

Neutrophil apoptosis and the resolution of infection

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Abstract Polymorphonuclear leukocytes (PMNs) are the most abundant white cell in humans and an essential component of the innate immune system. PMNs are typically the first type of leukocyte recruited to sites of infection or areas of inflammation. Ingestion of microorganisms triggers production of reactive oxygen species and fusion of cytoplasmic granules with forming phagosomes, leading to effective killing of ingested microbes. Phagocytosis of bacteria typically accelerates neutrophil apoptosis, which ultimately promotes the resolution of infection. However, some bacterial pathogens alter PMN apoptosis to survive and thereby cause disease. Herein, we review PMN apoptosis and the ability of microorganisms to alter this important process.

Keywords Neutrophil · Cell death · Apoptosis · Phagocytosis · Pyroptosis · Autophagy · Macrophage · Inflammation · Pathogen · Innate immunity

Introduction

Polymorphonuclear leukocytes (PMNs or neutrophils) are the most abundant leukocyte in humans and typically the first type of white cell recruited to sites of infection. Neutrophils are the primary cellular defense against bacterial and fungal infections. PMNs ingest microbes by a process known as phagocytosis, and the ingested microorganisms are destroyed by the combination of reactive oxygen species (ROS) and cytotoxic components of granules [1–4] (Fig. 1). This task is facilitated by the numerous receptors enriched on the cell surface and proinflammatory stimuli.

On the other hand, PMNs contain or produce many cytotoxic molecules that can cause significant damage to host tissues/organs if the inflammatory response is not tightly regulated. Resolution of the inflammatory response is a complex process and includes production of

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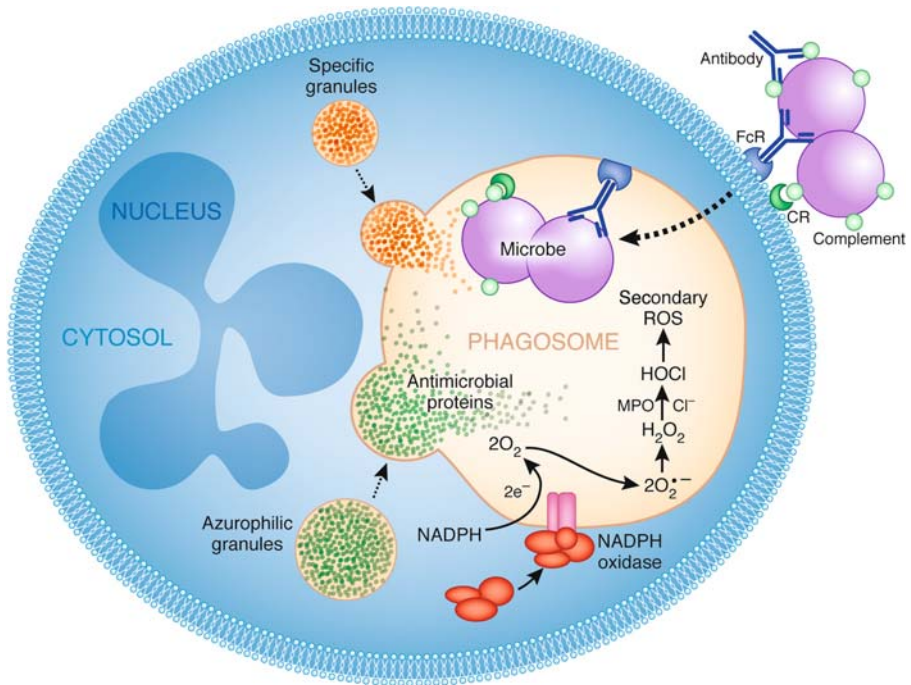


Fig. 1 Neutrophil phagocytosis and microbicidal activity. Following phagocytosis, microbes are destroyed by ROS and antimicrobial proteins released from granules. See text for details. FcR, Fc receptor; CR, complement receptor; MPO, myeloperoxidase

host-derived anti-inflammatory mediators and apoptosis of PMNs. Specifically, PMN apoptosis and removal of apoptotic PMNs by macrophages is a mechanism to clear effete neutrophils and ultimately facilitate the resolution of inflammation.

This review highlights two areas of neutrophil biology related to immune system homeostasis and the innate host defense, namely, PMN apoptosis and the ability of microbes to manipulate this process, and the resolution of the acute inflammatory response, including clearance of apoptotic cells by macrophages.

Granulocytes, granulopoiesis, and the development of myeloid cells

Granulocytes (basophils, eosinophils, and neutrophils) can be differentiated physically from lymphocytes and other circulating leukocytes based upon multiple phenotypes, such as the presence of characteristic granules in the cytoplasm, a multilobed nucleus and specific cell surface markers (Fig. 2, top panels). Development of granulocytes involves transcription factors distinct from those that facilitate lymphocyte maturation [5–9]. Many of these transcription factors are required for granule protein maturation and ligands essential for PMN activation, as different granules appear during separate stages of PMN development ([9], reviewed in [1, 10]). Secondary or specific granules predominate in mature neutrophils, whereas the relative number of azurophilic or primary granules decreases during granulocyte maturation [11].

Circulating leukocytes and other cells of the immune system develop from pluripotent stem cells located in bone marrow [12]. Granulocytes differentiation proceeds through a

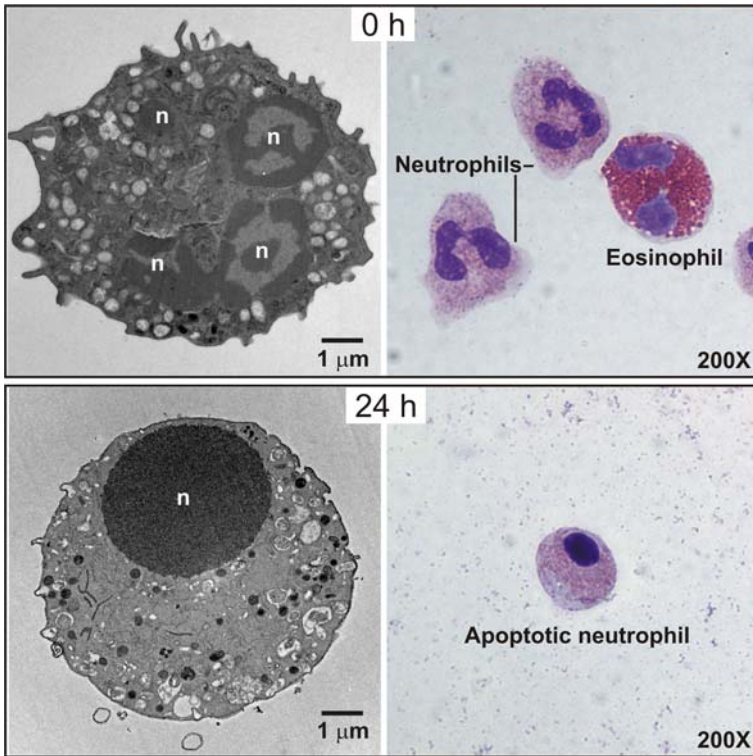


Fig. 2 Neutrophil apoptosis. Human neutrophils isolated from peripheral blood have multilobed nuclei when fresh (0 h), whereas those cultured for 24 h *in vitro* have condensed nuclei of apoptotic cells (24 h). Left panels, transmission electron micrographs. Right panels, brightfield micrographs of PMNs stained with a modified Wright-Giemsa. Nucleus, n. Transmission electron microscopy was performed by David W. Dorward at Rocky Mountain Laboratories, NIAID

concerted series of steps that include granule formation and ultimately exit of mature PMNs from bone marrow to peripheral blood [1, 9–11, 13–18]. This maturation and differentiation process takes ~6.5 days and is called granulopoiesis, i.e., the formation of granulocytes [11]. Although PMNs have a relatively short life span, the greatest percentage of hematopoiesis is committed to the production of neutrophils—nearly 60% of all leukocytes in bone marrow are granulocyte precursors. Mature PMNs are terminally differentiated cells and circulate in the bloodstream for ~10–24 h before migrating into tissues [15–18]. Neutrophils may function for an additional 1–2 days in tissues before undergoing apoptosis [11, 17] and are then cleared by macrophages [19, 20]. Remarkably, PMN turnover is on the order of 10^{11} cells per day in the average adult human [15].

Recruitment of neutrophils from the bloodstream

Neutrophil recruitment, priming, and extravasation

Neutrophils survey venules and lymphatic organs for signs of invading microorganisms and/or host tissue injury and inflammation. These “signs” are often chemoattractants, host- or

microorganism-derived molecules recognized by receptors on the cell surface. Chemoattractants recruit PMNs to sites of infection or areas of tissue damage. Most chemoattractants, such as IL-8, lipopolysaccharide (LPS), and N-formylated peptides (e.g., fMLP), also prime neutrophils for enhanced function (see Table 1 in [21]).

