3 CHAPTER 3 Neutrophils and Their Products

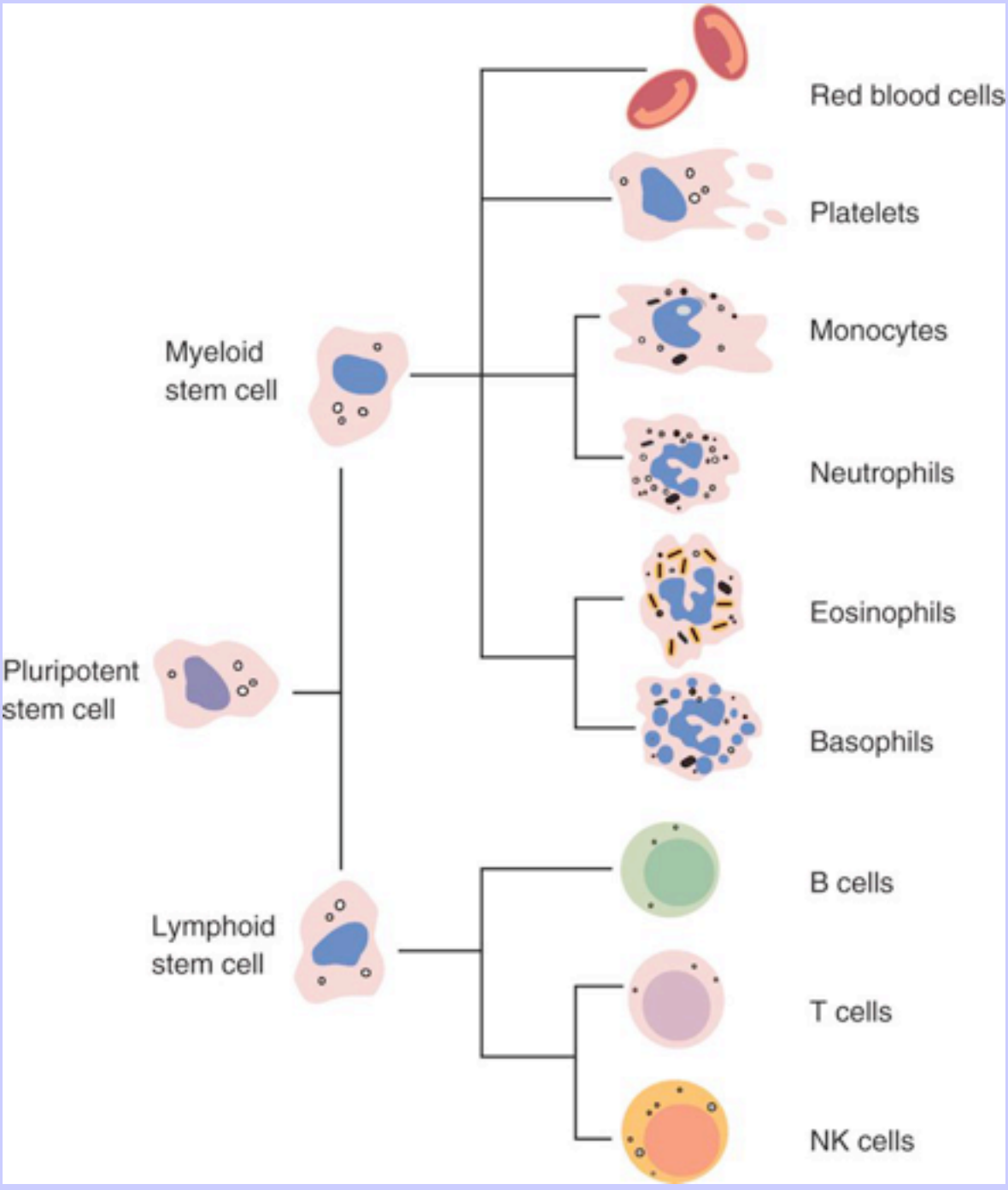
3.1 KEY POINTS

- The major cell type attracted to sites of inflammation is the neutrophil.
- Inflammatory cytokines activate vascular endothelial cells so that neutrophils in the bloodstream will adhere to them before migrating towards sites of microbial invasion and tissue damage.
- Neutrophils will bind and phagocytose invading microorganisms.
- Microorganisms need to be opsonized before they can be efficiently ingested and killed. The most effective opsonins are antibodies and complement.
- Ingested microorganisms are killed by potent oxidants through a process called the respiratory burst, by antibacterial proteins called defensins and by lytic enzymes.
- Neutrophils are short-lived cells that cannot undertake prolonged or multiple phagocytosis.

Although physical barriers such as the skin exclude many organisms, such barriers are not impenetrable, and microbial invaders often gain access to body tissues. These invaders must be promptly attacked and destroyed. Some are killed by antimicrobial peptides or complement, but many are eaten and killed by cells. This eating of microbes by cells is called phagocytosis (Greek for “eating by cells”). Phagocytosis is central to the whole inflammatory process.

The defensive cells of the body circulate in the bloodstream, where they are called leukocytes (white cells). The blood cells of mammals derive from myeloid stem cells located in the bone marrow (*myelos* is Greek for “bone marrow”) (Figure 3-1). All types of leukocyte originate from myeloid stem cells, including neutrophils, monocytes, lymphocytes, and dendritic cells, and all help defend the body. Two types of leukocytes are specialized for killing and eating invading microorganisms. These cells, called neutrophils and macrophages, originate from a common stem cell but look very different and have different, but complementary, roles. Thus neutrophils respond and eat invading organisms very rapidly but are incapable of sustained phagocytic effort. Macrophages, in contrast, move more slowly but are highly effective phagocytes and are capable of repeated phagocytosis. In this chapter we will review the properties of neu

FIGURE 3-1 The origin of cells from the bone marrow. Note that lymphoid cells originate from different stem cells than the cells of the myeloid system. Note too that cells such as eosinophils and basophils are probably closely related despite significant morphological differences.



trophils and their role in inflammation and innate immunity.

3.2 LEUKOCYTE CLASSIFICATION

Examination of a stained blood smear reveals that there are several different types of leukocyte. Some leukocytes have a cytoplasm filled with granules; these are called granulocytes (Figure 3-2). Granulocytes have characteristic lobulated, irregular nuclei and are thus described as “polymorphonuclear” (as opposed to the single, rounded nuclei of “mononuclear” cells such as macrophages). Granulocytes are classified into three populations based on the staining pro-perties of their granules. Cells whose granules take up basic dyes such as hematoxylin are called basophils; those whose granules take up acidic dyes such as eosin are called eosinophils; and those that take up neither basic nor acidic dyes are called neutrophils. All play important roles in the defense of the body.

3.3 NEUTROPHILS

The major cell blood leukocyte is the polymorphonuclear neutrophil granulocyte, otherwise called the neutrophil (Figure 3-3). Neutrophils are formed from stem cells in the bone marrow at a rate of about 8 million per minute in humans, migrate to the bloodstream, and about 12 hours later move into the tissues. They die after a few days and must therefore be constantly replaced. Neutrophils constitute about 60% to 75% of the blood leukocytes in most carnivores but only about 50% in the horse and 20% to 30% in cattle, sheep, and laboratory rodents. There are two pools of neutrophils in blood: a circulating pool and a pool of cells sequestered in capillaries. During bacterial infections the numbers of circulating neutrophils may increase tenfold as they are released from the bone marrow and the sequestered pool.

Toll-like receptors are expressed on myeloid stem cells. Binding to these receptors, microbial pathogen-associated molecular patterns such as lipopolysaccharides (LPS) trigger them to produce more

FIGURE 3-2 Differentiation and nomenclature of the cells found in blood. Thus leukocytes are first differentiated on the basis of their nuclear shape. Polymorphonuclear cells are then differentiated on the basis of their granule staining. Lymphocytes and macrophages are differentiated on the basis of nuclear shape and extent of cytoplasm. Note that it is not possible to differentiate the different subpopulations of lymphocytes on the basis of their morphology.

