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Neutrophils: Many Ways to Die

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Neutrophils or polymorphonuclear leukocytes (PMN) are key participants in the innate immune response for their ability to execute different effector functions. These cells express a vast array of membrane receptors that allow them to recognize and eliminate infectious agents effectively and respond appropriately to microenvironmental stimuli that regulate neutrophil functions, such as activation, migration, generation of reactive oxygen species, formation of neutrophil extracellular traps, and mediator secretion, among others. Currently, it has been realized that activated neutrophils can accomplish their effector functions and simultaneously activate mechanisms of cell death in response to different intracellular or extracellular factors. Although several studies have revealed similarities between the mechanisms of cell death of neutrophils and other cell types, neutrophils have distinctive properties, such as a high production of reactive oxygen species (ROS) and nitrogen species (RNS), that are important for their effector function in infections and pathologies such as cancer, autoimmune diseases, and immunodeficiencies, influencing their cell death mechanisms. The present work offers a synthesis of the conditions and molecules implicated in the regulation and activation of the processes of neutrophil death: apoptosis, autophagy, pyroptosis, necroptosis, NETosis, and necrosis. This information allows to understand the duality encountered by PMNs upon activation. The effector functions are carried out to eliminate invading pathogens, but in several instances, these functions involve activation of signaling cascades that culminate in the death of the neutrophil. This process guarantees the correct elimination of pathogenic agents, damaged or senescent cells, and the timely resolution of the inflammation that is essential for the maintenance of homeostasis in the organism. In addition, they alert the organism when the immunological system is being deregulated, promoting the activation of other cells of the immune system, such as B and T lymphocytes, which produce cytokines that potentiate the microbicide functions.

Keywords: cell death, apoptosis, pyroptosis, necroptosis, necrosis, NETosis, autophagy, neutrophils

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INTRODUCTION

Neutrophils, also denominated microphages or polymorphonuclear leukocytes (PMN), are granulocytes that are the most abundant leukocyte type in the peripheral human blood, constituting from 40 to 70% of the total white blood cells under normal conditions (1). Their half-life is short, from 8 to 20 h in circulation, although after migration into tissues, their life is considerably prolonged (1–4 days) (2). They comprise the first cells of the immune system to arrive at the

infection site and are characterized by their potent antimicrobial and antifungal capacity, which is granted by their cellular components: antimicrobial peptides, neutrophil-specific proteolytic enzymes, as well as their production of ROS and neutrophil extracellular traps (NETs) (3–5).

Neutrophils contain an extraordinary array of receptors for the recognition of pathogens, including membrane receptors such as scavenger receptors (6, 7), mannose receptors (8), Dectin-1 (9), CD14 (10, 11), C1qR (12), receptors for IgG (FcγR) (13–16), C3b/C3biRs (CR1,CR3) (17), collectins (CD91-calreticulin complex) (18, 19), and Toll-like receptors (TLRs) (20); they also express intracellular receptors such as NOD-like receptors (NLRs) (21). Through these receptors, known as pattern recognition receptors (PRRs), neutrophils are activated to respond to infections or to damage signals. PMNs also contain enzymatic machinery for the production of free radicals that allows a much more effective elimination of pathogens (22). At the same time or after performing their effector functions, neutrophils initiate mechanisms of cell death such as autophagy, NETosis, or apoptosis. Cell death of neutrophils is an indispensable event required to maintain the number of neutrophils in infection or inflammation and in an organism in homeostasis (23–25).

The regulation of neutrophil death rate is an essential mechanism for maintaining homeostasis under physiological conditions. Their accelerated death, either idiopathic or acquired (e.g., drug-induced), leads to a decrease in the total count (neutropenia) increasing the probability of contracting bacterial or fungal infections and negatively affecting their resolution (26, 27). In contrast, delays in the death of neutrophils raises their count (neutrophilia) (28). Neutrophilia is a classic indicator of acute inflammation related to acute atherosclerotic events or trauma. For these reasons, the regulation of neutrophil survival is essential in the inflammatory process. The following review brings together recent findings related to the mechanisms that regulate the death of neutrophils, as well as the intrinsic and extrinsic signals that initiate these processes.

REVIEW

Processes of Cell Death

Apoptosis

The cells of an organism are capable of dying through a mechanism of programmed cell death denominated apoptosis, characterized by changes in the cell structure, such as the acquisition of an elongated shape, retraction of the pseudopods, reduction of the cell volume (pyknosis), and chromatin condensation, with scarce or null structural modification of the cytoplasmic organelles. Other changes include DNA fragmentation with or without oligonucleosomal fragmentation, and usually activation of caspases (29–31). Apoptosis occurs with minimal damage to the surrounding tissue.

In neutrophils, this process is initiated by either of two distinct signaling pathways: the intrinsic or the extrinsic apoptotic pathway. In the intrinsic pathway, permeabilization of the mitochondrial membrane, due to the absence of antiapoptotic factors, leads to the release of cytochrome C to the cytoplasm. Once in the cytoplasm, the cytochrome C participates in the assembly of a multimeric complex denominated apoptosome and the subsequent activation of the effector caspase (caspase-3). On the other hand, the extrinsic pathway is activated in response to extracellular ligation of death receptors, resulting in the activation of caspase-3 by means of the activation of caspase-8 (28, 29, 32, 33).

In this first phase of the death process, the neutrophils are no longer functional since their chemotaxis, respiratory burst, and degranulation are impaired (34, 35), in addition to a decrease in the expression of surface receptors and adhesion molecules (36, 37) and a significant reduction in the release of chemokines (38).

It is also known that neutrophil apoptosis can be delayed or accelerated depending on specific cellular stimuli. According on the inflammation conditions, the neutrophil response to microorganisms can change significantly: while some pathogens initiate the spontaneous apoptotic process, others engage in such tight interactions that cause apoptosis followed by cell lysis (39, 40). The apoptotic death process, as opposed to the necrotic death process, maintains membrane integrity to limit the release of harmful neutrophil contents. The exposure of phosphatidylserine (PS) residues on the cell membrane allows for greater recognition by macrophages, thereby enhancing the clearance of the cells. It also increases the production of IL-10 and TGF-β in macrophages, cytokines associated with resolving the inflammatory response and promoting tissue repair (41, 42).

In contrast, the life span of neutrophils can be considerably extended under certain inflammatory conditions, such as in the presence of granulocyte-colony stimulating factor (G-CSF), granulocyte-monocyte colony-stimulating factor (GM-CSF), Interleukin 8 (IL-8), and C reactive protein, among other proinflammatory mediators (43–46).

Apoptosis is a non-inflammatory process of cell death activated in senescent neutrophils by cytokines and growth factors inducing the PI3-K/Akt, BCL-2/BCL-X_L, and p53/p21 pathways, in a similar way as it occurs in other cells (32, 47, 48). However, this is not the only way to activate apoptosis in neutrophils. It is recognized that apoptosis pathways can be activated in neutrophils after phagocytosis and ROS production, or during inflammatory processes when molecules such as extracellular matrix proteins (49), lipopolysaccharide (LPS), complement fragments (44), and cytokines, among others, favor the activation of TNF receptors and FaS/CD95 through JNK and NF-kB (50, 51).