Neutrophil priming was originally defined as the ability of a primary agonist such as LPS to enhance superoxide production by a secondary stimulus [22]. Secretory vesicles, gelatinase-containing granules, and a limited population of specific granules fuse with the plasma membrane during priming [23], thereby enriching the cell surface with receptors important for host defense and the inflammatory response. Primed cells can be differentiated from unprimed cells in part by distinct cell morphology, e.g., cell size and/or granularity by flow cytometry, or increased expression of surface receptors such as CD11b [24]. Some priming agents are agonists or ligands of TLRs, and in vitro and in vivo experiments have shown that TLRs are critical for PMN priming as well as pathogen recognition [25].

Neutrophils enter sites of infection by exiting peripheral circulation through the endothelial wall in a process called extravasation. This process is initiated by selectins, a C-type lectin family of glycoproteins that are expressed on activated endothelial cells (E- and P-selectin), platelets (P-selectin), and primed/activated PMNs (L-selectin, CD62). As part of the inflammatory response, E- and P-selectin become up-regulated on endothelial cells and interact with L-selectin expressed on the surface of neutrophils. This initial interaction, called “tethering,” facilitates subsequent rolling of PMNs along the surface of the endothelium. Rolling is followed by firm adhesion promoted by CD11b/CD18 [26] and CD54 [27], and transmigration is mediated in part by CD31 [28], CD54 [27], CD44 [29], and CD47 [30]. Following extravasation, there are several possible fates for PMNs related to apoptosis and turnover, and these are discussed in detail below.

PMN microbicidal activity

PMNs destroy pathogens through a series of well-coordinated steps that result in the production of ROS and enrichment of phagosomes with cytotoxic molecules from cytoplasmic granules (called degranulation) (Fig. 1). ROS are derived from superoxide produced by a multisubunit enzyme complex called NADPH oxidase (reviewed in [3]). The phagocyte NADPH oxidase is composed of at least 7 proteins that reside in cytosolic and membrane compartments in resting PMNs. During cell activation, cytosolic oxidase components translocate to the phagosome membrane and associate with the membrane component, flavocytochrome b_{558} , to form the assembled NADPH oxidase. NADPH oxidase assembly and activation is regulated by multiple signaling events, including phosphorylation of one of the cytosolic proteins, $p47^{phox}$, and several SH3-domain interactions [3, 31]. NADPH oxidase catalyzes the formation of superoxide (O_2^-), which dismutates rapidly to H_2O_2 , although O_2^- and H_2O_2 are weakly microbicidal [2].

As the phagosome forms/matures, granule components such as myeloperoxidase (MPO) accumulate in the phagosome [11]. MPO catalyzes a reaction with H_2O_2 and chloride to form hypochlorous acid (HOCl) and other secondarily derived ROS such as OH- and singlet oxygen [32]. Neutrophil cytoplasts—neutrophils lacking nuclei and depleted of cytoplasmic granules—retain a respiratory burst, but lack MPO. Odell and Segal reported that cytoplasts phagocytose *Staphylococcus aureus*, but bacteria are not killed unless they are coated with MPO [33]. MPO activity is inhibited by azide, and to a lesser extent cyanide, and this inhibition decreases killing of bacteria by neutrophils [34].

Landmark work by Rosen and Klebanoff [35] demonstrated that the key antimicrobial components of human PMNs, NADPH oxidase and the MPO-halide system, serve as redundant mechanisms to kill ingested microbes. Consistent with this notion, MPO deficiency in humans does not necessarily correlate with morbidity from infections (reviewed in [4]). PMNs from MPO-deficient individuals retain microbicidal activity against several bacterial pathogens, although at a lower rate than PMNs from normal donors [4]. Collectively, these findings suggest that a combination of mechanisms is necessary for optimal killing of microbes.

In addition to the intracellular proteins that degrade the ingested microbes, the surface of PMNs is decorated with multiple receptors that bind to foreign particles, including those that bind pathogen associated molecular patterns (PAMPs). Many pattern recognition receptors (PRRs) do not promote phagocytosis directly, although they do prime phagocytes for enhanced uptake/activation or function as co-receptors. For example, TLRs recognize numerous PAMPs [36], but there is little data available to show that TLRs promote phagocytosis directly (reviewed in [37]). By comparison, neutrophil dectin-1 [38], a C-type lectin PRR, binds β -glucan residues on fungi and this interaction triggers phagocytosis [39]. Phagocytosis of unopsonized β -glucan particles is morphologically different than phagocytosis of complement-opsonized zymosan, suggesting that signal transduction following these receptor-ligand interactions is distinct [39]. Importantly, dectin-1 mediated phagocytosis elicits ROS production and degranulation, and ultimately killing of fungi [39].

PMN apoptosis and the resolution of the acute inflammatory response

Normal turnover of cells, regardless of type or tissue location, is governed by apoptosis, and defects in apoptosis lead to profound and devastating conditions ranging from cancer to autoimmune diseases. Neutrophils undergo constitutive or spontaneous apoptosis as a mechanism to maintain immune system homeostasis [19]. Importantly, PMN apoptosis is accompanied by a general decrease in cell function and proinflammatory capacity, which is key to non-inflammatory removal of effete cells [40, 41]. Neutrophil apoptosis can be initiated by extrinsic pathway stimuli (extracellular, e.g., tumor necrosis factor- α (TNF α) or FAS ligand) or intrinsic pathway stimuli (intracellular, e.g., ROS and/or that mediated by mitochondria). Neutrophil apoptosis is a non-inflammatory process characterized by loss of cytoplasmic granules, rounding of the nucleus, and condensation of nuclear heterochromatin [19, 20] (Fig. 2, lower panels). PMNs also undergo apoptosis after phagocytosis, a process also known as phagocytosis-induced cell death (PICD, see below). Apoptotic neutrophils are subsequently ingested by macrophages, providing the means to resolve the inflammatory response without releasing cytotoxic molecules that would otherwise damage host tissues. Phagocytosis of apoptotic PMNs was first observed by Elie Metchnikoff in the 19th century [42].

Spontaneous (constitutive) PMN apoptosis

PMN apoptosis can be induced through multiple mechanisms (Fig. 3a, b). Spontaneous neutrophil apoptosis is an intrinsic process, meaning that the signal to initiate apoptosis originates from within the cell (Fig. 3a). Many proteins and/or molecules are known to regulate constitutive PMN apoptosis and only a few examples are provided here. For example, MCL1 is a key BCL2-family protein located in the nucleus and cytoplasm of PMNs [43–45].

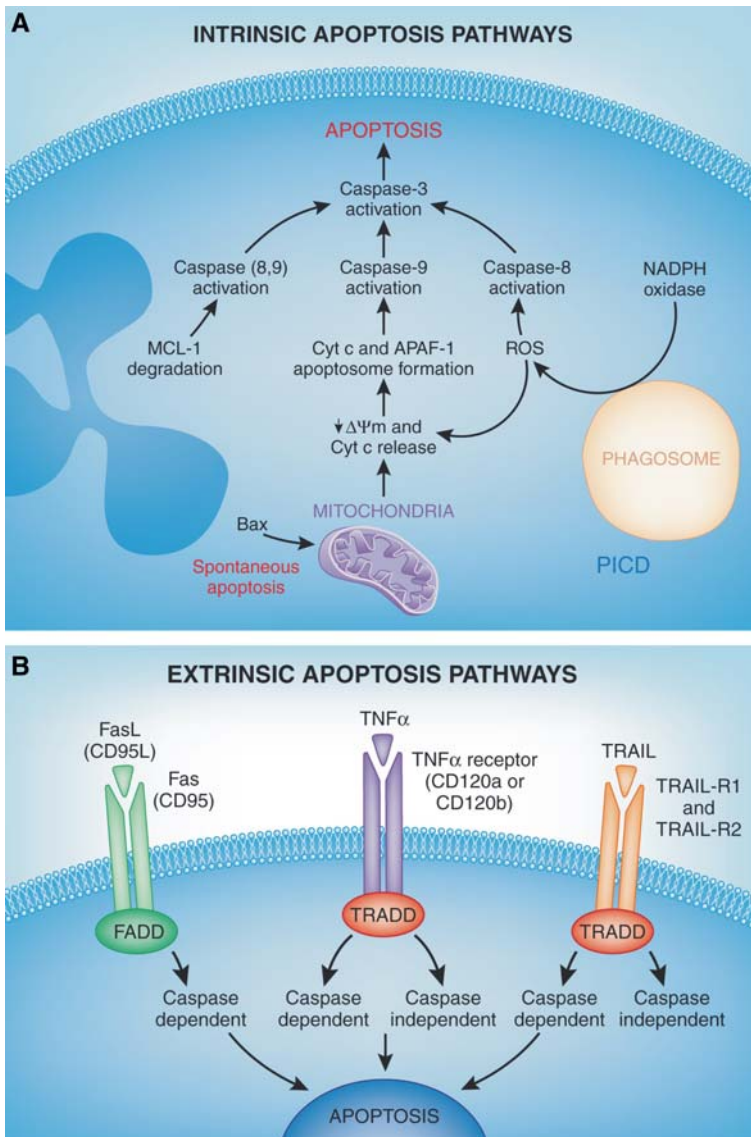


Fig. 3 Mechanisms of PMN apoptosis. (a) Intrinsic pathways of apoptosis result from intracellular signals, while extrinsic pathways (b) originate from extracellular signals

As senescent neutrophils undergo apoptosis, MCL1 levels decline rapidly, presumably degraded by the proteasome (and there is a concurrent decrease in *MCL1* mRNA), suggesting neutrophil survival is regulated by MCL1 expression [46].