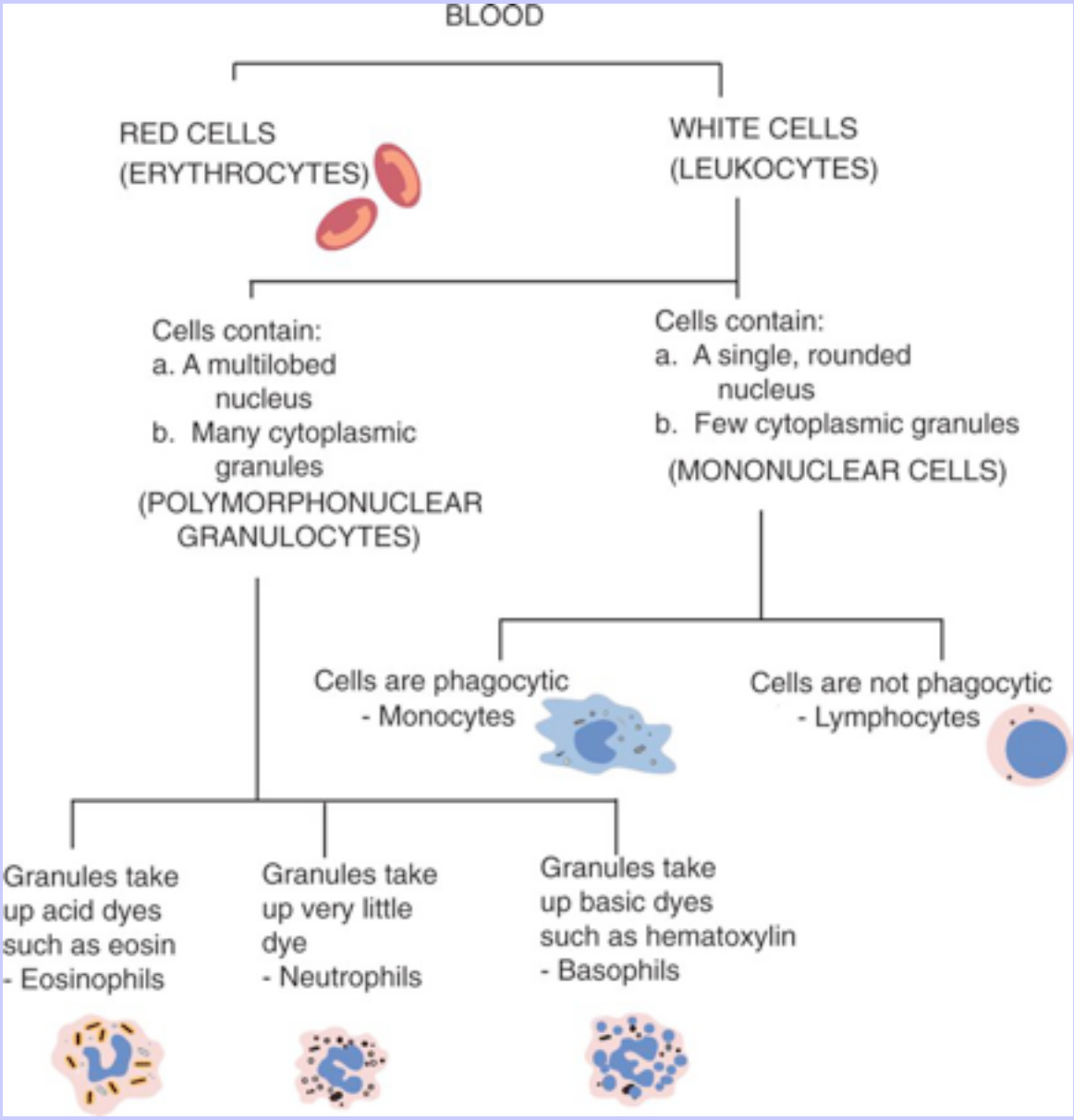
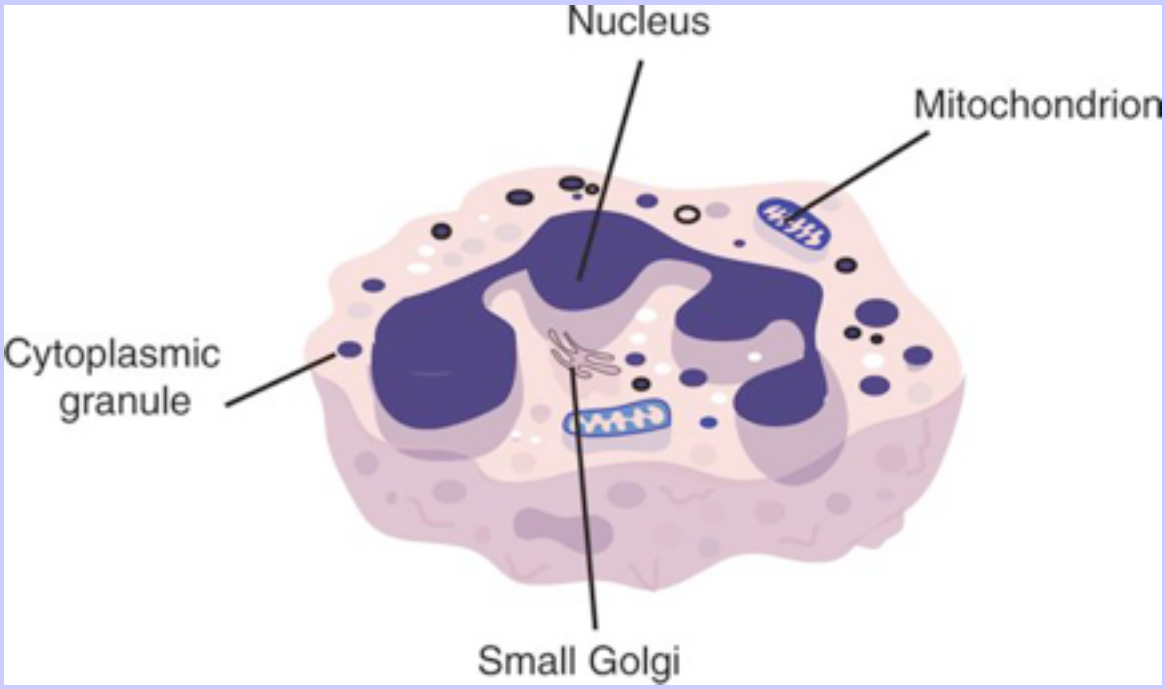


FIGURE 3-3 Major structural features of a neutrophil.



neutrophils. Toll-like receptors thus provide a pathway whereby the cells of the innate immune system may be rapidly replenished in response to infections.

3.3.1 Structure

Neutrophils suspended in blood are round cells about 10 to 20 μm in diameter. They have a finely granular cytoplasm at the center of which is an irregular sausagelike or segmented nucleus (Figure 3-4). Because the chromatin in the nucleus is compacted, neutrophils cannot divide. Electron microscopy shows many different types of enzyme-rich granules in their cytoplasm (Figure 3-5). Some of these granules contain enzymes such as myeloperoxidase, lysozyme, elastase, β-glucuronidase, and cathepsin B. Other granules lack myeloperoxidase but contain lysozyme and collagenase and the iron-binding protein lactoferrin. Mature neutrophils have a small Golgi apparatus, some mitochondria, and a few ribosomes or rough endoplasmic reticulum.

3.4 CHANGES IN VASCULAR ADHERENCE

Neutrophils are normally confined to the bloodstream and circulate with the other blood cells. If they are to defend tissues against a microbial invasion, they must leave the bloodstream. In normal tissues, neutrophils are carried along by the flow, like other blood cells. In

FIGURE 3-4 Neutrophils in peripheral blood smears. **A,** Horse. **B,** Cat. **C,** Dog. These cells are about 10 µm in diameter. Giemsa stain. (Courtesy Dr. M.C. Johnson.)

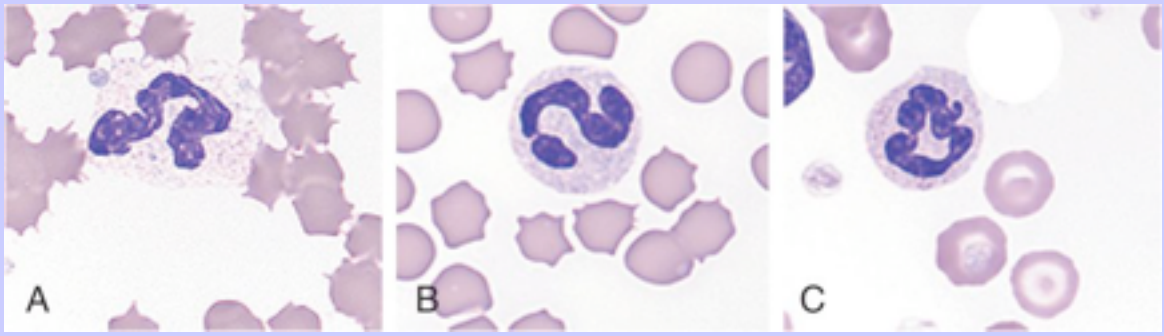


FIGURE 3-5 Transmission electron micrograph of a rabbit neutrophil. Note the two lobes of the nucleus and the granule-filled cytoplasm. (Courtesy Dr. S. Linthicum.)

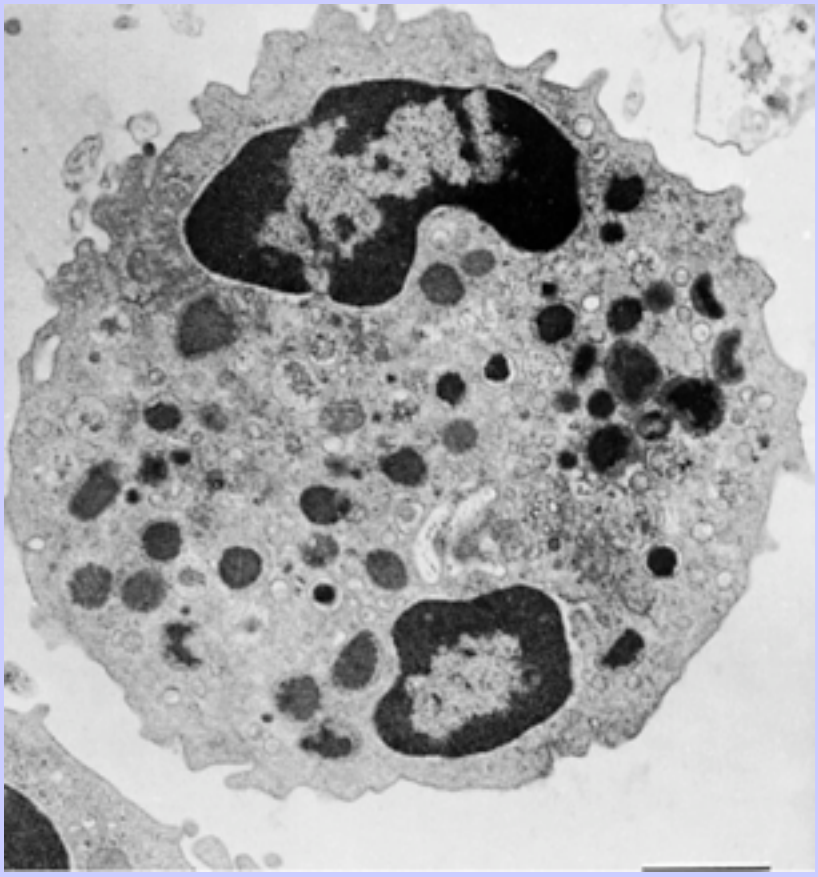
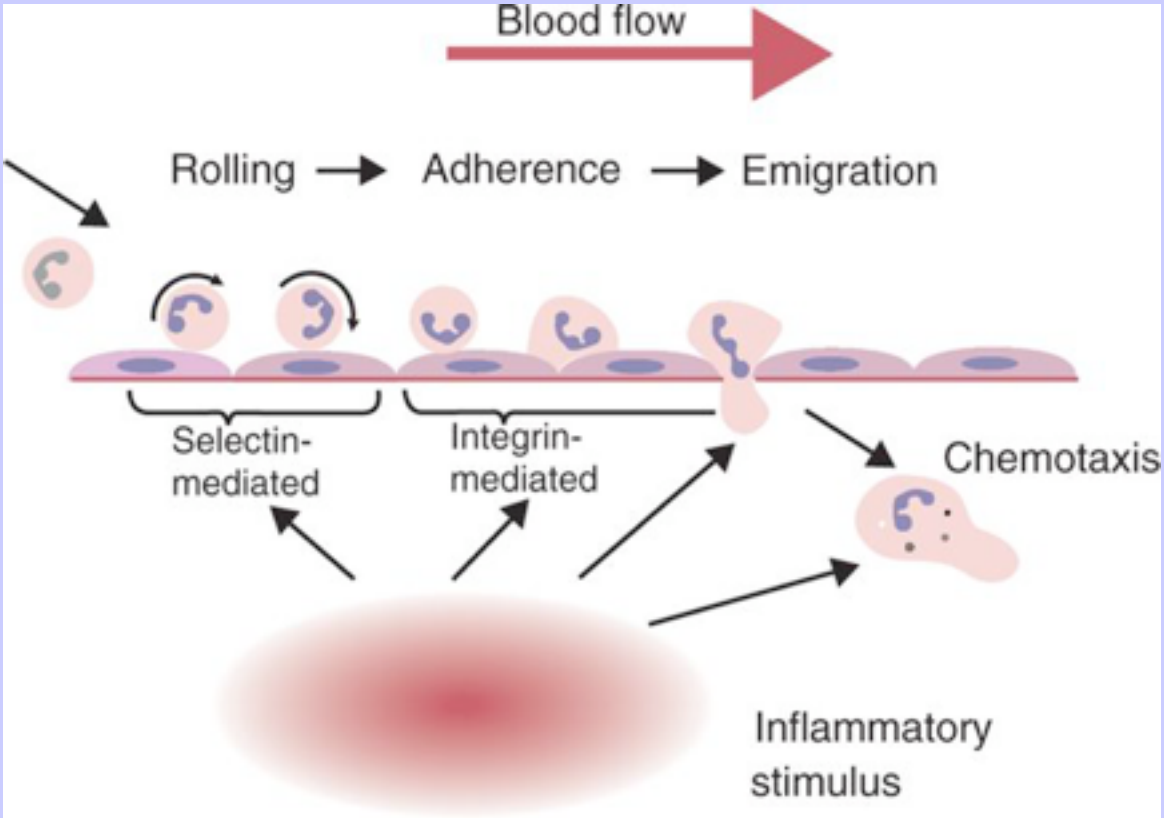


