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The role of neutrophils in host defense and disease

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Abstract

Neutrophils, the most abundant circulating leukocyte, are critical for host defense. Granulopoiesis is under the control of transcriptional factors and culminates in mature neutrophils with a broad armamentarium of antimicrobial pathways. These pathways include NADPH oxidase, which generates microbicidal reactive oxidants, and non-oxidant pathways that target microbes through several mechanisms. Activated neutrophils can cause or worsen tissue injury, underscoring the need for calibration of activation and resolution of inflammation when infection has been cleared. Acquired neutrophil disorders are typically caused by cytotoxic chemotherapy or immunosuppressive agents. Primary neutrophil disorders typically result from disabling mutations of individual genes that result in impaired neutrophil number or function, and provide insight into basic mechanisms of neutrophil biology. Neutrophils can also be activated by non-infectious causes, including trauma and cellular injury, and can have off-target effects in which pathways that typically defend against infection exacerbate injury and disease. These off-target effects include acute organ injury, autoimmunity, and variable effects on the tumor microenvironment that can limit or worsen tumor progression. A greater understanding of neutrophil plasticity in these conditions is likely to pave the way to new therapeutic approaches.

Capsule Summary

Neutrophils mediate pathogen defense and inflammation through multiple mechanisms. We provide a discussion of the current understanding of neutrophil biology, host defense mechanisms, and the role of neutrophils in inflammatory, autoimmune and malignant diseases.

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Keywords

neutrophils; neutrophil extracellular traps; granulopoiesis; innate immunity; phagocytosis; degranulation; neutrophil dysfunction

Introduction

Neutrophils are the most plentiful leukocyte type found in human peripheral blood, constituting about 60% of white blood cells in human blood. They are a major cellular contributor to inflammation, mediating the early phases of inflammatory responses. They can eliminate microbes via multiple mechanisms, and are critical for host defense. However, the same pathways that kill pathogens and amplify inflammation can also cause injury to the host. Therefore, calibration of neutrophil responses to defend against pathogens while averting excessive tissue injury is required.

The following are key requirements for neutrophil-mediated host defense: (i) adequate number of circulating neutrophils; (ii) trafficking to sites of infection and injury; (iii) sensing of microbes; phagocytosis; (iv) killing of pathogens (or containing infection); and termination of inflammation after pathogen clearance. Impairment of these pathways can result from a number of causes, including disabling mutations in critical genes and acquired immunodeficiencies (e.g., bone marrow disorders and immunosuppressive therapy). This review will focus on normal neutrophil biology and diseases associated with neutrophil dysfunction.

Neutrophil Development

In the bone marrow, multipotent progenitor cells (MPPs) differentiate into common myeloid progenitor cells (CMPs). Granulopoiesis is regulated by specific transcriptional factors that are activated at different stages of development. The CMPs generate granulocyte-macrophage progenitor cells (GMPs). These GMPs, when exposed to granulocyte colony-stimulating factor (G-CSF), differentiate into myeloblasts, committing to neutrophil generation. Neutrophils arise from myeloblasts over about a 14 day period[1]. First the myeloblast differentiates into a promyelocyte, followed by a myelocyte, both of which have a round nucleus. At the metamyelocyte stage, the nucleus changes into a kidney shape. Metamyelocytes mature to band cells with a band-shaped nucleus, and finally these mature into mature neutrophils (polymorphonuclear granulocytes), with their classic segmented nucleus.

Neutrophil granule formation occurs progressively throughout the different stages of neutrophil maturation. Primary (azurophilic) granules are found at the myeloblast to promyelocyte stage. Secondary (specific) granules are first observed at myelocyte and metamyelocyte stages. Tertiary (gelatinase) granules are not found until the band cell stage. Finally, secretory vesicles are seen only in mature neutrophils[2] (Figure 1).

During neutrophil maturation, integrin α4β1 (VLA4) and chemokine receptor CXCR4 are downregulated, while CXCR2 and Toll-like receptor 4 (TLR4) are upregulated. The bone

marrow stromal cells express vascular cell adhesion molecule 1 (VCAM1), a ligand for VLA4, and CXCL12, a ligand for CXCR4, to retain myeloid progenitor cells in the bone marrow. G-CSF downregulates CXCR4 on neutrophils and its ligand CXCL12 on bone marrow stromal cells, allowing mature neutrophils to leave the bone marrow and enter the peripheral circulation, mediated by interactions of CXCR2 with CXCL1 and CXCL2[3].

Neutrophil Biology

Neutrophils are granular, large leukocytes (about 12-15um in diameter) with a nucleus that is segmented into three to five lobules. Human neutrophils are abundant, with adult humans producing over 1 $x10^{11}$ neutrophils per day[4]. They have been conventionally considered a very short-lived cell in the peripheral blood, with a half-life in circulation of approximately 6-8 hours[5]. This concept has been challenged in a study using *in vivo* stable isotope labeling with heavy water (^2H_2O)that estimated the *in vivo* half-life of circulating neutrophils is much longer, about 3.7 days[6]. However, a more recent study also using ^2H_2O labeling as well as deuterium-labeled glucose support older concepts of a shorter half-life on the magnitude of hours rather than days[7]. Neutrophils express a large number of selectins, chemokine receptors, and integrins that allow them to be rapidly recruited from the circulation to a site of tissue injury or infection. Once activated in tissues, neutrophils mediate host defense via multiple mechanisms including phagocytosis of pathogens, production of antimicrobial and proinflammatory enzymes, oxidative burst to generate toxic reactive oxygen species, and release of neutrophil extracellular traps (NETs) into the extracellular space. Like many immune cell types, neutrophils display circadian rhythmic variations in many of these key functions, including adhesion molecule and chemokine receptor expression, superoxide production, and phagocytic activity,[8–10] though the relative expression of circadian clock genes may be lower in neutrophils than other immune cell types[8].