Src homology 2 domain-containing inositol 5'-phosphatase (SHIP-1) is an intracellular protein critical for signal transduction during PMN apoptosis [47]. The molecule is a negative regulator of immune receptor and cytokine and growth factor receptor signaling [48]. SHIP-1 is recruited to the plasma membrane of myeloid cells after ligation of CD18 [49], and this initiates an anti-apoptotic signaling cascade that requires the Src kinase Lyn and

the anti-apoptotic protein, Akt [48]. *Ship-1^{-/-}* mice have increased numbers of myeloid cells and form lung granulomas after infection [50]. It was proposed that the granulomas fail to resolve because *Ship-1^{-/-}* cells display enhanced survival and proliferation [50].

BCL2A1 is another protein important for PMN survival. Like MCL1, BCL2A1 is a member of the BCL2 family of proteins, and *BCL2A1* mRNA is constitutively expressed in human neutrophils [51]. PMN *BCL2A1* is up-regulated by G-CSF or LPS, agonists known to promote neutrophil survival [51]. Promyelocytic HL-60 cells have increased *BCL2A1* mRNA after neutrophilic differentiation with all-trans retinoic acid [51], suggesting that the protein has an important role in mature neutrophils.

BCL2-associated X protein (BAX) interacts with the mitochondria, an intracellular organelle known to regulate PMN survival [52, 53]. In contrast to BCL2A1, BAX is a BCL2-family member that promotes neutrophil apoptosis. Upon translocation to the mitochondria, BAX is cleaved by calpain-1 to an 18-kDa fragment unable to interact with BCL2 family members, thereby promoting apoptosis [54, 55]. Inhibition of BAX cleavage in vivo leads to increased and prolonged inflammation, indicating that the protein is required for the resolution of acute inflammation [52]. Using BAX antisense gene silencing, Dibbert et al. showed that PMN apoptosis is delayed in several human inflammatory diseases and there is an associated decrease in BAX protein expression [56].

Soluble extracellular factors also influence spontaneous PMN apoptosis [57]. For example, macrophage migration inhibitory factor is a proinflammatory cytokine that delays cleavage of BAX and ultimately neutrophil apoptosis [58]. PMNs stimulated with G-CSF or GM-CSF have decreased expression of BAX, suggesting the anti-apoptotic effect of these cytokines is in part related to inhibition of BAX. GM-CSF and G-CSF also preserve the chemotaxis and phagocytosis capacity of PMNs aged in vitro [40, 59], indicating inhibition of apoptosis is tied to the preservation of PMN function.

In addition to BAX activity, mitochondria regulate their transmembrane potential, which is critical for cell survival. As depicted in Fig. 3a, ROS and oxidative stress can lead to PMN apoptosis through disruption of mitochondria transmembrane potential [60–65]. Neutrophil mitochondria contain calpain-1 and its release can activate pro-apoptotic factors or degrade anti-apoptotic proteins [66]. Although human neutrophils express low levels of cytochrome *c*, it plays an important role in apoptosis [65]. As cytochrome *c* is released from the mitochondria, it associates with apoptotic protease-activating factor 1 (APAF-1) and initiates apoptosis [53].

Several distal effector proteins participate in apoptosis. Caspase 3, caspase 8, and caspase 9 trigger proteolytic cascades that result in the apoptotic death of human neutrophils [67] (Fig. 3b). The importance of neutrophil caspases in inflammation is illustrated in a recent study by Tanejo et al. [68], in which it was reported that individuals with bacterial sepsis have significant reduction of caspase-3 and caspase-9 activities and decreased neutrophil apoptosis.

These are only a few specific examples of the molecules known to regulate spontaneous neutrophil apoptosis and overlap exists with extrinsic pathway stimuli as described below.

FAS/FAS ligand-mediated apoptosis

In contrast to spontaneous PMN apoptosis, FAS-mediated, TNF α -mediated and TRAIL-mediated apoptosis proceed by an extrinsic pathway, as an extracellular signal initiates apoptosis (Fig. 3b). Extrinsic pathway signals are usually soluble protein factors that bind to membrane-bound receptors on the cell surface and trigger apoptosis. FAS (CD95) is the nidus of the death-inducing signaling complex (DISC) and is expressed on the surface of neutrophils as well as on virtually all other cells in humans [69]. Neutrophils also express

FAS ligand (FASL, CD95L) [70], and FASL–FAS interaction initiates PMN apoptosis in a caspase-dependent manner. Because PMNs express FAS and soluble FASL, PMN apoptosis is triggered through autocrine and paracrine pathways. FAS-mediated PMN apoptosis can be suppressed by GM-CSF, G-CSF, interferon-gamma ($\text{IFN}\gamma$), $\text{TNF}\alpha$, and the tyrosine kinase inhibitors herbimycin A and genistein [70], thus highlighting the complexity of signaling pathways involved in regulating neutrophil turnover.

FAS is a transmembrane protein containing a FASL-binding extracellular domain and FAS-associated death domain (FADD) on its intracellular region. Interaction of FASL with FAS leads to clustering of FADDs on the cytoplasmic side of the plasma membrane, which, in turn, leads to caspase activation and apoptosis. As with many other signaling receptor complexes, e.g., T- and B-cell receptors, current evidence indicates that FAS accumulates in cholesterol-rich membrane domains called lipid rafts and that clustering of these receptors leads to caspase-8 activation and, ultimately, cell death. Macrophages also secrete FASL in response to phagocytosis [71]. Secretion of FASL by macrophages induces leukocyte apoptosis in a paracrine manner and assists further with the resolution of inflammation [71]. For example, Jimenez et al. studied patients with systemic inflammatory response syndrome, and PMNs from these patients have decreased FAS-mediated apoptosis and accompanying tissue damage following bacterial infections, presumably from the accumulation of activated neutrophils that release tissue degrading enzymes [72].

TNF α receptor (TNFR)-mediated apoptosis

The TNFR is a transmembrane protein containing an extracellular TNF α binding domain and a TNFR-associated death domain (TRADD) in the cytoplasmic region of the protein [73] (Fig. 3b). Neutrophils express two TNFRs, TNFRSF1A (55-R, CD120a, or TNFRI) and TNFSFR1B (75-R, CD120b, or TNFRII), and each has a slightly different role in PMN apoptosis. Gon et al. showed that TNFRI is required for TNF α -mediated PMN apoptosis and its ability to promote apoptosis is enhanced by TNFRII [74]. Blocking TNFRI but not TNFRII with specific antibodies inhibits neutrophil apoptosis [74]. Additional work has shown that TNFRI is dominant using TNFR-selective mutants [75]. As with FADDs, TRADDs cluster in lipid rafts during TNFR–TNF α binding. This clustering of signaling molecules leads to the recruitment of adaptor molecules and activation of transcription factors. Although similar to FAS/FASL-mediated apoptosis in many ways, one major difference is that TNF α receptor-mediated apoptosis does not depend solely on caspase activation [76].

