLs, restores meropenem antibiotic activity both *in vitro* and *in vivo* [18].

Interestingly, when the standard macrolide azithromycin, which lacks activity against Gram-negative bacilli in standard laboratory assays, was tested in tissue culture medium that mimics the host environment, there was a bactericidal effect *in vitro* and protection *in vivo* in mice. Further, it synergized with cationic antimicrobial peptides including human cathelicidin LL-37, which was otherwise inactive, and with colistin [19].

63.1.1.8 Defensins and Antibacterial Peptides

Defensins, antimicrobial peptides derived primarily from PMNs, have long been known to have immunomodulatory activity [20]. Three subfamilies of defensins, alpha, beta, and theta, have similar structure. Clinical development of defensins has been hampered by the difficulty in generating sufficient amounts for *in vivo* evaluation of their therapeutic potential, as well as their toxicity. Theta defensins were identified in rhesus macaques have an 18-residue cyclic peptide encoded by genes similar to those for alpha defensins, although a premature stop codon results in peptides with 12 residues that form cyclized nonapeptides. In humans, an additional stop codon results in a pseudogene that is express as mRNA, but the sequence has been used to resurrect the putative coding sequence. The resulting peptide has been named retrocyclin [20]. The peptide has high potency and a broad range of antimicrobial activity, including Gram-positive, Gram-negative bacteria, *B. anthracis* bacilli and spores, viruses, and fungi. Recently, Lu and colleagues developed a synthetic chemical process by which large amounts of retrocyclins, can be produced. These compounds have high stability and low toxicity [21].

Several decades ago, Peter Elsbach and Jerry Weiss discovered that a neutrophil protein which they named bactericidal permeability inducing protein (BPI) not only was bactericidal for Gram-negative bacilli but also directly neutralized endotoxin [22]. This discovery led to the development of a recombinant protein from the endotoxin-binding portion of the molecule. A clinical trial in children with meningococcal sepsis was unable to demonstrate protective efficacy but did find a reduction in gangrene among survivors. The delay in obtaining parental consent for entry into the trial most likely explained the lack of protective efficacy in this fulminant infection [23]. Interestingly, BPI is being reexamined as a potential antimicrobial agent not prone to the mechanisms of antibiotic resistance [24]. Additional LPS-binding peptides have been evaluated for LPS-neutralizing activity. Two 34-mer peptides were derived from the core LPS-binding region of Factor C from the horseshoe crab, a serine protease with multiple high-affinity LPS-binding sites [25].

63.1.2 Targeting DAMPs and the Host Response to DAMPs and PAMPs

Pathogen-associated molecular pattern (PAMPs) molecules comprise a set of highly conserved structural components of microbial pathogens such as

lipopolysaccharide (LPS), bacterial lipoproteins, flagellin, and viral, fungal, or bacterial genomic features that are distinct from human structures, and are recognized by pattern recognition receptors (PRRs). Remarkably, many of these same PRRs also recognize damaged elements arising from human tissues when released into the circulation following cell necrosis. These damage-associated molecular pattern (DAMP)-related molecules come in two basic forms: (i) conserved molecules usually confined to the intracellular or intranuclear space (e.g., histones, mitochondrial DNA, HMGB1, and actin filaments) or (ii) conserved elements within the interconnecting ground substances between cells that are disrupted during cell death (e.g., hyaluronic acid, heparan monomers, and biglycans) [26].

Collectively PAMPs and DAMPs are referred to as signal 0 of the acute inflammatory response. Signal 1 consists of antigen recognition and class I and II major histocompatibility complex; signal 2 is provided by costimulatory receptors and ligands (CD28 on T cells and CD80/86 on antigen presenting cells). Signal 3 is dendritic cell-mediated polarization of the emergent T cell response and signal 4 is differential integrin expression on activated T cells that instruct T cell trafficking into tissues during the inflammatory response [27]. Therapeutic targets and novel agents to modulate the initial host response to PAMPs and DAMPs are summarized in Table 63.2.

63.1.2.1 Statins

While the 3-hydroxy, 3-methyl glutaryl coenzyme A (HMG–CoA) inhibitors have become first line, lipid lowering agents to treat atherosclerosis and their immunomodulatory effects have been equally impressive and likely accounts for their many beneficial therapeutic effects. Statins promote the expression of eNOS (endothelial nitric oxide synthase) with its capacity to protect the microcirculation, limit neutrophil and monocyte adherence to endothelial cells, and support endothelial barrier function [28]. These anti-inflammatory effect and endothelial protective effect are mediated by statin-induced inhibition of the isoprenoid GGPP (geranyl geranyl pyrophosphate). GGPP mediates the post-translational modification of a variety of RAS-like proteins such as Rho. Decreased Rho activity improves endothelial barrier function and decreases NFκB formation [29].

Another recent finding that might explain a number of the salutary effects of statins during systemic inflammation is their capacity to activate endothelial cyclooxygenase (COX)-2 by S-nitrosylation. COX2 activation followed by extracellular 5-lipoxygenase activity catalyzes the synthesis of 13-series resolvins [30]. These host-protective, short lipid signaling molecules, along with related resolvins and protectins, promote resolution of inflammation and trafficking of inflammatory cells to regional lymphatic tissues.

A number of clinical trials have been undertaken to test the capacity of statins to provide benefit in septic patients and ARDS. The results have been mixed and generally disappointing [31]. As is always the case with anti-inflammatory agents, questions on timing, dosing and patient selection persist as to whether statins will hold promise as an adjuvant therapy in prevention and treatment of septic shock and related inflammatory states.

Table 63.2 Agents targeting host responses to DAMPs and PAMPs.

Target	Potential therapy	Mechanism(s) of action	Development status
DAMPs/PAMPs	Immunomodulatory and endothelial protection from statins	limit neutrophil binding to endothelial cells, inhibit isoprenoid synthesis and promote resolvin actions	Used clinically for cardioprotection [28–31].
Histones	Inter-alpha inhibitor protein (IaIp); non-anticoagulant heparin	IaIp and heparins possess GAGs that neutralize histones	Preclinical and clinical trials [32–34]
Histones	Soluble thrombomodulin (sTM)	sTM binds histones and blocks p rotein C activation; giving sTM repairs histone inhibition	In late clinical trials [35,36]
DAMPs and PAMPs	TLR 4 inhibitors and signaling inhibitors: innate immune response regulators	Limit signal transduction of innate immune activation from DAMPs/PAMPs	Preclinical studies [36–40]
DAMPs and PAMPs	Receptor for advanced glycation end products (RAGE) inhibitors	Soluble RAGE decoy receptors and mAbs to RAGE limit continued immune activation	Preclinical studies [41–44]
PAMPs DAMPs and actin filaments	Plasma gelsolin (pGSN)	pGSN therapy removes actin and can replete loss of circulating gelsolin during acute inflammation	preclinical and early clinical studies [45–47]
DAMPs (HMBG-1, HSPs)	Triggering receptor expressed on myeloid cells (TREM)-1 inhibitors	Soluble TREM and TLT-1s (TREM like transcript small variant) block TREM-1 potentiation of TLR signals	Preclinical studies [48–51]
High mobility group box 1 (HMGB1)	HMGB1inhibitors	block HMGB-1- mediated reduced endothelial and epithelial barrier function and might help reverse PCI	Preclinical studies [52–58]
DAMPs PAMPs	Resolvins, proctectins	Transcellular lipoxygenase products are proresolving and anti-inflammatory	Preclinical and early clinical studies [30,59]
DAMPs PAMPs	Proprotein convertase subtilisin/kexin (PCSK)-9 mAb	LPS and pathologic lipids are cleared by LDLr; PCSK9 mAb improves LDLr clearance function	Preclinical studies, clinical trials in cardiology [60–62]

GAG: glycosaminoglycans; LPS: lipopolysaccharide; mAb: monoclonal antibody; PCI: persistent critical illness; LDLr: low-density lipoprotein receptor.

63.1.2.2 Histone Inhibitors

Extracellular histones are highly injurious as DAMPs and can induce a potentially lethal inflammatory state in experimental animals [32–34,63–67]. Histones are strongly positively charged to interact closely the heavily negatively charged phosphoryl groups on DNA in tightly packed nucleosomes within chromosomes. Histones are released upon cell death by necrosis and during the extracellular release in the formation of neutrophil extracellular traps (NETs). NETs develop in the microcirculation during bloodstream infection as a final attempt to clear systemic bacterial infection [33,34,64–69].

Therapies that appear to serve as inhibitors of the inflammatory potential of histones include a family of human plasma proteins known as inter-alpha inhibitor proteins [32] and heparin [33,34], even modified forms of heparin that lack significant anticoagulant activity. Clinical trials are contemplated with such agents to limit the toxicity of extracellular histone release. Thrombomodulin (TM) will also bind to histones and can, at high histone concentrations, interfere with TMs capacity to activate protein C. A soluble form of recombinant TM is in clinical trials in sepsis at present to determine if restoring TM levels can improve survival [35,36].

63.1.2.3 Extracellular and Intracellular Signaling Inhibitors

The dramatic increase in antibiotic resistance and diminished antibiotic development pipeline, there has been considerable effort to develop host-oriented therapies, interventions directed at modifying the host response to infections. If successful, these therapeutics would have the advantage of not being subject to antibiotic resistance mechanism and not inducing such resistance. One approach is under development whereby small molecule inhibitors directed at the cytoplasmic TIR (Toll/interleukin-1 receptor) domains of Toll-like receptors (TLRs) have shown inhibitory activity in preclinical testing both *in vitro* and *in vivo* [37]. Cell-penetrating peptides comprised of the translocating segment of Drosophila antennapedia homeodomain fused with BB loop sequences of several TLR–BB peptides inhibited NFkB translocation following several TLR agonists. Since the TIR domains are highly conserved among multiple TLRs, such interventions may be more broadly applicable than small molecules directed at the TLR4 ectodomain (but not TLR3 or TNF-alpha signaling).

Following the novel finding that a peptide inhibitor of MD2, Eritoran, was protective in experimental influenza infection, these peptides were shown to improve survival in influenza-infected mice [37]. Additional peptides that inhibit MyD88 dimerization and recruitment of IRAK1 and IRAk4 have also been found to inhibit intracellular signaling [38]. High-throughput computational screening to discover inhibitors of host MyD88 protein–protein interactions implicated in the lethal immune response to staphylococcal enterotoxin identified a binding site for MyD88 on the dimeric model of Toll-interleukin receptor (TIR). Biochemical analysis and cell-based reporter assays found that the most promising compound, T6167923, disrupted MyD88 homodimeric formation, and that a single dose of this compound protected mice from lethal SEB-induced toxic shock [39]. These promising lead compounds have yet to be testing in human systemic inflammatory states.

Another class of small molecule synthetic peptides, innate defense regulators (IDR), targets intracellular signaling pathways [39]. Innate defense regulators (IDRs) are synthetic immunomodulatory versions of natural host defense peptides that mediate protection against bacterial challenge in the absence of direct antimicrobial activity. They broadly modulate a spectrum of immune responses. These antibacterial peptides that restrict excess innate immune responses are active in experimental sepsis models and might prove to be effective in clinical medicine.

63.1.2.4 Receptor for Advanced Glycation End Products (RAGE) Inhibitors

RAGE is an attractive target for therapeutic manipulation in systemic inflammatory states for a number of reasons. RAGE is the quintessential pattern recognition receptor that binds to and signals upon recognition of a myriad of endogenous DAMPs and even some PAMPs [41,42]. RAGE is a proinflammatory receptor that activates transcriptional programs for acute systemic inflammation. As opposed to many other inflammatory mediators, which have their receptors downregulated upon activation, RAGE is continuously upregulated during inflammation. RAGE and is a viable target even after systemic inflammation is established and ongoing. RAGE inhibitors include soluble decoy receptors lacking intracellular domains or monoclonal antibodies [43,44].

63.1.2.5 Recombinant Gelsolin and TREM-1

Plasma levels of gelsolin, a ubiquitous human plasma protein, fall rapidly in severe sepsis and the loss of this protein impairs actin clearance, reduces phagocytosis, and promotes inflammatory cytokine generation [45]. Actin is a DAMP molecule released into the circulation during cell injury or death. Low plasma levels are correlated directly with worsening disease severity. Recombinant gelsolin is available and could be a potential novel therapy for severe sepsis [46]. Extensive animal data and pilot studies in human sepsis with gelolsin appear promising and the potential therapeutic effects of gelsolin should be tested in septic patients [47].

Another attractive target for therapeutic manipulation during sepsis and other systemic inflammatory states is the triggering receptor expressed on myeloid cells (TREM)-1 [48]. TREM-1 belongs to a family of TREM molecules that partner with a transmembrane signaling molecule known as DNAX activation protein 12 kDa in size (DAP-12). DAP-12 possesses a ITAM (immuno-receptor tyrosine-based activation motif) that is proinflammatory signal to amplify cell activation events induced by TLR signaling. TREM-1 is a receptor for as yet not fully characterized repertoire ligands, including heat shock proteins, HMGB-1 (high mobility group box-1), and likely other DAMPs [27].

A natural inhibitor exists that effectively blocks TREM-1 potentiation of TLR signaling known as TLT-1 (triggering receptor expressed on myeloid cell-like transcript-1) [48,49]. Alternatively spliced short variants of TLT-1 that also have inhibitory activity [50]. Short sequences of peptides from the extracellular domain of TLT-1 have been identified that could be used as an inhibitory therapeutic agent [50,51]. These agents are effective in animal experiments in sepsis and could be viable candidates for clinical trials in the near future.

63.1.2.6 High Mobility Group Box 1

High mobility group box 1 (HMGB1) is a DNA binding protein found in the nucleus that is actively secreted in small amounts from activated immune effector cells and is passively released in large amounts from necrotic cells [52–54]. The disulfide form of HMGB1 signals innate immune cells through TLR4 [54]. Circulating HMGB1 in the bloodstream is a highly injurious molecule that damages endothelial barriers, and induces the release of IL-1 alpha and IL-1 beta from endothelial cells [55–57]. HMGB1 induces major defects in gut epithelial barrier function and contributes to increased mucosal permeability in prolonged shock [52]. HMGB1 also induces mitochondrial injury and cellular apoptosis during septic shock [54].

HMGB1 is now recognized as a major contributor in perpetuation of septic shock in its later phases. This pathologic state is known as persistent critical illness (PCI). PCI is a distressingly common problem in modern ICUs where prolonged need for ICU supportive care costs dearly in terms of resources and patient lives. Remarkably, HMGB1 also appears to play a role in the rather common occurrence of cognitive decline as a late complication of severe sepsis, at least in experimental mouse models [58]. Antibodies to HMGB1 and other inhibitory strategies to block HMGB1 actions are protective in experimental sepsis. Feasibilty studies with HMGB1 inhibitors should hopefully be initiated in human clinical trials in the near future.

63.1.2.7 Proresolving Agents

Serhan and colleagues have carefully investigated the biochemical signals that contribute to resolution of tissues damaged by injury and or infection and have discovered that repair and clearance of inflammatory foci is not a passive process of simply the lack of ongoing inflammatory makers [59]. Resolution of infection is an active process following an ordered sequence of lipoxygenase-mediated proresolving lipids. Many of these host-protective and proresolving lipid-mediated molecules have been identified (called resolvins, protectins, etc.) and are now in early clinical trials to increase resolution of tissue injury and improve outcomes [30].

Another remarkable recent discovery in cardiology of the role of proprotein convertases in removal of damaging phospholipids might have more general application to include systemic inflammatory states including sepsis [60]. A human protein called PCSK9 (proprotein convertase subtilisin kexin 9) has recently been shown to impair the clearance of low-density lipoproteins associated with atherosclerosis [61]. The same enzyme has been found to limit clearance of bacterial lipopolysaccharide and other pathologic lipid mediators found in sepsis [62]. Monoclonal antibodies to PCSK9 can recover the capacity to clear LPS efficiently and might serve as a novel strategy to improve the outcome in human sepsis.

63.1.3 Epithelial Membrane Barriers and Microbiome as a Target for Sepsis

Loss of epithelial mucosal barrier protection contributes to the initiation and perpetuation of the systemic immune dysfunction in sepsis [70,71]. Mucosal ischemia and inflammatory changes are commonplace is septic shock patients

and these pathologic processes alter the complex interactions between the gut mucosa and resident microflora in health and disease. Epithelial membranes play an essential role in normal physiology of separating and defending the parenchymal tissues from the external milieu. Epithelial membranes are polarized with an apical surface and a basolateral surface. This feature of selective semipermeable membranes regulates water, solute, and macromolecule flow rates, and segregate the resident microbiota from host tissues [71].

The microbiome of the gut is best known but similar microbiomes are now recognized to colonize in the lower airways, oropharynx, skin, and genitourinary tract [72–74]. The gut microbiome generates 100-fold more expressed genes than the entire complement of expressed human genes. The microbiome consists of over 10^{14} bacterial cells with is home to thousands of different species of bacteria, viruses, Archaea, fungi, and commensal protozoan parasites. The microbiome is now considered the "second human genome" that needs to be understood and accounted for in health and disease. Changes in the microbiome certainly occur in critical illness and the details and relevance of these alterations on the human host are only beginning to be unraveled. Attempts to regulate and reestablish a healthy microbiome might represent a novel strategy to promote the resolution of sepsis in patients with persistent critical illnesss (PCI) [71,72,74].

This might be feasible by probiotics, defined as the placement of favorable bacterial strains or fungi along the epithelium to reestablish a healthy microbiome. Another strategy is the use of prebiotics and the process of placing nonabsorbable complex polysaccharides or other macromolecules to alter the substrates for metabolism and structure of the microbiome [75–80]. These efforts have already produced some limited success, particularly in preventing necrotizing enterocolitis in critically ill neonates and preventing recurrent respiratory infections in children [78]. Many other possible benefits might accompany regulating or restoring a healthy microbiome. Some preliminary studies suggest that probiotics or prebiotics could be useful to prevent colonization and infections from multidrug resistant, nosocomial Gram-negative bacterial pathogens and recurrent *Clostridium difficile*-associated colitis [81–83]. However, studies designed to manipulate the microbiome to prevent ventilator-associated pneumonia have largely been unsuccessful to date [84]. Much more work will be necessary to fully understand the potential value and the limitations of regulating patient microbiomes [71,72].

Another therapeutic option is to repair damaged epithelial cells or reestablish the structure and function at the junctions between adjacent epithelial cells. In the gut epithelium, cells are held together by intercellular adhesion molecules, including occludin, ZO-1, and claudin [71,74]. Growth factors, such as granulocyte-macrophage colony-stimulating factor [85] and hepatocyte growth factor [86], support enterocyte growth and function. Specific enterocyte trophic cytokines, including interleukin-11 [87], IL-22 [88], and other epithelial growth factors [89,90], can reduce epithelial apoptosis, repair tight junctions, stimulate enterocyte growth, and retain epithelial barrier function.

Oral adminstration of protease inhibitors to prevent increased gut permeability from pancreatic enzymes are in clinical trials at present [91]. Nonabsorbed agents, such as tranexamic acid and related protease inhibitors, remain in the gut lumen

Table 63.3 Agents targeting the epithelium and the microbiome in inflammatory states.

Target	Potential agent	Mechanism(s) of action	Development status
Maintain healthy luminal microbiota	Regulate the microbiome with probiotics and prebiotics	Tightens epithelial junctions, stimulates intestinal immunity, colonization resistance, limits MDR pathogens and *C. difficile*	In clinical studies [75–84]
Epithelial membrane barriers	Hepatocyte growth factor; Interleukin-11; interleukin-22	Epithelial growth factors, cytoprotective for intestinal epithelium in acute inflammatory states	Preclinical studies, early clinical trials [85–88]
Protect epithelial barrier function	Insulin-like growth factor-1; TNFAIP3	Prevents sepsis-induced excess gut epithelial permeability and apoptosis	Preclinical studies [89,90]
Enzymatic breakdown of epithelial barrier	Orally administered pancreatic enzyme inhibitors-tranexamic acid	Prevents breakdown of tight junctions and epithelial barrier during sepsis	In clinical trials [91]

GM–CSF: granulocyte-macrophage colony-stimulating factor; MDR: multidrug resistant; TNFAIP3: tumor necrosis factor alpha-induced protein 3; I/R-ischemia reperfusion injury.

and protect the intestinal epithelium from luminal proteases that induce loss of membrane function and excess intestinal permeability during septic shock.

A summary of new epithelial barrier defensive strategies to prevent or to treat sepsis and other acute inflammatory syndromes are present in Table 63.3.

63.1.4 Targeting the Vascular Endothelium in Acute Inflammatory States

The endothelial surface is at the interface between intravascular inflammation and extravascular inflammatory responses during sepsis [71,92]. The innate immune system and coagulation pathways coevolved to collaborate in protecting the host from the simultaneous risk of exsanguination and invasive microbial infection following any break in the integument. The interface between clotting and inflammation is particularly critical in sepsis [92–94]. Dysfunction or disruption of the endothelial barrier by injury or apoptosis exposed the fluid phase of the blood compartment to subendothelial sources of tissue factor (TF), the main initiator of coagulation activation in sepsis. Activated monocytes and macrophages are major sources of TF in severe sepsis. Even activated endothelial cells themselves can express TF along their cell surface when exposed to high-levels TNF or interleukin-1β. TF binds circulating factor VII and this TF:FVIIa complex and activates factor X with generation of thrombin and fibrin. TF can also reside on microparticles (MPs) shed from hematopoietic and endothelial cells. MPs can activate both coagulation and inflammation in sepsis [92].

