REVIEW

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Neutrophils at work

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In this Review we discuss data demonstrating recently recognized aspects of neutrophil homeostasis in the steady state, granulopoiesis in 'emergency' conditions and interactions of neutrophils with the adaptive immune system. We explore *in vivo* observations of the recruitment of neutrophils from blood to tissues in models of blood-borne infections versus bacterial invasion through epithelial linings. We examine data on novel aspects of the activation of NADPH oxidase and the heterogeneity of phagosomes and, finally, consider the importance of two neutrophil-derived biological agents: neutrophil extracellular traps and ectosomes.

Neutrophils were identified more than 100 years ago, yet knowledge of how they are recruited to sites of infections and interact with other cells of the immune system has expanded tremendously during the past few years, in part due to emerging techniques that permit *in vivo* observations of neutrophils in tissues. Here we present and discuss these novel aspects of neutrophil biology.

Production of neutrophils

Only mature neutrophils are normally released from the bone marrow. Hematopoietic stem cells locate to bone marrow niches provided mainly by perivascular cells that express a membrane-bound form of stem cell factor[1] and the chemokine CXCL12 (SDF-1)[2], which are ligands for the stem cell antigen CD117 (c-Kit) and chemokine receptor CXCR4, respectively. CXCR4 delivers retention signals and gradually disappears as myeloid cells such as neutrophils mature, whereas CXCR2, the receptor for the chemokines CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8 (ref. 3), relays release signals and progressively increases in abundance over time, which leads to the egress of myeloid cells from the bone marrow. Inhibitors of CXCR4 signaling have been developed to assist the mobilization of hematopoietic stem cells for use in stem cell transplantation[4].

Dendritic cells (DCs) regulate the distribution of neutrophils between the bone marrow and peripheral organs and blood[5], and conventional DCs (cDCs) control production of the growth factor G-CSF and the chemokines CXCL1 (KC), CCL2 (MCP-1) and CXCL10 (IP-10). Depletion of cDCs in mice increases the concentration of these cytokines, which leads to loss of neutrophils from the bone marrow and secondary neutrophilia[6]. Neutrophilia from such depletion occurs in the absence of infection, which indicates that cDCs participate in neutrophil homeostasis. Unidentified factors present in the plasma of mice depleted of cDCs also augment the capacity of neutrophils for phagocytosis and NADPH oxidase activity[7]. As DC numbers are reduced in sepsis[8], these processes may enhance the capacity to eliminate microorganisms during severe infection.

Regulation of neutrophil production by intrinsic and extrinsic factors has been reviewed[9,10]. Central to the regulation of steady-state granulopoiesis, the ingestion of apoptotic neutrophils by tissue macrophages activates transcription factors of the LXR family that in turn suppress the production of proinflammatory cytokines[11]. Such macrophages ingesting cells by 'efferocytosis' decrease their production of the inflammatory cytokine interleukin 23 (IL-23) and thereby reduce the stimulus for IL-17 production by T lymphocytes and the downstream production of G-CSF[12,13]. In contrast to steady-state granulopoiesis, 'emergency' granulopoiesis refers to events during microbial challenge, when bacterial products (such as endotoxin) and increased amounts of IL-1, tumor-necrosis factor (TNF) and the growth factors G-CSF and GM-CSF stimulate granulopoiesis and the release of neutrophils into the circulation. Whereas the transcription factor C/EBP-α is fundamental for steady-state granulopoiesis, C/EBP-β drives emergency granulopoiesis[14] and is thus particularly responsive to GM-CSF[15]. Progenitors of neutrophils identified by the antigen c-Kit may also be recruited to sites of infection and establish local production of neutrophils that help clear the infection, as has been demonstrated in mice[16].

One study has questioned the mechanistic basis for the distinction between steady-state granulopoiesis and emergency granulopoiesis[17]. Even in the absence of overt infection, healthy mice maintained under normal conditions have more circulating neutrophils than do genetically identical mice raised aseptically. Antibody-mediated depletion of neutrophils from mice increases G-CSF and granulopoiesis in the bone marrow of mice maintained in normal or aseptic conditions. In Toll-like receptor (TLR4)-deficient mice that are unresponsive to endotoxin, neutropenia increases neither G-CSF nor granulopoiesis. On the other hand, depletion of neutrophils from mice that lack T cells, including the subsets that produce IL-17, increases G-CSF. Together these observations undermine the general validity of the proposal that a peripheral IL-23–IL-17 axis provides feedback regulation of granulopoiesis[12,13] and instead indicate that signaling through pattern-recognition molecules modulates steady-state granulopoiesis as well as emergency granulopoiesis[17,18].

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The well-known circadian fluctuations in the number of circulating neutrophils have been shown to rely on a short feedback circuit that involves selectins, chemokine receptors, and β_2 integrins. As circulating neutrophils age, downregulation of the expression of L-selectin (CD62L), upregulation of the expression of CXCR4 and CD11b and nuclear hypersegmentation occur irreversibly and peak every 24 hours. $CXCR4^{hi}$ neutrophils home to the bone marrow, where they are ingested by local macrophages. Activation of the transcription factor LXR promotes macrophages to downregulate expression of CXCL12 in the bone marrow, which in turn liberates $CD62L^{hi}CXCR4^{lo}$ neutrophils, again with a peak every 24 hours in counterphase with oscillation to the $CD62L^{lo}CXCR4^{hi}$ phenotype[19]. Circadian fluctuations in the recruitment of neutrophils to sites of inflammation and of neutrophil precursors to bone marrow endothelial niches depend on signals that are initiated by the intensity of light and are transmitted through the sympathetic nervous system to influence the expression of CXCL12, P- and E-selectins, and the integrin ligands VCAM-1 and ICAM-1 endothelial cells[20]. These elegant studies[19,20] were of mice, and *in vivo* studies of humans are lacking, but human neutrophils do increase their CXCR4 expression during aging *in vitro*[21].

