Neutrophils in the innate immune response

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Source of support: by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases.

Summary

Polymorphonuclear leukocytes (PMNs or neutrophils) are an essential component of the human innate immune system. Circulating neutrophils are rapidly recruited to sites of infection by host- and/or pathogen-derived components, which also prime these host cells for enhanced microbicidal activity. PMNs bind and ingest microorganisms by a process known as phagocytosis, which typically triggers production of reactive oxygen species and the fusion of cytoplasmic granules with pathogen-containing vacuoles. The combination of neutrophil reactive oxygen species and granule components is highly effective in killing most bacteria and fungi. Inasmuch as PMNs are the most abundant type of leukocyte in humans and contain an arsenal of cytotoxic compounds that are non-specific, neutrophil homeostasis must be highly regulated. To that end, constitutive PMN turnover is regulated by apoptosis, a process whereby these cells shut down and are removed safely by macrophages. Notably, apoptosis is accelerated following phagocytosis of bacteria, a process that appears important for the resolution of infection and inflammation. This review provides a general overview of the role of human neutrophils in the innate host response to infection and summarizes some of the recent advances in neutrophil biology.

Key words: neutrophil • phagocytosis • inflammation • infection • apoptosis

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GRANULOPOIESIS

Cells of the immune system are derived from pluripotent stem cells in bone marrow\textsuperscript{14, 15, 41}. At an early stage in granulopoiesis, these hematopoietic stem cells give rise to unipotent myeloblasts (committed stem cells)\textsuperscript{96}, which differentiate subsequently into granulocytes (reviewed by Cline\textsuperscript{34}). Based on in vivo radiolabeling studies in humans\textsuperscript{40, 146, 198}, differentiation from myeloblasts to myelocytes normally requires ~7.5 days and from myelocytes to mature polymorphonuclear leukocytes (PMNs, neutrophils, or granulocytes) ~6.5 days (summarized by Bainton et al.\textsuperscript{7}). From these studies it is estimated that granulocyte precursors comprise ~60% of the nucleated cells in bone marrow\textsuperscript{7}. Myeloblasts lack granules ultimately present in mature PMNs, but have numerous ribosomes and mitochondria\textsuperscript{7}. Azurophilic and specific granules are formed during the promyelocyte and myelocyte stages of differentiation, respectively\textsuperscript{7}. As myeloid precursors become mature neutrophils, they sequentially acquire the receptors and proteins needed for innate host defense (reviewed by Faurou and Borregaard\textsuperscript{59}). Mature PMNs are released into the bloodstream where they circulate with a typical half-life of 6–8 h\textsuperscript{6, 34, 40, 62}. Although PMNs have a relatively short life-span, the greatest percentage of hematopoiesis is committed to the production of neutrophils, and PMN turnover is on the order of 10\textsuperscript{11} cells per day in the average adult human\textsuperscript{6}.

NEUTROPHIL RECRUITMENT AND PHAGOCYTOSIS

Recruitment

Active recruitment of neutrophils to sites of infection is fundamentally important to the innate immune system. This multi-step process involves mobilization of PMNs from circulation and bone marrow reserves in response to host- and pathogen-derived chemotactic factors. Neutrophils roll along the walls of postcapillary venules, surveying connective tissue, mucosal membranes, skeletal muscle, and lymphatic organs for signs of tissue distress and the presence of chemoattractants\textsuperscript{109}. There are numerous host-derived factors that trigger recruitment of neutrophils. One of the most potent neutrophil chemoattractants is the chemokine interleukin (IL)-8\textsuperscript{196, 211}. IL-8 is produced by many cell types during inflammatory states such as that accompanying infection. These cells include monocytes, macrophages, mast cells, epithelial cells, keratinocytes, fibroblasts, endothelial cells, and neutrophils (discussed below). Bacteria also produce molecules that directly recruit PMNs (e.g. N-formylated peptides)\textsuperscript{170, 177}. An important property of chemoattractants is that they can prime neutrophils for enhanced function, thereby facilitating host defense.