Platelet adhesion to activated endothelial cells is increased in sepsis by alterations in von Willebrand factor. Final assembly of von Willebrand factor multimers occurs on endothelial surfaces. Large multimers of von Willebrand factor occur in

sepsis that avidly bind to platelet glycoprotein Ibα under conditions of shear force [94]. These large von Willebrand factor multimers are normally cleaved by a protease known as ADAMTS 13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13). Sepsis induces an acquired deficiency of ADAMTS13, resulting in ultralarge von Willebrand factor multimers and platelet adhesion to injured endothelium [94–97].

Thrombin and other activated clotting factors are recognized by a set of seven-transmembrane receptors known as protease-activated receptors (PARs) [98]. Four PARs exist in humans can either promote or disrupt endothelial barrier function, depending on which G-protein-linked intracellular signaling pathway is activated. Thrombin bound to PAR1 in early sepsis contributes to endothelial dysfunction by GTPase RhoA-dependent cytoskeletal derangements in endothelial cells and induces endothelial cell contraction and rounding [98,99]. Endothelial cell contraction disables extracellular membrane barrier function thereby increasing vascular permeability. Macromolecules, such as large proteins and white cells, can now exit the circulation into the extravascular space. Over time thrombin–PAR1 complexes transactivate PAR2 and form PAR1–PAR2 heterodimers on endothelium. This reverses the net effect of thrombin generation to an endothelial barrier protection effect. This is mediated by PAR1– PAR2 switching of intracellular GTPase signaling from the RhoA-mediated barrier disruptive pathway to a PAR2-mediated Rac1 endothelial barrier protective function. This two-way competing pathway can be manipulated to a therapeutic advantage with agents that target specific PARs [98,99].

Endothelial barrier function prevents vascular leak, maintains an anticoagulant surface, and prevents macromolecules from exiting the microcirculation. Endothelial barriers are maintained by adherens junctions, consisting of vascular endothelial (VE) cadherin, and tight junctions (the zona occludens), formed by occludins and claudins [94]. Intracellular actin filament networks maintain endothelial cell apposition and this is further supported by transmembrane Robo4-slit proteins [100,101].

Several treatment options designed to retain endothelial barrier function are now under preclinical and clinical investigation. Selepressin is a vasopressin analog that is specifically targets the V1a vasopressin receptor. In addition to its vasoconstrictive properties, this vasoactive peptide limits endothelial leakage of fluid into the extra-vascular space. This drug is now in clinical trials in septic shock [102]. Another vasoactive drug strategy is monoclonal antibody to adrenomedullin [103]. Adrenomedullin is a 52-amino-acid vasodilatory peptide produced by endothelial cells that counteracts the effects of alpha adrenergic vasoconstrictive agents. Adrenomedullin antibody can restore catecholamine responsiveness and serve as an adjuvant therapy in septic shock [103].

63.1.5 Immunomodulators and Other Promising new Therapeutic Targets

63.1.5.1 Immune Reconstitution as a Molecular Target

A myriad of immunologic findings clearly demonstrate that sepsis-induced immune dysfunction occurs in many patients with prolonged sepsis and

PCI [71,104]. Reduced T-cell and B-cell functions, reduced lymphocyte proliferative capacity, excess apoptosis, T-cell exhaustion, and dysregulated innate immune responses are readily demonstrable and have significant adverse clinical consequences [105]. Reduced expression of major histocompatibility complex (MHC) class II antigens on antigen presenting cells is frequently observed in sepsis [104]. Opportunistic viral infections are reactivated in the majority of patients with severe sepsis after prolonged ICU care [106,107] and closely follow the occurrence of systemic opportunistic viral infections observed in organ transplant recipients [108]. Disseminated cytomegalovirus infection and herpes simplex infection occur regularly in severe sepsis patient. Opportunistic bacterial and fungal infections also occur at high frequency in septic patients [107].

63.1.5.2 Immune Reconstitution Strategies as a Therapy for Sepsis

A number of therapeutic strategies to limit immune dysfunction or reconstitute immune function following sepsis are now in early clinical development. These include the T-cell growth factor and thymic conditioning factor known as thymosin alpha 1 [109], inhibitors of regulatory control mechanisms such as anti-programmed cell death ligand (PDL1) antibody or anti-B and T lymphocyte attenuator (BTLA) [104], or other immune support and T-cell growth factors such as interleukin-7 and IL-15 [110,111]. Immunostimulant regimens in patients with systemic inflammation is not without risk (immune reconstitution inflammatory syndrome) and use of these therapies will need to be closely monitored in these critically ill patients [112].

63.1.5.3 Mesenchymal Stem Cells as a Therapy for Sepsis

Mesenchymal stem cells (MSC) do not express class 1 or class 2 antigens and thus are well tolerated when infused into patients with a variety of inflammatory diseases. MSCs have the remarkable capacity to traffic to sites of tissue injury and deliver anti-inflammatory cytokines in a paracrine manner to inflamed tissues. They also promote bacterial clearance and assist in remodeling and tissue repair. Their capacity to benefit patients with sepsis or acute lung injury is just beginning to be tested in clinical studies [113].

Specific immunostimulatory oligodeoxynucleotides appear capable of restoring immune competence in experimental models of sepsis by expanding the generation of endogenous MSCs. This might be another way of providing MSCs to damaged tissues in acute inflammatory states [114,115].

63.1.5.4 Mitochondrial Sparing agents and Immunometabolism as a Target for Sepsis

Other novel sepsis therapies are designed as mitochrondrial sparing agents to prevent intrinsic pathway activation of cellular apoptosis [116,117]. Sirtuins and situin activators have mitochondrial growth stimulant activity that is protective against lethality in experimental models of sepsis [118].

Considerable interest has recently developed in readily available methods of regulating the host inflammatory status by directing the immunometabolism of

Table 63.4 Immunomodulators and other potential targets in systemic inflammation.

Target	Potential therapeutic agent	Mechanism(s) of action	Development status
Sepsis-induced immune suppression	Thymosin	Thymic growth factor, supports T cell expansion and function in immune compromised patients	Currently in clinical trials for sepsis [109]
Immune reconstitution	Programmed cell death ligand-1 monoclonal antibody	Inhibit co-inhibitory signaling between lymphocytes and Antigen presenting cells	Clinical trials in oncology and pilot clinical studies in sepsis [105]
Inflammation-induced immune suppression	Interleukin (IL)-7, IL-15	T-cell growth factors that reverse lymphocyte exhaustion and promote T-cell receptor diversity	Clinical trials in oncology and pilot studies in sepsis [110,111]
Inflammation -induced organ injury	Mesenchymal stem cells (MSCs)	MSCs traffic to damaged tissue and provide local anti-inflammatory signals and promote tissue repair	Early clinical trials in acute lung injury and other indications [112]
Sepsis-induced immune suppression	Immunostimulatory oligodeoxynucleotides	Promote synthesis and release of MSCs and clear bacterial pathogens	Preclinical studies and pilot clinical studies [113]
Inhibition of apoptosis in systemic inflammation	Mitochondrial sparing agents; SIRT-1 activators	Improves cellular energetics and limits endogenous apoptosis	Preclinical investigations [74,75]
Metabolic switch to glycolysis	Immunonutrition strategies to switch to oxidative metabolism	Can induce inhibitory phenotype of macrophages	Preclinical investigations [76,77]
Macrophage activation syndrome	Interleukin-1 receptor antagonist (IL-1ra)	Blocks IL-1-induced inflammation, organ injury and cytopenias	Pilot clinical trials, phase 2 studies are contemplated

LPS: lipopolysaccharide; mAb: monoclonal antibody; HMGB1: high mobility group box 1; MDR: multidrug-resistant bacteria.

inflammatory cells [119,120]. Converting monocytes away from anaerobic glycolysis to aerobic metabolism changes the inflammatory phenotype to an anti--inflammatory functional cell type. This is an attractive mechanism to regulate the inflammatory system and will need to be testing in clinical studies to determine if it could be a clinically viable option.

63.1.5.5 CD28 Costimulatory Signal Peptide Inhibitors as a Novel Therapy for Sepsis

Short synthetic peptide inhibitors of the CD28 homodimer interface during cell activation are being developed to limit the costimulatory signals on antigen presenting cells and T lymphocytes [121]. One such peptide inhibitor looks promising in experimental studies in toxic shock syndromes and necrotizing soft tissue infections [122]. A recent phase II trial with a CD28 inhibitory peptide known as AB103 was successful in a phase II trial in patients with necrotizing fasciitis and is now in phase III trials [123].

63.1.5.6 The Return of Interleukin-1 Receptor Antagonist (IL-1ra) in Systemic Inflammation

Macrophage activation syndrome (MAS) is a component of the hemophagocytic syndromes and consists of a shock-like state with evidence of hemophagocytosis in the bone marrow, acute severe inflammation, hyperferritinemia, multiorgan failure, and poor clinical outcomes. IL-1ra has been tested in pilot studies in refractory cases of MAS with some successful treatment outcomes. A retrospective reanalysis of a phase 3 sepsis trial with examination of a subgroup of patients who met MAS criteria suggested a survival benefit when they received IL-1ra [124]. A prospective, randomized trial focused on MAS patients is planned in the near future to determine if this approach can be confirmed in a large clinical trial (see Table 63.4 for a detailed summary of immunomodulators in clinical development).

63.2 Conclusion

With the ever-increasing knowledge of the molecular and cellular mediators that contribute to sepsis pathophysiology, a new generation of therapeutic opportunities are now available for detailed investigation. Recovery and maintenance of epithelial and endothelial barriers are rational targets to assist recovery in patients with acute systemic inflammatory state, be they sterile inflammation or infection-related sepsis. Other experimental agents currently in development promote cell survival by targeting mitochondria and cellular energetics. New strategies that bolster immune defenses should be tested in this era of progressive antimicrobial resistance among bacterial and fungal pathogens. These novel agents targeting new molecular mediators need to be linked with rapid molecular diagnostic studies to identify patients likely to benefit from specific interventions [125]. Innovations in genomic medicine will bring a new generation of adjuvant therapies to the bedside to the benefit our patients.

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64

Inflammation and Microbiota and Gut Reconditioning

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"Everything in excess is opposed to Nature. All disease begins in the gut."
Hippocrates

64.1 A Dramatic Increase in Incidence of Chronic Diseases

The burden of chronic diseases is steadily increasing all around the world and is forecasted to continue to do so for at least another 50 years. While the increase in chronic diseases in Western countries, although accelerating in the last 50 years, had been ongoing for more than 100 years, it is mainly in the period after the last world war that more pronounced increases in incidences of chronic diseases have occurred, especially outside the Western hemisphere. Pronounced increases are, for example, reported from Japan from the period of the first 50 years after the last world war (1948–1998) – dramatic increases in incidences of diseases such as breast, ovarian, prostatic, and testicular, much in parallel to the Japanese population adapting Western food habits, to a large extent complementing or eventually also replacing traditional horticultural and aqua-cultural foods with processed Western-type agricultural foods. During these 50 years, for example, prostatic cancer in Japan increased by no less than 25 times, much in parallel to an increase in intake of egg 7 times, meat 11 times, and dairy products 20 times [1,2]. Similar, although slightly delayed, developments are also reported both from other continents and from neighboring Asian countries to Japan [3]. Seemingly this epidemic dominated by obesity and associated diseases has its epicenter in the southern United States,[1] with states such as Alabama, Louisiana, and Mississippi having the highest incidence of obesity and chronic diseases in the United States

1 http://www.cdc.gov/obesity/data/trends.html and http://www.cdc.gov/diabetes/data/trends.html.

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and the world. These diseases are rapidly spreading across the world – to the west to New Zeeland and Australia, to the north to Canada, to the west to Western Europe and the Arab world, and to the south, particularly Brazil, and Mexico.

64.2 The Epidemic Forecasted to Continue – Also in the Western World

Recent studies forecast that by 2050 at least a doubling has occurred in incidence of diabetes [4] and a tripling in incidence of attention deficit hyperactivity disorder (ADHD), Alzheimer disease [4], and cancer [5] in most countries, including both the United Kingdom and the United States, already heavily burdened by obesity and chronic diseases. A most interesting recently published study looked at the future development of disease pattern in the United States and the United Kingdom, two countries together representing approximately 5% of the world's population and two countries that already have the highest rates of obesity and chronic diseases. The study suggest that these countries combined will, by 2030, see another 76 mill obese adults, additional 6–8.5 mill cases of diabetes, 6–7 mill cases of cardiovascular disease, 492 000–669 000 new cases of cancer, leading to loss of 26–55 mill quality-adjusted life years, and a dramatic increase in costs of care – calculated to be $50–68 billion/year [6]. Other rather recent studies suggest that the increase will continue beyond 2030, if dramatic preventive measures are not instituted. While predicting future disease, especially cancer, might be fraught with uncertainty; predictions are necessary aids to health planners and others and must be done. The general experience is that the statistical models have, over the years, been refined and today these models are capable of providing accurate predictions.

64.3 Developing Countries Soon to Take Over the "Yellow Jersey"

During the last two to three decades, noncommunicable diseases (NCD) have taken over as the leading cause of death in countries such as Mexico, Brazil, Saudi Arabia, and India. Today the same development is seen in sub-Saharan Africa. It is promising that the rates of cardiovascular disease (CVD) including coronary heart disease (CHD) and stroke, have in recent years been declining in some industrialized countries, including the United States and the United Kingdom, but instead they are fast rising in most developing countries. For example the rate of mortality in stroke in urban Tanzania is, much in contrast to rural Tanzania, reported to be threefold higher than in the United Kingdom [7,8]. Recent studies suggest that 75% of the world's hypertensive population are to be found in developing countries [9] – a severe condition, which 40 years ago did not almost exist in developing countries [10]. Today more than one-third of adults living in Africa are hypertensive [11].

64.4 Hypertension Associated with Dysbiosis

Lifestyle-induced hypertension is today often accompanied by obesity and deranged sugar and fat metabolism and is today close to smoking, the leading cause of death on the Earth. Lifestyle-induced diseases are together responsible for the largest number of deaths in the United States [12] as probably in most other countries around the world, especially and certainly so in most developing countries. Increasing evidence suggests that for example hypertension is accompanied by a chronic low-grade chronic inflammation that perpetuates the hypertensive state and contributes to the often observed end-organ damage [1,13,14].

Hypertension, like most other chronic disorders, is often preceeded by and always accompanied by malfunctioning intestinal microflora – a condition named dysbiosis. A recent experimental study reports significant decrease in microbial richness, diversity, and evenness in rats with spontaneous and induced hypertension [15]. Similar observations are done in a small cohort of human hypertensive patients (n = 10), who compared to control patients (n = 7) demonstrate a much reduced richness and diversity of microflora, characterized by significant increase in the ratio of Firmicutes/Bacteroidetes but also a significant decrease in acetate- and butyrate-producing bacteria [15], important to prevent a leaky gut.

64.5 Inflammation – A Mother of Disease

A few years after having achieved the status of Professor Emeritus, I felt a strong desire to better understand the pathogenic mechanisms underpinning various chronic diseases, the incidence of each having increased significantly during my lifetime years. I did spend endless hours during almost one year to study and try to understand the pathogenesis of various diseases from ADHD, allergy and Alzheimer's disease to Zollinger–Ellison syndrome and Zuska's disease. It became a great surprise to me that the processes leading to the various diseases were almost indistinguishable – the great majority of the chronic diseases demonstrated clear signs of a most often discrete chronic inflammation accompanied by increased activation of proinflammatory cytokines such as Il-6, acute phase reactants such as C-reactive protein (CRP), PAI-1 and increased levels of free fatty acids much similar to what is seen in acute diseases, such as trauma or sepsis. This observation lead me at that time to call the process leading to the various chronic diseases "chronic phase reaction" and to publish a review entitled "Acute and "chronic" phase reaction – a mother of disease" [16,17]. Observations already in those days indicated a strong involvement of the gut and microflora in the process leading to development of chronic inflammation and associated metabolic manifestations. Among the informations early available was the observations that key proinflammatory cytokines are synthesized by enterocytes in response to the damaging endotoxin, to the largest extent produced by the gut microflora [18–20].

64.6 The Gut Harbors the Majority of the Immune System

The intestine is not only a major site for entry of pathogens, it is also an organ exposed to an enormous amounts of food antigens. It contains under normal conditions trillions of commensal bacteria and a collective genome at least 150 times larger than our own. Immunoglobulin A plays a major role in neutralization invasive pathogens and maintenance of a strong presence of noninvasive commensal bacteria, is crucial for maintaining good health. Multiple mechanisms are suggested to be involved [21,22]. The Norwegian immunologist Peter Brandtzaeg suggested in 1989 that the gut, in contrast to what was earlier believed the spleen and the lymph nodes, is the major location of "the immune system" – 70–90% of IgA immunocytes (plasma cells and plasma blasts) are found in the enteric mucosa [23].

The intestinal mucosa is densely populated by various recognition cells of which the dendritic cells (DCs) in the lamina propria (LP) are especially and strongly associated with both adaptive and innate immunity. The recognition that dietary nutrients and microbial communities in the intestine influence both mucosal and systemic immune cell development and function and development of immune-mediated disease has in recent years led to a growing interest in the functionality of intestinal recognition cells, particularly in DCs, and an explosion in information in mucosal immunology.

64.7 Strong Association Between Composition of Microbiota and Chronic Diseases

The epidemic of a plethora of chronic inflammatory NCD, all documented to have in common an underlying low-grade inflammation, constitutes a major threat to future development and particularly to the economy of modern Societies. The epidemic include early-onset NCDs, such as allergy, asthma, and some autoimmune diseases, as well as later-onset NCDs, such as CVD, metabolic disease, and neurodegenerative disorders, which all appear to share common environmental risk factors and genetic risk variants – see Reference [24] for further detail. Although the pathways to disease are multifactorial, the lifestyle and eating habits and associated severely impaired gut colonization pattern with decreasing microbial numbers and diversity are clearly central to the observed physiologic, immunologic, and metabolic dysregulations seen in most NCDs. A dysbalance between two main phyla is observed in almost all NCDs, a dysbalance observed between Bacteroidetes, comprising gram-negative bacteria, and Firmicutes, comprising gram-positive bacteria [25]. As Firmicutes and Bacteroidetes account for more than 90% of the bacterial population in the colon [26], alterations within and between these two phyla are most likely of the greatest pathogenic significance. It was originally believed that the composition of the intestinal microbiota was relatively stable from early childhood; however, recent evidence suggests that poor diet can rapidly – within 24 h – cause dysbiosis, and alterations in the composition of the microbiota could fast lead to aberrant and most negative immune responses. Studies in recent years have repeatedly demonstrated a great

sensitivity of microbiota to lifestyle changes, particularly food habits; studies performed on large supply of fat, large supply of sugar, large supply of fat and sugar, fiber-reduced, calorie-restricted, complex carbohydrates, refined sugar, vegetarian diet, high n-6 PUFA, animal milk fat, all demonstrating significant alterations in composition of microflora – for review see [27]. It is of great interest that a dysbalance in favor of gram-positive Firmicutes is associated with a significantly increased extraction of energy [28].

64.8 Paleolithic Forefathers Had a Much Different, Much More Diverse and Richer Microbiota

The microbiota of some ancient remains on the Earth with lifestyle similar to our human ancestors, having lifestyle and food habits, which in most aspects date back thousands if not millions of years are especially precious to investigate. The most representative of such groups was until recently most likely the Hadzas, living in the Rift Valley ecosystem around the shores of Lake Eyasi in north-western Tanzania, who belong to the few last remaining hunting and gathering communities in the world. The Hadza diet consists of wild foods – meat from hunted animals, berries, fruit and seeds from the baobab tree, honey, and tubers.

The Hadzas practice no cultivation or domestication of plants and animals and eat minimal amounts of agricultural products (< 5% of calories) from external sources. They consume 100–150 g of fiber each day, which is about 10 times more than typically Americans and Europeans eat. A detailed study of the microbiota of 27 Hadza, aged 8–70 years, mean age 32 years and compared to 16 urban Italians age 20–40 years, mean age also 32 years, was recently published [29]. Although Firmicutes and Bacteroidetes are the dominant phyla in both Hadza and Italian gut microbiota, Hadza are characterized by a relatively higher abundance of Bacteroidetes and a lower abundance of Firmicutes. The two gut microbiota ecosystems are remarkably different with respect to subdominant phyla (< 10% relative abundance). The microbiotas of the Hadzas are largely enriched in Proteobacteria and Spirochaetes, which are extremely rare in the Italian gut microbiota, while Actinobacteria, an important subdominant component of the Italian gut microbiota, are almost completely absent from the Hadza microbiome. It is important to stress that in addition to those mentioned above many hitherto unclassified genera belonging to Bacteroidetes, Clostridiales, and Ruminococcaceae were observed in the Hadza gut microbiota. Furthermore, it was observed that the Hadza gut microbial ecosystem is profoundly depleted in Bifidobacterium and comprises an unusual arrangement of Clostridiales. This arrangement is defined by a general reduction of well-known butyrate producers, members of the Clostridium clusters IV and XIVa22 and a corresponding increase in unclassified Clostridiales and Ruminococcaceae. Detailed recent information on the health status of Hadza is not available but early studies suggest that the Hadza have low rates of infectious disease, metabolic disease and nutritional deficiencies in comparison to other settled groups in the northern Tanzania and south-eastern Uganda region [30,31].

