The Multifaceted Functions of Neutrophils

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Abstract

Neutrophils and neutrophil-like cells are the major pathogen-fighting immune cells in organisms ranging from slime molds to mammals. Central to their function is their ability to be recruited to sites of infection, to recognize and phagocytose microbes, and then to kill pathogens through a combination of cytotoxic mechanisms. These include the production of reactive oxygen species, the release of antimicrobial peptides, and the recently discovered expulsion of their nuclear contents to form neutrophil extracellular traps. Here we discuss these primordial neutrophil functions, which also play key roles in tissue injury, by providing details of neutrophil cytotoxic functions and congenital disorders of neutrophils. In addition, we present more recent evidence that interactions between neutrophils and adaptive immune cells establish a feed-forward mechanism that amplifies pathologic inflammation. These newly appreciated contributions of neutrophils are described in the setting of several inflammatory and autoimmune diseases.

INTRODUCTION

Multicellular organisms face a constant challenge of surviving in an environment containing unicellular pathogens. Phagocytes have evolved as specialized cells that engulf and kill invading pathogens to protect the host against microorganisms. They are the major cellular arm of the innate immune system, which is common to species throughout the evolutionary tree. Indeed, the survival of primitive organisms—for example, insects, which lack adaptive immune cells such as lymphocytes—relies on the function of their innate immune phagocytes (1). In humans, neutrophils account for 50% to 70% of all circulating leukocytes, and they are the first line of host defense against a wide range of infectious pathogens including bacteria, fungi, and protozoa. Neutrophils are generated at a rate of 10^{11} per day, which can increase to 10^{12} per day during bacterial infection. Thus, not surprisingly, 55% to 60% of the bone marrow is dedicated to their production (2). Neutrophils are terminally differentiated and relatively short lived. Traditional estimates based on ex vivo survival in culture or on half-life after adoptive transfer suggested that these cells survive for only 8–12 h in the circulation and up to 1–2 days in tissues, with their turnover delayed or accelerated during the inflammatory response (3–5). More modern approaches using deuterium labeling methods in vivo suggest that under homeostatic conditions, human neutrophils may have a circulatory life span up to 5 days (6). Although this dramatically different view of neutrophil half-life is somewhat controversial (7, 8), these types of more updated immunological investigations are changing our overall perception of neutrophil function in immunity. Whereas researchers once believed that neutrophils were present only during the acute phase of the inflammatory response, functioning only as pathogen killers, we now appreciate that neutrophils can shape the immune landscape by communicating with macrophages, dendritic cells (DCs), and cells of the adaptive immune response through direct cell-cell contact or soluble mediators (9–11).

In this article, we aim both to revisit well-established principles of neutrophil function during inflammation and host defense—including their production, recruitment, and killing capacities—and to illuminate many of the newer findings on the contribution of these cells to other aspects of immunity. The purpose of this exercise is to renew and stimulate discussion on how processes fundamental to neutrophil function may uniquely influence disease progression. We focus on those studies that reveal the growing appreciation of the complexity of neutrophil function both in normal immune responses and during immune-mediated disease states.

NEUTROPHIL HOMEOSTASIS

Neutrophils are formed within the bone marrow during hematopoiesis in response to several cytokines, principally granulocyte colony–stimulating factor (G-CSF) (12). The major determinants of the total number of neutrophils in the body are their rate of production, their storage in and egress from the bone marrow, and their survival in and clearance from the blood. Entry into the tissues during inflammatory responses can also affect overall neutrophil numbers. The ability of an organism to maintain a balance between neutrophil production and turnover, while adapting to environmental challenge, implies that there must be a molecular process for measuring neutrophil numbers at any given time. The existence of some form of a "neutrostat" that measures neutrophil numbers and adjusts them accordingly remains very controversial (13). However, recently described feedback loops certainly contribute to neutrophil homeostasis under resting and inflammatory disease conditions.

One such feedback loop was discovered in studies of adhesion molecule [CD18, E/P/L-selectin, or CD11a (LFA-1)]–deficient mice. In these animals, neutrophil egress from the peripheral blood is reduced; hence, the animals display significant neutrophilia. Under steady-state conditions, senescent neutrophils are

engulfed by tissue macrophages [primarily in the liver, spleen and bone marrow (14)], which then initiate anti-inflammatory signals via expression of PPARγ (peroxisome proliferator–activated receptor γ) and LXR (liver X receptor) (15). These anti-inflammatory signals, in turn, lower the steady-state production of interleukin (IL)-23 by macrophages. IL-23 is a well-established inflammatory cytokine that induces IL-17 production by T lymphocytes, natural killer (NK) cells, and natural killer T (NKT) cells, which, in turn, induces production of G-CSF and granulocyte macrophage colony–stimulating factor (GM-CSF) by stromal cells, driving granulopoiesis and inflammation (16). Adhesion molecule–deficient mice have high steady-state levels of IL-23 and IL-17, due to reduced egress of neutrophils out of the blood and hence reduced neutrophil uptake by macrophages, leading to elevated G-CSF (17). Genetic depletion of IL-23 in CD18 knockout mice reverses their neutrophilia, supporting the model that the rate of phagocytosis of apoptotic neutrophils regulates their production via an IL-23–IL-17 axis (18). Similarly, mice lacking the LXRα and LXRβ receptors also display significant neutrophilia associated with high levels of IL-23- and IL-17-producing T cells (19).

The IL-23–IL-17–G-CSF feedback loop is clearly not the only mechanism controlling neutrophil production, given that mice lacking T lymphocytes and NK cells (i.e., the major sources of IL-17) have normal neutrophil numbers (20, 21). Moreover, antibody-mediated depletion of neutrophils in mice, through use of the Gr-1 monoclonal antibody, leads to a significant increase in serum GM-CSF and G-CSF, which, in the absence of other inflammatory cytokines, triggers neutrophil progenitor proliferation (21). This observation suggests that neutrophil numbers are regulated, at least in part, simply according to the available space within the bone marrow, in a fashion referred to as density-dependent or neutrophil-mass sensing. A molecular understanding of how bone marrow stroma, which is the major site of G-CSF production, can sense low neutrophil numbers remains unclear. However, very recent results suggest that this sensing pathway depends on the innate immune receptor Toll-like receptor (TLR) 4 and its signaling adapter Toll/interleukin-1 receptor (TIR) domain–containing adapter-inducing interferon-β (TRIF), because antibody-mediated depletion of neutrophils fails to cause G-CSF elevation or progenitor proliferation in mice lacking TLR4 or TRIF (20).

Most studies have focused on G-CSF and GM-CSF as the ultimate cytokines that regulate neutrophil production, but this is clearly too simple a view, given that mice lacking one or both of these cytokines still have approximately 20% of the normal level of mature neutrophils in their blood (22). Indeed, these cytokine-deficient animals can also mount increased neutrophil production in response to inflammatory stimuli, a process termed emergency granulopoiesis (23). That hematopoietic progenitors can ramp up production of granulocytes in response to inflammatory or pathogen challenge, even in the absence of canonical granulopoiesis-stimulating cytokines, indicates that other signaling pathways must exist to regulate granulopoiesis. New studies clearly demonstrate that hematopoietic progenitor cells proliferate upon sensing pathogen molecules through a host of innate immune receptors such as the TLRs and Nod-like receptors (24). The combination of consumption of neutrophils in the periphery—in the process of fighting pathogens—and exposure of progenitor cells to pathogen molecules could lead to a synergistic stimulation of granulopoiesis during these emergency situations (25). The observation that steady-state granulopoiesis depends completely on the transcription factor C/EBP (CCAAT/enhancer-binding protein) α, whereas emergency granulopoiesis requires C/EBPβ, also suggests that these are two separate pathways (26, 27). Finally, the observation that mice lacking commensal organisms—that is, germ-free animals—have dramatic neutropenia, with neutrophil levels even lower than those in combined G-CSF and GM-CSF knockout mice, also suggests

Granulocyte macrophage colony–stimulating factor (GM-CSF): a classical cytokine that promotes granulopoiesis and primes neutrophils for enhanced cytotoxic responses

Toll-like receptors (TLRs): a family of intracellular and extracellular PRRs that engage the intracellular transcriptional machinery to promote synthesis and secretion of pro-inflammatory cytokines

ROS: reactive oxygen species

Neutrophil extracellular traps (NETs): formed by the release from neutrophils of decondensed chromatin that is covered with antimicrobial components and potential self-antigens

that hematopoietic progenitor cells are tuned to respond directly to environmental cues (20).

In the end, neutrophil homeostasis is likely influenced by all these processes—phagocytic uptake in the periphery, cell mass in the bone marrow, and presence of pathogen (or commensal) stimuli—in a complex concert. Under any given condition, one pathway may dominate over the others, but a clear understanding of regulation of neutrophil numbers (especially during disease states) will require further research.

NEUTROPHIL RECRUITMENT

After their birth in the bone marrow, mature neutrophils reach sites of tissue inflammation or infection via the vasculature. The exit of neutrophils from the blood, primarily via postcapillary venules, follows an ordered process referred to as neutrophil recruitment (28–31). The neutrophil recruitment cascade is mediated by the sequential interaction of receptors present on neutrophils with ligands induced on the surface of the activated (i.e., inflamed) endothelium. The classical multistep adhesion cascade consists of the following steps: (*a*) initial attachment of the neutrophil to the endothelium (capture), (*b*) rolling of the neutrophil along the endothelium, (*c*) firm arrest of the neutrophil with accompanying cell spreading, (*d*) crawling of the neutrophil along the endothelium, and (*e*) transmigration of the neutrophil into the tissue (**Figure 1**), where full neutrophil activation leads to phagocytosis and killing of pathogens through the production of reactive oxygen species (ROS), degranulation (**Figure 2**), and generation of neutrophil extracellular traps (NETs) (**Figure 3**).

The molecular requirements for the multistep paradigm of neutrophil recruitment, depicted in **Figure 1**, have primarily been derived from the real-time analysis of postcapillary venules in the transparent cremaster muscle or mesentery using intravital microscopy (IVM) (32). However, in autoimmune diseases, the contribution of humoral immunity components, including immune complexes and complement activation products, needs to be taken into account. Fcγ receptors (FcγRs), which are receptors for immunoglobulin (Ig) G immune complexes, are a case in point. In models of antibody-mediated glomerulonephritis and arthritis, mice that lack activating FcγRs exhibit no neutrophil accumulation. However, tissue recruitment is restored by the selective expression of human FcγRIIA and/or FcγRIIIB on neutrophils (33, 34). IVM of murine neutrophils expressing human FcγRIIA and FcγRIIIB shows that these receptors trigger both slow rolling and adhesion in the presence of deposited immune complexes (33). IgG immune complexes can also trigger complement activation, leading to production of complement component 5a (C5a), a potent neutrophil chemoattractant. C5a lowers the threshold of FcγR-mediated neutrophil activation (34, 35) and increases macrophage-1 antigen (Mac-1) [complement receptor (CR) 3] activity (36), which may potentially influence neutrophil accumulation. Thus, in the presence of immune complexes, the principles of neutrophil recruitment (i.e., slow rolling, adhesion, and chemokine-directed migration) may not fundamentally diverge from those that follow simple cytokine stimulation but involve additional neutrophil receptors that link the inflammatory insult, in this case immune complexes and complement, to neutrophil accumulation.