Priming

Neutrophil “priming” was described by McPhail et al.\textsuperscript{133} in the early 1980s as the ability of a primary agonist, typically at a sub-stimulatory concentration, to influence/enhance superoxide production triggered by a second stimulus. Since that time, many studies have shown that neutrophils can be primed for enhanced adhesion, phagocytosis, production of reactive oxygen species (ROS), cytokine secretion, leukotriene synthesis, degranulation, and bactericidal activity (see Table 1 for a comprehensive list of priming agents). Many neutrophil priming agents are host derived and include cytokines, chemokines, growth factors, and lipid-derived signaling molecules (Table 1). Cell-cell contact\textsuperscript{115} and adherence/adhesion\textsuperscript{71, 88, 123} also prime cells for enhanced function (Table 1). As implied above, microorganisms produce numerous factors that prime PMNs for enhanced responses to subsequent stimuli\textsuperscript{30, 53, 54, 65, 74, 119, 124, 133, 205} (Table 1). Many priming agents are Toll-like receptor (TLR) agonists, and TLRs are thus critical components in the pathogen-mediated priming process (see below). A distinction can be made between primed cells and those that are fully activated\textsuperscript{133}. Priming typically includes mobilization of secretory vesicles and secretion of cytokines, but fails to induce complete degranulation or elicit production of superoxide. Arguably, the single most important role for PMN priming is to promote efficient clearance of invading microorganisms.

Neutrophil rolling, tethering, and adhesion

PMN “rolling” in blood vessels is mediated by a family of C-type lectin glycoproteins known as selectins\textsuperscript{109}. Selectin levels are modulated on the surface of cytokine-activated endothelial cells (E- and P-selectin), activated platelets (P-selectin), and on primed or activated neutrophils (L-selectin)\textsuperscript{184}. For example, P-selectin is up-regulated on the surface of endothelial cells following exposure to leukotriene B4, histamine, or complement peptide C5a\textsuperscript{64}. E- and P-selectins interact with neutrophil CD162 (P-selectin glycoprotein ligand-1) to facilitate a process known as “tethering”, which mediates rolling\textsuperscript{137}. Selectin-supported tethering precedes firm adhesion mediated by leukocyte adhesion molecules such as β1 (VLA-4) and β2-integrins (Mac-1 or LFA-1)\textsuperscript{116, 194}. Once firmly bound, several neutrophil surface molecules, including CD31\textsuperscript{139}, CD54\textsuperscript{31}, CD44\textsuperscript{93}, and CD47\textsuperscript{78}, facilitate transmigration through the endothelium into tissues.
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Phagocytosis

At the site of infection, neutrophils bind and ingest invading microorganisms by a process known as phagocytosis. PMNs directly recognize surface-bound or freely secreted molecules produced by bacteria, including peptidoglycan, lipoproteins, lipoteichoic acid, lipopolysaccharide (LPS), CpG-containing DNA, and flagellin. These pathogen-derived molecules, known as pathogen-associated molecular patterns, interact directly with a number of pattern-recognition receptors expressed on the cell surface, including TLRs (reviewed by Akira and Takeda\cite{2}) and CD14\cite{207, 208}. Recent studies by Hayashi et al.\cite{78} demonstrated that TLRs 1, 2, and 4–10 are expressed in/on human neutrophils, extending earlier findings\cite{141}. Ligation of neutrophil TLRs, especially TLRs 2 and 4, activates signal transduction pathways that ultimately prolong cell survival\cite{164}, facilitate adhesion\cite{164} and phagocytosis\cite{78}, enhance release of cytokines, chemokines, and ROS\cite{78, 108, 164}, and promote degranulation\cite{17, 124}, thereby contributing to microbicidal activity\cite{17}. Peptidoglycan-recognition protein (PGRP) is another recently described pattern-recognition molecule that plays a role in the intracellular killing of Gram-positive bacteria by neutrophils\cite{55, 92, 120, 121}. There are four known isoforms of PGRP in mammals, and neutrophils express PGRP-short (PGRP-S, also known as PGLYRP1), a protein that binds peptidoglycan and Gram-positive bacteria\cite{55, 121}. PGRP-S contributes directly to bactericidal activity rather than promoting pathogen recognition and uptake\cite{120}. Neutrophil phagocytosis is also facilitated by several other host pattern-recognition molecules known as the collectins, which are reviewed elsewhere\cite{125}.