The high concentrations of reactive oxygen species produced by the NADPH oxidase are an important marker for triggering apoptosis or necrosis. In resting neutrophils, the NADPH is inactive and its components are distributed between the cytosol and cellular membranes. Upon neutrophil activation, the cytosolic components (p47phox, p67phox, p40phox) and the Rho family GTPase Rac-2 migrate toward the membranes of intracellular granules and the cell membrane (52) to participate in the assembly of the NADPH oxidase complex, which mediates the metabolic burst and ROS production. In the last years, it has been suggested that ROS produced by the NADPH oxidase participate as signaling molecules and it has been shown that the

molecular pathways leading to apoptosis of neutrophils depend on ROS generation (53–55).

Kinases such as AKT and JNK regulate apoptosis-NETosis pathway in a ROS production-dependent manner. Activated AKT leads to NETosis, however its inhibition lead neutrophils to apoptosis (56) while JNLK is an important molecular sensor that initiates NETosis in response to increasing concentrations of LPS- and Gram-negative bacteria (57, 58). Recent studies show that apoptosis can occur along with NETosis, when neutrophils are stimulated with ultraviolet (UV) irradiation, calling it ApoNetosis. This type of death activates caspase 3 and is NOX-independent, though this interesting development requires further investigation (59).

Necroptosis

While apoptosis is one of the most studied forms of cell death in different cellular lineages, necroptosis is another type of programmed cell death that is not so well-known. This type of death is a regulated process, which depends on receptor interacting protein kinase-3 (RIPK3) and mixed lineage kinase domain-like protein (MLKL) (60–62).

While necroptosis is a process of programmed cell death, it does not involve the precise morphological changes characteristic of apoptosis and, importantly, it does not involve DNA fragmentation. Diverse forms of activation of neutrophils have been found to culminate in this process. Stimuli such as the activation of cell death receptors like TNFR1, ligation of TLRs, IFNGR, adhesion receptors such as CD11b, CD15, or CD18, the presence of monosodium urate (MSU) crystals, and the phagocytosis of *Staphylococcus aureus*, have been shown to initiate activation pathways that culminate in the activation of the RIPK3-MLKL complex (62–67). The signaling events upstream of the activation of the RIPK3-MLKL complex are different depending on the receptor involved. The activation pathway initiated through the TNFR1 is probably the best-characterized one (68). The activation of the receptor induces the formation of a complex with RIPK1, whose ubiquitination through diverse proteins, such as the cellular inhibitors of apoptosis [cIAP] and the linear ubiquitin chain assembly complex [LUBAC]) (69–71), activate the NF-κB survival factor, thus contributing to cell survival, proliferation, and production of proinflammatory cytokines; if ubiquitination of RIPK1 is inhibited, a different complex is formed that recruits caspase-8, inducing apoptosis (72); however, if the activity of caspase-8 is inhibited, then RIPK1 recruits RIPK3 in a complex called the necrosome. This complex can now recruit the caspase-8 in order to give rise to the necroptotic process (73–75).

In the case of activation of adhesion receptors or the presence of MSU crystals, the activation of necroptosis depends on the formation of ROS inside the cell, although the mechanism by which ROS induce the activation of the RIPK3-MLKL complex has not been not established. However, intracellular production of ROS is indispensable for necroptosis, as neutrophils of patients with chronic granulomatous disease are incapable of entering into necroptosis through the adhesion molecules (62, 76). In the case of MSU, there is evidence that relates necroptosis with the release of neutrophil extracellular traps (NETs); this stimulus is linked with the formation of ROS by inducing the assembly and activity of the NADPH oxidase complex. It was observed that the inhibition of RIPK3, as well as the action of other necroptosis inhibitors, diminished the formation of NETs induced by MSU crystals, thus confirming the relation of necroptosis with NETosis, at least after this stimulus (77).

Necrosis

Adverse environmental conditions, such as lack of oxygen or essential nutrients, high temperature, toxic compounds, and mechanical stress, can initiate necrosis. In contrast with apoptosis, there is a gain in cell volume (oncosis), rupture of the plasma membrane, and release of intracellular material to the surrounding milieu during necrosis (78, 79); similarly, there is swelling of cytoplasmic organelles (mitochondria, endoplasmic reticulum, and Golgi), moderate chromatin condensation, the absence of the activation of caspases, release of cytochrome c, and fragmentation of oligonucleosomal DNA. Morphologically, these have been defined as the spectrum of post-mortem changes of a tissue due to the progressive action of the enzymes released by the damaged structures (79). The morphology of the necrotic cells results from the denaturation of proteins and the autolytic or heterolytic enzymatic digestion (30, 31, 79).

The habitual participants in necrotic cell death, independently of the stimulus, are Ca^{2+} ions and ROS. During necrosis, the levels of cytosolic Ca^{2+} rise, leading to an overload of calcium in the mitochondria and the activation of proteases and phospholipases (80). ROS generate damage to lipids, proteins, and DNA; consequently, there is mitochondrial dysfunction, deregulation of the ionic equilibrium, and loss of membrane integrity. Destabilization of the membrane during necrosis is also mediated by other factors, such as acid sphingomyelinase (SMA), phospholipase A (PLA), and the calpains. In addition, necrotic cells release immunomodulatory factors that lead to their recognition and trapping by phagocytes, which can promote a subsequent immunological response.

Necrotic neutrophils, as well as other cells such as monocytes and dendritic cells, can activate NF-κB in neighboring cells (like fibroblasts or macrophages), stimulating the production of IL-6, IL-8, and TNF-α (81). The stimuli that induce this type of cell death of neutrophils are mainly pathogens that prompt a ligand–receptor response, giving rise to a high concentration of intracellular ions, as well as to the release of proinflammatory cytokines since the external stimulus. TNF-α secreted by activated neutrophils or other cells can activate neutrophil necrosis, although membrane proteins can regulate this with enzymatic activity, such as aminopeptidase N (CD13) (82, 83).

NETosis

In 2004, the discovery of neutrophils extracellular traps (NETs) established a mechanism of effector response specific of these cells (84). However, it was later demonstrated that all granulocytes possess this capacity after treatment with IL-5 and IFN-γ or with GM-CSF and C5a (85). The release of NETs is part of a programmed cell-death process denominated NETosis. In contrast with apoptosis or necrosis, the nuclear

membrane and the cellular membrane disintegrate; however, the cytoplasm contents remain integrated for a moment, allowing the interaction of the antimicrobial granule proteins with the DNA (86, 87). On releasing its genetic material, the extracellular chromatin functions for immobilizing the bacteria and preventing its dispersion through the host tissues. Simultaneously, these traps are capable of degrading virulent bacterial products like IcsA, a key virulence factor of the human pathogen *Shigella flexneri* (88) or IpaB (invasive plasmid antigen B) (89, 90). These bacterial products and their subproducts can induce inflammation and diverse chemotactic signals that result in the recruitment of monocytes and other cells to the infection site (84, 86, 91).

The process of NETosis can be induced by an extensive number of stimuli that include pathogenic microorganisms, activated platelets (92), pathogen's structures, formylated peptides, and cytokines, among others. Signals characteristic of apoptosis, such as the condensation of genetic material, the presence of phosphatidylserine (PS) in the membrane, and activation of caspases, are not observed during NETosis (93).