Neutrophils are unique among leukocytes in their ability to roll along vascular endothelium at significantly high shear stress (i.e., in larger vessels with higher blood pressure). This ability likely evolved to allow neutrophil recruitment to occur in a broader range of tissue areas. Most leukocytes roll along postcapillary venules at shear stresses of 0–3 dyn/cm^2, whereas neutrophils can roll on vessels at shear stresses of 6–10 dyn/cm^2 (31). Neutrophils accomplish this resistance to fluid shear force by four mechanisms: (*a*) flattening out over the endothelium to engage more adhesive molecules, (*b*) increased use of selectin family molecules and their receptors to form catch bonds that tend to become stronger with increasing force,

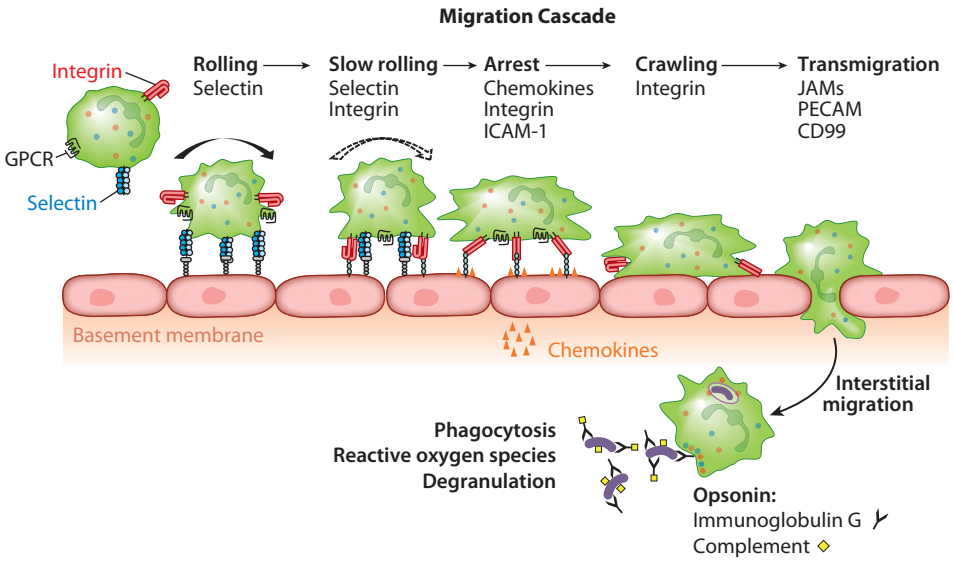

Figure 1

Multistep adhesion cascade of neutrophil recruitment. The initial steps of neutrophil attachment and rolling on the endothelium are supported by the transient interaction of P-, E- and L-selectins with their fucosylated ligands. These low-affinity interactions decelerate the flowing neutrophil, allowing it to roll and thus sample the local microenvironment for inflammatory cues such as host-derived chemokines or pathogen molecules. A secondary signal from these chemoattractants, some of which are immobilized on endothelial proteoglycans, induces β_2 and β_1 surface integrins on the neutrophil to undergo a conformational unfolding, allowing them to interact with ligands (primarily ICAM-1 and ICAM-2) expressed on the inflamed endothelium. This inside-out activation of integrins is mediated by intracellular signaling molecules such as Kindlin-3, Talin, and guanine exchange factors for small GTPases (253). Ligand engagement of integrins slows the rolling velocity and promotes the firm arrest of the neutrophil along the endothelium. Exit from the vessel into the interstitium is preceded by intraluminal crawling, a process that directs neutrophils to preferred sites of emigration. Interestingly, neutrophils can migrate upstream, against the flow of blood, during intraluminal crawling (254). Neutrophils usually begin diapedesis across the endothelium at the tricellular junctions of endothelial cells, at sites where endothelial cell ICAM-1 expression is often the highest (255). Within the vessel, these sites are usually at vessel branch points within postcapillary venules. Sites of preferred transmigration are also dictated by the composition of the underlying basement membrane (i.e., low expression of laminin, collagen, and nidogen-2), allowing easier exit from the vessel. Transmigration of neutrophils occurs predominantly via endothelial cell-cell junctions (paracellular transmigration) and in some cases through the endothelium (transcellular transmigration). Neutrophils are guided into tissue by local gradients of chemoattractants (53, 256) in a process that requires them to switch from sensing chemokines on the apical endothelial surface to sensing those emanating from the tissue source of inflammation. Once in the interstitial space, neutrophils migrate toward the offending stimulus. Cell mobility in the interstitium was long accepted to require integrin-based adhesive interactions with the extracellular matrix. However, a recent study using mice lacking all integrins on their immune cells suggests that although integrins are indispensable for neutrophil anchoring to the endothelial surface, they are dispensable for migration in collagen gels (257). In the 3D context of extravascular tissue, neutrophil migration is an actin-driven protrusive process characteristic of amoeboid locomotion with coordinated protrusions at the leading front and actinomyosin-dependent retractions at the trailing edge. These data predict that in patients, integrin deficiency or blockade prevents initial cell recruitment to sites of inflammation but has no effect on chemotaxis of transmigrated neutrophils, a prediction with obvious implications for integrin-based therapies. Abbreviations: ICAM, intercellular adhesion molecule; JAM, junctional adhesion molecule; PECAM, platelet endothelial cell adhesion molecule.

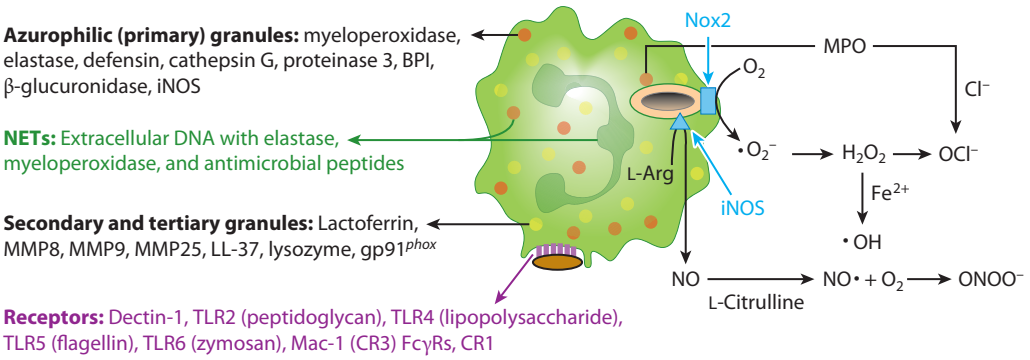

Figure 2

Cytotoxic functions of neutrophils. Three categories of granules are recognized on the basis of their enzyme content and function, but there is some overlap (12, 258). Primary or azurophilic granules contain the antibacterial enzyme myeloperoxidase (MPO), numerous antimicrobial peptides (e.g., defensins), β-glucuronidase, lysozyme, and serine proteases (e.g., elastase/neutrophil elastase, cathepsin G, and proteinase 3). Secondary or specific granules contain large amounts of lactoferrin, which sequesters free iron to prevent bacterial growth and increases permeability to lysozyme to facilitate breakdown of the bacterial cell wall. Secondary granules also contain matrix metalloproteinases (e.g., MMP8, also known as collagenase). Tertiary granules contain MMP9, also known as gelatinase. Both secondary and tertiary granules contain components of NADPH oxidase, $p22^{phox}$ and $gp91^{phox}$. In addition to the classical granules, neutrophils contain highly mobilizable secretory vesicles that serve as a reservoir primarily for plasma membrane receptors. These include receptors for lipopolysaccharide (CD14), complement (CR1 and CR3/Mac-1), urokinase-type plasminogen activator, immune complexes (FcγRs), and chemoattractants (e.g., to formyl peptides) (10). NADPH oxidase is an electron transport chain that provides electrons from NADPH that reduce O_2 to form superoxide ($\cdot O_2^-$), which spontaneously dismutates into various reactive oxygen species such as hydrogen peroxide, singlet oxygen, hydroxyl radicals, and possibly ozone (259). Abbreviations: BPI, bactericidal/permeability-increasing protein; CR, complement receptor; FcγR, Fcγ receptor; iNOS, inducible nitric oxide synthase; Mac-1, macrophage-1 antigen; NET, neutrophil extracellular trap; TLR, Toll-like receptor.

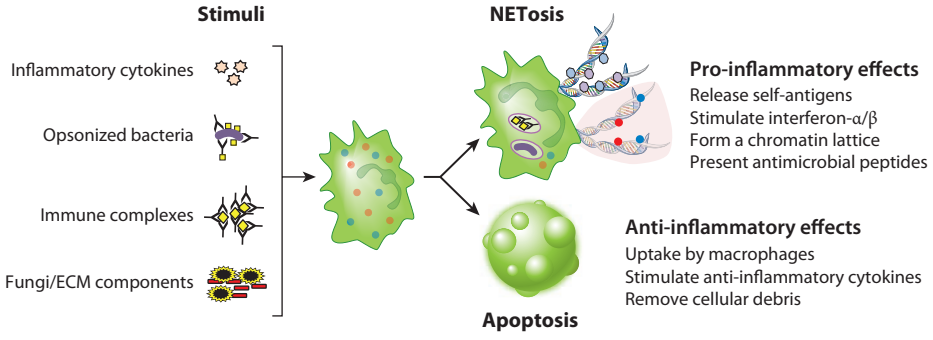

Figure 3

Balancing apoptosis versus NETosis. In response to numerous inflammatory stimuli, including combinations of inflammatory cytokines, pathogens, immune complexes, and extracellular membrane (ECM) components, neutrophils can be activated to undergo either NETosis or apoptosis. We propose that the balance between these two outcomes is a function of the strength or presentation of the stimulus—neutrophils that are highly primed by cytokines and subsequently exposed to opsonized microbes may undergo apoptosis, whereas weaker stimuli may lead to NETosis. Whether neutrophils undergo NETosis or apoptosis dictates the subsequent immune responses. NETosis tends to be pro-inflammatory and prothrombotic and to lead to the release of novel self-antigens, such as deiminated proteins, which stimulate autoimmunity. By contrast, apoptosis is anti-inflammatory, in that it leads to the promotion of M2 macrophage development associated with wound healing and tissue repair processes.

(*c*) formation of membrane tethers that can extend up to several cell diameters behind the rolling neutrophil to slow it down, and (*d*) formation of newly described slings of membrane, which extend in front of neutrophils rolling at high shear rates and help resist the high fluid pressure (37). Novel microscopic techniques (quantitative dynamic footprinting using total internal reflection fluorescence microscopy) have allowed the visualization of such membrane fragments that make up the tethers and slings.