Although pattern-recognition receptors play a role in the recognition of microbes by neutrophils, the efficiency of phagocytosis by neutrophils is markedly enhanced if microbes are opsonized with serum host proteins, such as complement and/or antibody. Activation of complement promotes the deposition of complement components C3b, iC3b, and Clq on microbial surfaces\cite{144}. Complement-opsonized microbes are efficiently recognized by complement surface receptors on PMNs, including ClqR\cite{56}, CD35 (CR1)\cite{153, 163}, CD11b/CD18 (CR3)\cite{45, 81}, and CD11c/CD18 (CR4)\cite{142} (reviewed by Nauseef and Clark\cite{144}). Antibody-coated microbes are recognized by neutrophil receptors specific for the Fc-region of antibody, such as CD23 (FceRI, IgE receptor)\cite{73}, CD89 (FccR, IgA receptor)\cite{4}, CD64 (FcyRI, IgG receptor), CD32 (FcyRIIa, low-affinity IgG receptor)\cite{129, 144}, and CD16 (FcyRIIIb, low-affinity IgG receptor)\cite{61, 129}. Binding of antibody and complement receptors at the PMN surface triggers phagocytosis of microorganisms (Fig. 1).

Production of cytokines and chemokines by activated neutrophils

Recent studies have shown that phagocytosis of bacteria triggers synthesis of neutrophil genes encoding many immunomodulatory agents, including IL-1α, IL-1β, IL-1ε, IL-1RN, IL-6, IL-8, IL-10, IL-12β, IL-15, IL-18, CCL2 (MIP1α), CCL3 (MIP1β), CXCL1 (GROα), CXCL2 (MIP2α), CXCL3 (MIP2β), CXCL12 (SDF1), CCL20 (MIP3α), tumor necrosis factor (TNF)-α, vascular endothelial cell growth factor, and oncostatin M\cite{23, 102, 103, 169}. In addition to recruiting more PMNs and modulating subsequent neutrophil functions, these molecules potentially coordinate early responses of monocytes, macrophages, dendritic cells, and lymphocytes during inflammatory states. Importantly, production of cytokines and chemokines by PMNs serves as a first
link between the innate and acquired immune responses. This topic is reviewed in detail by Scapini et al.169.

Immune evasion strategies employed by bacteria

Although most bacteria are recognized and destroyed by PMNs, some pathogenic bacteria have developed clever strategies to inhibit PMN recruitment and phagocytosis. *Streptococcus pyogenes* is a human pathogen that employs several novel mechanisms to circumvent destruction by neutrophils (reviewed by Voyich et al.195). For example, *S. pyogenes* produces a 130-kDa serine endopeptidase aptly called C5a peptidase that specifically cleaves the complement chemoattractant C5a to inhibit PMN recruitment33. In addition, strains of *S. pyogenes* that cause necrotizing fasciitis or “flesh-eating” infections release a trypsin-like protease that degrades IL-882. Reduced levels of IL-8 may contribute to the observed paucity of neutrophils at the site of *S. pyogenes* soft-tissue infections82. The pathogen disrupts other critical aspects of PMN function including deposition of opsonins and phagocytosis195. For example, the surface M protein of *S. pyogenes* impedes the binding of opsonic fragment C3b to its surface by inhibiting complement regulatory proteins, such as C4b-binding protein, factor H, and factor H-like protein19. *S. pyogenes* also secretes Mac, a host-receptor mimetic of the leukocyte β2-integrin Mac-1, which interacts with CD16 and Mac-1 at the neutrophil plasma membrane to inhibit opsonophagocytosis112. Streptococcal inhibitor of complement is a protein secreted by *S. pyogenes* that blocks PMN phagocytosis by presumably altering normal PMN cytoskeleton function83. Although the molecules involved in these mechanisms of immune evasion are specific to *S. pyogenes*, the general strategies, i.e. inhibition of phagocytosis and chemotaxis, are commonly used by other bacterial pathogens.