FIGURE 3-6 Stages of neutrophil adhesion and emigration from blood vessels. Selectins on endothelial cells tether neutrophils and stimulate them to roll. When they come to a halt, integrins bind them firmly to vascular endothelial cells and signal them to emigrate into tissues.



inflamed tissues these fast-moving cells must slow down, stop, bind to blood vessel walls, and then leave blood vessels by emigration through the vessel walls. This emigration is triggered by changes both in the endothelial cells that line blood vessel walls and in the neutrophils themselves.

3.4.1 Changes in Endothelial Cells

Bacterial products such as LPS, or alarmins from damaged tissues such as thrombin or histamine, cause capillary endothelial cells to express a glycoprotein called P-selectin (CD62P). P-selectin is normally stored in granules, but it moves to the endothelial cell surface within minutes after cell stimulation. Once expressed on the endothelial cell surface, the P-selectin binds to a protein called L-selectin (CD62L) on the surface of passing neutrophils. This binding is transient because the neutrophils readily shed their L-selectin. Nevertheless, the neutrophils gradually slow down and roll along the endothelial cell surface as they lose speed and eventually come to a complete stop (Figure 3-6).

3.4.2 Changes in Neutrophils

As the neutrophils roll along the endothelial surface, the second stage of adhesion occurs. Platelet-activating factor secreted by the endothelial cells activates the rolling neutrophils so that they express a protein called CD11a/CD18 or LFA-1 (leukocyte function-associated antigen-1). LFA-1 is an adhesive protein or integrin, and it binds strongly to a glycoprotein called intercellular adhesion molecule-1 (ICAM-1 or CD54) expressed on the endothelial cells. This strong binding makes the neutrophil come to a complete stop and attaches it firmly to the vessel wall despite the shearing force of the blood flow. Adherent neutrophils also secrete small amounts of elastase. The elastase removes CD43 (leukosialin), an antiadhesive protein, from the neutrophil surface, which allows

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FIGURE 3-7 Simplified view of the proteins and their ligands engaged in neutrophil-vascular endothelial cell binding. Selectins are carbohydrate-binding proteins that bind other glycoproteins through a carbohydrate called sialyl Lewis^X (SLe^X). This selectin-mediated binding is weak and temporary. Subsequently integrins on leukocytes, especially CD11a/18, bind strongly to their ligand intercellular adhesion molecule-1 (ICAM-1) on vascular endothelial cells.

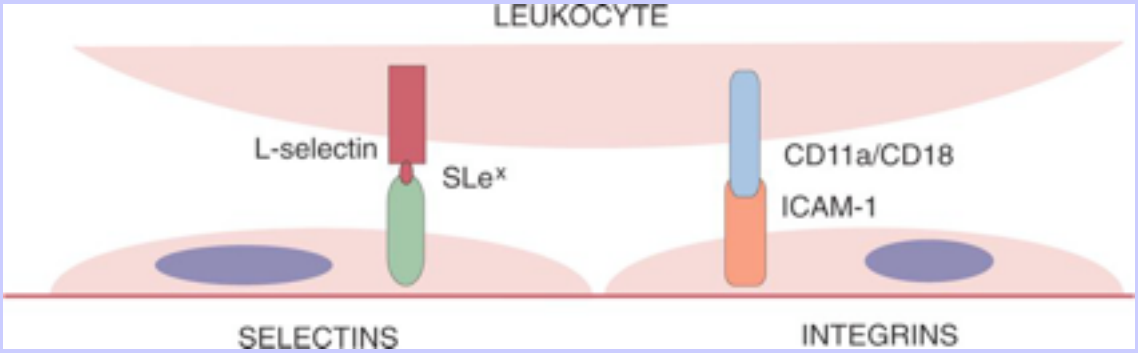
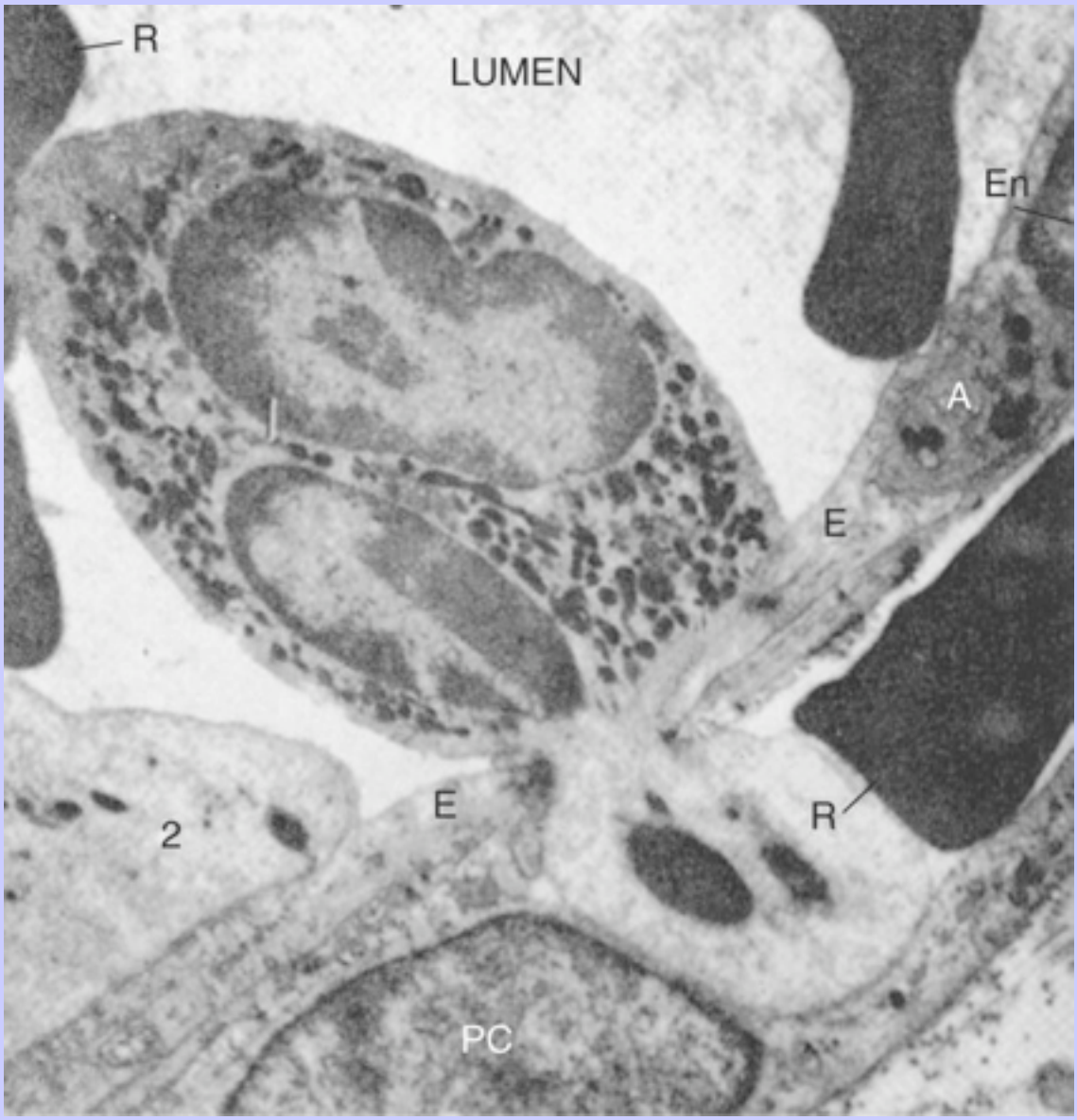


FIGURE 3-8 Inflamed venule of a rat. Cell 1 is a neutrophil pushing its way through a capillary wall to reach the surrounding tissues. R, Red blood cells; E, endothelium; PC, periendothelial cell; cells 2 and 3 are also neutrophils. (From Marchesi VT, Florey HW: Q J Exp Physiol 45:343, 1960.)



the neutrophils to bind to the endothelial cells even more strongly.