Neutrophils migrate through the endothelial cell barrier in two fashions: via a paracellular (between endothelial cells, as shown in **Figure 1**) or a transcellular (through endothelial cells) route. Most transmigration occurs via the paracellular route, although the transcellular route is favored when endothelial expression of intracellular adhesion molecule (ICAM)-1 is high (38). Paracellular migration depends on the formation of endothelial domes (also known as transmigratory cups), which are membrane protrusions rich in adhesion molecules [ICAM-1 and vascular cell adhesion molecule (VCAM)-1] that extend from the endothelial cell to surround the neutrophil (39–41). Endothelial adhesion molecules interact with neutrophil integrins [predominantly lymphocyte function–associated antigen (LFA)-1] to form a tight seal, or ring, within the dome (42). Formation of these domes is thought to limit vascular leak (i.e., permeability) during neutrophil egress across the endothelium (43). The actual steps of transmigration via both paracellular and transcellular routes depend on homophilic interactions between additional adhesion molecules, such as platelet endothelial cell adhesion molecule (PECAM)-1 and CD99, which are expressed on both the leukocyte and the endothelial cell (30). Interactions between the junctional adhesion molecules (JAM-A, JAM-B, and JAM-C) and leukocyte integrins (Mac-1) also play a significant role in transmigration. Most of these roles have been demonstrated in knockout mouse models, in which deletion of one or more of these molecules specifically blocks transmigration. Many of the adhesion molecules are located in a specific membrane compartment on endothelial cells, termed the lateral border recycling compartment (44). This specific subcellular region on endothelial cells is thought to provide the additional membrane components needed to form the large domes that surround the transmigrating neutrophil. Additional molecules within the lateral border recycling compartment, such as the poliovirus receptor (CD155), activated leukocyte cell adhesion molecule (ALCAM/CD166), and integrin associated protein (IAP/CD47), are also required for normal transendothelial migration (30). These proteins potentially affect the movement of membrane and adhesion molecules on endothelial cells or the loosening of adhesion junctions between endothelial cells that is required for efficient leukocyte transmigration. Not surprisingly, most of these molecules play a role in both paracellular and transcellular migration. One potential difference between these two routes of transmigration is the lack of transmigratory cup formation on endothelial cells during transcellular migration, which is instead characterized by formation of invasive podosomes on the leukocyte that probe the apical (vascular) surface of the endothelial cell (45, 46). Transcellular migration may also be favored when endothelial junctions are particularly tight—for example, in the blood-brain barrier or when leukocytes are highly activated, potentially by direct exposure to inflammatory cytokines or chemokines present on the apical side of the endothelium (47). Unifying models of paracellular and transcellular transendothelial migration have recently been proposed by Muller (30).

Over the years, improvements in leukocyte labeling strategies and the advent of multiphoton IVM imaging have unveiled unique leukocyte behaviors in specific vascular beds of solid organs such as the lung, liver, and kidney (32, 48). In the lung, neutrophil extravasation occurs mainly in the small capillaries surrounding the alveoli and not in postcapillary venules (49). Lung capillaries are particularly narrow, which leads to low blood flow velocity and forces

ICAM-1: intercellular adhesion molecule 1

neutrophils to change shape to pass through, prolonging transit time of cells through the vasculature. Recent work suggests that a tissue-resident pool of patrolling neutrophils is rapidly recruited into the lung following an inflammatory insult that depends on monocyte-derived factors (50). Neutrophil recruitment across the liver sinusoidal endothelium differs significantly from that in other tissues simply due to the porous nature of this vasculature (51). Although this porosity allows easy exchange of solutes between the vasculature and hepatic cells, the vessels are narrow, as in the lung, resulting in a large marginated pool of cells and a relative absence of selectin-mediated rolling during hepatic injury (52). In the liver, the anatomic location and stimulus dictate the molecular mechanisms employed for neutrophil recruitment. For example, leukocyte recruitment in the portal and central venules is similar to that in the mesentery and cremaster. However, in the sinusoidal capillaries, certain hepatic inflammatory conditions lead to neutrophil crawling guided by an endothelial cell immobilized chemokine, macrophage inflammatory protein (MIP)-2, which is then superseded by a gradient of formyl peptide receptor ligands released by necrotic hepatocytes (53). Moreover, the molecular mechanisms of recruitment in the liver differ depending on the stimulus. Neutrophil sequestration in the sinusoids in response to lipopolysaccharide relies on the binding of neutrophil glycoprotein CD44 to the liver sinusoidal endothelial hyaluronan (54), but in response to sterile injury, the β_2 integrin Mac-1 and endothelial ICAM-1 are required (53). Real-time imaging in the kidney has not traditionally been approachable by IVM due to the organ's complex structure and optical inaccessibility, although work in the hydronephrotic kidney, in which tubule atrophy results in a thin tissue preparation, has circumvented some of these issues (55). Recent work using multiphoton imaging of the intact kidney provides evidence that neutrophils and monocytes constitutively patrol the capillaries of the glomerulus. Upon inflammation induced by antibody against glomerular basement membranes, these cells exhibit a greater dwell time and increased ROS generation. These processes are dependent on Mac-1 (56), consistent with the requirement for this integrin in frank nephritis in this disease model (57). An underlying characteristic of neutrophil recruitment in these solid organs is the increased transit time through capillaries of small diameter, which leads to increased potential for vascular injury. The arrest of neutrophils in these small vessels may be linked to activation-induced changes in their cytoskeleton that yield decreased deformability and subsequent sequestration (58). These studies illustrate the ability of state-of-the-art confocal multiphoton-fluorescence microscopy to provide new insights into neutrophil recruitment under different inflammatory states.

Compared with transendothelial migration, transepithelial migration of neutrophils has been less well studied (59–61). Transepithelial migration has an obvious impact on inflammatory diseases at epithelial surfaces, such as acute lung injury and inflammatory bowel disease. Most studies examining the mechanisms of transepithelial migration have used in vitro cell culture models. Transepithelial migration proceeds in the opposite direction from transendothelial migration; that is, neutrophils migrating through the interstitial tissues first interact with molecules on the basal (i.e., the basement membrane side) of the epithelium and then migrate out toward the apical side of the epithelium. Epithelial cells tend to be much larger than endothelial cells, and hence the pathway through which neutrophils must pass is much longer (20 μm or more for epithelial cells, compared with 2–4 μm for endothelial cells). Transepithelial migration occurs only through the paracellular route; there is no evidence for transcellular transepithelial migration. Even in cell culture models, transepithelial migration is often associated with more significant neutrophil activation and epithelial cell injury than is transendothelial migration, resulting in increased permeability across the epithelial barrier. Neutrophil transmigration induces a repair program in epithelial cells,

mediated by β-catenin signals, that is required for reestablishment of a permeability barrier (62). Transepithelial migration depends on a slightly different repertoire of adhesion molecules than does transendothelial migration. For example, the leukocyte integrins (Mac-1 and LFA-1) are implicated, but the ICAM/VCAM molecules likely play no role because they are present only on the apical side of the epithelium. Instead, these adhesion molecules probably facilitate retention of neutrophils on the epithelial apical surface. JAM-C is implicated in neutrophil transepithelial migration, potentially as a ligand for the neutrophil integrins (Mac-1). Neutrophil-expressed CD47 and its ligand SIRP-α are also implicated in transepithelial migration, primarily across intestinal epithelia (63). Very recent evidence has implicated the triggering receptor expressed on myeloid cells (TREM)-1 in transepithelial migration during acute lung injury (64). Interestingly, during experimental *Pseudomonas* pneumonia, neutrophils from TREM-1-deficient mice can complete transendothelial migration out of the pulmonary vasculature but get stuck in the interstitium and fail to cross the epithelial cell barrier. Hence, the TREM-1 mutant mice have significantly increased mortality to this pathogen. Clearly, what is lacking in experimental examination of transepithelial migration are sensitive IVM and two-photon imaging techniques to visualize this process in vivo, as has been done with transendothelial migration.

NEUTROPHIL ACTIVATION

Circulating neutrophils are quiescent—their activation is a defining step in the inflammatory response. Neutrophil activation is usually a multistep process. It begins with the partial activation of cells as they transit through the vascular endothelium during the recruitment process. After entry into the inflammatory tissue site, in response to pro-inflammatory stimuli in the tissue, neutrophils become fully activated, a state characterized by release of granule proteins, acquisition of phagocytic capabilities, and production of NETs, all of which are designed to enhance the cells' pathogen-destruction capacity (**Figure 2**). Neutrophils are relatively nonresponsive to a single stimulus, but exposure to one stimulus [e.g., lipopolysaccharide, tumor necrosis factor (TNF), chemokines, growth factors, adhesion] enhances the ability of the cell to mount an enhanced activation response to a second individual stimulus (65–67). This effect, referred to as neutrophil priming, allows rapid and maximum neutrophil activation, including enhanced phagocytosis and radical oxygen generation (67–69). However, the activation mechanisms that are beneficial for pathogen killing can also be detrimental in the context of sterile injury such as autoimmune and other chronic inflammatory diseases. Thus, an understanding of the mechanisms that control neutrophil killing is important for developing therapeutics that can prevent neutrophil-mediated damage to host tissues.

Pattern-recognition receptor (PRRs): recognize PAMPs associated with microbes or cell stress as well as DAMPs released by injured cells

Neutrophil Activation by Pathogen Molecules or Cell Damage–Associated Proteins

Neutrophils recognize pathogens via classes of cell surface and intracellular receptors (**Figure 2**) that bind to microbe-specific molecules. Neutrophils also use numerous receptors that recognize host proteins (such as IgG and complement) opsonizing the microbe. These receptors induce intracellular signals that lead to full pathogen-killing capacity. The magnitude, quality, and duration of the elicited response are dictated by the repertoire of receptors engaged at any one time, which in turn defines the set point (i.e., activation status) of the neutrophil.

Pattern-recognition receptors. Pathogen-associated molecular patterns (PAMPs; e.g., lipopolysaccharide, peptidoglycan and lipoteichoic acids, double-stranded viral RNA, bacterial DNA) are recognized by neutrophil pattern-recognition receptors (PRRs). Many of these receptors are also engaged by damage-associated molecular patterns (DAMPs), which are released by necrotic cells (e.g., high-mobility group protein B1,

mitochondrial formyl peptides, mitochondrial DNA) during sterile inflammation such as in burns or hypoxia. Engagement of PRRs by either PAMPs or DAMPs likely has similar outcomes. Obesity-related DAMPs resulting from low-grade cell death may contribute to the observed nonresolving inflammation in this disorder. In thermal injury, released mitochondrial formyl peptides serve as DAMPs that contribute to inflammation-induced tissue damage (11).

In neutrophils, the primary endocytic PRRs are the C-type lectin receptors, the most important of which is Dectin-1, which recognizes fungal β-glucan. Dectin-1, together with the integrin Mac-1, internalizes and eliminates fungal pathogens (70, 71). Expression of TREM-1, another endocytic receptor that binds various pathogens (72), is increased by bacterial and fungal stimuli and is strongly upregulated on peritoneal neutrophils of patients with microbial sepsis. Antibody-mediated blockade of TREM-1 protects against endotoxic shock in mice (73). The major type of nonphagocytic PRRs on neutrophils is the TLRs, which recognize lipids, carbohydrates, peptides, DNA, and single- and double-stranded RNA (74). At the RNA level, neutrophils express TLR1, -2, -4, -5, -6, -8, and -10 (and, after GM-CSF treatment, TLR9) (75). TLR engagement primes neutrophils for enhanced responses to other stimuli, thus augmenting their phagocytic capacity, stimulating increased cytokine release, and slowing neutrophil apoptosis (76). Other signaling PRRs include the cytosolic microbial sensors NOD1 and NOD2, which recognize peptidoglycan-related molecules of gram-negative and gram-positive bacteria, respectively (77). Neutrophils express NOD2, which, when triggered by the appropriate proteoglycan, can lead to IL-8 release (78).