TNF α can elicit inflammatory or anti-inflammatory effects depending on the type of cell with which it interacts. Notably, TNF α has been shown to have both pro-apoptotic and anti-apoptotic effects toward neutrophils. Van den Berg et al. showed that this bi-polar effect is concentration dependent [77]. At low concentrations (≤ 0.1 ng/ml), TNF α delays PMN apoptosis and elicits production of proinflammatory cytokines, whereas at higher concentrations TNF α initiates apoptosis [77]. Consistent with these observations, high concentrations of TNF α (10–100 ng/ml) override the ability of $\text{IFN}\gamma$ and GM-CSF to delay apoptosis [77]. Moreover, the ability of TNF α to induce neutrophil apoptosis was linked to ROS production, since PMNs from patients with chronic granulomatous disease (CGD, see below) and PMN cytoplasts fail to undergo apoptosis in the presence of high concentrations of TNF α [76, 77]. Individuals with TNFR-associated periodic syndrome (TRAPS) have a defect in the TNFR and, therefore, diminished TNF α -induced PMN apoptosis [78]. Patients with TRAPS experience recurrent fever attacks lasting >1 week that is associated with abdominal pain, severe arthromyalgias, rash, and periorbital edema. However, TRAPS has not been associated with increased infection [78].

TNF-related apoptosis-inducing ligand (TRAIL)

Our understanding of TRAIL-induced PMN apoptosis is relatively recent compared to that of FAS- or TNF-receptor-mediated apoptosis. Five TRAIL receptors (TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and TRAIL-R5) have been identified and PMNs express TRAIL-R2 and TRAIL-R3 constitutively [79, 80]. Of the five TRAIL receptors, only TRAIL-R1 and TRAIL-R2 have the ability to induce pro-apoptotic, caspase-dependent signaling (Fig. 3b), whereas TRAIL-R3 and TRAIL-R4 are decoy receptors that lack the ability to induce apoptosis [81]. TRAIL-R3 and TRAIL-R4 do not have intact death domains and these receptors have been proposed to serve as “decoys” that inhibit apoptosis either by sequestering TRAIL or by complexing with TRAIL-R1 or TRAIL-R2 upon binding to the ligand. Either mechanism would inhibit apoptosis, but there is paucity of data to provide conclusive support to this notion. TRAIL-induced apoptosis appears important for senescent PMNs that preferentially home to bone marrow after circulating in peripheral blood [82]. *Trail*^{-/-} mice have an enhanced susceptibility to autoimmune diseases [83], and Trail blocked experimentally in animal models exacerbates inflammation caused by type 1 diabetes [84] and autoimmune encephalomyelitis [85]. PMNs also express and secrete TRAIL, which has been implicated in host defense against virus-infected cells and tumor cells [86]. IFN γ up-regulates TRAIL expression and IFN γ -activated PMNs store TRAIL in an intracellular pool that is mobilized following exposure to proinflammatory mediators [79, 87, 88]. TRAIL expressed and secreted by PMNs acts in an autocrine manner to induce apoptosis [87].

Down-regulation of proinflammatory capacity

PMNs produce numerous cytokines and chemokines following phagocytosis, including TNF α and IL-8, which contribute to the inflammatory response [89–91]. Production of these molecules is regulated in part at the level of gene expression [92, 93]. Microarray-based studies have shown that phagocytosis initially up-regulates neutrophil transcripts encoding proteins that play an important role in the acute inflammatory response, such as IL-1 α , IL-1 β , IL-1 ϵ , IL-1RN, IL-8, IL-10, IL-12 β , IL-15, TNF α , vascular endothelial growth factor (VEGF), oncostatin M (OSM), IL-6, GRO β , and GRO γ [21, 94–97]. Taken together, these processes are critical for defense against invading microorganisms. However, once phagocytosis is complete and microbes are destroyed, these PMN functions are down-regulated coincident with the induction of apoptosis [95, 98–102]. For example, key proinflammatory or signal transduction molecules, including CXCR1 and CXCR2, are down-regulated during the initial stages of apoptosis [94]. Walcheck et al. have shown recently that phagocytosis-induced neutrophil apoptosis is accompanied by shedding of L-selectin and concomitant increased surface expression of ADAM17, a metalloprotease that likely plays an important role in the down-regulation of neutrophil function [103, 104]. In general, most primary PMN functions, including chemotaxis, phagocytosis, superoxide production, and degranulation, decrease significantly with apoptosis [40, 41].

IL-6 receptor (IL-6R) transsignaling

IL-6 signals through a receptor system composed of the IL-6R, an 80 kDa transmembrane protein that binds the signaling molecule gp130. Although the expression of IL-6R is restricted to a small subset of cells, gp130 is expressed ubiquitously. IL-6 can also elicit responses through a pathway involving soluble IL-6R (sIL-6R) and this phenomenon is

known as IL-6R transsignaling. sIL-6R is generated by either shedding from the cell surface by proteolytic cleavage or possibly alternative splicing of *IL-6R* mRNA [105, 106]. Chalaris et al. demonstrated that apoptosis is accompanied by the release of IL-6R from cells, a phenomenon mediated by ADAM17 [103, 104]. sIL-6R recruits mononuclear phagocytes to the site of infection, which are then either directly involved in the removal of apoptotic PMNs or differentiate into macrophages that carry out this task [107]. This process links the acute inflammatory stage of disease and resolution of the inflammatory response.

Resolvins and lipoxins

Endogenous mediators termed resolvins and protectins actively participate in the dampening of host responses to orchestrate resolution of inflammation. These endogenous mediators lend more evidence that the resolution of inflammation is an actively regulated program rather than a passive termination of the immune response. After the initial inflammatory response, these molecules promote recruitment of macrophages to sites of inflammation and initiate the resolution of the acute inflammatory response [108]. One of these mediators is known as lipoxin A4 (LXA4), which is generated from arachidonic acid via the lipoxygenase pathways. Together with the aspirin-triggered 15-epi-LXA4 (ATL), these molecules are among the first to down-regulate PMN infiltration and begin the “slow down” during the course of inflammation [109–111]. For example, lipoxins drive the resolution of inflammation by stimulating nonphlogistic uptake of apoptotic PMNs by fixed-tissue macrophages [112].

Removal of apoptotic PMNs by macrophages

As with removal of undesirable, autoreactive T-cells in the thymus, apoptosis is a mechanism to clear effete PMNs. Apoptotic cells maintain membrane integrity for a fixed period of time and need to be cleared quickly to prevent secondary necrosis and the release of cytotoxic molecules that would otherwise cause inflammation and/or damage to host tissues. Clearance of apoptotic neutrophils has been studied mainly with human monocyte-derived macrophages [113] and mouse alveolar macrophages [114]. Although clearance of apoptotic cells is not completely understood, several macrophage receptors have been implicated in this process (Fig. 4). These molecules include the phosphatidylserine (PS) receptor [114, 115], complement receptors [116], scavenger receptors and lectins [117], the $\alpha_v\beta_3$ /CD36/thrombospondin recognition system [118, 119], CD14 [119], and CD44 [121–123]. The redundancy of removal mechanisms suggests that clearance of apoptotic neutrophils is a critical process to preserve immune system homeostasis.

Phosphatidylserine receptor (PSR)

Transposition of PS from the inner leaflet of the plasma membrane to the outer leaflet is a traditional hallmark of cells undergoing apoptosis. Recently, Park et al. have shown that stabilin-2 serves as a novel PSR and that blocking stabilin-2 on macrophages inhibits phagocytosis of apoptotic cells and causes release of proinflammatory cytokines [124]. Miyanishi et al. identified Tim4 as a PSR through the use of an antibody library and showed that blocking Tim4 in vivo resulted in an accumulation of apoptotic cells in the thymus and the development of autoantibodies [125]. The transcript encoding the Psr is

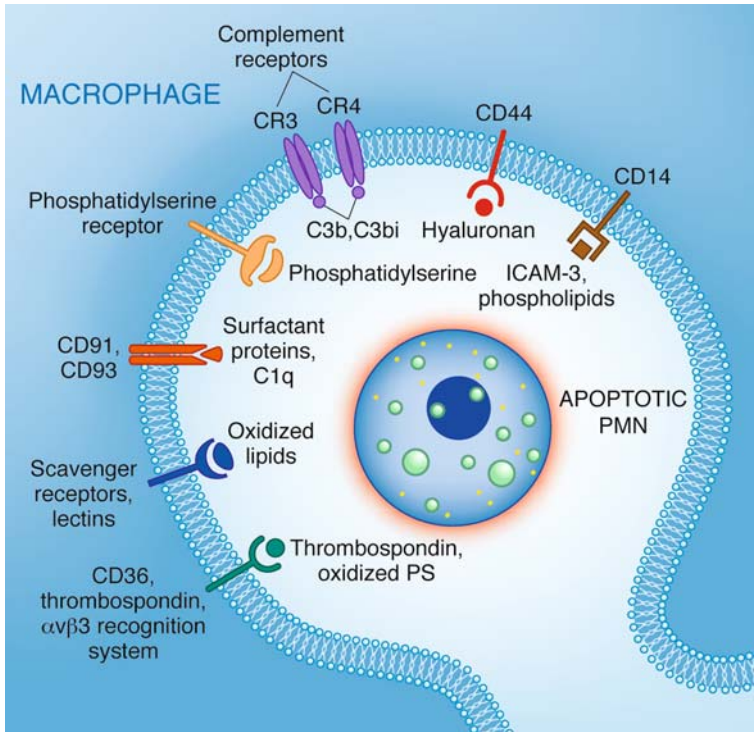


Fig. 4 Receptors on macrophages that bind antigens on apoptotic cells. Apoptotic neutrophils are recognized by specific receptors present on mononuclear phagocytes. See text for details

expressed in several tissues in mice [126]. Human and mouse macrophages express the Psr and it is required for clearance of apoptotic cells *in vitro* and *in vivo* [126–128]. Consequently, mice genetically deficient in the gene encoding Psr have accumulation of apoptotic cells in the lung and brain during embryogenesis and develop autoimmune diseases [126, 129]. It is possible that the generation of autoimmune diseases in these models results from the pool of apoptotic cells that releases autoantigens over time.

