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# Cell intrinsic functions of neutrophils and their manipulation by pathogens

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Neutrophils are a crucial first line of defense against infection, migrating rapidly into tissues where they deploy granule components and toxic oxidants for efficient phagocytosis and microbe killing. Subsequent apoptosis and clearance of dying neutrophils are essential for control of infection and resolution of the inflammatory response. A subset of microbial pathogens survive exposure to neutrophils by manipulating phagocytosis, phagosome-granule fusion, oxidant production, and lifespan. Elucidating how they accomplish this unusual feat provides new insights into normal neutrophil function. In this review, we highlight recent discoveries about the ways in which neutrophils use cell-intrinsic mechanisms to control infection, and how these defenses are subverted by pathogens.

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## Introduction

Neutrophils are the most abundant white blood cell in humans and are a critical first line of defense against infectious challenge. In response to signals that are released in the context of infection, neutrophils migrate into tissues, where they release a variety of components for efficient phagocytosis and microbe killing. The components of this defense arsenal and enzymes that produce them are housed in subcellular organelles called granules that are mobilized in the activated neutrophil to fuse with the nascent phagosome (Figure 1). These components include toxic oxidants, degradative enzymes, metal sequestration proteins, and cationic antimicrobial proteins and peptides (Table 1). The subsequent apoptosis and clearance of dying neutrophils by macrophages are essential for control of infection and resolution of inflammation.

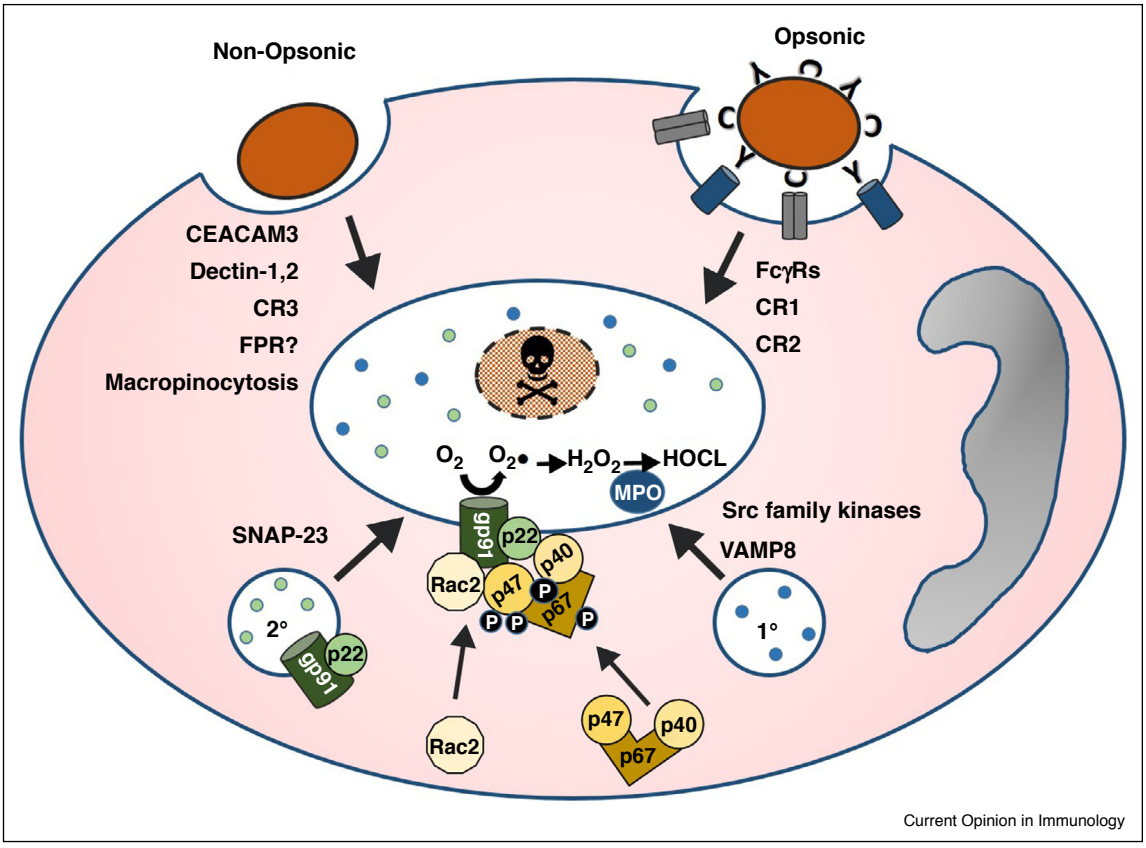
Given neutrophils' potent antimicrobial activity, it is remarkable that a subset of microbial pathogens evade elimination. Elucidating how this unusual feat is accomplished provides new insights into normal neutrophil function. In this review, we highlight recent discoveries about the ways in which neutrophils use intracellular mechanisms to control infection, and how these defenses are subverted by pathogens. For those interested in extracellular activities of neutrophils, cell ontogeny, coordination of immune responses, and migration, we refer readers to excellent recent reviews [1–4].