This process is regulated, among others, by peptidyl arginine deaminase 4 (PAD4), an enzyme that contains a putative sequence of nuclear localization, but that resides principally in cytosolic structures in resting neutrophils, although it can also enter into the nucleus and citrullinate the histones and diverse transcription factors. Therefore, it can participate in the epigenetic regulation of gene expression and cellular differentiation (94, 95). PAD4 is associated with the cytosolic subunits $p47^{phox}$ (also known as neutrophil cytosolic factor 1, NCF1) and $p67^{phox}$ (NCF2) that form part of the NADPH oxidase complex involved in the respiratory burst. Activation of the cell leading to the elevation of intracellular Ca^{2+} to physiological levels do not result in activation of PAD4 enzymatic activity. However, high levels of intracellular calcium (higher than those of physiological neutrophil activation, such as those observed by disruption of the cell membrane) lead to activation of PAD4 and rapid citrullination of p47phox/NCF1 and p67phox/NCF2, as well as their dissociation from PAD4. Citrullination of NCF1 and NCF2 prevents the assembly of NADPH oxidase complex (95). Originally, it was reported that the presence of NADPH oxidase and myeloperoxidase (MPO) was necessary for the correct formation of NETs; until recently, all the activators capable of inducing NETs formation were known to require in some manner the presence of ROS (96). However, this has been a controversial theme in recent years due to the description of novel NETosis activation pathways that appear to be independent of NADPH oxidase (81, 97, 98).

PAD4 citrullinates chromatin, specifically histones, catalyzing the deimination of the arginine at the amino terminal end of the H3 subunit. This reduces the attraction of heterochromatin protein HP1B to H3-adjacent lysine 9, resulting in the formation of dispersed chromatin. In addition to this, neutrophil elastase (NE) and MPO complement the decondensation of chromatin. Both NE and MPO are stored in azurophilic granules that are released once ROS are formed in the cell. NE is a PMN-specific serine protease that degrades virulence factors and is bactericidal. However, during the process of NETosis, it enters the nucleus and cleaves the histones, promoting greater chromatin decompactation (99). Later during the NETosis process, when the NE have already acted, MPO binds to the chromatin prompting even a greater decompactation. The synergic process of these enzymes for the formation of dispersed chromatin reaches the point at which the nuclear and cellular membranes break and NETs are released (100). Interestingly, the most abundant component of the NETs are histones and these are one of the most potent antimicrobial factors (96, 101).

Activated neutrophils produce large amounts of superoxide through the NADPH oxidase. Superoxide is then converted into hydrogen peroxide (H_2O_2) leading to the formation of a variety of toxic oxygen derivatives. MPO catalyzes the oxidation of halides by means of hydrogen peroxide. Both, the NADPH oxidase and MPO, have been implicated in regulating NETs formation. It is now known that the ROS generated through the NADPH oxidase prevent activation of caspases (102) and that NADPH oxidase is necessary for the formation of NETs through many (though probably not all) stimuli.

Participation of the Raf-MEK-ERK pathway in the formation of NETs has been identified by the phosphorylation of ERK1, ERK2, and c-Raf. Since the inhibition of the NADPH oxidase does not block the phosphorylation of these proteins induced by PMA, this suggests that activation of the Raf-MEK-ERK pathway is found upstream of NADPH oxidase activation (103, 104).

NETs formation depends directly on the intensity of the stimulation of the cell and on the time of exposure to the stimulus. Currently, NETosis continues to be considered a process of cell death, although some studies have demonstrated a process known as vital NET formation, in which neutrophils release mitochondrial DNA, which does not compromise their viability (105, 106).

Pyroptosis

This pathway of cell death is dependent on caspase-1. This caspase is not involved in apoptotic cell death and its function is to process the precursors of the IL-1β and IL-18 inflammatory cytokines through the multiprotein complex called inflammasome (107, 108). This type of cell death is observed, e.g., in cells infected with *Salmonella*, in which the activation of caspase-1 is produced by effector substances released into the cytoplasm of host0 cells through the *Salmonella* Pathogenicity Island 1 Type III Secretion Systems (SP1 T3SS).

The assembly and function of an inflammasome are essential for the process of pyroptosis; these oligomeric complexes serve as a molecular platform for the key step of the process, that is, the cleavage of pro-CASP-1 zymogen into active caspase-1. Canonical inflammasomes are composed of cytoplasmic pattern recognition receptors (PRR), which include NLRs, absent in melanoma 2 (AIM-2)-like receptors (ALRs), adapter protein apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1 (109, 110). Numerous canonical inflammasomes have been recognized in pyroptosis including NLRP1, NLRP3, AIM2, NLRC4, and Pyrin inflammasomes (111–116).

Activation of caspase-1 results in the formation of pores in the plasma membrane and the cell becomes permeable to

small-molecular-weight colorants such as 7-aminoactinomycin (7-AAD), ethidium bromide (EtBr), and propidium iodine (PI). In contrast, the cell membrane in an apoptotic cell remains intact and the cells fragment into apoptotic bodies that are not stained with 7-AAD nor with PI (108). Activation of caspase-1 leads to DNA fragmentation and to cell lysis by separate pathways. DNA fragmentation is carried out by an unidentified caspase 1-activated nuclease that does not involve the degradation of ICAD (inhibitor caspase-activated deoxyribonuclease) (117, 118) that, together with rearrangements of the actin cytoskeleton, are events required for the formation of membrane pores of between 1.1 and 2.4 nm in diameter (119). In addition, caspase-1 cleaves the precursors to produce biologically active IL-1B and IL-18, which come out easily through the pores. Dissipation of the cellular ionic gradients leads to a flow of water with cellular tumefaction and osmotic lysis (120), releasing the intracellular (proinflammatory) contents. Even so, according to some researchers, pyroptosis comprises nothing more than a caspase-1-dependent necrotic-type death.

Pyroptosis has been mainly described in macrophages and dendritic cells. However, there is evidence of caspase-1 activity in other cell types. Many pathogens can survive and replicate within the macrophages; however, very few pathogens can do this in neutrophils. This is probably due to intrinsic differences between the two cell types. Macrophages have a longer life but reduced microbicidal activity as compared to neutrophils, which converts them into a more infection-prone cellular objective. Neutrophils, on the other hand, are highly microbicide and short-lived, which makes them poor targets for intracellular pathogens.

During acute Salmonella infection, neutrophils can activate the NLRC4 inflammasome to produce IL-1β, without undergoing pyroptosis, and thus they can continue their inflammasome-independent antimicrobial effector functions while maintaining IL-1β secretion (121, 122). This can suggest that neutrophils do not undergo pyroptosis in response to proteins of some intracellular pathogens (such as flagellin or the proteins making up T3SS) that activate assembly of NLRC4 inflammasome. However, it has been determined that neutrophils can undergo pyroptosis, activating a non-canonical infammasome pathway through P2X7 receptors in absence of NOX2 in pseudomonal infections (105, 123, 124).

Autophagy

The term autophagy derives from the Greek to eat (*phagy*) oneself (*auto*), that is, self-digestion. This is a highly conserved process in evolution that takes place in all eukaryotic cells, from yeasts to mammals. It occurs in response to different forms of stress, such as lack of nutrients, absence of growth factors, infectious processes, or hypoxia. Therefore, it is possible that the main function of this process is to provide necessary nutrients to maintain the vital functions of the cell in situations of energy or nutrient deprivation. Additionally, some studies point out that the process denominated "selective autophagy" is also utilized to remove "dangerous" material in the cytoplasm, such as damaged mitochondria or protein aggregates (125). To date, three distinct forms of autophagy have been identified, macroautophagy, microautophagy, and selectiveautophagy (126).

