Trends in Immunology

Review

How Neutrophils Meet Their End

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Neutrophil death can transpire via diverse pathways and is regulated by interactions with commensal and pathogenic microorganisms, environmental exposures, and cell age. At steady state, neutrophil turnover and replenishment are continually maintained via a delicate balance between host-mediated responses and microbial forces. Disruptions in this equilibrium directly impact neutrophil numbers in circulation, cell trafficking, antimicrobial defenses, and host well-being. How neutrophils meet their end is physiologically important and can result in different immunologic consequences. Whereas nonlytic forms of neutrophil death typically elicit anti-inflammatory responses and promote healing, pathways ending with cell membrane rupture may incite deleterious proinflammatory responses, which can exacerbate local tissue injury, lead to chronic inflammation, or precipitate autoimmunity. This review seeks to provide a contemporary analysis of mechanisms of neutrophil death.

Neutrophil Homeostasis

The greatest percentage of hematopoiesis is committed to the production of neutrophils, with an estimated 60% of all leukocytes in the bone marrow comprising granulocyte precursors [1]. This results in the generation of a staggering 100 billion neutrophils every day by the average human adult [2]. Once in circulation, neutrophils are relatively short-lived with a half-life between 18 and 19 h [1]. Therefore, to preserve homeostatic balance, equal numbers of neutrophils must routinely be turned over. Due to the nature of their cytotoxic contents, however, appropriate developmental and clearance mechanisms must be firmly established to protect the host against unintended inflammatory injury. Nonlytic forms of neutrophil death pathways are predominant during steady state, promoting anti-inflammatory immune responses due to retention of granular and cytoplasmic components intracellularly. By contrast, certain infectious or inflammatory etiologies can trigger lytic forms of neutrophil death, which allow the release of noxious proinflammatory chemokines, cytokines, and granular proteins that if unregulated may instigate or worsen local tissue injury [3]. Neutrophil homeostasis is maintained through a meticulous balance between neutrophil development (Box 1), bone marrow storage and release, migration into vascular compartments and peripheral tissues, cell aging, and death [1]. Because diverse neutrophil death pathways exist, understanding mechanistic differences among them is vital in engineering targeted, specific therapies to treat a variety of related diseases (Figure 1, Key Figure). In this contribution, we explore various pathways of neutrophil cell death, describe how neutrophil aging and the host microbiome contribute to cell death mechanisms, and highlight consequences of aberrant death pathways in human health and disease.

Pathogen-Induced Cell Death and Efferocytosis

Pathogen-induced cell death (PICD; see Glossary) is a primary form of neutrophil apoptosis and an essential mechanism for preserving steady-state granulopoiesis, which couples microbial killing to accelerated neutrophil clearance in a process termed **efferocytosis** (see Box 2) [4,5]. Neutrophil apoptosis via PICD renders the cell unresponsive to extracellular stimuli and enables the recognition and clearance of dying cells by professional phagocytes [6]. Failure to effectively clear apoptotic neutrophils by this death pathway can cause cellular necrosis and subsequent

Highlights

Neutrophils are the most abundant immune cells in humans and the first to respond to infection or inflammation. Their rapid generation and secretion of proinflammatory mediators and toxic granular substances require cell death mechanisms to protect against tissue injury.

Neutrophil death occurs via diverse pathways regulated by an internal diurnal cell timer, interactions with commensal or pathogenic microorganisms, environmental exposures, as well as cell aging.

Neutrophils may undergo apoptosis (less inflammatory), necroptosis, or pyroptosis (both proinflammatory), influencing immune responses and kinetics of disease resolution.

Nonapoptotic forms of neutrophil cell death include NETosis or senescence with autophagic degradation of subcellular components.

Manipulation of neutrophil death pathways may enable the discoveries of novel therapies to treat certain infectious, autoimmune, and congenital neutrophil disorders.

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Box 1. Neutrophil Development

Mammalian neutrophil development begins between the myelocyte and promyelocyte stages of development and proceeds over the subsequent 4–6 days in a process termed 'targeting by timing model' [77]. Specifically, granule protein production occurs in a continuum during all stages of neutrophil development with proteins packed into granules as they are produced [77]. Protein synthesis is therefore dependent only upon cell maturity, such that **azurophilic granule proteins** are generally synthesized at the promyelocyte developmental stage, specific granule proteins at the myelocyte stage, and gelatinase/ficolin-1 granule proteins at the metamyelocyte and band stages of neutrophil maturation, after which granule formation concludes, and secretory vesicles form [78]. Subsequently, segmented, mature neutrophils are formed and create a reserve pool within the bone marrow from which cells can be released into the peripheral circulation as polymorphonuclear neutrophils to maintain homeostasis. Alternatively, this neutrophil storage pool can be depleted upon acute exposure to infectious or inflammatory mediators to facilitate a rapid rise in neutrophil numbers in the bloodstream [79].

Severe congenital neutropenia (SCN) is a life-threatening congenital condition caused by defects in neutrophil production. More than half of all SCN cases occur due to mutations in genes encoding neutrophil elastase (*ELA2*) and HCLS1 associated protein X-1 (*HAX1*) [80,81]. Genetic defects of *ELA2* lead to neutrophil elastase misfolding, prompting its intracellular accumulation and mislocalization [80]. This deformation leads to endoplasmic reticulum stress and induction of apoptosis through activation of the **unfolded-protein response** at the earliest phases of neutrophil differentiation, or at the myeloblast to promyelocyte maturational stage [80]. The *HAX1* gene, conversely, directly controls mitochondrial proteases that regulate the accumulation of BCL-2-associated X protein (BAX), a proapoptotic protein in the outer mitochondrial membrane [81]. Mitochondrial functions, indispensable for cell survival, are abrogated when cytoplasmic amounts of BAX exceed those of their antiapoptotic counterparts MCL-1 [81]. This facilitates BAX oligomerization, leading to the formation of pores in the outer mitochondrial membrane, release of cytochrome c into the cytoplasm, and cell death through caspase activation via the 'intrinsic cell death pathway' [82].

release of noxious granular proteins and **damage-associated molecular pattern** (DAMP) biomolecules extracellularly, which act to exacerbate local inflammatory responses and perpetuate surrounding tissue injury [7]. A murine pneumoseptic model of *Klebsiella pneumoniae* infection illustrates this process, wherein a pathogen-induced reduction of the 'eat me' signal, **phospholipid phosphatidylserine** (PS), on the neutrophil cell surface results from the bacterium's ability to directly increase the activity of phospholipid transporter **flippases** [8]. Retention of PS within the inner leaflet of the plasma membrane impairs efferocytosis and drives the neutrophil toward proinflammatory necroptosis-mediated death machinery [8].