## Phagocytosis

Neutrophils readily phagocytose microbes that are opsonized with complement and/or IgG, but possess fewer receptors than macrophages for nonopsonic uptake (Figure 1). Phagocytic receptors are abundant in secretory vesicles and tertiary granules and are upregulated at the cell surface as neutrophils migrate to sites of infection. Neutrophils nonopsonically ingest a subset of pathogens [5••,6–9]. *Neisseria gonorrhoeae* and *Helicobacter pylori* bind carcinoembryonic antigen-related cell adhesion molecule 3 (CEACAM3), expressed exclusively in humans by neutrophils and other granulocytes. CEACAM3 signaling is proinflammatory and may have evolved to counteract anti-inflammatory signals from the ubiquitously expressed CEACAM1 [10•]. Complement receptor 3 (CR3; CD11b/CD18) binds β-glucan and other microbial components to drive nonopsonic uptake of microbes particularly by human neutrophils, including the pathogenic fungi *Aspergillus* and *Candida* [11–13]. In mice, the C-type lectins dectin-1 and dectin-2 recognize β-glucans and mannose to drive nonopsonic uptake of fungi and some bacteria [14]. Recently, formyl peptide receptors 1 and 2, best known for their roles in neutrophil chemotaxis, were proposed to promote phagocytosis [15], presumably by engaging N-formylated proteins on the surface of bacteria. Like macrophages, neutrophils may use receptor-independent, micropinocytosis-like processes for phagocytosis [14], but additional studies are needed to define how F-actin dynamics are regulated.

Not surprisingly, evasion of phagocytosis is a common strategy used by pathogens to avoid intracellular killing, and this occurs in a variety of ways. Capsular polysaccharides can prevent opsonin binding, as recently shown for

**Figure 1**




Phagocytic killing of microbes by neutrophils. Neutrophils phagocytose microbes that are opsonized with complement (C) and/or antibody (Y) using complement and Fc gamma receptors, respectively (upper right). They also phagocytose microbes that not opsonized (upper left). Fusion with primary (1°) and secondary (2°) granules delivers antimicrobial proteins and peptides (blue and green circles) into the phagosome, or at the phagocytic cup (not shown). See Table 1 for antimicrobial peptides and proteins in these granules. Secondary granule fusion delivers the integral membrane subunits of NADPH oxidase, gp91$^{phox}$ and p22$^{phox}$ (flavocytochrome $b_{558}$), to the phagosome. Upon cellular activation, the p40$^{phox}$/p47$^{phox}$/p67$^{phox}$ complex is phosphorylated (P) and Rac2 GTPase undergoes GDP → GTP exchange. These cytosolic subunits translocate to the phagosomal membrane and co-assemble with gp91$^{phox}$ and p22$^{phox}$ to create the NADPH holoenzyme. Superoxide ($O_2^{\bullet}$) produced by NADPH oxidase dismutates into hydrogen peroxide ($H_2O_2$), which is converted into hypochlorous acid (HOCl) by the primary granule enzyme myeloperoxidase (MPO). Fusion with endocytic compartments delivers the vacuolar H+-ATPase to the phagosomal membrane, where it modestly acidifies the phagosome (not shown). Oxidative and non-oxidative components work in concert to kill microbes within the phagosome. As described in the text, certain pathogens avoid intraphagosomal killing by preventing phagocytosis, preventing or redirecting granule fusion with the phagosome, limiting oxidant production, and manipulating neutrophil cell death pathways.