A third stage of increased leukocyte–endothelial cell adhesion takes several hours to develop and is mediated by cytokines and chemokines. Thus endothelial cells activated by interleukin-1 (IL-1), interleukin-23 (IL-23), or

tumor necrosis factor-α (TNF-α) express E-selectin (CD62E) (Figure 3-7), which enhances neutrophil adhesiveness even further. IL-1 and IL-23 also induce the production of the chemokine CXCL8 by endothelial cells, and this attracts still more neutrophils. TNF-α stimulates endothelial cells to secrete IL-1. It also promotes vasodilatation, procoagulant activity, and thrombosis, and increases both expression of cell adherence proteins and the production of chemotactic molecules.

3.4.3 Integrins

Many cell surface proteins make cells stick together, but the most important of these are the integrins. There are several families of integrins. Each consists of paired protein chains (heterodimers) using a unique a chain linked to a common β chain. For example, three β_2-integrins are found on neutrophils. The a chain, called CD11a, b, or c, is linked to a common β_2 chain (CD18). So these three integrins are called CD11a/CD18, CD11b/CD18, and CD11c/CD18. As described above, LFA-1 expressed by activated neutrophils binds to ICAM-1 expressed on capillary endothelial cells. CD11b/CD18 also binds leukocytes to endothelial cells and is a receptor for some components of the complement system (complement receptor 3 [CR3]) (see Chapter 5).

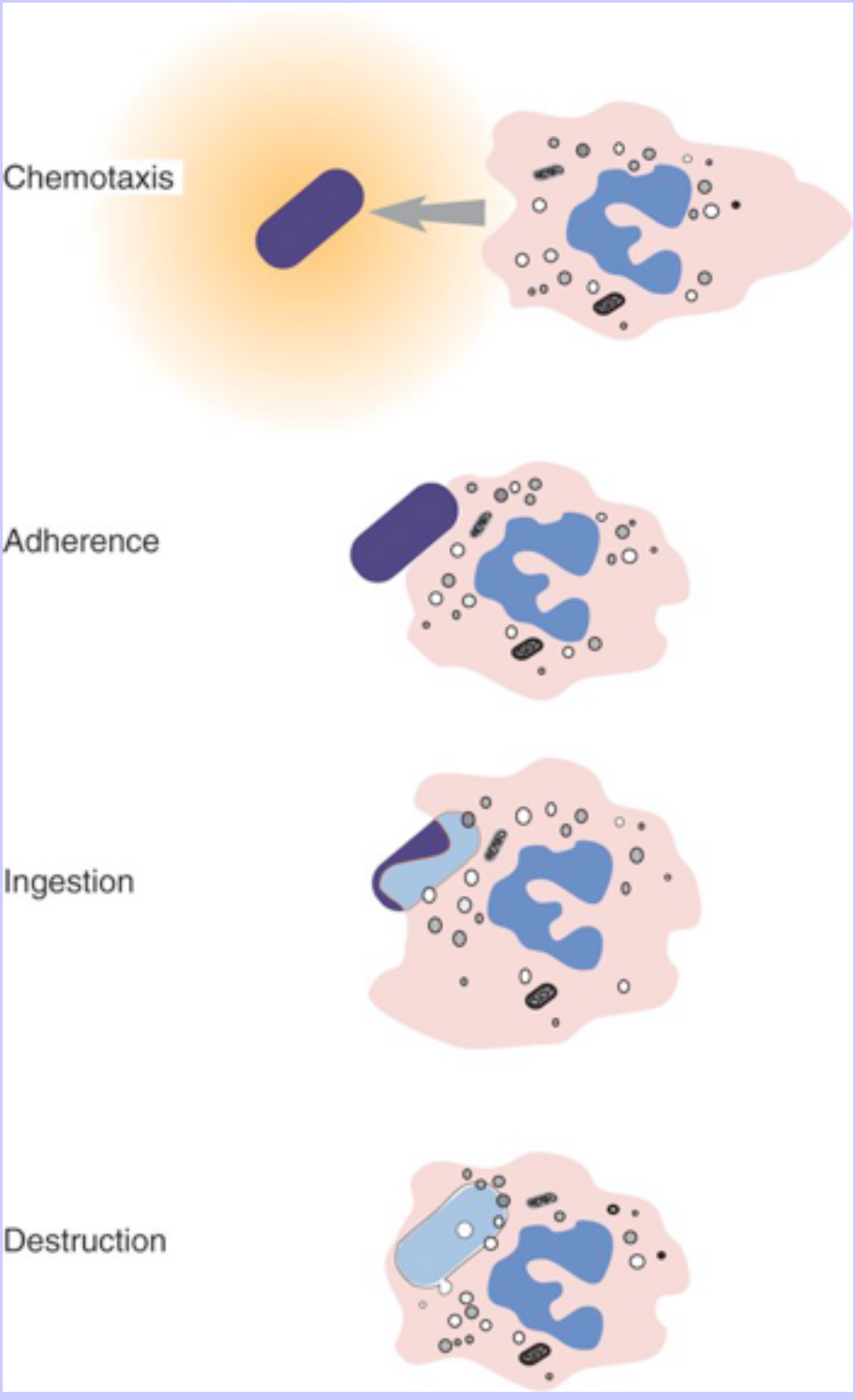
3.4.4 Emigration

After binding to blood vessel walls and coming to a complete stop, the neutrophils emigrate into the surrounding tissues under the influence of chemoattractants (Figure 3-8). The migrating neutrophils squeeze between the endothelial cells and the basement membrane. This process has been called diapedesis or transmigration. They then crawl towards any invading microbes. Since neutrophils are the most mobile of all the blood leukocytes, they are the first cells to arrive at the damaged tissues.

3.5 PHAGOCYTOSIS

Once they reach sites of microbial invasion, neutrophils eat and destroy foreign particles such as invading bacteria through phagocytosis. Although a continuous process, phagocytosis can be divided into discrete stages: activation, chemotaxis, adherence, ingestion, and destruction (Figure 3-9).

FIGURE 3-9 Different stages in the process of phago-cytosis.



3.5.1 Activation

Neutrophils attack and destroy invading organisms after they have become “activated.” Thus, when neutrophils receive the dual signal of integrin binding together with stimulation by TNF-α, CXCL8, or C5a, they secrete elastase, defensins, and oxidants. The elastase released promotes their adhesiveness. The oxidants activate tissue metalloproteases, which in turn cleave more TNF-α from macrophages. The TNF-α in turn attracts more neutrophils.

3.5.2 Chemotaxis

Neutrophils do not wander randomly but crawl directly towards invading organisms and damaged cells in a process called chemotaxis. Microbial invasion and the resulting tissue damage generate many different attractants. These include a peptide called C5a, generated by activation of complement (see Chapter 5); a peptide called fibrinopeptide B, derived from fibrinogen; and a peptide called azurocidin related to the defensins. Other chemoattractants include many different chemokines (see Chapter 2) and lipids such as leukotriene B_4. Invading bacteria release peptides with formylated methionine groups that are very attractive to the neutrophils of some mammals. Thus migrating neutrophils receive a multitude of signals attracting them to sites of invasion and tissue damage.

Not all animals have equally responsive neutrophils. For example, some cows with a specific genotype of the chemokine receptor CXCR2 show reduced neutrophil migration compared to cows with other genotypes. Cows with this specific genotype also show reduced expression of the integrin chains CD18 and CD11b and decreased resistance to mastitis (infections of the udder).

As chemotactic molecules diffuse from sites of a microbial invasion, they form a concentration gradient. When neutrophils detect these molecules, they crawl toward the area of highest concentration—the source of the material. The moving cells generate projections (lamellipodia) at their leading edge. Chemoattractant receptors are distributed over the neutrophil surface, but the formation of lamellipodia is driven by the higher concentration of attractants at the cell's leading edge.

3.5.3 Adherence and Opsonization

Once a neutrophil encounters a bacterium, it must “catch” it. This does not happen spontaneously, because both cells and bacteria suspended in body fluids usually have a negative charge (zeta potential) and so repel each other. The charge must be neutralized, which requires that the bacteria be coated with positively charged molecules. Molecules that coat bacteria in this way and so promote phagocytosis are called opsonins. This word is derived from the Greek word for “sauce,” implying perhaps that they make the bacterium “tastier” for the neutrophil. Examples of such charged molecules include innate molecules such as mannose-binding lectin and complement components and acquired molecules such as antibodies (see Chapter 14).

The surface of phagocytic cells is also adorned with many receptors that can recognize their ligands on the surface of infectious agents. These receptors may recognize a particle directly or recognize opsonins. Ingestion may or may not depend on opsonization. Thus neutrophils have some cell surface receptors such as mannose receptors or integrins that bind directly to bacteria.

Antibody receptor–mediated phagocytosis (or type I phagocytosis) is triggered by the binding of antibody-coated bacteria to antibody receptors on the neutrophil surface (Figure 3-10). CD32 is an example of such an antibody receptor. When antibody-coated microbes

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FIGURE 3-10 Opsonization of a bacterium by antibodies and complement. The combination of these ligands with their appropriate receptors triggers ingestion and the respiratory burst. The antibody receptor is called CD32, and the complement receptor is called CD35. Type 1 phagocytosis is mediated by antibodies through CD32. Type 2 phagocytosis is mediated by complement through CD35.