Complement receptors

The human complement cascade can be activated by antibodies (classical pathway) or by oligosaccharide antigens on microbes or apoptotic cells (lectin and alternative pathways) (reviewed in [130]). Each of the complement cascades is a sequential, proteolytic cascade that results in opsonization of targets with C3b(i) and/or lysis of opsonized cell by the membrane attack complex (MAC). Complement proteins, specifically C3b and its breakdown products, bind to apoptotic cells, possibly through the exposure of PS [116], and altered lipid and carbohydrate moieties. This process facilitates removal of apoptotic cells from tissues (reviewed in [131]). The majority of complement receptor phagocytosis is mediated through one of the four receptors that bind to complement component C3; CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18), and CR4 (CD11c/CD18). Macrophages express CR3 and CR4 as well as the C1q receptor, which binds to the first component of the classical complement pathway. The importance of C3 and C1q in apoptotic cell clearance

has been shown in mice with targeted deletion of the genes encoding these receptors [129, 132]. Grevnik et al. reported that although systemic lupus erythematosus patients have reduced total complement hemolytic activity in their sera, complement-mediated phagocytosis of apoptotic cells remained unaffected [133]. This observation can be explained by differential depletion of specific complement components in the patients, i.e., reduced levels of C9 and normal levels C3.

Vitronectin ($\alpha_v\beta_3$)/CD36/thrombospondin recognition system

Vitronectin ($\alpha_v\beta_3$ integrin), CD36, and thrombospondin are believed to function in a complex on macrophages and promote binding and uptake of apoptotic cells. Cooperation among these three antigens occurs during recognition of apoptotic PMNs by human monocyte-derived macrophages [118] and murine bone marrow-derived macrophages [113]. Thrombospondin functions somewhat like C3b in that thrombospondin binds to an unknown ligand on apoptotic cells, which then is recognized by the CD36/vitronectin complex expressed on macrophages. CD36 is an 88 kDa multifunctional glycoprotein, a B-type scavenger receptor, expressed by several cell types including monocytes, endothelial cells, epithelial cells, and several tumor cell lines [134]. In addition to thrombospondin, oxidized PS has recently been shown to be a ligand for CD36 [135]. Macrophage CD36, along with $\alpha_v\beta_3$ integrin, is important for the removal of apoptotic cells during the resolution of viral infections [119]. The domain on CD36 that is critical for the removal of apoptotic cells has been localized to amino acids 155–183 [136].

CD44

CD44 is a ~90 kDa surface protein that binds to hyaluronic acid. Ligation of CD44 generates intracellular signals that specifically augment clearance of apoptotic PMNs [121, 122]. For example, blocking CD44 with anti-CD44 Fab' antibodies decreases macrophage-mediated phagocytosis of apoptotic bodies [121]. Furthermore, CD44-deficient mice have reduced capacity to clear apoptotic PMNs post-inflammation [137].

CD14

CD14 is a 53–55 kDa protein expressed on monocytes, on macrophages, and at relatively low levels on granulocytes. Although CD14 is a co-receptor for LPS/LPS-binding protein [138], recent evidence indicates CD14 also binds intercellular adhesion molecule-3 (ICAM-3) on leukocytes [139]. CD14 can be expressed in soluble form (sCD14) and may bind apoptotic cells and subsequently react with a sCD14 receptor on phagocytes [140]. Little is known about the role of CD14 in the removal of apoptotic PMNs; however, there is good evidence that CD14 is crucial for the removal of apoptotic lymphocytes [141, 142].

Scavenger receptors and lectins

In addition to the flipping of PS from the inner leaflet to the outer leaflet of the plasma membrane, sugars and oxidized lipids that are exposed on the surface of apoptotic cells are modified and recognized as “non-self” by macrophages. These modifications are recognized by scavenger receptors and lectins expressed on the surface of macrophages and fibroblasts [143].

Non-inflammatory removal of apoptotic PMNs

Neutrophil apoptosis and the removal of apoptotic neutrophils is a nonphlogistic process. Phagocytosis of apoptotic cells by macrophages, monocytes, or dendritic cells causes these cell types to produce anti-inflammatory cytokines [144, 145]. For example, macrophages exposed to apoptotic cells secrete TGF- β , which suppresses LPS-mediated release of inflammatory cytokines by infected cells [146]. Macrophages also secrete factors that contribute to PMN apoptosis (e.g., soluble FASL [71]) and increase expression of anti-inflammatory factors [147]. For example, in the airway (bronchi and lungs), apoptotic PMNs are ingested by alveolar macrophages without release of inflammatory mediators (reviewed in [148]). Moreover, engagement of apoptotic cells by CD36 on macrophages inhibits production of proinflammatory cytokines TNF α , IL-1 β , and IL-12, but increases secretion of TGF- β and IL-10, both anti-inflammatory cytokines [149].

In addition to the autologous signals that contribute to the anti-inflammatory process, steroids—specifically glucocorticoids—promote a non-inflammatory environment during phagocytosis of apoptotic cells. Human and murine macrophages exposed to glucocorticoids for 24 h have enhanced ability to phagocytose apoptotic leukocytes [150]. Compared to macrophages, peripheral blood monocytes have a much lower phagocytic capacity toward apoptotic cells, but incubation of human monocytes with glucocorticoids induces the phenotype of monocyte-derived macrophages much more competent to ingest apoptotic cells [19, 151]. The ability of monocytes to phagocytose apoptotic leukocytes after incubation with glucocorticoids has been linked to 11 β -hydroxysteroid dehydrogenase (11 β -HSD), a molecule not present in monocytes [152, 153]. Cytokines also influence phagocytic capacity of macrophages toward apoptotic cells. For instance, IFN γ inhibits the glucocorticoid-induced ability of macrophages to clear apoptotic PMNs [154].

Factors that prolong neutrophil survival

Although neutrophil apoptosis is critical for granulocyte homeostasis and the resolution of inflammation, many proinflammatory molecules extend PMN survival during the initial stages of the inflammatory response. For example, neutrophils that migrate to sites of tissue inflammation have a prolonged lifespan and become resistant to both FAS- and TNF-induced apoptosis [155]. This delay in apoptosis likely promotes robust early response to infection or inflammatory insult and is important because it would increase the window of time during which neutrophils can be recruited to such sites and remain fully functional.

Host-derived factors as well as bacterial and fungal products serve to delay neutrophil apoptosis. Cytokines, such as IL-1 β , TNF α , GM-CSF, G-CSF, and IFN γ , recruit PMNs to sites of infection and each of these molecules can delay PMN apoptosis [57, 156, 157]. Notably, most of the host-derived cytokines that delay apoptosis also prime neutrophils for enhanced function to a second agonist. Complement component C5 is cleaved to form C5b, a member of the membrane attack complex, and C5a, a powerful chemotactic agent for neutrophils [158]. C5a protects PMNs from apoptosis and this process involves BCL2-antagonist of cell death (BAD) and phosphoinositol-3 kinase [159]. Bacterial products, especially LPS and lipoteichoic acid (LTA), are potent inhibitors of PMN apoptosis [57].

In addition to soluble factors, the extracellular environment can signal PMN apoptosis. For instance, acute hypoxia enhances the inflammatory response and causes a decrease in PMN apoptosis that has an additive effect with GM-CSF [160]. This effect is also BCL2-independent, suggesting that another intracellular, pro-survival signaling molecule is

involved in this process [161]. Hypoxia also increases TLR4 expression and enhances the pro-survival effects of LPS toward human PMNs [162].