Pathogen recognition and phagocytosis

Human neutrophils express a wide variety of pattern recognition receptors (PRRs) including Toll-like receptors TLR1 through TLR10 (with the exception of TLR3 and TLR7), C-type lectin receptors (e.g., Dectin-1), Nod-like receptors, and others, enabling them to initiate various important immune responses upon recognition of pathogen-associated molecular patterns (PAMPs)[11]. In neutrophils, phagocytosis may be initiated by PRR-PAMP interaction, but more effective phagocytosis is mediated via neutrophil Fcγ receptors or complement receptor 3 (CR3; CD11b/CD18; Mac-1) receptors binding to IgG- or C3bi-opsonized microbes, respectively. CR3 plays a dual role in complement-mediated phagocytosis of microbes and neutrophil adhesion to endothelial cells required for trafficking. Activation of neutrophils by pathogens and other stimuli increases surface expression of CR3, thereby amplifying the capacity of neutrophils to phagocytose pathogens and to traffic to sites of infection.

Pathogens are engulfed into a cell membrane-derived vacuole called the phagosome. Phagosomal maturation occurs via the fusion of the phagosome with secretory vesicles and granules, to acquire antimicrobial enzymes and components of the NADPH oxidase

complex[12]. Additionally, PRR activation initiates the process of neutrophil degranulation, releasing the proinflammatory contents of neutrophil primary, secondary, tertiary and secretory granules (discussed below). Fusion of secondary granules with the plasma membrane results in increased surface expression of cytochrome b558 ($p22^{phox}$ and $gp91^{phox}$ heterodimer), a component of the NADPH oxidase complex, which enhances extracellular reactive oxidant generation (discussed below).

Neutrophil Granules

Three types of neutrophil granules are present in mature neutrophils, and these granules are all filled with pro-inflammatory contents (Table I). The function of these preformed neutrophil granular constituents is to rapidly respond to infectious threats by activation of multiple host defense pathways. Primary granules are also called azurophilic granules and their major protein content is myeloperoxidase (MPO). Because of this, they have also been termed peroxidase-positive granules. Primary granules also contain defensins, serine proteases, proteinase 3, cathepsin G and C, bactericidal permeability-increasing protein (BPI), neutrophil elastase, CAP37 (azurocidin), and NSP4. Secondary granules, also called specific granules, contain lactoferrin, hCAP-18 (cathelicidin), collagenase (MMP8), β2-microglobulin, haptoglobin, pentraxin-3, NGAL and SLPI. In the unstimulated neutrophil, cytochrome b558 is principally expressed in secondary granules. Tertiary granules contain gelatinase (MMP9), arginase I, and ficolin I. The presence of gelatinase, and absence of lactoferrin or NGAL distinguishes tertiary granules from secondary granules, both of which are peroxidase-negative. MMP9 likely plays a role in neutrophil-mediated remodeling of extracellular matrix enabling neutrophils to migrate to sites of infection within tissue following extravasation, while the arginase pathway likely plays a role in wound healing. Nearly all proteins in primary granules are proteolytically processed prior to storage in the granules. In contrast, proteins in secondary and tertiary granules are stored unprocessed and inactive[13].

Neutrophil Oxidative Burst

In neutrophils, the activated NADPH oxidase converts oxygen into reactive oxygen species (ROS), oxidizing products that can kill microbes. The NADPH oxidase complex is comprised of a membrane-bound heterodimer, $gp91^{phox}$ (*CYBB* or *NOX2*) and $p22^{phox}$ (*CYBA*), and 3 cytosolic subunits, $p47^{phox}$ (*NCF1*), $p67^{phox}$ (*NCF2*), and $p40^{phox}$ (*NCF4*). Upon activation, the cytosolic subunits assemble upon the scaffold of the membrane-bound portion to make a functional, 5-subunit oxidase complex. Complex assembly can occur at the plasma membrane, or at the phagosomal membrane during ingestion of particles. The small GTP-binding protein Rac2 dissociates from its inhibitor GDI and binds GTP, joining with the complex to enhance oxidase activation and superoxide formation[14]. The NADPH oxidase transfers one electron from cytosolic NADPH to molecular oxygen. The product of this reaction, superoxide anion ($O_2^{\bullet -}$), is then converted to other reactive oxygen metabolites, including hydrogen peroxide (H_2O_2). H_2O_2 can combine with chloride to form hypochlorous acid (HOCl), a potent antimicrobial, in a reaction catalyzed by myeloperoxidase (MPO)[15] (Figure 2a). Hydroxyl anion (OH^-) can be generated from superoxide anion via the iron-dependent Fenton reaction. In addition, reactive oxidant and nitrogen intermediates can react to generate microbicidal radicals, e.g. peroxynitrite

anion. In addition to the direct antimicrobial effects of NADPH oxidase-generated reactive oxygen species (ROS), activation of NADPH oxidase can amplify host defense through other pathways. In neutrophils, NADPH oxidase activation can result in solubilization and activation of granular proteases that mediate host defense [16] and in the generation of neutrophil extracellular traps (described below).

Finally, NADPH oxidase activation is not only injurious to pathogens, but can also injure host cells; therefore, termination of NADPH oxidase activation and induction of cytoprotective pathways that limit neutrophilic injury are required for host protection [17]. As an example, nuclear erythroid-related factor 2 (Nrf2) is a transcriptional factor that is activated by ROS and electrophiles, and induces the activation of numerous ROS-scavenging pathways that limit oxidative stress and cellular injury [18–20].