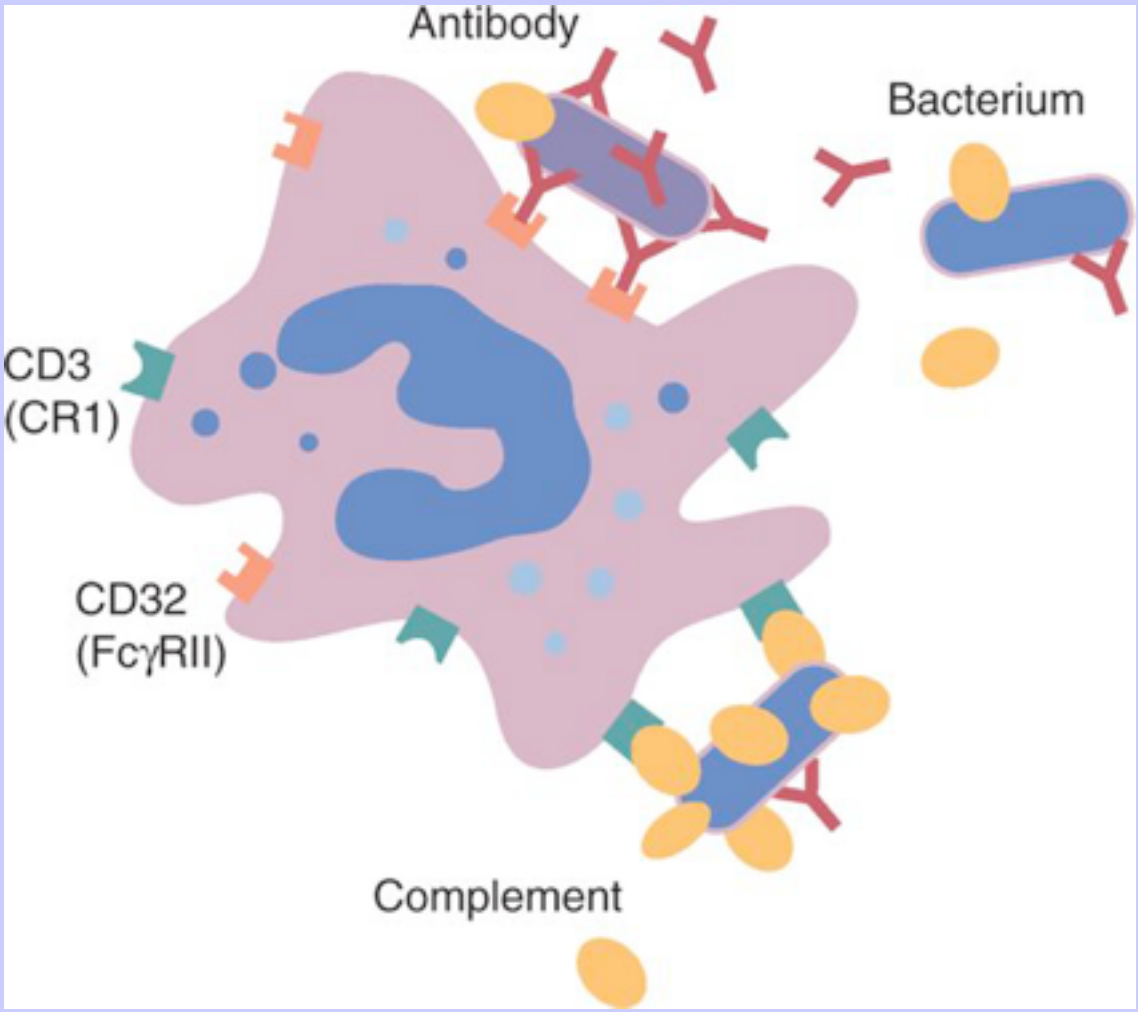
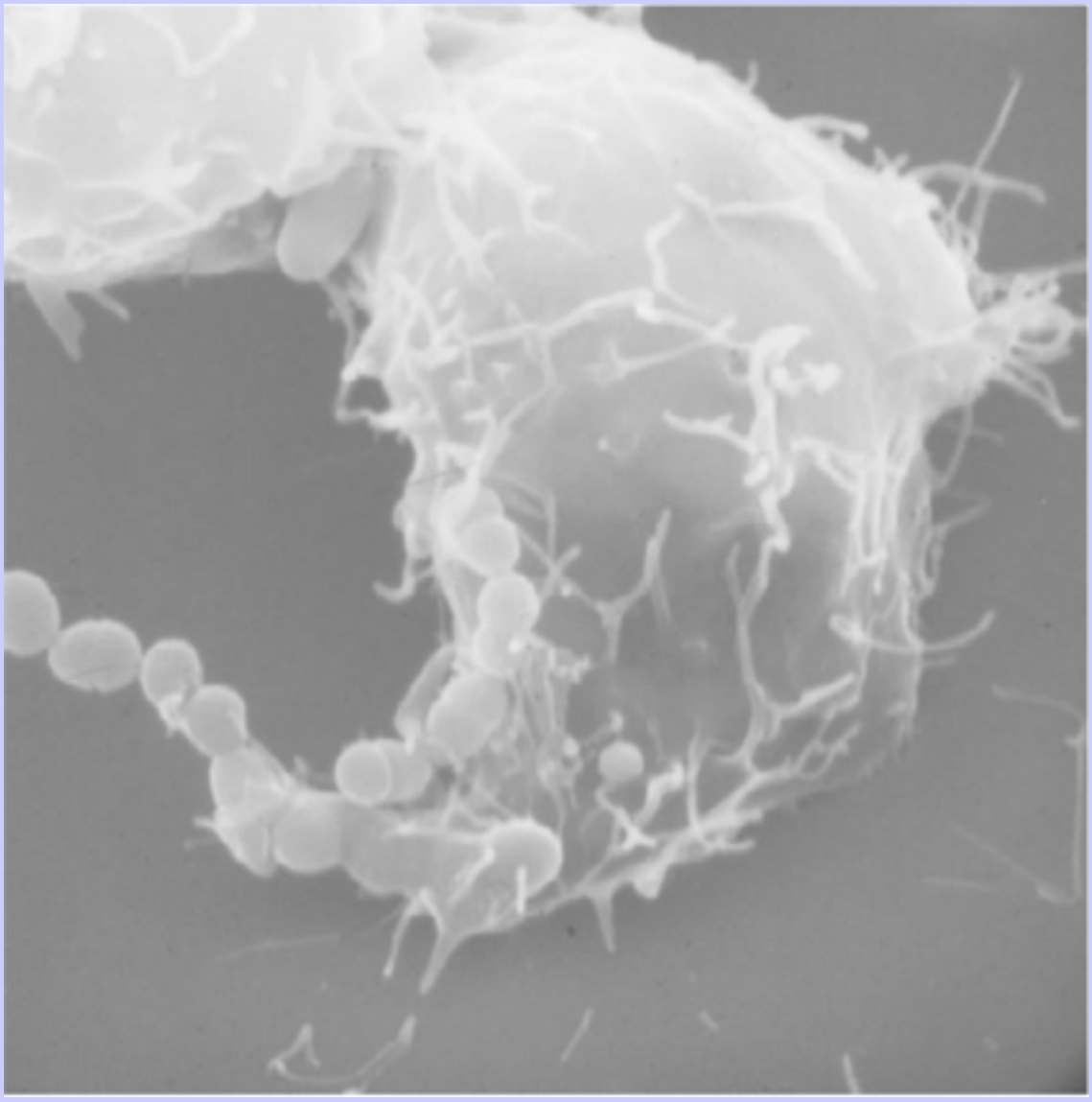


FIGURE 3-11 Scanning electron micrograph of a bovine milk neutrophil ingesting Streptococcus agalactiae. Note how a film of neutrophil cytoplasm appears to flow over the surface of the bacterium. Original magnification ×5000.



bind to CD32, they trigger polymerization of F-actin. As a result, F-actin-rich lamellipodia extend from the cell to engulf the particle. The ligand of CD32 is a site on the Fc region of antibody molecules (see Chapter 13). CD32 is therefore an example of an Fc receptor (FcR). (Since there are several different Fc receptors, CD32 is classified as FcγRII.)

In complement-mediated phagocytosis (type II phagocytosis), particles sink into the neutrophil without lamellipodia formation, suggesting that the ingestion process is fundamentally different from the antibody-

mediated process. CD35 (or complement receptor 1 [CR1]) is a receptor for the complement component C3b. CR1 is found not only on neutrophils but also on other granulocytes, monocytes, red cells, and B cells. Binding of C3b-coated particles to neutrophil CD35 leads to their attachment but may not necessarily trigger ingestion.

Antibodies, the major proteins of the acquired immune system, are by far the most effective opsonins. They coat bacteria, link them to receptors on phagocytic cells, and trigger their ingestion. However, as pointed out previously, antibodies are not produced until several days after the onset of an infection, and the body must therefore rely on innate opsonins for immediate protection.

Another important mechanism that promotes contact between bacteria and neutrophils is trapping. Normally bacteria are free to float away when they encounter a neutrophil suspended in blood plasma. If, however, a bacterium is lodged in tissues, or trap-ped between a neutrophil and another cell surface and thus prevented from floating away, it can be readily ingested. This process is called surface phagocytosis.

Although it has generally been accepted that neutrophils ingest bacteria before killing them, they can also trap and kill extracellular bacteria. After activation by IL-8 or LPS, neutrophil granule proteins and chromatin are released into the extracellular fluid, where together they form a network of extracellular fibers. Not only can these fibers physically capture bacteria, but they can also kill them and destroy their virulence factors. These neutrophil extracellular traps (NETS) are abundant at sites of acute inflammation and are found, for example, in mastitic milk.

3.5.4 Ingestion

As neutrophils crawl toward a chemotactic source, a lamellipod advances first, followed by the main portion of the cell. The cytoplasm of the neutrophil lamellipodia contains a filamentous network of actin and myosin whose state determines the fluidity of the cytoplasm. When a neutrophil meets a bacterium, its lamellipod flows over and around the organism and binding occurs between opsonins on the organism and receptors on the neutrophil surface (Figure 3-11).

Binding of these receptors enables a cuplike structure to cover the particle. The bacterium is eventually drawn into the cell; as it is engulfed, it becomes enclosed in a vacuole called a phagosome. The ease of this ingestion depends on the properties of the bacterial surface. Neutrophil cytoplasm readily flows over lipid surfaces so that hydrophobic bacteria, such as *Mycobacterium tuberculosis,* are readily ingested. In contrast, *Streptococcus pneumoniae,* a cause of pneumonia in humans, has a hydrophilic capsule. It is poorly phagocytosed unless made hydrophobic by opsonization. The progressive covering of a particle by the linkage of cell receptors with ligands on the particle has been likened to a zipper. An alternative process is called coiling phagocytosis. In this case a single lamellipod may wrap itself several times around the organism. This is associated with bacteria such as *Legionella pneumophila* and *Borrelia burgdorferi*.

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3.5.5 Destruction

Destruction of the ingested bacterium occurs through two distinct processes. One, the respiratory burst, involves the generation of potent oxidants. The other involves release of lytic enzymes and antimicrobial peptides from intracellular granules.

