



# HHS Public Access

Author manuscript

*Immunol Rev.* Author manuscript; available in PMC 2024 March 01.

Published in final edited form as:

*Immunol Rev.* 2023 March ; 314(1): 210–228. doi:10.1111/imr.13163.

## Microbes and the Fate of Neutrophils

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### Summary

Neutrophils or polymorphonuclear neutrophils (PMNs) are an important component of innate host defense. These phagocytic leukocytes are recruited to infected tissues and kill invading microbes. There are several general characteristics of neutrophils that make them highly effective as antimicrobial cells. First, there is tremendous daily production and turnover of granulocytes in healthy adults—typically  $10^{11}$  per day. The vast majority (~95%) of these cells are neutrophils. In addition, neutrophils are mobilized rapidly in response to chemotactic factors and are among the first leukocytes recruited to infected tissues. Most notably, neutrophils contain and/or produce an abundance of antimicrobial molecules. Many of these antimicrobial molecules are toxic to host cells and can destroy host tissues. Thus, neutrophil activation and turnover are highly regulated processes. To that end, aged neutrophils undergo apoptosis constitutively, a process that contains antimicrobial function and proinflammatory capacity. Importantly, apoptosis facilitates nonphlogistic turnover of neutrophils and removal by macrophages. This homeostatic process is altered by interaction with microbes and their products, as well as host proinflammatory molecules. Microbial pathogens can delay neutrophil apoptosis, accelerate apoptosis following phagocytosis, or cause neutrophil cytolysis. Here, we review these processes and provide perspective on recent studies that have potential to impact this paradigm.

### Keywords

Bacteria; cytolysis; neutrophil turnover; phagocytosis; apoptosis; granulopoiesis

## 1 | INTRODUCTION

### 1.1. | Neutrophil development and lifespan

**1.1.1 | Development**—Neutrophils are the most abundant white blood cell in mammals and play an essential role in host defense against the multitude of pathogens encountered throughout life.<sup>1</sup> On the other hand, neutrophils have a very short lifespan in circulation, so they must be replaced in large numbers on a daily basis ( $\sim 10^{11}$ /day) in order to perform

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#### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

their host defense surveillance function. Neutrophils are produced in the bone marrow by a process known as granulopoiesis, and ~55-60% of the bone marrow is dedicated to the production of this one cell type.<sup>2</sup> Granulopoiesis involves a series of stages originating from common myeloid progenitor cells (CMPs) and is principally regulated by granulocyte colony stimulating factor (G-CSF).<sup>3-5</sup> CMPs differentiate into granulocyte-macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs), which in turn give rise to mature myeloid cells and platelets or red blood cells. Stimulation of GMPs with granulocyte macrophage colony-stimulating factor (GM-CSF) leads to the production of monoblasts and myeloblasts, which are unipotent stem cells. Myeloblasts differentiate into promyelocytes, which then are committed to form neutrophils. Promyelocytes are similar in shape to myeloblasts and have a round nucleus; however, they contain many cytoplasmic azurophil granules that contain myeloperoxidase. In addition, promyelocytes have microperoxisomes, which contain catalase and suggests that even at this early stage in development neutrophils express protective antioxidant enzymes.<sup>6</sup> These cells are classified as blast cells and can still actively divide. Promyelocytes give rise to myelocytes, which are smaller than promyelocytes and have oval nuclei that are localized eccentrically to one side of the cell. Myelocytes reside in the bone marrow and are characterized by the appearance of secondary or specific granules containing lactoferrin. Myelocytes are also considered blast cells and continue to divide and contribute to the neutrophil mitotic pool. Myelocyte mitosis dilutes the azurophil granule contents in each daughter cell, and specific granules eventually become more numerous than the azurophil granules. Precursors at the next stage are known as metamyelocytes and are no longer able to undergo mitosis—they can only develop into mature neutrophils. Metamyelocytes have kidney bean-shaped nuclei and contain ~67% specific granules and 33% azurophil granules. These cells are much smaller than myelocytes and eventually develop into band cells, which are the penultimate immature neutrophil form. Band cells are smaller than metamyelocytes and contain eccentric curved nuclei that are not lobular and have the same distribution of azurophil and specific granules as metamyelocytes, as well as a third granule type known as gelatinase granules. Band cells can now enter the bloodstream, and approximately 2-6% of cells in circulation are bands cells, although their circulating numbers can increase significantly during severe infection or inflammatory events in a process known as bandemia. Interestingly, bandemia has been correlated with an increasing likelihood of bacteremia and in-hospital mortality.<sup>7,8</sup> The final stage of neutrophil development is maturation into segmented cells or polymorphonuclear neutrophils (PMNs or neutrophils). PMNs are characterized by segmented nuclei connected by thin strands of chromatin. While PMN nuclei normally have 3-5 lobes, neutrophil hypersegmentation has been observed where 6 or more segments are present and is one of the earliest signs of megaloblastic anemia.<sup>9</sup> On the other hand, PMN hypersegmentation can also be seen under other situations that are not as well understood.<sup>10,11</sup> Throughout the course of granulopoiesis, additional changes in surface marker expression, cytoskeletal elements, and granule contents also occur. For example, *N*-formyl peptide receptors are not expressed on myeloblasts but gradually appear as these cells mature through later stages of development.<sup>12</sup> Mature PMNs contain secretory vesicles in addition to the three granule types found in band cells. Secretory vesicles are regulated exocytic vesicles similar to granules but contain plasma proteins and membrane receptors, and it has been suggested that they are formed in mature PMNs by endocytosis.<sup>13</sup> As expression of granule proteins

changes during maturation from promyelocytes to band cells, the granules formed at each stage also seem to acquire different cargo, which may account for the different subsets of granules that are observed.<sup>14</sup> Note that new stores of microbicidal components are not resynthesized in PMNs, and new granules do not form in mature cells, which is consistent with a terminally differentiated end cell that has a short lifespan. That said, circulating neutrophils retain some capacity for gene expression and protein synthesis, which is needed to maintain functional capacity for extended periods (hours) and facilitate apoptosis and turnover.<sup>15</sup>

Bone marrow contains a large reserve of PMNs, and their rate of release is a major determinant of the number of circulating neutrophils present. PMNs must migrate across the sinusoidal endothelium that separates the hematopoietic compartment from the circulation, and migration of PMNs, as well as egress of some band cells, appears to be regulated by the relative levels of activation or egress signals versus retention signals. For example, PMN CXCR2 activation promotes mobilization of PMNs into the circulation, whereas interaction of CXCL12 (a.k.a. stromal cell-derived factor 1, SDF-1) with PMN CXCR4 functions to retain band cells and PMNs in the bone marrow.<sup>16</sup> Thus, the CXCR4 axis is considered to play a key role in PMN egress, and it has been proposed that CXCR4 upregulation on senescent PMNs leads to preferential homing of these cells back to the bone marrow where they are cleared from circulation by bone marrow stromal macrophages.<sup>17,18</sup>

The relatively short PMN lifespan explains the need for high steady-state production of these cells by the bone marrow. In healthy adults, neutrophil development time in the bone marrow prior to entering circulation (i.e., maturation from myeloblast to PMN) is estimated to be 5-8 days. Subsequently, PMNs circulate in the bloodstream for less than a day, with a circulating PMN half-life ranging from 6-19 hours, depending on how the analysis was performed.<sup>19</sup> Circulating PMNs function as sentinels to detect sites of infection or tissue damage and are among the first-responder cells that are rapidly recruited to these sites, where they utilize an array of oxygen-dependent and oxygen-independent mechanisms to protect and defend the host.<sup>20,21</sup> The circulating PMN surveillance function is aided by their ability to marginate and roll along the vessel walls<sup>3</sup>, and it has been estimated that ~49% of the PMN circulating pool is freely flowing in the blood, whereas the remaining 51% is in the marginated pool, which can be readily mobilized into the bloodstream if necessary for recruitment to other sites in the body for host defense.<sup>22</sup>

**1.1.2 | Apoptosis and turnover**—Since innate host responses are not specific to individual pathogens, host tissue can be damaged along with insulting microorganisms and, if chronic in nature, contribute to the pathogenesis associated with inflammatory diseases. Thus, PMNs are programmed for rapid cell death by constitutive or spontaneous apoptosis in an effort to prevent excessive inflammation and disease.<sup>23,24</sup> This is the default fate of all neutrophils. Moreover, PMNs are programmed to aid in the resolution of inflammation and thus produce proresolving mediators relatively soon after they initiate the inflammatory response.<sup>25</sup> Indeed, insufficient activation of proresolving mechanisms contributes to an excessive or prolonged inflammatory response to the initial insult.<sup>26-28</sup> These observations support the concept that the innate immune system has developed to not only protect the

host from infection or damaging insult, but also to confine this response and resolve it as soon as possible and thereby avoid collateral host tissue damage.<sup>26-28</sup>