*Yersinia pestis* [16]. Similarly, *Pseudomonas aeruginosa* rugose small-colony variants that emerge in cystic fibrosis patients impair phagocytosis by overproducing biofilm-associated and aggregate-inducing exopolysaccharides [5••]. Pathogens including *Neisseria meningitidis*, *Candida albicans*, and *Aspergillus fumigatus* limit complement-mediated phagocytosis by targeting Factor H or degrading C3 [17–21]. Recently discovered virulence factors that degrade C3 are the serine proteases ScpA from *Streptococcus pyogenes* [22] and Pra1 from *C. albicans* [23]. Whereas anticapsular antibodies overcome phagocytosis inhibition for some pathogens including the 'superbug' ST258 lineage of *Klebsiella pneumoniae*, *Staphylococcus aureus* Protein A and *S. pyogenes* Protein G bind the Fc portion of IgG and prevent antibody-mediated phagocytosis. Additionally, *S. aureus* SElX blocks phagocytosis through the neutrophil IgG receptor CD16b (FcγRIIIb), independent of its superantigen activity [24]. Finally, *Porphyromonas gingivalis* proteases RgpA and RgpB cleave CR3, components of the Arp2/3 complex, Cdc42, and other proteins contributing to phagocytosis, with protease stability increased by citrullination from a bacterial peptidylarginine deiminase [25•].

## Phagosome maturation

Phagosome maturation in neutrophils is mediated by fusion with secondary and primary granules, which can occur at forming phagocytic cups or with sealed phagosomes [26] (Figure 1). The efficacy of this response impacts antimicrobial activity, but can also cause tissue

**Table 1**

**Antimicrobial proteins of neutrophil primary and secondary granules**

| | |
|---|---|
| Primary granules | |
| Elastase | Serine protease |
| Cathepsin G | Serine protease |
| Proteinase 3 | Serine protease |
| Proteinase 4 | Serine protease |
| α-defensins | Cationic antimicrobial peptides |
| Bactericidal/permeability-increasing protein | Binds lipopolysaccharide |
| Myeloperoxidase | Reactive oxygen species |
| Secondary granules | |
| Lysozyme | Peptidoglycan degradation |
| hCAP18 | LL-37 cationic antimicrobial peptide precursor |
| Calprotectin | Metal sequestration |
| Lactoferrin | Metal sequestration |
| Lipocalin-2 | Metal sequestration |
| $gp91_{phox}/p22_{phox}$ | Reactive oxygen species |

damage if granules are instead exocytosed at the plasma membrane. Primary and secondary granule components work cooperatively for optimal microbe killing, using proteases, antimicrobial peptides and proteins, oxidant generators (see below), and nutrient scavengers (Table 1) [27,28]. However, the signals mediating mobilization of these granules are distinct. For instance, Src family kinase signaling is required for fusion of primary granules but not secondary granules with phagosomes, and a strong intracellular $Ca^{2+}$ flux mediates primary granule fusion with the phagocytic cup [26]. The proteins that regulate fusion of these two granule subsets also differ. SNARE complex protein VAMP-8 is important for fusion of primary granules with phagosomes, enabled by Munc13-4, whereas secondary granules use SNAP-23 [29]. The Rab GTPases that regulate phagosome-granule fusion are currently undefined but do not include Rab27a, which is critical for granule exocytosis.

Despite our limited understanding of how neutrophil phagosome maturation is regulated, a subset of pathogens manipulate this process as part of their virulence strategy. In some cases, blockade of phagosome maturation requires live microbes. Most phagosomes containing live *Filifactor alocis* appear to exclude primary and secondary granules, whereas compartments containing heat-killed bacteria do not [30]. *Yersinia pseudotuberculosis* secretes effectors through its type III secretion system that not only block phagocytosis, but also prevent phagosome-granule fusion [27,29,31]. In contrast, phagosomes containing live *Y. pestis* accumulate CD63 but not elastase, even though both are primary granule proteins [16]; whether granule heterogeneity, phagosome remodeling or some other mechanism accounts for this remains to be determined.