Opsonic receptors. The classical opsonins, IgG and the C3 complement activation product C3b, are critical for neutrophil-mediated pathogen killing—neutrophil responses to many types of pathogens are ineffective in their absence. In the context of sterile inflammation, as in autoimmune diseases, tissue accumulation of these opsonins is a major trigger of neutrophil-mediated tissue damage. Engagement of complement- and/or IgG-opsonized pathogens leads to rapid uptake of microbes and strong stimulation of neutrophil killing mechanisms. Resting neutrophils express two types of complement receptors, CR3 (also known as Mac-1) and CR4, which recognize targets opsonized by the complement activation product C3bi (79). In addition, expression of CR1 (CD35), which recognizes C3b/C4b complexes and the mannan-binding lectin, increases severalfold on the neutrophil surface via mobilization from granule pools during neutrophil activation (80). Neutrophils express both low- and high-affinity receptors for the Fc portion of IgG, termed FcγRs (81). Resting human neutrophils express the low-affinity activating FcγRIIA and FcγRIIIB, which have low affinity for monomeric IgG but high affinity for immune complexes, whereas activated neutrophils upregulate expression of the high-affinity FcγRI for IgG. The inhibitory FcγRIIB inhibits intracellular signaling pathways initiated by activated FcγRs when coengaged with immune complexes (82). However, FcγRIIB is not consistently detected on the surface of murine neutrophils (83, 84). Moreover, in human neutrophils, it is barely detectable in individuals with the 2B.1 promoter haplotype and is present at low levels in the infrequent 2B.4 haplotype (85). These findings predict that, in contrast to other immune cells, neutrophils regulate the intracellular signaling pathways of activating FcγRs via inhibitory receptors other than FcγRIIB. Nonetheless, a recent study demonstrated a function for FcγRIIB on neutrophils in downregulating responses to complement: FcγRIIB inhibited signaling through the complement receptor C5aR when coengaged with Dectin-1 by highly galactosylated IgG (86).

The pentraxin molecules, a family of secreted PRRs, represent a third major type of opsonin that engages both FcγRs and CRs (87). The best-studied pentraxins are C-reactive protein (CRP) and serum amyloid P

component (SAP), both of which are produced in the liver during states of inflammation. CRP and SAP can opsonize microbial pathogens through recognition of PAMPs. Association of CRP or SAP with a microbe induces rapid complement activation on the pathogen surface, facilitating recognition of the pathogen by neutrophil CRs. Additionally, both CRP and SAP are directly recognized by FcγRs to induce neutrophil activation (87).

G protein–coupled receptors. Neutrophils express a large repertoire of G protein–coupled receptors (GPCRs) that recognize bacterial products (e.g., formyl peptides) as well as endogenous molecules released during inflammation (e.g., leukotrienes; chemokines such as IL-8, C5a, and adenosine) (88–90). These GPCRs are involved mainly in guiding neutrophil migration. However, signaling through GPCRs can also prime neutrophil activation in response to other activating agents or, at a high enough concentration, lead to full cellular activation. Neutrophils can detect and respond to complex gradients of chemoattractants, which provide graded intracellular signaling responses, allowing the cells to reach the site of pathogen invasion (91, 92). Some of these GPCRs, such as the formyl peptide receptors, also recognize host cellular products released during cell injury or death (93).

Pathogen Killing by Neutrophils

The process by which neutrophils kill invading pathogens depends on three primary mechanisms: receptor-mediated uptake of the pathogen into a vacuole within the cell; production of highly toxic ROS in the pathogen-containing vacuole; and fusion of neutrophil granules, containing various antimicrobial mediators, to the vacuole (**Figure 2**). These steps may also contribute to inflammatory diseases in which ligands are deposited on tissue components.

Phagocytosis. Uptake, or phagocytosis, of the opsonized microbe depends on engagement of opsonic receptors, such as FcγRs and C-type lectin receptors, which enclose the pathogen within a defined vacuole referred to as the phagosome (94, 95). Neutrophil phagocytosis is very rapid—uptake of IgG-opsonized particles occurs in less than 20 s (96). Uptake is followed by fusion of the phagocytic vacuole with preformed granules within the cell to form the phagosome, in a process referred to as phagosomal maturation. These granules contain hydrolytic enzymes and NADPH oxidase subunits that initiate killing mechanisms (see below). Phagocytosis in neutrophils differs from that in other professional phagocytes such as macrophages, in which both particle uptake and phagosome maturation are much slower (97). Additionally, the fully mature neutrophil phagosome has a neutral pH, whereas in macrophages the phagosome is highly acidic (98). This difference may reflect the effects of the oxidative burst, which is massive in neutrophils compared with macrophages, on vacuolar pH. Efficient phagosomal maturation in neutrophils also depends on cytosolic calcium, whereas in macrophages, fusion between lysosomes and the phagosome is calcium independent (99, 100).

The speed with which the neutrophil can engage, engulf, and kill pathogens is an obvious advantage in terms of host defense. However, phagocytosis and phagosomal maturation are not perfect processes, as granules may fuse with the phagosome before it is completely sealed. This leads to the release of cytolytic contents and oxidative products outside the neutrophil, where damage to other cells may occur (101). Neutrophil phagocytic receptors may also be engaged by immune complexes or complement deposited along large surfaces, such as the vascular endothelium, which the neutrophil cannot completely engulf. This so-called frustrated phagocytosis leads to the release of granule contents and oxidative products, causing extensive tissue injury. A prime example of this phenomenon is thrombohemorrhagic vasculitis, in which overproduction of inflammatory mediators such as TNF leads to significant complement (C3) deposition along the vascular endothelium. The deposited

MPO: myeloperoxidase

C3 activates the neutrophil CR3 (Mac-1) receptors, leading to massive granule release, which in turn causes vascular breakdown and hemorrhage (102). Uncontrolled release of neutrophil granules or oxidative products is one of the major causes of tissue injury in a variety of infectious and inflammatory diseases.

Generation of reactive oxygen species. Coincident with particle binding and phagocytosis is the dramatic increase in oxygen consumption (the respiratory burst) associated with the ROS generated by the activation of NADPH oxidase. The assembly of a functional NADPH oxidase requires the inducible translocation of the cytosolic NADPH oxidase components $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ to the membrane, where $gp91^{phox}$ (NOX2), $gp22^{phox}$, and the GTPase Rac2 (or Rac1) reside (103). The primary granule protein myeloperoxidase (MPO) catalyzes the formation of hypochlorous acid (HOCl; the active ingredient in bleach) through reaction of hydrogen peroxide with chloride (104). These oxygen derivatives play a critical role in the killing of pathogenic bacteria and fungi. However, whether they kill directly (through decarboxylation, deamination, or peroxidation of proteins and lipids) or indirectly (through modulation of phagocyte proteinase activity) is still debated (105). Against certain pathogens, such as *Aspergillus*, NADPH oxidase is critical for host defense independently of proteinases (106), and its importance is revealed in that patients who lack any one of the oxidase subunits suffer from chronic granulomatous disease (CGD) (see **Table 1**).

Nitric oxide (NO), a short-lived (half-life of a few seconds), highly reactive molecule, is produced by inducible nitric oxide synthase (iNOS), which is present in primary granules and is induced upon neutrophil priming (via TNF, IL-1, or IFN-γ) (107) and during bacterial infection (108). NO production complements ROS production by neutrophils; mice lacking both NAPDH oxidase and iNOS ($gp91^{phox-/-}$; $Nos2^{-/-}$) develop spontaneous infections caused by commensal flora, whereas singly deficient mice do not (109).

Degranulation. Neutrophils store proteinases and antimicrobial peptides in granules that fuse with the phagosome during pathogen uptake. Granule fusion with the plasma membrane, causing extracellular release of contents, also occurs during neutrophil activation. In general, extracellular release of secretory vesicles and tertiary vesicles happens with modest activation, whereas release of secondary and primary granules occurs mainly through inadvertent leakage from the phagosome, most commonly during frustrated phagocytosis. Fusion of secretory vesicles with the plasma membrane results in the presentation of adhesion and chemotactic receptors that promote neutrophil recruitment (**Figure 2**). Mobilization of tertiary and secondary granules during transmigration may facilitate degradation of collagen in the basement membrane and thus removal of a physical barrier to neutrophil egress.

The contribution of neutrophil antimicrobial peptides to host defense is an especially rich area of current investigation. Neutrophils contain mainly cationic peptides—α-defensins and cathelicidins (110). Cationic antimicrobial peptides provide microbicidal activity through interaction with the negatively charged membrane components of pathogens, resulting in formation of pores, induction of nonspecific membrane permeabilization, binding to intracellular targets to inhibit DNA and/or RNA biosynthesis, and disruption of bacterial biofilms. Cathelicidins, the best studied of which is LL-37, are proteolytically processed from larger proteins. Besides their direct antimicrobial activity, many antimicrobial peptides (especially LL-37) also have clear immunomodulatory functions such as stimulation of neutrophil chemotaxis, induction of chemokine receptor expression, induction of cytokine production, and suppression of neutrophil apoptosis. LL-37 can also potentiate functions of other immune cells, such as DCs (111). The cellular receptors and signaling pathways by which these peptides mediate their immunomodulatory function constitute an active area of research.

Table 1 Features of select congenital phagocyte dysfunction syndromes

Disease	Cause(s)	Laboratory findings: neutrophil defects	Clinical manifestations
Disorder of neutrophil homeostasis			
Severe combined neutropenia (138)	Dominant mutations in neutrophil elastase (~60% of cases) Recessive mutations in HAX-1 or G6PC3 (~30% of cases)	Marked neutropenia (<500 cells/μl of blood) Neutrophil maturation arrest with accumulation of promyelocytes or other immature myeloid cells in the bone marrow	Recurrent infections (bacterial and fungal) Leukemia incidence ~25% after 20 years Associated with acquired G-CSF receptor mutations
Disorders of neutrophil recruitment			
Leukocyte adhesion deficiency I (260)	Mutations in CD18 (*ITGB2*) integrins resulting in CD18 deficiency or surface expression of dysfunctional integrins	Abnormalities in chemotaxis, adhesion, aggregation, immune complexes, and complement-dependent phagocytosis	Marked leukocytosis (and neutrophilia), recurrent severe infections, periodontal disease, delayed separation of umbilical stump
Leukocyte adhesion deficiency II (260)	Mutation in Golgi complex GDP-fucose transporter (SLC35C1) resulting in a defect in fucose metabolism; fucose is required for synthesis of functional selectin ligands (addition of fucose to *O*-linked glycan structures)	Absence of neutrophil rolling on selectins	Marked leukocytosis (and neutrophilia), recurrent infections, periodontal disease Non-phagocyte-related defects due to absence of fucose on all glycoproteins: mental retardation, Bombay blood type, lack of Lewis blood group
Leukocyte adhesion deficiency III (260)	Mutation in Kindlin-3 (*FERMT3*), which is involved in regulation of β integrin activation	Absence of CD18 integrin–mediated neutrophil firm adhesion and migration; selectin-mediated rolling is unaffected	Marked leukocytosis (and neutrophilia), recurrent severe infections, delayed separation of umbilical stump, bleeding tendency (due to defects in platelet integrins)
Disorders of neutrophil activation			
MyD88 and IRAK-4 deficiency (261)	Recessive mutations in MyD88 (*MYD88*) and IRAK-4 (*IRAK-4*) leading to loss of expression; MyD88 and IRAK-4 are responsible for transducing signals from TLRs	Strongly impaired adhesion molecule (L-selectin and CR3) expression, oxidative burst, cytokine production, and cell survival in response to agonists for TLR1/2, TLR2/6, TLR4, and TLR7/8 Normal TLR9 response (262) Impaired activation of NADPH oxidase in primed neutrophils (263)	Recurrent pyogenic infections with pus formation Invasive disease, most often meningitis and septicemia, and localized bacterial diseases Life-threatening infections not documented after adolescence, potentially due to a more proficient adaptive immune response (264)
Dectin-1 deficiency (261, 265)	Recessive mutation in Dectin-1 (*CLEC-7A*) leading to loss of expression; Dectin-1 recognizes β-1,3-glucans in the fungal cell wall and unknown components of *Mycobacterium tuberculosis*	Defective production of cytokines by human peripheral blood monocytes; neutrophil functions not evaluated	Mucocutaneous fungal infections less severe than the classical chronic mucocutaneous candidiasis and/or onychomycosis