64.9 Different Microbiota in Infants in Hunting and Gathering Communities

Fecal microbiota of 14 healthy children, aged 1–6 years, from the Mossi ethnic group living in the small village of Boulpon in Burkina Faso (BF) were studied and compared with that of 15 healthy European children (EU) living in the urban area of Florence, Italy [32]. A significant depletion in Firmicutes accompanied by a unique abundance of bacteria from the genus *Prevotella* and *Xylanibacter*, known to contain a set of bacterial genes for cellulose and xylan hydrolysis, was observed in the BF children, but completely lacking in the EU children. A significantly greater production of short-chain fatty acids (SCFAs) was also observed in BF children in comparison to the EU children. The microbiota of the BF children was significantly richer in potentially anti-inflammatory gram-positive bacteria, while the microbiota of the EU children had a much greater component of potentially inflammation-inducing gram-negative bacteria. It was especially observed that Enterobacteriaceae (*Shigella* and *Escherichia*) are significantly underrepresented in BF compared to EU children. As concluded by the authors, gut microbiota seems to have coevolved with the polysaccharide-rich diet of BF individuals, allowing them to maximize energy uptake from fibers but also protecting them from inflammation-induced noninfectious diseases.

The gut microbiota of 44 rural Malawian 6-month-old infants was in another important study compared to that of 31 Finnish infants of the same age [33]. The Finnish children were breast-fed to 68%, the Malawian to 100% (no formula, no complementary food at 6 months of age, just as the World Health Organization (WHO) and other authorities recommend today. The Malawian microbiota comprised Bacteroides-Prevotella (17 vs. 5%) and *Clostridium histolyticum* (4.4 vs. 2.8%; P¼ 0.01), respectively. The species *Bifidobacterium adolescentis*, *Clostridium perfringens*, and *Staphylococcus aureus* were absent in Malawian but not in Finnish infants. Bifidobacteria were dominant at 6 months of age in all of the infants, although to a greater proportions in Malawian (71%) than in Finnish infants (47%). The preponderance of these special bacterial species might reflect the risk of developing allergy; it is well known that allergic infants are more often colonized by *C. difficile* and *S. aureus* and less by bifidobacteria. Higher levels of clostridial species and staphylococci, common in Western microbiota, are known to be associated with obese and overweight infants.

64.10 The Most Robust Microbiota Ever Seen

A most recent report describe the microbiome of a previously unknown group – the Yanomamis [34], a group of villagers living in complete isolation in the Amazon, and as suggested, with no contact with the outside world for an estimated 11 000 years. The Yanomami were originally mountain people, who were first contacted in the mid-1960s and who continue to live a semi-nomadic hunter-gatherer lifestyles in the Amazon jungle in the high Orinoco state of the vast mountainous Yanomami territory, Venezuela, uninfluenced by anybody from

the outside world. These villagers are observed to eat wild bananas and seasonal fruits, plantain, palm hearts, and cassava. Their hunting for food focuses mostly on birds and small mammals but also small crabs, frogs, small fish from nearby streams, and also, although very occasionally, peccary, monkey, and tapir. Samples of the microbiota of oral cavity, forearm skin, and feces were recently obtained from 34 of 54 villagers at the time of the first medical expedition in 2009 to this isolated and previously uncharted village.

The microbiotas of the villagers were analyzed and reported to have the highest diversity ever reported in humans (~40% more in Westerners), but also when compared to the rural Africans discussed above [34] – an important observation indicating that a large proportion of important ingredients of our early microbiota have become extinct during the process of Westernization. The Yanomamis harbor high amounts of *Prevotella*, *Helicobacter*, *Oxalobacter*, and *Spirochaeta* but also high levels of parasites that are absent or significantly reduced in industrialized humans. A most unexpected finding was that the gut and oral bacteria also contained genes coding for antibiotic resistance. Though these genes were inactive, they confer resistance, not only to natural antibiotics found in the soil but, and most likely, also to other chemicals including pharmaceuticals. The observations support the assumption that a large proportion of Western diseases, rare in rural Africa and South America, are results of lifestyle and profound diet changes. The medical workers reported that villagers were healthy and did not suffer from autoimmune disorders, diabetes, or high blood pressure.

64.11 Rural Lifestyle Prevents Western Diseases

The British physician Dennis Burkett should be credited for alerting the world to the need of a much higher intake of fiber to maintain health. He reported some 40 years ago a strong association between low-fiber content in the diet and a significantly higher risk of colorectal cancer, but also of other diseases such as CHD and diabetes, all based on his observations in Uganda, Africa. He reported that the shortage in plant fibers in Western diet was associated with poor gastroenteral function – both a 10 times reduction in stool weight (United Kingdom 60 g/day, Uganda 600 g/day) and a 5 times increase in transit time (United Kingdom: 100 h., Uganda: 20 h) in Western Societies (United Kingdom) in comparison to what was observed in Uganda [35–39]. The lack of fibers in diet was at that time reported especially low in special groups of the Western Societies and especially so in the foods served at hospitals – a shocking transit time of more than 14 days observed and reported in half of the investigated British geriatric patients [40].

Studies in other ancient remains of humans of the world, such as Abkhazia, Vilcabamba och especially Hunza – all living almost totally on nonprocessed plant food: Hunza och Vilcabamba 99% and Abkhazia on 90% provide most interesting results. They seem to harvest energy better than Westerners as they despite hard work seem to live and maintain weight on only 17–1800 calories, obtained from 100% nonprocessed plant foods. Obesity is said to never be seen, the incidence of disease is remarkably low and the numbers of centenarians are greater than observed in most places on the Earth – for review, see Reference [41].

64.12 Endotoxin (LPS) – A Major Inducer of Inflammation and Disease

The most obvious and harmful consequences of reduced microbiota, dysbiosis, are high levels of endotoxin in plasma (endotoxemia), which both in experimental and clinical studies are strongly associated with inflammation and risk of obesity and almost all chronic diseases. Endotoxins are integral components of the outer membrane of gram-negative bacteria such as *Enterobacteriaceae* and *Pseudomonadaceae*, and essentially composed of proteins and lipids, in addition to the toxic and inflammation-inducing lipopolysaccharides (LPS). LPS is known to have exceptionally strong ability to induce inflammation via the so-called toll-like receptors 2 (TLR2) and 4 (TLR4). Volunteers living for 1 month on a Western-style diet demonstrated, in a crossover study, a 71% increase in plasma levels of endotoxin activity (endotoxemia) when compared to those consuming, what the authors called, and a "prudent-style diet," who, in turn, demonstrate a 31% reduced level of endotoxin [42]. A positive correlation between sedentary lifestyle and higher levels of endotoxin levels, and a negative correlation to the degree of physical exercise is reported [43]. High-fat-content foods, rather than high-carbohydrate-content foods, correlate with high levels of endotoxemia. Mice fed with a high-energy diet (either high-fat diet or high-carbohydrate diet) demonstrate a significant increase in plasma LPS; however, again, a high-fat diet contributes more than a high-carbohydrate diet to a high endotoxin level [44].

64.13 Meals Rich in Long-Chain Fatty Acids Induce Endotoxemia

Fat in foods influences negatively microflora and its replication, but the most pronounced negative effects are observed with high consumption of animal-origin fat, which facilitates translocation of LPS – the chylomicrons serving as vehicle for translocation of endotoxin, through the mucosa and into the circulation, a process referred to as transcellular transportation. This process facilitates higher concentrations and a long-lasting presence of both long-chain fatty acids and endotoxin in the general circulation – see further below. Strong correlations between plasma levels of endotoxin and numbers of parameters of metabolic syndrome as well as between persistent levels of high endotoxin/plasma and "prospect of life" have been observed – especially large differences being reported between the first and fourth quartiles [42]. Among the diseases associated with increased presence of fat and endotoxin in plasma are particularly; Alzheimer's disease [45,46] and cognitive impairment [47], arteriocoronary disease [47,48] and stroke [49], diabetes 1 [50,51], diabetes 2 [52], cancer [52,53], but also allergy [54], ALS [55], autism [56], autoimmune diseases [57], bipolar disease [58], chronic fatigue syndrome [59], chronic obstructive pulmonary disease (COPD [60], minimal encephalopathy [61,62], fibromyalgia [63], HIV [64], liver cirrhosis [62,65], macular degeneration [66], nephropathies [67], obesity [68,69], osteoarthritis [70], paradontosis [71], Parkinson's disease [72,73], rheumatoid

diseases [74,75], psychiatric and cognitive disorders [47,76,77], stress [78,79], and uveitis [80].

64.14 Humans Are Especially Sensitive to Endotoxin

Humans are known to be extremely sensitive to endotoxin exposure and reported to show signs of inflammation at extremely low doses of LPS, reported to be at least 250-fold lower than what is required, for example, in mice [81]. This is especially important to remember as modern humans, much more than other primates, as an unfortunate consequence of modern living, are exposed to high amounts of LPS both at outdoor and indoor living. Tobacco smoking is a major source of LPS but also dust from home is rich in endotoxin, often occurring at home, workplace and at school. Industries such as agriculture, textile, and wood industries are especially recognized as bad environment with often extremely high levels of endotoxin exposure.

64.15 Fresh Fruits and Vegetables Contain Minimal Levels of Stimulants of Toll-Like Receptors (TLRs)

The fact that the food we eat often contains unacceptably high levels of endotoxin is often neglected. Cooking the food makes little difference as LPS is heat-resistant and both LPS and dead bacteria maintain their ability to induce inflammation. The majority of fresh and raw whole vegetables should normally contain only minimal or undetectable levels of stimulants of TLR2 or TLR4 [82]. However, certain raw and minimally processed vegetables (MPVs) are very sensitive to storage and might occasionally contain considerable quantities of bacteria and endotoxins; especially bean sprouts, diced onions, and chopped root vegetables such as carrots and onions [82]. Beef, pork and turkey increase within a few days of storage their content of TLR2- and TLR4-stimulants remarkably, even when stored at below 5 °C, especially when exposed to air [83]. The accumulation of TLR2- and TLR4-stimulants is minimized by storage of meat in intact rather than minced forms, and when stored under a modified atmosphere, rather than exposed to air [83]. Very little data exists about the health hazards of game meat, which despite its favorable nutritional profile, compared to farmed meat, is often kept hanging for weeks and, as a consequence, is especially rich in bacteria and endotoxins.

64.16 Several Proteotoxins Stimulate Also Toll-Like Receptors (TLR) and Induce/Enhance Inflammation

Different food ingredients and particularly proteins may enhance the inflammatory properties of human diets. Many peptides and proteins, to which modern humans are daily exposed, possess the ability to induce inflammation and activate

transforming growth factor-β (TGF-β) and TLRs. Among these are various lectins, especially glutenoids, and also caseins. Novel molecules, induced by heating of food or longer storage at room temperature, are collectively called Maillard products and consist in so-called "advanced glycation end products" (AGEs) and "advanced lipoxidation end products" (ALEs). The "invention of fire" in Stone Age increased dramatically the possibilities for proinflammatory food products to be produced, and the introduction of gluten-containing grains, which occurred about 10 000 years ago with the advent of agriculture, did further increase human exposure to dysfunctioning proteotoxins – proinflammatory molecules – developments, which might be regarded as unfortunate "mistakes of evolution."

The main reasons for the large exposures during human history to glutenoids might have been that it was mainly the members of the *Triticeae* grass tribe (wheat, rye, barley) and the *Pooideae* subfamily (including even oats) that grew well at higher latitudes. Modern plant breeding technology has made it worse as modern bread contains 15–20 times more gluten, compared to bread produced in the past. An unexpected consequence of high consumption of wheat, rye, and barley, is that modern will suffer from a series of highly unwanted human disorders that relate to exposure to glutenoids, particularly gluten (wheat), but also to secalins (rye) and hordeins (barley), which all have strong ability to induce inflammation and increase intestinal permeability [84]. Oats is distantly related to wheat, rye and barley, but its active peptides, the avenins, are rarely reported to give stronger reactions, either inflammation or allergy [85].

64.17 Gluten-Sensitivity a Common and Newly Detected Inflammation-Inducing Disorder

It has become increasingly apparent that "classic" celiac disease (CD) represents only "the tip of the iceberg" of an overall large glutenoid-associated disease burden. We are increasingly aware that, besides those rather few, who suffer CD and classic wheat allergy, most individuals, if not all, also suffer very discrete, but always negative reactions to glutenoids. Despite the fact that few direct and immediate allergic reactions or autoimmune mechanisms are involved with exposure to wheat, rye, or barley, consumption of these foods will almost always or always add to the general burden of inflammation in the body. A rather recently made observation has lead to recognition and acceptance of a new disorder called gluten sensitivity (GS) [86]. Although LA-DQ8 is present in almost all CD patients, these genes are only present in about half of patients with GS. Some of the individuals with GS may have no symptoms at all, others will suffer well-defined chronic diseases, and the majority "only" more ill-defined distresses; fatigue, depression, encephalopathy/"foggy mind," lack of energy, diffuse abdominal pain, bloating, diarrhea, eczema and/or rash, undefined headache, numbness in the legs, arms or fingers, joint pain and many other manifestations. More or less all affected individuals report that, when turning to gluten-free diets, increased well-being and frequently also improved clinical signs and symptoms will occur.

64.18 Gluten-Free Foods Have Led to a New and Successful Industry

Experiences such as these have made the gluten-free diet the number one health trend in the world,[2] growing faster than both low carbohydrate diets and "fat-free" diets, and rapidly fueling the gluten-free product market, which has grown in the United States with 44% in less than 3 years, from 2011 to 2013, and is expected to continue to grow further – the US market from $10.5 to $15.6 billion, and the UK market from $395 to $931 million by 2017. A parallel development is also observed in the growth of alternative milk – the global growth of soy, oat, almond, and so on based alternative milks constitute seemingly around 6% per year of the combined milk and alternative milk products.[3]

A fast increase in alternative sources of flours is also observed. Among the alternatives flours for bread-baking are ancient grains, several of them known to grow particularly, if not exclusively, well in Africa; amaranth, arrowroot, brown rice, buckwheat, chia, chickpea, corn, hemp, maize, millet, oat, potato, quinoa, sesame, sorghum, soya, tapioca, teff, and white rice, of which sorghum is the fifth most common grain in world and especially attractive due to its extremely high content of antioxidants, low content of energy [87], and ability to resist heat-induced protein glycation [88,89], but also due to its high versatility and cost-effectiveness.

64.19 Glutenoids Show Endotoxin-Mimicking Abilities

Glutenoids, which demonstrate endotoxin-mimicking abilities, are capable of lowering the threshold for immune responses, attracting leukocytes and increasing their reactive state, in a manner similar to that of endotoxin. Nearly 10 μg/mL of wheat gluten induces the equivalent effects of 1 ng/ml endotoxin LPS [90], increasing DC maturation as well as chemokine secretion [90]. A study investigating fecal samples from 76 symptom-free, nonceliac, first-degree CD relatives and compared to samples from 91 aged-matched healthy controls reported significantly lower level of acetic acid and other SCFAs) in parallel to significantly increased levels of *i*-butyric acid and fecal tryptic activity in the asymptomatic CD-relatives [91].

The information that removal of gluten from the diet of the nonobese diabetic mouse could attenuate the intensity of autoimmunity and reduce the incidence of diabetes led to a cross-over study, where 17 first-degree relatives were kept on a gluten-free diet for the first 6 months followed by another 6 months on a standard gluten-containing diet [92]. The acute insulin response to intravenous glucose tolerance test rose significantly in 12/14 patients after the first 6 months of gluten-free diet when complying with the diet ($P = 0.04$) and decreased again in 10/13 during the following 6-month period on a standard gluten-containing diet ($P = 0.07$) [92].

2 https://www.glutenfree.com/#/articles/gluten-free-a-global-perspective.
3 http://blog.euromonitor.com/2014/09/whitewaves-acquisition-of-so-delicious-dairy-free-should-cement-its-lead-in-non-dairy-milk-alternati.html.

64.20 Similar Effects Observed on Gluten Restriction in Individuals with ADHD

A similar outcome is reported from a crossover study in which 100 individuals suffering ADHD, aged 4–8 years, who were randomly assigned to 5 weeks on a restricted elimination diet (not planned as but in fact a gluten-restricted diet) or to what was referred to as a healthy diet, followed by another 5 weeks on the alternative diet. All parameters' total score, inattention, hyperactivity, and abbreviated Connor scale scores (ACS) improved significantly on the restricted diet but deteriorated again during the subsequent period on a "normal," although supposedly healthy diet [93]. Another study focused on thirty-four individuals with irritable bowel syndrome, 56% of them with human leukocyte antigen (HLA)-DQ2 and/or HLA-DQ8 genes, during 6 weeks on a gluten-free diet [94]. Statistically significant improvements, compared to controls, were reported in the gluten-free group in 3/6 parameters studied: abdominal pain ($P = 0.02$), satisfaction with stool consistency ($P = 0.03$), and tiredness ($P = 0.001$), but no improvement being observed in overall symptoms ($P = 0.15$), wind ($P = 0.08$), and nausea ($P = 0.69$), and no differences observed between individuals with or without DQ2/DQ8 genes [94]. Another rather recent study reported disappearance of signs of malabsorption and intractable therapy-resistant seizures in three young girls (ages 18, 19, 23) when placed on gluten-free diet [95]. Similarly, individuals with symptoms of neuropsychiatric diseases; Alzheimer's disease/cognitive decline [96], autism [97], and schizophrenia [98] are reported in the literature as symptom-free on a gluten-free diet.

64.21 Gluten Sensitivity Associated with Severe Dysbiosis, Leaky Gut and Increased General Inflammation

Typical symptoms of nonceliac GS are fatigue, sleep disturbances (insomnia and/or nonrestorative sleep), morning stiffness and cognitive problems, but also gastrointestinal symptoms are frequent – a clinical picture almost identical to fibromyalgia, 50–70% of patients with fibromyalgia are reported to suffer nonspecific gastrointestinal symptoms such as abdominal distension (59%), dyspepsia (50%), nausea (21%), alternating diarrhea-constipation (63%), constipation (12%), and diarrhea (9%) [99].

All gluten-sensitive diseases as mentioned above are strongly associated with severe dysbiosis, leaky gut and permanent general chronic inflammation. A recent study was aimed to clarify how cultured peripheral blood mononucleated cells (PBMC) obtained from nonceliac gluten-sensitive (NCGS) patients responded to contact with wheat proteins. Wheat protein was observed to induce an overactivation of the proinflammatory chemokine CXCL10 and decrease the transepithelial resistance of monolayers of normal colonocytes by diminishing the mRNA expression of cadherin-1 (CDH1) and tight junction protein 2 (TJP2), two primary components of the tight junction strands. Furthermore, the induction of

inflammation was greater with proteins extracted from modern compared to when extracted from ancient wheat genotypes [100].

64.22 Proteotoxin-Induced Low Threshold for Immune Response

Inclusion of industrially made foods ingredients and particularly those of protein nature might both enhance and diminish the inflammatory properties of the diet. This is also true for various lectins and frequently observed with various bovine milk-derived proteins, and particularly with powdered milk. The synthesis in the brain of serotonin (5HT) and melatonin is dependent on access to their precursor amino acid, the essential amino acid L-tryptophan (TRP), normally released in the gut by microbial fermentation of plants rich in this amino acid [101]. As it is believed that some food proteins might block release of some amino acids such as L-tryptophan, an animal study was undertaken to compare the effects of five different such proteins: zein (corn), gluten (wheat), soy protein isolate, casein, lactalbumin or no protein. An eightfold variation was observed in levels of cortex tryptophan: a marked decline followed zein ingestion, slightly more modest reductions were observed after casein or gluten application, all paralleled by reductions in cortical and hippocampal hypothalamic 5-hydroxytryptophan (5HTP) [101].

Fermented milk such as Kefir contains more than 250 peptides, mainly released from β-casein, of which several have significant biological effects: ACE-inhibitory, antioxidant, antithrombotic, mineral binding, antimicrobial, immunomodulating, and opioid activity [102]. It is most likely that the same peptides are released during microbial fermentation in the gut, which might explain why intake of various dairy products are associated with dysbiosis, increased general inflammation and also why dairy-free diet often leads to reduced general inflammation and clinical improvements, and also explain the observed association between larger intake of dairy and increased incidence of diseases including Alzheimer's disease and dementia [103–107].

64.23 Heat- and Storage-Induced Inflammation-Inducing Proteins

Heat- and storage-induced dysfunctioning proteins are of special interest, as they are shown to have strong ability to induce inflammation [108,109]. It is thought that certain dietary AGEs are indigestible by human gut enzymes and most likely enter the colon, where they will act as a growth substrate for detrimental bacteria such as some Clostridium and Bacteroides species [110,111]. Therefore, it is most conceivable that individuals who consume highly processed diets (which contain large quantities of AGEs) may adversely alter their colonic microbial composition and potentially enhance the risk for the development of metabolic diseases such as obesity and type 2 diabetes [112].