**NEUTROPHIL MICROBICIDAL ACTIVITY**

Human neutrophils use oxygen-dependent and oxygen-independent mechanisms to kill ingested microorganisms. Phagocytosis of microorganisms triggers generation of superoxide radicals and other secondarily derived ROS, such as hydrogen peroxide, hypochlorous acid, hydroxyl radical, and chloramines31, which are potent microbicidal agents100. Concomitant with the production of ROS, cytoplasmic granules fuse with bacteria-containing phagosomes (a process known as degranulation), thereby enriching the vacuole lumen with antimicrobial peptides and proteases (Fig. 1).

**NADPH oxidase and the production of superoxide**

In activated neutrophils, a membrane-bound NADPH-dependent oxidase generates high levels of superoxide, a process traditionally called “respiratory burst” (reviewed by Quinn and Gauss149) (Fig. 1). In unstimulated neutrophils, components of the NADPH oxidase complex are separated in cytosol (p40phox, p47phox, p67phox, Rac2)114, 122, 192, 193, 204 and membrane compartments (flavocytochrome b558, Rap1A)24, 150, 172. During phagocytosis, the cytosolic components translocate to the plasma and/or phagosome membrane and associate with flavocytochrome b558, a transmembrane heterodimer comprised of gp91phox (Nox2) and p22phox, thereby forming the active oxidase32, 49, 80, 192. The oxidase transfers electrons from cytosolic NADPH to intraphagosomal molecular oxygen, thus producing superoxide. Superoxide anion is short-lived and dismutates rapidly to hydrogen peroxide and forms other secondary products, such as hypochlorous acid, hydroxyl radical, and singlet oxygen, which are effective microbicidal compounds98, 99, 160, 161. The importance of NADPH oxidase and the production of ROS is exemplified by a rare hereditary disorder known as chronic granulomatous disease (CGD; for recent reviews see refs.113, 162). Individuals with CGD have recurrent bacterial and fungal infections due to defects in the NADPH oxidase113, 162. These infections are often life threatening, although advances in the therapeutic interventions for CGD, such as treatment with interferon-γ or itraconazole, have significantly improved prognosis66, 130.

**Myeloperoxidase-halide system**

Early studies by Klebanoff and coworkers demonstrated that the microbicidal activity of NADPH oxidase-derived ROS is augmented significantly by myeloperoxidase (MPO), an abundant hemoprotein stored within neutrophil azurophilic granules98–100, 160, 161. During cell activation, MPO is targeted to forming phagosomes and catalyzes a reaction with chloride and hydrogen peroxide to produce hypochlorous acid, a potent microbicidal agent98–100 (Fig. 1). This reaction comprises what is known as the MPO-halide system100. Curiously, individuals with hereditary deficiency of MPO typically lack increased susceptibility to infection143, and it has been proposed that superoxide and hydrogen peroxide, and granule components, may be sufficient to compensate for the lack of the MPO-halide system and its products under certain conditions108, 143, 144. Despite overwhelming evidence, which indicates ROS and neutrophil granules are independently microbicidal98–100, 143, 160, 161, recent work by Segal and coworkers sug-
gests ROS may have limited capacity for direct microbicidal activity. Further work is necessary to elucidate the basis for these provocative observations.

Degranulation

Neutrophil phagocytosis triggers mobilization of cytoplasmic granules, which fuse either with the plasma membrane (exocytosis) or with phagosomes (Fig. 1). The regulatory mechanisms underlying granule mobilization and targeting following activation are incompletely defined, but involve in part calcium-and/or ceramide-mediated signal transduction, Src family tyrosine kinases FGR and HCK, p38 MAP kinase, and soluble N-ethylmaleimide sensitive factor attaching proteins (SNAPs) and SNAP receptors. Importantly, fusion of azurophilic granules with phagosomes enriches the vacuole lumen with numerous anti-microbial peptides, including α-defensins, cathepsins, proteinase-3, elastase, azurocidin, and lysozyme. Neutrophil α-defensins, known as HNP 1–4 (reviewed by Ganz), comprise up to 50% of the protein in azurophilic granules and have potent antimicrobial activity. Neutrophil α-defensins modulate a number of other functions in innate host defense, including chemotaxis, wound repair, and histamine release, and are clearly important mediators of human innate host defense. Degranulation also enriches phagosomes with components of the specific granules, such as flavocytochrome b558 and lactoferrin, further augmenting anti-microbial potential. Lactoferrin sequesters iron needed for growth of many microorganisms and, conversely, supplies iron required to generate neutrophil hydroxyl radical. Notably, lactoferrin has direct microbicidal activity against bacteria that involves membrane permeabilization.