(*Continued*)

Table 1 (*Continued*)

Disease	Cause(s)	Laboratory findings: neutrophil defects	Clinical manifestations
CARD9 deficiency (265)	Recessive mutations CARD9 (*CARD9*) leading to loss of expression; CARD9 is an intracellular adapter molecule that relays Dectin-1-induced intracellular signals, leading to NF-κB activation and chemokine generation	Not determined	Mostly mucocutaneous fungal infections with some susceptibility to develop into disseminated candidiasis, particularly meningitis
Disorders of pathogen killing			
Chronic granulomatous diseases (103, 266)	Mutations in components of NADPH oxidase (gp91^{phox}, p47^{phox}, and more seldom p22^{phox}, p67^{phox}, p40^{phox})	Absence of oxidative burst	Severe infections with catalase-positive bacteria such as *Staphylococcus aureus* and fungi such as *Aspergillus* and *Candida* Excessive and/or persistent inflammation, pus, granulomas
Chédiak-Higashi syndrome (266, 267)	Recessive loss-of-function mutations in LYST (*LYST*), a lysosomal trafficking regulator	Large abnormal granules due to lysosomal trafficking defects	Neutropenia; susceptibility to pyogenic infections, especially with *S. aureus*; periodontal disease Non-phagocyte-related defects include neurologic dysfunction, bleeding disorders, lymphoproliferative disorder
Neutrophil-specific granule deficiency (266)	Recessive mutations in either C/EBP (*CEBPE*) or GFI-1 (*GFI1*) leading to loss of these transcription factors, which results in impaired specific granule development	Defects in chemotaxis; reduced releases of myeloperoxidase (MPO) and other granule proteins	Recurrent cutaneous, ear, and sinopulmonary bacterial infections
MPO deficiency (268)	Recessive mutations in MPO (*MPO*) causing loss of expression	Absent MPO	Minimal unless combined with another defect; then, *Candida albicans* or other fungal infections

Abbreviations: CARD9, caspase recruitment domain 9; CR3, complement receptor 3; G6PC3, glucose 6-phosphatase 3; G-CSF, granulocyte colony–stimulating factor; HAX-1, hematopoietic cell–specific Lyn substrate 1 (HCLS1)-associated protein X-1; IRAK-4, interleukin-1 receptor–associated kinase 4; LYST, lysosomal trafficking regulator; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor κB; TLR, Toll-like receptor.

Neutrophil extracellular traps. By far the most explosive area of research in neutrophil biology over the past five years has been the description of NETs (**Figure 3**) (112–114). In 2004, Zychlinsky and colleagues (115) described a process (since coined NETosis) by which neutrophils extrude a meshwork of chromatin fibers decorated with granule-derived antimicrobial peptides and enzymes such as neutrophil elastase and MPO. They suggested that NET formation was a novel form of extracellular bacterial killing. Though NETosis was initially greeted with skepticism, many others have gone on to validate it as an active process in neutrophil host defense that, when perturbed, increases susceptibility to pathogen infection. Moreover, like many neutrophil host defense functions, NETosis

also has a dark side—untended tissue injury and stimulation of undesirable immune reactions.

Mechanisms of NETosis. NETosis is a distinctive form of cell death in which decondensed chromatin and associated granule products are released into the extracellular space as a result of dissolution of nuclear and granule membranes. NETs are defined by the colocalization of chromatin and granule proteins (such as MPO), visualized by immunostaining, in a meshwork outside the cell (116). NETting neutrophils are distinguished from apoptotic cells by the lack of "eat me" signals on the cell surface. As a result, NETting neutrophils are not cleared by other phagocytes, and instead, the residual chromatin is disassembled mainly by nucleases (117). NETosis is stimulated by a variety of inflammatory mediators (e.g., TNF, IL-8, immune complexes) and PAMPs from a wide range of microbes (bacteria, fungi, and protozoa). Similar to other host defense functions, the degree of NETosis varies with the strength and combination of stimuli. However, unlike ROS production and degranulation, NETosis is a slow process, usually taking place over 2–4 h (118). NETosis stimulated by microbial products requires NADPH oxidase activity—the process does not occur in neutrophils from patients with CGD (119). However, NETosis can also be stimulated by soluble immune complexes or *Candida albicans* hyphae immobilized to the extracellular matrix component fibronectin in the absence of ROS production (**Figure 3**) (84, 120). How ROS production stimulates NETosis is controversial. ROS may inactivate intracellular caspases to inhibit apoptosis and induce autophagy, which would promote the breakdown of cellular membranes during NETosis (121). Deimination of histones (mainly conversion of arginine side chains to citrullines) is thought to be required for disassembly of chromatin to allow full NET dispersion (122). Knockout mice lacking PAD4 (peptidylarginine deiminase 4), the enzyme required for histone deimination, have impaired NETosis and reduced host defense to infection (123).

Antimicrobial function of neutrophil extracellular traps. NETs provide antimicrobial function both by localizing and trapping pathogens within a sticky meshwork of chromatin and by exposing pathogens to highly concentrated antimicrobial peptides and enzymes trapped within the chromatin. Besides MPO and neutrophil elastase, NETs are a concentrated source of LL-37, S100A, and lactoferrin-chelating proteins. Moreover, histones themselves have significant antimicrobial activity (124). When these antimicrobials are concentrated along the chromatin network, the potential for synergistic action is enhanced (125). Indeed, some staphylococcal and streptococcal strains express nucleases that degrade NETs to free the bacterium from the chromatin meshwork (126). Others modify their outer polysaccharide capsule to reduce binding to NETs (127). Although the expulsion of DNA to form NETs may be considered an end point for neutrophils, recent work suggests that anuclear neutrophils can migrate and retain the necessary components to kill bacteria through conventional mechanisms (128). If so, then NETosis is certainly distinct from apoptosis, a form of cell death that results in reduced neutrophil function and changes in membrane constituents (i.e., expression of "eat-me" signals) to promote cell clearance (**Figure 3**). What stimuli drive NETosis as opposed to apoptosis is not particularly clear, as ROS can play a major role in both processes. For example, ROS can promote NETosis, but ROS produced during complement- or IgG-mediated phagocytosis also triggers apoptosis associated with caspase activation (3) and induction of proapoptotic genes (129). The molecular mechanisms that differentiate between these outcomes await further investigation. The magnitude and duration of ROS production triggered by the stimulus may be factors in determining the fate of the neutrophil. The nature and/or presentation of the stimulus may also be important determinants. The following

Extracellular matrix: proteins that make up basement membranes and intracellular structures in tissues; critical in neutrophil recruitment, interstitial migration, and cellular activation

Systemic lupus erythematosus (SLE): a chronic, multiorgan autoimmune disease primarily affecting females; characterized by high titers of circulating immune complexes (e.g., to nuclear antigens)

is a case in point: Precipitating immune complexes and antibody-opsonized targets lead to marked apoptosis, whereas soluble immune complexes instead trigger NETosis when endocytosed (84, 130). Understanding the mechanisms that tip the balance between apoptosis and NETosis (**Figure 3**) will be important for predicting the outcomes of inflammation and represents a fruitful area for future investigation.

The dark side of NETosis. Excessive NET formation is linked to various neutrophil-mediated pathologies, including vasculitis (131), sepsis (118), and systemic lupus erythematosus (SLE) nephritis (132). NETs are a rich source of pro-inflammatory molecules (such as chromatin–LL-37 complexes) and autoantigens (133). NETs also induce platelet procoagulant activation, which can lead to significant thrombosis and vascular injury (134). Excessive NET formation and endothelial cell activation are also associated with preeclampsia of pregnancy (135). The mechanisms of tissue injury by neutrophils and NETs are expanded below.

DISORDERS OF NEUTROPHILS

Most of our current knowledge of neutrophil function is based on in vitro studies on human neutrophils and models of disease in genetically engineered mice. Given that immunological processes in mice and humans differ (136), and given the caveats with knockout approaches (e.g., compensation), it is important to validate in humans some of the concepts developed in murine systems. Congenital abnormalities in human patients involving leukocyte recruitment have helped define the molecular underpinnings of neutrophil recruitment and activation. Genetic deletions in components of the NADPH oxidase and MPO pathways have enriched our understanding of neutrophil cytotoxic functions, while deletions in PRRs such as Dectin-1 and downstream adapter molecules of TLRs have aided in our understanding of the relative contributions of these PRRs in neutrophil function and host defense. **Table 1** summarizes some of these congenital abnormalities along with associated laboratory findings and clinical manifestations (137). One would predict from mouse studies that some of these mutations might protect patients from development of certain types of sterile inflammatory diseases, although proof for this concept is not forthcoming because of the rarity of these disorders.

Homeostasis

Though rare, numerous human mutations have been defined that give rise to defects in neutrophil maturation. These syndromes are collectively known as severe congenital neutropenia (SCN) (138). Patients with SCN typically present with recurrent life-threatening infections in the first few months of life and have neutrophil counts of less than 500 cells/μl of blood for at least 3 months. The neutrophils of patients with SCN often show a characteristic maturation arrest at the promyelocyte stage of differentiation. Approximately 60–75% of SCN cases are due to mutations in the gene encoding neutrophil elastase. These mutations often act in a dominant manner and are thought to lead to accumulation of misfolded elastase molecules, causing an endoplasmic reticulum stress (unfolded protein) response in maturing neutrophils that induces apoptosis (139). Recessive mutations in the genes encoding the mitochondrial antiapoptotic protein HAX-1 [hematopoietic cell–specific Lyn substrate 1 (HCLS1)-associated protein X-1] and the glycosylation enzyme G6PC3 (glucose 6-phosphatase 3) also lead to a range of neutropenias (140, 141). Nearly one-third of SCN patients have acquired mutations in the G-CSF receptor, which commonly lead to expression of a truncated protein that lacks signaling capacity. Surprisingly, studies in cell lines and mouse models demonstrate that these mutations cause increased signaling responses to G-CSF, potentially leading to apoptosis. Indeed, patients with this form of SCN often develop myeloid leukemia (142).

Recruitment

Inherited defects in neutrophil recruitment in patients with leukocyte adhesion deficiency (LAD) highlight the importance of selectins (LADII); β_2 integrins (LADI); and integrin activation, specifically Kindlin-3 (LADIII) in getting cells to the site of inflammation. Patients with LADI, LADII, or LADIII present with infections without pus formation, a reflection of poor neutrophil accumulation. Importantly, mice with deficiencies in the β_2 integrins, selectins, or integrin activation exhibit profound defects in neutrophil recruitment (28). Ironically, the initial confusion concerning the underlying gene mutations that cause LADIII (which was originally thought to be caused by mutations in a gene next to Kindlin-3) also taught us an important lesson about genotype-phenotype correlation (143). Finally, though extremely rare, patients have been found with mutations in the Rac2 GTPase, which lead to impairment in chemokine signaling and actin remodeling that result in recruitment defects (144).