The administration of endotoxin to healthy humans results in the appearance of $CD62L^{lo}CD11b^{hi}$ neutrophils with nuclear hypersegmentation that are able to suppress T cells and thus are characterized as a unique population of myeloid-derived suppressor cells (the so-called 'G-MDSCs')[22]. We consider it very likely that this neutrophil subset represents normal aged neutrophils that have previously homed to the bone marrow but are released from that site by endotoxin, along with $CD62L^{hi}$ cells with band-cell morphology as the typical left-shift response to microbial challenge that have also been identified in circulation after challenge with lipopolysaccharide[22].

Whereas aging neutrophils in the circulation clearly adopt an altered phenotype, shedding CD62L and acquiring CXCR4 to home back to the bone marrow, human aging affects the functional capacity of neutrophils as well. In contrast to neutrophils from the young, neutrophils from people 60 years of age and older maintain the ability to move but lose chemotactic focus and 'spill' more proteases on their way, which results in less-efficient bacterial clearance and more inadvertent tissue destruction. Higher constitutive phosphatidylinositol-3-OH kinase activity underlies the compromised function of neutrophils from elderly people, as decreasing that signaling pathway 'rejuvenates' the neutrophils and restores their chemotactic accuracy[23].

Neutrophil trafficking

Evolution prudently invested considerable effort into fortifying local defenses at skin and mucosal linings, where exposure to normally colonizing microorganisms and the risk of invasion through a barrier defect are both constant. Even without antibiotic therapy, defenses at the periphery usually successfully combat and constrain infections locally. In contrast, failure of local defenses allows microbes access to circulation and increases the risk of frequently fatal sepsis. We view recruitment of circulating neutrophils to various organs in this context (**Fig. 1**).

The marginated neutrophil pool

Early studies of neutrophil kinetics[24,25] demonstrated that epinephrine (adrenalin) doubles within minutes the number of circulating neutrophils as a result of their liberation from the pulmonary vascular bed[26,27]. Neutrophils marginated in the pulmonary bed differ from mature neutrophils in bone marrow[28]. The CXCR4 inhibitor plerixafor recruits into the circulation neutrophils marginated in the pulmonary bed without concomitant mobilization of bone marrow neutrophils[29]. Consistent with the notion that rapid mobilization of neutrophils from the lungs relies on CXCR4, pulmonary vascular endothelial cells express CXCL12, the ligand for CXCR4. Because CXCR4 expression increases as circulating neutrophils age, it is possible that aging neutrophils populate the pulmonary reservoir. The relative propensity of marginated neutrophils to extravasate into tissue during infection and inflammation has not been assessed. In addition to their redistribution in the lungs, neutrophils may exit the circulation in the spleen and reenter the blood in response to epinephrine, although the size of this splenic pool in the absence of splenomegaly has not been accurately determined[30].

Neutrophil extravasation in response to invading microbes

Responding to invading microorganisms, local resident macrophages and mast cells secrete TNF, IL-1β and several other cytokines that

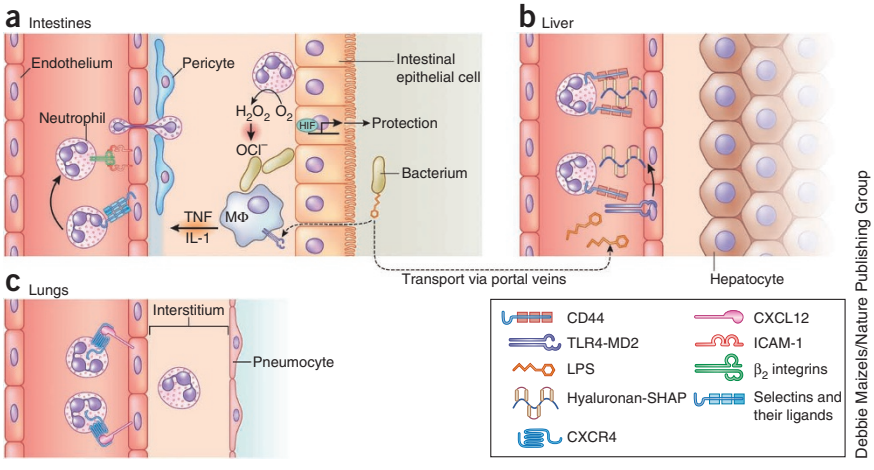

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Figure 1 Tissue-specific recruitment of neutrophils. Three different mechanisms underlie the recruitment of neutrophils to tissues. (**a**) The gastrointestinal tract illustrates the process of neutrophil recruitment after disruption of the normal mechanical barrier provided by epithelial cells. Local tissue macrophages (MΦ) detect invading microorganisms and release cytokines that, together with bacterial products, create a chemotactic gradient and activate endothelial cells, which capture neutrophils by selectin-mediated interactions and induce firm adhesion mediated by ICAM-1 and β_2 integrins. Pericytes support the migration of neutrophils across the epithelial lining. Neutrophils activated to kill microorganisms by reactive oxygen species generated from NADPH oxidase create a hypoxic environment that stimulates HIF-1α-mediated protection of mucosal epithelial cells. (**b**) The recruitment of neutrophils by blood-borne microbes is exemplified here by events in the liver. Bacteria or bacterial products (lipopolysaccharide (LPS)) engage TLR4 and its coreceptor MD2 and activate epithelial cells. As a result, hyaluronan associates with SHAP and thereby greatly increases its affinity for CD44 on neutrophils. (**c**) The recruitment of neutrophils to pulmonary endothelial cells results in a marginated pool mediated by CXCL12 (SDF-1) and CXCR4, even in uninfected lungs. Under experimental conditions, antagonists of CXCR4 release these marginated neutrophils, and physiological mediators such as adrenalin probably prompt the same redistribution of cells. Even in the absence of inflammation or infection, neutrophils are found in the pulmonary interstitium.