Neutrophil Extracellular Traps

Upon activation, neutrophils can generate neutrophil extracellular traps (NETs)[21]. NETs are web-like extracellular structures of DNA covered with histones and enzymes such as neutrophil elastase (NE) and myeloperoxidase (MPO), and can be abundant at inflammatory sites. NET generation can be achieved through a cell-death process called NETotic cell death[22], which is distinct from apoptosis or necrosis. Alternatively NET formation can occur in living cells when mitochondrial DNA is used as the NET scaffolding[23]. NETs bind bacteria, fungi and other microbes, providing a high local concentration of antimicrobial molecules to kill pathogens and a barrier to prevent their dissemination[24,25]. However the strongly alkaline histones and degradative enzymes contained in NETs also have a high cytotoxic potential, and may contribute to host cell death and chronic tissue injury. NET formation can be stimulated by a variety of signals, among them microbial products such as LPS and fungal elements, and by non-infectious triggers including immune complexes and urate crystals[26]. To initiate NETotic cell death, neutrophils arrest their movement and depolarize. The nuclear envelope then breaks apart and nuclear chromatin is released into the cytoplasm.

NET generation can occur through NADPH oxidase-dependent and –independent pathways [27]. ROS generated by NADPH oxidase can trigger MPO to translocate neutrophil elastase (NE) from the primary granules to the cytoplasm[28,29]. MPO and NE together facilitate decondensation of chromatin, and the chromatin mixes in the cytoplasm with cytoplasmic and granular proteins[30]. Two other proteins shown to aide in the chromatin decondensation process are DEK[31] and peptidylarginine deiminase-4 (PAD4)[32], an enzyme that citrullinates arginine residues on histones. The cell membrane finally permeabilizes and the NETs extrude into the extracellular space, a process resulting in neutrophil death (Figure 2b). Depending on the stimulus of NET development, NET formation can be independent of PAD4, NE, MPO, and ROS[33–36]. ROS also appears to be required for mitochondrial DNA release for NET formation in live cells, driving a reversible actin and tubulin glutathionylation and cytoskeletal rearrangement essential for mitochondrial-sourced NET extrusion[37].

Neutrophil Dysfunction

Neutrophils are the primary mediators of innate immunity targeting bacterial and fungal pathogens. They function by phagocytosing pathogens, generating toxic superoxide and its metabolites, releasing antimicrobial peptides, and forming neutrophil extracellular traps (NETs). They also recruit additional immune cells to fight infection via the release of cytokines and chemokines. Neutrophil disorders should be considered in patients with recurrent or severe bacterial or invasive fungal infections. Immune deficiencies involving neutrophils may be divided into quantitative and qualitative disorders.

Quantitative Neutrophil Defects

Quantitative neutrophil disorders include drug-induced, autoimmune, and genetic neutropenias. Multiple genetic defects may lead to a phenotype of severe congenital neutropenia (SCN; also referred to as Kostmann disease), characterized by severe chronic peripheral blood neutropenia, defined as counts <200 neutrophils/μL, with maturational arrest of granulocyte precursors at the promyelocyte or myelocyte stage. Patients with SCN develop severe infections in the first several months of life, including omphalitis, skin infections, pneumonia, deep-seated abscesses, and sepsis. In addition to infections, a significant percentage of patients with SCN develop myelodysplastic syndrome or acute myelogenous leukemia, caused by somatic mutations in *CSF3R* and *RUNX1*[38]. Autosomal dominant mutations in *ELANE*, which encodes for neutrophil elastase, are the cause of the majority of SCN cases in Caucasians[39]. Autosomal dominant *GFI1* mutation may also cause SCN. Activating mutations in the *WAS* gene cause SCN inherited in an X-linked manner. In this defect, neutropenia is caused by myeloid cell apoptosis related to dysregulated polymerization of the actin cytoskeleton[40]. Autosomal recessive SCN may be caused by mutations in *G6PC3*[41], *VPS45*[42], *HAX1*, or *AK2*. A subset of patients with HAX1 deficiency have seizures and developmental delay in addition to severe neutropenia[43]. AK2 deficiency causes reticular dysgenesis with a phenotype of severe combined immunodeficiency in conjunction with severe neutropenia.

WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome, caused by autosomal dominant gain-of-function *CXCR4* mutation, leads to severe neutropenia in conjunction with lymphopenia and monocytopenia, despite the presence of a hypercellular bone marrow. The mutated CXCR4 chemokine receptor is oversensitive to the CXCL12 chemokine, leading mature neutrophils to remain in the bone marrow instead of mobilizing into the peripheral blood[44]. The CXCR4 antagonist, plerixafor, has shown promise for the treatment of myelokathexis in WHIM syndrome [45,46], and is currently in Phase III clinical trials for this condition[47].

Cyclic neutropenia is caused by autosomal dominant mutations in the *ELANE* gene, at different locations than those causing severe congenital neutropenia. These mutations don't appear to carry the leukemogenic potential as SCN-causing *ELANE* mutations[48]. Children with cyclic neutropenia develop episodes of severe neutropenia typically occurring every 21 days, though ranges between 14-40 days have been reported[49]. These episodes may become milder with age[50]. During times of neutropenia, patients develop fever, oral ulcers

and bacterial infections. Sepsis, especially with *Clostridium* spp., has also been reported[51]. Gingivitis and oral abscesses can cause tooth loss in patients with cyclic neutropenia.