Role of toll-like receptors (TLRs) in PMN apoptosis

TLRs in combination with other receptors, e.g., complement receptors, promote rapid and efficient recognition of pathogens by neutrophils [163, 164]. To date, ten TLRs are expressed in human tissues and all but TLR3 are expressed in neutrophils [165]. Ligands for TLRs include lipoproteins (TLR1/TLR2) LTA (TLR6), LPS (TLR4), flagellin (TLR5), double-stranded DNA (TLR3), single-stranded RNA (TLR7), and unmethylated CpG DNA of bacteria and viruses (TLR9) [166]. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the surface of PMNs, whereas TLR7/9 is localized to intracellular compartments and recruited to the plasma membrane during PMN priming/activation [166–169].

Although all TLRs play an important role in the innate immune response, only TLR2, TLR4, and TLR6 seem to modulate PMN apoptosis directly. Of these TLRs, TLR4 seems to be more tightly coupled to PMN survival than either TLR2 or TLR6. TLR4 binds to LPS and this interaction up-regulates *IL-8* mRNA and leads to prolonged neutrophil survival that involves monocytes [170]. This observation can be explained in part by the ability of monocyte CD14 to bind LPS, thereby signaling release of a factor that acts in a synergistic manner with TLR4 to prolong PMN survival [171]. The monocyte-dependent delay in PMN apoptosis can be reconstituted by adding peripheral blood mononuclear cells (PBMCs) to highly purified PMNs [172]. *Sta. aureus* peptidoglycan and LTA, agonists for TLR2, also delay PMN apoptosis [173, 174]. In PMN preparations that have been depleted of monocytes by magnetic bead isolation, TLR4 ligation to LPS is the principal TLR survival signal. However, the LPS-mediated delay in apoptosis in the absence of monocytes is relatively short (~4 h), as opposed to the 22-h delay observed in the presence of monocytes [43, 175].

Phagocytosis-induced cell death (PICD)

Neutrophil apoptosis differentiation program

Although a widely accepted view was that mature PMNs have little biosynthetic capacity, recent evidence clearly indicates that this previous view is incorrect. Human PMNs express ~12,000–14,000 transcripts and have significant biosynthetic potential. Changes in PMN transcript levels (and associated proteins) during phagocytosis are an important component to the resolution of the inflammatory response [89, 92, 93, 95, 97, 176–180]. Furthermore, phagocytosis of bacterial pathogens, including *Burkholderia cepacia*, *Borrelia hermsii*, *Sta. aureus*, *Str. pyogenes*, and *Listeria monocytogenes*, is followed by global changes in neutrophil gene expression concurrent with induction of apoptosis [94]. These findings provide an explanation for the increased RNA synthesis in neutrophils following phagocytosis that was originally reported by Martin Cline in the 1960s [181]. Bacteria-induced apoptosis or PICD correlates well with alterations in transcripts encoding factors that promote or repress cell death, influencing the fate of PMNs [94]. Furthermore, PICD is accompanied by down-regulation of genes encoding proinflammatory molecules [94, 177]. Based on these findings, it was proposed that bacteria induce an apoptosis differentiation program in human PMNs that facilitates timely neutrophil turnover during infection. The neutrophil apoptosis differentiation program represents the final stage of neutrophil maturation.

tion and regulates in part multiple processes in human neutrophils, including apoptosis and associated decreased function.

Bacteria-induced cell death

Phagocytosis-induced cell death (PICD) is a mechanism to clear tissues of spent neutrophils containing dead or partially digested microbes, thereby facilitating the resolution of infection [94, 182–185]. As with spontaneous neutrophil apoptosis, timely removal of neutrophils—although in this case after phagocytosis—would prevent release of cytotoxic molecules into surrounding host tissues, a phenomenon mediated by necrotic lysis [186]. This process is important because neutrophils and neutrophil products are known to contribute to inflammatory diseases [187–191]. The mechanism of neutrophil PICD remains incompletely defined, but progress has been made.

Watson et al. were the first to demonstrate that phagocytosis accelerates PMN apoptosis [192]. Since that time, many bacteria have been shown to trigger neutrophil apoptosis or PICD (Table 1). Importantly, the work by Watson et al. linked PMN ROS with apoptosis [192] and ROS play a crucial role in neutrophil apoptosis [3, 47, 60–62, 95, 161, 182, 188, 193–199]. The bacteria: PMN ratio dictates in part the level of ROS produced in vitro, which in turn influences cell fate. For example, interaction of *E. coli* with human PMNs at a 1:1 ratio (1 bacteria per PMN) delays PMN apoptosis, whereas increasing this ratio to

Table 1 Bacteria that modulate cell death in neutrophils and other phagocytes

Bacterial pathogen	Leukocyte affected	References
<i>Anaplasma phagocytophilum</i>	Neutrophils	[176, 221–223, 230, 265]
<i>Borrelia hermsii</i>	Neutrophils	[94, 99]
<i>Bordetella pertussis</i>	Macrophages, neutrophils	[266–274]
<i>Burkholderia cepacia</i>	Macrophages (J774.2), neutrophils	[94, 275–277]
<i>Chlamydia pneumoniae</i>	Macrophages, neutrophils, promyelocytic cells (U937, THP-1)	[224, 231, 278–280]
<i>Chlamydia trachomatis</i>	Macrophages, neutrophils	[281, 282]
<i>Clostridium difficile</i>	Macrophages, eosinophils	[283, 284]
<i>Escherichia coli</i>	Macrophages, monocytes, neutrophils	[182, 206, 285–293]
<i>Fusobacterium necrophorum</i>	Neutrophils	[294]
<i>Fusobacterium nucleatum</i>	Neutrophils, peripheral blood mononuclear cells	[295]
<i>Haemophilus somnus</i>	Neutrophils	[296]
<i>Helicobacter pylori</i>	Macrophages, monocytes, neutrophils	[205, 297–300]
<i>Listeria monocytogenes</i>	Dendritic cells, macrophages, neutrophils	[251, 301–306]
<i>Mannheimia (Pasteurella) haemolytica</i>	Neutrophils	[307–309]
<i>Mycobacterium bovis-BCG</i>	Macrophages, monocytes, neutrophils	[310–315]
<i>Mycobacterium tuberculosis</i>	Macrophages, monocytes, neutrophils	[184, 210, 316–337]
<i>Neisseria gonorrhoeae</i>	Neutrophils	[199]
<i>Photobacterium damsela</i> subsp. <i>Piscicida</i>	Macrophages, neutrophils	[338]
<i>Porphyromonas gingivalis</i>	Neutrophils, promyelocytic cells (THP-1)	[339, 340]
<i>Pseudomonas aeruginosa</i>	Dendritic cells, macrophages, neutrophils	[158, 259, 341–349]
<i>Shigella dysenteriae</i>	Macrophages, neutrophils	[350, 351]
<i>Staphylococcus aureus</i>	Neutrophils	[61, 173, 174, 185, 214, 352–354]
<i>Streptococcus pneumoniae</i>	Macrophages, neutrophils	[207, 211, 355–362]
<i>Streptococcus pyogenes</i>	Promyelocytic cells (U937), neutrophils	[218, 220, 363, 364]

10:1 triggers apoptosis/PICD (see Fig. 2a of ref. [192]). Simons et al. found similar results with *Neisseria gonorrhoeae* using low (1:1) bacteria: PMN ratios, although the readout was caspase activity rather than ROS production [200]. These results underscore the delicate balance between factors that delay apoptosis (e.g., LPS) and those that trigger PICD (ROS). Inasmuch as pathogen-induced apoptosis is complex process involving a multitude of signaling molecules and bacterial components, the process is likely dictated by the specific microbe and/or number of microbes ingested.

Coxon et al. showed that neutrophils from *Cd11b/Cd18^{-/-}* mice have decreased ROS production and delayed apoptosis [195]. Work by Kobayashi et al. used PMNs from 6 X-linked CGD patients to demonstrate that ROS are essential for PICD and a microarray-based approach was used to investigate the role of ROS in PICD [188]. Zhang et al. reported that ROS production triggered by ligation of CD11b/CD18 ultimately activates initiator caspases 3 and 8 in PMNs and that PICD occurs independent of typical death receptors (e.g., FAS/FASL) [60]. Additional links between ROS production and PMN apoptosis were identified in myeloperoxidase-deficient mice and may involve generation of hypochlorous acid [64, 201]. The importance of ROS in the resolution of infection is exemplified by studies in CGD mice, which revealed that neutrophils fail to undergo apoptosis after interaction with pathogens and are thus not removed from the site of infection [202]. Together, these studies revealed that NADPH oxidase-derived ROS are an important intracellular link between the destruction of bacteria and the resolution of infection.

Although most proinflammatory cytokines and many bacteria-derived factors delay apoptosis [57, 174, 203–206], a complex signaling system exists such that bacteria-induced apoptosis or PICD overrides any delay in apoptosis due to these factors [207, 208].