PICD is also dependent upon the local production of reactive oxygen species (ROS), as demonstrated by persons suffering from **chronic granulomatous disease** (CGD). Specifically classified as a primary immunodeficiency disorder, CGD occurs secondary to genetic defects in an essential catalyst of ROS production, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [9,10]. NADPH oxidase is required for the formation of hydrogen peroxide, which combines with chloride and myeloperoxidase from azurophilic granules, to generate large amounts of the ROS superoxide, a noxious substance that is indispensable for neutrophil-mediated killing of pathogens [9,10]. Patients with CGD may fail to resolve certain bacterial and fungal infections with such neutrophil defects, and can suffer from sustained proinflammatory responses and ongoing tissue damage, which may be clinically reflected by the development of **granulomas** [9].

Deficient neutrophil efferocytosis has also been described in the pathogenesis of human atherosclerosis, the hallmark lesion of cardiovascular disease. Atherosclerosis typically begins when circulating lipoproteins accumulate in focal areas within the subendothelial matrix of medium-sized and large arteries [11]. These lipoproteins subsequently become oxidized, leading to an influx of leukocytes to the inflamed site [12]. Oxidized lipoproteins may also proteolytically cleave and inactivate the macrophage efferocytosis receptor c-Mer tyrosine kinase (MerTK) [12]. MerTK silencing leads to impaired efferocytosis clearance mechanisms and inhibition of positive-feedback signaling for inflammation resolution [12]. Monocyte-derived macrophages consume the deposited lipoproteins in an effort to contain the lesion, leading to their transformation into '**foam cells**' [13,14]. Once containment measures fail, the inundated foam cells secrete proinflammatory

Glossary

Acute respiratory distress syndrome (ARDS): rapidly progressive pulmonary disease in critically ill or septic patients. Proinflammatory mediators cause respiratory capillaries to become leaky, allowing fluid to enter alveolar air sacs; this may impair oxygen and carbon dioxide exchange and lead to respiratory distress or frank failure.
Apoptosis: cell death occurring as a normal and controlled part of cellular homeostasis, or as a means to enable the growth and development of an organism.
Azurophilic granule proteins: 'primary' granule proteins; confer potent antimicrobial activity through both oxidative and non-oxidative pathways (e.g., myeloperoxidase, α-defensins, bactericidal/permeability-increasing protein, elastase, proteinase-3, and cathepsin G).
Chronic granulomatous disease (CGD): diverse group of hereditary diseases in which certain cells of the immune system have difficulty forming reactive oxygen compounds (e.g., the superoxide radical due to defective phagocyte NADPH oxidase) used to kill certain ingested pathogens.
Crohn's disease: inflammatory bowel disease caused by chronic inflammation of the gastrointestinal tract. Symptoms may include abdominal pain, diarrhea, weight loss, anemia, and fatigue.
Damage-associated molecular patterns (DAMPs): host biomolecules that can initiate and perpetuate a noninfectious inflammatory response.
Death receptors: belong to the TNF superfamily, and provide a rapid and efficient route to apoptosis. Possess cysteine-rich extracellular domains and an intracellular cytoplasmic sequence, the 'death domain'. Ligands generally include TNF, FasL, Apo3L, TRAIL, TRADD (TNFR1-associated death domain protein).
**Disseminated intravascular coagulopathy:** small blood clots develop throughout the bloodstream, blocking small blood vessels. Increased clotting depletes platelets and clotting factors needed to control bleeding, which may result in severe hemorrhaging.
Efferocytosis: process by which dying/dead cells are removed by phagocytic cells (burying).
Flippases: enzymes that transport lipids from the external surface toward the cytosolic surface of the plasma membrane.

Key Figure

Primary Pathways of Neutrophil Death
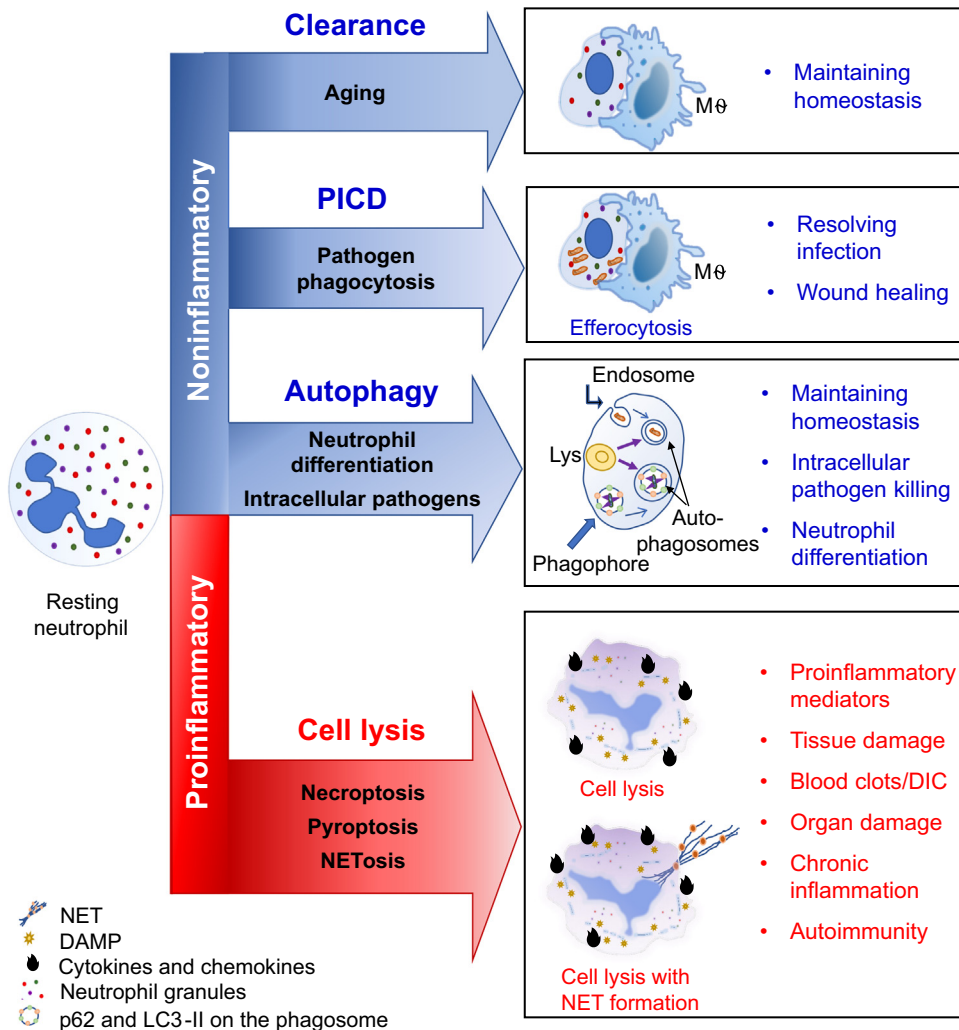
Figure 1. Noninflammatory death pathways include the following: clearance, which occurs when senescent, aged neutrophils return to the bone marrow, where they complete programmed apoptosis and are consumed by macrophages. Pathogen-induced cell death (PICD), which occurs following microbial ingestion into the neutrophil's phagosomes. This form of cell death initiates efferocytosis, or ingestion of the dying neutrophil by macrophages (M), to dampen proinflammatory processes and promote wound healing. Autophagy is triggered when viable intracellular pathogens are engulfed by either endosomes or phagophores. Phagophores form as a result of cytosolic ubiquitination of the microorganism's outer cell membrane and subsequent recognition by p62 and light-chain 3-II (LC3-II). Endosomes and phagophores can then fuse with lysosomes (Lys) to form autophagosomes, which induce cell death. Proinflammatory death pathways involve cell membrane lysis and include necroptosis, pyroptosis, and neutrophil extracellular trap (NET) formation (NETosis). Necrotic cell death and membrane lysis result in the unregulated release of neutrophil-derived toxic granular proteins and proinflammatory mediators, including damage-associated molecular pattern (DAMP) biomolecules, that may adversely affect host well-being. Cell membrane lysis can also provide a robust cell death mechanism by which powerful antimicrobial intracellular contents are extruded to