Among foods rich in AGEs and ALEs are dairy products, especially powdered milk (frequently used in enteral nutrition and baby formulas, as well as in numerous other industrially produced foods), and also especially rich in high-temperature prepared fried and grilled meat and poultry, but also fish (especially deep-fried and oven-fried), drinks including coffee and colas, Asian sauces, such as Chinese soy sauces, balsamic products as well as smoked and cured foods in general [113–115]. Consumption of such foods, often main constituents of fast foods, has increased dramatically in recent decades, much in parallel to the endemic of chronic diseases. Higher levels of AGEs such as methylglyoxal derivatives in serum (sMG) and/or other AGEs are strongly associated with a faster rate of cognitive decline in elderly individuals [116], neurodegenerative diseases [113], premature aging and cognitive decline [114], diabetes type 1 and 2 [115], diabetic nephropathy [116], obesity [117], liver disease, particularly liver steatosis and liver fibrosis [118], lung disease, particularly COPD [119], various cancers especially breast cancer [120], colorectal cancer [121], esophageal [122], gastric [123], lung [122], ovarian [122], pancreatic [124], prostatic [125], renal [126], and leukemia [127].

64.24 Numerous Proinflammatory Mediators Involved

A wide range of proinflammatory mediators, including TNF-α, IL-1b, IL-6, IL-8, and the nuclear protein high mobility group box-1 (HMGB1), are implicated in the pathogenesis of the above-mentioned chronic diseases. HMGB1 is one of the important mediators known to signal effects of the AGEs, particularly RAGEs, and through the TLR2 and TLR4. Activation of these receptors will ultimately result in activation of NF-κB, known to induce up-regulation of leukocyte adhesion molecules and production of proinflammatory cytokines and angiogenic factors in both hematopoietic and endothelial cells, thereby promoting inflammation [128]. A recent review suggests that particularly HMGB1, TLR and RAGE constitute a functional tripod with high ability to promote inflammation [129].

64.25 Short-Chain Fatty Acids – A Magic Bullet?

The gut microbiota is, as already discussed, the key to gut/immune homeostasis and health to the host. Increasing evidence suggests that this is achieved, at least to a large extent, through the release SCFAs, which are the main microbial metabolites produced by specific colonic anaerobic bacteria in the gut fermentation of dietary fiber and resistant starches. Deficiencies in SCFAs will strongly affect the pathogenesis of a diverse range of diseases, from allergies and asthma to cancers, autoimmune diseases, metabolic diseases, and neurological diseases.

Therapeutic manipulation of the gut microbiota and restoration of normal microbiota (a condition called eubiosis) could potentially reduce the levels of circulating AGE levels and improve the metabolic health of individuals at risk for the development of type 2 diabetes or other metabolic syndrome-associated diseases. Regular consumption of fiber-rich/prebiotic-rich foods to promote the growth of beneficial gut bacterial flora is a most promising possibility. Prebiotics/

nondigestable plant-derived carbohydrates confer health benefits to the host by acting as a fermentation substrate in the colon, stimulating the preferential growth and activity of a limited number of beneficial microbial species [130]. Prebiotic-induced stimulation of gut fermentation increases the levels of intestinal Bifidobacterium species, known to attenuate the production of ROS and markers of inflammation in individuals consuming high fat diets [131]. Supplementation of the human diet with prebiotic fructans such as inulin or fructo-oligosaccharides alters the bacterial composition of the large intestine and favors a selective proliferation of beneficial lactic acid-producing species such as bifidobacteria and lactobacilli.

64.26 Consumption of Plant Fibers Crucial to Eubiosis and Good Health

It is increasingly understood that different fibers have different impacts on microbiota composition and metabolism. When the effects on human fecal microbiota of six dietary fibers: pectin, guar gum, inulin, arabinoxylan, b-glycan, and resistant starch was investigated, pectin was found to induce increases in Actinobacteria (more specifically bifidobacteria), guar gum increased levels of Bacteroidetes and reductions in Firmicutes and Proteobacteria, resistant starch increased levels Bifidobacterium adolescentis type-2 and Blautia wexlerae, while inulin increased Actinobacteria [132]. Supplementations of guar gum and inulin resulted in the largest production of butyrate [132]. Apple pectin is reported to be an effective apple component improving the fecal Environment – stimulating growth of Bifidobacterium, Lactobacillus, Enterococcus, and the Bacteroides fragilis group but not microbes such as *Escherichia coli*, *Collinsela aerofaciense*, *Eubacterium limosum*, and *C. perfringens* [133].

Fibers such as pectin and guar gum are known to inhibit weight gain, adiposity, fatty liver, and high blood glucose levels in experimental animals fed a high-fat diet. A recent study demonstrated significantly increased levels of butyric acid in cecum and blood after supply of guar gum with medium and high viscosity as well as of high methoxylated pectin [134]. These fibers reduce to varying extent levels of blood cholesterol, liver steatosis, and blood glucose levels – much depending on the degree of methoxylation and viscosity of the particular fiber. The medium viscosity guar gum was the most effective fiber to prevent a diet-induced hyperlipidemia and liver steatosis. Guar gum is shown to have distinct bifidogenic effects independent of viscosity, and increases the cecal abundance of bifidobacterium about 10-fold [134].

64.27 Prebiotic Fibers Generally Resistant to Digestion in the Small Intestine

It is characteristic of prebiotic fibers that they, to the greatest extent, are resistant to digestion in the human small intestine and that they consequently will largely

pass intact into the colon, where they increase viscosity and bulking of the fecal matters. It is preferably in the proximal half of the large intestine that the dietary fibers undergo fermentation by the resident anaerobic colonic microbiota and produce SCFAs. SCFAs consist in a subset of saturated fatty acids containing six or less carbon molecules that include formic acid(C1), acetate (C2), propionate (C3), butyrate (C4), pentanoic (valeric) acid (C5), and hexanoic (caproic) acid (C6). Recent advances in the information about SCFAs, favorably acetate, propionate, and butyrate, highlight their much important effects on metabolism at both cellular and molecular levels.

Butyrate is mainly utilized by colonic mucosa, while acetate and propionate reach the liver solely through the portal vein. Propionate is fastly metabolized by hepatocytes, while acetate will either remain in the liver or be released to the peripheral venous system and tissues. Of the SCFAs only acetate can be detected as circulating in the body.

64.28 It Is the Luminal Levels of SCFAs, Which Make the Difference

A significant association exists between composition of the microbiota and luminal levels of SCFAs. Higher luminal concentrations and lower colonic pH are observed in proximal colon where fermentation is highest (pH = 5.5–6.5), compared to the distal colon with its significantly higher pH (pH = 6.5–7.0). SCFAs, especially butyrate – the primary energy source for the colonic mucosa – is credited for its unique and important ability to inhibit growth of ptotential pathogens, gram-negative Enterobacteriaceae, especially pathogens such as *Salmonella* spp. and *E. coli* [135,136].

Diets containing low amounts of dietary fibers are strongly associated with distinct long-term negative effects on composition of the intestinal microbiota and production of SCFAs. All SCFAs have significant anti-inflammatory and antiproliferative properties. Consumption of a diet rich in meat and low in fiber promotes proteolytic fermentation and production of uremic compounds and branched-chain fatty acids, which all are proinflammatory and increase the risk of development of disease, particularly cancer.

64.29 Not only SCFAs but MCFAs Have Strong Ability to improve Immunity and Prevent Disease

The documented strong ability of SCFAs to control inflammation, reduce excessive production of radical oxygen species and mitochondrial dysfunction [137], and to regulate immune functions particularly regulating T-cell functions [138] should offer hope for various therapeutic applications, not only by SCFAs (1–6 carbon atoms) but also by medium-chain fatty acids (MCFAs) (6–12 carbon atoms), which are more easy to administer. However, attempts to infuse various SCFAs and MCFAs have not been met with the success,

which was primarily expected. The most significant benefits obtained this far were obtained by direct application of bioactive fibers – administer to facilitate derived production of SCFAs at the mucosal surface. However, intake of plant fibers is not always possible, especially in critically ill, which somewhat limits the applicability of this otherwise successful treatment alternative.

64.30 Stress Increases Microbial Growth and Virulence

It is well documented that the immune system and central nervous system are involved in cross-talk to maintain homeostasis under normal and disease conditions. Acute and chronic stress induces release of a variety of hormones, neurochemicals, and neuropeptides, which directly or indirectly lead to severe impairment of immune functions [139]. Nearly all types of cells, especially immune cell possess receptors for the stress-related neurohormones adrenaline and noradrenaline. Sympathetic nerve fibers do also extensively innervate "immune"/lymphoid organs, such as the bone marrow, thymus, spleen and lymph nodes, and terminate in close proximity to lymphocytes [139]. The large intestine has by far the greatest number of nerve fibers when compared to other organ in the body – the total number of enteric neurones estimated to be at least 500 million [140]. Stress hormones such as norepinephrine and dopamine are shown to dramatically increase the virulence of pathogenic hormones [141]. Noradrenaline, adrenaline, dopamine, and the synthetic catecholamine inotropes dobutamine and isoprenaline are all able to increase virulence and microbial growth by up to 100 000-fold over controls [142].

64.31 Early-Life Conditions Influence Long-Term Health

Early-life conditions play important role in the establishment of microbiota and immune functions, which often influences stress reactions: allergic and immunological response neurodevelopmental later in life. Babies born via cesarean section are born by mothers with high levels of stress. Meta-analyses suggest a 20% increase in incidence of asthma among babies born with cesarean section [143]. Cesarean section, especially unplanned cesarean section, is perceived as severely stressful [144,145] with significantly increased glucorticoid and catecholamine neuroendocrine hormone levels [146,147]. Breast-fed babies, fed by a different and unique prebiotic human milk fibers, oligosaccharides [148], acquire maternal intestinal bacteria from breast milk [149] to digest the fibers and have during this period a much different, more "beneficial" gut microbiota [150,151]. Maternal separation, a common early-life stressor, has been shown to cause a significant decrease in fecal lactobacilli on day 3 post separation, returning to normal by day 7 [152]. It is suggested that early-life stress might have long-term effects on the microbiome [153]. Analysis of the 16 S rRNA diversity in adult rats exposed to maternal separation for 3 h/day from post-natal days 2–12 reveal a significant and permanently altered fecal microbiome, when compared to nonseparated control animals [154].

64.32 Psychological Stress Impairs Microbiota

Psychological stress has long been known, both clinically and experimentally, to cause significant bowel problems. In a study published in 1976 the fecal contents of three astronauts were examined during a 5-month period, when housed under significantly stressful conditions isolated in Skylab [155]. Bacteroides fragilis subspecies thetaiotaomicron was found to be significantly increased in all three astronauts investigated. A slightly later similar study in astronauts reports a distinct decrease in the numbers of Bifidobacterium and of Lactobacilli and a substantial increase in numbers of the pathogens E. coli and of Enterobacteria during preparation for flight, particularly significant within the period immediately before take-off. The numbers of potentially pathogenous Enterobacteria and Clostridia were substantially increased and numbers of Lactobacilli decreased also after the flight. Other studies at the same time conclude that preparation for flights is associated with about 50% reduction in beneficial bacteria, lactobacilli and Bifidobacteria [156]. A detailed analyses of the microflora of 24 Russian astronauts during preparation for flight performed a few years later reported dramatic reductions in various Lactobacilli and significant increases in potentially pathogens [157]. The composition of intestinal [157], oral [158,159] and nasal [158] microbiota was significantly deranged after spaceflights, changes that remained at least for weeks after spaceflights [160], and most likely also for months if not years. See further a recent review [161]. Another interesting study collected saliva samples and fecal samples from 23 healthy undergraduate students during two separate 1-week periods: during the beginning of semester (low-stress baseline condition) and during the first week of exams (high-stress condition). The density of microbes in feces fell in connection with exams from a baseline level of 6×10^7–1×10^7 and remained at this low level during the following 5 days [162].

64.33 Physical Exercise Increases Microbiota Numbers and Diversity

Numerous studies report that physical activity decreases the risk of many chronic diseases and increases longevity. Current US, UK, Australian, and WHO guidelines recommend that significant health benefits can be obtained through about 150 min of moderate activity or 75 min of vigorous activity per week – see further a recent review [163]. Large meta-analyses of cohort studies have reported an app 30% difference in mortality when comparing groups with the lowest and highest amount of physical activity [164,165]. Both volume and intensity of physical exercise have been shown to exert significant influence on gastrointestinal health status. It is demonstrated that exercise reduces the transient time of foods in the gastrointestinal tract, hereby reducing the prolonged contact of pathogens with the gastrointestinal mucus layer and also with circulatory system [166]. Exercises have been shown to have capacity to change gut microbiota diversity both quantitatively and qualitatively and a significant increase of butyrate concentrations is reported in animals after voluntary running exercise [167]. A recent study

in elite rugby players reports significant increases in gut microbiota richness and diversity after physical exercise [168].

It is clearly demonstrated that the diversity of gut microbiota correlates positively with protein intake and creatine kinase concentrations, suggesting that diet and exercise are drivers of biodiversity in the gut. As expected, athletes and controls differ significantly in levels of plasma creatine kinase (a marker of extreme exercise), as in levels of inflammatory and metabolic markers. Furthermore, athletes are shown to have significantly higher diversity of gut microorganisms, representing 22 distinct phyla, which in turn positively correlate with protein consumption and creatine kinase. These results provide evidence for a beneficial impact on gut microbiota diversity of exercise, but it is most likely also influenced by the better diet, often rich in fibers characteristic to elite athletes. For further information – see recent review [169].

64.34 Exercise Has Positive and Negative Effects on Immunity Depending on Its Intensity

Exercise might though have both positive and negative effects on immune function and also health, much depending on intensity. Regular moderate-intensity exercise enhances immune functions significantly above those typically found in sedentary individuals [170]. These functions include the potentiation of T-cell-mediated immunity, natural killer (NK) cell cytotoxicity, proinflammatory cytokine production, and a stronger Th1 reaction as demonsdtrated both in human and animal models. Very prolonged strenuous bouts of exercise with periods of intensive, very strenuous training and strong competition may, however, impair immune functions and increase susceptibility to acute disorders such as upper respiratory tract infections (URTI) by decreasing saliva secretory immunoglobulin A (S-IgA) secretion, NK-cell activity, and proinflammatory cytokine production. Comparisons were recently made between patients, who reported that they exercised 3–6 h/week (LOW), 7–10 h/week (MED) or 11 or more h/week (HIGH). The HIGH and MED groups had more URTI episodes than the LOW group (2.4 ± 2.8 and 2.6 ± 2.2 vs. 1.0 ± 1.6, respectively: $P < 0.05$) [171]. The HIGH group demonstrated approximately threefold higher levels of interleukin (IL)-2, IL-4 and IL-10 (all $P < 0.05$) in antigen-stimulated whole blood cultures compared to the LOW group while the MED group showed a twofold higher IL-10 levels than the LOW group ($P < 0.05$) [170]. Although to my knowledge no studies exist on alterations in composition of microbiota in immediate connection with strenuous exercise, it is likely that increasing load of exercise is accompanied by increasing exhaustion also of the microbiome.

64.35 Postprandial Inflammation a "Deadly" Threat to Long-Term Health

Postprandial lipemia has emerged as a potential candidate to induce postprandial inflammation. It is well demonstrated that ingestion of a high-fat meal causes

systemic increases in a wide range of inflammatory mediators. Frequent episodes of postprandial inflammation has for years been recognized as a key factor behind the development of arteriosclerosis [172] and various other chronic diseases, especially metabolic syndrome [173], diabetes [174], and hepatosteatosis [171]. Every meal rich in fat is associated with significant derangement of microbiota [27], cascades of markers of inflammatory and oxidative stress [175], particularly tumor necrosis factor-α (TNF-α) [176] and significant endothelial dysfunction [177]. Plasma endotoxin levels were studied in 12 healthy men (20–58 years, mean age: 32 years) after exposure to no meal, three cigarettes, a high-fat meal, or a high-fat meal plus three cigarettes [176]. The high-fat meal with or without cigarettes, but not the no meal or only smoking, reduced significantly ($P < 0.05$) the plasma endotoxin neutralization capacity – an indirect measure of endotoxin exposure The levels of endotoxin/plasma increased with a mean of 50% ($P < 0.05$) and remained significantly elevated for about one and a half-hour, while the triacylglycerol concentrations remained statistically significantly elevated as long as 4 h after the meal [176]. This study provides strong support to the hypothesis of endotoxemia and postprandial inflammation as major contributors to endothelial activation and to the development of atherosclerosis. It is also observed that simultaneous intake of sugars significantly potentiates the postprandial inflammation induced by a high fat meal [177]. The inflammatory responses of the vascular endothelial cells are shown to vary directly and much in parallel to the patient's postprandial serum triglyceride level and waist circumference [178].

64.36 Abdominal/Visceral Obesity Enhances Postprandial Inflammation

Visceral obesity was extremely rare among our forefathers and remained so until only a few decades ago. When anthropometric studies using NMR were performed in 54 otherwise healthy female volunteers with BMI ranging from 19 to 57 kg/m^2, it was demonstrated that visceral fat content can vary from only a few ml in lean individuals to over 6 L in morbidly obese [179]. A strong correlation exists between visceral amount of fat, waist circumference and waist-to-hip ratio [179]. It is of the greatest interest that when in stress, visceral adipocytes will per time unit, compared to subcutaneous fat cells, secrete significantly more of free fatty acids and also about three times as much of proinflammatory factors such IL-6 and PAI-1 per gram tissue, observations that might well explain the high risk of acute disease episodes in individuals with visceral obesity [180]. As a matter of fact, the stress-induced load on the vascular endothelium and particularly those the brain, heart and lungs can be extreme, as proinflammatory and procoagulant molecules can vary up to 1000 times in blood concentrations. Observations such as these provide strong support to what has been called the "portal theory," proposing that the liver when exposed to larger amounts of free fatty acids and proinflammatory factors, released from visceral depots of fat into the portal vein especially in obese patients, promotes development of liver steatosis, hepatic insulin resistance, and enhances the development of metabolic

syndrome, and subsequent development of type 2 diabetes [181]. Dysbiosis and metabolic endotoxemia is strongly associated with obesity and insulin resistance both in mice and humans, supporting the strong linkage between gut microbes, gut barrier function, acute and chronic inflammation, adipose tissue inflammation, endothelial inflammation and dysfunction, diet-induced obesity, chronic inflammation and insulin resistance and various chronic diseases.

64.37 Postprandial Inflammation Are Induced by Long-Chain but Not Medium-Chain Fatty Acids

The metabolism in the body of consumed MCFAs (C6–C12) is fundamentally different from that of long-chain fatty acids (LCFAs) (C12–C21) – observations that have not yet received enough interest. Medium-chain triglycerides (MCT) are, much in contrast to long-chain triglycerides (LCT), much more readily hydrolyzed. MCFAs are known to be absorbed from the small intestine by a more direct and much more rapid pathway, the portal vein than that of LCFAs. Since several decades ago it is well known that MCTs undergo a rapid hydrolysis by the gastric, salivary or pancreatic lipases, most likely due to much better solubility and motility of the MCT lipid droplet. MCFAs are therefore rapidly transported as nonesterified fatty acids into the portal blood stream to reach the liver, while LCFAs after long-lasting transport as chylomicrons via the thoracic duct and after hours remaining in the general circulation are redistributed as LCTs by hepatic lipoproteins to nonhepatic tissues – see References [182,183] and Figure 64.1 for further detail.

The clinical effects of chain length were studied in a most interesting experimental study. Wale Wistar rats were fed isocaloric high-fat diets containing triacylglycerols composed of either MCFAs or LCFA. Insulin sensitivity was

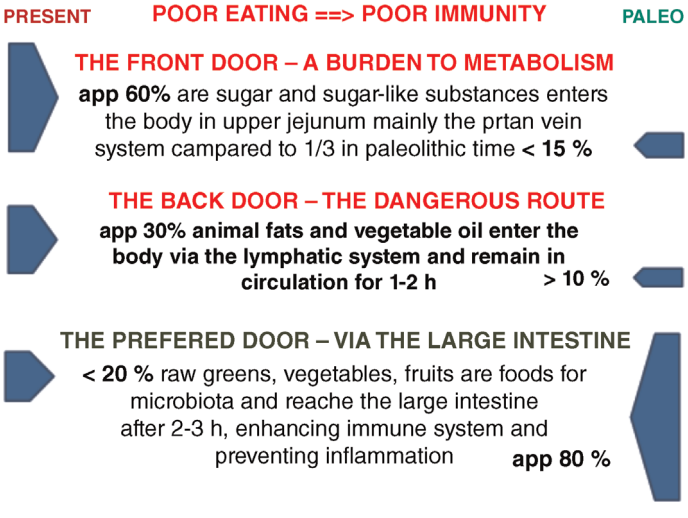


Figure 64.1 Comparing overview of eating habits and immune system.

demonstrated to already after 4 weeks be reduced by 30% in the LCFA group, while it remained nonaffected in the MCFA group [184]. Triacylglycerol concentrations in muscle were higher in both the high-fat groups in comparison to controls. No diet-induced changes were found in acyl-CoA oxidase (ACO) activity (liver and muscle) in the MCFA group, while feeding LCFAs significantly raised carnitine palmitoyltransferase activity. It was concluded that the chain length of saturated fatty acids clearly affect whole-body insulin sensitivity and mitochondrial fatty acid uptake even in absence of obesity. It was also observed that MCT compared to LCT feeding resulted in lowered fasted and post-prandial triglyceride concentrations [184]. Feeding sows with diets containing 15% MCTs resulted in a significantly lower mortality of newborns and promoted development, particularly of underweight piglets [185,186].