Bystander tissue damage and disorders linked to neutrophil-mediated inflammation processes

Although the cytotoxic components produced by neutrophils kill ingested microorganisms efficiently, host tissues can be damaged inadvertently by PMNs through the action of degradative enzymes and the production of ROS. Cell activation and granule exocytosis, prolonged acute inflammatory responses, tissue remodeling, and PMN lysis at the site of infection can each contribute to inflammatory disorders. For example, rheumatoid arthritis has been linked to the production of ROS by neutrophils following activation by immune complexes in the synovial fluid of joints. Oxidation of low-density lipoproteins (LDLs) by superoxide and neutrophil-derived ROS likely contributes to atherosclerosis. These oxidized LDLs are recognized by resident macrophages, thereby facilitating processes that lead to atherosclerosis. This idea is supported by the finding that neutrophils activated by hypochlorous acid-oxidized LDL adhere to epithelial cells and subsequently activate to produce ROS. Host tissue damage caused by some types of Staphylococcus aureus pneumonia (necrotizing pneumonia) is presumably due to neutrophil lysis, although the hypothesis remains to be tested directly. Thus it is critical that neutrophil homeostasis and turnover are highly regulated processes during infection and inflammation.

NEUTROPHIL APOPTOSIS

AND THE RESOLUTION OF INFECTION

Overview of the importance of neutrophil apoptosis

Apoptosis is an important mechanism for maintaining homeostasis of the human immune system. For example, apoptosis plays a critical role in shaping both T and B cell repertoires by deleting unproductive as well as potentially autoreactive immune cells. In addition, programmed cell death plays a major role in the normal turnover of cells of the innate immune system. Although the process of apoptosis is ubiquitous in immune cells, there is considerable difference in the rate of turnover of various cell types and the impact on resolution of inflammation (reviewed in refs. and). Since neutrophils are the most numerous type of leukocyte in humans, with rapid turnover and an abundance of potent cytotoxic components, there is potential for tissue injury and trauma associated with inflammatory disease (see above). Hence the importance of appropriate neutrophil apoptosis is underscored by inflammatory potential and possible host tissue damage should they undergo lysis.

Normal turnover of aging neutrophils occurs in the absence of activation through a process known as spontaneous apoptosis. The intrinsic ability of neutrophils to undergo apoptosis is essential for maintaining appropriate cell numbers in circulation. Notably, the process of neutrophil apoptosis is significantly accelerated upon activation by phagocytosis. Together, these processes limit inappropriate inflammatory potential and are critical to the resolution of infection. It is therefore conceivable that bacterial pathogens have evolved mechanisms to exploit this essential cellular process to promote pathogenesis.
**Apoptosis as a critical component of the resolution phase of inflammation and infection**

The onset of human bacterial disease is accompanied by the active recruitment of massive numbers of neutrophils to infected tissues. PMNs remove bacteria via phagocytosis, and initiation of the neutrophil-mediated acute-phase inflammatory response is crucial for the resolution of infection. On the other hand, the timely removal of activated PMNs from affected sites is paramount to the resolution of inflammation. Thus, the resolution phase of both inflammation and infection requires a delicate balance between both neutrophil survival and death signals.