Foam cell: type of 'engorged' macrophage that localizes to fatty deposits on the walls of blood vessels and ingests low-density lipoproteins.

Gasdermin D (GSDMD): protein that serves as a specific substrate of inflammatory caspases and as an effector molecule for the lytic and highly inflammatory form of programmed cell death known as pyroptosis.

Granuloma: mass of granulation tissue produced in response to infection and inflammation.

Inflammasome: multiprotein intracellular complex detecting pathogenic microorganisms and sterile stressors; activates the highly proinflammatory cytokines IL-1β and IL-18. Inflammasomes also induce pyroptosis.

Intracellular DNA and RNA sensors: widely expressed mammalian host intracellular pattern recognition receptors that bind components of pathogen DNA or RNA and include TLRs, NOD-like receptors, RIG-1-like receptors, and a group of intracellular DNA sensors, for example, cyclic GMP–AMP synthase and interferon-γ-inducible protein 16.

LC3-II (light-chain 3-II): cytosolic autophagy marker recruited to the phagosomal membrane; binds p62 to mediate rapid fusion of phagosome and lysosome to create the autophagosome.

Left-shift: increase in the number of immature granulocytes circulating in the bloodstream.

Low-density granulocytes: neutrophilic granulocytes that remain in the fraction of peripheral blood mononuclear cells after density-gradient separation.

Melioidosis: or Whitmore's disease; infectious disease of humans or animals caused by the bacterium *Burkholderia pseudomallei*; it predominates in tropical climates, especially Southeast Asia and northern Australia.

Methotrexate: chemotherapy agent and immunosuppressant used to treat cancers, autoimmune diseases, ectopic pregnancy, and certain medical abortions; antimetabolite of the antifolate type.

Mixed lineage kinase-like (MLKL) protein: plays a crucial role in TNF-induced necroptosis, via interaction with receptor-interacting protein 3 (RIP3).

Necrotic core: portion of an advanced atherosclerotic plaque consisting of lipids, dead cells, and calcifications.

Box 2. Pathogen-Induced Cell Death

To distinguish themselves from viable cells, dying human neutrophils typically generate and secrete 'find me' signals, such as soluble annexin 1, which is recognized by lipoxin A4 receptors on surveying resident macrophages [83]. Following pathogenic encounters in mice, these signals, in addition to secreted neutrophil granular proteins (such as cramp or human cathelicidin LL-37, cathepsin G, and azurocidin), function as chemoattractants and activators of professional phagocytes that, in turn, enhance neutrophil cytokine release and bacterial phagocytosis [84]. In mice, engulfment of dying neutrophils is further enabled by macrophage cytoskeletal reorganization, which is mediated by the Rho family of small GTPases, including RhoG/Rac1, and is induced by cellular engagement of phosphatidylserine receptors [85].

Biologically, local reduction of **tissue oxygen tension** to extreme hypoxia, commonly associated with infectious and inflammatory lesions, facilitates PICD mechanisms [86]. Hypoxia is a potent antiapoptotic neutrophil stimulus that prolongs cell survival to enable bacterial clearance and expedite proinflammatory responses [86]. In the absence of oxygen, neutrophil energy production relies on anaerobic glycolysis, using glucose as a substrate [87]. Neutrophil survival can, therefore, be prolonged and proinflammatory responses heightened by increasing glucose metabolism [87], or via induction of the oxygen-sensitive prolyl hydroxylase (Phd3) [86]. As observed in a $Phd3^{-/-}$ knockout murine model of LPS-mediated lung disease and colitis, loss of Phd3 was associated with upregulation of the proapoptotic mediator Siva1, loss of its binding target Bcl-x$_L$, and increased neutrophil apoptosis relative to WT mice [86].

cytokines, such as tumor necrosis factor (TNF)-α, attracting even more neutrophils to the lesion [13]. This accrual of activated neutrophils boosts local ROS production, further intensifying lipid oxidation and exacerbating inflammation [15]. Failure of neutrophil efferocytosis by foam cells results in the buildup of secondarily necrotic cells and the formation of a highly inflammatory '**necrotic core**', which is pathognomonic for advanced atherosclerotic plaques – the primary culprit triggering heart attacks and strokes [16]. **Statins** are commonly prescribed to combat cardiovascular disease by lowering blood cholesterol concentrations [17]. These drugs are also used to target and block the actions of RhoA – an inhibitor of macrophage efferocytosis – thus increasing the removal of dead or dying neutrophils [17].

To summarize, efferocytosis is a vital, noninflammatory death pathway that utilizes reduced oxygen tension within inflamed or infected tissue to prolong neutrophil survival to enhance pathogen consumption. Efferocytosis is dependent upon ROS, with congenital absence of NADPH oxidase leading to a debilitating, chronic condition known as CGD. Deficient efferocytosis is also observed in the pathogenesis of atherosclerosis and cardiovascular disease, and is targeted by commonly prescribed statins, which also function to lower bloodstream cholesterol concentrations.

Proinflammatory Neutrophil Death Pathways

Necrotic neutrophil death pathways involving cell membrane lysis comprise a fail-safe mechanism designed to eradicate intracellular pathogens that escape PICD-mediated anti-inflammatory pathways [18]. By eliminating the pathogen's replicative niche and destroying infected cells, necrotic death mechanisms may also function to curtail proinflammatory signaling [19]. Unregulated release of neutrophil-derived toxic granular proteins and proinflammatory mediators, however, may also adversely affect host well-being [20]. Here, we detail cell-lysis-mediated death mechanisms including necroptosis, pyroptosis, and the formation of **neutrophil extracellular traps** (NETs).