3.5.5.1

The Respiratory Burst

Within seconds of binding to a bacterium, neutrophils increase their oxygen consumption nearly 100-fold. This increase is a result of activation of a cell surface enzyme complex called NADPH oxidase (NOX). The components of the NOX complex are separated in resting cells. When a neutrophil binds cytokines such as TNF-α or is exposed to other inflammatory stimuli, the NOX complex is assembled (Figure 3-12). Once assembled, the activated NOX converts NADPH (the reduced form of NADP, nicotinamide adenine dinucleotide phosphate) to $NADP^+$ with the release of electrons. A molecule of oxygen accepts a donated electron, resulting in the generation of a superoxide anion (the dot in $^{\bullet}O_2^-$ denotes the presence of an unpaired electron):

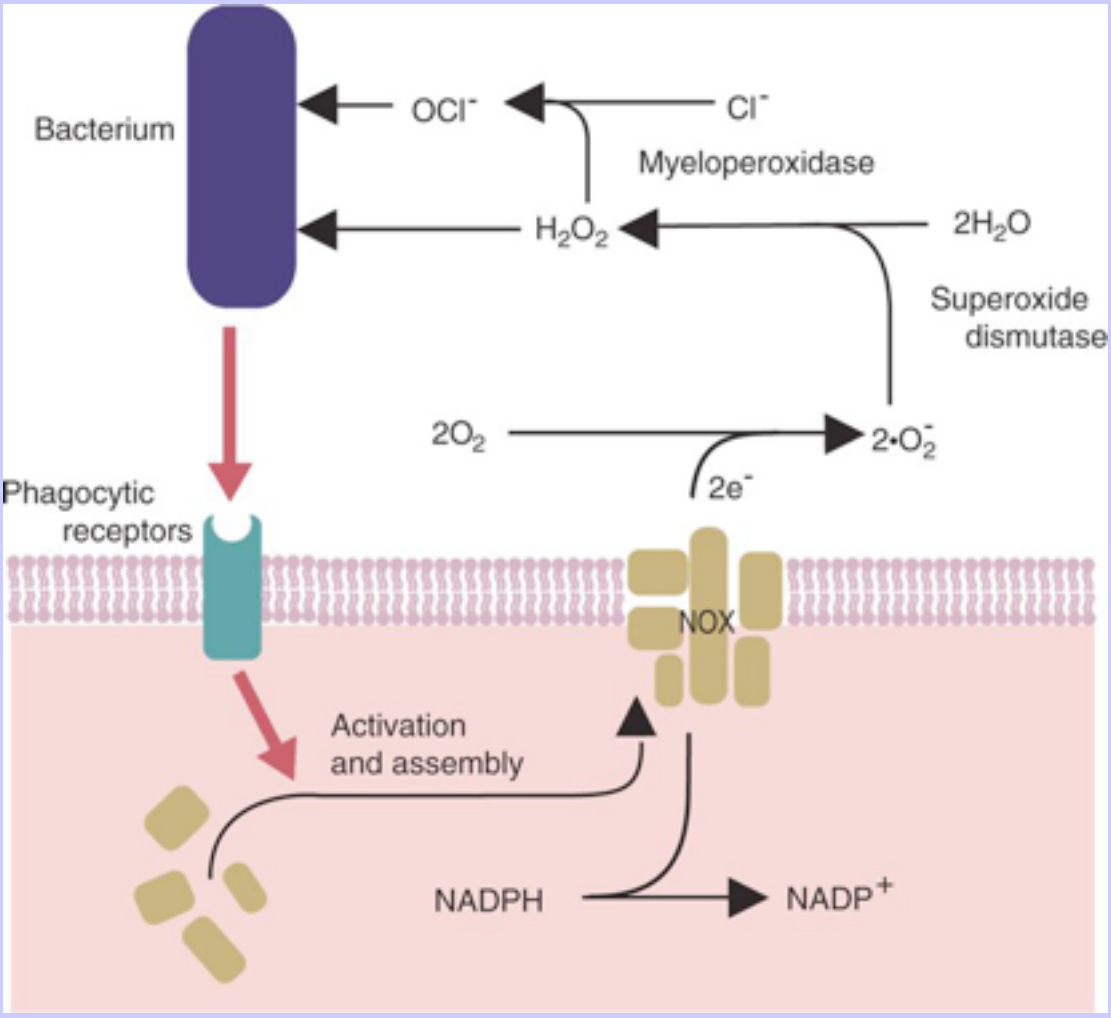
$$NADPH + 2O_2 \xrightarrow{NOX} NADP^+ + H^+ + 2\,{}^{\bullet}O_2^-$$

The $NADP^+$ accelerates the hexose monophosphate shunt, a metabolic pathway that converts glucose to a pentose and CO_2 and releases energy for use by the cell. The two molecules of $^{\bullet}O_2^-$ interact spontaneously (dismutate) to generate one molecule of H_2O_2 under the influence of the enzyme superoxide dismutase:

$$2\,{}^{\bullet}O_2^- + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

Because this reaction occurs so rapidly, superoxide anion does not accumulate but H_2O_2 does. The hydrogen peroxide is converted to bactericidal compounds through the action of myeloperoxidase, the most significant respiratory burst enzyme in neutrophil granules. Myeloperoxidase catalyzes the reaction between hydrogen peroxide and intracellular halide ions (Cl^-, Br^-, I^-, or SCN^-) to produce hypohalides:

FIGURE 3-12 The major features of the respiratory burst pathway in neutrophils. The process is triggered by cytokines such as TNF-α or by binding of opsonized bacteria to phagocytic receptors. It results in the assembly of the multicomponent enzyme NADPH oxidase (NOX) in the membrane of the phagosome. Once assembled, NOX catalyses the generation of bactericidal products such as hydrogen peroxide (H_2O_2) and hypochloride ions (OCl^-).



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$$H_2O_2 + Cl^- \xrightarrow{\text{myeloperoxidase}} H_2O + OCl^-$$

Plasma Cl^- is probably used at most inflammatory sites except in milk and saliva, where SCN^- is also employed. OCl^- is the major product of neutrophil oxidative metabolism. Because of its reactivity, OCl^- does not accumulate in biological systems but disappears in multiple reactions. As long as H_2O_2 is supplied, and neutrophils can generate H_2O_2 for up to 3 hours after triggering, myeloperoxidase will use plasma Cl^- to generate OCl^-. OCl^- kills bacteria by oxidizing their proteins and lipids and enhances the bactericidal activities of the lysosomal enzymes. (Remember that HOCl is the active ingredient of household bleach and is commonly used to prevent bacterial growth in swimming pools.) There are minor quantitative differences in neutrophil activity between the domestic species, especially in the intensity of the respiratory burst. For example, sheep neutrophils appear to produce less superoxide than human or bovine neutrophils. Neutrophils also have safety mechanisms to detoxify oxidants and minimize collateral damage. Thus they contain large amounts of glutathione, which reduces the oxidants. Redox-active metals such as iron can be bound to lactoferrin to minimize OH formation, and antioxidants such as ascorbate or vitamin E interrupt these reactions.

3.6 ANTIMICROBIAL MOLECULES

3.6.1 Lytic Enzymes

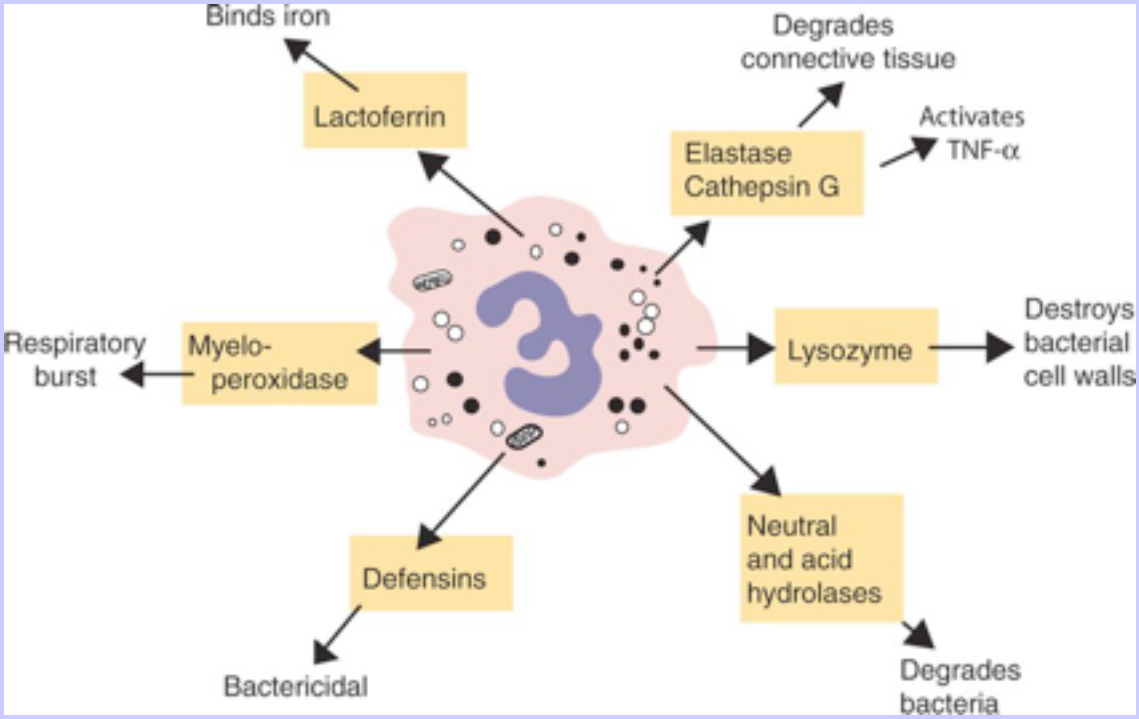
While many bacteria are killed by oxidation, the phagosomes continue to mature. They progressively acidify their contents, reducing their pH to a level optimal for granule proteases.