Activation

Numerous disorders linked to alterations either in pathogen sensing or in the molecules involved in intracellular signaling downstream of pathogen-sensing receptors have now been defined in patients. Defects in TLRs or TLR signaling pathways [MyD88 (myeloid differentiation primary response gene 88) or IRAK-4 (IL-1 receptor–associated kinase 4)] impair bacterial sensing, leading to reduced neutrophil activation. Patients with these defects manifest pyogenic infections. By contrast, patients with inborn deficiencies in the receptors or signaling molecules involved in fungal sensing [Dectin-1 and CARD9 (caspase recruitment domain 9)] present almost exclusively with fungal (often *Aspergillus*) infections. Although studies in neutrophils of these patients are not comprehensive, mice deficient in these molecules have defects in neutrophil cytotoxic responses (70, 145). These observations highlight the point that the type of pathogen infection is often a significant clue to the molecular defect involved. Indeed, increased susceptibility to herpesvirus encephalitis has been mapped to mutations in TLR3, the primary virus-sensing TLR, though these mutations affect antigen-presenting cells more than neutrophils (146).

Pathogen Killing

The most common type of neutrophil functional defect is caused by genetic deficiency of any one of the subunits of NADPH oxidase, which results in CGD. The most common form of CGD is due to loss of $gp91^{phox}$, which is encoded on the X chromosome. Boys with this disease present early in life with chronic infections that often lead to formation of tissue granulomas. Autosomal-recessive forms of CGD result from deficiencies of the other NADPH oxidase subunits; these disorders tend to have a better prognosis than the X-linked form. Importantly, many patients with CGD develop chronic intestinal inflammation, mimicking Crohn's disease or ulcerative colitis, due to bacterial overgrowth and inability to maintain barrier function in the gut (147). This observation illuminates the frequent overlap between immunodeficiency diseases and autoimmunity—often the former causes the latter.

Other disorders of pathogen killing can arise from defects in granule formation and/or loss of granule enzymes. Chédiak-Higashi syndrome (CHS) manifests in many granule-containing cells because of mutations in the *LYST* (lysosomal trafficking regulator) gene, which encodes a protein essential for lysosomal trafficking. CHS patients also have defects in cytolytic T cell killing. Surprisingly, patients with loss of the granule enzyme MPO are only modestly affected, suggesting that other neutrophil killing mechanisms are adequate to provide host defense in these patients. Another extremely rare disorder of neutrophil killing is neutrophil-specific granule deficiency (SGD). Neutrophils from SGD patients lack secondary and tertiary granules due to mutations in genes encoding transcription factors C/EBPε or Gfi-1 (growth factor independent 1) (148).

Finally, numerous other disorders that affect many cell types can also result in neutrophil functional defects and impaired pathogen killing. A prime example is Wiskott-Aldrich syndrome (WAS), which is due to loss of the WAS protein, which is involved in actin remodeling. Neutrophils from WAS patients show migratory and bacterial killing defects (149).

Rheumatoid arthritis (RA): an autoimmune disease characterized by inflammation of the joint-lining membranes associated with autoantibodies (e.g., to citrullinated epitopes)

IMMUNOMODULATORY FUNCTION OF NEUTROPHILS

There is emerging evidence from a number of groups indicating that neutrophils not only are involved in the killing of extracellular pathogens but also contribute to the immune response to intracellular pathogens and viruses through complex cross talk with other immune cells, such as DCs, lymphocytes, and NK cells. Much of this cross talk is mediated by the ability of neutrophils to secrete a host of cytokines or express a large number of cell surface molecules that directly interact with other immune cells (9). These findings are changing our traditional view of neutrophils from terminally differentiated effectors to transcriptionally and functionally active partners in the entire immune response.

Regulation of Dendritic Cells

Many neutrophil products, including lactoferrin, α-defensins, and chemokines (such as CCL3), are chemotactic for DCs and are required for rapid DC recruitment to sites of infection (150, 151). Direct binding of neutrophils to DCs promotes maturation of DCs into more effective antigen-presenting cells and provides DCs access to neutrophil-captured pathogen products (152, 153). Neutrophil-DC interactions such as these have been defined in the colonic mucosa of Crohn's disease patients (154). Neutrophils also play a significant role in activating DCs in autoimmune diseases, such as SLE and diabetes (155, 156). NETs, containing chromatin complexed with LL-37 peptides, induce IFN-α production from plasmacytoid DCs, which in turn drives formation of self-reactive lymphocytes that recognize chromatin–LL-37 complexes, leading to production of autoantibodies. Type I interferons, such IFN-α, also stimulate neutrophils, further promoting NET formation and thereby establishing a self-amplifying loop of inflammation between neutrophils and DCs.

Regulation of T and B Cell Function

Neutrophils and T cells modulate each other at several levels. Neutrophils can serve as antigen-presenting cells. Upon IFN-γ stimulation, neutrophils express low levels of major histocompatibility complex class II and co-stimulatory molecules, which can facilitate Th1 and Th17 differentiation (157). These markers of antigen-presenting function have been detected on neutrophils from patients with active Wegener's granulomatosis (158) and rheumatoid arthritis (RA) (159) and in a mouse model of chronic colitis (160). Interestingly, neutrophils can also carry antigens to lymph nodes by migrating through lymphatics, where they either directly present the antigen to T cells or deliver it to DCs (161, 162). They may also carry viral antigens from the skin directly to the bone marrow to promote establishment of $CD8^+$ memory T cells (163). Neutrophils can also have immunosuppressive effects. They can inhibit the proliferation of IFN-γ-producing T cells through an NO-dependent mechanism. Liberation of arginase by activated or dying neutrophils depletes extracellular L-arginine, thereby inhibiting T cell proliferation (164). More recently, a subset of neutrophils was shown to inhibit T cell proliferation by releasing ROS in the immunological synapse (165).

Neutrophils are a major producers of the cytokines BAFF (B cell–activating factor; also known as BLyS) and APRIL (a proliferation-inducing ligand), which are required for B cell survival and activation (166). Activation of splenic neutrophils by microbial PAMPs leads to significant production of BAFF, APRIL, and IL-21, which in turn directly activate splenic marginal zone B cells and facilitate production of antibodies to T cell–independent antigens

(167). Interestingly, circulating neutrophils cannot directly stimulate B cells, suggesting that specific clues within the splenic microenvironment are required for formation of B cell–helper neutrophils.

Regulation of Natural Killer Cells

Numerous interactions between neutrophils and NK cells have recently been defined. Neutrophils regulate terminal NK cell maturation under steady-state conditions both in patients and in a mouse model of neutropenia (168). These studies used neutrophil depletion (via either monoclonal antibody treatment or genetic mutation of the myeloid transcription factor Gfi-1) in mice or clinical observations in autoimmune-neutropenic patients to correlate neutrophil counts with normal development and function of NK cells. How this cross talk is mediated in the steady state remains to be determined. However, during infectious disease, release of cytokines from neutrophils directly activates NK cell function—for example, neutrophil-derived IL-18 is required for IFN-γ production by NK cells during *Legionella* infection in mice (169). IL-12 production by DCs is also required for full activation, suggesting a three-way interaction between these cells. Indeed, colocalization of neutrophils, NK cells, and DCs in inflammatory lesions in Crohn's disease patients has been observed and may be involved in IFN-γ production by NK cells during active inflammation (170). Importantly, many NK cell–derived cytokines, such as IFN-γ and GM-CSF, act back on neutrophils, either priming them or prolonging survival, thereby enhancing inflammatory responses.

Regulation of Macrophages

Neutrophil-macrophage interactions are important in both the initiation and resolution phases of the inflammatory response. Recruited neutrophils contribute to monocyte influx at sites of inflammation by secreting chemokines (e.g., CCL2, CCL3, CCL19, CCL20) and granule proteins (e.g., S100A and various antimicrobial peptides). Neutrophil primary granule proteins also enhance the antimicrobial activity of macrophages by increasing their ability to phagocytose and elaborate cytokines (10, 171). A prime example is the demonstration that the murine cathelicidin peptide CRAMP (cathelicidin-related antimicrobial peptide; homologous to human LL-37) stimulates monocyte and macrophage recruitment into inflammatory atherosclerotic lesions by activation of formyl peptide receptors (172). During the resolution phase of inflammation, uptake of apoptotic neutrophils by macrophages leads to a decrease in IL-23 production by the macrophages, which diminishes IL-17 secretion by T cells and hence reduces G-CSF and neutrophil production (see Neutrophil Homeostasis, above). Phagocytosis of apoptotic neutrophils by macrophages also stimulates the macrophages to produce IL-10 and downmodulate IL-12, thus assuming an M2-like phenotype, to promote tissue repair during resolution of inflammation (173).

Effects on Endothelial and Epithelial Cells

Interactions between neutrophils and endothelial or epithelial cells during inflammatory responses can have significant effects on the inflammatory and barrier functions of the latter cell types (174). During the process of transmigration, particularly under more severe inflammatory conditions, neutrophils can induce signaling changes in endothelial or epithelial cells, causing them to contract and thereby generating intercellular gaps. This allows serum proteins (such as cytokines, antibodies, and complement) to pass through the endothelial or epithelial barrier, generating edema fluid. The effects on epithelial barrier integrity during neutrophil transepithelial migration are particularly adverse and contribute to fluid accumulation on the luminal side of epithelial cells, such as in the lung (causing pulmonary edema) or the gut (exacerbating inflammatory diarrhea). Numerous soluble

factors derived from neutrophils, such as vascular endothelial growth factor (VEGF), serine proteinases, and lipid mediators such as leukotriene A4, act on their cognate receptors [VEGF receptor, various proteinase-activated receptors (PARs), and Cox1 or Cox2 enzymes that metabolize leukotriene A4 to thromboxane] on endothelial or epithelial cells, inducing changes in the actin cytoskeleton that result in cell contraction and loss of barrier integrity. Direct interaction between neutrophil integrins (Mac-1 and LFA-1) and endothelial ICAM-1 induces intracellular signaling pathways that lead to cell contraction. Release of the neutrophil-derived heparin-binding protein (HBP), present in both secretory and azurophilic granules, causes significant changes in endothelial cell barrier integrity through binding to cell surface proteoglycans (175). Inactivation of HBP prevents neutrophils from inducing endothelial cell hyperpermeability both in cultured cells and in vivo (176). Indeed, the serum level of HBP has been used as a biomarker to predict clinical outcome in patients with pulmonary edema and severe sepsis or shock (177). Production of ROS by neutrophils also directly affects endothelial cell integrity, reducing barrier function (178). Many of these same interactions induce inflammatory cytokine and chemokine production by endothelial and epithelial cells, which only furthers neutrophil recruitment and activation, thus establishing a self-amplifying loop of inflammation. For example, release of mitochondrial DAMPs during tissue injury acts directly on both endothelial cells and neutrophils to exacerbate inflammation-associated increases in vascular permeability (179). Targeting mechanisms of changes in endothelial or epithelial cell permeability function has obvious therapeutic potential.