64.38 Reduced Intake of LCFAs and Increased Intake of MCFAs Good for Microbiota and Health

Numerous studies have demonstrated significant beneficial impacts of feeding MCFAs on both composition of the intestinal microbiota and inhibitory effects on bacterial concentrations in the digesta, mainly of Salmonella and coliforms. Apart from the specific nutritional and metabolic effects of MCTs and MCFA, their rapid digestion, passive absorption and obligatory oxidation, provide these fatty acids with especially strong immunomodulary effects. While LCTs and LCFAs have meat and dairy as their dominating food source, MCTs and MCFAs are typical plant fats. The content of thesefats is especially high in coconut oil – caprylic acid (C8), 3.2–15% of capric acid (C1), and 41–56% of lauric acid (C12). High contents of caprylic (2.4–6.2%), capric (2.6–7.0%), and lauric acid (41–55%) are also found in palm kernel oil. Cuphea seeds (family of loosestrife) have a broad species-dependent diversity in MCFAs. Oils from Cuphea seeds are known for their extraordinarily high content of MCFAs; Cuphea lanceolata and Cuphea ignea oils containing over 80% capric acid and are often used for nutrition of domestic animals, particularly piglets [186].

This far too few studies are undertaken in humans. One study reports impressive effects in patients with severe hypertriglyceridemia after 7-day treatment with a formula diet rich in omega-3 fatty acids and medium-chain triglycerides: plasma triglycerides decreased from a mean of 1601–554 mg/dL ($P < 0.05$), total cholesterol levels reduced from 417 to 287 mg/dL ($P < 0.001$), and slight decreases also observed in fasting glucose (−8%) and uric acid levels (−12%) [187]. Significant improvements of supplementation of MCTs och MCFAs are also reported in conditions such as:

- Obesity with significantly reduced body weight, waist line and insulin resistance [188,189]
- Mild to moderate dementia in Alzheimer [190] – improved cognition without adverse effects, and improvements in adrenergic or symptomatic responses to hypoglycemic
- Type 1 diabetic patients [191]

- Premature aging and arteriosclerosis [192]
- Inherited cardiomyopathy [193,194]
- Chronic pancreatitis [195]

64.39 Gut Reconditioning – An Important Tool to Maintain Optimal Health

Supplementing live bioactive bacteria constitutes in addition to eating large amounts of raw plant fibers a powerful instrument to control inflammation and protect health. It is important to stress that only a small minority of bacteria sold as probiotics on the market have any significant probiotic clinical effects, even if they sopmetimes carry the same name as one demonstrated to be effective. Only a few products on the market have done proper preclinical and clinical studies – the three leading being, in addition to my own synbiotic product, Synbiotic 2000 – see below – are Lactobacillus GG (Valio Ldt), Lactobacillus reuteri (Biogaia Ltd), and VSL 3 (Sigma-Tau Ltd.). Exciting results are obtained when probiotics, which are properly tested preclinically; *in vitro*, in experimental animals are tried in controlled clinical trials. About two dozens human controlled studies, most of them published in the last few months are presented below. They demonstrate statistically significant and impressive clinical effects: improved gut colonization and immune system [196], reduced allergic manifestations [197], reduced risk of ADHD [198], reduced disease activity in rheumatoid arthritis [199], reduced clinical symptoms and improved quality of life in colorectal cancer survivors [200], reduced diarrhea in children with tropical diarrhea [201], clinical improvement in full-term infants with critical illness [202], improved safety and clinical effects in very low-birth-weight infants (VLBW) [203], reduced translocation and inflammation in HIV patients [204], reduced insulin sensitivity, blood lipids, and inflammation in metabolic syndrome [205], relief of symptoms in irritable bowel syndrome (IBS) [206,207], improved eradication of *Helicobacter pylori* when used as supplement to antibiotic therapy [208], reduced inflammation in CD [209], reduced negative effects when exposed to hepatotoxins and carcinogens [210], reduction in fatty infiltration and inflammation in non-alcoholic fatty liver disease (NAFLD) [211], reduced accumulation of urea in chronic kidney disease [212], reduced inflammation in dialysis patients [213], increased reduction of uremic waist products in patients undergoing renal dialysis [214], reduced number of days with URTIs [215,216], reduced degree of inflammation in cystic fibrosis (CF) [217], and prevention of toxic effects of environmental toxic metal exposure [218] – just to mention a few of very many nteresting studies presented in the litterature.

Here follows brief summaries of these selected studies.

64.39.1 Allergies

Improved gut colonization and development of the immune system – 241 mother-infant pairs with history of allergic disease and atopic sensitization were randomly

assigned to receive either (1) *Lactobacillus rhamnosus* LPR and *Bifidobacterium longum* BL999 (LPR + BL999), (2) *L. paracasei* ST11 and *B. longum* BL999 (ST11 + BL999), or (3) placebo, beginning 2 months before delivery and continuing during the first 2 months of breast-feeding [216]. Altogether 205 infants completed the follow-up up to 24 months and were included in the analyses. The risk of developing eczema during the first 24 months of life was significantly reduced in infants of mothers who received LPR + BL999 ($P < 0.001$) and ST11 + BL999 ($P < 0.001$), the odds ratios (ORs) for chronically persistent eczema described to be 0.30 ($P = 0.016$) and 0.17 ($P = 0.003$), respectively [196].

Reduced allergic manifestations –48 patients were randomized to receive for 12 weeks a combination of two probiotics: *Lactobacillus salivarius* LS01 and *Bifidobacterium breve* BR03 or maltodextrin as placebo and studied at baseline, at the end of therapy, and 2 months later [197]. The patients receiving probiotics showed a significant improvement in clinical parameters, Severity Scoring of Atopic Dermatitis Index (SCORAD) ($P < 0.0001$) and in Dermatology Life Quality index (DLQ) ($P = 0.021$). The probiotics reduced significantly microbial translocation ($P = 0.050$), immune activation ($P < 0.001$), improved T-helper cell – regulatory T cell (Treg) ($P = 0.029$) and Th1/Th2 ($P = 0.028$) ratios. None of these changes were observed in the placebo group [197].

64.39.2 ADHD

Reduced risk of developing/elimination of ADHD – 75 infants were randomized to receive *L. rhamnosus* GG (ATCC 53103) or placebo during the first 6 months of life and were thereafter followed for 13 years [198]. The children were reinvestigated at the age of 13 years by specialist in child neurology or child psychiatry. ADHD and Asperger's syndrome (AS), based on ICD-10 diagnostic criteria, were found in 6/35 (17.1%) of the children in the placebo-treated and in *none* in the perinatally probiotic-treated children ($P = 0.008$). The mean numbers of *Bifidobacterium* species bacteria in feces during the first 6 months of life was found to be significantly lower in the children who developed ADHD and AS – 8.26 (1.24) log cells/g compared to healthy children 9.12 (0.64) log cells/g; ($P = 0.03$) [198].

64.39.3 Autoimmune Diseases

Reduction in disease activity in Rheumatoid arthritis – 46 female patients with established rheumatoid arthritis (RA) for more than 1 year, and on stable medication for at least 3 months, were in a randomized double-blind clinical trial randomly allocated to receive for 8 weeks either 10^8 colony-forming units (cfus) of *L. casei* 01, or a placebo. Twenty-two patients received the probiotic and 24 patients the placebo [199]. *L. casei* 01 supplementation decreased significantly serum high-sensitivity C-reactive protein (hs-CRP) levels, and reduced tender and swollen joint counts, and improved global health (GH) score and Disease activity score-28 (DAS28) ($p < 0.05$). Significantly more patients in the *L. casei* 01 group, at the end of the study, as based on the European League Against Rheumatism (EULAR) criteria, demonstrated clinical response to the probiotic treatment ($P < 0.01$). Statistically significant differences in favor of the probiotic

group were also demonstrated in activity of IL-10, IL-12, and TNF-α ($P < 0.05$) [199].

64.40 Cancer

Reduction of bowel symptoms and improving quality of life in colorectal cancer survivors – A double-blind, randomized, placebo-controlled trial was undertaken with 32 participants receiving twice daily for 12 weeks Lacidofil (Institut Rosell's *L. helveticus* and *L. rhamnosus.*) and 28 participants receiving placebo [200]. Lactofil did significantly decrease the numbers of patients suffering from irritable bowel-like symptoms ($P = 0.03$), improve cancer-related quality of life ($P = 0.04$), and improve fatigue-related quality of life ($P = 0.02$) as well as patients' health-9 (PHQ-9) scores ($P = 0.01$) [200].

64.41 Childhood Diarrhea

Reducing tropical childhood diarrhea – An open-labeled, randomized controlled trial was undertaken in 2000 children with acute severe watery diarrhea, aged between 6 months and 5 years [201]. The children received for 5 days either *Lactobacillus* GG (LGG) in dose of 10 billion cfus/day or no probiotic medication. Median duration of diarrhea was significantly shorter in children in LGG group (60 vs. 78 h, $P < 0.001$) and the consistency of stool returned to normal significantly faster than in the control group (36 vs. 42 h, $P < 0.001$). Furthermorre, significant reduction in the average number of stools per day was observed in the treated children ($P < 0.001$). The benefits of probiotics were also seen rotavirus-positivite children [201].

64.41.1 Critical Illness

Improved safety and clinical effects in critically ill neonates – A double-blind, randomized controlled trial was conducted in 100 full-term critically ill infants, assigned at birth to duriong 8 days three times daily receive a total of 30 billion of L casei, *L. acidophilus*, *Bacillus subtilis*, and *Enterococcus faecalis* or placebo [206]. Significant reduction in the rate of nosocomial pneumonia (18 vs. 36%) and in multiple organ dysfunction syndrome (6 vs. 16%) was observed in the probiotic-treated group compared with the placebo group ($P < 0.05$). Significantly higher levels of IgA than the placebo group was observed in the probiotic-treated infants ($P < 0.05$). Furthermore, a significantly reduced hospital stay was reported in the probiotic-treated group ($P < 0.05$) [202].

Prevention or reduction of disease activity in necrotizing enterocolitis (NEC) in VLBW – A multicentre, prospective, randomized, controlled trial was conducted in 400 VLBW infants, who during 8 weeks and until discharge or death were supplemented either *B. lactis*, the prebiotic inulin, or a combination of *B. lactis* and inulin (= synbiotics) [203]. Maternal exposure to antibiotics increased significantly the risk of the babies developing NEC. The rate of NEC was lower

in both *L. lactis*-treated (2.0%) and *L. lactis* plus inulin-treated (4.0%) compared to only inulin (12.0%) or placebo (18.0%) treated children ($P < 001$). The time to reach full enteral feeding was significantly shorter ($P < 0.001$), the rate of clinical nosocomial sepsis was lower ($P = 0.004$), the stay in the neonatal intensive care unit shorter, ($P = 0.002$), and the mortality rate lower ($P = 0.003$) in the infants receiving active treatments compared to controls [203].

64.41.2 HIV – Virologic Suppression

Reduce translocation and inflammation in HIV patients – double-blind, randomized, placebo-controlled trial was conducted in 44 nonconsecutive HIV-1-infected patients with viral load of <20 copies/mL during the last 2 years, who received either Saccharomyces boulardii or placebo [204]. A significant decrease in lipopolysaccharide-binding protein (LBP) was observed (57.9 vs. 6.2% $P = 0.002$) and also in IL-6 (−0.60 vs. +0.78 pg/mL, $P = 0.002$) [204].

64.41.3 Metabolic Syndrome and Diabetes

Reduced insulin sensitivity, blood lipids, and inflammation – A probiotic composition consisting in 112.5×10^9 cfus of three strains of bifidobacteria (*B. longum*, *B. infantis*, and *B. breve*), four strains of lactobacilli (*L. acidophilus*, *L. paracasei*, L. delbrueckii subsp. bulgaricus, and *L. plantarum*), and one strain of *Streptococcus salivarius* subsp. thermophilus. (VSL 3) with or without added omega-3 fatty acid was tried in 60 overweight (BMI > 25), healthy adults, aged 40–60 years, who for 6 weeks received either placebo, only omega-3 fatty acid, only probiotic VSL 3, or combination of omega-3 and VSL 3 [205]. Patients with low HDL, higher insulin resistance, and high hsCRP had significantly lower total lactobacilli and bifidobacteria count and higher *E. coli* and bacteroides count. VSL 3 supplementation resulted in significant reductions in total cholesterol, triglyceride, LDL, and VLDL, and increase in HDL ($P < 0.05$). The VSL 3 treated patients demonstrated significantly improved insulin sensitivity ($P < 0.01$), decreased hsCRP, and an improved gut microbiota. Also the Omega-3 treated demonstrated significant effect on insulin sensitivity and hsCRP, but no effects on gut microbiota. Combination of VSL 3 with omega-3 fatty acid attenuated the effects on HDL, insulin sensitivity, and hsCRP [205].

64.41.4 Gastrointestinal Disorders

Relief of symptoms in patients with irritable bowel syndrome (IBS) – 50 patients suffering from IBS were randomized to receive daily for 4 weeks either *L. casei rhamnosus* LCR35 in a dose of 6×10^8 cfus (n = 25) or placebo (n = 25) [206]. No general improvements were reported, but the subgroup dominated by diarrhea demonstrated significant improvement with LCR35 treatment (reduction with 37%) compared to placebo (freduction with 3%) and also significant reduction in abdominal pain severity score (reduction with 36%). On the other hand, a slight improvement in favor of placebo was observed in patients with severe constipation; more reduced with placebo reduction with 41%) than in the LCR35-treated

(reduction with 20%) [206]. Another randomized, double-blind, placebo-controlled trial was undertaken in adult patients with symptomatic IBS with a probiotic composition consisting in *L. rhamnosus* NCIMB 30174, *L. plantarum* NCIMB 30173, *L. acidophilus* NCIMB 30175, and *Enterococcus faecium* NCIMB 30176 or placebo (1 mL/kg/day), administered for 12 weeks [207]. Total 152 patients completed the study. The mean reduction in IBS scores was 63.3% in the probiotic-treated versus 28.3% in placebo ($P = 0.019$). No improvements in IBS quality of life scores could, however, be seen [207].

Improved clinical effects as supplement to antibiotic therapy to eradicate H. pylori. – A multicenter, prospective, randomized, placebo-controlled, and double-blind study in totally 650 patients, who received for 14 days, twice a day, at least 2 h prior to or after the administration of antibiotics, capsules with probiotics containing either 10^8–10^{10} of *L. rhamnosus* GG (LGG®) and *Bifidobacterium (*BB-12®) or placebo capsules [208]. A significantly larger proportion of successfully cured patients were observed with probiotics added to the antibiotic treatment than without (87.38 vs. 72.55%; $P < 0.001$). The average intensity for symptoms induced by the antibiotic therapy was significantly reduced when probiotics were added (0.76 vs. 0.55; $P < 0.001$). Supplementation of probiotics reduced in average 7 of the following 10 symptoms: presence and intensity of epigastric pain, bloating, flatulence, taste disturbance, loss of appetite, nausea, vomiting, heartburn, rash, and diarrhea [208].

Significant reduction of inflammation in CD – The potential effects of *B. longum* CECT 7347 in children with newly diagnosed CD were evaluated in a double-blind, randomized, placebo-controlled trial in 33 children, who received daily for 3 months in addition to a gluten-free diet either10^9 cfus *B. longum* CECT 7347 or placebo [209]. The *B. longum* CECT 7347 treated demonstrated compared to placebo greater height percentile improvements ($P = 0{\cdot}048$), decreased peripheral $CD3^+$ T lymphocytes ($P = 0{\cdot}004$), and slightly reduced TNF-α concentration ($P = 0{\cdot}067$). Significant decreases in $CD3^+$ ($P = 0{\cdot}013$), in human leukocyte antigen (HLA)-DR^+ T lymphocytes ($P = 0{\cdot}029$), and slightly reduced TNF-α concentration ($P = 0{\cdot}085$) were also reported in the *B. longum* CECT 7347-treated patients [209].

64.41.5 Liver Disease

Reduced uptake of hepatotoxins and carcinogens – 90 healthy young men were enrolled in a study aimed to determine whether administration of probiotic bacteria could block the intestinal absorption of a dangerous poison aflatoxin B(1) and reduce urinary excretion of aflatoxin B(1)-N(7)-guanine (AFB-N(7)-guanine), a marker of aflatoxin exposure [210]. The patients received for 5 weeks twice daily either a mixture of *L. rhamnosus* LC705 and *Propionibacterium freudenreichii* subsp. shermanii strains or a placebo. The percentage of samples with negative AFB-N(7)-guanine values were after the 5-week intervention higher in the probiotic group than in the placebo group period ($P = 0.052$), and the decrease in urinary AFB-N(7)-guanine was statistically significantly reducecd in the probiotic-treated group compared to placebo ($P < 0.05$). The observed reduction was as high as 36% after 3 and 55% after 5 weeks – the geometric means for the

probiotic and placebo groups reported to be 0.24 and 0.49 ng AFB-N(7)-guanine/mL respectively ($P = 0.005$) [210].

Reduced fatty infiltration in NAFLD – A double-blind clinical trial supplementing a probiotic composition consisting in 112.5×10^9 cfus of three strains of bifidobacteria (*B. longum*, *B. infantis*, and *B. breve*), four strains of lactobacilli (*L. acidophilus*, *L. paracasei*, *L. delbrueckii* subsp. *bulgaricus*, and *L. plantarum*), and one strain of *Streptococcus salivarius* subsp. *thermophilus*. (VSL 3) or placebo, supplemented to obese children with biopsy-proven NAFLD [211]. Fourty-four patients (22 VSL 3 and 22 placebo) completed the study. At baseline, moderate and severe NAFLD were present in 64% and 36% of placebo children and in 55% and 45% of VSL 3 children. The probability that children supplemented with VSL 3 had in comparison none, light, moderate, or severe fatty liver at the end of the study was 21 versus 0%, 70 verus 7%, 9 versus 76%, and 0 versus 17% group ($P < 0.001$). No differences were detected in triglycerides, homeostasis model assessment (HOMA) and alanine transaminase (ALT) while BMI decreased and glucagon-like peptide 1 (GLP-1) and activated GLP-1 (aGLP-1) increased in the VSL 3 group ($P < 0.001$ for all comparisons) [211].

64.41.6 Renal Disease

To reduce the accumulation of urea in chronic kidney disease – Patients with chronic kidney disease (CKD) show an increase in bowel aerobic bacteria that produce uremic toxins and decreased anaerobic bacteria as bifidobacteria and lactobacillus [212]. Two different strains of *L. casei shirota* (LcS) were tried for 8 weeks in CKD patients in aimple randomized, controlled clinical trial in two doses: Group A: 8×10^9 cfu and group B: 16×10^9 CFU. The larger dose was most effective, inducing a statistically significant no less than 10 times reduction in blood urea concentrations [212].

To reduce inflammation in dialysis patients – Peritoneal dialysis (PD) patients received daily for 6 months one capsule of a probiotic composition containing 10^9 cfu Bifobacterium bifidum A218, 10^9 cfu Bifidobacterium catenulatum A302, 10^9 cfu *B. longum* A101, and 10^9 cfu *L. plantarum* A87, or maltodextrin as placebo [213]. 39 patients completed the study. The serum levels of proinflammatory cytokines TNF-α, IL-5, IL-6, and endotoxin were significantly decreased after 6 months of treatment, while serum levels of anti-inflammatory cytokine IL-10 were significantly increased, whereas no significant changes in levels of serum cytokines and endotoxin were observed in the placebo group. Most importantly, the residual renal function was well preserved after 6 months in patients receiving probiotics [213].

To reduce uremic waist products in patients with in patients with chronic kidney disease – In a 6-month crossover trial 46 outpatients were supplemented a probiotic formulation consisting in a gel capsule containing a mix of L. acidophilus KB27, B. longum KB31, and S. thermophilus KB19, totally 1.5×1010 CFU/day [214]. Significant improvements were observed: enhanced well-being (quality of life – QLT) ($P < 0.05$), absence of serious adverse effects and impressive reductions in blood urea nitrogen (BUN) ($P < 0.05$ – BUN levels reported to be decreased by 63%, creatinine levels by 43% and uric acid levels by 33%. The improvement in QOL was estimated to be no less than 86% ($P < 0.05$) [214].