The circulating pool of PMNs is freely able to move through tissues in their role as sentinels, and it has been estimated that PMNs migrating into tissues under homeostatic conditions may reside there for 2-3 days before they undergo apoptosis and are removed by the reticuloendothelial system.<sup>29</sup> Although apoptosis and clearance of apoptotic cells was proposed by Kerr et al., the removal process for neutrophils was characterized by Newman et al., who demonstrated that aged or senescent neutrophils are ingested by inflammatory macrophages.<sup>30,31</sup> Subsequent studies by Savill et al. reported that aged human neutrophils undergo apoptosis within 24 h in culture and are then phagocytosed by macrophages.<sup>23</sup> These two landmark studies served as the springboard for many lines of investigation over the next three decades that have resulted in our current understanding of this essential homeostatic process. Healthy cells have receptors that signal “don’t eat me”.<sup>32</sup> By comparison, phagocytosis of apoptotic neutrophils involves ligation of receptors that facilitate the nonphlogistic recognition and uptake by macrophages and other mononuclear phagocytes.<sup>32</sup> This process is known as efferocytosis (Fig. 1).<sup>32</sup> Consistent with the non-inflammatory removal process, neutrophil proinflammatory and antimicrobial capacities decrease concomitant with apoptosis.<sup>24</sup> For example, Whyte and colleagues found that human PMNs undergoing apoptosis had reduced responses to receptor-mediated stimuli, and decreased capacity for chemotaxis, degranulation, and phagocytosis.<sup>24</sup> Kobayashi et al. verified this decreased function and extended the findings to include decreased proinflammatory capacity at the level of the neutrophil transcriptome.<sup>33,34</sup> These processes work in concert to promote safe removal of ~10<sup>11</sup> effete neutrophils on a daily basis. For more detail on specific mechanisms of apoptosis and efferocytosis, we refer the reader to reviews on these topics.<sup>32,35</sup> It is noteworthy that the non-inflammatory removal of effete neutrophils by apoptosis contrasts sharply with the proinflammatory outcomes that are caused by neutrophil cytolysis.

In the absence of infection or injury, which results in the focused migration of PMNs to sites of infection or inflammation and an extended lifespan, PMNs exit the circulating/marginating pool and enter the liver, spleen, and bone marrow in approximately equal proportions where they undergo spontaneous apoptosis and are removed by macrophage efferocytosis (Fig. 1).<sup>36</sup> Note that recognition and ingestion of apoptotic neutrophils by macrophages also induces macrophage G-CSF generation, which in turn stimulates granulopoiesis to replenish the pool of neutrophils.<sup>3,18</sup> Thus, return of senescent PMNs to the bone marrow (and ultimate removal) is an essential process to maintain a robust pool of fresh PMNs and is facilitated by the upregulation of CXCR4 on these senescent cells that enhances homing to the bone marrow. Under homeostatic conditions, turnover rates from one body compartment to the next seem to be relatively stable. However, under acute inflammatory conditions or infections, PMNs are activated and rapidly mobilized from the blood compartment by chemoattractants, such as interleukin 8 (CXCL8), leukotriene B<sub>4</sub> (LTB<sub>4</sub>), platelet-activating factor (PAF), and N-formyl peptide, which are generated at sites of infection/inflammation.<sup>1</sup> This process may result in transient neutropenia that triggers an associated increase in release of PMNs and expansion of the circulating PMN pool or even bacteremia in cases of severe infection to supplement the increased tissue demand

until the inflammation/infection is controlled.<sup>7,8</sup> The mechanisms controlling inflammation-associated circulating neutrophilia are not well understood; although acute mobilization of PMNs from the bone marrow seems to require the coordinated actions of G-CSF and CXC chemokines, with disruption of the CXCR4-SDF-1 retention mechanism and CXC chemokines stimulating neutrophil migration from the bone marrow.<sup>17,18,37</sup> PMN priming is also associated with delayed apoptosis and enhanced lifespan, and there are a number of factors produced during the inflammatory response that can extend a PMN's lifespan, including cytokines (e.g., G-CSF, GM-CSF), damage-associated molecular patterns (DAMPs), and pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS).<sup>38</sup> Subsequent to the resolution of inflammation, which is facilitated by recruited PMNs themselves as well as the process of efferocytosis, activated neutrophils recruited to address the insult and clean up the associated tissue damage undergo apoptotic or necrotic cell death and are removed, whereas bone marrow PMN production returns to homeostasis.<sup>25,39,40</sup>

### 1.2 | Neutrophil microbicidal mechanisms

Neutrophils utilize an extensive array of microbicidal mechanisms to destroy and remove infectious agents, and these mechanisms effectively utilize the numerous granule-packaged products developed during granulopoiesis. Neutrophil microbicidal mechanisms have been reviewed extensively (e.g., see refs.<sup>1,41,42</sup>) and are only briefly outlined here.

PMNs generate microbicidal reactive oxygen species (ROS) through the activation of a multi-protein enzyme complex known as the NADPH oxidase.<sup>43</sup> The NADPH oxidase is expressed in neutrophils, as well as all other granulocytes, and plays an essential role in host defense against pathogenic microorganisms.<sup>43-45</sup> It is generally accepted that the core NADPH oxidase enzyme complex is composed of five proteins (p22<sup>phox</sup>, p40<sup>phox</sup>, p67<sup>phox</sup>, and gp91<sup>phox</sup>) and a small GTPase (Rac1/2).<sup>43,46-49</sup> Current nomenclature for the phagocyte NADPH oxidase proteins uses the suffix *phox*, which refers to *phagocyte oxidase*. The one exception is gp91<sup>phox</sup>, which is also known as NOX2. In resting cells, these proteins are segregated from each other in specific granule and secretory granule membranes, as well as cytosolic compartments, to protect the host from untimely oxidase activation and the production of toxic ROS.<sup>50-52</sup> The neutrophil NADPH oxidase is activated in response to a wide range of stimuli, including bacteria, fungi, protozoan parasites, viruses, soluble proinflammatory molecules, and other foreign antigens. Depending on the stimulus, NADPH oxidase assembly and activation can occur at the plasma membrane (e.g., activation by soluble stimuli such as N-formyl peptides following priming) or in the phagosomal membrane (e.g., activation following phagocytosis of a pathogen or foreign antigen) (Fig. 2).<sup>53,54</sup> Activation of the NADPH oxidase ultimately results in the formation of extracellular or intraphagosomal superoxide anion (O<sub>2</sub><sup>•-</sup>), a process known as the respiratory burst. Although high concentrations of O<sub>2</sub><sup>•-</sup> can accumulate in phagosomes where the pH is low and can be directly toxic to some pathogens by virtue of its ability to oxidize iron-sulfur clusters present in bacterial enzymes, it is generally thought that O<sub>2</sub><sup>•-</sup> represents the precursor to more powerful ROS that play more prominent roles in pathogen killing.<sup>44</sup> The initial production of O<sub>2</sub><sup>•-</sup> is absolutely essential to human health, as demonstrated by chronic granulomatous disease (CGD), which is a rare immunodeficiency resulting from

NADPH oxidase defects. Individuals with CGD are susceptible to severe, recurrent bacterial and fungal infections, as well as hyperinflammation.<sup>55-57</sup> At physiologic pH,  $O_2^{\bullet-}$  is rapidly converted to hydrogen peroxide ( $H_2O_2$ ) through spontaneous or enzymatic dismutation by superoxide dismutase (SOD). SOD is abundant in PMNs and plays an essential role in protecting cells and tissues from excessive ROS, as do catalase and glutathione peroxidase. In addition to the production of ROS, PMN activation also results in the release of cytoplasmic granule contents into the phagosome, including high concentrations of MPO delivered from azurophil granules, which catalyzes the production of potent microbicidal ROS, such as hypochlorous acid (HOCl).<sup>44</sup> Additional ROS and reactive nitrogen species (RNS) are also generated during the inflammatory response and are important in host defense, as well as in the pathogenesis of various inflammatory diseases.<sup>44,58</sup>