Neutrophil-Derived Microparticles as Modulators of Inflammation

Recent studies have highlighted the potential immunomodulatory function of membrane microparticles derived from activated neutrophils in a variety of disease settings (180, 181). Microparticles are membrane fragments released from a variety of cell types by blebbing (similar to apoptotic blebs) during cellular activation. These particles are small (usually <1 μm), their membranes are rich in negatively charged phosphatidylserine, and they contain surface proteins that reflect the cell from which they were derived (neutrophil-derived microparticles are often described as containing CD11b, CD62L, and CD66). Originally described as a by-product of platelet activation, microparticles were initially believed to be inert. It is now clear that they contain functional ligands and receptors that can confer modulatory effects on other cells. Neutrophil-derived microparticles have both immune-activating functions (on platelets and endothelial cells) and immune-suppressive functions (on macrophages) (182, 183). Microparticles influence cellular function directly, through receptor-ligand interactions, or by being internalized by the target cell, by fusing membranes with the target cell, and even by delivering nucleic acids. The generation of neutrophil-derived microparticles has been visualized in vivo during neutrophil transendothelial migration (184). Neutrophil-derived microparticle activation of platelets, which is mediated by binding of neutrophil Mac-1 and PSGL-1 to platelets, display of platelet activating factor (PAF), and accumulation of tissue factor, has been best characterized as a major contributor to coagulopathies that accompany severe inflammatory responses (185). The anti-inflammatory properties of neutrophil microparticles on monocytes are mediated by expression of annexin 1 and by their ability to induce macrophage transforming growth factor β (TGF-β) production (183, 186). Neutrophil-derived microparticles can also elicit inflammatory cytokine production by endothelial cells. Very recent proteome profiling of neutrophil-derived microparticles, generated by activating neutrophils under adhesive conditions or in suspension with soluble agonists, revealed that microparticles can have diverse compositions depending on how they were generated (187). These

differences are also reflected in comparing neutrophil-derived microparticles found in the serum of septic patients with those found in inflammatory exudate from skin blisters. Functionally, neutrophil-derived microparticles can even mount limited ROS production and chemotaxis responses to chemokine gradients (187). Neutrophil-derived microparticles are observed in the serum, exudate fluid, and cerebrospinal fluid of patients with a variety of inflammatory diseases, often at levels that correlate with disease severity (181), suggesting that their presence may have a physiologically relevant immunomodulatory effect. Clearly further work in this area is justified.

NEUTROPHILS IN CHRONIC DISEASES

The role of neutrophils in acute inflammation leading to tissue injury is well established. However, several studies suggest that neutrophils also play a significant role in both initiating and shaping the immune response during more chronic inflammatory diseases, such as atherosclerosis, autoimmune disease, and even cancer. These new functions for neutrophils, which highlight their multifaceted capabilities, are discussed below.

Atherosclerosis

Atherosclerosis is a chronic disease that results from the deposition of pro-inflammatory lipids in the vasculature, which leads to poorly controlled blood vessel inflammation (188). Until recently, the potential contribution of neutrophils was largely neglected in this disease because they were not detected in atherosclerotic lesions. However, more sensitive immunohistochemical methods with markers restricted to granulocytes have allowed neutrophils to be detected in both early and more developed atherosclerotic lesions in humans (189) and in mouse models of the disease (190–192). MPO-generated ROS that promote endothelial cell apoptosis, tissue factor expression, low-density lipoprotein nitration, and lipid peroxidation may advance lesion development (193). Many neutrophil granule proteins, including azurocidin, LL-37, and α-defensins, are also found in human atherosclerotic lesions, suggesting that activated neutrophils may directly contribute to lesion development (194, 195). Studies in mouse models of atherosclerosis and indirect evidence in human samples suggest that many of these neutrophil-derived factors attract monocytes and influence their activation status (196). NETs, which contain cathelicidin-chromatin complexes, have also been suggested to drive vessel inflammation by recruiting monocytes (172). Neutrophils may also promote matrix degradation through proteinase 3 (PR3) and matrix metalloproteinases (MMPs), leading to weakening of the fibrous cap on vascular atherosclerotic lesions (196). Manipulation of circulating neutrophil numbers or neutrophil recruitment affects lesion development in mouse models. In particular, neutropenia induced by CXCR2 deficiency results in reduced lesion size (197), whereas neutrophilia triggered by disruption of the CXCR4-CXCL12 axis, important for retaining granulocytic precursors within the bone marrow, leads to increased atherosclerotic burden (198). In addition to the roles of neutrophils in the initiation and progression of atherosclerotic lesions, the chronic presence of neutrophils in these lesions may contribute to thrombosis, myocardial infarction, and stroke, as NET formation and serine protease release promote intravascular thrombus growth (199). Deposition of activated platelets in the vessel wall in turn attracts more neutrophils through the display of platelet adhesion molecules such as P-selectin or the production of platelet-derived chemokines such as CCL5. Mice genetically deficient in these molecules, and those rendered platelet deficient, have reduced atherosclerotic lesion development (190, 200). Taken together, the histological evidence in human lesions and the functional analysis in murine models imply a complex interaction between neutrophils, platelets, and monocytes that drives chronic inflammation in atherosclerotic lesions.

Autoimmune Diseases

Antineutrophil cytoplasmic antibodies (ANCAs): a group of antibodies against intracellular antigens in neutrophils and monocytes that present on the surface of activated cells

Neutrophils have been implicated in the pathogenesis of numerous autoimmune diseases, both as effector cells that mediate tissue injury and, more recently, as immune-modulating cells that affect the function of other cells. Much more is known about how neutrophils mediate tissue injury in autoimmune disease, mainly as a result of poorly controlled neutrophil activation, which leads to stimulation of effector functions normally used to control pathogen infection. However, with the increasing recognition that neutrophils can modulate the function of other immune cells, via cytokine production or direct interaction, it is now appreciated that neutrophils can play more complex roles in autoimmune disease development. In some autoimmune diseases, neutrophils have now been defined as the major source of autoantigens that drive disease pathogenesis. Here, we focus primarily on examples of IgG-mediated autoimmune disease as IgG immune complexes directly elicit neutrophil cytotoxic responses.

Direct activation of neutrophils by autoantibodies. Numerous vasculitides, including Wegener's granulomatosis, microscopic polyangiitis, Churg-Strauss syndrome, and renal-limited vasculitis, are caused by the development of autoantibodies that bind to neutrophil antigens and induce cellular activation (201). These diseases are characterized by neutrophil and macrophage localization to small and medium-sized vessels without detectable immune deposits in the tissue. Neutrophil activation at these sites is associated with severe vessel damage in organs including the lungs and kidneys. The antineutrophil cytoplasmic antibodies (ANCAs) are directed against cytoplasmic neutrophil products such as MPO, PR3, and Lamp-2 (lysosome-associated membrane protein 2), which are exposed at the cell surface of primed/activated or apoptotic neutrophils (202, 203). Convincing evidence of the pathogenic potential of ANCAs and the role of neutrophils in disease pathogenesis was provided in animal models wherein anti-MPO antibodies in the absence of functional T cells caused vasculitis and glomerulonephritis that were significantly reduced by depletion of neutrophils (204, 205). The Fab portion of ANCAs can trigger robust neutrophil activation, whereas the Fc portion engages neutrophil FcγRIIA to promote degranulation, cytokine release, and ROS production (206). The cumulative stimulation of neutrophils with TNF, complement (induced by TNF and the Fc portion of ANCAs), FcγRs, and β_2 integrins leads to increasing levels of activation and subsequent sequestration within vessels in the absence of classical selectin-mediated recruitment (207). ANCA-activated neutrophils can set into motion a feed-forward cascade of increasing inflammation and autoantibody production by inducing thrombosis (and hence platelet-mediated neutrophil activation) or by releasing BAFF to promote B cell differentiation and enhance autoantibody production (208–210). ANCA-activated neutrophils also produce NETs—chromatin-MPO complexes have been found in glomeruli and in the interstitium of renal biopsies from small-vessel vasculitis patients (131). The autoantigen MPO in the tissue-deposited NETs may instigate further immune-complex deposition and thus more inflammatory cell stimulation.

Activation of neutrophils by tissue immune complexes. A number of disease states lead to production of autoantibodies to host antigens, which form tissue-deposited immune complexes that directly activate neutrophils, leading to tissue injury, often through the process of frustrated phagocytosis. Many of these diseases, such as RA, have been modeled in mice and depend on FcγR- and integrin-mediated neutrophil activation. SLE is another well-studied autoimmune disease in which neutrophil recognition of tissue immune complexes (mainly in the kidney) leads to neutrophil recruitment and subsequent tissue damage. Although the role of neutrophils in the effector phases of immune complex–mediated diseases is well recognized, neutrophil activation in these diseases has only

recently been shown to both initiate and amplify the disease process by providing sources of self-antigens and directly stimulating other immune cells.

For decades, rheumatoid factor (an IgG that recognizes self-IgM antibodies) was considered the main pathogenic autoantibody in RA. More recently, investigators demonstrated that autoantibodies that recognize citrullinated proteins are more specific indicators of RA. Autoantibodies to deiminated filaggrin, fibrin, vimentin, and collagen are all defined in RA (211). These same types of anticitrullinated antibodies are also found in patients with Felty syndrome, a severe form of arthritis that progresses to neutropenia and susceptibility to repeated infection (212). The primary source of these autoantigens is NETs. As discussed above, NETosis depends on deimination of histones (mainly conversion of arginine residues to citrullines by the enzyme PAD4) to promote disassembly of chromatin. Many other neutrophil proteins are deiminated during NETosis and are then picked up by antigen-presenting cells and presented to self-reactive lymphocytes to drive the formation of autoantibodies and immune complexes. Serum from patients with RA and Felty syndrome can directly activate human neutrophils in vitro, leading to NETosis (213). Hence, neutrophils not only respond to immune complexes in RA but are a major source of the antigens that drive the disease, again establishing a self-amplifying loop of inflammatory pathology.

Recent studies suggest that neutrophils play a major role in initiating systemic autoimmune diseases such as SLE. Neutrophils have long been recognized in renal SLE lesions, and their presence is associated with increased disease severity (214); stimulation of NETosis by tissue immune complexes is now believed to be a major driver of type I interferon (IFN-α and IFN-β) production in these patients (133). A major feature of mononuclear and tissue cells from SLE patients is a gene expression profile that makes them appear as if they were stimulated by type I interferons; this profile is referred to as the interferon signature (215). Animal models support the role of type I interferons as major stimulators of both antigen-presenting cells and self-reactive lymphocytes in systemic autoimmunity. Several studies demonstrate that neutrophils from SLE patients readily undergo NETosis in response to numerous stimuli and have an enhanced ability to strongly activate type I interferon production by plasmacytoid DCs (155, 216). Within the NETs, the cathelicidin peptide LL-37 is particularly important in inducing IFN-α production. These interferons can act on other immune (and nonimmune) cells to stimulate inflammatory cytokine expression, upregulate adhesion molecules, and produce chemokines, establishing a feed-forward amplification loop for disease pathogenesis. Besides production of NETs, the severity of SLE in subsets of patients is correlated with reduced ability to clear NETs, mainly due to autoantibodies that block DNase I–mediated NET breakdown in tissues (132). A direct correlation between reduced ability to break down NETs and disease severity has recently been demonstrated (217).