In other cases, phagosome maturation is dictated by the receptors engaged independent of bacterial viability. For example, *N. gonorrhoeae* that express CEACAM3-binding opacity (Opa) proteins reside almost exclusively in phagosomes that have fused with primary and secondary granules [32]. Phagosome maturation can be blocked by masking Opa proteins or inhibiting Src family kinase signaling, and the majority of *N. gonorrhoeae* that switch off Opa expression by phase variation reside in phagosomes that accumulate secondary but not primary granule proteins, regardless of bacterial viability at the start of infection [33]. Thus, for unopsonized *N. gonorrhoeae*, phase-variation of Opa proteins to the OFF state reduces phagocytic killing [32,34], underscoring the ability of mechanism of entry to dictate microbes' fate, as was first demonstrated by comparisons of Fc receptors versus CR3 and the mannose receptor in macrophages [35].

## Oxidative host defense mechanisms

A hallmark of activated neutrophils is production of reactive oxygen species (ROS) that include superoxide anion, hydrogen peroxide ($H_2O_2$), and hypochlorous acid (HOCl) (Table 1). The importance of ROS production to host defense is underscored by the repeated, life-threatening infections that occur in individuals with chronic granulomatous disease. A detailed discussion of the phagocyte NADPH oxidase, regulation of its activity and the chemistry that occurs in the phagosome lumen is described elsewhere in this volume [36] (Figure 1). In brief, in resting neutrophils, NADPH oxidase is disassembled and inactive, with 85% $gp91/p22^{phox}$ heterodimers in the membranes of secondary granules and the remainder in tertiary granules, secretory vesicles and the plasma membrane. Rac2 and a complex of $p47/p67/p40^{phox}$ are in the cytosol. Signaling downstream of phagocytic receptors triggers holoenzyme assembly and activation on forming phagosomes, detectable within 30 s of microbe binding. NADPH oxidase complexes are highly enriched on phagosomes for 20–30 min and then disassemble to terminate the respiratory burst. Phagosomes that fuse with primary granules contain myeloperoxidase (MPO), which converts NADPH oxidase-derived $H_2O_2$ into HOCl.

Pathogens such as *Francisella tularensis*, *H. pylori*, and *N. gonorrhoeae* use diverse strategies to modulate neutrophil oxidant production that include disrupting NADPH oxidase assembly at the phagosome via effects on one or more subunits, inhibiting the activity of the assembled holoenzyme, or diverting the enzyme to other subcellular sites, such as the plasma membrane [37,38]. While these strategies may enhance a pathogen's survival inside neutrophils, they can also be exploited to exacerbate tissue damage as a means of nutrient acquisition, as posited for *H. pylori* [37]. The first description of a pathogen targeting MPO was recently described: intraphagosomal *S. aureus* secretes SPIN (staphylococcal inhibitor of

myeloperoxidase), which blocks the MPO active site to ensure that some organisms escape oxidative killing [39].

Signals that control the duration of NADPH oxidase assembly at the phagosome were recently identified. The PX domain of $p40^{phox}$ binds phosphatidylinositol 3-phosphate (PI3P) on the phagosomal membrane. When PI3P levels fall due to changes in kinase/phosphatase balance, $p40^{phox}$ dissociates and superoxide production stops [40•]. In contrast, $p40^{phox}$ appears to be dispensable for NADPH oxidase assembly or activity at the plasma membrane [41]. The importance of PI3P to phagosome oxidant production is revealed by the *P. aeruginosa* type III secretion system effector ExoS, which ADP-ribosylates Ras to inhibit activation of PI 3-kinase and production of PI3P, leading to impairment of $p47^{phox}$ phosphorylation and phagosome retention of $p40^{phox}$ [42].