Although significantly diminished in capacity, CGD neutrophils are still able to kill a variety of microorganisms through the application of an array of oxygen-independent microbicidal antimicrobial peptides and enzymes that developed and were packaged into granules during granulopoiesis.<sup>4,55</sup> In addition to their high levels of MPO, neutrophil azurophil granules contain four neutral proteinases (cathepsin G, elastase, NSP4, and proteinase 3), and to date all but NSP4 have demonstrated antimicrobial activity. Lysozyme is present in both the azurophil and specific granules and cleaves bacterial wall peptidoglycan and can participate with complement in bacterial permeabilization. Azurophil granules also contain several microbicidal cationic proteins, including bactericidal/permeability-increasing protein (BPI) and the  $\alpha$ -defensins, which can permeabilize and kill a range of Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses. Specific granules store unsaturated lactoferrin and lipocalin-2 to sequester iron and limit bacterial growth, transcobalamin II to sequesters cyanocobalamin from bacteria, lysozyme, collagenase, phospholipase A<sub>2</sub>, and hCAP-18, which is cleaved by elastase to generate bactericidal LL-37. Additionally, neutrophil specific granules contain a number of membrane proteins that are transported to the plasma or phagosomal membranes upon neutrophil activation. Altogether, it is clear that PMNs can bring to bear a powerful array of microbicidal mechanisms during an inflammatory response, yet these same mechanisms have the potential to cause significant host damage and must be contained or rapidly removed to maintain health of the host.<sup>42,52,59</sup>

## 2 | MODULATION OF APOPTOSIS BY MICROORGANISMS

Neutrophil apoptosis is not an immutable process and can be delayed to promote enhanced clearance of microbial pathogens or induced/accelerated to facilitate turnover and resolution of infection and inflammation (Fig. 1). The ability of neutrophils to decipher and respond to environmental signals is paramount for their success as professional phagocytes. As PMNs are recruited to sites of infection, many of the signals they receive function as priming stimuli (reviewed in ref.<sup>60,61</sup>). Neutrophil priming agents—including many proinflammatory molecules—enhance microbicidal capacity and in general delay apoptosis. Although the delay of PMN apoptosis can be beneficial to the host by enabling increased bactericidal activity, the potential neutrophil accumulation comes with the risk of enhanced inflammatory capacity and damage to host tissue. Acute and chronic inflammatory disorders are often associated with excessive neutrophil accumulation. The infection site inflammatory milieu is complex, and neutrophils must decipher a multitude of signals from both host

and bacteria-derived molecules that can be pro-survival or pro-death. In addition, bacterial pathogens can delay neutrophil apoptosis following uptake to facilitate intracellular survival or accelerate the process to evade PMN killing.

## 2.1 | Inflammatory molecules that promote neutrophil survival

**2.1.1 | Cytokines, chemokines and proinflammatory molecules—**Neutrophils receive a multitude of signals to indicate the presence of bacterial pathogens and to recruit them to sites of infection. Host immune cells in inflamed tissue produce proinflammatory mediators that generally operate as neutrophil priming agents and are known to delay PMN apoptosis. Begley et al. reported that neutrophil survival is enhanced by culture with GM-CSF and G-CSF.<sup>62</sup> This observation was followed by studies showing that these proinflammatory cytokines, in addition to interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor (TNF), IL-6, interferon- $\gamma$  (IFN- $\gamma$ ) and complement factor 5a (C5a), extend neutrophil survival by inhibiting programmed cell death / apoptosis.<sup>38,63</sup> Indeed, it is now known that numerous proinflammatory molecules, including IL-8, IFN- $\alpha$ , GRO $\alpha$ , PAF, fMLF, leptin, and LTB<sub>4</sub> can delay neutrophil apoptosis.<sup>64-69</sup> Interestingly, the effects of TNF $\alpha$ , an important PMN priming agent, are antiapoptotic at low concentrations (0.1 ng/mL) whereas high doses (10 ng/mL) result in induction of apoptosis.<sup>70</sup> Neutrophil priming with TNF elicits multiple signaling pathways including p38, extracellular signal-regulated kinase (ERK) 1/2, phosphoinositide-3-kinase (PI3K) and nuclear factor- $\kappa$ B (NF- $\kappa$ B).<sup>71</sup> High concentrations of TNF $\alpha$  trigger caspase-dependent acceleration of myeloid cell leukemia-1 (Mcl-1) turnover, whereas low concentrations stimulate expression of the antiapoptotic molecule Bfl-1.<sup>72</sup>

GM-CSF and other proinflammatory mediators delay neutrophil apoptosis largely by stabilizing Mcl-1, an anti-apoptotic member of the Bcl-2 family.<sup>73</sup> Mcl-1 has a short half-life and is targeted rapidly by proteasomal degradation.<sup>74</sup> Mcl-1 activity is sustained through activation of signaling pathways that involve PI3K/Akt and ERK.<sup>75</sup> GM-CSF treatment results in enhanced expression of Mcl-1 in addition to the anti-apoptotic Bcl-2 family members Bcl-X<sub>L</sub> and Bcl2A1.<sup>76</sup> Of note, bronchiole alveolar lavage (BAL) neutrophils from patients with neutrophilic inflammatory lung disease demonstrate reduced intracellular levels of pro-apoptotic Bax and accompanying delayed neutrophil apoptosis compared to blood neutrophils from the same patients.<sup>77</sup> Moreover, the BAL cells from patients released large amounts of G-CSF and GM-CSF compared to control individuals.

### 2.1.2 | Host molecules and environmental factors at infection sites—

Neutrophil trafficking from circulation to sites of infection involves signaling through a chemotactic gradient provided by proinflammatory cytokines and bacterial molecules. Interaction of neutrophil adhesins with cellular ligands can modulate apoptosis. For example, interaction of the neutrophil integrin  $\alpha_9\beta_1$  with endothelial cell molecule VCAM-1 delays both spontaneous and Fas-induced apoptosis.<sup>78</sup> In addition, engagement of  $\beta_2$  integrins on the neutrophil surface delays spontaneous apoptosis and involves activation of survival pathways through Akt and MAPK-ERK.<sup>79,80</sup> Neutrophil apoptosis is also delayed by ligation of soluble fibrinogen to CD11b by activation of NF- $\kappa$ B through ERK1/2-dependent signaling.<sup>81</sup> Marginated neutrophils are exposed to a diversity of new

stimuli in the inflammatory milieu at sites of infection, many of which delay apoptosis. Low oxygen tension is encountered in inflamed tissue and this hypoxic environment is well appreciated to delay neutrophil apoptosis and thereby extend survival.<sup>82,83</sup> Neutrophil apoptosis is also delayed by mild extracellular acidosis.<sup>84</sup> In addition, there are several reports that describe the anti-apoptotic effects of potentially neutrophil derived molecules such as heme, ATP, myeloperoxidase, and lactoferrin.<sup>85-88</sup>

## 2.2 | Bacterial molecules that promote survival or extend the PMN lifespan

Neutrophils detect the presence of bacterial pathogens at least in part through ligation of surface receptors to microbial shed and secreted molecules. Several of these bacterial-derived components, such as LPS, lipoteichoic acid (LTA), peptidoglycan, CpG DNA and flagellin, have been reported to delay neutrophil apoptosis.<sup>89</sup> The effects of LPS on neutrophil biology have been studied extensively, and endotoxin is classically described to be a potent neutrophil priming agent.<sup>90</sup> LPS is recognized by a TLR4/MD2/CD14 complex, and LPS stimulation of neutrophils contributes to a delay in spontaneous apoptosis through a process involving NF- $\kappa$ B and PI3K activation.<sup>91-93</sup> In addition, several bacterial toxins have been reported to delay neutrophil apoptosis, including *S. epidermidis* phenol soluble modulins (PSM), staphylococcal enterotoxins (A, B) and toxic shock syndrome toxin, *E. coli* O157:H7 verotoxin 2, *E. coli* Shiga toxin, and *H. pylori* water soluble surface protein.<sup>94-98</sup>

## 2.3 | Influence of microorganisms on PMN fate

**2.3.1 | Bacteria that delay neutrophil apoptosis**—The neutrophil *raison d'être* is to recognize and eliminate microbial pathogens by phagocytosis. The ability of inflammatory mediators and bacterial-derived products to enhance this process through extending neutrophil survival is critical to maximize host defense. On the other hand, the induction of neutrophil apoptosis following phagocytosis of microbes accounts for safe removal of effete PMNs through efferocytosis and is essential for resolution of infection and inflammation (Fig. 1). Although different types of microbial pathogens (e.g., bacteria, fungi, protozoa, and viruses) can influence PMN apoptosis directly or indirectly, for simplicity our discussion is focused primarily on bacterial pathogens.