64.41.7 Respiratory

To reduce the number of days with URTI in academically stressed students – Randomized, double-blind, placebo-controlled study examined the effect of three potentially probiotic bacteria on the proportion of healthy days over a 6-week period in academically stressed undergraduate students (n = 581) who received one of the following probiotics; Lb helveticus R0052, *B. longum* ssp. infantis R0033, *B. bifidum* R0071 or placebo [215]. The percentage of participants reporting ≥ 1 day of cold/flu during the 6-week intervention period was significantly lower only with *B. bifidum* ($P < 0{\cdot}05$), while no effects were observed with supplementing *B. infantis* or *L. helveticus* [215]. The efficacy of daily dietary consumption of *L. brevis* KB290 (KB290) to prevent against influenza also in elementary schoolchildren, was studied in an open-label, parallel-group trial conducted 8-week periods [216]. The incidence of influenza was 16% with probiotic protection and 24 without ($P < 0{\cdot}001$). The reduction in the incidence of influenza by KB290 consumption was especially pronounced in nonvaccinated individuals [216].

To reduce inflammation in CF patients – 22 children with CF children were enrolled in the study. The CF children were found to suffer severe intestinal dysbiosis with significant reductions in *Eubacterium rectale*, *Bacteroides uniformis*, *Bacteroides vulgatus*, *B. adolescentis*, *B. catenulatum*, and *Faecalibacterium prausnitzii* [217]. Significant elevations in fecal calprotectin (CLP: 184 vs. 52 ± 46 μg/g, $P =< 0.01$) and in rectal nitric oxide (rNO) levels (184 vs. 52 ± 46 μg/g, $P =< 0.01$)) were reported in children with CF compared to healthy controls. The degree of dysbiosis was especially increased in children with CF, who were taking antibiotics. A significant correlation was observed between the treated groups in favor of the probiotic-treated group both in degree of inflammation and increase of microbial richness of intestinal microbiota. Administration of 6×10^9 cfus of Lactobacillus GG (LGG) reduced significantly fecal CLP and restored, at least partially, the intestinal microbiota [217].

64.41.8 Toxic Exposure

To prevent effects of environmental toxic metal exposure – A study in Tanzania investigated the efficacy of probiotic foods to prevent consequences of high local toxic metal exposures. A group of 44 school-aged children was followed over 25 days, and 60 pregnant women were followed over their last two trimesters until birth [218]. A yogurt containing 10^{10} CFU *L. rhamnosus* GR-1 per 250 g was administered, while control groups received either whole milk or no special foods. Changes in blood metal levels were assessed, and the gut microbiomes of the children were profiled by analyzing 16 S rRNA sequencing via the Ion Torrent platform. The children and pregnant women at the beginning of the study found to have significantly higher blood levels of lead and mercury when compared to age- and sex-matched Canadians. Consumption of probiotic yogurt provided a statistically significant tool to protect against increases in blood levels of both mercury (3.2 nmol/L; $P = 0.035$) and arsenic (2.3 nmol/L; $P = 0.011$) particularly in the pregnant women, a trend, which was not statistically significant in the children, at least not after only treated for 25 days [218].

64.42 Fecal Microbiota Transplantation (FMT) – A Giant Step Forward

Reconditioning of the gut by transfer of microbiota from a healthy individual to an often very sick patient, marks a new époque in the history of restitution a healthy gut and attempts to reduce the burden of disease. It is unfortunate that in the Western world most, if not all of the donors, also suffer a much reduced microbiota, compared to those still living a rural forefather-like lifestyle such as the Hadzas [29], the Burkina Fasons [32], the Yanomamis [34], whose microbiota demonstrate around 50% higher diversity – and worse, recent data suggest that the deterioration of human microbiota is a continuous process [219]. It seems reasonable to assume that the most effective health-promoting bacteria are already extinct in Western population due to lack of proper foods. Maybe one day such rich stools from groups such as those may be available deep-frozen for FMT. Under all circumstances it will not last long before deep-frozen stools are a reality – to begin with stools from the individual saved already at an early age. It must be emphasized though that the best results from reconditioning of the gut is obtained when combined with a dramatic change in lifestyle and food habits – and that forever. Recent studies demonstrate that it takes less than 24 h to destroy microbiota if the microbes do not receive for them suitable nutrients [220]. For information about recent progress with FMT – please read some recent excellent reviews [221–225].

64.43 Synbiotics – A Less Invasive Alternative

Oral or rectal supplementation of a synbiotic formula offers simple and less cumbersome alternatives – constitutes attempts to produce "synthetic feces."

For the most recent 15 years my personal focus has been on trying:

1) To identify lactobacilli (LAB) on growing plants that might have unique inflammation-reducing abilities, based on the belief that the strongest anti-inflammatory microbes might be extinct in human microbiome.
2) To provide strong fibers with documented ability to serve as substrate for microbial fermentation (favored nutrients for microbes) and in doses of at least 10 g – adding about 40% more fiber to the average fiber consumption, which is very low in Westyerners.
3) To provide large doses of LAB than commonly used (exemption VSL 3).

I took initiative in 1999 to develop and produce a synbiotic formula based on these principles Since 1999, all my efforts in this field have concentrated on a four LAB/four fiber composition, consisting of either a mixture of either 4×10^{10} (40 billion LAB in a Standard version – Synbiotic 2000™) or a mixture of 10^{11} (400 billion, Forte version – Synbiotic 2000 Forte™). From studies of about 500 lactobacillus species (about 200 collected from plants) the following four LAB, which demonstrating unique anti-inflammatory abilities [226,227] were chosen for the composition: *Pediococcus pentosaceus* 5–33:3, *Leuconostoc mesenteroides* 32–77:1, *Lactobacillus paracasei* subsp paracasei 19, and *Lactobacillus plantarum* 2362. To the four LAB was added 10 g of documented bioactive fibers:

4 × 2.5 g of each of the following four fermentable fibers: betaglucan, inulin, pectin and resistant starch, a formula that is currently a product produced by Synbiotic AB, Sweden.

Below is summarized the experience from some of the human randomized controlled studies, using Synbiotics 2000:

To prevent postoperative sepsis in extensive pancreatic surgery – A prospective, randomized, double-blind trial was performed in 80 patients undergoing pylorus-preserving pancreatoduodenectomy (PPPD) who received, twice daily, either Synbiotic 2000™ (2 × 40 billion LAB, i.e., 80 billion LAB/day) or only the fibers in composition from the day before surgery and during the first seven post-operative days [228]. A highly significant difference in infection rate ($P = 0{,}005$) was observed – only 5/40 patients (12.5%) in the Synbiotic 2000-treated group suffered infections (4 wound and one urinary tract infection) in sharp contrast to 16/40 (40%) in the fiber-only group (6 wound infections, 5 peritonitis, 4 chest infections, 2 sepsis, and 1 of each of urinary tract infection, cholangitis, and empyema). The number of infecting microorganisms were also statistically significantly reduced ($P = 0{,}005$) as was the use of antibiotics (mean: Synbiotic 2000; 2 ± 5 days, only fibers; 10 ± 14 days) [228].

To prevent postoperative sepsis in abdominal cancer operations – 45 patients undergoing major surgery for abdominal cancer were randomized into three treatment groups: 1. enteral nutrition (EN) supplemented with Synbiotic 2000 (LEN), 2. EN supplemented with only the fibers in the same amounts (20 g) (20 g) as in Synbiotic 2000™ (FEN) and 3. Standard parenteral nutrition (PN). All treatments lasted for 2 preoperative and 7 days postoperative days. The incidence of postoperative bacterial infections was 47% with PN, 20% with FEN and only 6.7% with Synbiotic 2000-based LEN ($P < 0.05$). The numbers of infecting microorganisms were statistically and significantly reduced. Significant improvements were observed with both Synbiotic 2000 and only-fiber-based treatments (LEN, FEN), in prealbumin, CRP, serum cholesterol, serum endotoxin, and with only Synbiotic 2000 in white cell blood count and in IgA (Han Chun Mao, personal information).

To prevent postoperative sepsis in orthotopic liver transplantation – 66 human orthotopic liver transplant patients were randomized to either receive Synbiotic 2000 or selective bowel decontamination (SBD) [229]. The treatment continued from the day before surgery until 14 days after surgery. The Synbiotic 2000 treatment did almost extricate postoperative sepsis. After 1-month follow-up had only one patient in the Synbiotic 2000-treated group developed an infection – a benign *E. faecalis*-based asymptomatic catheter-induced urinary infection 1/33 (3%) compared to 17/33 (51%) in the SBD-treated patients [229]. The use of antibiotics was on average 0.1 ± 0.1 days in the Synbiotic-treated patients and 3.8 ± 0.9 days in the SBD-treated patients [229].

To reduce infections and complications to severe acute pancreatitis – 62 patients with severe acute pancreatitis (SAP) were supplemented twice daily during the first 14 days after arrival at the hospital with Synbiotic 2000™ (80 billion + 20 g fibers) or the same amounts of fibers (totally 20 g/day) as in Synbiotic 2000™ [230]. 9/33 patients (27%) in the Synbiotic 2000-treated group and 15/29 patients (52%) in the fiber-only treated group developed infections.

8/33 (24%) of the Synbiotic 2000-treated and 14/29 (48%) of the fiber-only treated patients developed SIRS, MOF or both ($P < 0.005$). A total of seven pathogenic microorganisms were detected in the Synbiotic-treated group compared to seventeen in the fiber-only group [230] Another study supplemented Synbiotic 2000 Forte (400 billion Lab + 10 g fibers) from within 24–48 hrs of symptoms of sickness to patients with severe acute pancreatitis and compared to 32 patients on a control formula considered as anti-inflammatory [231]. Synbiotic-2000 Forte treatment lead to a lower infection rate including less pancreatic and peripancreatic necroses; secondary infections (2 vs. 9, $P = 0.0001$), septicemia (2 vs. 7, $P = 0.03$), lower rate of surgical interventions, (3 vs. 12, $P = 0.005$), shorter stay in ICU (8 vs. 16 days $P = 0.05$), shorter hospital stay (23 vs. 36 days, $P = 0.03$), and reduced mortality (0 vs. 17 patients, $P = 0.02$) [231].

To reduce infections and complications to major trauma – Two prospective randomized trials with either Synbiotic 2000™ or Synbiotic 2000 Forte™ were undertaken. In the first study [232] 52 patients were randomly allocated into 4 groups with different commercial nutrition formulas: Group A: Alitraq (Abbott-Ross) B: Nova Source (Novartis) Group C: Nutricomp peptide (*B. braun*) plus Synbiotic 2000) Group D: Nutricomp standard (*B. braun*) plus Synbiotic 2000. Nova Source and Nutricomp peptide did not reduce the levels of proinflammatory cytokines at all, while Alitraq and Nutricomp standard + Synbiotic 2000 significantly down-regulated Il-6, but not Il-8 and TNF- α. The groups containing Synbiotic 2000 demonstrated significant reductions in both total infections and in chest infections; the total number of infections being reduced with about two thirds ($P < 0.005$) and the number of chest infections being reduced to about half ($P < 0.005$) [232]. In a series of studies 65 polytrauma patients were randomized to receive once daily for 15 days following major trauma, either Synbiotic 2000 Forte (400 billion LAB + 10 g of fibers) or maltodextrine as placebo [233–235]. Significant reductions were observed between the groups in the number of deaths (5/35 vs. 9/30, $P < 0{,}02$), severe sepsis (6/35 vs. 13/30, $P < 0.02$), chest infections (19/35 vs. 24/30, $P < 0.03$), central line infections (13/32 vs. 20/30, $P < 0.02$), and days on artificial ventilation (average 15 vs. 26 days). A total of 54 pathogenic microorganisms were cultivated in the Synbiotic treated group compared to 103 in the placebo group. Repeat analyses revealed that serum levels of endotoxin (LPS) were significantly decreased and the time to occurrence ofbloodstream infection significantly prolonged in patients treated with Synbiotic 2000 Forte $P < 0.02$) [233–235].

64.44 Less Suffering for the Patients – Great Savings for Society

Figure 64.2 summerizes the gains in reduced numbers of infections obtained after Synbiotic 2000 treatment in various severe conditions – reductions varying between 60 and 94% depending on disease/trauma/surgical treatment demonstrated. Figure 64.3 summarizes the savings in use of costly antibiotics, savings varying between 80 and 97%, savings on costly artificial respiration of about 20% savings on reduced time in costly ICUs vaying between 14 and 67% and also savings on the average in hospital time varying from 3 to 24% depending on treated condition.

REDUCTIONS IN INFECTIONS/POSITIVE BLOOD CULTURES

Liver transplantation – 66 patients[236]	
Patients with postop. infections	16 => 1 = 94%
Patients with pos. blood cultures	11 => 1 = 91%
Pancreatdoudenectomy for cancer – 80 patients[235]	
Patients with postop. infections	16 => 5 = 69%
Patients with pos. blood cultures	27 => 5 = 82%
Severe pancreatitis – 62 patient[237]	
Patients with infections	15 => 9 = 40%
Patients with pos. blood cultures	17 => 7 = 59%
Severe trauma, treated with synbiotic 2000 standard – 52 patients[239]	
Patients with infections	23/30 (77 %) => 17/35 (49 %)
Severe trauma, treated with synbiotic 2000 Forte – 72 patients[239-241]	
Patients with post-trauma infections	13 => 5 = 62%
Patients with pos. blood cultures	13 => 5 = 62%

Figure 64.2 Reduction in infections/positive blood cultures after Synbiotic 2000 treatment.

REDUCTIONS IN USE OF ANTIBIOTICS, ARTIFICAL RESPIRATION, TIME IN ICUs, AND IN HOSPITAL

Liver transplantation – 66 patients[236]	
Days on Antibiotics	3.8 => 0.1 = 3.7 (97 %)
Days in ICUs	10.2 => 8.8 = 1.4 (14 %)
Days in Hospital	27.9 => 27.8 = 0.1 (3 %)
Pancreatdoudenectomy for cancer – 80 patients[235]	
Days on Antibiotics	10 => 2 = 8 (80 %)
Days in ICUs	6 => 2 = 4 (67 %)
Days in Hospital	22 => 17 = 5 (23 %)
Severe acute pancreatitis – 62 patient[237]	
Days in Hospital	19.7 => 14.9 = 4.8 (24 %)
Severe trauma treated with synbiotic 2000 Forte – 65 patients[239-241]	
Days on Artificial Respiration	24 => 19 = 5 (21 %)
Days in ICUs	41.3 => 27.7 = 13.6 (33 %)

Figure 64.3 Reduction in use of antibiotics, artificial respiration, time in ICUs, and in hospital after Synbiotic 2000 treatment.

64.45 Ecobiological Functions – Same but Different Response Compared to So-Called Biological Drugs

Inflammation is said to involve about 1200 genes and affecting an enourmously wide range of effector molecules; proinflammatory cytokines such as IL-1β, IL-6, TNF-α and IL-18, chemokines such as IL-8, IP-10, MCP-1, MIP-1 and RANTES, MMPs such as MMP-1, -3, -9, and -13, and metabolic proteins such as Cox-1, Cox-2, and iNOS, and many more. The so-called biological drugs are made to target exactly the same genes, but mainly one at the time. Ecobiologicals;

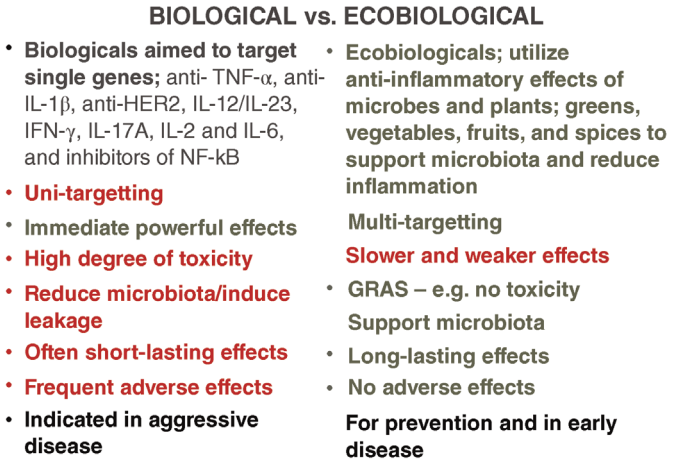


Figure 64.4 Comparison of effects of biologicals versus ecobiologicals.

probiotic bacteria, plant fibers, plant antioxidants and so on produce an accumulated response targeting all the genes much in parallel but slower and weaker (Figure 64.4).

Especially affected are the two special tissues, strongly involved in inflammation; the intestinal mucosa and the vascular endothelium organ, both effective barriers, effective metabolic, effective endocrine, and also important immune organs, both covering similarly wide surfaces: the intestinal mucosa that of a soccer field – greater than 7000 m^2 and consisting in greater than 10 000 billion cells and weighing greater than 1 kg each and both intimately involved in inflammations such as postprandial inflammation [171–185]. The total endothelial tissue of the body, which cover a similarly sized surface, has the unique ability to activate extraordinary many genes and factors involved in inflammation such as VCAM-1, ICAM-1, E-selectin, PDGF, TGF-β, TNF-α, IGF-1, MCP-1, CTGF, Il-6, PAI-1, RAGE, VEGF, ANG II-dependent cell activation factors, type IV collagen expression factors, toll-like stimulatory factors, cell cycle progression factors, and fibronectin [236].

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65

Natural Products as Source of Anti-Inflammatory Drugs

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65.1 Introduction

Natural products have been an invaluable resource for drug discovery over centuries [1–5]. Most information on medicinal properties of natural products is based on the traditional systems of medicine such as traditional Chinese medicine (TCM), Ayurveda, and other forms of complementary and alternative medicine (CAM) [6–10]. This knowledge was derived from the use of indigenous natural products, such as crude plant extracts, by local healers/practitioners as recipes for the maintenance of health and treatment of diseases. Subsequently, application of advanced methodologies to natural products research allowed the fractionation of crude extracts and isolation of bioactive molecules from them. This, in turn, paved the way for drug discovery from natural products obtained from plants, microbes, and animals [2,6,10,11].

Over the past few decades, scientific developments in the characterization and quality control, in defining the mechanism of action, and in improving the efficacy and safety of natural products have broadened the scope of natural products research [1–5,12]. Typically, drug discovery from natural products involves the following steps: the screening of natural products using *in vitro* cell culture models and/or *in vivo* experimental animal models for their therapeutic effects; the identification of a lead multi component product and determining its chemical constituents; the screening of bioactive compounds under controlled conditions and studying their mechanisms of action; the refining of drug delivery approaches for selected compounds; and the testing of one or more lead compound(s) in clinical trials in patients with a particular disease [2,10–13]. This approach is similar to the mainstream "forward pharmacology" in which a

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large library of synthetic compounds is screened to identify a lead compound having the desired functional attributes and then taking it through different steps to final testing in the clinic. In contrast to this, a reverse approach ("reverse pharmacology") is increasingly being applied to natural products, such as medicinal plants in the traditional systems of medicine (e.g., TCM), whose extracts are already known to be effective against various diseases [14,15]. Such products are first thoroughly characterized for their bioactive principles, and then the structure-function relationships and toxicity profiles of select compounds are further improved in the laboratory before testing the modified compound(s) back in a clinical setting.

Natural products also offer an opportunity for multitarget drug discovery [6–11]. The multiplicity of therapeutic targets of natural products is an asset, and it can be viewed in context of multiple bioactive components in a mixture, for example, compounds in an extract from the leaves of a medicinal plant, as well as of more than one target of a compound purified from that mixture. The availability of a well-defined bioactive compound from a natural product offers certain advantages, for example, the ease of both quality control and examination of its mechanism of action, when compared with a crude extract. In some cases, a single bioactive compound can replicate a vital biological activity or therapeutic benefit of the whole extract, and therefore in those situations such a compound can be pursued further for development of a pharmaceutical lead compound/drug. However, there are some likely disadvantages to consider as well. When a bioactive compound is removed from other compounds in the same crude extract, it can display unexpected effects in terms of its tolerability, bioavailability, toxicity, and even biological activity. These effects, in turn, can be attributed to the additive/synergistic versus counterbalancing activity of the accompanying compounds. Awareness of these issues can guide the process of drug discovery to meet the goals of improved therapeutic efficacy coupled with reduced toxicity. In addition, the knowledge of bioactive compounds from different natural products and the use of these compounds in specific combinations can be exploited for developing an effective multitarget natural product for inflammatory diseases [16].