Autophagy is characterized by the formation of autophagic intracellular vesicles and the degradation of cellular contents, including essential organelles such as mitochondria, inside the vesicles. Many of the PRRs, including TLRs and NLRs, induce autophagy in macrophages (127). Similarly, neutrophils undergo autophagy in response to TLR ligands. Apparently, autophagy in neutrophils can be induced dependently or independently of phagocytosis (128, 129).

Autophagy in the neutrophil is a mechanism of survival. Pliyev and Menshikov (130) demonstrated that inhibitors of autophagy, such as 3-methyladenine (MA) and chloroquine (CQ), notably accelerate the spontaneous apoptosis of neutrophils as evidenced by exposure of phosphatidylserine, DNA fragmentation, and the activation of caspase-3. It has also been observed that human neutrophils can be induced to autophagy by simultaneous stimulation by sialic acid binding immunoglobulin-like lectin-9 (Siglec-9) ligation and certain cytokines with survival-promoting activity in neutrophils (131). Some studies have demonstrated that adhesion molecules induce death in neutrophils independently of caspases. The process is characterized by great cytoplasmic vacuolization and the fusion of organelles, thus, it has been associated with autophagy (132) Neutrophils with morphological signs of autophagy have been observed in septic shock, cystic fibrosis, rheumatoid arthritis, and various skin diseases, suggesting that the induction of autophagy in polymorphonuclear leukocytes comprises a general phenomenon in their response to inflammation (133). It has also been demonstrated that the inhibition of autophagy diminishes NETosis, avoiding chromatin decondensation and resulting in a cell death characterized by signs of apoptosis (134, 135).

Autophagy in neutrophils is activated by phagocytosis of pathogens or activation of pattern recognition receptors (PRRs) that induce non-canonical selective autophagy during bactericidal activity by pathogen-derived toxins and molecules that damaged organelles such as mitochondria or peroxisomes resulting in specific fusion of lysosomes with these organelles (136–139).

The fact that LPS or phagocytosis of opsonized particles can induce autophagy in neutrophils independently of the formation of NETs, suggests that it is an additional effector mechanism that is independent of other responses to pathogens (140); however, the molecular mechanisms responsible for this remain unclear and in dispute. In this manner, bacteria that could escape from the phagolysosome may become exposed to the degradative proteases present in autophagosomes (132).

Phagocytosis

Phagocytosis is an important physiological mechanism that allows the elimination of excess cells or damaged cells and, in cases of infection by pathogens, their elimination. Because many of the eat-me signals are displayed on necrotic cells or cells in the process of apoptosis, it was thought that phagocytes only feed on dead cells or cells condemned to die (141). However, there is now evidence that phagocytosis of viable cells can cause their death, a process that has been termed primary phagocytosis or phagoptosis (142, 143).

The process of phagocytosis of apoptotic neutrophils is normally initiated with exposure of the phospholipid, phosphatidylserine (PS) (the "eat-me" signal). In healthy cells, PS is found nearly exclusively in the internal leaflet of the plasma membrane but, in apoptotic cells, it becomes exposed on the outer side through the action of a phospholipid scramblase. Exposure of PS can take place because of the following diverse processes:

The increase of intracellular Ca^{2+} ions that stimulates the scramblase and inhibits the translocase of phospholipids (flippase).
Depletion of ATP, which inhibits the translocase.
Oxidative stress, which stimulates the scramblase and inhibits the translocase.
The fusion of intracellular vesicles with the plasma membrane.
Necrosis due to the rupture of the plasma membrane, an increase of intracellular calcium or depletion of ATP (144, 145).

Activated macrophages can induce the exposure of PS on viable neutrophils (independently of apoptosis). PS exposure can then promote their phagocytosis, which is facilitated by the PS-binding proteins MFG-E8 (Milk fat globule-EGF factor 8 protein) and Annexin 1 (146).

The plasminogen activator inhibitor 1 (PAI-1) acts as a "don't-eat-me" signal in neutrophils as phagocytosis of viable neutrophils of PAI-1 knockout mice by macrophages was increased when compared to that of neutrophils from wild type mice. On the other hand, when PAI-1 or its receptor (CD47) are blocked, calreticulin (CTR) acts as the main "eat-me" signal and the lipoprotein receptor-related protein (LRP) (147) mediates phagocytosis.

Changes in the microenvironment of the neutrophil after having initiated a phagocytic process can lead to cell death. Neutrophils are exceptionally efficient phagocytes and are capable of gobbling up a prey opsonized with IgG in <20 s, in comparison with macrophages, which need up to several minutes to ingest similar amounts of opsonized particles (148). In neutrophils, the recruitment of NADPH oxidase during phagosome maturation is significantly higher than in macrophages due to the fusion of cytoplasmic granules with the phagosome (149, 150). These, and probably other mechanisms, result in the neutrophil phagosome to retain a pH close to neutral for longer periods of time, in comparison with the maturation of the phagolysosome in macrophages, in which acidification can reach pH values of 4.5–5 (151, 152).

It was reported that after phagocytosis of heat-killed *Escherichia coli*, the intracellular pH is crucial to determine the fate of the neutrophil and that the pH depends on the pathogen: phagocyte ratio; mild acidification of pH leads to cell apoptosis, but conditions of high bacterial concentrations that induce a larger decrease in pH lead to necrosis (153). In the case of virulent *Staphylococcus aureus* strains, it was shown that its phenol-soluble modulin (PSM) alpha proteins cause necrosis of neutrophils after phagocytosis, increasing the survival rate of the bacteria (154, 155). It is known that TNFα, influenza virus, as well as the virulence factors of *E. coli*, *Mycobacterium tuberculosis*, and other microorganisms, promote the death of neutrophils (156–158). In the majority of cases, this occurs after certain virulent factors are able to escape from the phagosome, activating cell death pathways such as necrosis or pyroptosis. As mentioned earlier, pyroptosis is mediated by the inflammasome, which is assembled after the phagocytized bacteria escape from the phagosomes and the bacterial products are detected by PRRs in the cytosol (159, 160).

INTRACELLULAR MEDIATORS OF THE CELL DEATH OF NEUTROPHILS

Free Radicals

In a normal process and under the appropriate stimuli, neutrophils are capable of releasing cytotoxic mediators, such as ROS and RNS, generating damage to pathogens but also to the host's tissues. After the elimination of the proinflammatory stimuli, repaiment of the damaged tissue is necessary to return the tissue to a homeostasis state. At this point, anti-inflammatory signals start to be released contributing to the resolution of inflammation, and neutrophils in the tissue should enter apoptosis and be ingested by macrophages in order to clean the inflamed area. Apoptosis of neutrophils is regulated by intracellular mediators and extracellular signals; among the intracellular mediators are the ROS, mainly produced by the NADPH oxidase of the activated neutrophils (161, 162), although some reports show that ROS can be generated by mechanisms that are independent of the NADPH oxidase. Thus, it was reported that the production of superoxide and hydrogen peroxide can be mediated by low-conductance, calcium-activated potassium channels known as SK (small conductance) channels (163, 164). Another way of generating ROS in the neutrophil is through the accumulation of electrons of the respiratory chain, linked to a low activity of complex IV due to the low levels of cytochrome c (16, 165–167). Decreasing intracellular ROS levels by reduced glutathione (GSH), as well as by catalase, inhibits the death of neutrophils (39, 168, 169).