The relatively short neutrophil half-life is not conducive to long-term survival strategies employed by many intracellular pathogens. Notwithstanding, a limited number of bacterial pathogens have been shown conclusively to delay neutrophil apoptosis as a mechanism of replication and survival. *Anaplasma phagocytophilum*, the etiologic agent of human granulocytic anaplasmosis, was the first bacterial pathogen reported to delay PMN apoptosis.<sup>99</sup> *A. phagocytophilum* is an obligate intracellular bacterium and is uniquely tropic for granulocytes, where it multiplies strictly within membrane-bound inclusions.<sup>100,101</sup> In addition to *A. phagocytophilum*, several *Chlamydia* spp., including *C. pneumoniae*, *C. trachomatis*, and *C. psittaci*, have been reported to delay PMN apoptosis.<sup>102-104</sup> Mechanisms underlying the delay in neutrophil apoptosis caused by *A. phagocytophilum* and *C. pneumoniae* are similar, and include increased phosphorylation of p38 mitogen-activated protein kinase (MAPK), activation of PI3K/Akt, and maintained expression of the antiapoptotic protein Mcl-1.<sup>105,106</sup> *Coxiella burnetii*—the causative agent of the zoonotic disease Q fever in humans—also delays neutrophil apoptosis through stabilization of



Mcl-1.<sup>107</sup> Likewise, the facultative intracellular pathogen *F. tularensis* delays neutrophil apoptosis in a process specifically dependent on the activity of NF- $\kappa$ B, PI3K $\alpha$ , and p38 MAPK.<sup>108,109</sup> Several other important bacterial pathogens, including *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Klebsiella pneumoniae*, *Treponema pallidum*, and *Mycobacterium tuberculosis*, have also been reported delay neutrophil apoptosis.<sup>110-116</sup>

Some of the bacterial pathogens that prolong neutrophil survival either inhibit NADPH oxidase assembly and/or activation or have mechanisms to evade ROS-mediated killing. For example, intracellular *A. phagocytophilum* elicit negligible production of PMN ROS, which is consistent with reports that the bacterium interferes with assembly and activation of the NADPH oxidase in the inclusion membrane.<sup>117-120</sup> *C. burnetii* disrupts assembly of the NADPH oxidase and produces an acid phosphatase that prevents translocation of p47<sup>phox</sup> to the phagosome membrane.<sup>121,122</sup> *F. tularensis* disrupts the NADPH oxidase by multiple mechanisms including inhibition of oxidase assembly on the phagosome and diminished phosphorylation of p47<sup>phox</sup> and other PKC substrates.<sup>123,124</sup> Thus, inhibition of the NADPH oxidase is a mechanism used by bacterial pathogens to subvert neutrophil microbicidal activity and thereby facilitate intracellular survival. These findings are compatible with multiple lines of evidence that suggest ROS are important in promoting phagocytosis-induced cell death (PICD).<sup>89</sup>

**2.3.2 | Phagocytosis-induced cell death (PICD)**—Although neutrophils possess significant microbicidal capacity and kill most ingested microbes, at some point this capacity can be spent fully, and effete neutrophils must be removed safely. To that end, neutrophils can undergo PICD, a form of apoptosis dependent on production of NADPH oxidase-derived ROS. Watson et al. discovered that neutrophils undergo rapid apoptosis following phagocytosis of *Escherichia coli*.<sup>125</sup> Induction of apoptosis in these studies was dependent on bacteria-to-neutrophil ratios of at least 10:1, was dose-dependent, and the level of neutrophil ROS produced correlated with apoptosis. Notably, apoptotic neutrophils underwent necrotic lysis after 24 h, findings consistent with the default outcome for these terminally differentiated cells in a closed system.<sup>125</sup> Studies published later that year by Coxon et al. found that phagocytosis-induced apoptosis, later termed neutrophil PICD, requires CD11b/CD18 and NADPH oxidase-derived ROS.<sup>126</sup> Subsequent work by that research group revealed PICD involves caspases 3 and 8, and the process could be modulated (enhanced or suppressed) by TNF or GM-CSF.<sup>127</sup> Gamberale et al. then showed that precipitating immune complexes and antibody coated erythrocytes also triggered apoptosis in an ROS-dependent manner, albeit phagocytosis was not required.<sup>128</sup>

To gain a more comprehensive view of the molecular processes that occur following phagocytosis, we used a microarray-based approach to identify human PMN genes that accompany antibody- and complement-receptor mediated phagocytosis.<sup>15</sup> Unexpectedly, the studies revealed that genes involved in apoptosis and cell fate were differentially expressed several hours after phagocytosis and were accompanied by neutrophil PICD. This led to the hypothesis that PICD is regulated in part at the level of gene expression and facilitates resolution of the inflammatory response.<sup>15</sup> Based on these findings, we proposed that changes in the PMN transcriptome accompanying PICD comprise an apoptosis-differentiation program, a final stage of differentiation that promotes safe turnover of effete

neutrophils.<sup>129</sup> This idea was then extended to phagocytosis of bacterial pathogens.<sup>130</sup> Indeed, phagocytosis of diverse bacterial species induced a common remodeling of the neutrophil transcriptome (the apoptosis-differentiation program) and ultimately PICD.<sup>130</sup> PICD was induced by pathogens that were phagocytosed readily and killed by neutrophils. These findings provide strong support to the idea that the apoptosis-differentiation program and PICD contributes to the resolution of bacterial infection (Fig. 1). However, it is important to note that some bacterial pathogens alter or circumvent this process. For example, the epidemic USA300 strain (*S. aureus*) causes rapid neutrophil cytolysis after phagocytosis (see Section 3).

#### 2.4 | Inflammatory disease and immune disorders

Although delayed neutrophil apoptosis facilitates initiation of host defense against bacterial infection, failure to remove accumulating cells contributes to the pathology of inflammatory disease.<sup>131</sup> For example, bacterial sepsis and sequelae, such as acute respiratory distress syndrome (ARDS), are associated with a profound delay in neutrophil apoptosis.<sup>132-134</sup> Although the mechanisms responsible for the delay remain to be fully elucidated, pro-inflammatory cytokines such as GM-CSF are purportedly involved in sepsis.<sup>135,136</sup> In addition, the pro-survival factor Mcl-1 and regulators, such as myeloid nuclear differentiation antigen (MNDA) and PD-L1, contribute to delay of PMN apoptosis during sepsis.<sup>137-139</sup> Delayed neutrophil apoptosis is also described as a feature of other lung diseases, including chronic obstructive pulmonary disease (COPD), severe asthma, cystic fibrosis, and bronchiectasis.<sup>140-142</sup> Neutrophil apoptosis is also delayed in blood and synovial fluid in patients with rheumatoid arthritis (RA).<sup>143,144</sup> Both blood and synovial fluid from RA patients generally contain elevated levels of pro-inflammatory cytokines such as GM-CSF.<sup>145</sup> In addition, RA blood neutrophils have higher levels of Mcl-1 and the hypoxic synovial environment promotes Mcl-1 expression thus facilitating survival.<sup>143,146</sup> In conjunction with delayed neutrophil apoptosis during inflammatory disease, dysregulated neutrophil clearance contributes to disease. For example, efferocytosis is impaired in ARDS and contributes to lung pathology confounded by accumulation of neutrophils.<sup>147</sup>

### 3 | NEUTROPHIL LYSIS (CYTOLYSIS)

#### 3.1 | Neutrophils and host tissue damage

Each of the aforementioned processes are coordinated to an extraordinary degree to regulate neutrophil homeostasis and turnover. This regulation is especially important during infection and other disease conditions. These processes provide multiple and often redundant mechanisms to prevent damage to host tissues that can be caused by cytotoxic molecules released during neutrophil activation or cytolysis.

##### 3.1.1 | Fundamental concepts for neutrophil-mediated host tissue damage

—The path from quiescent to fully activated neutrophils normally occurs as a graded or stepwise process, whereby chemotactic molecules such as IL-8 or bacterial products including LPS prime neutrophils for enhanced function or activation by subsequent stimuli. These proinflammatory molecules cause mobilization of secretory vesicles and a subset of specific granules but do not typically activate neutrophils to release primary granules (which



radicals, albeit the inhibition is context-dependent.<sup>153-155</sup> Neutrophils also employ redox antioxidant systems that include catalase, SOD, peroxiredoxin, glutathione, and thioredoxin metabolism.<sup>129,156-161</sup> There are also multiple host protease inhibitors, including  $\alpha$ 1-antitrypsin (SERPINA1), leukocyte elastase inhibitor (present in neutrophil cytoplasm and also known as SERPINB1), and  $\alpha$ 2-macroglobulin, which inhibit neutrophil elastase. The importance of  $\alpha$ 1-antitrypsin in protection against host tissue injury by neutrophil elastase is exemplified by genetic deficiency of  $\alpha$ 1-antitrypsin, in which case affected individuals often have severe inflammatory diseases, such as pulmonary emphysema and adult respiratory distress syndrome.<sup>162,163</sup> Outside of genetic deficiency, elastase-mediated tissue injury can occur during inflammatory responses in which protease inhibitors are overwhelmed by neutrophil elastase and/or  $\alpha$ 1-antitrypsin is inactivated by PMN oxidants.<sup>148</sup> Studies by Weiss and colleagues demonstrated that activated neutrophils produce ROS in quantities sufficient to inactivate protease inhibitors in an area surrounding the cell.<sup>164</sup> Thus, a strict balance between active and inactivated elastase is an important component of the inflammatory response.