Once a bacterium is attached to the neutrophil membrane, the granules (or lysosomes) migrate through the cytoplasm, fuse with the maturing phagosome, and release their enzymes. (The complete vacuole is then called a phagolysosome.) The rise in ionic strength within phagosomes releases enzymes such as elastase and cathepsin G from their sulfated proteoglycan matrix (Figure 3-13). Other lysosomal enzymes include lysozyme, proteases, acid hydrolases, and myeloperoxidase. The enzymes that accumulate in phagosomes can digest bacterial walls and kill most microorganisms, but, as might be expected, variations in susceptibility are observed. Gram-positive bacteria susceptible to lysozyme are rapidly destroyed. Gram-negative bacteria such as *Escherichia coli* survive somewhat longer, since their outer wall is relatively resistant to digestion. Lactoferrin, by binding iron, may deprive bacteria of this essential nutrient and so limit bacterial growth. Some organisms such as *Brucella abortus* and *Listeria monocytogenes* can interfere with phagosomal maturation in such a way that they do not come into contact with the lysosomal enzymes and can therefore grow inside phagocytic cells. Neutrophil enzymes released into tissues cleave membrane-bound TNF-α from macrophages. The TNF-α attracts and activates yet more neutrophils.

3.6.2 Peptides

Antimicrobial peptides are widely distributed throughout the plant and animal kingdoms, and more than 800 have been identified to date. Although structurally diverse, these peptides have a net cationic charge due to the presence of multiple arginine and lysine residues and the ability to form amphipathic structures;

FIGURE 3-13 Some of the enzymes and other antibacterial molecules found in the diverse cytoplasmic granules of neutrophils.



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that is, they have both hydrophobic and hydrophilic regions. The hydrophobic regions can insert themselves into the lipid-rich membranes of bacteria, whereas the other regions can form channel-like pores or simply cover the membrane. This results in membrane disruption and microbial death.

The cationic antimicrobial peptides can kill most species of bacteria as well as some fungi, protozoa, enveloped viruses, and tumor cells. The fact that they kill microorganisms rather than host cells is thought to be due to their interactions with microbial phospholipids, LPS, or teichoic acids.

Antimicrobial peptides are concentrated in sites where microbes are most likely to be encountered. These include intracellular locations such as within neutrophils and macrophages (see Chapter 4) and within lymphoid organs (see Chapter 10). Epithelial cells of the skin and respiratory, alimentary, and genitourinary tracts also synthesize antimicrobial peptides.

The defensins are typical antimicrobial peptides. Defensins contain 28 to 42 amino acids arranged in a β-sheet that contains three or four disulfide bonds. More than 50 different mammalian defensins have been identified. The vertebrate defensins are classified as a, b, or q defensins based on their origin and on the number and position of these disulfide bonds. The a defensins account for about 15% of the total protein in neutrophil granules. In cattle at least 13 different a defensins are produced by neutrophils alone. They are also found in the granules of Paneth cells in the small intestine; β defensins are expressed in many different tissues including the epithelial cells that line the airways, skin, salivary gland, and urinary system. Theta defensin is a circular peptide that is found only in primate neutrophils. Defensins can be produced at a constant rate (constitutively) or in response to microbial infection. Some defensins attract monocytes, immature dendritic cells, and T cells. All defensins identified so far

can kill or inactivate some bacteria, fungi, or enveloped viruses. Some defensins can also neutralize microbial toxins such as the toxins of *Bacillus anthracis, Corynebacterium diphtheriae,* and staphylokinase from *Staphylococcus aureus*.

Although present in normal tissues, defensin concentrations increase in response to infections. For example, calves infected with *Cryptosporidium parvum* or *Mycobacterium paratuberculosis* show a significant increase in cryptdin production. *Mannheimia hemolytica* infection in bovine lungs induces increased defensin expression in airway epithelium.

The second major classes of antibacterial peptides in neutrophil granules are the cathelicidins. These are peptides ranging from 12 to 80 amino acids in size with a broad range of antibacterial activity. They are all stored within cells in an inactive form attached to a conserved precursor N-terminal peptide of about 100 amino acids and released following cleavage of the precursor molecule. They are named using acronyms or amino acid symbols followed by the number of amino acids they contain. Humans and mice have only one cathelicidin gene while the pig, cow, and horse have multiple cathelicidin genes. Porcine cathelicidin PR-39 has been shown to promote wound repair, angiogenesis, and neutrophil chemotaxis. The bovine cathelicidin BMAP-28 induces apoptosis in some cells and may serve to get rid of unwanted cells. Many of these peptides have been given their own specific names such as protegrins, novispirin, and ovispirin.

Other antibacterial peptides include the serprocidins and the granulysins. Serprocidins are antimicrobial serine proteases found in the primary granules of neutrophils. Granulysins are peptides produced by cytotoxic T cells and natural killer cells (see Chapters 16 and 30). In addition to their antibacterial functions, granulysins are chemoattractants and activate macrophages. Two other important antibacterial proteins include bactericidal permeability-increasing protein (BPI) and calprotectin. BPI is a major constituent of the primary granules of human and rabbit neutrophils. It kills Gram-negative bacteria by binding to LPSs and damaging their inner membrane. Calprotectin is found in neutrophils, monocytes, macrophages, and epidermal cells. It forms about 60% of neutrophil cytoplasmic protein and is released in large amounts into blood and tissue fluid in inflammation.

3.6.3 Lysozyme

The enzyme lysozyme cleaves the bond between N-acetyl muraminic acid and N-acetyl glucosamine and so destroys the peptidoglycans of Gram-positive bacteria. Lysozyme is found in all body fluids except cerebrospinal fluid and urine. It is absent from bovine neutrophils and tears. It is found in high concentrations in tears of other mammals and in egg white. Although many of the bacteria killed by lysozyme are nonpathogenic, it might reasonably be pointed out that this susceptibility could account for their lack of pathogenicity. Lysozyme is found in high concentrations in some neutrophil granules and so accumulates in areas of acute inflammation, including sites of bacte-rial invasion. Lysozyme is also a potent innate opsonin, binding to bacterial surfaces and so facilitating phagocytosis in the absence of specific antibodies and under conditions where its enzyme activity is ineffective.

3.6.4 Lectins

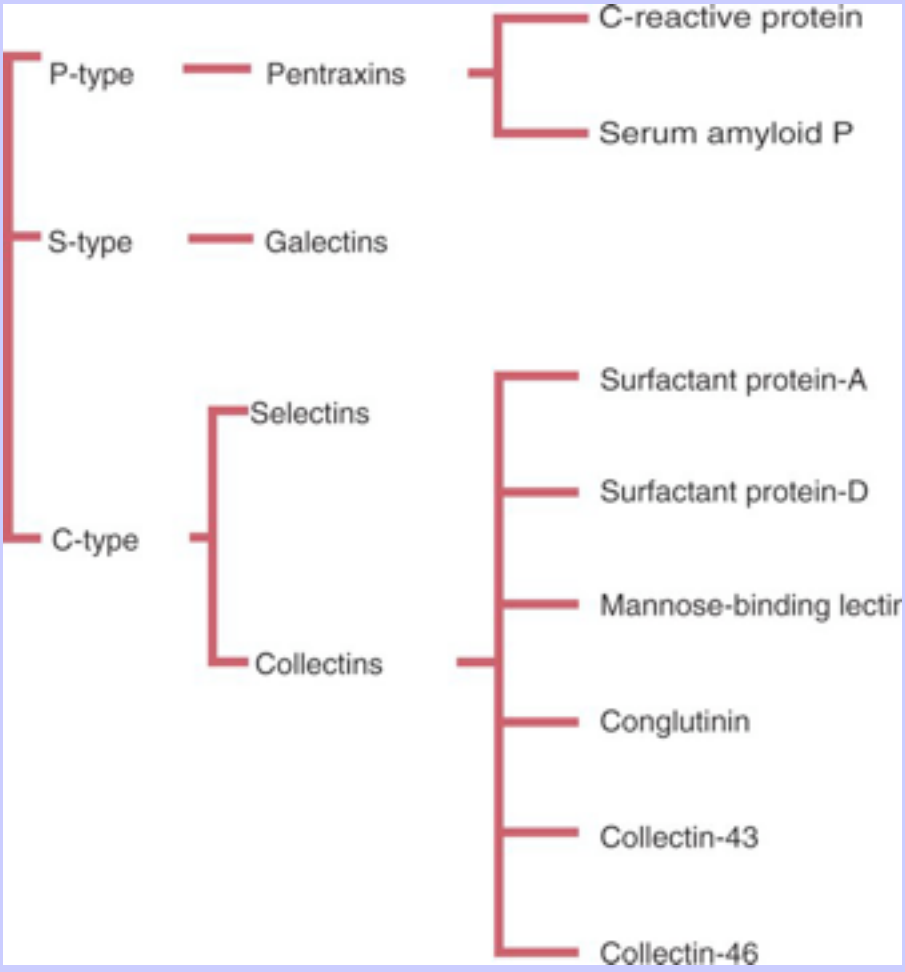
Lectins are proteins that bind carbohydrates. Given that carbohydrates are major components of bacterial cell walls, lectins often bind to bacteria. Mammalian lectins are classified into P-, S-, and C-type lectins (Figure 3-14).

The pentraxins are P-type lectins. They include two important molecules: C-reactive protein (CRP) and serum amyloid P (SAP). These are called acute-phase proteins because their blood levels climb greatly during infections

or following trauma. Pentraxins have multiple biological functions, including activation of the complement system and stimulation of leukocytes. Pentraxin molecules consist of five protein subunits arranged in a ring. Pentraxins bind to carbohydrates such as bacterial LPS in a calcium-dependent manner. Both CRP and SAP can activate the classical complement pathway by interacting with C1q (see Chapter 5). They also interact with neutrophils, monocytes/macrophages, and natural killer cells and augment their activities. For example, CRP not only binds to phosphocholine, a molecule found in all cell membranes, but its major receptor on leukocytes is FcγRII (CD32). It can bind to invading organisms and promote their phagocytosis. SAP binds to galactose polymers and glycosaminoglycans.

The galectins are S-type lectins. Their name derives from their specificity for galactosides. They play a role in inflammation by mediating the binding of leukocytes to extracellular matrix.

FIGURE 3-14 Carbohydrate-binding proteins called lectins play important roles in the defense of the body. They can bind to microbial carbohydrates and then serve as opsonins or activators of other innate defenses. They are diverse, and they belong to multiple protein families.



C-type lectins require calcium to bind to carbohydrates. They include the selectins involved in leukocyte adherence to blood vessel walls and the collectins that are key components of the innate immune system. Each end of a collectin molecule has a distinct function: the C-terminal domain binds to bacterial carbohydrates whereas the N-terminal domain interacts with cells and complement components, thereby exerting the biological effect. Their ability to recognize foreign carbohydrates makes collectins a part of the innate host defense system.