Cell-Lysis-Mediated Death Pathways

A form of programmed cell death, **necroptosis** is induced when caspases fail to become activated and is generally triggered by pathogens that exploit infected cells for replication and

capture and kill pathogens released extracellularly via NETosis. NET release and cell lysis can have detrimental consequences to the host, resulting in multiorgan damage/failure or even host death. This injury may be caused by microvascular clotting, development of disseminated intravascular coagulopathy (DIC), the exacerbation of local tissue damage, or through the development of long-term complications such as autoimmune disorders or chronic inflammation.

Neutrophil extracellular traps (NETs): networks of extracellular fibers, primarily composed of DNA from neutrophils and proteins; bind pathogens.

p62: protein that facilitates the binding of ubiquitinated intracellular microorganism's outer cell membrane to LC3-II, located within the phagosomal membrane to create the autophagosome.

Pathogen-induced cell death (PICD): host immune pathogen-induced defense mechanism whereby infected cells are sacrificed for the benefit of remaining cells.

Phagophore: double membrane organelle; encloses and isolates intracellular pathogens or other cytoplasmic components during autophagy.

Phospholipid phosphatidylserine (PS): a component of the cell membrane that when exposed plays a key role in cell signaling, particularly as an 'eat me' stimulus (here triggering macrophage efferocytosis).

Pore-induced intracellular traps (PITs): structures conceptually parallel to the NETs; coordinate innate immune responses via complement and scavenger receptors to drive recruitment of and efferocytosis by neutrophils.

Pyroptosis: highly inflammatory form of programmed cell death; occurs most frequently upon infection with intracellular pathogens and is likely to form part of the antimicrobial response.

RIP homotypic interaction motif (RHIM): an interacting domain involved in virus recognition. RHIM is necessary for the recruitment of RIP and RIP3 by the IFN-inducible protein DNA-dependent activator of IRFs (DAI) [DLM-1 or Z-DNA-binding protein (ZBP1)]. Both RIP kinases contribute to DAI-induced nuclear factor-κB activation.

RIPK1 and RIPK3: proteins that interact to form an amyloid spine that functions in a variety of cellular pathways related to both cell survival and death. They play a vital role in apoptosis and necroptosis.

Secondary granules: 'specific' granules; peptides include cytotoxic molecules (e.g., alkaline phosphatase, lactoferrin, lysozyme, and NADPH oxidase).

Statins: class of drugs that help lower blood cholesterol concentrations.

Swarming: coordinated neutrophil movement in response to acute tissue inflammation or infection.

immune evasion, such as intracellular viruses and parasites [18,21] (Box 3). In human peripheral blood neutrophils, necroptosis can be induced by activation of **death receptors**, **Toll-like receptors** (TLRs), and **intracellular DNA and RNA sensors**, as well as via receptor agonism for interferon (IFN)-α, adhesion molecules (including CD44, CD11b, CD18, and CD15), or granulocyte–macrophage colony-stimulating factor [22]. The significance of neutrophil necroptosis in human pathogenesis remains unclear, as a possible overlap between **RIPK1 and RIPK3** signaling to other cell death processes (such as **apoptosis** and **pyroptosis**) may occur and needs to be completely assessed.

By contrast, pyroptosis is activated by cytosolic **inflammasome** perturbations by intracellular pathogens, such as *Shigella* sp., *Francisella tularensis*, *Salmonella enterica*, and *Yersinia* sp., or from intracellular exposure to lipopolysaccharide (LPS) [23–25]. Pyroptosis-like cell death can be triggered by inflammatory caspase or neutrophil serine protease (NSP) cleavage of **Gasdermin D** (GSDMD) into its functional forms: (i) N-terminal domain (GSDMD-N) that binds membrane lipids (phosphatidylinositol phosphates and phosphatidylserine) to enable membrane pore formation (Box 3); and (ii) C-terminal fragment that has an autoinhibitory effect on the intrinsic pyroptosis-inducing activity of the N terminus [23,26]. The N-terminal fragment may also directly kill intracellularly trapped bacteria by binding cardiolipin in the pathogen's inner and outer membrane leaflets and perforating their cell membrane [23,27].

Box 3. Mechanisms of Cell Lysis-Mediated Neutrophil Death

Necroptosis

In mammalian myeloid-derived cells, necroptosis is propagated by receptor-interacting protein kinase-1 (RIPK1) and receptor-interacting protein kinase-3 (RIPK3), which associate with one another through **RIP homotypic interaction motif** (RHIM). This association usually leads to the phosphorylation of the pore-forming protein **mixed lineage kinase-like** (MLKL) by RIPK3 [88]. Both RIPK3 and MLKL are constitutive nucleocytoplasmic shuttling proteins that are activated in the nucleus following the induction of necroptosis and which translocate to the cytosol, where they jointly contribute to necrosome (also known as ripoptosome) formation. Phosphorylation of MLKL by the necrosome prompts MLKL oligomerization, enabling MLKL to insert into and permeabilize the plasma membrane [89]. The importance of RIPK3 in this cell death pathway is demonstrated by murine models designed to inhibit necroptosis. Whereas *Ripk3*$^{-/-}$ knockout mice exhibit enhanced protection against ischemic injury, sterile shock, and increased susceptibility to infection with viruses, *Mlkl*$^{-/-}$ animals do not [90,91]. Moreover, the absence of *Ripk1* in mice exacerbates cell death by necroptosis, indicating that this form of cell death is not dependent upon the presence of RIPK1 [92].

Pyroptosis, Pore-Induced Intracellular Traps, and Gasdermin D

Pyroptosis caspase-1-mediated membrane pores, termed PITs, are sized sufficiently to allow the release of soluble cytosolic contents only, retaining larger bacteria and organelles intracellularly [47]. Conceptually similar to NETs, PIT formation is mediated by IL-1β, IL-18, and eicosanoids. The two, however, act independently of each other. PITs define a mechanism by which trapped bacteria within the pyroptotic cell corpse can be killed through ROS reactions with subsequent efferocytosis [47]. Mice with impaired NETosis (*Mpo*$^{-/-}$ and *Elane*$^{-/-}$) can effectively clear bacteria trapped in PITs, while NETosis is not inhibited by the pan-caspase inhibitor z-VAD-fmk [47].