Qualitative Neutrophil Defects

Primary immune deficiencies with qualitative neutrophil defects include those causing defective chemotaxis, impaired neutrophil oxidative function, or defects in neutrophil granules. Defects in the adhesion molecules necessary for neutrophil rolling and firm adhesion along the vascular endothelium and extravasation from the blood stream into infected tissues underlie a group of three disorders termed leukocyte adhesion deficiencies. The most common, LAD1, is still very rare. It is caused by homozygous mutations in *ITGB2*, the gene which encodes CD18, the common chain of the three β2 integrins on neutrophil surfaces, LFA-1, CR3, and CR4. Neutrophils in LAD1, with low or absent β2 integrin expression, cannot bind to ICAM-1 and -2 on vascular endothelial cells, and are unable to firmly adhere to the endothelial cells or transmigrate into infected tissues[52]. As CR3 is a complement receptor for iC3b, phagocytosis of complement-coated pathogens is impaired in LAD1 as well[53,54]. Disease severity correlates inversely with residual CD18 expression[53], and the severe phenotype presents with leukocytosis, delayed umbilical cord separation with omphalitis, poor wound healing, periodontitis, and skin, respiratory or gastrointestinal infections with *Staphylococcus aureus* or gram-negative bacteria, in which pus formation is absent.

LAD2 is a very rare autosomal recessive disease of impaired fucosylation due to mutations in *SLC35C1.* Fucosylated proteins are absent, including Sialyl-Lewis^x, the ligand for selectins on the surface of leukocytes, which prevents tethering and rolling of neutrophils on the vascular endothelium[55]. The infectious phenotypes of LAD2 is similar to LAD1 though milder, but additional features are present including developmental and growth delay, dysmorphic facies, and the Bombay blood type, caused by a lack of fucosylated antigen H on erythrocytes[56]. LAD3 is an autosomal recessive disease caused by mutations in *FERMT3*, which encodes for kindlin-3, required for activation of all β-integrins[57]. Impaired integrin function on neutrophils leads to severe leukocytosis and recurrent infections, while impaired β3-integrin platelet aggregation causes a bleeding disorder[58].

Chronic granulomatous disease (CGD) is caused by defects in NADPH oxidase. Pathologic mutations in any of the 5 genes encoding the subunits of the NADPH oxidase system can result in CGD, but a mutated *CYBB* (gp91^{phox}) gene, located on the X chromosome, is the most common cause of CGD. While Rac2 also associates with the NADPH oxidase complex, *RAC2* mutation actually presents with a clinical phenotype closer to leukocyte adhesion deficiency rather than CGD, given the additional role of Rac2 in mediating chemotaxis and rolling, via its regulation of the actin cytoskeleton[59].

Patients with CGD are at increased risk for a subset of catalase-producing pathogens. Major pathogens in CGD include the following, with common manifestations in parentheses: *Staphylococcus aureus* (severe soft tissue infections, liver abscess, pneumonia) *Serratia marcescens* (bone infection), *Burkholderia cepacia* (pneumonia), nocardiosis (pneumonia, central nervous system), and rare bacterial pathogens (e.g., *Granulibacter bethesdensis* and *Chromobacterium violaceum*); invasive aspergillosis (pneumonia, dissemination) and other

molds are major causes of mortality in CGD [60]. The specific spectrum of pathogens to which CGD patients have increased susceptibility points to the requirement for NADPH oxidase in defense against specific pathogens while being dispensable for others.

Catalase appears not to be critical for the virulence of these organisms in hosts with CGD as studies in which the catalase gene was deleted in either *Staphylococcus aureus* or *Aspergillus nidulans* did not demonstrate altered virulence in mouse models of CGD[61,62]. Neutrophils of patients with CGD have variable impairment in their ability to generate ROS ranging from 0.1% to 27.0% of the normal range. Patients with greater residual ROS production typically have less severe disease activity and better survival[63].

In contrast to CGD, deficiency in myeloperoxidase (MPO) is very common, yet patients with MPO deficiency are often asymptomatic, without increased frequency of infections. Myeloperoxidase is released from the azurophilic granules into the phagosomes, where it converts hydrogen peroxide to hypohalous acid, killing phagocytosed microbes. However, redundant MPO-independent microbicidal activities make up for lack of MPO in most deficient individuals. Superficial or invasive candidiasis have been reported in patients with MPO deficiency, but the presence of a comorbid immunosuppressive condition such as diabetes mellitus is typically observed [64].

CARD9 is a signaling adaptor protein in phagocytes downstream of the Dectin-1, Dectin-2, Dectin-3, and Minacle fungal-sensing pattern recognition receptors[65]. Autosomal recessive CARD9 deficiency often presents with candidal meningitis and deep dermatophytosis, in which dermatophytes initially infect the epidermis and nails, but then invade the dermis and disseminate into underlying tissues including bone, lymph nodes and brain[66,67]. CARD9 deficiency leads to defects in Th17 differentiation through impaired IL-1β and IL-6 production from monocytes[68]. However, in contrast to other Th17 defects associated with superficial mucocutaneous candidiasis, CARD9-deficient patients exhibit impaired neutrophil recruitment to the CNS in response to fungal infection, and impaired killing of fungi, which appear to contribute to the invasive fungal infections characteristic of the disorder. Neutrophils from CARD9-deficient patients have normal oxidative burst, but have impaired IL-8 release in response to *Candida albicans*, and have impaired ROS-independent killing of unopsonized *Candida* spp. through the CR3-Syk-PI3K pathway [68,69]. Additionally, CARD9 is essential for microglial production of IL-1β and CXCL1 in response to Candida, which recruit neutrophils to the CNS during candidal infection[70].