activate endothelial cells, which then capture circulating neutrophils[31,32]. A well-described series of events follows, whereby neutrophils are caught via selectin-mediated binding, which activates and polarizes the captured neutrophil and triggers 'inside-out' activation of CD11a and CD18 located on the surface and CD11b and CD18 translocated from intracellular stores. To examine these events under the conditions of high shear stress that would exist *in vivo* in some vascular beds, a technique known as 'quantitative dynamic footprinting' has been developed[33]. In a variety of experimental systems, neutrophils display membrane extensions or tethers at the rear of the cells (the uropod) that contribute to the stability of the rolling neutrophil[34]. In addition to tethers, rolling neutrophils in venules display membrane extensions in front of the cell. Referred to as 'slings', these structures wrap around the neutrophils and provide a path for rolling neutrophils to traverse endothelium, despite the mechanical challenges of the ongoing high shear stress, generally in excess of 0.6 Pa, that occurs in postcapillary venules and arterioles[33].

Once slowed, the rolling neutrophil flattens, becomes polarized and crawls along the endothelial cells in search of an escape route from the vessel lumen, either between endothelial cells or through them[35,36]. Integrins binding to the adhesion molecule ICAM-1 on endothelial cells mediate firm attachment of neutrophils to endothelial cells and direct the motion of neutrophils to points of egress from the vascular lining, either by the paracellular route at points where three or more endothelial cells join or by a transcellular route through so-called 'endothelial cups' that accommodate migrating neutrophils[32,37–40]. Endothelial cells are not passive bystanders during neutrophil transmigration, as the aggregation of ICAM-1 on the endothelial cell surface that is prompted by contact with neutrophils initiates intracellular signaling pathways, including Src and NO synthase[41], that promote migration.

Pericytes (contractile perivascular cells found on the abluminal surface of capillaries and post-capillary venules[42]) interact with endothelial cells and contribute to vascular homeostasis. At inflammatory sites, neutrophils migrate along pericytes interdigitated in the vessel wall, exiting through gaps between neighboring pericytes[43]. Variability in the morphology and distribution of pericytes in different vascular beds[42] may contribute to the tissue-specific features of the recruitment of neutrophils to individual viscera[44,45]. In addition to the pericytes, tissue mast cells[46] and macrophages[47] contribute to the regulation of neutrophil migration into specific tissues. The transmigration cascade culminates as the elongated uropod of the emigrating neutrophil detaches from the vessel wall and the neutrophil migrates into tissue[48].

The process of emigration from the circulation alters the functional state of the neutrophil. Tissue neutrophils exhibit augmented NADPH oxidase activity in response to agonists such as formylated peptides[43,49], contain and produce more proinflammatory cytokines such as CXCL8 (IL-8)[50–52] and become resistant to antiapoptotic stimulation[50]. The reprogramming of neutrophils during their transformation from circulating cells to tissue phagocytes suits their adaptation to a new setting. As with other cells, context molds the phenotype and activity of neutrophils to meet functional needs, as in wound healing and in modulating the immune response[52,53].

The classic view of neutrophil diapedesis noted above has been delineated largely by *in vitro* and *in vivo* studies of a mouse cremaster muscle model, but the characteristics of inherited leukocyte adhesion deficiencies (LADs) confirm the principles therein. In LAD II, deficiency in fucose transferase disables the capture of ligand by selectins and causes a universal defect in neutrophil extravasation. Likewise, both LAD I, which is due to lack of CD18 (the common β-chain of β_2 integrins), and LAD III, caused by mutations in the gene encoding kindlin-3 and an inability to activate integrins, are characterized by a profound inability to extravasate neutrophils during infection[54–56]. Although they are not as prominent in pulmonary vascular beds as in other vascular beds, endothelial selectins figure notably in neutrophil extravasation in the lungs as well[57]. The mechanisms elucidated for neutrophil diapedesis noted above apply to neutrophils migrating up a gradient of chemoattractant generated by or in response to microbes that have accessed tissue through breaks in the skin or mucosal barriers to prevent lymphatic or hematogeneous spread. A different scheme is probably in effect in the liver, brain, spleen and other organs without external exposure. In such settings, other interactions regulate the recruitment of neutrophils from the circulation.

Neutrophil trafficking in response to blood-borne microbes

The development of dual-photon spinning-disc laser microscopy and the use of transgenic and knockout mice have made it possible to study neutrophil trafficking in organs *in vivo* and to describe the contributions of individual types of cells and specific proteins.