Compelling evidence that neutrophils directly contribute to end organ damage in SLE comes from human genome-wide association studies. These studies have identified polymorphisms in genes that are highly expressed in neutrophils as potential SLE susceptibility loci, including the genes that encode FcγRIIA, FcγRIIIB, and Mac-1 (CD11b/CD18) (219). Recent work suggests that the nonsynonymous Mac-1 R77H single-nucleotide polymorphism, one of the strongest genetic risk factors in human SLE, is associated with a reduction in Mac-1 function in neutrophils (220). In vivo, passive transfer of human SLE lupus serum containing immune complexes promoted renal neutrophil accumulation and nephritis in mice expressing the human FcγRIIA on neutrophils. Interestingly, this occurred only when mice additionally lacked Mac-1. Evidence was provided that Mac-1 negatively regulates FcγRIIA-mediated neutrophil accumulation. The influx of macrophages was minimal, and their depletion did not affect disease progression in this model (221). Together,

these studies demonstrate an important role for neutrophils and their receptors in the regulation of end organ damage in SLE.

Allergic Diseases

If there is any area in medicine in which the potential contribution of neutrophils to disease pathogenesis is unappreciated, it is allergy and anaphylaxis. However, recent studies highlight a role for neutrophils in these diseases as well. Although allergy is typically considered to depend on IgE and mast cells, there are clearly roles for IgG and other immune cells in development of allergic diseases. These roles were recently demonstrated in a mouse model of anaphylaxis, both through passive administration of IgG and through administration of antigen to sensitized mice (222). In both models, depletion of neutrophils or neutrophil FcγRs protects mice from anaphylaxis. Amazingly, adoptive transfer of human neutrophils into the FcγR-deficient mice restored the response, suggesting that human cells can induce systemic anaphylactic reactions in response to IgG. In this model, anaphylaxis is mediated not by histamine but by neutrophil-derived platelet-activating factor, a known vasoactive lipid. By contrast, neutrophil-derived histamine is the major contributor to pulmonary allergic inflammation in chronic mycoplasma infection (223). Neutrophils may also contribute to the sensitization phase of allergic skin diseases. This idea is suggested by the surprising finding that depletion of neutrophils protects mice from the development of contact dermatitis, which suggests that these cells are important in facilitating the development of allergen-specific T cell responses (224).

Inflammatory Bowel Disease

Neutrophils play a clear role in the pathophysiology of inflammatory bowel disease (IBD), both Crohn's disease and ulcerative colitis. Neutrophils make up a significant proportion of the inflammatory infiltrate in the intestinal walls of patients with IBD; the degree of neutrophil infiltration correlates with the clinical severity of the disease (225). In animal models, depletion of neutrophils or blockade of their ability to respond to chemokine stimuli (i.e., in CXCR2-deficient mice) reduces disease severity in experimental colitis (226). In both animal models and humans, promotion of neutrophil expansion through the IL-23–IL-17–IL-22 axis plays a significant role in the development of IBD (227). Much of the pathology of neutrophils in IBD is correlated with their effects on epithelial barrier function, as the process of transepithelial migration is associated with a significant breakdown of epithelial integrity. In cell culture, neutrophil transmigration across intestinal epithelia leads to changes in the epithelial actin cytoskeleton, causing cell retraction and loss of barrier function. Similarly, release of neutrophil serine proteases, such as elastase or PR3, can directly activate epithelial PARs, leading to cell retraction and reduction of barrier function (228). Increased levels of neutrophil-derived MMP8, MMP9, and prolyl endopeptidase have been found in the intestinal walls of patients with IBD. Besides causing direct cellular damage, these proteases can degrade collagen to produce chemotactic peptides that further drive neutrophil recruitment to the bowel. Antibody-mediated blockade of collagen-derived peptides reduces disease in experimental colitis (229). Production of ROS by neutrophils also has a direct tissue-damaging effect in IBD. Mice lacking the gp91^{phox} subunit of NADPH oxidase are protected from experimental colitis (although curiously, gp47^{phox} mutant animals respond normally) (230). The overproduction of ROS by neutrophils has been linked to the inflammatory induction of gastrointestinal cancer that is common in IBD patients, likely through a direct mutagenic effect on epithelial cells or through stimulation of additional epithelium-mediated inflammatory responses (60). Finally, the development of ANCAs (in particular anti-PR3 antibodies), which can directly activate neutrophils and thus exacerbate inflammation, is also observed in many IBD patients (231). Indeed, these antibodies, along with neutrophil secretion

products (such as the neutrophil gelatinase-associated protein lipocalin), are useful serum biomarkers of inflammatory activity in IBD patients (232). Hence, as in other inflammatory diseases, targeted therapies that diminish neutrophil activity should have a significant effect in IBD.

Cancer

The relationship between neutrophils and cancer pathogenesis is a burgeoning area of research (233–235). It is also a confusing area, because it appears that tumor-associated neutrophils (TANs) can have either protumorigenic or antitumorigenic effects, depending on the tumor type and/or the model system used. Most clinical observations suggest that the presence of abundant neutrophils within a tumor bed is associated with increased tumor growth and hence a poor prognosis (236). Many tumors produce chemokines that attract neutrophils to the tumor bed, including CXCL6, CXCL8, and CCL3 (and in murine systems the orthologs GCP-2, KC, and MIP-1α) (237, 238). In some model systems, the initial recruitment of neutrophils to the tumor bed leads to their activation, resulting in the production of neutrophil-derived chemokines (such as CXCL1, CXCL2, and CCL3) that further amplify inflammatory cell recruitment (239). Expression profiling of TANs in mouse models confirms that mRNAs for various chemokines are significantly (>75-fold) increased (240). Within the tumor bed, neutrophils can produce factors that facilitate tumor growth, including proteinases such as neutrophil elastase, MMP8, and MMP9; proangiogenic factors such as VEGF; and agents such as oncostatin M that act directly on tumor cells. Most of the data supporting these observations come from animal models—for example, the reduced growth of tumors of various types in mice lacking elastase or MMP9 (241, 242). Similarly, antibody-mediated depletion of neutrophils leads to loss of many of these factors within the tumor bed, such as VEGF within transgene-induced pancreatic islet tumors (243). In a melanoma tumor model, neutrophils are suggested to promote metastasis by directly binding to tumor cells (via tumor cell ICAM-1 binding to neutrophil Mac-1) and facilitating their entry into the vasculature (244). Release of elastase from neutrophils may also facilitate metastasis by degrading basement membranes and allowing egress of tumor cells into the bloodstream. Finally, TANs have a direct immunosuppressive effect on cytolytic $CD8^+$ T cells, potentially through the secretion of arginase 1, which degrades arginine and thereby limits T cell activation (245). Consistent with this effect, depletion of neutrophils in tumor-bearing mice can result in activation of $CD8^+$ effector T cells to promote antitumor immunity (246). The presence of tumor-associated myeloid cells with immune-suppressor function has been recognized in mouse models for many years and was recently defined in human tumor isolates (234). There appear to be two general types of myeloid-derived suppressor cells (MDSCs): a monocytic variety and a granulocytic variety. The relationship between granulocytic MDSCs and straightforward TANs is somewhat murky, though recent gene expression profiling suggests that they represent a spectrum of activated neutrophils (240). In fact, many of the properties ascribed to TANs, such as high arginase 1 production, are also ascribed to MDSCs; hence, these may just be differentially activated variants of the same cell type (247). However, because these suppressive neutrophils have similar characteristics to M2-like macrophages (which are involved in tissue repair and are also associated with high arginase production), several investigators refer to them as N2 neutrophils (246).

In contrast to these immunosuppressive neutrophil functions, numerous observations suggest that inflammatory neutrophil infiltration provides an antitumor effect in some forms of cancer (233). Neutrophils can directly kill tumor cells, mainly in vitro through production of ROS that induce oxidative

damage in the tumor cell. The production of ROS may differentially affect metastatic cells, thereby limiting tumor spread, as suggested in one study of "tumor entrained neutrophils" in breast cancer patients (248). Activated neutrophils also express FAS, which, through interactions with FAS ligand on the tumor cell, can induce apoptosis. Activated neutrophils are, of course, pro-inflammatory and hence can secrete cytokines that augment cytolytic $CD8^+$ T cell function. Activated tumor-associated neutrophils may also either directly present tumor antigens to $CD8^+$ T cells or facilitate presentation by DCs. Finally, many of the antibody therapies against tumor cells depend on FcγR function and cytolytic activity of neutrophils, as demonstrated in animal models using neutrophil-depletion approaches. Because many of these functions are associated with the pro-inflammatory, host-defense function of neutrophils, some investigators refer to these types of TANs as N1 neutrophils (246).

The factors that drive the balance between protumorigenic N2 neutrophils and antitumorigenic N1 cells remain enigmatic. Obviously, if one could change this balance—driving N2-like cells to an N1-like phenotype, potentially by modulating the cytokine balance (e.g., TGF-β) in the tumor microenvironment—one could develop novel immunotherapies for cancer.

CONCLUSIONS AND PERSPECTIVES

Despite two decades of research uncovering destructive neutrophil functions in inflammatory diseases, there has been little progress in specifically targeting these processes for therapeutic purposes. Limiting drug targets to the cytotoxic functions of neutrophils while sparing their recruitment and accumulation is likely ideal, because recent studies, albeit primarily in vitro, suggest that neutrophil soluble mediators and contact with other adaptive immune cells can influence the immune response. Moreover, neutrophils may contribute to the resolution of inflammation and tissue repair. Thus, a scalpel approach to debilitating specific cytotoxic functions, tailored to specific inflammatory conditions, has the greatest possibility for limiting the tissue-destructive potential of neutrophils while only minimally immunocompromising the patient. Advances in this area will require better tools. The literature is replete with studies in knockout mice that suggest roles for proteases and oxygen radicals in disease, but the challenge is to decipher whether neutrophils are the source of these products. Difficulty arises with the short life span and easy activation of neutrophils, which preclude in vitro genetic manipulations that are possible in other immune cells. Innovative in vivo approaches that are less labor intensive than the traditional and conditional knockout approaches are needed. There have been some developments in this area; recent technological advances include transgenic short hairpin RNA (shRNA) silencing in neutrophils in vivo (249), retroviral transduction of bone marrow progenitors with reconstitution (250), and reconstitution of *Gfi1*$^{-/-}$ mice that genetically lack neutrophils with mature neutrophils (251). Finally, zebra fish models expressing the green fluorescent protein transgene in neutrophils, coupled with forward genetic screens and live imaging, are now used to identify candidate regulators of neutrophil-mediated inflammation and resolution (252); this model system could identify new genetic pathways and hypotheses. In any case, the old view of neutrophils as mere first responders in the army of the immune system, with no other function than to seek out pathogens within tissue sites and then kill them, is clearly incorrect. We are seeing an explosion of new roles for these cells in immune function in a variety of settings. Of course, learning to use that information therapeutically will be the next challenge. Neutrophil-specific targeting of signaling molecules downstream of activating receptors or approaches to changing the balance of activating versus suppressive function of these cells are interesting ideas.

SUMMARY POINTS

1. Neutrophil production is coordinated through cytokine production by adaptive immune cells.
2. Neutrophil recruitment to sites of infection involves unique molecular interactions in different tissues.
3. Recognition of pathogens by neutrophils involves coordination between a repertoire of cellular receptors.
4. Killing of pathogens is achieved through the production of toxic metabolites and the release of nuclear contents.
5. Heritable disorders of neutrophils provide key insights into molecular mechanisms of neutrophil function.
6. Neutrophils play a central role in coordinating the response of other immune effector cells.
7. Pathologic interactions between adaptive immune cells and neutrophils are a major contributor to many autoimmune and inflammatory disease states.
8. Neutrophils play both positive and negative roles in cancer progression.

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Errata

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