In murine models, both caspase-1 and caspase-11 are able to cleave pro-IL-1β into functional IL-1β, although caspase-11 is more efficient [30]. Caspase-11-induced NETosis is also morphologically similar to classical neutrophil elastase (NE)-driven NETosis, suggesting that while these pathways are triggered in response to different challenges via distinct signaling mechanisms, they can converge on the common executioner protein GSDMD [30]. GSDMD cleavage by NSPs provides an alternative mechanism for lytic neutrophil death to pyroptosis, with the generation of similar quantities of proinflammatory cytokines, such as IL-1β [26]. NSPs, including NE, proteinase 3 (PR3), cathepsin G (CG), and serine protease-4 (NSP4), promote inflammation through cleavage-mediated activation or inhibition of cytokines, opsonins, and receptors [26]. Even though the ability to successfully cleave GSDMD has not been demonstrated by all NSPs, those with GSDMD-cleaving capabilities produce a wide range of immunologic effects [26]. While CG can cleave GSDMD and initiate cell death via membrane lysis in both murine and human cell lines, PR3-mediated GSDMD cleavage can produce fragments that are rapidly degraded in a serine protease (*Sb1a.Sb6a*$^{-/-}$) knockout mouse model [26]. Because of their opposing actions, concomitant release of PR3 and CG might hypothetically cancel each other and block GSDMD fragmentation and cell death [26]. This divergence might partly explain recent evidence suggesting that CG, not GSDMD, was vital for both neutrophil death and cytokine release upon LPS challenge [26].

Similar to necroptosis, pyroptosis eliminates the niche used by intracellular pathogens and promotes the dysregulated release of toxic neutrophil substances extracellularly. In particular, the release of proinflammatory cytokine interleukin-1β (IL-1β) appears to escalate proinflammatory responses and propagation of local tissue injury, as demonstrated in a murine model of **melioidosis** [28,29]. Unlike other myeloid cells, caspase-1 does not readily trigger pyroptosis in neutrophils, even though production of IL-1β is maintained [30]. This resistance to caspase-1-mediated pyroptosis prolongs neutrophil survival, enabling this cell type to continue to perform its classic antimicrobial functions, including phagocytosis and pathogen killing at inflamed sites [30]. Therefore, whether or not neutrophils die by classical pyroptosis continues to be debated.

Cell death mechanisms may also overlap, as recently demonstrated by community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA); specifically, this pathogen employs components of necroptosis, pyroptosis, and NSP-mediated cell death. Following phagocytosis by ultrapure human polymorphonuclear neutrophils (>99.8%), CA-MRSA remains viable and triggers IL-1β, but not IL-18, production [31]. Moreover, the generation of IL-1β and cell death appear to result from induction of RIPK3 (but not RIPK1) and NSPs, but has been found to be independent of canonical NLRP3 inflammasome and caspase-1 activation in these human neutrophils [31]. Therefore, events in this alternate pathway suggest that defined cell death mechanisms might not act in isolation [31].

Neutrophil Extracellular Trap Formation (NETosis)

Neutrophils can initiate first-line innate immune responses against bacteria, fungi, and protozoa through the formation of NETs (Box 4). First described in 2004 [32], NET formation involves the expulsion of the neutrophil's intracellular contents extracellularly for the entrapment and eventual killing of pathogens. The 'NETome' is composed of nuclear chromatin, citrullinated histones [33], elevated concentrations of highly potent bactericidal azurophilic and specific granular proteins –

Box 4. Mechanisms of Neutrophil Extracellular Trap (NET) Formation

Classical

Although extrusion of neutrophil contents extracellularly occurs in conjunction with autophagy, necroptosis, pyroptosis, and oncosis, NET formation generally occurs via two processes dependent upon cell viability during chromatin extraction. NET formation following neutrophil necrosis is initiated in response to LPS or TNFα and involves the expansion of nuclear material, chromatin decondensation, nuclear envelope disintegration, subsequent mixing of cytoplasmic and nuclear components, followed by plasma membrane rupture, and release of the chromatin material [35]. This form of 'NETosis' requires activation of NADPH oxidase through the Raf–MEK–ERK pathway, ROS production, and activation of the receptor-interacting protein kinase-3 (RIPK3)–mixed lineage kinase-like (MLKL) cascade [35].

Conversely, NET formation can occur in approximately 20%–25% of viable human neutrophils in a ROS-independent manner following exposure to certain bacteria, such as *S. aureus* [93]. This type of NET formation is initiated by the complement system [94], TLR2, and/or fibronectin [95]. It involves the release of chromatin material from an intact plasma membrane through intracellular vesicles that fuse with the outer membrane in a RIPK3–MLKL-independent fashion [96]. This allows trapping of bacteria in NETs, while the anuclear neutrophil maintains its phagocytic and pathogen killing abilities for a period thereafter [97].

Gasdermin D-Mediated NET Formation

Noncanonical, GSDMD-mediated NETosis differs from classical NET formation pathways in that intracellular LPS or Gram-negative bacteria promote inflammasome assembly and caspase cleavage of GSDMD, whereas classical NETosis is caspase independent, requiring ROS activation of serine proteases that cleave GSDMD [25,30]. In this pathway, caspase-11 in mice (or caspase-4 and caspase-5 in humans) and GSDMD-mediated pores target and lyse neutrophil granule membranes, releasing neutrophil elastase (NE) and myeloperoxidase during early NETosis. Release of these granular proteins initiates nuclear delobulation, DNA expansion, histone degradation, chromatin condensation, and nuclear membrane permeabilization [30]. NE and GSDMD engage in a feed-forward loop wherein NE initially activates GSDMD. Functional GSDMD, in turn, enhances the granular release of NE by forming granular pores, leading to a surge in cytosolic NE concentrations that further cleave GSDMD in a reiterative process [30]. Functional GSDMD is also critical for late stages of NETosis and is responsible for creating pores in the neutrophil cell membrane to enable DNA extrusion and creation of the extracellular 'NETome'.

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proteolytic enzymes that degrade bacterial virulence factors [34] – as well as enzymatically active myeloperoxidase [35]. NETs can bind both Gram-negative [36] and Gram-positive bacteria [37] and cleave bacterial flagella [38].

The release of proinflammatory cytokines and chemokines from NET-mediated cell lysis can result in the recruitment of substantial numbers of activated neutrophils and monocytes to areas of infection [39]. This buildup of stimulated immune cells, in combination with continued NET production, can exacerbate local tissue damage, lead to the formation of blood clots within the bloodstream, or trigger the onset of **disseminated intravascular coagulopathy** from vascular occlusion [40]. Collectively, these adverse events can promote the development of multiorgan dysfunction syndrome, result in organ failure, or cause host death [41].