Optimized for metabolic purposes, the portal circulation brings blood from the gastrointestinal tract to liver sinusoids but may deliver microbes from the gastrointestinal tract as well, which makes the liver the internal organ most routinely exposed to enteric microorganisms or their products. Endothelial cells of the liver sinusoids capture neutrophils via CD44-hyaluronan, an interaction involved in the recruitment of circulating T cells to virus-infected cells and to inflamed tissues, such as the joints in rheumatoid arthritis and the central nervous system in encephalomyelitis[58–60]. Like selectins in their ability to bind carbohydrates, the transmembrane protein CD44 binds hyaluronan[61,62], a widely expressed extracellular proteoglycan. Inter-α-trypsin inhibitor, a complex of two heavy chains coupled covalently to the chondroitin sulfate on the serine protease inhibitor bikunin and synthesized in hepatic cells, can undergo transesterification, whereby the heavy chains transfer to hyaluronan, releasing bikunin and replacing chondroitin sulfate with hyaluronan. The product of those events, SHAP (serum-derived hyaluronan–associated protein), augments the affinity of hyaluronan for CD44 on neutrophils[60]. Intravenous administration of endotoxin induces the modification of hyaluronan by SHAP on hepatic sinusoidal endothelial cells, where neutrophils then attach by mechanisms dependent on TLR4 signaling in the endothelial cell[58]. Whereas the accumulation of neutrophils in the liver helps reduce viral infection through the formation of platelet-rich intravascular neutrophil extracellular traps (NETs)[63], hyaluronan-mediated adhesion of neutrophils may be deleterious and might induce the capillary leak typical of sepsis.

Neutrophils in tissues

Whereas the migration of neutrophils across the endothelial cell lining is strictly dependent on activated β_2 integrins, migration in tissues distant from the site of injury is not. After leaving the circulation, neutrophils aggregate around the focus of infection or injury in two waves of recruitment. Initially, neutrophils adjacent to the focus of injury migrate toward the nidus, followed by a second 'swarm' of neutrophils recruited from more than 200 μm from the site of tissue injury. Neutrophils in the center of the reaction generate leukotriene B4, which drives the second, long-distance migration of neutrophils by engagement of their receptor for this leukotriene[64]. Furthermore, that receptor and other G protein–coupled receptors support aggregation of the clustering neutrophils at the injury site. Thus, the signaling between neutrophils mediated by neutrophil-derived chemoattractants makes possible the long-distance migration seen in the tissues.

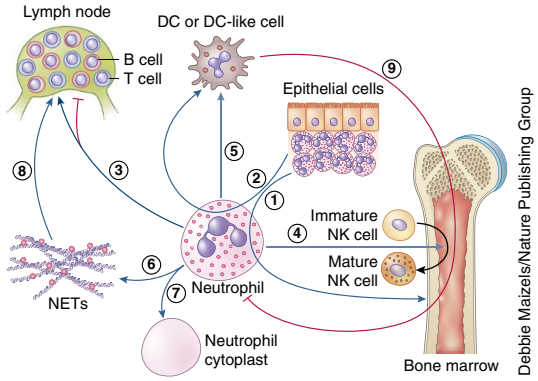

Figure 2 Interfaces between neutrophils and elements of adaptive immunity. Neutrophils interact with several distinct elements of adaptive immunity: they can carry antigens from the epithelium to the bone marrow (1) or to DCs or DC-like cells (2); they inhibit the proliferation of T cells in lymph nodes (3, blunt-ended red line); and they stimulate maturation of antibody-producing B cells (3, arrow). Neutrophils are essential for the generation of NK cells (4) and can transform into functional DC-like cells that maintain essential neutrophil antigens (5). Neutrophils can release NETs (6) and thereby transform into anuclear cytoplasts (7). NETs can stimulate autoimmunity by eliciting immune responses to the NET-associated nuclear antigens and granule proteins (red particles) (8). Reciprocally, elements of the adaptive immune system can modulate neutrophil activity, as DCs can inhibit the generation and function of neutrophils (9, blunt-ended red line).

Even uninfected lungs have a sizable pool of neutrophils, both marginated (i.e., adjacent to the luminal endothelial surface) and also in tissues (i.e., having traversed the endothelial lining in the lungs). During infection, monocytes in the pulmonary parenchyma promote the redistribution of neutrophils, stimulating them to extravasate into the lungs and to swarm to form clusters of neutrophils, as has been shown in peripheral tissues, as discussed above[28]. In the absence of monocytes, neutrophils adhere to but do not traverse the endothelium.

Neutrophils in lymphoid organs

It has become clear that neutrophils enter lymphoid organs, where they can modulate the adaptive immune response. The chemokines CCL19 and CCL21 released from lymph nodes engage their receptor CCR7 on neutrophils to stimulate migration to lymph nodes[65]. Whereas this receptor-ligand set guides DCs to lymph nodes, neutrophils seem to inhibit rather than augment the response of $CD4^+$ T cells and B cells to immunization[66]. Lymph node–infiltrating neutrophils produce the prothrombotic factor thromboxane A_2 and both reduce the contact between DCs and T cells and inhibit the further migration of T cells to more central lymphatic nodes, possibly by thromboxane A_2–mediated vascular constriction[67]. Myeloperoxidase (MPO), a major protein of neutrophil azurophil granules that is essential for the production of microbicidal hypochlorous acid (HOCl)[68], inhibits the proliferation and activation of DCs, thereby decreasing their numbers in draining lymph nodes. Consequently, $CD4^+$ T cells in the lymph nodes exhibit reduced activity and diminished responses to antigen. In support of the concept that MPO influences the activity of $CD4^+$ T cells, MPO-deficient mice develop more joint inflammation in a model of autoimmune inflammatory arthritis dependent on the activation of $CD4^+$ T cells[69]. Such suppressive effects of neutrophils on the adaptive immune system may be viewed as a feed-forward brake for reducing tissue destruction.

Neutrophils enhance local adaptive immunity during pulmonary infection with *Mycobacterium tuberculosis*. The first cells to accumulate and phagocytose *M. tuberculosis* during infection, neutrophils both secrete CCL19 and possibly CCL21 to attract DCs that might ingest *M. tuberculosis*–laden neutrophils and prime DCs to express CCR7 for faster migration to the draining lymph nodes and activation of $CD4^+$ T cells[70] (**Fig. 2**).