ROS have the capacity to damage DNA generating cell death through direct or indirect activation of p53 (170). ROS can also induce activation of the inflammasome, formed by NLRs, an adaptor molecule, and caspase-1 (171, 172). However, ROS can directly alter the activity of the intracellular signaling pathways implicated in the death and survival of neutrophils, such as NF-κB and MAPK (mitogen-activated protein kinases) (173, 174). In fibroblasts, ROS cause oxidation and inhibition of JNK-inactivating phosphatases, promoting JNK activation, release of cytochrome c, and the subsequent activation of caspase-3 (175, 176). Similarly, it has been demonstrated that the accumulation of ROS results in the grouping of death receptors in lipid rafts and the activation of caspase-8, independently of the Fas ligand (29). The generation of intracellular (but not phagosomal) ROS, caused by neutrophil activation, triggers the process of apoptosis, while the production of intraphagosomal ROS during phagocytosis does not have this effect. This suggests that the location of the production of ROS is fundamental for inducing the death of neutrophils (177). Among the molecules involved in

the regulation of ROS production after cell activation, is Bruton's tyrosine kinase (Btk). The absence of this kinase induces a greater production of ROS by hyper phosphorylation and activation of phosphatidylinositol-3-OH-kinase (PI3K) and protein tyrosine-kinases (TKPs) (178). In addition, the levels of free radicals can be elevated by nitric oxide synthase (NOS) through the production of nitric oxide (NO) (177, 179). In recent years, it has been demonstrated that NO has also the capacity to induce apoptosis in neutrophils (180). It has been shown that the derivative of oxatriazol-5-amine, PGE 3162, and SIN-1 increase the rate of apoptosis in human neutrophils, correlating with data of the greater DNA fragmentation and cell death in neutrophils treated with exogenous NO (181, 182). Interestingly, high levels of ROS or RNS in neutrophils inhibits the activity of the caspases, suggesting the existence of an alternative, caspase-independent cell-death pathway (177, 179, 183, 184).

Caspases and Calpains Involved in Apoptosis

Most stimuli that lead to apoptosis converge in the mitochondria inducing the permeabilization of their external membrane. With permeabilization, a series of proteins are released that activate the caspases (185), which carry out the majority of the proteolytic events of apoptosis and are considered as the ultimate responsible for cell death. Caspases are localized in the form of procaspases in the cytoplasm and the intermembrane space of the mitochondria. Procaspases are activated by cleavage and act as executors cleaving cellular survival molecules triggering processes that induce cell death. Caspases are regulated at the post-translational level and possess a classic structure consisting of a prodomain (N-terminal), a small subunit (p10) in the C-terminal, and a large subunit (p20) that contains the active center with cysteine within a QACXG-conserved motif (186, 187).

The initiator caspases possess prodomains larger than effector caspases. The latter contain caspase recruitment domains (CARD), as in the case of caspase-2 or caspase-9, or cell death effector domains (DED), as in the case of caspase-8 and caspase-10, which permits them to interact with other molecules that regulate their activation. The stimulus for cell death induces the grouping of the initiator caspases (Scaffold-Mediated Activation), allowing them to transactivate by cross-cleavage and, thus, proceed to activate the effector caspases. In all the studied cases, the mature enzyme is a heterotetramer that contains two p20/p10 heterodimers and two active centers (186). In neutrophils, caspases 3 and 10 play an important role in the induction of cell death (188). In *Helicobacter pylori*-infected neutrophils, the activity of caspase-1 is increased, promoting its association into inflammasomes to participate in the triggering of pyroptosis (189). In neutrophils, the activity of caspase-9 also increases drastically during apoptosis (167).

The calpains are calcium-dependent cysteine proteases that can be divided into two subfamilies according to the cation concentrations necessary for their activation: micromolar for the μ-calpains and millimolar for the m-calpains. They play an important role in cell proliferation, progression of the cell cycle, cell migration, and programmed cell death (190). Calpains are present in the cytosol as inactive enzymes that are activated in response to increases in the cellular concentration of Ca^{2+} ions. Once activated, they can modify transcription factors, cytoskeletal proteins, kinases, and proapoptotic proteins, such as Bid and Bax, into fragments incapable of interacting with antiapoptotic proteins, inducing the release of cytochrome c and activation of caspase-3 leading to apoptosis (191, 192). In human neutrophils, calpains do not only play a role in apoptosis, but calpains are also involved in the adhesion of TNF-α-stimulated cells, and play a role in migration regulated by the cytosolic Ca^{+2} concentration (193, 194).

Bcl2 and IAP (Apoptosis-Regulatory Proteins)

The process of apoptosis in human cells is regulated by a family of diverse pro-apoptotic and anti-apoptotic proteins, with protein Bcl-2 as the prototype protein. Members of this family are grouped into three subfamilies as follows: the antiapoptotic proteins (Bcl-2, Bcl-Xl, Mcl-1, and others), the "multidomain"-type proapoptotic proteins (Bax and Bak), and the "BH3-only"-type proapoptotic proteins (Bid, Bim, Bad, among others). The balance among these three groups determines susceptibility to cell death or survival as shown by the increased resistance to apoptosis of certain tumors related to the overexpression of the antiapoptotic proteins.

Another family of proteins with the ability to inhibit apoptosis (denominated IAPs) regulate the cytochrome c-caspase pathway. In humans, the following three members have been characterized: XIAP: c-IAP 1, and c-IAP 2, which bind to caspase-9 preventing its activation. In neutrophils, caspase-9 increases its activity during apoptosis, even though the levels of cytochrome c in these cells are very low (165–167, 195). Data obtained by Murphy et al. (196) suggest that neutrophils possess a lower threshold of cytochrome c for the assembly of functional apoptosomes and their low content of cytochrome c can be compensated for by the elevated expression of apoptotic protease-activating factor 1 (Apaf-1). These further suggest that neutrophils retain a low expression of cytochrome c for the assembly of functional apoptosomes rather than for oxidative phosphorylation.

Neutrophils contain few mitochondria and these are found as tubular networks, and are grouped and depolarized under apoptotic conditions. Despite the relatively low levels of cytochrome c in these cells, the mitochondrial death pathway is functional (167, 197). In neutrophils, the expression of antiapoptotic molecules, such as Mcl-1 and Bcl-xl, is preferential in comparison with those of the proapoptotic protein family (198).

One of the mechanisms that regulate the transcription of the antiapoptotic proteins in neutrophils is stimulation with GM-CSF and TNF-α, which prevents the time-dependent nuclear localization of Mcl-1, and an increase in the transcription and translocation of Bcl-Xl, via stimulation of the NF-κB pathway. However, these cytokines also participate in the increase and maintenance of RNAm levels of BH3-only proapoptotic proteins. GM-CSF is a survival factor for neutrophils that promotes the transcription of Bim and the expression of BimeL (199). During neutrophil cell death by apoptosis, Mcl-1 levels diminish gradually, leading to the release of Bax and to its later

translocation to the mitochondrial membrane (200). The amount of Mcl-1 can be regulated at the transcriptional level through NF-κB and PI3K (201). Another mechanism of regulation of the neutrophil mitochondrial integrity is the flow of potassium (K). A high concentration of extracellular K^+ promotes the survival of neutrophils through the prevention of mitochondrial dysfunction and the release of proapoptotic factors.