The most important lectin in the innate immune system is mannose-binding lectin (MBL), a collectin found in serum. MBL has multiple carbohydrate-binding sites that bind oligosaccharides, such as N-acetylglucosamine, mannose, glucose, galactose, and N-acetylgalactosamine. The binding is relatively weak, but multiple binding sites give a high functional activity. Most of the ligands of MBL are present at high levels on microbial surfaces. As a result, MBL binds very strongly to bacteria such as *Salmonella enterica, L. monocytogenes, Haemophilus influenzae,* and *Neisseria meningitidis*. It binds to Streptococcus and E. coli with moderate affinity, but it does not bind to encapsulated *N. meningitidis, H. influenzae,* or *Streptococcus agalactiae*. MBL binds strongly to yeasts such as *Candida albicans* and *Cryptococcus neoformans*. It can bind viruses such as human immunodeficiency virus and influenza A as well as the protozoan parasite *Leishmania*. MBL plays an important role in activating the complement system (see Chapter 5). There are two forms of mannose binding lectin in the pig called MBL-A and MBL-C. These can bind to *Actinobacillus suis* and *Haemophilus parasuis*. Some European pig breeds may express very low levels of MBL-C and hence suffer from increased disease susceptibility.

Collectins such as MBL can bind to leukocytes, platelets, endothelial cells, and fibroblasts. Bacteria coated by MBL are readily ingested by phagocytic cells through interaction with surface receptors. The collectins are especially important in young animals whose acquired immune system is not capable of mounting an efficient response. As a result, a congenital deficiency of MBL makes children highly susceptible to infections. Six different collectins (conglutinin, MBL, pulmonary surfactant proteins [SP-A, SP-D], and collectin-46 [CL-46] and CL-43) have been identified in mammals. However, conglutinin, CL-46, and CL-43 have only been identified in bovidae.

3.6.5 Iron-Binding Proteins

One of the most important innate factors that determines the success or failure of bacterial invasion is the availability of iron. Many pathogenic bacteria, such as *S. aureus, E. coli, B. anthracis, Pasteurella multocida,* and *M. tuberculosis,* require large amounts of iron for growth given that iron is the key catalytic site for many enzymes. However, animal hosts also require iron to survive. As a result, both microbe and host compete for the same metal. Iron concentrations within animal tissues are normally very low. Mammalian blood has just 10^{-26} M free iron since almost all available iron is bound to the iron-binding protein transferrin. Thus one effective defensive strategy is to remove as much iron as possible from sites of bacterial invasion. Within the body, iron is associated with several iron-binding proteins including transferrin, lactoferrin, siderocalin, haptoglobin, and ferritin. When bacteria invade the body, intestinal iron absorption ceases. Liver cells are stimulated to secrete transferrin and haptoglobin, and there is increased incorporation of iron into the liver. This effectively reduces the availability of iron still further. A similar situation occurs in the mammary gland when, in response to bacterial invasion, milk neutrophils release their stores of lactoferrin. The lactoferrin binds free iron and makes it unavailable to the bacteria. In spite of the reduced availability of iron, some bacteria, such as *M. tuberculosis, B. anthracis,* and *E. coli,* can successfully invade the body because they produce potent iron-binding proteins (siderophores) that can remove the iron from transferrin or lactoferrin. In effect, the body and the bacteria engage in a “tug-of-war” for iron molecules. Mycobacteria use their siderophore carboxymycobactin to strip iron from mammalian ferritin. The outcome of this competition may determine the outcome of the infection. When serum

iron levels are elevated, as occurs following red cell destruction, animals may become more susceptible to bacterial infections.

Mammals can also capture iron by stealing bacterial siderophores. Thus during bacterial infections the mammalian liver, spleen, and macrophages synthesize a protein called lipocalin 2. Lipocalin 2 (also called siderocalin) binds the bacterial siderophore enterochelin with very high affinity. Lipocalin 2 is essential for limiting the growth of enterochelin-producing bacteria such as *E. coli* but does not affect the growth of bacteria that use other methods of acquiring iron.

3.6.6 Complement

The complement system consists of a complex mixture of enzymes, regulatory proteins, and receptors that plays a major role in innate antimicrobial immunity. This system, described in detail in Chapter 5, can be activated simply by exposing microbial cell walls to serum proteins. It can also be activated by MBL bound to microbial walls, or even by antibodies on microbial cell walls. Once activated, complement components, especially C3 (the third component), bind irrevers-ibly to bacteria and initiate bacterial killing or phagocytosis.

3.6.7 Cytokines

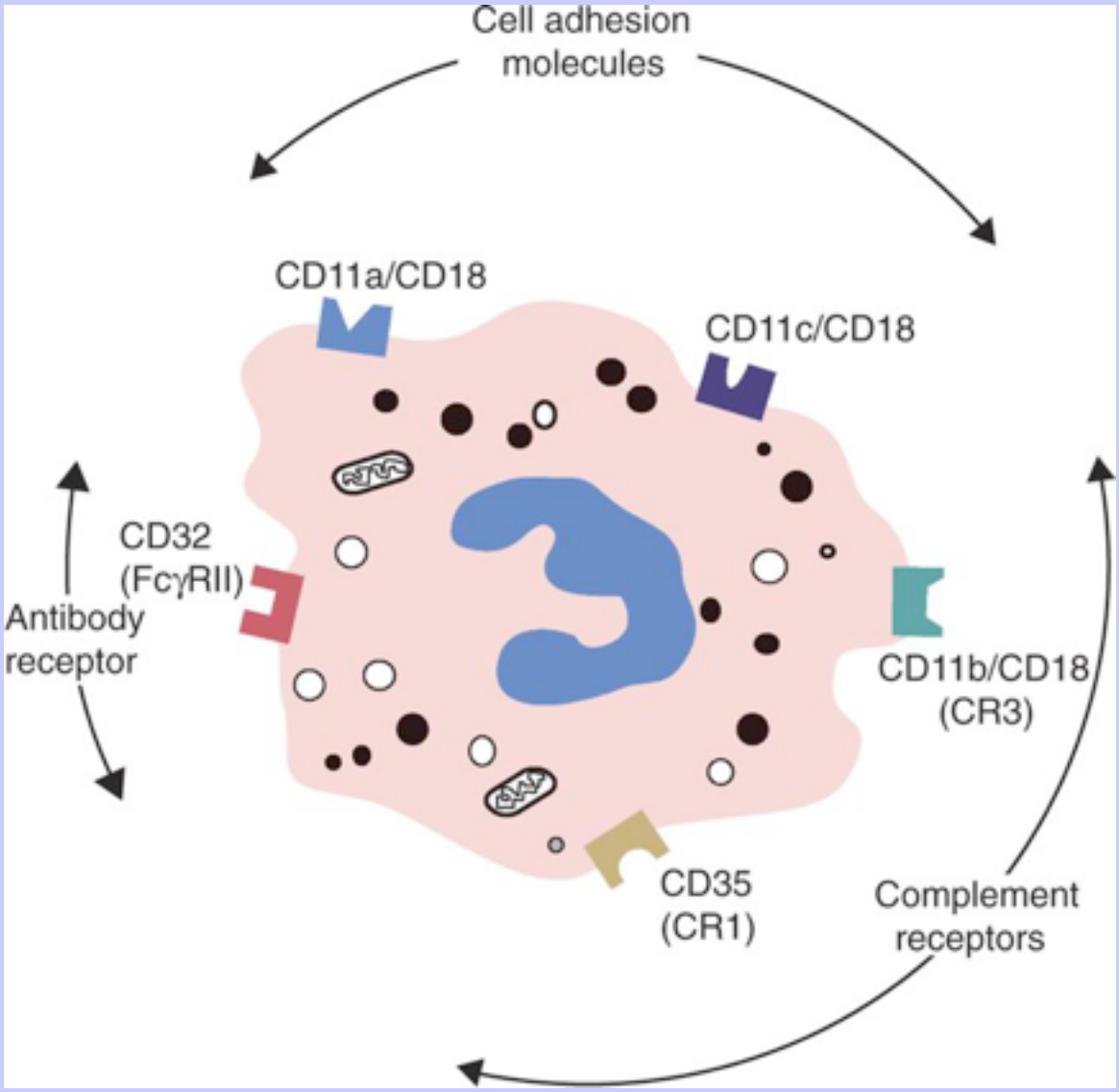
Under the influence of bacterial products such as LPS, neutrophils can secrete many different cytokines such as IL-1α, IL-1β, IL-1RA, TNF-α, IL-6, CXCL8 (IL-8), IL-10, and transforming growth factor-β. Although they produce only small quantities of these cytokines, neutrophils invade inflammatory sites in large numbers, so their total contribution may be significant.

3.7 SURFACE RECEPTORS

Cells must interact with many molecules in their environment. To this end they express many different cell surface receptors. As mentioned in Chapter 2, cell surface glycoproteins are classified by the cluster of differentiation (CD) system. Neutrophils carry many different CD molecules on their surface (Figure 3-15). The most relevant of these proteins are the receptors for opsonins and those that mediate neutrophil attachment to blood vessel walls. Other neutrophil surface molecules include receptors for inflammatory mediators such as leukotrienes, complement components such as C5a, chemokines, and cytokines.

A recent finding that tends to confuse rather than clarify cell identification is the fact that cells may exchange fragments of surface membrane together

FIGURE 3-15 Some of the major surface receptors on neutrophils and their functions.



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with their receptors. For example, integral membrane proteins can be rapidly transferred to bovine neutrophils from a variety of apoptotic and necrotic cells. These include major histocompatibility complex class II molecules and CD3 from macrophages and T cells. This transfer is mediated by the fusion of membrane fragments or microvesicles to the neutrophil membrane and not by phagocytosis of cell fragments. These studies not only complicate studies on neutrophil cell membrane phenotypes, but they may well have implications for neutrophil functions.

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3.8 FATE

Neutrophils have a limited reserve of energy that cannot be replenished. They are therefore active immediately after being released from the bone marrow but are rapidly exhausted and can undertake only a limited number of phagocytic events. The vast majority of neutrophils survive for only a few days. Thus they may be considered a first line of defense, moving rapidly toward invading organisms and destroying them promptly but being incapable of sustained effort. The second line of defense is the mononuclear phagocyte system.

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