In the mantle zone of the spleen resides a specialized population of neutrophils conditioned by IL-10 from local endothelial cells and macrophages exposed to endotoxin. Called 'B cell helper neutrophils', these cells secrete the B cell–activating and survival factors BAFF, APRIL and IL-21, induce immunoglobulin class switching of B cells and extend membrane protrusions that directly contact B cells[71].

Although it is not a lymphoid organ itself, bone marrow contains $CD8^+$ memory T cells and, under certain circumstances, neutrophils may participate more directly in antigen presentation. An elegant study has demonstrated that neutrophils may acquire antigen from dermis and transport it to the bone marrow, bypassing draining lymph nodes, as part of the CXCR4-dependent homing described above. The return of antigen-loaded neutrophils from the dermis to the bone marrow strictly depends on the chemokine receptor CCR1. Resident macrophages in the bone marrow ingest antigen-laden neutrophils and present antigen to $CD8^+$ T cells for the generation of memory T cells[72].

Neutrophils can directly adopt structural and functional characteristics of DCs, including cytoplasmic projections, round nuclei, and expression of CD11c and the antigen-presenting major histocompatibility complex class II molecules and T cell–costimulatory molecules CD80 and CD86, while maintaining attributes typical of mouse neutrophils, such as the expression of L-selectin and Ly6G and the ability to phagocytose microorganisms that far exceeds that of normal DCs[73,74]. This phenotypic transition affects only a minority of tissue-infiltrating neutrophils and requires stimulation by GM-CSF, which is known to transform monocytes into DCs[75]. In certain settings, some human neutrophils recovered from tissue express the DC marker CD83 as well as major histocompatibility complex class II molecules[76], which indicates that the same plasticity reported in mouse systems exists in humans.

Neutrophils also regulate the maturation of natural killer (NK) cells, as a mouse mutagenesis study has identified a mutation in the gene encoding Gfi-1, a transcription factor known to be involved in granulopoiesis, that results in a strain with diminished NK cell activity[77] (**Fig. 2**). These so-called 'Genista' mice are also neutropenic, and the hypothesis that neutrophils are essential for normal NK cell development has been confirmed by analysis of the NK cell repertoire of both wild-type mice with induced neutropenia and patients with severe congenital neutropenia[77].

Neutrophil microbicidal activities

Accumulating data have provided novel insights into events critical to effecting optimal antimicrobial action in neutrophil phagosomes. We now focus our attention on new insights into events that modulate the responses of neutrophils to ingested microbes trapped within phagosomes.

The assembly and activity of NADPH oxidase in phagocytes, essential for neutrophil microbicidal activity[78], requires translocation of a cytoplasmic complex containing $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$. Despite its membership in this essential ternary complex[79,80], the function of $p40^{phox}$ has eluded firm definition. Like $p47^{phox}$, $p40^{phox}$ contains a PX domain, a specific motif that supports the binding of proteins to phosphoinositides on the inner leaflet of membranes[81]. Studies with a variety of experimental systems have identified phosphatidylinositol-3-phosphate, a product of class III phosphatidylinositol-3-phosphate kinase, as the target for the PX domain of $p40^{phox}$ on phagosomal

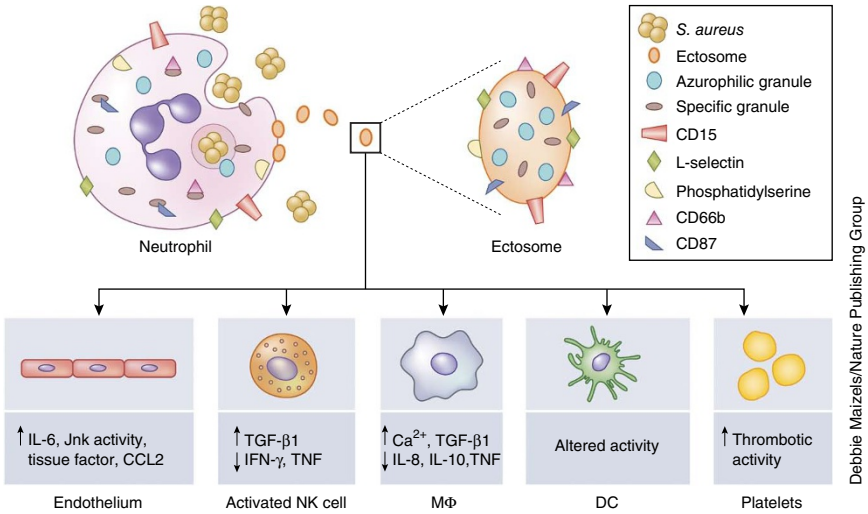

Figure 3 Neutrophil ectosomes provide long-range signaling to modulate inflammation. The specific agonist that triggers the release of ectosomes dictates both the composition and the functional capacity of neutrophil ectosomes. In the example here, ectosomes released from the plasma membrane of neutrophils during stimulation with *S. aureus* have surface receptors from the plasma membrane (for example, CD15 and L-selectin), phosphatidylserine from the inner leaflet of the plasma membrane, proteins from specific granule membranes (for example, CD66b and CD87), and matrix proteins from granules (for example, MPO, elastase, proteinase 3, defensins, lactoferrin and collagenase I). Depending on the surface proteins and intravesicular contents, tissue targets that engage ectosomes can respond in many ways. For example, endothelial cells interacting with ectosomes activate the kinase Jnk pathway and increase production of IL-6, tissue factor and CCL2; macrophages flux calcium and release TGF-β1, thereby modifying DC function while decreasing their production of IL-8, IL-10 and TNF; platelets increase prethrombotic activity; and IL-2- and IL-10-treated NK cells produce an anti-inflammatory cytokine profile, with increased release of TGF-β1 and decreases in interferon-γ (IFN-γ) and TNF.