Regarding the source of natural products for drug discovery, different plants, animals, and terrestrial microorganisms have contributed significantly to the pool of products for this purpose [2,6,10,11]. However, the potential of the microorganisms from the marine environment as well as that of underexplored terrestrial microbial species remains to be fully tapped for diversifying the sources of natural products [5,6]. Advancements in biotechnology and other areas of research including, high-throughput screening, combinatorial biosynthesis, metabolic engineering, microarrays, systems pharmacology, and nanotechnology-based drug delivery systems (Figure 65.1) are making a significant impact on the process of drug discovery from natural products [2,4]. Furthermore, hopefully the recent awarding of the 2015 Nobel Prize in Physiology or Medicine to William C. Campbell, Satoshi Ōmura, and Youyou Tu for the discovery of avermectins and artemisinin for anti-parasite therapy

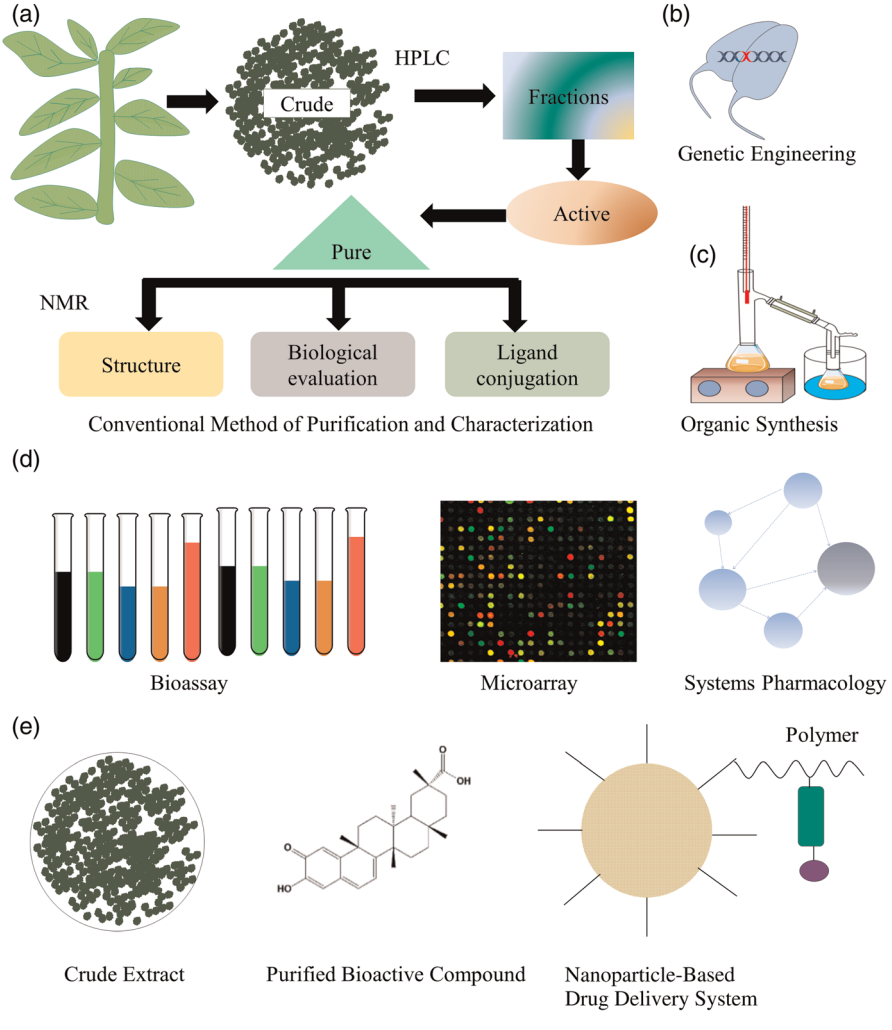


Figure 65.1 Advancements in the methods for the production, characterization, and delivery of natural products. (a) Conventional scheme for the fractionation and purification of bioactive compounds from crude extracts of natural products; (b) genetic engineering; (c) organic synthesis; (d) common screening methods (e.g., bioassays), microarrays, and systems pharmacology; and (e) nanotechnology for delivery of natural products.

would further rejuvenate interest in, and research efforts into, natural products drug discovery.

In this chapter, we have presented a brief summary of the key pathways involved in the process of inflammation; of natural products as the source of compounds/drugs with anti-inflammatory activity for use in the treatment of inflammatory, autoimmune, and other immune-mediated diseases; and of the

modern approaches for, and recent advancements in, the production, characterization, and delivery of natural products. (Although inflammation is also linked with cancer, but that aspect of natural product therapy is not covered here.)

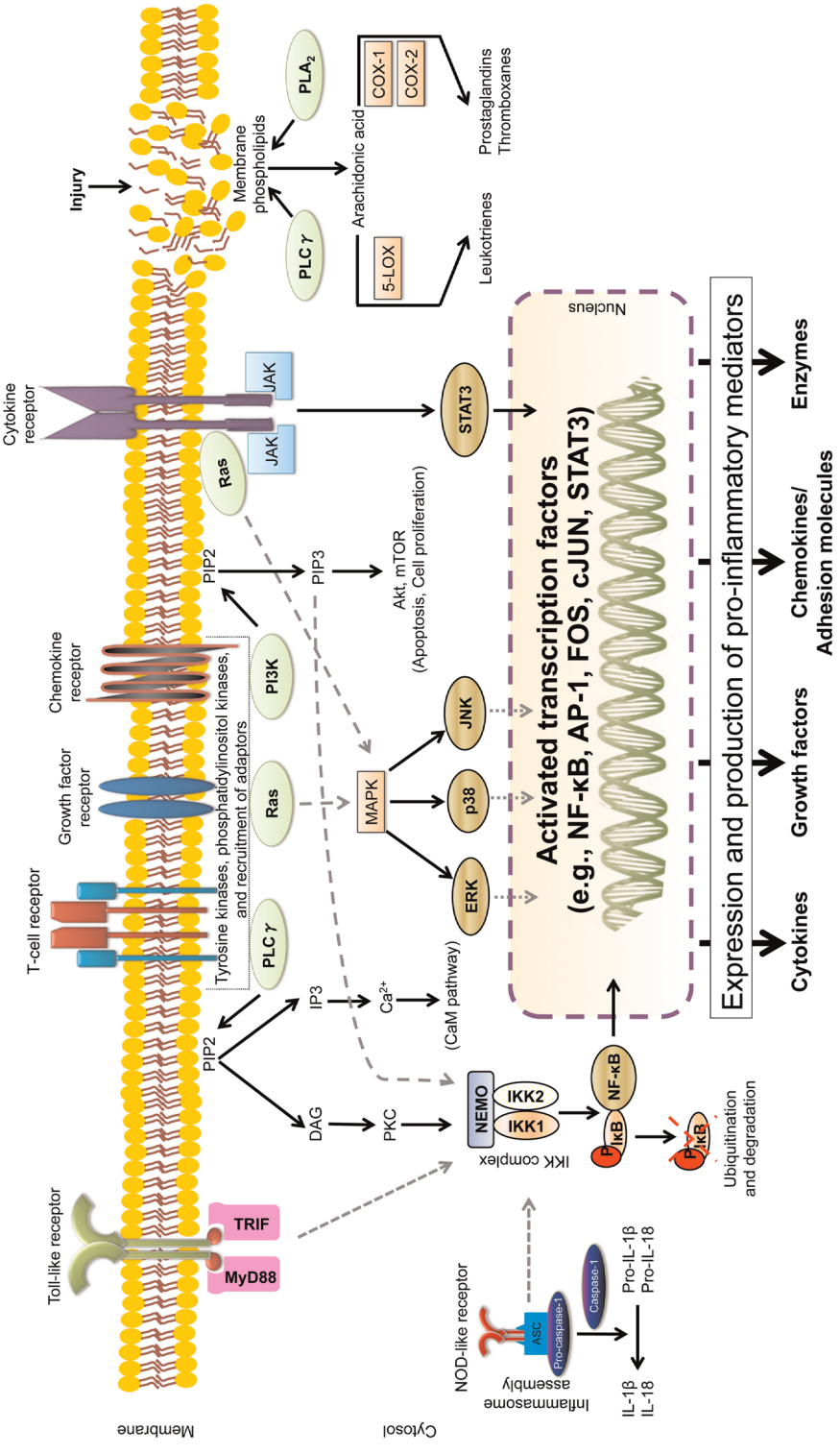
65.2 Cellular, Molecular, and Biochemical Pathways of Inflammation

Inflammation is a complex process involving a variety of cellular, molecular, and biochemical mediators that are activated/induced in response to inflammatory stimuli. The mechanisms of induction and amplification of the key inflammatory events are briefly described below. Anti-inflammatory drugs, including those derived from natural products, combat inflammation by targeting one or many of these pathways (Figure 65.2).

65.2.1 Inflammation Triggered by Innate Immune Responses

Macrophages, dendritic cells, fibroblasts, and mast cells are among the cellular components of the innate immune system. Pattern recognition receptors (PPRs) expressed on these cells recognize pathogen-associated molecular patterns (PAMPs), such as microbial lipoproteins, carbohydrates, and nucleic acids. Another category of PRRs is the damage-associated molecular patterns (DAMPs) released from injured cells [17]. The description below is mainly for PAPMs, although certain DAMPs can also use these pathways. Toll-like receptors (TLRs) are the major PPRs expressed on the cell surface (Figure 65.2). More than 10 different TLRs have been identified in humans and mice. Specific ligand-induced activation of TLRs, which interact with adapter molecules such as myeloid

Figure 65.2 A schematic representation of major cell signaling pathways associated with inflammation that are targeted by natural products. Activation of cell surface receptors such as the toll-like receptors (TLRs) and receptors for antigens, cytokines, chemokines, and growth factors occurs following the binding of respective ligands. This results in the activation of receptor complex-associated tyrosine kinase activity, the recruitment of adapter proteins, and/or the activation of other enzymes. These early events in turn lead to the activation of various intracellular signaling cascades culminating in the production of various mediators of inflammation. Similarly, injury and tissue damage initiate a cascade of reactions that lead to the production of various biochemical mediators of inflammation such as prostaglandins, thromboxanes, and leukotrienes. A number of agents derived from natural products are known to interact with one or more of these pathways and exhibit anti-inflammatory activity (Tables 65.1 and 65.2). (*Abbreviations*: AP-1, activator protein 1; ASC, associated speck-like protein containing CARD; CaM, calmodulin; COX; cyclooxygenase, DAG, diacyl glycerol; ERK, extracellular signal-regulated kinase; IKK, IκB kinase; IP3, inositol triphosphate; JAK, janus kinase; STAT3, signal transducer and activator of transcription; JNK, c-Jun N-terminal kinase; LOX, lipoxygenase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; MyD88, myeloid differentiation primary response gene 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-kappa B; NOD, Nucleotide oligomerization domain; PI3K, phosphatidylinositol-3-kinase; PIP2, phosphatidylinositol bisphosphate; PKC, protein kinase C; PLA_2, phospholipase A2; PLCγ, phospholipase C gamma; TRIF, TIR-domain-containing adapter-inducing interferon-β.)


Injury
Membrane phospholipids
PLA2
PLC γ
Arachidonic acid
COX-1
COX-2
5-LOX
Prostaglandins Thromboxanes
Leukotrienes
Cytokine receptor
JAK
JAK
STAT3
Ras
PIP2
PIP3
Akt, mTOR (Apoptosis, Cell proliferation)
Chemokine receptor
PI3K
Growth factor receptor
Ras
MAPK
JNK
p38
ERK
T-cell receptor
Tyrosine kinases, phosphatidylinositol kinases, and recruitment of adaptors
PLC γ
IP3
Ca2+
(CaM pathway)
PIP2
DAG
PKC
NEMO
IKK2
IKK1
IKK complex
NF-κB
P IκB
P IκB
Ubiquitination and degradation
Nucleus
Activated transcription factors (e.g., NF-κB, AP-1, FOS, cJUN, STAT3)
Expression and production of pro-inflammatory mediators
Cytokines
Growth factors
Chemokines/ Adhesion molecules
Enzymes
Toll-like receptor
TRIF
MyD88
NOD-like receptor
Inflammasome assembly
ASC
Pro-caspase-1
Caspase-1
Pro-IL-1β
Pro-IL-18
IL-1β
IL-18
Membrane
Cytosol

differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF) at their cytosolic domain, activates MyD88- and TRIF-dependent signaling pathways. Activation of these pathways then results in the activation of transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the activator protein 1 (AP-1) [18,19]. Nucleotide oligomerization domain (NOD)-like receptors (NLRs) are cytosolic PRRs. Activation of NLRs by microbial products or aberrant self components of the host leads to NF-κB activation [20] and may also induce inflammasome activation. Inflammasome assembly typically consists of a sensor (an NLR), an adapter (apoptosis-associated speck-like protein (ASC) containing an N-terminal pyrin domain (PYD) and a C-terminal caspase activation and recruitment domain (CARD), and an effector (CARD and caspase) component [21]. Activation of the inflammasome leads to the activation of caspases, which play an important role in the induction and propagation of inflammation [22]. For example, activated caspase-1 causes the maturation of proinflammatory cytokines such as IL-1β and IL-18 by proteolytic cleavage of pro-IL-1β and pro-IL-18, respectively (Figure 65.2) [23,24]. These cytokines and other biochemical and immunological mediators contribute to the initiation and propagation of inflammation. The above-mentioned PRRs are attractive targets for the control of diseases associated with inflammation including, infections, autoimmunity, and cancer. Several agents targeting TLRs [25–28] and NLRs [29,30] are undergoing preclinical/clinical evaluation for the prevention and treatment of these disorders.

65.2.2 Inflammation Triggered by Adaptive Immune Responses

The T cells and B cells are vital components of the adaptive arm of the immune response. An imbalance in adaptive immune responses may lead to inflammation and autoimmunity. Activation of the antigen receptors (T-cell receptor (TCR)/B-cell receptor (BCR) on T/B cells, respectively, occurs following antigen recognition [31–35]. (In the case of T cells, TCR recognizes an antigenic peptide presented by the major histocompatibility complex (MHC). The signal transduction by the TCR and BCR has broad similarities and involves the activation of protein kinases, phospholipase C-gamma (PLCγ), and of small G proteins (Figure 65.2) [31–35]. The binding of antigen to cell surface receptors activates receptor-associated protein kinases at the inner surface of the cell membrane, which leads to the activation of other cytosolic targets including the enzyme PLCγ. PLCγ cleaves phosphatidylinositol bisphosphate (PIP2) to yield diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 then increases intracellular Ca^{2+} concentration, activates calcineurin pathway, and results in the activation of nuclear factor of activated T cells (NFAT) and related transcription factors. Similarly, DAG and Ca^{2+} activate protein kinase C (PKC), which then leads to activation of NF-κB and other transcription factors. In addition, the kinase activity associated with the activated TCR/BCR complex recruits and activates guanine-nucleotide exchange factors (GEFs) with the help of adapter proteins. GEFs then activate small G proteins, which in turn trigger MAPK pathway. MAPK pathway then activates AP-1 and related transcription factors. All these activated transcription factors

move into the nucleus and regulate gene expression of a variety of mediators of inflammation (Figure 65.2).

65.2.3 Cell Signaling Pathways involved in inflammation

65.2.3.1 The Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-kB) Pathway

Mediators such as proinflammatory cytokines, growth factors, and enzymes play a central role in the induction and amplification of inflammation. The NF-κB pathway is a master regulator for the synthesis of these mediators, which then participate in inflammation associated with infection or autoimmunity [36–38]. Initial signals for activation of the classic NF-κB pathway come from the binding of specific ligands to their respective cell surface receptors such as receptors for cytokines, growth factors, or microbial products. The downstream event of these interactions is the activation of the IKK complex, which consists of I kappa B kinase 1 (IKK1), I kappa B kinase 2 (IKK2) and NF-κB essential modulator (NEMO) (Figure 65.2). The activated IKK complex phosphorylates IκB, a component of the inactive cytosolic NF-κB complex, which consists of inhibitory IκB and a heterodimeric NF-κB/RelA complex. The phosphorylated IκB undergoes ubiquitination and proteasomal degradation. The degradation of IκB in turn activates NF-κB, which then translocates into the nucleus, binds to DNA, and induces the expression of specific target genes [37,38]. Phosphorylation and subsequent degradation of IκB is critical for the activation of NF-κB. Agents that inhibit either the activation of the IKK complex or the phosphorylation of IκB or enhance the proteasomal degradation of IκB can thereby help retain NF-κB in an inactive state in the cytoplasm. Such NF-κB inhibitors are one of the prominent groups of anti-inflammatory agents capable of controlling inflammation in a wide variety of diseases [39]. Many natural product-derived molecules have been shown to inhibit NF-κB pathway in various *in vitro*, preclinical, or clinical settings. These include aspirin, curcumin, resveratrol, quercetin, celastrol, and others (Tables 65.1 and 65.2).

65.2.3.2 Mitogen-Activated Protein Kinase (MAPK) Pathway

MAPKs are a family of serine/threonine protein kinases, which are activated by the activation of small G proteins in response to various proinflammatory stimuli [108,109]. Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK represent three defined MAPKs (Figure 65.2) [108]. Activation of ERK leads to the activation of transcription factors such as c-Jun, c-Fos, and activating transcription factor 2 (ATF-2). Similarly, JNK activation in turn activates c-Jun and ATF-2. The p38 MAPK mediates both anti-inflammatory and proinflammatory processes, and its action involves transcription factors cAMP response element-binding protein (CREB) and ATF-2 [108,110,111]. The inhibition of ERK and JNK pathway has been shown to have greater efficiency than p38 MAPK in attenuating inflammation associated with autoimmune/inflammatory diseases [112–115]. Examples of natural product-derived agents that regulates MAPK pathway, and which have been tested *in vitro* and in preclinical/clinical settings, are given in Tables 65.1 and 65.2.

Table 65.1 Natural product-derived bioactive compounds with anti-inflammatory activity.

Lead agent	Chemical class	Natural source category	Targets: cellular/molecular mediators and pathways	Health maintenance/ therapeutic applications used/tested	References
Acetylsalicylic acid (aspirin) (modified, synthetic)	2-(acetoxy) benzoic acid	Based on salicylic acid	COX-1, COX-2, MAPK signaling, NF-κB pathway, adenosine release	Pain, fever, RA and other types of inflammation, cardiovascular disease prevention	[16,40–42]
Salicylic acid (natural)	Beta hydroxy acid	Plant (*Salix alba*)			
Curcumin	Diarylheptanoid	Plant (*Curcuma longa*)	NF-κB pathway, Nrf2, STAT3 signaling, COX-1, 5-LOX, protein kinase C, glycogen synthase kinase-3P, histone acetyltransferases, p53	RA, OA, and other types of inflammation	[16,43–46]
Epigallocatechin-3-gallate (EGCG)	Catechin	Plant (*Camellia sinensis*)	NF-κB pathway, JAK/STAT, PI3K/Akt, COX-1, 5-LOX, proteasome, CDK, dihydrofolate reductase, MMPs	RA, MS	[16,47–49]
Resveratrol	Stilbenoid	Plant (*Vitis vinifera, Polygonum cuspidatum*)	PI3K/Akt signaling, NF-κB pathway, MAPK signaling, 5-LOX, COX-1, Nrf2, PPARy, AMPK, Wnt/p-catenin signaling, SIRT signaling	Diabetes, metabolic disorders, coronary artery disease, skin diseases	[16,50–52]
Quercetin	Flavonol	Plant (Many fruits, vegetables, grains)	PI3K/Akt signaling, MAPK signaling, NF-κB pathway, JAK3, P-catenin signaling, CDK, serine/threonine protein kinases, PDGF receptor	OA, RA, prostatitis	[16,53–56]
Capsaicin	Capsaicinoid	Plant (*Capsicum sps*)	TRPV1 agonist	Local pain, OA	[57,58]
Myriocin (ISP-1)	Amino fatty acid	Fungus (*Mycelia sterilia, Isaria sinclairii*)	Serine palmitoyltransferase, T cell proliferation, immunosuppression		[59]
Fingolimod (FTY720)	Modified myriocin	Based on myriocin	Phosphorylated drug acts as an agonist of G protein-coupled receptor for sphingosine-1-phosphate (S1P) and blocks egress of lymphocytes from thymus and secondary lymphoid tissues, immunosuppression	MS	[60,61]

Mizoribine (MZB)	Imidazole nucleoside	Fungus (*Eupenicillium brefeldianum*)	Inosine monophosphate (IMP) dehydrogenase and guanosine monophosphate (GMP) synthetase, purine biosynthesis, T- and B-cell proliferation, immunosuppression.	RA, SLE, and other rheumatic and renal diseases	[62,63]
Cyclosporine A (CsA)	Cyclic peptide (with a single D-amino acid)	Fungus (*Tolypocladium inflatum*)	Calcineurin inhibitor, NFAT, IL-2, T-cell activation, immunosuppression	Organ transplant rejection, psoriasis, RA, keratitis, uveitis	[64–69]
Tacrolimus (FK506)	Macrolide lactone	Bacteria (*Streptomyces tsukubaensis*)	Calcineurin inhibitor, NFAT, IL-2, T-cell activation, immunosuppression	Organ transplant rejection, inflammation associated with ulcerative colitis, eczema	[67,69,70]
Sirolimus (Rapamycin)	Macrocyclic lactone	Bacteria (*Streptomyces hygroscopicus*)	mTOR, IL-2, T- and B-cell activation, immunosuppression	Organ transplant rejection, ocular diseases, atopic dermatitis	[71,72]
Mycophenolic acid	Small molecule, antibiotic	Fungus (*Penicillium Roquefort* and other penicillium species	Purine nucleotide biosynthesis, IMP dehydrogenase, T- and B-cell proliferation, immunosuppression	Organ transplant rejection, pemphigus vulgaris, psoriasis,	[73,74]
Mycophenolate mofetil (MMF)	Mofetil ester of mycophenolic acid	Based on mycophenolic acid		SLE	[75–77]

Examples of lead compounds in their native or derivatized/modified form that have proved beneficial following preclinical and clinical testing are listed with their source, chemical composition, molecular targets, and therapeutic applications. (*Abbreviations*: AMPK, Activated protein kinase; CDKs, Cyclin-dependent kinases; COX, cyclooxygenase; ERK, extracellular signal-regulated kinase; HIF, hypoxia-inducible factor; IBD, inflammatory bowel disease; IL-2, interleukin 2; iNOS, inducible nitric oxide synthase; JAK, janus kinase; JNK, c-Jun N-terminal kinase; LOX, lipoxygenase; MAPK, mitogen-activated protein kinase; MMPs, matrix metalloproteases; MS, multiple sclerosis; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; NOS, nitric oxide synthase; Nrf-2, nuclear factor erythroid 2-related factor 2; OA, osteoarthritis; PI3K, phosphatidylinositol-3-kinase; p-JNK, phospho c-Jun N-terminal kinase; PDGF, Platelet-derived growth factor; PPAR, Peroxisome proliferator-activated receptor; RA; rheumatoid arthritis; SLE, systemic lupus erythematosus; STAT, signal transducer and activator of transcription 3; Th17, T helper 17; TNFα, tumor necrosis factor alpha; TRPV1, transient receptor potential vanilloid receptor; Treg, regulatory T cell.)