Bacterial infections are accompanied by important host responses, including release of proinflammatory cytokines, which alter neutrophil apoptosis. In general, cytokines such as TNF-α, IL-1β, IL-6, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor delay spontaneous PMN apoptosis. In addition, bacteria-derived products such as LPS and a number of bacterial toxins are known to delay neutrophil apoptosis. Together, these observations suggest that enhanced neutrophil survival is a desirable consequence during early stages of inflammation and promotes the clearance of bacterial pathogens. It is increasingly clear that the process of phagocytosis significantly accelerates the rate of apoptosis in human PMNs. Consistent with this idea, neutrophil ingestion of *Escherichia coli*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Burkholderia cepacia*, *Borrelia hermsii*, and *Listeria monocytogenes* significantly accelerates the rate of PMN apoptosis. Importantly, phagocytosis significantly increases the rate of PMN apoptosis irrespective of any delay in cell fate imparted by cytokines or bacteria-derived factors. Neutrophil apoptosis is also associated with an overall decrease in cellular function. For example, apoptotic neutrophils have diminished capability to chemotax, produce ROS, secrete cytokines, adhere, and phagocytose. In addition, proinflammatory capacity is down-regulated during neutrophil apoptosis. As a critical step toward resolution of inflammation, PMNs undergo cell surface changes that include the external expression of components such as phosphatidylserine. Apoptotic neutrophils are subsequently recognized and ingested by either macrophages or neighboring cells in the absence of proinflammatory consequence. Thus, PMN apoptosis plays a central role in the resolution of infection and the control of inflammation.

**Altering apoptosis as a mechanism of pathogenesis**

The ability of neutrophils to undergo apoptosis is essential for the maintenance of human health. Thus it is not surprising that bacterial pathogens are capable of exploiting this critical process in order to survive and cause disease. However, the relatively short life-span of neutrophils is not conducive to long-term survival of most intracellular pathogens. In order for intracellular pathogens to replicate and persist in PMNs, the ability of these pathogens to alter neutrophil apoptosis would facilitate their survival in these short-lived granulocytes. Although several bacterial pathogens have been reported to survive in PMNs, only two have been shown conclusively to delay neutrophil apoptosis. *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis, was the first described bacterial pathogen to both replicate and delay PMN apoptosis. Although not sharing the same tropism for granulocytes as *A. phagocytophilum*, the obligate intracellular bacterial pathogen *Chlamydia pneumoniae* has recently been shown to multiply in neutrophils and delay spontaneous apoptosis. Thus, intracellular bacterial pathogens are able to extend neutrophil life-span by altering normal apoptosis. The specific cellular mechanisms responsible for pathogen-mediated inhibition of neutrophil apoptosis are unclear, and further research on this topic will likely reveal insight into bacterial pathogenesis.

As described in the previous section, accelerated neutrophil apoptosis after phagocytosis of bacteria is a normal and desirable phenomenon that contributes to the resolution of infection. However, recent evidence suggests that bacterial pathogens such as *S. pyogenes* can additionally alter neutrophil apoptosis in a manner that ultimately results in rapid cell lysis. In the absence of a normal progression of apoptosis in activated neutrophils, the inability of macrophages to safely remove PMNs prior to lysis would presumably lead to unfettered inflammation at infection sites. These findings are consistent with the clinical presentation of necrotic lesions and gross inflammation often associated with *S. pyogenes* infections. Thus, the ability of pathogens to alter neutrophil fate by either blocking apoptosis to facilitate intracellular pathogen survival or promoting rapid lysis to eliminate neutrophils represents plausible mechanisms of virulence. However, the complex association of neutrophils, macrophages, and secreted pathogen and host factors at infection sites complicate this rather simplistic model. Appropriate *in vitro* and *in vivo* model systems will prove invaluable for providing specific molecular mechanisms for the role of neutrophil apoptosis in bacterial pathogenesis.
CONCLUSIONS

Neutrophils form the first and most prominent line of cellular defense against invading microorganisms. The importance of neutrophils in the immune system is exemplified by patients with neutropenia or defects that impair neutrophil function. Such individuals are predisposed to serious infections that are typically life threatening. The processes of PMN recruitment, transmigration, phagocytosis, and activation are highly coordinated to prevent or eliminate human infections. Although most bacteria are killed by neutrophils in healthy individuals, there are a number of human pathogens that circumvent PMN killing by neutrophils in neutropenic patients. An alternative mechanism of priming by-diacylglycerol. Independence from activation or calcium ionophore (A23187) stimulated human neutrophils from calcium ionophore (A23187) stimulated human neutrophils.

ACKNOWLEDGMENT

The authors thank Mark T. Quinn (Montana State University, Bozeman, MT, USA) for critical review of the manuscript.

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