Notwithstanding the mechanisms that dampen the tissue destructive potential of neutrophils, these host leukocytes are the primary contributors to inflammatory diseases, including those caused by infection. It is important to reiterate that neutrophils are terminally differentiated leukocytes and undergo cytolysis if they are not removed from tissues by other phagocytic cells. In the absence of such removal, neutrophil cytolysis is inevitable, regardless of the cell death pathway (e.g., secondary necrosis after apoptosis, programmed necrosis/necroptosis, non-specific lysis, etc.). Therefore, if the influx of neutrophils exceeds the capacity for nonphlogistic removal by macrophages, they will lyse in tissues, causing host damage and triggering further inflammation. This problem is borne out in numerous inflammatory diseases, and is intriguing that this long-known inflammatory process is now conflated widely with formation of neutrophil extracellular traps (NETs) and the accompanying specialized form of cytolytic neutrophil death termed “NETosis”.

### **3.1.2 | Neutrophil extracellular traps (NETs), NETosis, and cytolysis—**

Brinkmann et al. reported that neutrophils stimulated with IL-8, LPS, or phorbol myristate acetate (PMA) produce extracellular traps.<sup>165</sup> NETs in these studies were comprised of extracellular DNA, histones, and proteins from specific and azurophilic granules. In the original study, the authors demonstrated that NETs ensnare bacteria, including *S. aureus*, *Shigella flexneri*, and *Salmonella typhimurium*, and bacterial virulence molecules were degraded by the associated granule proteases.<sup>165</sup> Brinkmann et al. also proposed the idea that NETs might moderate host tissue damage by containment of neutrophil cytotoxins.<sup>165</sup> This initial report indicated NETs can form within 10 min of activation and in the absence of cell lysis (i.e., with viable neutrophils). However, subsequent work by the same research group showed that NETs form as the cell membrane ruptures.<sup>167</sup> This cell death process was later termed “NETosis”, and at that time was reported as distinct from apoptosis and necrosis. As originally described by Fuchs et al., NETosis requires ROS.<sup>167</sup> In contrast to the rapid formation of NETs (within 10 min) described in the first study by the same research group, *S. aureus* triggered formation of NETs only after an extended period of time *in vitro* (~120 min).<sup>167</sup> Indeed, the authors found that within the first 60 min, neutrophils

killed *S. aureus* following phagocytosis rather than forming NETs.<sup>167</sup> Whether *S. aureus*-induced formation of NETs as reported by Fuchs et al. is distinct from lysis of human neutrophils that occurs following phagocytosis of *S. aureus* is discussed below. Although these two original reports by the same research group (Brinkmann et al. and Fuchs et al.) are seemingly discordant with regard formation of NETs from viable versus dead neutrophils, it is possible they report distinct phenomena. That is, NETs can form as an active process from viable neutrophils<sup>167</sup> or they form as a result of neutrophil cytolysis, which can occur by multiple mechanisms. In keeping with the purpose of this article, which relates to the influence of microbes on neutrophil fate, our discussion here is limited to processes that involve neutrophil lysis. For a much more detailed view of live versus dead neutrophil NET formation, we refer the reader to recent articles by Yousefi et al. and Boltz et al.<sup>168,169</sup>

To gain an enhanced understanding of the stimuli that can trigger formation of NETs, Kenny et al. demonstrated that NETs form after neutrophil exposure to diverse stimuli.<sup>170</sup> These stimuli activate distinct signaling pathways and each caused formation of NETs and concomitant cytolysis.<sup>170</sup> There are also phenotypes associated with known cell death pathways that accompany or are the underlying basis of NET formation. For example, Desai et al. and Schreiber et al. reported programmed necrosis (necroptosis) leads to formation of NETs, whereas subsequent work by Amini et al. reported that NETs can form in the absence of RIPK3 and MLKL signaling (a hallmark of necroptosis).<sup>171-174</sup> There is certainly no paucity of conditions and stimuli that have been linked to NET formation—and many of these reports are at variance with one another. For example, formation of NETs have been reported to be triggered by IL-8, LPS, PMA, calcium and potassium ionophores, bacteria, fungi, viruses, and protozoan parasites.<sup>170,175-177</sup> ROS may or may not be required to trigger NETs, depending on the conditions or stimuli used. Interestingly, studies conducted many decades ago by Min-Fu Tsan demonstrated that neutrophil “autotoxicity” following activation with PMA requires ROS, a finding compatible with recent findings on PMA-induced cell death and NET formation.<sup>178</sup> Studies that report the involvement of (or requirement for) MPO, elastase, and PAD4 for formation of NETs are also at variance.<sup>179-184</sup> However, the unifying phenomenon for these studies is neutrophil cytolysis. Intriguingly, this is the default pathway for these terminally differentiated leukocytes. That is, in the absence of nonphlogistic removal, all neutrophils eventually undergo cytolysis. Indeed, the vast majority of studies report formation of NETs as occurring via neutrophil cytolysis.

Mechanisms of NET formation have been determined primarily by using *in vitro* assays with human neutrophils, and/or with mouse knock-out models. As with most *in vitro* approaches, it is difficult to reproduce conditions in humans, and this includes modeling inflammatory diseases in animal models. In addition, mouse disease models do not necessarily corroborate conditions in human disease. In the context of inflammatory diseases, this is in part due to differences between mouse and human neutrophils. Indeed, there are notable differences in NET formation *in vitro* between mouse and human neutrophils.<sup>185</sup> Study of neutrophil processes, such as formation of NETs is feasible *in vitro*, but difficult or not possible in humans *in vivo*. Extracellular DNA, MPO, elastase, and citrullinated histones are commonly reported as markers of NETs in human tissues. However, the presence of these molecules in tissues does not indicate a specific process or the mechanism by which they were

released. As stated above, MPO, elastase, and other granule constituents are exocytosed onto tissues by fully activated neutrophils. There can be multiple sources of extracellular DNA in inflamed tissues, including DNA released by lysed neutrophils. Thus, it is likely that a significant percentage of studies reporting NETs in inflammatory diseases are in fact reporting components of activated neutrophils and/or simply the remains of dead neutrophils. This notion is borne out in recent studies of severe COVID-19 cases.

Although neutrophils are known to contribute to severe respiratory diseases, the SARS-CoV-2 pandemic prompted much renewed interest in the specific role played by these host phagocytes. This is due largely to the observation that ICU patients with severe COVID-19 associated pneumonia have significantly increased neutrophil counts,<sup>186,187</sup> which in turn led to the idea that neutrophilia observed in the patients with severe COVID-19 might contribute to formation of NETs.<sup>165,188</sup> Indeed, subsequent studies demonstrated an association of COVID-19 severity and presence of NETs or at least components of NETs in the lungs.<sup>189,190</sup> The confounding issue in this recent literature is the synonymous use of “NETosis” and formation of NETs. These are not synonymous terms, and we concur with Yousefi et al. in that such interchangeable use of the terms is inaccurate or misleading.<sup>169</sup> Further, Boltz et al. and Galluzi et al. (and the Nomenclature Committee on Cell Death 2018) suggested the term “NETosis” should be avoided.<sup>168,191</sup>

### 3.2 | Destruction of neutrophils by microbes

There are a relatively limited number of microbes known to kill neutrophils directly. However, if we consider formation of NETs by cytolysis, then by extension many varied microbial species influence killing of neutrophils. This topic is expansive, and a comprehensive discussion on each is beyond the scope of this review. Therefore, our discussion on this topic will focus solely on one model human pathogen that has well-known cytolytic capacity—the bacterium *S. aureus*.