membranes[82–86]. The identification and characterization of a patient with chronic granulomatous disease (CGD) due to a missense mutation in the gene encoding $p40^{phox}$ that results in replacement of arginine at position 105 with glutamine[87] has clearly demonstrated that stable assembly and activation of the phagocyte oxidase at phagosomes requires the docking of $p40^{phox}$ via its PX domain. In neutrophils of this affected patient, the presence of this substitution in the PX domain undermined the stable association of $p40^{phox}$ on phagosomes and culminated in profoundly depressed phagosomal generation of oxidants, as well as defective killing of ingested *Staphylococcus aureus*. In contrast to the failure to generate oxidants in phagosomes, the mutant neutrophils produced nearly normal amounts of reactive oxygen species in response to soluble agonists, such as phorbol myristate acetate and formyl methionylleucylphenylalanine; this demonstrates the specialized role of $p40^{phox}$ in targeting oxidase assembly at phagosomes.

The clinical presentation of the first $p40^{phox}$-deficient patient differed markedly from that characteristically seen in patients with CGD. Whereas recurrent and severe infections with a few selective organisms typify the clinical phenotype of CGD[88], the $p40^{phox}$-deficient patient suffered from refractory inflammatory colitis but lacked recurrent infections with organisms that characterize CGD (*S. aureus*, *Serratia marcescens*, *Burkholderia cepacia*, *Nocardia* and *Aspergillus*). Furthermore, in two different experimental models, $p40^{phox}$-deficient mice have been shown to exhibit increased colonic injury, with increased neutrophil recruitment, production of cytokines, and upregulation of CCR1 expression[89]. Although as many as 20% of patients with CGD present with signs and symptoms that resemble inflammatory bowel disease[90], infectious complications of defective neutrophil function predominate.

The clinical picture of the $p40^{phox}$-deficient patient discussed above highlights the widely recognized but incompletely understood link between neutrophil biology and gut health[91]. One study has described how oxygen consumption, rather than oxidant production, by neutrophils transmigrating across gut mucosa stabilizes the hypoxia-inducible factor HIF-1α in intestinal cells, protects the epithelium and promotes the resolution of inflammation[92]. As neutrophils from patients with CGD fail to consume oxygen, mucosal HIF-1α will not be stabilized and inflammation will persist. Increased HIF-1α has also been demonstrated in biopsies from patients with inflammatory bowel disease, which demonstrates the clinical relevance of the mouse model[92]. This dysfunction in CGD compounds the excess activity of granule proteins released in the absence of H_2O_2 and HOCl that otherwise would oxidize and inactivate them[93]. The combined effects of excess protease activity and absence of HIF stabilization in the gut mucosa of patients with CGD may underlie the inflammatory colitis commonly seen in such patients.

Ion flux and phagocyte oxidase

Operating as an electron transferase, the NADPH oxidase in phagocytes redistributes negative charges into the phagosome lumen and thereby depolarizes the phagosomal membrane[94]. In the absence of charge compensation, electron flow across the NADPH oxidase NOX2 and $p22^{phox}$ would cease when the membrane potential reached the equilibrium potential for electron movement[95]. Most of the charge compensation occurs via Hv1, a voltage-gated proton channel[96]. Situated in the plasma membrane and membranes of peroxidase-negative granules[97], Hv1 associates with nascent phagosomes and thereby localizes together with but does not directly associate with NOX2-$p22^{phox}$ (refs. 97,98). Neutrophils from mice that lack Hv1 produce less superoxide anion and H_2O_2 (refs. 99,100), which demonstrates the requirement for Hv1 in sustained oxidase activity. Furthermore, Hv1 contributes to the maintenance of a neutral pH within neutrophil phagosomes[101].

Like proton flux, the transport of chloride into phagosomes supports biochemistry integral to the optimal antimicrobial activity of neutrophils. Efficient killing by the MPO-H_2O_2-halide system requires a source of chloride ions, as the little chloride incidentally internalized during phagocytosis would be rapidly consumed by the generation of HOCl[68]. The cystic fibrosis transmembrane conductance regulator (CFTR) mediates the bulk of chloride transport into neutrophil phagosomes[102,103], tapping into the relatively large reservoir of chloride ions in the neutrophil cytoplasm[104]. Located in the secretory vesicles of resting neutrophils, CFTR becomes incorporated into phagosomal membranes during phagocytosis. Neutrophils that lack CFTR exhibit defective killing of ingested *Pseudomonas aeruginosa*[104], which emphasizes the clinical relevance

of both HOCl production and CFTR-dependent chloride transport in neutrophils. Two additional transporters, ClC-3 (ref. 105) and KCC3 (ref. 106), contribute to the redistribution of chloride in neutrophils, although to a much lesser extent than does CFTR. Abnormal neutrophil function in cystic fibrosis extends beyond defective antimicrobial action, as affected neutrophils exhibit delayed apoptosis[107], which could underlie the observed prolonged inflammation seen in patients with cystic fibrosis. Identification of the array of neutrophil disturbances in cystic fibrosis[108] may prompt novel therapeutic interventions that target the immunomodulation of neutrophils in this common inherited disorder.