Organ dysfunction related to excessive NET formation is best exemplified by the rapid onset of **acute respiratory distress syndrome** (ARDS) in patients with severe infection, inflammation, or sepsis. ARDS, a life-threatening lung injury that presents clinically as respiratory difficulty or failure, is caused by leaky pulmonary capillaries that impair normal gas exchange by permitting fluid entry into alveolar spaces [42]. Employing a murine model of MRSA ventilator-associated pneumonia, investigators observed striking neutrophilia in bronchoalveolar lavage (BAL) fluid (>90% BAL cells) 10–24 h after infection [42]. As the infection progressed, excessive numbers of accumulating neutrophils within the alveolar space amplified NET production, exacerbated local tissue injury, and worsened clinical symptoms relative to controls [42]. Neutrophil death via NET formation might also contribute to the development of autoimmune disorders by exposing intracellular endogenous components, such as chromatin, to cells of the immune system, which can potentially heighten the inflammatory response and provoke the development of autoantibodies, as demonstrated in **low-density granulocytes** derived directly from systemic lupus erythematosus patients (SLE) [35,43,44].

Chronic NET production has also recently been linked to the pathogenesis of atherosclerosis [45], as demonstrated by using a hypercholesterolemic (apolipoprotein E-deficient or $Apoe^{-/-}$) mouse model genetically depleted of a neutrophil survival factor ($Apoe^{-/-}Ly6g^{cre}Mcl1^{flox/flox}$), or genetically altered to enhance systemic neutrophilia via inhibition of CXCR4 ($Apoe^{-/-}Lyz2^{cre}Cxcr4^{flox/flox}$) [46]. Compared with modified mice, activated neutrophils of wild-type (WT) controls were attracted to regions of arterial tissue damage and sterile inflammation, where they increased local concentrations of ROS and completed NETosis when efferocytotic pathways capabilities were exceeded [46]. In this study, however, the normal extracellular extrusion of citrullinated histone H4 during NET formation unexpectedly caused arterial smooth muscle cell death via rapid, receptor-independent membrane pore formation [46]. This lytic arterial smooth muscle cell death further contributed to vascular tissue damage, plaque destabilization, and greatly increased the imminent risk of myocardial infarction relative to controls [46]. Therefore, attenuating the ability of neutrophils to accumulate in atherosclerotic plaques or preventing their death via NETosis might be exploited to develop novel candidate pharmacologic therapies for the prevention of heart attacks and strokes.

Gasdermin D: A Link between Pyroptosis, NETosis, and Efferocytosis?

GSDMD is believed to participate in pyroptosis, **pore-induced intracellular traps (PITs)** formation (Box 3), efferocytosis, and NET-mediated (Box 4) cell death pathways. Differences in GSDMD processing may link these cell death mechanisms, although it is yet to be determined: (i) which pathways are employed to defend against microbial invasion or sterile inflammation, and (ii) which are dependent upon the initial processing of GSDMD into its lethal fragment and additional factors. Inflammatory caspase-mediated pyroptosis and NSP-activated NETosis can cleave GSDMD at different sites to produce functional GSDMD-N fragments with variable lengths [23,25,26,30].

Thus, during an infectious challenge, it is possible that pathogen ensnarement by NETs might occur following extracellular release by pyroptotic neutrophils. Simultaneously, PITs may enhance the accumulation of neutrophils and macrophages to the inflamed site through release of inflammatory cytokines, while containing the intracellular bacteria within the dying cell's corpse [47]. By activating efferocytosis, PITs might also function to impair pathogen replication and escape, while also moderating proinflammatory responses [47]. Therefore, GSDMD-mediated neutrophil death may have context-dependent proinflammatory or anti-inflammatory effects and may offer a unique target for anti-inflammatory or antibacterial pharmacologic therapies, although further investigations are necessary to clearly define its role [23].

Autophagy: A Noninflammatory Cell Death

Autophagy is an evolutionary conserved intracellular degradation and energy recycling system that aims to maintain cell homeostasis in response to cellular stress or starvation [48,49]. Autophagy represents a mechanism by which the cytosol and organelles can be sequestered within double-membrane vesicles (autophagosomes) and be delivered to lysosomes and vacuoles for enzymatic degradation and recycling via self-cannibalization, for example, in cases such as combatting cancer [50,51] and/or bolstering host defenses [48,52,53]. By mobilizing intracellular lipid stores, autophagy also provides an essential energy source to support mitochondrial respiration during neutrophil differentiation and maturation within the bone marrow, which is necessary to facilitate the extensive cytoplasmic and nuclear remodeling that occurs during this process [54].

Triggered by phagocytosis-dependent and phagocytosis-independent (phorbol 12-myristate 13-acetate) mechanisms [53,55], the mammalian autophagic process represents an essential clearance mechanism for many internalized pathogens, such as *Streptococcus pneumoniae* [56], *Leishmania donovani* [57], and adherent invasive *Escherichia coli* [58], a phenomenon often termed **xenophagy**. Xenophagy is initiated either by (i) cytosolic ubiquitination of the microorganism's outer cell membrane and subsequent recognition by **p62** and light-chain 3-II (**LC3-II**) of the **phagophore** or (ii) canonical methods, in which bacteria enter neutrophils via endosomal compartments [58]. The phagophores or endosomes are subsequently engulfed by a double-membrane vesicle, leading to the formation of an autophagosome, which enables pathogen destruction via the cell's lysosomal machinery [58] (Box 5).

Box 5. Importance of Autophagy-Related Gene Proteins in Autophagy as Regulators of Neutrophil Function

At present, two evolutionarily conserved cell sensors are believed to control autophagy in mammals: (i) class I phosphatidylinositol 3-kinase (PI3K), which induces mammalian target of rapamycin (mTORC1) to inhibit autophagy through the promotion of nutrient uptake and metabolic activities and (ii) class III PI3K vacuolar protein sorting 34 (Vps34), which counterbalances mTORC1 to directly trigger autophagosome formation [98]. Autophagy-related gene (ATG) proteins, which are downstream components of mTORC1, are also essential regulators of autophagy [53]. Upon induction by autophagy-related stimuli, ATG proteins become activated and are recruited to begin autophagosome formation [53]. In murine models, Atg proteins exhibit control of important intrinsic neutrophil properties, including cellular differentiation and proper execution of innate immune responses [48,53].

As an essential regulatory mechanism of neutrophil function, autophagy directly impacts cellular differentiation, phagocytosis, cytokine production, degranulation, IL-1β production, bacterial killing, and cell death [48]. Whereas inhibition of mTORC1-induced autophagy blocks neutrophil differentiation, deletion of *Atg5* accelerates neutrophil proliferation and hastens cellular differentiation within murine bone marrow myeloid hematopoietic stem cell precursors [99]. Alternatively, murine neutrophils deficient for *Atg7* exhibit marked impairments of gelatinase and specific degranulation due to secretion (not production) defects, reductions in the severity of neutrophil-mediated inflammatory responses, and decreased NADPH-mediated ROS generation [48]. Simultaneous deletions of *Atg5* and *Agt7* in murine models not only dampen NADPH oxidase-mediated ROS production and hinder degranulation, but also attenuate neutrophil-mediated inflammatory and autoimmune conditions [48]. Neutrophil maturation is also directly controlled by *Atg7* in mice, with knockouts exhibiting an accumulation of functionally and phenotypically immature neutrophil populations within the bone marrow [54]. Atg7 supports neutrophil differentiation through autophagy-mediated lipid degradation and fatty acid oxidation, which provides the necessary energy to facilitate mitochondrial respiration and enhance ATP production [54].