Neutrophil specific granule deficiency (SGD) is a rare autosomal recessive disease caused by *C/EBPε* mutations[71]. Neutrophils from patients with SGD have atypical, bilobed nuclei and lack specific/secondary granules and their contents including lactoferrin, transcobalamin I, gelatinase B, and collagenase. They also have decreased defensin proteins in their primary granules. Multiple neutrophil functions are impaired in SGD including neutrophils chemotaxis, receptor upregulation, and oxidative burst. Eosinophils have been shown to be affected in SGD in addition to neutrophils. Patients with SGD develop severe pyogenic infections with bacteria including *Staphylococcus aureus* and *Pseudomonas aeruginosa*[72].

Role of Neutrophils in Pathogenesis

Neutrophils in Inflammation/Autoimmunity

Neutrophils have a pathologic role in various inflammatory processes that include acute organ injury, ischemia reperfusion injury, pathologic thrombosis, atherosclerosis, and autoimmunity. Activated neutrophils mediate inflammation by synthesizing and secreting cytokines, chemokines, leukotrienes and prostaglandins. In particular, neutrophils have been shown to synthesize and secrete the chemokine CXCL8, which in turn recruits more neutrophils[73]. Activated neutrophils also synthesize IL-1, IL-6, IL-12, TGF-β, and TNF-α, which can subsequently activate both neutrophils and other cells of the immune system[74]. Neutrophils are a significant source of leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2). LTB_4 is a neutrophil chemoattractant, but PGE_2 has a mainly anti-inflammatory effect on neutrophils.

It has long been recognized that dysregulated neutrophil activation is an important mediator of inflammation and organ damage in certain autoimmune disorders, with rheumatoid arthritis (RA) being the focus of much study. Recently, a number of studies have also observed that neutrophils are a major source of autoantigens in these diseases[75,76] (see discussion below “NETs in autoimmunity”).

Upon migration into RA joints, activated neutrophils encounter aggregates of immunoglobulins. These complexes of immunoglobulins engage Fcγ receptors on the surface of the neutrophil, which attempts to phagocytose the IgG-coated joint tissue, as it would an opsonized microbe. This process, termed “frustrated phagocytosis” then triggers degranulation and production of ROS[77]. Neutrophil granule contents are implicated in the destruction of the collagen matrix within cartilage[78]. Oxidative stress as a result of inappropriate release of ROS by neutrophils is implicated in the pathology of RA, causing damage to DNA, and oxidation of lipids, proteins and lipoproteins[79].

NETs in tissue injury and thrombosis

NETs have an important role in pathogen defense that include restricting spread of infection and pathogen killing. However, NETs can also lead to injurious effects in the host. In the lungs, NETs can kill epithelial and endothelial cells, with NET histones playing a key role in this NET-mediated cytotoxicity[80]. NETs have been proposed as a mechanism of airway tissue damage in multiple lung diseases including transfusion-related acute lung injury (TRALI)[81] and cystic fibrosis[82]. Patients with cystic fibrosis often have *Pseudomonas aeruginosa* colonization of their lungs. *P. aeruginosa* is a strong inducer of NET generation, yet patient-derived isolates of this bacteria are often resistant to killing by NETs[83]. NET formation is also associated with lung damage in mouse models of acute lung injury from methicillin-resistant *Staphylococcus aureus* or *P. aeruginosa*, and NETs are associated with the development and severity of acute respiratory distress syndrome (ARDS) in critically ill patients with pneumonia or sepsis[84].

NETs promote occlusion of the vasculature and thrombosis. During thrombosis, the endothelium releases P-selectin which recruits neutrophils and promotes NET extrusion[85]. Platelet recruitment then occurs and activated platelets can enhance NET formation[86]. NETs

released from the neutrophils provide a scaffold on which the thrombus forms[87], and also recruit Factor XIIa, driving the contact pathway of coagulation[86]. Treatment of mouse models with DNase and PAD4 inhibitor blocks formation of deep vein thrombosis[88,89].

NETs in autoimmunity

NETs expose autoantigens such as nucleic acids and proteins in an inflammatory microenvironment that can initiate an autoimmune response in predisposed individuals. The association of increased NET formation and autoimmunity was first described in ANCA-associated vasculitis, but has subsequently been described in anti-phospholipid antibody syndrome, rheumatoid arthritis and systemic lupus erythematosus (SLE)[76,90–92]. Proteins and nucleic acids found in NETs may be the source of key autoantigens, such as MPO, proteinase 3, and dsDNA. In addition to being a potential source for autoantibodies, NET formation in SLE can also be driven by exposure of neutrophils to anti-ribonucleoprotein autoantibodies. NETs have been demonstrated in in vitro and animal models of SLE to drive plasmacytoid dendritic cells to produce high levels type I interferons[93,94]. IFN-α is an important inflammatory cytokine associated with SLE[95], and itself has been shown to promote further NET formation, resulting in a positive-feedback loop[93]. In SLE, increased NETs are associated with increased disease activity and presence of kidney disease, suggesting that NETs could be a disease activity marker[96,97]. NET inhibitors have been advancing through studies as therapies for SLE and other autoimmune diseases in which NETs appear to play a role. These investigational drugs include PAD4 inhibitors (in preclinical studies)[98,99] and anti-type I IFN monoclonal antibodies[100].

Interestingly, while antimalarials such as hydroxychloroquine have long been mainstays of SLE treatment, part of their mechanism of action may be the inhibition of autophagy required for NET formation[101]. Chloroquine inhibits NETs in control and SLE neutrophils in vitro[102] as well as in neutrophils in a mouse model of pancreatic cancer[103].