Table 65.2 Medicinal herbs, nutraceuticals, and endogenous products possessing anti-inflammatory activity.

Natural product	Active component(s) identified	Targets: cellular/ molecular mediators and pathways	Therapeutic applications	References
A. Extracts of medicinal herbs				
Tripterygium wilfordii Hook F (Thunder God Vine)	Triptolide, Celastrol	NF-κB pathway, MAPK signaling, iNOS	RA, SLE, other inflammatory diseases	[78–83]
Boswellia serrata (Indian frankincense)	Boswellic acids	5-LOX, proinflammatory cytokines	RA, OA, bursitis, tendinitis inflammatory bowel disease (IBD), asthma	[84–86]
Urtica dioica (Stinging nettle)	Flavonoid glycosides, terpenoids	PLA2, NF-κB pathway	OA, RA, colitis, allergies	[86,87]
Harpagophytum procumbens (Devil's claw)	Harpagoside, harpagide	NF-κB, MAPK signaling, COX-2, NO	OA, back pain	[86,88,89]
Uncaria tomentosa (Cat's claw)	Mitraphylline	NF-κB pathway, proinflammatory cytokines, antioxidant	OA	[90,91]
Rosa canina (Dog rose)	Carotenoids, organic acids	COX-1 and COX-2	OA	[92,93]
B. Commonly used nutraceuticals				
Curcuma longa (Turmeric)	Curcumin	NF-κB, MAPK signaling, JAK/ STAT, arachidonic acid pathway, and iNOS	RA, OA, other autoimmune diseases	[43,86,94]
Zingiber officinale (Ginger)	Zingerone, gingerol	NF-κB pathway, COX-2	OA and RA	[95,96]
Fish oil	Omega-3 fatty acids	Inflammatory cytokines and prostaglandins	RA, SLE, psoriasis, other inflammatory conditions	[91,97,98]
Perna canaliculus (New Zealand Mollusc)	Omega-3 fatty acids	5-LOX, Leukotrienes production	RA and OA	[91,99,100]

Table 65.2 (*Continued*)

Natural product	Active component(s) identified	Targets: cellular/ molecular mediators and pathways	Therapeutic applications	References
C. Naturally occurring compounds in the body				
S-adenosyl-L-methionine	S-adenosyl-L-methionine	ERK and STAT3 pathway	OA, fibromyalgia, bursitis, tendinitis, chronic low back pain	[101,102]
Dehydroepiandrosterone	Dehydroepiandrosterone	Pro-inflammatory cytokines	SLE, RA, MS	[103–105]
Melatonin	Melatonin	NF-κB pathway, MAPK signaling, HIF, Nrf2 signaling	Fibromyalgia, irritable bowel syndrome (IBS)	[106,107]

Examples of different categories of natural products that have anti-inflammatory effects are listed along with their source, chemical composition, molecular targets, and therapeutic applications. Abbreviations are same as in Table 65.1.

65.2.3.3 The Janus Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) Pathway

Inflammatory signals from the activated NF-κB and MAPK pathways result in the production of various effector molecules, including cytokines. The binding of certain cytokines to their receptors rapidly transduces signals through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway. JAK enzymes are associated with the cytoplasmic domain of cytokine receptors. Cytokine-cytokine receptor interaction leads to phosphorylation of JAKs and STATs. Activated STATs form a dimer, and these dimers then translocate into the nucleus (Figure 65.2). Within the nucleus, the STATs differentially modulate the expression of specific cytokine-responsive genes [116]. The seven mammalian STATs have distinct roles [117], for example, in the differentiation of naïve T cells into a specific T-cell subset (e.g., Th1, Th2, Th17, and Treg). The inhibition of JAK-STAT pathway, particularly the Th17-related STAT3-dependent signaling, is important to control Th17-associated inflammation in autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), and others [118–120]. Several natural product-derived agents, including curcumin, sirolimus, epigallocatechin-3-gallate (EGCG), quercetin, and celastrol, have been shown to modulate the JAK/STAT pathway (Table 65.1) [121–126].

65.2.3.4 The Phosphatidylinositol-3-Kinase (PI3K)/Akt/Mammalian Target of Rapamycin (mTOR) Pathway

The PI3K/Akt/mTOR pathway is another regulator of events of cell metabolism and cell survival, and it plays a role in the pathogenesis of immune-mediated disorders [127–133]. This pathway can be activated by environmental changes, including growth factors, cellular stress, and other inflammatory stimuli. Furthermore, this pathway has been shown to regulate angiogenesis, cell proliferation, and cell survival in cancer [134]; insulin resistance, adipogenesis, and T-lymphocyte activation in type 2 diabetes (T2D) [134]; and osteoclast differentiation, and survival and proliferation of synovial fibroblast-like cells in rheumatoid arthritis [135,136]. Several natural products such as sirolimus, EGCG, resveratrol, and quercetin have been shown to inhibit PI3/Akt/mTOR pathway, and this inhibition contributes to their anti-inflammatory and immunosuppressive/immunomodulatory activities (Tables 65.1 and 65.2).

65.2.3.5 The Arachidonic Acid Pathway

Prostaglandins, thromboxanes, and leukotrienes are biochemical mediators that act as short-lived hormones to induce inflammation and pain at the local site [137,138]. These mediators regulate vasodilation, platelet aggregation, and clot formation to increase inflammatory responses. Arachidonic acid is released by the action of activated phospholipase A2 (PLA2) on membrane phospholipids following mechanical trauma or tissue destruction (Figure 65.2). Arachidonic acid is then transformed into prostaglandins and thromboxanes by a series of enzymatic reactions following the action of cyclooxygenases (COX) [137]. Similarly, 5-lipoxygenase (5-LOX) activity results in the transformation of arachidonic acid into leukotrienes [139]. Two isoforms of COX, COX-1 and COX-2, have been identified. COX 1 is constitutively expressed in the gastrointestinal tract (GIT), whereas COX-2 is inducible and primarily expressed at sites of inflammation. This information has led to the development of selective inhibitors of COX-2 to limit some of the side effects associated with inhibitors of COX-1 only or of both enzymes [137,140]. Inhibition of the arachidonic acid pathway has the potential to suppress inflammation and pain by blocking the generation of downstream mediators [141]. Aspirin, an esterified compound of salicylic acid isolated from the bark of the Willow tree (*Salix Alba*), is a well-known drug that inhibits the synthesis of prostaglandins and thromboxanes. It irreversibly inhibits both COX-1 and COX-2, with a predominant effect on COX-1 compared with that on COX-2. Subsequently, several other inhibitors of arachidonic acid pathway have been found to be beneficial following preclinical/clinical testing (Tables 65.1 and 65.2).

65.2.3.6 Nitric Oxide (NO), Inducible Nitric Oxide Synthase (iNOS), and Inflammation

Free radicals are a group of highly reactive species, which can differentially display beneficial or deleterious effects depending on the environmental conditions [142]. NO is one of the free radicals and plays a role in inflammatory reactions [143]. NO is synthesized from the guanidino group of L-arginine during its conversion to L-citrulline by NO synthases (NOS) [144]. Under normal conditions, NO is continually generated by constitutively expressed endothelial cell NOS (eNOS)

and brain NOS (bNOS), and it has beneficial effects [145]. However, NO is produced at higher rates under inflammatory conditions by the activity of inducible NOS (iNOS), which is expressed in macrophages, neutrophils, granulocytes, and many other cells in response to proinflammatory signals [146]. NO produced under these conditions helps propagate inflammatory responses [147]. The role of NO in inflammatory conditions including autoimmune disorders has been studied extensively [148–150], and the inhibition of NO has been shown to mediate anti-inflammatory effects. Tacrolimus and cyclosporine A are examples of inhibitors of iNOS and NO production [151], and both these drugs have been shown to be effective in treating immune-mediated inflammation (Table 65.1). Similarly, other natural products such as *Curcuma longa*, *Tripterygium wilfordii* Hook F., and *Harpagophytus procambuns* have been shown to inhibit NO production (Table 65.2).

65.2.3.7 Other Pathways Associated with Inflammation

Inflammation alters multiple cellular functions as well as the response of cells to environmental conditions. Furthermore, there is a cross-talk between different cellular pathways that are operative concurrently in an inflammatory milieu. The pathways that mediate cell proliferation, cell migration, cell apoptosis, glucose metabolism, hypoxia, and oxidative stress also contribute, directly or indirectly, to the process of inflammation and in turn are affected by it. Therefore, the mediators and regulators of these pathways also constitute additional targets for therapeutic control of inflammation. Many of the natural products targeting such mediators are described below (Tables 65.1 and 65.2). For example, the nuclear factor erythroid 2–related factor 2 (Nrf2), which upregulates cytoprotective genes to restore redox homeostasis, is regulated by curcumin and resveratrol [152,153]; cyclin-dependent kinases (CDKs), which regulate cell cycle, are targeted by EGCG [154]; and serine palmitoyltransferase (SPT), which is involved in sphingolipid metabolism, is inhibited by Myriocin [59].

65.3 Natural Products from Diverse Sources Possess Anti-Inflammatory Activity

Inflammation is an integral component of the pathological processes in a variety of diseases. During inflammation, there is a cross-talk among many of the effector molecules produced in different pathways described above, and these interplays can further augment inflammation. In this regard, natural products and synthetic compounds that can modulate one or more of these pathways have been used as anti-inflammatory agents for the control of inflammation and management of symptoms associated with it. Most natural products that have been used for medicinal purposes for their anti-inflammatory activity have originated from plants, terrestrial microorganisms such as fungi and bacteria, and marine animals. Such products include purified bioactive compounds in their native state or in synthetically modified forms (Table 65.1), extracts of medicinal herbs (Table 65.2A), commonly used nutraceuticals (Table 65.2B), and those that occur naturally in the body (Table 65.2C). Collectively, the chemical structures of

the major bioactive principles that endow anti-inflammatory activities to these natural products are vastly diverse. These natural products have been used for the treatment of a variety of chronic diseases including, inflammatory and autoimmune diseases (e.g., rheumatoid arthritis (RA), multiple sclerosis (MS), systemic lupus erythematosus (SLE) (also known as lupus), psoriasis, pemphigus vulgaris, uveitis); pain and swelling associated with other rheumatic diseases (osteoarthritis (OA), chronic low back pain, tendinitis, bursitis, fibromyalgia); inflammation and related immune-mediated events in organ transplant rejection (e.g., liver or kidney transplant), inflammatory bowel disease (IBD), colitis; other conditions associated with inflammation (e.g., dermatitis, prostatitis, keratitis); and other diseases such as T2D, metabolic disorders, irritable bowel syndrome, cardiovascular disease, and cancer.

The above-mentioned examples represent the potentially broad scope of the use of natural products, in one form or another, as anti-inflammatory agents. Of these products, synthetically modified compounds based on natural compounds (e.g., aspirin from salicylic acid, fingolimod from myriocin, mycophenolate mofetil from mycophenolic acid) and the unmodified bioactive compounds derived from natural products (e.g., cyclosporine A, tacrolimus, sirolimus, mizoribine) are already approved drugs for therapeutic use (Table 65.1). Another group of natural compounds (e.g., curcumin, resveratrol, EGCG, quercetin, capsaicin) includes promising anti-inflammatory drug candidates (Table 65.1). They have extensively been tested in clinical studies by different investigators. However, additional scientifically controlled clinical trials and comparative studies with established mainstream anti-inflammatory drugs are required before one or more of these candidates are approved for therapeutic use. Similarly, besides extensive experience through the traditional systems of medicine (e.g., TCM and Ayurveda), the extracts of medicinal herbs (e.g., *T. wilfordii* Hook F, *Boswellia serrata*, *Urtica dioica*, *Harpagophytum procumbens*, *Uncaria tomentosa*, *Rosa canina*) have been subjected to extensive testing in several clinical trials for their efficacy and safety (Table 65.2A). The results of these trials have been promising, yet additional studies are warranted before approval is granted for therapeutic use of one or more of these herbal products. For example, *T. wilfordii* Hook F (Thunder God Vine) has shown promising results in patients with rheumatoid arthritis, and the effects of this herbal preparation were comparable to, or better than, those of the mainstream drugs sulfasalazine and methotrexate [78–81,155]. However, the toxicity profile still remains a concern for the therapeutic use of this product.

The remaining groups of natural products (Table 65.2B and C) being explored for their anti-inflammatory activity include commonly used nutraceuticals (e.g., turmeric and ginger that are used as spices in food; fish oil; and New Zealand mollusk), and naturally occurring substances (e.g., hormones such as dehydroepiandrosterone and melatonin, and other compounds such as S-adenosylmethionine). (Turmeric contains *Curcumin longa*, the source of curcumin discussed above.) These products are widely consumed for general maintenance of health and prevention of inflammation, but additional controlled studies are needed to fully assess their utility for the treatment of inflammatory and immune-mediated diseases.

65.4 Technological Advancements in the Production, Characterization, and Delivery of Natural Products

Using a typical conventional scheme, the processing of a plant extract involves drying, grinding, boiling, and distillation or sublimation. The mixture of compounds thus obtained is subjected to fractionation using high-performance liquid chromatography (HPLC) and mass spectrometry (MS). Select compounds are then tested for their functional activity using bioassays. Bioassays are used for the detection, isolation, and functional characterization of new or unknown compounds in a mixture [2,12,156]. Such bioassays include those that measure enzyme activity, the activity of transcription factors, or the interaction of specific molecules with inflammatory cells [156,157]. However, certain limitations exit. For example, extracts of some natural products may emit endogenous fluorescence or possess certain components that may bind to proteins and form complexes with them, or denature them, and thereby interfere with the readout of the bioassays [158]. Following functional validation, a pure compound of interest can be subjected to nuclear magnetic resonance (NMR) to elaborate its structural features. Technological advances have significantly improved the efficacy of isolation and purification of natural compounds from a crude extract of a natural product (Figure 65.1) [2,5,12].

65.4.1 Semisynthesis, Combinatorial Biosynthesis, and Metabolic Engineering

Isolation of bioactive compounds from natural sources such as plants is influenced by soil conditions and seasonal variations. Depending on the structural complexity, the synthesis of a compound originally derived from a natural product can be undertaken in the laboratory using "total synthesis" or "semisynthesis" (partial synthesis) method. The latter method uses pre-existing natural product intermediates as templates for generating the final products that replicate the original, functional natural product-derived compounds. These synthetic approaches have successfully been used for the production of a variety of natural products, for example, rottnestol and herboxidiene [159], alkyne-containing jadomycin [160], aeruginosin [161,162], and epicoccin [163].

Combinatorial biosynthesis and metabolic engineering represent advanced techniques to produce complex organic compounds using genetically modified organisms (GMO), such as bacterial strains that have been genetically engineered to produce sets of compounds. Combinatorial biosynthesis is aimed at producing a library of structurally diverse compounds, including hybrid structures, by genetically introducing into an organism a set of genes, or sets of different combinations of genes, from specific biosynthetic pathways [164,165]. Furthermore, deliberate modifications introduced into enzymatic domains and the use of different enzyme-substrate combinations can produce structurally diverse compounds. Products of combinatorial biosynthesis are then subjected to screening procedures described below to identify functionally relevant compounds. In metabolic engineering, a similar approach is used to deliberately modify a particular biosynthetic

pathway for a structurally complex compound to generate a single compound, or few variants of that compound, with functional attributes that represent improvements over the original natural compound. Another application of this approach is to enhance the yield of a known product. The leads for targeting particular pathways or enzymes are derived from mathematical modeling of chemical networks of biosynthetic pathways and by identifying sites where changes are required. Thereafter, appropriate genetic modifications are introduced to alter the reaction pathway or to overcome any constraints.

Synthesis of alkynes such as polyketides [166,167] and terpenoids such as tetrahymanol [168] has been achieved using above methods. It is anticipated that these approaches would permit biosynthesis of several natural products of medicinal value, such as resveratrol, curcumin, paclitaxel, and artemisinin, in microbial hosts, in large amounts and in a consistent manner to minimize batch-to-batch variations for clinical testing and eventual therapeutic use.

65.4.2 High-Throughput Screening (HTS) and Virtual Screening

HTS, a laboratory-based method, uses automation to simultaneously examine a collection (library) of a large number of compounds against a set of defined targets with the purpose of identifying compounds that induce inhibition or activation of a specific biological target, for example an enzyme or a cell-surface receptor [169–171]. In addition, such a screening procedures can be used to determine the pharmacokinetic and pharmacodynamic attributes of a compound. *In vitro* and cell-based bioassays and microarray analysis are among the commonly used methods for screening of natural products and for defining their mechanism of action. Virtual screening, an *in silico* approach using defined computer algorithms for protein docking or ligand-protein interactions, is now increasingly being used in conjunction with, or in place of, actual laboratory-based screening of a large number of compounds of interest [2,8]. Compounds identified via virtual screening are then validated using appropriate *in vitro* and *in vivo* laboratory methods.

65.4.3 Microarrays

Microarray technology platform can also be utilized as a bioassay, besides its use for high throughput screening or biosynthesis. Microarrays use a chip-based platform to test the reactivity of a biological sample with a vast number of molecules (proteins, DNA or RNA) in a single screen. The readouts of such assays can directly or indirectly provide information about the expression level of RNA/DNA or the measurement of the biological activity of proteins, for example enzyme activity [172], anti-mitotic effects [170], or toxicological profile [171].

65.4.4 Systems Pharmacology

Systems pharmacology extends the application of the principles of systems biology to the study of drug actions, responsiveness to drugs, adverse effects of drugs, and identification of new targets (Figure 65.1). These aspects are examined in the context of network of interactions of drugs with other molecules

(e.g., proteins, genes, signaling components, etc.) within cells; with different types of cells within an organ; with different organs; and with the whole organism. These analyses use computational networks in combination with functional readouts of cellular metabolites (metabolomics), transcripts (transcriptomics), genes (genomics), or proteins (proteomics) [173–176]. In addition, bioinformatics and statistics are integral parts of these interpretations. Besides advancing our knowledge of drug actions, systems pharmacology can contribute significantly to better understanding of differences in therapeutic efficacy as well as risk of adverse effects, and to developing polypharmacy for complex diseases, including inflammatory and autoimmune disorders.

65.4.5 Use of Nanotechnology to Deliver Natural Products for Therapeutic Purposes

Nanoparticles are of nanoscale (up to 100 nm) size and are used to deliver hydrophilic drugs, hydrophobic drugs, proteins, and other biological products [177]. Nanoparticles are comprised of a drug tagged with a polymer and a targeting ligand, which together help in the targeting, delivery, solubility, bioavailability, and *in vivo* stability of that drug (Figure 65.1) [178–180]. Poly (lactic-co-glycolic acid) (PLGA) and polyglycolic acid (PGA) are examples of polymers that are often used as carriers of drugs [177,181]. PLGA has been approved by the Food and Drug Administration (FDA) for use with a therapeutic agent. The polymers can be designed so as to make them responsive to pH, temperature, or specific metabolites to further optimize drug delivery [177,181]. The main objective of developing nanoparticles for the delivery of natural products include improved targeting and delivery, enhanced bioavailability, reduced toxicity, and maintained or enhanced therapeutic effects [178–180]. Several natural products possessing anti-inflammatory properties have been formulated as nanoparticles, and these include, curcumin, EGCG, *Tripterygium wilfordii*, triptolide, quercetin, and ginger [180,182–185].

65.5 Concluding Remarks

The period between the 1940s and 1960s represented the "golden age" of drug discovery from natural products [5]. By 1990, approximately 80% of drugs for various categories of diseases had been estimated to be from natural products or their analogs [4]. However, over the past two decades, there has been a gradual decline in interest and effort on the part of major pharmaceutical companies in the process of drug discovery based on screening of natural products. Nevertheless, over 100 drugs based on natural products are being tested in clinical studies for many different diseases, and about 18 are at different stages of development for inflammation and related diseases [2,4,11]. Hopefully advancements in technology and applications of *in silico* approaches; expansion of the search of natural product-based drugs to marine microorganism and other underexplored microbes; and the recognition of the contribution of natural products research to drug discovery by the 2015 Nobel

Prize in Physiology and Medicine would reinvigorate the field of natural product drug discovery [4,5,16].

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