Because the dying neutrophil's cell membrane remains intact during autophagy, intracellular pathogens capable of subverting classical PICD killing mechanisms for immune evasion or intracellular replication can still be destroyed without exacerbating *in situ* inflammation [58]. Autophagic cell death may also function to control the influx of neutrophils to inflamed sites to safeguard against excessive tissue injury or the development of chronic inflammation or autoimmunity, as observed in models of **Crohn's disease** using adherent-invasive *E. coli* infection of neutrophil-like PLB-95 cells [58]. Moreover, mice engineered to exhibit impairments of autophagy via myeloid-specific deletion of autophagy-specific *Atg7* were shown to have an elevated number of circulating neutrophils, reduced proinflammatory responses with decreased NADPH oxidase-mediated ROS production, and impaired degranulation of **tertiary and secondary granules** relative to WT controls [48].

Neutrophil Aging, Functionality, and the Microbiome

Human and murine neutrophils exhibit natural circadian oscillations in both their phenotype and numbers in circulation under steady-state conditions [59]. Once neutrophils are released into the bloodstream from bone marrow stores, they begin the aging process and become progressively more proinflammatory (Box 6) [59]. Recent analysis of the neutrophil transcriptome of healthy human volunteers and mice suggests that neutrophil cell aging is regulated by the circadian-related gene *Bmal1* [brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1; encoded by *Arntl*] through the expression of chemokine Cxcl2 [59,60]. Cxcl2 induces chemokine receptor Cxcr2-dependent diurnal changes in both transcriptional and migratory properties of circulating neutrophils [59,60]. To formally test these genes, mice were generated with neutrophil-specific deficiencies in *Arntl*, *Cxcr2*, and *Cxcr4* [59] or timed pharmacological neutralization of Ccr2 [60]. Under normal, homeostatic conditions, blunting or absence of Bmal1 and Cxcr4 prevented the accumulation of 'primed', aged neutrophils into inflamed areas at night time, where their presence could have exacerbated local tissue injury [59]. However, these cells retained their ability to migrate into naïve tissues, where they could quickly mount a robust proinflammatory response against infectious stimuli [59]. Moreover, mice engineered with constitutive neutrophil aging through deletion of *Cxcr4*, a negative regulator of Cxcr2 signaling, displayed unrestrained neutrophil aging and improved survival against infection, but were predisposed to thrombo-inflammation and untimely death [59,60]. Thus, therapeutics that inhibit or slow neutrophil aging might provide innovative treatments for atherosclerosis, while

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Box 6. Neutrophil Clearance and 'Cloaking'

Most neutrophils never encounter a pathogen. As these cells age, their cell membrane L-selectin expression (CD62L) decreases, while CXCR4 (C–X–C chemokine receptor type 4) expression simultaneously increases [100]. These changes facilitate the cell's migration back to the bone marrow via the CXCL12 (C–X–C motif chemokine ligand 12 or SDF-1α)–CXCR4 chemoattractant pathway [100]. In addition, aging human neutrophils that remain senescent experience a concomitant decrease in cytoplasmic antiapoptotic MCL-1 amounts [101], prompting nonlytic TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis and clearance by bone marrow macrophages [101]. Because only nonapoptotic neutrophils can migrate back to the bone marrow [100], those that die in circulation are generally removed by Kupffer cells (liver-resident macrophages) that live immobilized within the liver vasculature [102] or by macrophages in the reticuloendothelial system of the spleen [103] in an IL-17/IL-23/G-CSF cytokine axis-dependent fashion [104]. All told, the bone marrow, liver, and spleen have each been shown to contribute to neutrophil clearance at steady state [101].

Mechanisms by which routine tissue homeostasis can be maintained without neutrophil activation and **swarming** have recently been described as neutrophil 'cloaking' [105]. Maintenance of tissue homeostasis and prevention of early neutrophil-mediated inflammatory damage are critical for local repair of cell injury, which occurs regularly in many organs due to mechanical and other stresses. Cloaking is a process by which tissue-resident macrophages can rapidly sense a small-scale, or even individual, cell death. By extending membrane processes around the dying corpse, activated macrophages can sequester the damage or area of inflammation. This acts to avert early chemoattractant signaling cascades that could produce neutrophil swarms to the inflamed site [105].

pharmacologic agents that promote aging might potentially offer adjuvant treatment options against certain types of infectious diseases.

In murine models of proinflammatory or endotoxemic conditions, fewer aged neutrophils are returned to the bone marrow than in controls, with trafficking instead targeting to inflamed tissue foci of infection [61]. Moreover, engagement of TLR4 and p38 MAPK-dependent pathways enable aged neutrophils in WT mice to induce conformational changes in β2 integrins that facilitate their sentinel role in the inflammatory response, compared with *Tlr4*-deficient mice [61]. In these circumstances, older neutrophils arrive instantly and much sooner than nonaged cells [61]. Therefore, neutropenia observed during severe sepsis episodes may result not only from depletion of bone marrow storage pools, but also from inhibition of bone marrow progenitor cells caused by reduced granulocyte colony-stimulating factor (G-CSF) stimulation and impaired TLR signaling due to decreased quantity and composition of the host microbiota following antimicrobial exposures [61].

Neutrophil homeostasis is regulated by interactions with the host microbiota through activation of TLRs and induction of myeloid differentiation factor 88-mediated (Myd88) signaling pathways [62]. By employing a relative germ-free experimental mouse model, investigators documented dramatic reductions in the number of circulating aged neutrophils compared with WT controls [62]. Germ-free mice also experienced attenuated pathogenesis and inflammation-related organ damage caused by endotoxin-induced septic shock relative to controls [62]. Neutrophil numbers in modified mice, however, rebounded to normal levels following restoration of the TLR4 ligand via intragastric gavage of LPS [62]. Further evidence was provided by adoptive transfer of *LysM-cre/myd88*$^{fl/fl}$ neutrophils into WT recipient mice that completely abrogated neutrophil aging; by contrast, aging kinetics remained unchanged when WT neutrophils were transferred into *LysM-cre/myd88*$^{fl/fl}$ recipient mice [62]. Additional support is offered by a study that obliterated the microbiota of murine pups via prolonged exposure to broad-spectrum antibiotics before and after birth [63]. Compared with controls, treated pups not only exhibited a reduced number and variability of intestinal microbes, but also experienced granulocytopenia with loss of biologically active aging neutrophils in circulation and suffered from a rise in *E. coli* sepsis-related mortality [63]. Restriction of progenitor cells in the bone marrow and decreased plasma G-CSF concentrations were shown to contribute to these findings [63].