Increases in the intracellular concentration of calcium (Ca^{2+}) drive several neutrophil processes pertinent to activities in the phagosome, including the activation of NADPH oxidase[109] and fusion of granules with phagosomes[110]. Published work has elucidated the mechanism and importance of the longstanding observation of localized increases in Ca^{2+} near phagosomes[111]. The Ca^{2+} sensor Stim1 serves an essential role in the regulation of store-operated calcium influx and calcium-release channels in human cells[112,113]. In mouse neutrophils, Stim1 promotes interactions of cisternae of the endoplasmic reticulum with Ca^{2+} channels on nascent phagosomes, which enables Ca^{2+}-dependent signaling and release of periphagosomal actin to support efficient phagocytosis[114]. Orai1, a store-operated calcium entry channel[113], promotes the flux of Ca^{2+} across phagosomal membranes and thus promotes phagocytosis[114,115], degranulation[115] and activation of NADPH oxidase[115,116]. The extent to which Stim1-dependent activities in neutrophils contribute to normal innate immune responses awaits further investigation, as published studies have focused attention on the importance of Stim1 and other store-operated Ca^{2+} channels in lymphocyte function[117].

Heterogeneity of phagosomes

While writing about macrophages, one author opined that there is structural and functional heterogeneity among phagosomes within an individual cell[118]. The same heterogeneity of phagosomes, the author noted, applies to other phagocytic cells. For example, the loss of fluorescence of green fluorescent protein–expressing *S. aureus* ingested by human neutrophils, a gauge of HOCl production in individual phagosomes, demonstrates the variability of phagosomes in the same neutrophils[119]. In such studies, at a multiplicity of infection of 1:1 (i.e., one colony-forming unit (or four cocci) for each neutrophil), a given neutrophil contains phagosomes with bleached bacteria as well as fluorescent bacteria[119]. Heterogeneity of neutrophil phagosomes could reflect, in part, variability in the assembly and activation of the NADPH oxidase on phagosomes, delivery of MPO by fusion of azurophilic granules and HOCl production, or both this variability and delivery. Whereas these events occur to some extent stochastically, specific biochemical events probably contribute to the variability observed.

Such phagosome diversity might reflect the variability among individual phagosomes in the lipid and protein composition of their membranes, the cytoplasmic enzymes to which abluminal targets on the phagosome would be subjected (for example, kinases, phosphatases and GTPases), and collisions with other intracellular organelles (for example, endosomes, lysosomes, endoplasmic reticulum and Golgi-derived vesicles). Consequently, the lipids and proteins in the phagosomal membrane could be envisioned as an organized network defined by its individual constituents, their specific post-translational modification and the downstream functional consequences of their presence or activation state.

In macrophages, a more tractable experimental phagocyte than neutrophils, the activity of NADPH oxidase and distribution of phosphatidylinositol on phagosomes demonstrate the heterogeneity of phagosomes[120]. The production of superoxide anion differs in individual phagosomes, not only between cells but also among phagosomes in the same cell. Although the phosphatidylinositol species of interest are the same, the activity of diacylglycerol kinase and the abundance of diacylglycerol differ among phagosomes, and this directly correlates with the production of superoxide anion. The design and application of sensitive biosensors that report biochemical changes in a dynamic and quantitative fashion promises to expand appreciation of the mechanistic basis for and functional consequences of phagosome heterogeneity.

Beyond phagosomes: NETs and ectosomes

Most direct antimicrobial activity by neutrophils occurs within phagosomes, but the involvement of neutrophils in innate immune response to infection extends to extracellular responses and the contributions of bioactive membrane vesicles released from activated neutrophils.

Since the first description of the phenomenon of NETs (extracellular strands of DNA bound to antimicrobial neutrophil-derived peptides and proteins)[121], this has been considered an alternative to death either by apoptosis (a process that supports the resolution of inflammation) or by pyroptosis, secondary necrosis or necroptosis[122] (events that can promote the release of proinflammatory cytokines from macrophages[123]). Although not yet fully characterized, the mechanisms that underlie 'NETosis' (as this death pathway has been named) have been partially defined *in vitro*, typically by analysis of neutrophils stimulated for 1–3 hours with phorbol myristate acetate, a potent activator of protein kinase C, under conditions without or with very low concentrations of serum proteins[124]. The citrullination of histone dependent on the peptidylarginine deiminase PAD4 seems to be essential for preparing chromatin for the release of DNA via NETosis[125,126]. NETosis under such experimental conditions depends on the presence of the major neutrophil serine protease elastase[80], MPO[127] and active NADPH oxidase, assembled via a pathway of the kinases Raf, MEK and Erk[128]. Consequently, NETosis would not be expected to occur in patients with MPO deficiency, a relatively common inherited disorder with a minor compromise in the ability to fight microbial infections[68], or in the CGD described above, which is a more severe immunodeficiency[129] characterized by the inability of neutrophils to produce reactive oxygen species[130]. Given the limited deleterious nature of the clinical phenotype of MPO deficiency, it seems that NETosis, as defined above, has a minor (if any) role in immunological defense. Similarly, patients with Papillon-Lefévre syndrome, whose neutrophils have neither elastase nor other serine proteases and consequently cannot support NETosis (Sørensen, O.E. *et al.* unpublished data), lack enhanced susceptibility to systemic infections and generally suffer only severe periodontal disease[131]. NETs have been shown to entrap bacteria[132], fungi[133] and even viruses[63] and may offer some protection to T cells against infection by human immunodeficiency virus[134]. Two independent studies have challenged the initial observation that NETs kill trapped bacteria and suggest instead that the inability to release bacteria from NETs is the basis for the failure to recover viable bacteria[135,136]. Nonetheless, trapping viable bacteria would restrain microbes and would thereby serve to prevent widespread metastases of infection.