Neutrophils in Asthma

Neutrophil infiltration in the lungs of asthmatics has been shown to be associated with asthma severity, and high burden of neutrophils versus eosinophils has been observed in the autopsy lung tissue of patients with fatal asthma[104]. Neutrophilic asthma has been classified as a specific asthma phenotype, associated with severe disease and steroid-refractoriness. Chronic obstructive pulmonary disease (COPD) is also characterized by high sputum neutrophils, and there is felt to be some overlap between neutrophilic asthma and COPD. It has been proposed that the pathophysiology of neutrophilic asthma may be related to a dysbiosis in the lung microbiome, where normal flora is replaced by *Tropheryma whipplei* and *Haemophilus influenzae*, with neutrophilia representing the host response to these infections[105]. However, neutrophilic infiltration in uncontrolled asthma has also been observed in the absence of infection[106].

In asthmatics, neutrophils can exert deleterious effects on the lungs via multiple mechanisms. While the release of NETs is important for pathogen elimination, they have also been proposed to contribute to airway inflammation in both asthma[107,108] and COPD[109]. The association of NETs with lung injury is discussed previously in this article.

Transforming growth factor-β (TGF-β) can be produced by multiple cell types in asthmatic airways, including neutrophils. TGF-β is a profibrotic cytokine, and has been implicated in airway remodeling in asthma. Peripheral blood neutrophils of asthmatics release more TGF-β than in normal controls, and there is are more TGF-β-producing neutrophils in the lungs of asthmatics than in non-asthmatic lungs[110]. Neutrophil elastase and matrix metalloprotease-9 (MMP-9) are neutrophil-derived mediators that contribute to airway inflammation. Neutrophil elastase increases CXCL-8 production by airway epithelium, recruiting more neutrophils into the lungs. It can also inactivate tissue inhibitor of metalloproteinase-1 (TIMP-1), an inhibitor of MMP-9[111]. MMP-9 itself is involved in extracellular matrix turnover and tissue repair, and may contribute to inflammatory cell migration[112,113]. An imbalance of MMP-9 compared to TIMP-1 has been observed in both adult and pediatric asthmatics[114,115], and elevated MMP-9 is seen in patients with cystic fibrosis (CF) and with non-CF bronchiectasis, with increased MMP-9 correlating with lower FEV1[116].

Neutrophils in the tumor microenvironment

A growing body of research has demonstrated distinct roles of neutrophils in the cancer microenvironment. Activated neutrophils can kill tumor cells through ROS generation[117,118] antibody-dependent cell-mediated cytotoxicity (ADCC). One exciting therapeutic approach involves enhancing ADCC of neutrophils and macrophages directed against tumor cells through inhibition of the SIRPα-CD47 “don’t eat me” pathway[119,120]. Neutrophil activation can also drive tumor progression and metastasis through a number of pathways, including stimulation of thrombosis and angiogenesis, stromal remodeling, and impairment of T cell-dependent anti-tumor immunity[121,122]. NETs can facilitate tumor progression in tumor-bearing mice, and are a potential therapeutic target[123–125]. In addition, neutrophils can bind to circulating tumor cells and enhance hematogenous metastasis through enhancing tumor cell cycle progression[126].

Although neutrophil heterogeneity has been recognized for decades [127], the concept of distinct neutrophil populations at the transcriptome and phenotypic level has been advanced over the past decade with better tools for molecular and cellular profiling. There is a growing appreciation of the plasticity of neutrophils in the tumor microenvironment at both the transcriptional [128] and metabolic levels [129] that can enhance or obstruct anti-tumor immunity. Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature immunosuppressive myeloid cells that can have granulocytic (PMN-MDSC) or monocytic features. MDSC are most extensively studied in the setting of advanced cancer where tumor-derived factors stimulate disordered myelopoiesis, resulting in the expansion of MDSC that suppress T cell activation and impair anti-tumor immunity [130]. Since neutrophil and PMN-MDSC have overlapping surface markers, PMN-MDSC are traditionally differentiated from neutrophils based on PMN-MDSC having a lower density and co-sedimenting with peripheral blood mononuclear cells after density-gradient centrifugation and PMN-MDSC suppressing T cell responses [131]. Distinct from PMN-MDSC, tumor-associated neutrophils (TAN) are divided into N1 (anti-tumorigenic) or N2 (suppressive and pro-tumorigenic) populations, with distinct transcriptional profiles and functional properties [132,133]. Though functionally similar to PMN-MDSC regarding

suppression of T cell responses, the phenotype of N2 neutrophils is driven by responses to TGF-β[133], and is not considered to result from disordered granulopoiesis. Singel et al. [122] recently showed that mature neutrophils are rendered immunosuppressive by exposure to ovarian cancer ascites and other malignant effusions, and that this suppressor function is dependent on multiple neutrophil effector functions, including complement signaling. Since mature neutrophils can phenocopy the suppressive function of PMN-MDSC [134], it is important to understand mechanisms driving the pro-tumorigenic or anti-tumorigenic properties of mature neutrophils distinct from disordered granulopoiesis. Neutrophil plasticity raises the possibility for therapeutically modulating neutrophils in multiple diseases, including cancer [128].

Conclusion

Neutrophils are an important component of the innate immune system, mediating both pathogen defense and inflammation, through multiple processes including phagocytosis, release of granular enzymes, oxidative burst, and NET formation. The future promises insight into additional poorly understood areas of neutrophil biology, including neutrophil heterogeneity, epigenetic regulation of neutrophil function, and the impact of the microbiome on neutrophils. Severe deficiencies in neutrophil number or function result in life-threatening immune deficiencies, while inappropriate or excessive activation of neutrophils cause host tissue damage in chronic inflammatory or autoimmune conditions. Neutrophils can have “off target” effects, where pathways that normally defend against infection are activated by non-infectious cues (e.g., products of cellular injury) and influence pathologic disorders ranging from autoimmunity to the tumor microenvironment. A greater understanding of neutrophil heterogeneity and plasticity in these conditions is expected to pave the way to new therapeutic approaches.