MODULATION OF CELL DEATH BY EXTRACELLULAR STIMULI

Trans endothelial migration (exiting the blood vessel to reach the inflammation site) is one of the situations that promote neutrophil survival. At the inflammation site, neutrophils encounter various cytokines and chemokines that promote their activation and survival. Inflammatory mediators delay the spontaneous apoptosis of neutrophils, contributing to the persistence of inflammation. The mechanisms responsible for this delay, and for their increased resistance to apoptosis induced by anti-Fas antibodies, are related to the inhibition of caspase activity (202). Interferons type I and II delay neutrophil death through the activation of STAT3 and the positive regulation of the expression of the inhibitor of apoptosis protein 2 (cIAP) (203).

TLRs ligands, such as LPS, R-848, and CpG, delay spontaneous neutrophil apoptosis through the activation of Toll-like receptors (TLRs). Delay of neutrophil cell death is associated with greater phosphorylation of Akt and an increase in the levels of the antiapoptotic proteins Mcl-1 and A1 (204). Stimulation with LPS also regulates the decreased expression of many other factors related to apoptosis, suggesting that gene regulation can play an essential role in the modulation of neutrophil death (205). The lifespan of neutrophils can be also prolonged by G-CSF and GM-CSF. GM-CSF can up regulate via PI3K/Akt the expression of antiapoptotic molecules, and down regulate the expression of proapoptotic molecules (206–208).

G-CSF can act by blocking Bid/Bax (195) and inhibiting the activation of caspase-3 (209). In a similar fashion, Bruno et al. (210) demonstrated in *in-vitro* experiments that the activation of the leptin receptors expressed on the membrane of neutrophils delayed their apoptosis. In addition, it was reported that high concentration of extracellular K+ ions prevents K+ efflux and delays neutrophil's apoptosis, suggesting that potassium released from damaged cells can function as a survival signal (211). Neutrophil death can also be delayed by activated endothelial cells through the release of GM-CSF and/or by direct interaction (212, 213). Similarly, neutrophil death is delayed by the interaction with natural killer (NK) cells, through the secretion of IFN-γ and GM-CSF by the latter cells (214). These studies showed that neutrophil death can be delayed by their interaction with other cells, not only by the action of secreted cytokines, but also as a consequence of integrin signaling induced by direct cell-cell interaction (215).

Another molecule with an antiapoptotic function on neutrophils is the plasminogen activator inhibitor-1 (PAI-1), which is present in severe inflammatory states and is associated with neutrophil activation. PAI-1 activates antiapoptotic-signaling pathways, including the positive regulation of PKB/Akt, Mcl-1, and Bcl-x (L) (216). On the other hand, TNF-α plays a dual role in neutrophils: it can induce either their survival or death, and the effect is dependent on the intracellular, as well as on the extracellular environment (201). Survival tends to be favored at low doses of TNF-α, while proapoptotic effects are observed at high concentrations (217–219).

Induction of cell death induced by TNF-α is mediated through its receptors by the activation of the JNK/MAPK pathways, release of ROS, and activation of caspase-8. However, cell death in the neutrophil can be caspase-independent (220). The TNF-α receptors can also promote survival in human neutrophils, inhibiting spontaneous death by inducing an increase in PKCδ and PI3K activity (221) and promoting the expression of the antiapoptotic molecules Al and Bcl-XL through an NF-κB-dependent pathway (222, 223).

CELL DEATH RECEPTORS

Apoptosis can be initiated by extracellular signals received through distinct membrane receptors known as death receptors or extrinsic pathway receptors. Two families of receptors have been identified as death receptors: the Fas protein (also called CD95 or APO-1) and receptors for TNF-α.

The transmembrane Fas protein has an intracellular death domain that, when Fas is aggregated, interacts with the adaptor protein FADD (Fas-associated protein with death domain), which participates in cell death activating caspases 8 and 10 (224). Additionally, the intracellular domain of Fas can activate another factor called DAP6 (death domain-associated protein 6, or Daxx). DAP6 activates a protein kinase pathway leading to the opposite effect, stimulating entering into the cell cycle and mitosis (225).

The Fas pathway remains inactive until the extracellular domain binds to Fas ligand (Fas L), a protein expressed on the membrane of a neighbor cell that initiates the apoptosis pathway in the cells expressing Fas. Since this apoptotic pathway does not require *de novo* protein synthesis, it acts very fast inducing apoptosis.

The membrane receptor 1 for TNF (TNFR1) is a member of the TNF receptors superfamily that has in its intracellular portion a death domain, thus being capable of inducing cell death. Upon activation, its intracellular domain interacts with proteins such as TRADD (TNF receptor associated death domain) and RAIDD (226) (receptor associated interleukin death domain), which activate the caspases that can initiate apoptosis (227). However, it can also associate with a different factor denominated TRAF (TNF receptor associated factor), which activates protein-kinases and stimulates cell proliferation instead of apoptosis (228).

Neutrophils constitutively express high levels of Fas and Fas L on their membrane; thus, they are highly susceptible to cell death. The activation of Fas leads to an increase in the activation of caspases 8 and 3, and increases mitochondrial permeability (229). Fas signaling also increases mitochondrial permeability through Bid, Bak, and Bax, accelerating the apoptosis of neutrophils, but they are not necessary for apoptosis to be produced. Apoptosis

triggered by Fas can be inhibited by Mcl-1 and Bcl-2 (230). The use of mitochondrial stabilizers significantly suppresses Fas-mediated cell death, suggesting that mitochondrial alteration is an essential step for neutrophil apoptosis induced by Fas (229). Nonetheless, despite the large body of data showing that activation of the Fas/FasL pathway accelerates the death of neutrophils, there are studies conducted in Fas (lpr)- or FasL (gld)-deficient mice that show that the deficiency of either of these molecules does not alter the rate of neutrophil death. This suggests that FasL/Fas-mediated apoptosis is not essential in determining the lifespan of neutrophils during an acute inflammatory response (231, 232). In agreement with this, the blockage of Fas/FasL with specific antagonists did not alter the rate of spontaneous death of neutrophils (233).

Neutrophils also express other receptors, such as TRAIL-R2 and R3 and its ligand, the TNF-related apoptosis inducing ligand (TRAIL) (234, 235). It has been demonstrated that the exposure of neutrophils to exogenous TRAIL accelerates the process of cell death and it has been suggested that this ligand suppresses cytokines with antiapoptotic effects (236–239).

In **Figure 1** we summarize the various pathways by which the neutrophil detects external signals through its PRRs and triggers effector functions, in addition to the activation of the different types of programmed cell death and the main signaling molecules involved.

NEUTROPHILS DEATH AND DISEASE

Neutrophil cell death has been found to participate in the pathophysiology of various diseases. The elucidation of the mechanisms involved is important to design prophylactic or therapeutic strategies to help patients. Given that great numbers of neutrophils are released daily to the circulation, a precise regulation of neutrophil death is essential to maintain the homeostatic equilibrium in the body. Neutrophils are of great importance in pathogen elimination due to their powerful antimicrobial arsenal. However, many of these microbicidal molecules can also enhance inflammation and tissue injury and can be detrimental for several inflammatory diseases.