**3.2.1 | *S. aureus* as a model human pathogen—***S. aureus* is among the most frequent causes of bloodstream, skin and soft tissue, and lower respiratory tract infections in much of the world.<sup>192,193</sup> The microbe is carried asymptomatically in the nose by approximately 30% of healthy humans and is a notorious opportunistic pathogen. The high percentage human interaction is compounded by antibiotic resistance and most notably by methicillin-resistant *S. aureus* (MRSA). Healthcare-associated MRSA infections are typical of individuals with predisposing risk factors and treatment is often difficult. In contrast, community-associated MRSA (CA-MRSA) cause disease in otherwise healthy individuals.<sup>194</sup> CA-MRSA emerged in the 1990s and then spread worldwide over the span of a decade. Although there has been a recent decrease in the number of hospital MRSA infections, the level of CA-MRSA infections, the vast majority of which are skin and soft tissue infections, has remained relatively constant.<sup>193</sup> The molecular basis for the high virulence potential and success of CA-MRSA strains such as the USA300 epidemic strain is incompletely defined, although significant progress has been made. It has been well-documented that USA300 produces cytolytic toxins that can kill neutrophils and/or cause lysis of neutrophils after phagocytosis. We suggested previously that the enhanced

ability of USA300 to circumvent killing by neutrophils, which includes neutrophil lysis, is linked to its success as a human pathogen.<sup>195</sup>

**3.2.2 | *S. aureus* cytotoxins and lysis of neutrophils**—*S. aureus* is notorious for the ability to produce a large number of secreted virulence molecules. Many of these molecules have seemingly redundant functions, including pore-forming cytolytic toxins.<sup>196</sup> Some of these cytolytic toxins such as  $\alpha$ -hemolysin ( $\alpha$ -toxin, Hla) and  $\alpha$ -type phenol-soluble modulins (PSM $\alpha$ ) kill a wide range of host cells, whereas others such as Pantan-Valentine leukocidin (PVL) and leukocidin GH/AB (LukGH/LukAB) primarily target myeloid cells.<sup>196,197</sup> Indeed, PVL and LukGH/AB are best known for their ability to cause neutrophil cytolysis. This host cell specificity is dictated by the expression of toxin surface receptors on the host cell.<sup>196</sup> Differential expression of host cell receptors among animal species contributes to the observed differences in cytolytic capacity toward neutrophils from different animal species *in vitro*.

PVL has been associated historically with a subset of skin and soft tissue infections, such as furuncles, or with severe necrotizing pneumonia following influenza A virus infection.<sup>198-200</sup> More recently, Shallcross et al. conducted a meta-analysis and found an association of PVL with skin and soft tissue infections (SSTIs) in general, but not with severe *S. aureus* infections (including pneumonia).<sup>201</sup> The results of this study brought into question the idea that PVL is associated primarily with invasive *S. aureus* infections that have poor clinical outcomes.<sup>201</sup> Consistent with these findings, Bae et al. analyzed data from a multinational phase three clinical trial and found presence of genes encoding PVL were not associated with clinical outcome in patients with complicated SSTIs.<sup>202</sup> Rather, patients infected with PVL-positive *S. aureus* strains had significantly better clinical outcomes.<sup>202</sup> Subsequent work by this group found that presence of PVL genes is not associated with clinical outcome in patients with hospital-acquired pneumonia caused by *S. aureus*.<sup>203</sup>

Although the relative contribution of PVL—if any—to *S. aureus* disease in humans remains incompletely determined, there is no question the toxin has the capacity to cause human neutrophil cytolysis *in vitro*.<sup>204</sup> At sublytic concentrations (~1 nM), PVL primes human neutrophils for enhanced function, presumably because of the interaction with neutrophil C5aR1.<sup>205-207</sup> At concentrations greater than 2 nM, purified PVL causes lysis of human neutrophils *in vitro*.<sup>206</sup> Pilszczek et al. originally reported the ability of PVL (~10 nM) to cause formation of NETs and more recently Mazzoleni et al. described formation of NETs by “PVL NETosis”, a cytolytic process distinct from the original description of NETosis and not necessarily dependent on formation of membrane pores.<sup>208,209</sup> This interesting finding merits further investigation. Given the capacity of PVL to cause lysis of neutrophils *in vitro*, one might predict this characteristic should translate to virulence *in vivo*. However, the translational component has been difficult to model from an animal infection standpoint, as *S. aureus* produces multiple virulence molecules, and PVL has tropism for human cells. Diep et al. used a rabbit model of severe USA300 respiratory tract infection to show that PVL-mediated lysis of neutrophils was the underlying cause of tissue destruction in the rabbit lung.<sup>210</sup> This end-stage model of necrotizing pneumonia utilized a high bacterial inoculum (~10<sup>10</sup> colony forming units) to demonstrate the potential of PVL to lyse neutrophils *in vivo* and in turn cause severe tissue damage.<sup>210</sup> Interestingly, PVL-mediated

neutrophil lysis data *in vitro* and tissue destruction in the rabbit severe pneumonia model do not corroborate the data with human clinical outcomes as described above. In the end, PVL is simply one of many *S. aureus* virulence molecules that have potential to contribute to human disease.

The contribution of LukGH/AB to human disease remains unknown, but patients with invasive *S. aureus* infections have elevated serum levels of anti-LukAB antibodies.<sup>211,212</sup> The leukocidin elicits inflammation following administration in rabbit or monkey skin, but fails to contribute to virulence in a rabbit model of USA300 SSTI.<sup>213</sup> Recently, LukAB was used as a component of a *S. aureus* toxoid vaccine that showed protection against primary and secondary infections.<sup>214</sup> *In vitro*, LukGH/AB has cytolytic capacity toward human neutrophils that is similar to that of PVL.<sup>213</sup> In addition, LukGH can cause formation of NETs by neutrophil cytolysis.<sup>215</sup> Like PVL, LukGH/AB is a bi-component pore-forming toxin, but its target receptor is CD11b and thus distinct from that of PVL.<sup>216</sup> Nonetheless, an attribute common to LukGH and PVL is formation of NETs by cytolysis. Importantly, Malachowa et al. demonstrated that non-specific pore formation caused by electropermeabilization of neutrophils led to formation of NETs that were identical to those caused by LukGH (i.e., comprised of DNA, MPO, and histones).<sup>215</sup> This observation brings into question the now long-held idea that a unique or specific form of cell death is required for formation of NETs.

The *S. aureus* PSM $\alpha$  peptides (PSMs) are amphipathic peptides produced at relatively high levels in strains with high-virulence phenotypes, such as CA-MRSA strains.<sup>217</sup> These peptide toxins are well characterized for their ability to cause neutrophil lysis by non-specific disruption of the plasma membrane.<sup>217</sup> However, at sublytic concentrations they bind formyl-peptide receptor 2 (FPR2) on the surface of neutrophils. FPR2 binding in turn elicits changes in human neutrophil gene expression that are mediated by early growth response protein 1 (EGR1).<sup>218</sup> PSMs prime neutrophils at low concentrations and promote neutrophil chemotaxis *in vivo* in mouse infection models.<sup>219</sup> Studies in multiple animal infection models demonstrate that PSMs contribute significantly to CA-MRSA virulence.<sup>217,219,220</sup> More recently, PSM production has been associated significantly with human skin and soft tissue infections.<sup>221</sup> Inasmuch as PSMs cause neutrophil cytolysis, it is perhaps not unexpected that they cause rapid formation of NETs.<sup>222</sup> Björnsdóttir et al. reported that PSM-mediated NET formation did not require active cell signaling (e.g., NADPH oxidase-derived ROS, MPO, or elastase). Rather, the authors suggested NETs were the result of a “passive outcome of membrane disturbances” caused by PSMs.<sup>222</sup>

**3.2.3 | Lysis of neutrophils after phagocytosis of *S. aureus***—Despite the capacity of *S. aureus* to produce dozens of molecules that can potentially dampen the innate immune response, most *S. aureus* are ingested and killed by neutrophils. Indeed, neutrophils are known widely as the primary cellular defense against *S. aureus* infections. However, some *S. aureus* strains, such as the epidemic USA300 strain, have increased ability to circumvent killing by neutrophils and can cause leukocyte lysis after phagocytosis.<sup>195,223</sup> This cytolytic phenomenon has the potential to influence virulence and strain success as a human pathogen.