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Box 1:

Key Points

- Neutrophils are plentiful but short-lived in the peripheral blood, and function as key mediators of innate immune responses against extracellular pathogens.
- Neutrophils exert antimicrobial effects through several processes, including toxic reactive oxygen species formation, enzymatic degradation, and NET formation
- NETs play a pathogenic role in inflammatory and autoimmune diseases. They can directly kill epithelial and endothelial cells, and provide a scaffold for thrombus formation. Granular proteins and nucleic acids in NETs may act as autoantigens initiating and driving autoimmune diseases.
- Impaired neutrophil immunity may manifest with predisposition to severe bacterial or invasive fungal infections. Genetic defects have been recognized leading to defects in neutrophil quantity, chemotaxis, oxidative function, granule proteins or release of granule contents.
- Continued research is needed to advance our understanding of areas such as neutrophil heterogeneity, epigenetic regulation of neutrophils, and the impact of the microbiome on neutrophils.

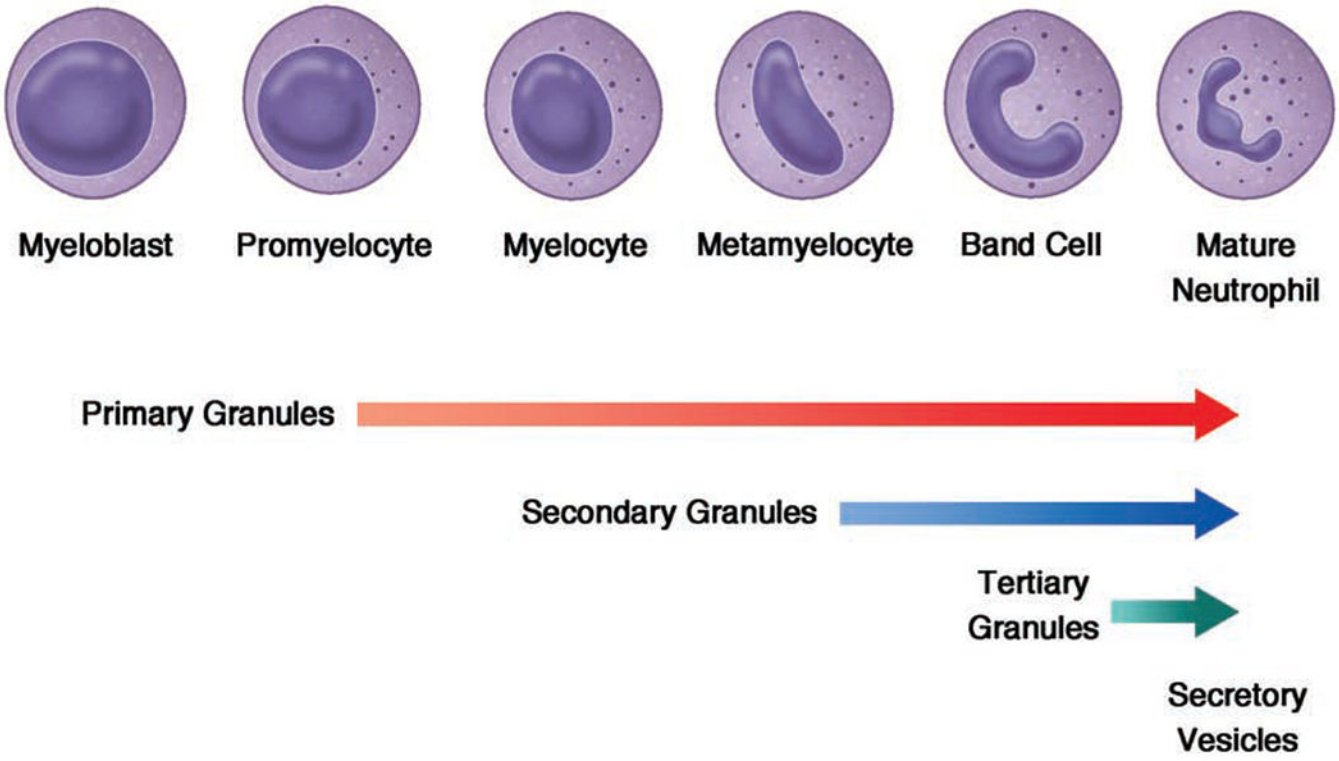

Figure 1:

Neutrophil development and granule formation. Myeloblasts are the first committed precursor cells of the neutrophil lineage. These differentiate into promyelocytes followed by myelocytes. These cells then proceed through maturation steps of metamyelocytes, band cells, and mature segmented neutrophils. As neutrophils mature, they develop granules which play a key role in neutrophil microbicidal activity. Primary (azurophilic) granules develop as neutrophils are differentiating from myeloblasts to promyelocytes. Secondary (specific) granules form in myelocytes and metamyelocytes. Tertiary (gelatinase) granules first appear in band cells. Secretory vesicles are small, easily exocytosed organelles, which are present only in mature, segmented neutrophils.