## Manipulation of neutrophil lifespan and cell death mechanisms

Neutrophils are programmed to undergo apoptosis ~24 hours after release into circulation, which is regulated by global changes in transcription [43]. Intracellular pathogens such as *Anaplasma phagocytophilum*, *Chlamydia pneumoniae* and *F. tularensis* significantly delay neutrophil apoptosis to sustain viability of their replicative niche. *F. tularensis* extends neutrophil lifespan by upregulating a subset of prosurvival regulatory factors, stabilizing mitochondria, and inhibiting caspases and calpains [44]. Contributing to this response are *F. tularensis* bacterial lipoproteins (BLPs), which act via TLR2/1 [45]. BLPs may function as surface-associated or secreted virulence factors [46], but their effect on apoptosis depends on the pathogen, as *Mycobacterium tuberculosis* (Mtb) BLPs are cytotoxic for neutrophils [47].

In contrast, extracellular pathogens such as *S. aureus* and *S. pyogenes* induce rapid neutrophil lysis to release intracellular viable bacteria [48]. Lysis by *S. aureus* is distinct from necroptosis as it requires RIPK3 activity, but not RIPK1, MLKL or TNFα [49]. Cell death is also associated with serine protease-mediated processing and secretion of pro-IL-1β that is inflammasome-independent [50]. These results underscore key differences in mechanisms of cytokine secretion by neutrophils and macrophages; it remains to be determined if *S. aureus*-induced lysis is a new mechanism of programmed necrosis.

An interesting interplay between cell death pathways is demonstrated by Mtb, which replicates in macrophages but triggers lytic death of neutrophils [51•]. Neutrophil necrosis requires MPO and the Mtb Esx-1 type VII secretion system. Extracellular live bacteria along with necrotic neutrophil debris are then phagocytosed by macrophages and avoid delivery to lysosomes. Mtb exhibit enhanced intracellular replication, and ultimately induce macrophage necrosis. In contrast, apoptotic neutrophils containing mutant Mtb are phagocytosed by macrophages and destroyed in phagolysosomes. These findings underscore that the mechanisms of cell death in neutrophils are complex and are manipulated by pathogens in intricate ways to enhance virulence.

## Conclusions and future outlook

Neutrophils have evolved to maximize the antimicrobial activity of their nascent phagosomes, while limiting the potential for collateral damage by spatially separating effectors and regulators and having a limited lifespan. Pathogens manipulating these processes can be used as tools to uncover the underlying mechanisms.

A major unresolved question in neutrophil biology is what explains the heterogeneity of response to pathogens. Even within a single cell, phagosome maturation and intraphagosomal oxidant accumulation vary [33,52–55], impacting the fraction of pathogens that survive intracellularly and sustain infection [39]. Overturning the longstanding assumption that neutrophils are a uniform population, neutrophils in fact exhibit functional and phenotypic variability. Neutrophil heterogeneity has been best described in cancer and inflammation, with subsets including 'N1' proinflammatory, antimicrobial cells and 'N2' pro-resolving, cancer-promoting cells [4,56,57]. In the context of microbial pathogenesis, mature human neutrophils infected *in vitro* with *H. pylori* differentiate into proinflammatory, cytotoxic 'N1-like' cells and exhibit profound nuclear hypersegmentation [58••]. Skin lesions of patients infected with *Leishmania braziliensis* parasites contain low density neutrophils that have normal, segmented nuclei, and are not immunosuppressive, but are positive for HLA-DR and costimulatory molecules [59], suggesting a potential for antigen presentation akin to neutrophil-DC hybrids [56]. Finally, genetically inherited differences affect neutrophil responses to infectious and inflammatory conditions. For example, a single nucleotide polymorphism in human *TLR1* changes TLR1 surface levels, thereby altering TLR2/1 heterodimer-dependent neutrophil responsiveness to *F. tularensis* BLPs [45]. Currently, the spectrum of neutrophil phenotypes in infectious conditions remain elusive. By integrating this capacity for neutrophil variation into experimental design and interpretation, we anticipate important nuances in the host-pathogen interface will be discovered as a result.

## Conflict of interest statement

Nothing declared.

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## Further reading


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