To a degree, NETs seem to contribute to autoimmune diseases in which the target antigens frequently are constituents of NETs (**Fig. 2**), including DNA as well as MPO and proteinase 3, as seen in systemic lupus erythematosus and Wegener's granulomatosis[137,138]. Clinically relevant flares of autoimmune diseases often associated with bacterial infections may reflect the consequences of acute activation of

neutrophils and the generation of more NETs and thus antigens[139]. However, other stimuli elicit a cellular process that shares features of NETosis and may have biological consequences. Endotoxin-activated platelets can induce NETosis (i.e., the presence of extracellular DNA and histones associated with neutrophil elastase and MPO), possibly involving mitochondrial DNA[140], and may be particularly relevant during sepsis, which often is associated with the activation of platelets. In addition, intravascular NETs may damage the endothelium and thus contribute to the capillary leak often associated with sepsis[141] and with acute lung injury[142].

It has been demonstrated that neutrophils undergoing active phagocytosis can extrude their DNA but still maintain their integrity and continue to migrate, albeit less efficiently. The dependence of this process, called 'vital NETosis'[143,144], on MPO, elastase or NADPH oxidase has not been demonstrated, although it does rely on signaling through TLRs, as neutrophils deficient in either the adaptor MyD88 or TLR2 do not initiate the response after microbial challenge[143]. Neutrophils undergoing vital NETosis lack nuclei and can be observed as anuclear cytoplasts. Vital NETosis differs in other ways from the phenomenon originally described as NETs: only complement-opsonized targets elicit the response, and it occurs mainly in extravasated neutrophils, both features of considerable biological relevance[143]. Furthermore, this cellular response occurs within minutes and limits bacterial dissemination *in vivo*. Such anuclear cytoplasts, which resemble the cytoplasts generated by high-speed centrifugation of cytochalasin B–treated neutrophils in a density gradient[145] and thermally induced neutrophil cytokinetoplasts[146], can be recovered from human abscesses. The subsequent fate of such anuclear cytoplasts remains unknown (**Fig. 2**).

A variety of names have been applied to the microvesicles derived from membranes, both endosomal (referred to as 'exosomes') and plasma (referred to as 'ectosomes'), of intact cells[147,148]. In human neutrophils, ectosome microvesicles are generated by stimulation with agents such as formyl methionylleucylphenylalanine and complement C5a[149]. In response to specific agonists, human neutrophils generate membrane vesicles 50–200 nm in diameter that arise by budding from plasma membrane[150] and express on their surface phosphatidylserine, as well as various membrane proteins, including CD15, CD64, CD66b and CD66c, and contain the matrix metalloproteinase MMP9, MPO, the proteinase PRTN3 and neutrophil elastase[151,152] (**Fig. 3**). The repertoire of proteins in and on ectosomes varies depending on the conditions under which they are generated[153]. Like the proteins expressed on the surface of neutrophils, ectosomes vary according to the initiating stimulus, and the consequences of their interactions with intact cells reflect the specific target cell and its array of surface receptors. For example, neutrophil-derived ectosomes exhibit proinflammatory properties in interactions with endothelial cells[154] and prethrombotic properties with platelets[155,156] but act as anti-inflammatory particles when they engage neutrophils or macrophages[147,157,158]. Neutrophil ectosomes participate in biology broadly related to innate immune responses, including events important for vascular homeostasis[151], and contribute to the pathophysiology of acute respiratory distress syndrome and acute lung injury[159]. Neutrophil ectosomes can modulate the activity of other phagocytes present during inflammation (macrophages and DCs) and thereby influence antigen presentation and the adaptive immune response. Phosphatidylserine on neutrophil ectosomes binds the receptor tyrosine kinase Mer and thereby increases signaling via phosphatidylinositol-3-OH kinase and the kinase Akt and decreases the transcriptional activity of the transcription factor NF-κB[147]. Consequently, TNF decreases down-modulation of the activity of DCs, but transforming growth factor-β1 (TGF-β1) increases this. Phosphatidylserine-dependent interactions with IL-2- and IL-12-treated NK cells shift their cytokine profile from proinflammatory to anti-inflammatory and provide a means by which neutrophil ectosomes can modulate the local environment during inflammation[160]. Present in the plasma of septic patients, ectosomes containing α_2-macroglobulin exert immunomodulatory effects, promoting neutrophil antimicrobial action and counteracting the endotoxin-driven downregulation of CXCR2 expression, thereby ameliorating in part the pathophysiological consequences of endotoxemia[161].

In addition to modulating the inflammatory microenvironment, neutrophil ectosomes exert direct antimicrobial action[162]. Ectosomes elicited from neutrophils challenged with either opsonized *S. aureus* or zymosan aggregate on the surface of specific microbes and, in some cases, kill the target bacteria[162]. For example, particle-induced neutrophil ectosomes retard the growth of *S. aureus* and *Escherichia coli* but not that of *Proteus mirabilis*. The bacteriostatic antimicrobial activity of such neutrophil ectosomes requires neither internalization of the bacteria nor the generation of oxidants by the NADPH oxidase in phagocytes. In the case of *S. aureus*, the adherence of ectosomes to the target depends on the uptake of glucose, functional β_2 integrin on the ectosome surface and reorganization of actin within neutrophils. Aggregating bacteriostatic neutrophil ectosomes can be recovered from bacteremic patients, which demonstrates that their generation and activities have biological relevance[162]. Although such ectosomes provide a vehicle with which to extend spatially the immunomodulatory and antimicrobial attributes of neutrophils, understanding the extent to which neutrophil ectosomes contribute to phagocyte-mediated host defense awaits additional investigation.

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