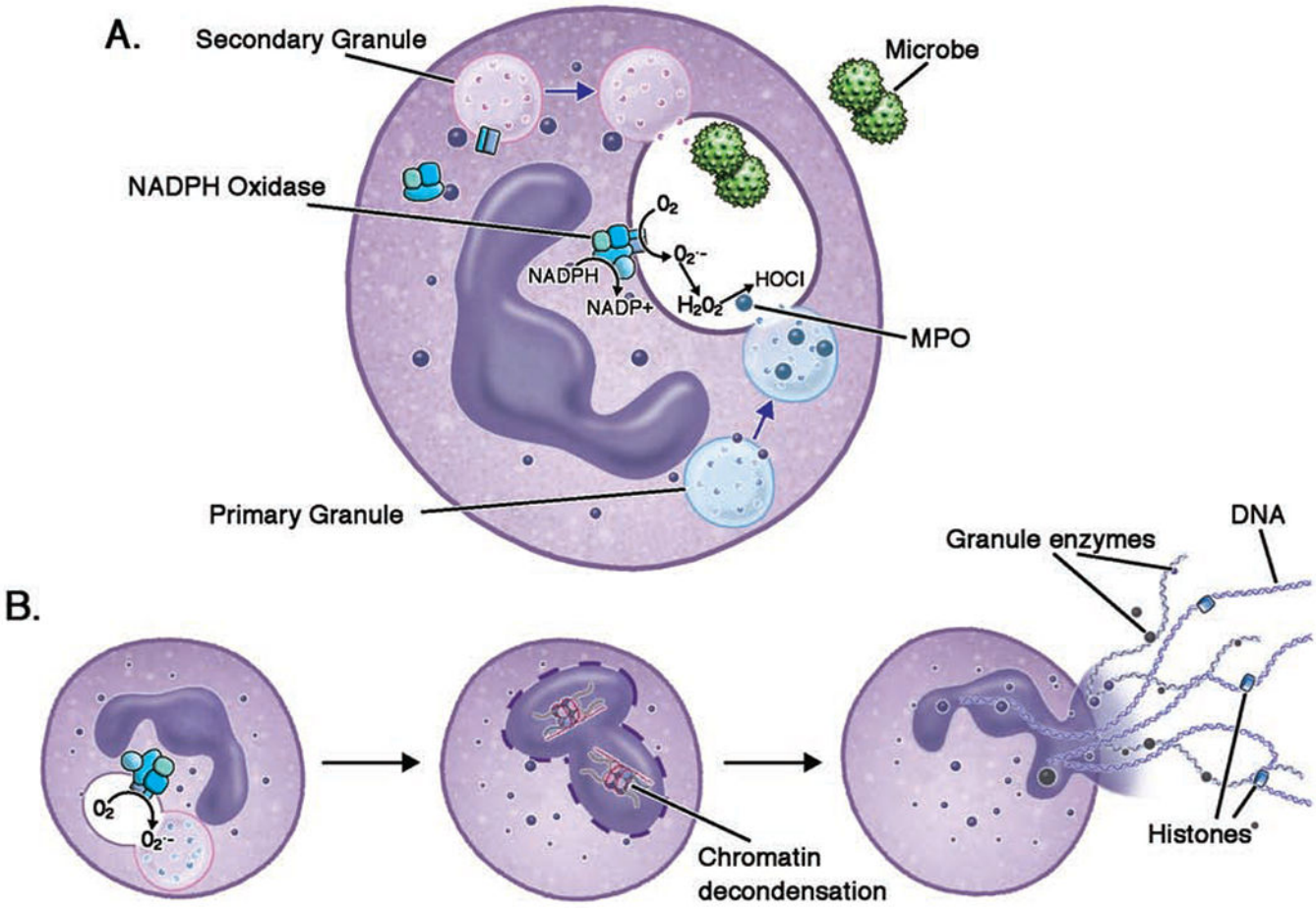

Figure 2:

Neutrophil microbicidal activities; a.) Following phagocytosis of microbes, fusion of neutrophil granules with the phagosome introduces antimicrobial granule contents into the phagosome. Non-azurophilic granules transport the membrane bound components of the NADPH oxidase into the phagosome, where they assemble with cytoplasmic components. The assembled NADPH oxidase transfers an electron from cytosolic NADPH to oxygen, forming O_2^-. O_2^- is converted into H_2O_2, and MPO combines H_2O_2 with Cl to form HOCl. b.) NETs are large, extracellular webs of microbicidal cytosolic and granule proteins assembled on a scaffold of decondensed chromatin or mitochondrial DNA (not shown). NET formation may be initiated by a variety of pathogenic triggers. In classic NADPH oxidase-dependent NETotic cell death, ROS trigger MPO to activate and translocate of NE from azurophilic granules to the nucleus, where NE disrupts chromatin packaging. MPO also works synergistically with NE in decondensing chromatin. Two nuclear enzymes are important in chromatin decondensation; DEK, a DNA-binding protein, and PAD4, which citrullinates histone arginine residues.

(H_2O_2: hydrogen peroxide; HOCl: hypochlorous acid; MPO: myeloperoxidase; NADPH: nicotinamide adenine dinucleotide phosphate; NE: neutrophil elastase; NET: neutrophil extracellular trap; $O_2^{\bullet-}$: superoxide; PAD4: protein-arginine deiminase type 4; ROS: reactive oxygen species)

Table I:

Neutrophil Granule Contents

Primary (Azurophilic) Granules	Secondary (Specific) Granules	Tertiary Granules
Myeloperoxidase	Lactoferrin	MMP9
Neutrophil elastase	Cathelicidin	Arginase I
Cathepsin G and C	MMP8	Ficolin I
Proteinase 3	β2-microglobulin	Cytochrome b558
Defensins	Haptoglobin	
CAP37	Pentraxin-3	
NSP4	NGAL	
BPI	SLP	
	Properdin	
	Cytochrome b558 (membrane-bound component of NADPH oxidase)	

Some of proteins are expressed in more than one granule subset. For example, lysozyme is expressed in primary, secondary, and tertiary granules.