Modulation of neutrophil cell death by bacterial pathogens

While phagocytosis-induced neutrophil apoptosis or PICD is desirable for the resolution of infection and prevention of excessive or chronic inflammation, many pathogens have devised means to alter apoptosis and promote pathogenesis [99]. Here, we provide a few examples of this phenomenon and refer the reader to recent reviews on the topic for a more comprehensive overview [99, 209].

Community-associated methicillin-resistant *Sta. aureus* (CA-MRSA) and *Streptococcus pyogenes* cause direct PMN lysis and/or accelerate bacteria-induced apoptosis to the point of secondary necrosis [94, 99, 204, 210–213]. The most prominent CA-MRSA strains produce leukotoxins known to cause lysis of human neutrophils, but at sublytic concentrations these toxins induce apoptosis [214]. It has been suggested that lysis of neutrophils by certain *Sta. aureus* strains promotes severe pathologic states such as necrotizing pneumonia or fatal sepsis, although this hypothesis remains to be tested. Inasmuch as PMNs are the primary cellular defense against staphylococcal infections, depletion of neutrophils seems a likely component of CA-MRSA pathogenesis [212, 215, 216]. However, *Sta. aureus* leukotoxins such as Panton-Valentine leukocidin (PVL) play little or no unique role in causing cell lysis after phagocytosis [213], a phenomenon that occurs rapidly after uptake of the most prominent CA-MRSA strains [212].

Str. pyogenes is associated with human infections ranging from mild pharyngitis to life-threatening necrotizing fasciitis [217]. Notably, *Str. pyogenes* accelerates neutrophil PICD, thereby causing necrosis [211, 217–220]. As with CA-MRSA, *Str. pyogenes* kills neutrophils to survive within the human host.

Pathogens that delay neutrophil apoptosis

In contrast to macrophages, few bacteria are known to survive and replicate within neutrophils. *A. phagocytophilum* is an obligate intracellular bacterium that delays neutrophil apoptosis to survive within PMNs [176, 221–225]. Although progress has been made, the mechanism(s) by which *A. phagocytophilum* delays apoptosis are not well understood. The pathogen is ingested by caveolae-mediated endocytosis and survives within inclusions/vacuoles that do not contain critical components of the NADPH oxidase [226, 227]. Moreover, *A. phagocytophilum* actively alters normal neutrophil granule trafficking and inhibits NADPH oxidase activation [176, 228, 229]. Notably, the pathogen fails to induce neutrophil apoptosis and dysregulates neutrophil gene expression to maximize survival and dissemination within the mammalian host [221–223, 230]. By prolonging the lifespan of neutrophils after uptake, *A. phagocytophilum* survives and disseminates in the host [223].

PMNs readily phagocytose *C. pneumoniae* and a small fraction of internalized bacteria remain viable for up to 90 h after uptake [224]. *C. pneumoniae* multiplies within neutrophils and delays constitutive apoptosis such that both phases of the *Chlamydia* biphasic life cycle occur within PMN inclusions [224, 231]. Delay of neutrophil apoptosis by *C. pneumoniae* can be as long as 3 days and is associated with decreased caspase-3 activity [224]. Prolonged PMN survival may be mediated by LPS and autocrine production of IL-8 [224]. Intracellular growth of *Chlamydia* is essential for pathogenesis, because the host cell supplies nutrients and shields the microbe from antimicrobial serum factors.

Mechanisms of cell death

Overview

The resolution of infection is complete when bacteria and spent host cells are cleared from tissues. Although phagocyte apoptosis or PICD has been reported after phagocytosis of many types of bacteria, the mechanism of cell death remains incompletely defined. For example, it is not always apparent if bacteria cause apoptosis after phagocytosis or if the observed cell death is a more cursory form of host cell lysis. Delineating the mechanism of host cell death is an important first step toward the development of better therapeutics for inflammatory disorders [232].

Initially, cell death was thought to proceed through either apoptosis or necrosis. However, necrosis is really the end result/equilibrium of cell death and thus not a cell death mechanism [233]. Several unique types of cell death are now known to occur depending on specific conditions, including the environment and state of the cell (Table 2). These conditions include extracellular factors, e.g., the presence of inflammatory markers or pH of the extracellular fluid, and intracellular factors such as the abundance of ATP [234]. For example, under conditions where ATP is depleted or limiting, cell death proceeds by oncosis, which entails cell swelling and lysis. However, when ATP is abundant, cell death will proceed via the more organized and non-inflammatory forms of cell death, such as apoptosis or autophagy. Importantly, a cohort of cell death mechanisms has been identified in cell populations in organs under stress [235]. Therefore, the resolution of bacterial infections through host cell death/turnover may encompass multiple mechanisms and not occur solely via apoptosis. As the different mechanisms of cell death receive more attention, the subcellular markers that distinguish these mechanisms have become more defined. Each of the

mechanisms can be distinguished from the others based on physical characteristics in combination with biochemical assays (Table 2).

Apoptosis

Apoptosis, also referred to as type I cell death, is defined as a non-inflammatory form of cell death characterized by membrane blebbing, nuclear condensation, and cytoplasmic condensation. As stated above, apoptosis can be initiated by extracellular signals or by intracellular signals. The end result is an orderly destruction of the infected/damaged cell that does not cause inflammation in the surrounding tissues.

Typically, apoptosis is detected by DNA degradation, caspase activation, and/or early exposure of PS on the cell surface. Nuclear fragmentation or DNA “laddering” can be visualized by transmission electron microscopy (condensation and fragmentation of the nucleus) or agarose gel electrophoresis. Caspases are expressed initially as pro-proteins that undergo activation by proteolytic cleavage. As such, activated caspases can be detected by Western blot or flow cytometry analysis (using a fluorophore labeled reagent that binds only to the activated caspase). The traditional marker for neutrophil apoptosis is nuclear condensation [19], as membrane blebbing does not appear to occur *in vitro* (Fig. 2).

Although other assays have been utilized to monitor apoptosis, several reports have shown that these methods may not be specific for apoptotic cells. For example, apoptotic DNA damage has also been monitored by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). However, Grasl-Kraupp et al. reported that TUNEL staining does not discriminate between apoptotic and late oncotic cells [236]. The plasma membrane of oncotic cells is extremely porous and TUNEL probably stains DNA via large holes in the cell membrane. During apoptosis the inner leaflet of the plasma membrane containing PS becomes exposed to the extracellular space and the exposed PS can be detected using labeled annexin V. As with TUNEL, recent evidence has shown that late oncotic cells stain positive for annexin V, thereby bringing into question the specificity of annexin V staining apoptotic cells [237, 238].

Autophagy-induced cell death

Autophagy, also known as Type II cell death, is a process whereby cells catabolize damaged components so that the essential building blocks, amino acids, lipids, carbohydrates, and etc., can be utilized for cellular repair, especially under times of cellular stress. Cells have the capacity to catabolize damaged proteins as well as entire organelles, including mitochondria. Autophagy was first observed in yeast and several genetic markers correlate with the formation of autophagic vacuoles in yeast and mammalian cells.

Autophagy-induced cell death is a programmed cell death that is distinct from apoptosis. Specifically, autophagy-induced cell death is induced under conditions of cellular starvation or endoplasmic reticulum (ER) stress [239]. Although the mechanism of autophagic cell death is not understood, two genetic markers, beclin 1 and ATG7, are critical for this non-apoptotic death pathway in mammalian cells ([240, 241] and reviewed in [242]). During autophagy-induced cell death, caspases are not activated, although there is cytoplasmic condensation and a reduction in cell size. The most defining feature of autophagy-induced cell death is the formation of multilamellar autophagosomes that engulf intracellular components. These large vacuoles are easily distinguished from other subcellular compartments using transmission electron microscopy. The formation of these large multilamellar

Table 2 Cell death mechanisms

Cell death	Characteristics/diagnostic markers	Caspase activation	ATP	Protein synthesis/changes in transcript levels	References
Apoptosis	Caspase activation Membrane blebbing PS exposure DNA fragmentation DNA Laddering/TUNEL+ Reduction in cell size Little inflammation Clearance of apoptotic bodies by phagocytes	Yes	Required		Numerous
Autophagy	Formation of multi-lamellar autophagosomes Reduction in cell size LC3-II TOR kinase activity Fusion of autophagosome with lysosome	No	Required	Protein synthesis required	[242, 243, 365]
Oncosis	Cell swelling Inflammation TUNEL+	No	Depleted	Protein synthesis not required	[234, 255, 257]
Pyroptosis	Cell swelling Inflammation Membrane damage/pore formation DNA damage TUNEL+ Caspase-1 activation Release of activated IL-1 β and IL-18	Yes	Required		[244, 245, 248, 250]
Phagocytosis-induced cell death (PICD)	Change in PMN transcriptome Dependent on ROS production	No		Modified PMN transcript levels	[94, 95, 100, 177, 188]
NETosis	Disintegration of nuclear envelope Mixing of nuclear and cytoplasmic material Disappearance of cytoplasmic organelles Dependent on ROS production	No			[259]

Abbreviations: PS, phosphatidylserine; LC3-II, microtubule-associated protein 1 light chain 3; TUNEL, terminal deoxynucleotide nick end labeling; TOR, Target of Rapamycin; ROS, reactive oxygen species

vacuoles may be cell type dependent and more studies need to be done with phagocytes to determine if these vacuoles form *in vitro* or *in vivo*. After autophagosome formation, intracellular components are degraded during autophagosome—lysosome fusion [243].