Clinical Implications With Apoptosis and Netosis

Apoptosis

In a normal inflammatory process, the apoptotic cells are recognized and removed by macrophages through efferocytosis, so that the inflammation process is regulated. Thus, it is important for neutrophils to undergo spontaneous apoptosis to contribute to the normal resolution of infections or inflammation. Conditions that delay or prevent neutrophil apoptosis result in neutrophilia and this can be a major contributor to chronicity. Gray et al. observed this phenomenon in cystic fibrosis patients, but a similar situation has been seen in many clinical conditions, especially in patients with lung diseases, such as lung cancer (240, 241), asthma (242, 243), chronic obstructive pulmonary disease (COPD) (244, 245), among others. Additionally, the extent of neutrophil apoptosis was found to be inversely proportional to the severity of sepsis in acute respiratory distress syndrome (ARDS). The mean percentage of apoptosis was significantly lower in sepsis-induced ARDS patients compared with uncomplicated sepsis (246).

A delay in neutrophil apoptosis has also been seen in inflammatory bowel disease (IBD) and the induction of apoptosis by the activation of different signaling pathways seems to be a possible solution for this disease. Zhang et al. observed that, in patients with IBD, treatment with TNF-α mAbs induces neutrophil apoptosis leading the resolution of inflammation in the intestinal mucosa (247). In a different study, Rossi et al. induced apoptosis of neutrophils using CDK inhibitors (that induce caspase dependent apoptosis) and found this facilitated the resolution of established neutrophil-dependent inflammation, suggesting this approach could be used to aid in the treatment of chronic inflammatory diseases (248).

On the other hand, a potentiated apoptosis could result in neutropenia and there are many pathological conditions related to this. Neutropenia is primarily associated with severe systemic bacterial infection. However, neutropenia due to excessive apoptosis can be found in systemic lupus erythematosus patients (249), patients undergoing hemodialysis (where apoptosis is related to the deficient biocompatibility of hemodialysis systems) (250), and congenital pathologies, such as myelokathexis, in which myeloid progenitors undergo spontaneous apoptosis due to the down regulated expression of *bcl-x*, an important regulator of apoptosis in hematopoietic cells (251).

NETosis

At the end of last century, the discovery of NETosis and its ability to kill bacteria gave rise to a whole new area of research. As the study of this phenomena advanced, it became evident that even though NETs are important effectors in the clearing of bacterial infections, they can also cause tissue injury (252–254). The precise regulation of NET release and the ensuing NETosis, the same as other cell death forms, is essential for maintaining the homeostatic equilibrium in the body. As mentioned before, a large array of factors that can cause inflammation are released during NETosis. Histones, e.g., are able to directly bind and activate platelets, accelerating thrombin production in a mechanism that is mediated by TLR2 and TLR4 (255, 256). In 2007, it was demonstrated in an experimental model of sepsis that TLR4-dependent platelet-neutrophil interactions induced NET formation and that NETs are able to trap circulating *E. coli*, but at the expense of injury to the endothelium and tissue (257).

NETosis has been shown to be involved in many inflammatory processes and diseases ranging from systemic lupus erythematosus (258) to the new COVID-19. Neutrophils increase dramatically in COVID-19 and ARDS: cytokines that induce NETosis are present in patients' lungs and serum during SARS-CoV-2 infection (259–261). In addition, it has been suggested that NETs could be used as a severity marker in COVID-19 (262).

Previous reports have correlated NETs with pulmonary diseases, particularly ARDS. It has been seen that NETs, and in particular certain components like histones, are present in much higher levels in ARDS than in healthy controls, potentiating the