Rogers and Tompsett demonstrated decades ago that coagulase-negative staphylococci (then *S. albus*; now *S. epidermidis*) and coagulase-positive staphylococci (*S. aureus*) are ingested rapidly by human PMNs in the presence of serum.<sup>224</sup> They found that PMN phagocytosis and killing of *S. epidermidis* and *S. aureus* were comparable at early time points (~30 min).<sup>224</sup> Subsequent work by Rogers and Melly reported similar ingestion within 60 min.<sup>225</sup> Despite similar overall uptake by PMNs, there were significant differences in the fate of these microbes at later time points.<sup>224</sup> Whereas *S. epidermidis* was killed and degraded by PMNs after phagocytosis, some of the ingested *S. aureus* survived within PMNs and caused leukocyte cytolysis within 3 or 4 h.<sup>224</sup> Subsequent work in a rabbit bacteremia model by Rogers demonstrated that *S. aureus* is sequestered rapidly by circulating PMNs, which harbor viable organisms that contribute to persistence in the host.<sup>226,227</sup> Studies conducted by Gresham et al. nearly 50 years later corroborated these findings with mouse neutrophils.<sup>228</sup> Mouse PMNs containing viable *S. aureus* had the ability to establish infection in naive animals.<sup>228</sup> Although PMN cytolysis was not evaluated, it is inferred as the means by which *S. aureus* escapes to cause infection. Therefore, this cytolytic process is potentially important for *S. aureus* pathogenesis and persistence in humans.

In studies published a number of years ago (c. 2005), we found that the epidemic USA300 CA-MRSA strain (named LAC in those studies) was ingested readily by human PMNs.<sup>195</sup> However, ~30-50% of these organisms survived, and PMNs containing them underwent cytolysis within 4 or 5 h.<sup>195</sup> These findings were compatible with the earlier studies by Rogers and colleagues. At the time, we hypothesized that the enhanced ability of USA300 to kill neutrophils after phagocytosis underlies the enhanced virulence phenotype this strain —i.e., the ability to cause infections in otherwise healthy individuals. We then characterized cellular and molecular features of neutrophils undergoing lysis after phagocytosis of USA300.<sup>223</sup> At early time points after *S. aureus* uptake, the cellular phenotype is similar to that of neutrophils undergoing apoptosis, although lysis is neither delayed nor blocked by caspase inhibitors. The phenotype included membrane blebbing within 3 h and nuclear rounding and condensation by 4 h. However, the nucleus ultimately expands to fill the cytoplasm concomitant with rupture of the cell membrane.<sup>223</sup> By microscopy analysis, the process bears striking resemblance to that of neutrophils forming NETs, as reported by Fuchs et al.<sup>166</sup> We also determined that PMN lysis after phagocytosis is not dependent on a functioning NADPH oxidase, as cytolysis is virtually identical in neutrophils from patients with X-linked CGD.<sup>223</sup> Transcriptome analyses were largely unrevealing to identify a specific mechanism, but neutrophil cytolysis was inhibited by actinomycin D or puromycin.<sup>223</sup> The possible effect of these chemical inhibitors on *S. aureus* function was not examined at that time.

Further insights into the mechanism have been reported more recently by Greenlee-Wacker and colleagues. First, the authors discovered that neutrophils containing *S. aureus* are diverted from normal phagocytosis-induced apoptosis, and macrophage efferocytosis is decreased significantly.<sup>229</sup> In addition, the authors demonstrated PMN lysis is in part inhibited by necrostatin-1, a RIPK-1 inhibitor often used to block necroptosis.<sup>229</sup> Although these data provided support to the idea that neutrophils containing USA300 undergo programmed necrosis (necroptosis), a subsequent study by Greenlee-Wacker et al. indicated this form of neutrophil cytolysis requires RIPK-3 rather than RIPK-1.<sup>230</sup> In any case, these

findings are consistent with the ability of *S. aureus* to disrupt neutrophil homeostasis and promote disease.

**3.2.3.2 | Contribution of *S. aureus* molecules to PMN lysis after phagocytosis:** Lysis of neutrophils after phagocytosis requires live *S. aureus*, and molecules produced by *S. aureus* contribute to the process.<sup>223</sup> Multiple experimental approaches have demonstrated neutrophil lysis can be triggered by internalized *S. aureus* as opposed to cytolytic toxins secreted in the culture media. Production of cytolytic toxins within the phagosome is possible, and ligand binding regions of PVL and LukGH/AB receptors are accessible. However, these same receptors on the plasma membrane are not accessible from the cytoplasm (incorrect topology). Consistent with this notion, USA300 isogenic mutant strains lacking genes encoding PVL retain full capacity to cause lysis after phagocytosis.<sup>231</sup> In contrast, *S. aureus* isogenic mutant strains lacking genes encoding LukGH/AB, PSMs or Hla have significantly reduced ability to cause neutrophil lysis after phagocytosis.<sup>232-236</sup> At present, it is not clear how these cytolytic toxins contribute to cytolysis caused by ingested *S. aureus*. Recent studies by Rungelrath et al. demonstrated that USA300 is retained in intact phagosomes until the point of PMN lysis.<sup>236</sup> Therefore, this cytolytic process does not require escape of *S. aureus* from the phagosome. It is possible that these *S. aureus* toxins elicit cell death signals from within the phagosome, but more work is needed to resolve a specific contribution of *S. aureus* molecules to lysis after phagocytosis.

**3.2.3.2 | Why is neutrophil lysis after phagocytosis of *S. aureus* important?:** The landmark study by Rogers and Tompsett was one of the first reports of PMN lysis after phagocytosis of *S. aureus*. The implications of the Rogers and Tompsett study were seemingly underappreciated at the time they were published. Based on those studies and work by Jensen, Rogers stated, “attempts to control the problem of staphylococcal disease in man by evoking or reinforcing specific humoral immunity is theoretically unsound and offers little hope of success”.<sup>237,238</sup> With regard to *S. aureus* opsonophagocytic vaccine approaches, the statement remains accurate. Human clinical trials that have been directed to promote *S. aureus* phagocytosis have failed or not met endpoint criteria. They have failed for two basic reasons: 1) phagocytosis of *S. aureus* is rapid and efficient in healthy individuals and 2) ingested *S. aureus* are not killed completely, and as a result some PMNs ultimately undergo cytolysis (Fig. 3). This is a major problem for a vaccine approach directed to enhance phagocytosis. In addition, lysis of neutrophils by ingested *S. aureus* likely contributes to (or is an underlying cause of) enhanced virulence phenotypes, persistent infections, and severe disease. Boosting the killing capacity of neutrophils toward ingested *S. aureus* would be ideal, but not practical or possible. Another solution is to moderate the severity of *S. aureus* infections by targeting virulence molecules. This vaccine approach has worked relatively well in animal infection models, and human clinical trials are in progress.

## 4 | CONCLUDING REMARKS

Neutrophil production and turnover are regulated to maintain immune system homeostasis and human health. Most bacteria are eliminated by neutrophils. However, pathogens such as *S. aureus* have the ability to alter normal neutrophil turnover, which in turn can have

a negative influence on human health. Based on our current understanding of neutrophil biology and function, several questions and points merit further consideration.

- **Formation of NETs as a mechanism of host defense.** Neutrophils cause host tissue damage and play a prominent role in severe inflammatory diseases. These processes are typically associated with neutrophil activation, ROS production, and exocytosis and/or cytolysis. The number of processes used by the host to regulate neutrophil activation and prevent cytolysis is extraordinary. Does a mechanism of host defense that requires neutrophil cytolysis fit with these key tenets of neutrophil biology?
- **Phagocytosis versus NETs in host defense.** This is in essence comparison of a high concentration of microbicides contained in the phagocytic vacuole versus a relatively low concentration of extracellular molecules that by diffusion alone are far less effective as microbicides. The later can also damage host tissues. There can be no question that phagocytosis remains the primary means by which neutrophils eradicate bacterial pathogens. This topic has been discussed recently by DeLeo and Allen.<sup>239</sup>
- **Neutrophils are terminally differentiated phagocytes and removed daily in large numbers by efferocytosis.** In the absence of efferocytosis and/or nonphlogistic removal, neutrophil will ultimately undergo cytolysis. This attribute might explain the ability of a wide range of stimuli and conditions to seemingly promote formation of NETs.

## ACKNOWLEDGMENTS

FRD and SDK are supported by the Intramural Research Program of the National Institutes of Allergy and Infectious Diseases (NIAID). MTQ is supported in part by National Institutes of Health IDeA Program Grant GM103474, USDA National Institute of Food and Agriculture, and the Montana State University Agricultural Experiment Station.

## DATA AVAILABILITY STATEMENT

Not applicable—no new data were generated.