Pyroptosis

Pyroptosis is a programmed cell death mechanism that results in cell lysis, tissue inflammation, and recruitment of host professional phagocytes. As with apoptosis, nuclear DNA is cleaved during pyroptosis, although this event is dependent on caspase-1-stimulated nuclease activity and not poly(ADP-ribose) polymerase [244]. Caspase-1 activation is a key event of pyroptosis and the enzyme activates IL-1 β and IL-18. These cytokines in turn recruit macrophages and PMNs to the site of infection. Caspase-1 activity also leads to the formation of pores in the eukaryotic cell membrane, allowing an influx of extracellular ions that causes cell swelling and lysis [244]. Shao et al. found that caspase-1 has enzymatic activity toward several proteins in the glycolytic pathway, suggesting a link between metabolism/ATP production and pyroptosis [245].

Analogous to the apoptosome complex that mediates apoptosis, pyroptosis is controlled by the formation of a large (~700 kDa) multiprotein complex known as the inflammasome. The inflammasome is composed of a sensor (NOD-like receptor) [246], an adaptor molecule (apoptosis-associated speck-like protein containing a CARD, ASC, or PYCARD), caspase-1, and caspase-5 [247]. Engagement of sensor molecules with ligands such as PAMPs triggers formation of the inflammasome complex [248]. It is interesting to note that activation of the inflammasome occurs in the host cell cytosol after bacteria escape from the phagosome. Bacteria that activate the inflammasome complex include *Shigella* [249], *Francisella tularensis* (reviewed in [250]), *L. monocytogenes* [251], *Yersinia* [252], and *Salmonella enterica* serovar Typhimurium [244, 253, 254].

Oncosis

Like pyroptosis, oncosis is a form of cell death characterized by cell swelling and lysis. Earlier research had defined cell swelling and lysis as necrosis, but necrosis is now widely accepted as the end result of cell death. Necrosis not only includes the lysis of infected cells but also the equilibrium or damage that is caused to surrounding tissues after a cell has lysed [233]. Oncosis, also known as Type III cell death, occurs through multiple mechanisms, but two of the most prominent involve pore forming toxins of pathogenic bacteria and complement-mediated lysis. Pore forming toxins such as alpha-hemolysin of *St. aureus* have the ability to lyse a variety of host cell types, and complement-mediated cell lysis occurs after the formation of the complement membrane attack complex on C3b-targeted cells. As a result, intracellular contents and inflammatory cytokines are released into the extracellular milieu, causing tissue damage and inflammation. Oncosis occurs when membrane integrity is damaged and an efflux of inorganic ions (e.g., Ca²⁺, Na⁺, Cl⁻) increases the intracellular and intraorganelle osmotic pressure [255, 256]. Depletion of ATP and loss of mitochondrial transmembrane potential accompanies this efflux of ions into the cell.

Bacteria such as *Brucella abortus*, *Campylobacter jejuni*, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium can cause host cell death after phagocytosis [257–260]. These bacteria escape from phagosomes and then alter cell metabolism through the expression of toxins that disrupt membrane integrity, thus causing oncosis. The invasive ability of *Cam. jejuni* has been associated with the expression of its cytolethal distending

toxin and subsequent ability to cause oncosis of epithelial cells [258]. Dacheux et al. showed that *P. aeruginosa* cystic fibrosis isolates induce rapid (within 60 minutes) oncosis of macrophages and PMNs that is type III secretion-dependent [259]. Expression of flagella proteins is required for *Sal. enterica*-mediated oncosis of and escape from human macrophages [260].

NETosis

A novel form of PMN death named NETosis has been described recently [261, 262] (Table 2). Neutrophil extracellular traps (NETs) are extrusions of plasma membrane and nuclear material composed of granule components and histones. NETs were initially reported to occur with viable PMNs [261, 262] and this finding was confirmed by others [263]. However, subsequent studies by Fuchs et al. suggest that the process of NET formation involves a type of cell death the authors called NETosis [261, 262]. NETosis occurs in an NADPH oxidase dependent manner [259] and bacteria deficient in DNases are susceptible to killing by NETs [264]. Although the physiological relevance in vivo has been debated, as has the issue of whether NETosis is distinct from other types of cell lysis, it is clear that in vitro NETs bind and kill extracellular microorganisms. Further research is needed to better understand these interesting structures.

Summary of neutrophil death mechanisms

Although there are many fates for phagocytes after interactions with microorganisms, the manner of cell death has serious repercussions that can impact severity and duration of disease. Pyroptosis and oncosis cause inflammation that recruits leukocytes to the site of infection, but if unchecked, these processes can cause necrosis that leads to the damage of surrounding cell and tissues. In contrast, apoptosis and autophagy are non-inflammatory forms of cell death that recruit phagocytes to the site of infection. A comprehensive understanding neutrophil apoptosis, death, and turnover is critical for a full understanding of bacterial pathogenesis mechanisms.

A paradigm for the resolution of infection

Neutrophil apoptosis is essential for maintaining normal immune system homeostasis and this process facilitates the resolution of inflammation. PMN apoptosis is mediated by both intrinsic and extrinsic mechanisms, and apoptotic cells are subsequently cleared by macrophages through multiple receptor-ligand interactions.

Neutrophil fate is altered by interaction with microorganisms. In most cases, the outcome of this interaction dictates whether the ingested organism will ultimately cause disease. Based upon our current knowledge, there are two possible outcomes for neutrophil-bacteria interactions (Fig. 5) [99]. On one hand, phagocytosis and killing of bacteria culminate with induction of apoptosis/PICD and the subsequent removal by macrophages, ultimately resulting in the resolution of infection (Fig. 5, top). Alternatively, pathogens such as *A. phagocytophilum*, *C. pneumoniae*, *Str. pyogenes*, and *Sta. aureus* alter neutrophil apoptosis to survive, and thereby disseminate and cause disease [176, 217, 219, 221, 222, 224] (Fig. 5, bottom). This relatively simple model will need refining and revision as we continue to accumulate new information.

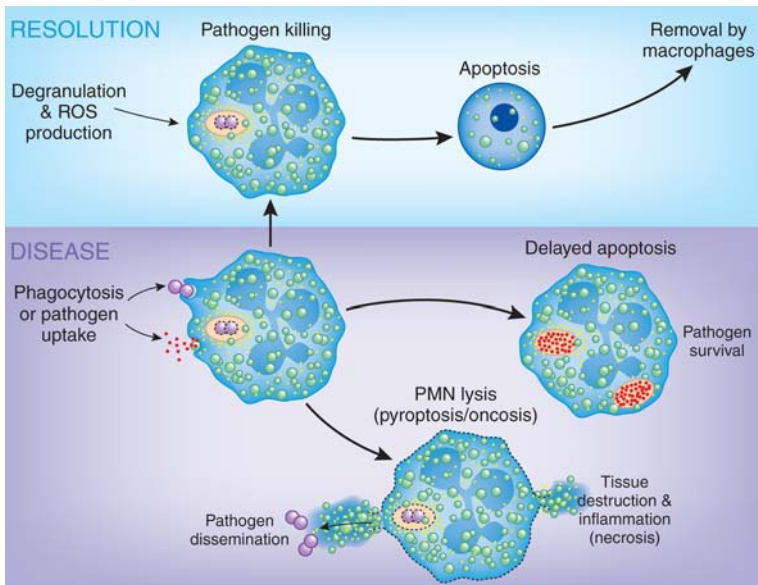


Fig. 5 A model schematic illustrating two possible outcomes of bacteria-neutrophil interaction. See text for details

Concluding comment

The use of genomics-based technologies has dramatically enhanced our discovery of mechanisms of bacterial virulence and fundamental immunology. A better understanding of innate host defense, host cell death, and bacterial pathogenesis will likely provide information important for development of vaccines, treatments, and prophylactic agents designed to prevent and/or control infections.

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