In summary, depletion of the host microbiota can reduce the number of biologically active aging neutrophils circulating in the bloodstream as well as inhibit bone marrow production of new cells due to low G-CSF concentrations [63]. These factors can lead to diminished innate immune responses required to resolve early infection [63]. By contrast, increased numbers of aged circulating neutrophils can result in resistance to infection, but their presence may predispose animals to thrombo-inflammation [59]. This finding may partly explain why circadian susceptibility to cardiovascular disease and worsening of clinical symptoms related to infection can be observed in mammals during overnight and early morning hours [59,64].

Deficient Neutrophil Clearance, Human Disease, and Current Therapeutics

Nearly 200–300 billion human cells are estimated to turn over every day, but the exact modalities involved in preserving homeostasis remain largely unknown due to our inability to track all dying cells, and the difficulty to account for the considerable number of phagocytic cells required for their clearance [5]. How neutrophils meet their end has different immunologic consequences. Whereas nonlytic forms of neutrophil death elicit anti-inflammatory responses due to containment of DAMPs, death pathways resulting in membrane rupture enable extracellular extrusion of DAMPs and toxic granular substances, leading to deleterious proinflammatory responses and

local tissue injury; these in turn, may progress to end-organ damage, development of chronic autoimmune disorders such as SLE [44] and rheumatoid arthritis [65], or even death [3].

Because of their short life span and large numbers in circulation, disturbances in the delicate balance between neutrophil production and clearance can be detrimental, potentially leading to inflammatory, infectious, and/or autoimmune conditions [5]. Cohen syndrome, for example, is a rare human genetic disorder resulting from an exaggerated rate of neutrophil apoptosis due to mutations in the *VPS13B* (*COH1*) gene in the absence of inflammation or infection [66]. Patients generally exhibit intermittent neutropenia and recurrent minor infections, although neutrophil functions appear normal [67]. Alternatively, certain autoimmune diseases, such as SLE, have been proposed to potentially develop when a high number of apoptotic neutrophils overwhelm their clearance mechanisms, thus resulting in necrosis or NETosis, along with expression of cell surface autoantigens and subsequent generation of autoantibodies [68]. Thus, nonsteroidal anti-inflammatory drugs, corticosteroids, and **methotrexate** are relied upon to attenuate disease severity, promoting neutrophil apoptosis and decreasing neutrophil migration to inflamed sites [68].

Blocking autophagy may also benefit patients afflicted with certain types of neutrophil-mediated autoimmune diseases [69]. The antirheumatic, antimalarial drugs hydroxychloroquine and chloroquine impair autophagy through inhibition of autophagosome–lysosome fusion and degradation of autophagosome contents [70]. These drugs are currently recommended for the treatment of active SLE [71] and can attenuate disease severity and improve patient survival, although prolonged use has also been shown to inhibit amyloid plaque degradation and might increase the risk of developing Alzheimer's disease [72].

Concluding Remarks

Neutrophil turnover is essential for maintaining normal cellular homeostasis and host health. At steady state, most neutrophils never encounter a pathogen or inflammatory process, remain quiescent as they age, and eventually complete noninflammatory programmed cell death by apoptosis with subsequent removal by professional phagocytes. Cell death pathways following neutrophil exposure to infectious or inflammatory mediators, however, can include lytic and nonlytic methods, or NET formation. Although these cell death pathways probably do not occur in isolation, the exact mechanisms of crosstalk among them remain unknown and may differ between species models, adding caution to human inferences.

Because neutrophil death is regulated by interactions with commensal and/or pathogenic microorganisms, understanding the coevolution between these cell death pathways and the host microbiota is important. Perturbations in newborn health that impede the natural development of the host microbiome, such as dietary practices or prolonged antibiotic therapy, can increase the infant's risk for the development of allergies, metabolic disorders (such as obesity and diabetes), inflammatory or autoimmune diseases, as well as neurodevelopmental defects [73,74]. Investigating how the developing microbiome influences the neutrophil's lifecycle is particularly advantageous during the neonatal period (first 28 days of life), a period in which host defense is heavily reliant upon innate immune responses due to a relative absence of adaptive immune responses from lack of antigen exposure *in utero*, and is an active field of inquiry (see Outstanding Questions).

The recent discovery of an intrinsic neutrophil timer, with strict diurnal regulation and increased proinflammatory responses with cell aging, has been hypothesized to contribute to the increased risk of human death from cardiovascular disease [75] and inflammatory conditions [76] during early morning hours, and is potentially amenable to pharmacologic manipulation. Humans

Outstanding Questions

How do various neutrophil death pathways overlap or function synergistically during times of infection? Can they be modified to prevent the overwhelming release of toxic neutrophil proinflammatory mediators that worsen tissue injury and facilitate septic shock?

Is the spectrum of neutrophil death pathways reflective of evolutionary selective pressures to contend with diverse microbes that have developed sophisticated strategies to exploit established biologic processes and/or evade particular microbicidal effectors?

How do differences in neutrophil maturation (promyelocytes, myelocytes, metamyelocytes, bands, and segmented forms) affect subsequent cell death mechanisms? Does the release of immature neutrophil forms during acute sepsis serve a specific function? or is it nature's attempt to 'throw in the kitchen sink' and get 'all hands-on deck'?

How do dietary choices and antibiotic exposure, leading to associated modifications in the host microbiota, change the neutrophil's ability to function as an immune sentinel and protect against infectious pathogens? For better or worse?

A large number of neutrophils are produced in the bone marrow each day. A generous storage pool of neutrophils also exists for immediate release into the bloodstream during acute infection, creating the classical 'left-shift'. In the absence of infection, what mechanism(s) are employed to turnover storage pool neutrophils?

typically experience a **left-shift**, or large influx of immature granulocytes into the bloodstream, during early stages of inflammation and/or infection. Understanding the role that early neutrophil precursors play in pathogen clearance and how maturity of neutrophils in the bloodstream skews cell death pathways or impacts disease resolution and healing may also offer additional innovative targets for therapeutic interventions. Illuminating how cell death mechanisms overlap, either synergistically or not, may also provide important targets for the putative treatment of conditions such as infection/sepsis, autoimmune or inflammatory diseases, cancers, organ/tissue transplantation, and/or neurodegenerative conditions.

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