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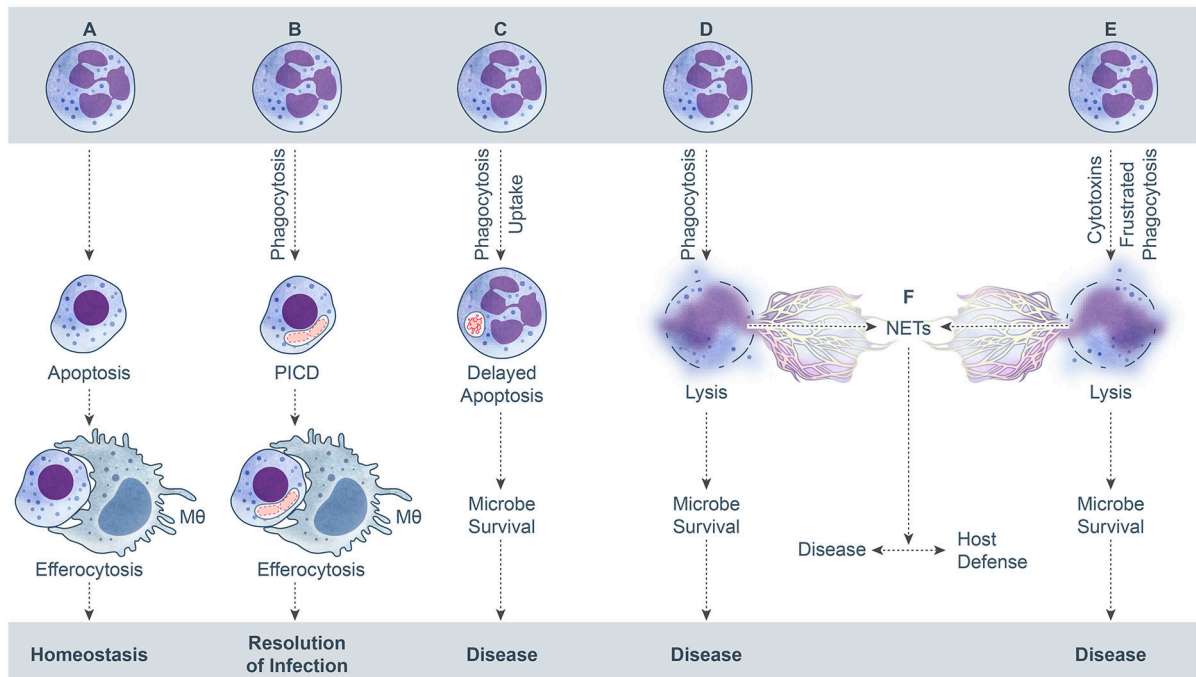
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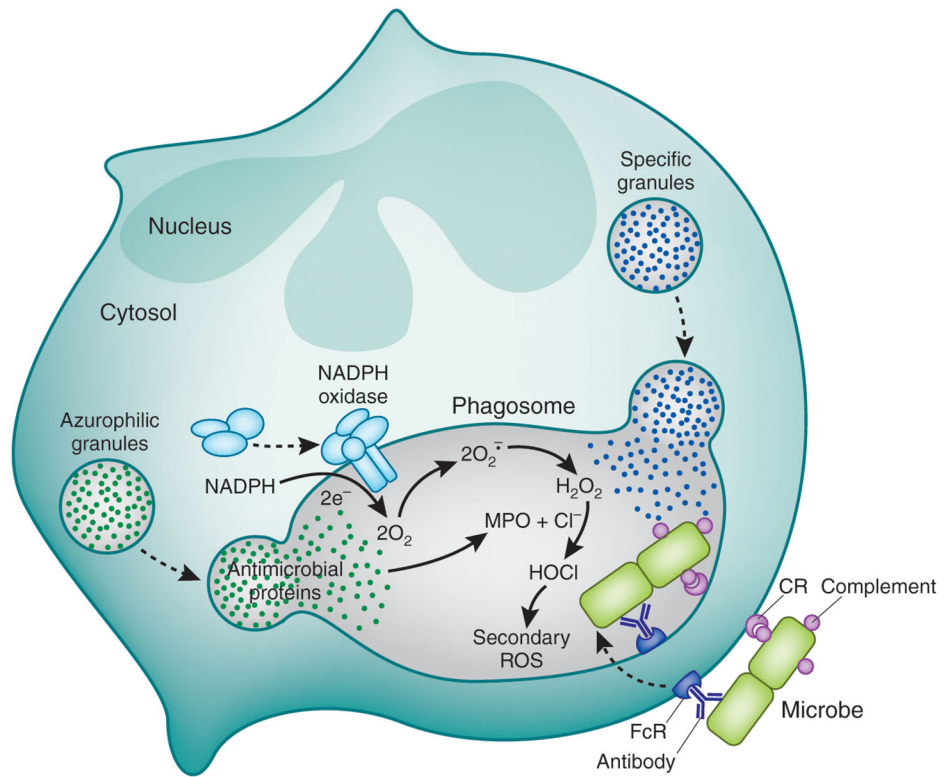
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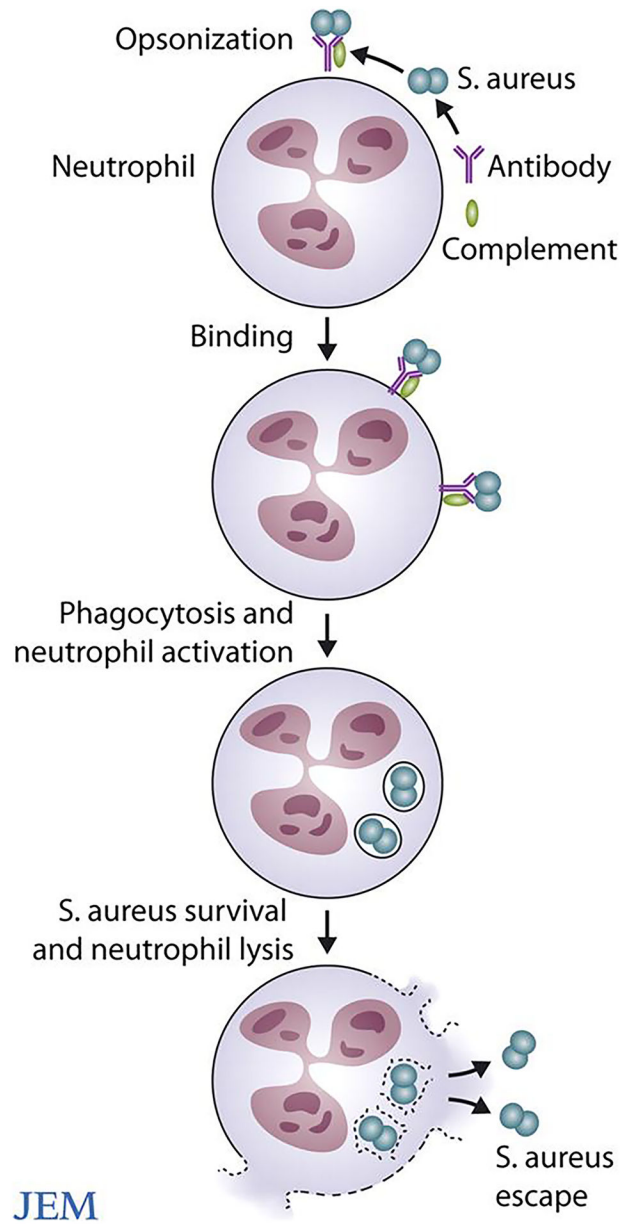
**FIGURE 1.**

Potential fates of neutrophils. (A) Apoptosis followed by efferocytosis. (B) PICD and efferocytosis. (C) Delayed neutrophil apoptosis and microbe survival. (D and E) Neutrophil cytolysis. (F) Formation of NETs by cytolysis. ©2017 Kobayashi, Malachowa, and DeLeo. Originally published in *Front Cell Infect Microbiol.* 2017; 7: 159. Published online 2017 May 1. doi:[10.3389/fcimb.2017.00159](https://doi.org/10.3389/fcimb.2017.00159).





**FIGURE 2.** Neutrophil activation during phagocytosis. ©2020 DeLeo and Nauseef. Originally published in *Granulocytic Phagocytes*. In Mandell, Douglas, and Bennett's *Principles and Practice of Infectious Diseases*, 9th Edition, Eds. Bennett JE, Dolin R, and Blaser M. (2020) Elsevier Limited, Oxford, UK. pp. 83-98.



**FIGURE 3.** Neutrophil cytolysis following phagocytosis of antibody and/or complement opsonized *S. aureus*. ©2008 DeLeo and Otto. Originally published in *J Exp Med.* 2008;205(2):271-274. doi:10.1084/jem.20080167.