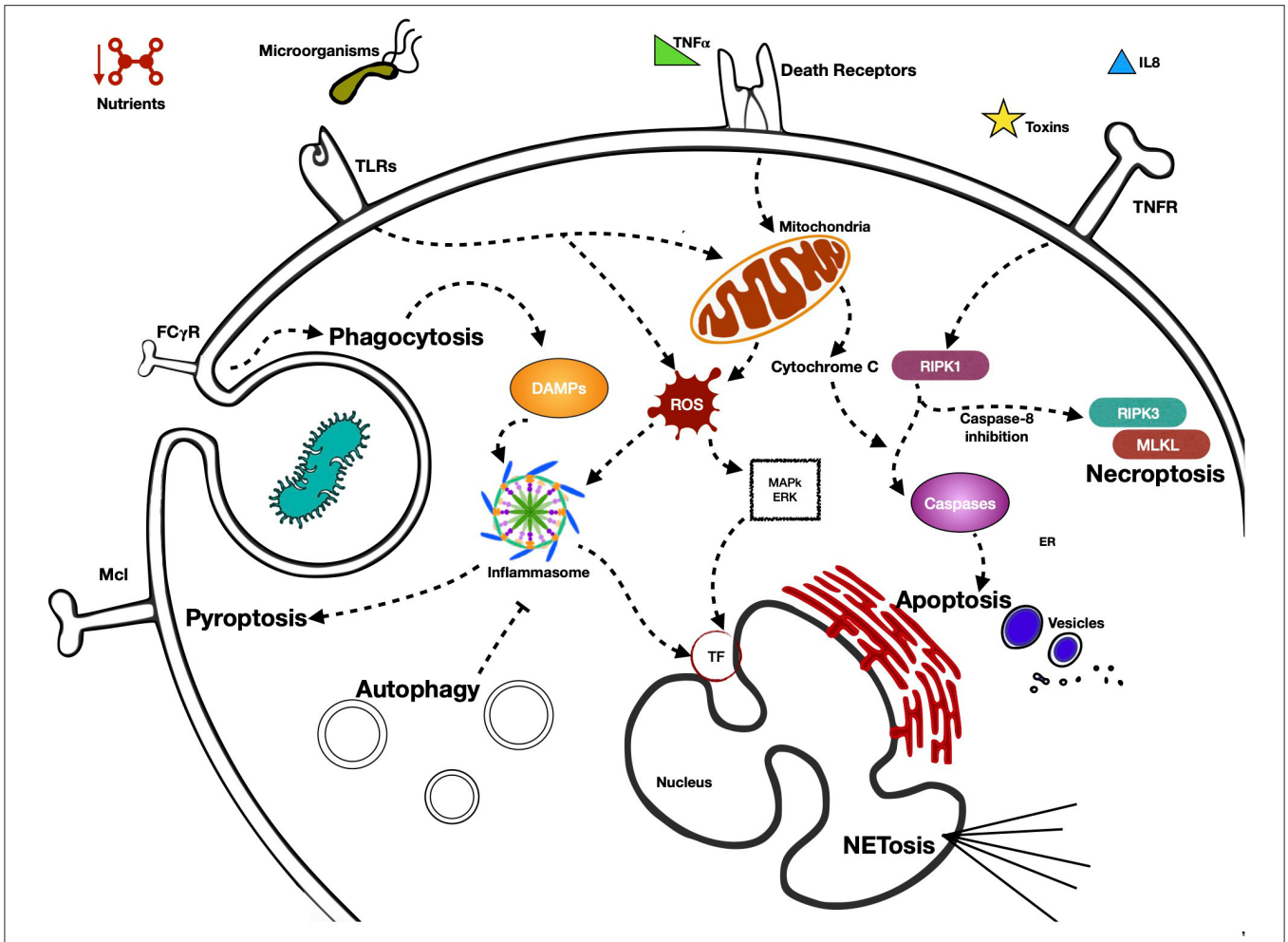

FIGURE 1 | Activation of different type of programmed cell neutrophil death, pathways by neutrophil senses external signals through its PRRs, and triggers effector functions. Neutrophils present in their cellular membrane, a great variety of receptors (pattern recognition receptors, PRR) that are capable of sensoring diverse MAMP (microorganisms associated molecular patterns) and DAMPs (damage-associated molecular patterns). Depending on the nature and intensity of the stimulus received, PRRs are capable of initiating signaling cascades that generally terminate in processes of cell death. These are mainly initiated by proinflammatory proteins, which possess effector functions that lead to the death of neutrophils by means of different mechanisms, such as NETosis, autophagy, necroptosis, or pyroptosis, among others. The receptor that is activated in the processes of cell death is very important, defining as it does the form of cell death that the cell takes. The processes of cell death generally begin with the receptor itself. However, the signaling cascade defines at some point what the fate of the neutrophil will be.

severity of the syndrome and patient's mortality (263–266). In addition, NETs from excessive NETosis makes mucus thicker and more viscous, facilitating bacterial infection, which in turn recruits more neutrophils and promotes further production of NETs. Consequently, the respiratory function of patients is progressively affected, increasing the severity of the disease (267).

NETs release by activated neutrophils in circulation can promote thrombosis in veins and arteries (268, 269). It has been shown in thrombosis animal models that coagulation may be enhanced by direct NET-dependent activation of the contact system (256, 270).

Moreover, a correlation has been demonstrated between NETs production and IL-1β and IL-6, associated with a pro-inflammatory state (271, 272) and the relationship between NETs and permanent organ damage in the cardiovascular and renal systems is well-known (273, 274). The potential of NETs to induce tissue damage underlines the importance of considering NETs evaluation in severe COVID-19 patients, it and suggests that targeting NETs may lessen the severe multi-organ consequences of COVID-19 (275).

Clinical Implications of Other Types of Neutrophil Death

Apoptosis and NETosis are very important neutrophil death forms in disease. Apoptosis due to the homeostatic regulation that must be accomplished in the body and its relation to neutrophils activity, and NETosis due to its ability to enhance inflammation, contributing to the pathophysiology of several diseases. The contribution of these two types of neutrophil death

TABLE 1 | Receptors involved in distinct mechanisms of cell death of neutrophils.

Apoptosis	Necroptosis	NETosis	Autophagy	Phagocytosis
TNFR (201, 219)	Death receptors:	FPR1/2	TLR	FCγR
FAS (APO-1/CD95) (232)	TNFR	FCgR (283)	NOD	
DR3 (APO-3/TRAMP) (201)	TLR	nAChR (284)	NLR	
DR4 (TRAIL-R1) (236)	INFAR	CR3 (285)	CLR	
DR5 (TRAIL-R2(236)		C5aR (when IFN-β is used as a priming cytokine) (286)		
DR6 (201)	Adhesion receptors:			
	CD11b			
	CD15			
	CD18 (62)			

TABLE 2 | Sensor proteins and signal cell death in distinct mechanisms of cell death of neutrophils.

Apoptosis	Necrosis	Necroptosis	Pyroptosis	NETosis
Caspase−2,−3,−6,−7,−8,−9,−10	HIF	RIPK1	Caspase−1,−11	Platelet-neutrophil interaction
Mitochondrial [Ca^{2+}] uptake	Nutrients	RIPK3	Inflammasome	
PIDDosome	Temperature	MLKL		
Cythocrome C, ROS	Mechanical stress	NADPH oxidase (via NETosis)		
	Bacteria			

to disease has received much attention, although other types of neutrophil cell death can also contribute to disease.

Autophagy could participate in disease in a similar way to apoptosis, since enhanced autophagy can result in neutropenia. Olcay et al. showed marked autophagy in neutrophils of patients with congenital dysgranulopoietic neutropenia (CDN), although it cannot be proved that the excess of autophagy may have caused the neutropenia (276).

Neutrophils are the first line of cellular protection against infection, so many of the clinical problems related to dysregulation of their programed cell death is reflected in relation to infection/sepsis diseases.

Pyroptosis is essentially a form of programmed cell death caused by intracellular pathogen infection. Although neutrophils are especially resistant to pyroptosis, it has been demonstrated that they can undergo this form of programmed cell death when there is an absence of a functional NADPH oxidase Nox2 during acute lung infection with *P. aeruginosa* (123), probably to compensate the absence of an antimicrobial pathway. Since neutrophils play an important role in sepsis, it has been suggested that the regulation of neutrophil pyroptosis may positively impact sepsis treatment (277).

Necrosis is usually considered a more pathological form of cell death. When neutrophils die by necrosis, their cytoplasmatic content may cause additional tissue damage or inflammation and it could promote chronic inflammation rather than the resolution of the problem (278, 279). Most of the clinically detrimental effects related to neutrophil necrosis are caused by the virulence of bacteria. This is the case of patients with chronic granulomatous disease, whose neutrophils undergo necrosis after *Burkholderia cenocepacia* infection (280) or the *Haemophilus influenzae* infection, commonly found in patients with COPD (281).

There are some studies that have related chronic inflammation to programmed neutrophil necrosis (282), but there is not much research about pathologies resulting specifically from neutrophil necroptosis. The most known cell types that are correlated with necroptosis-diseases in humans are epithelial liver (270), intestine, and skin cells (68). It was recently found that in patients with neutrophilic diseases, including cutaneous vasculitis, ulcerative colitis, and psoriasis, the migration of neutrophils to infection sites can trigger necroptosis through different adhesion molecules (76).

CONCLUSION

The humoral and cellular components of the innate and acquired immune response form a host-defense integrated system, in which numerous cells and molecules function collectively. Cells of the innate immune response are not only effector cells that can attack the invading microbe immediately, but they also influence the type of specific adaptive response that is subsequently developed. In this regard, neutrophils are very important participants in the immunological response. This is not only because they are important effector cells with great microbicidal activity, but also for the role their products play in the initiation of an adaptive immune response. Since the lifespan of neutrophils is short due to a constitutive program for apoptosis, cytokines and other stimuli at inflammatory sites can delay this apoptosis program. On the other hand, once the pathogenic threat is eliminated, neutrophil death and their

subsequent clearance by macrophages is extremely important for the resolution of inflammation. Dysregulation of the rate of neutrophils death can have deleterious effects. Accelerated death or neutrophils can lead to inefficient pathogen clearance, but delays in their death can result in exacerbated tissue damage and chronic inflammation. Thus, the study of the mechanisms regulating neutrophil death and survival, both in homeostasis and in inflammatory states, is of great interest.

It is noteworthy that all the mechanisms that trigger the death of neutrophils are important to provide signals that contribute to the initiation of an adaptive response, as well as to the recovery of the homeostasis of the tissue and the organism, which are also possible targets of inflammatory diseases (**Tables 1**, **2**). A better understanding of the mechanisms underlying the cell death of neutrophils will aid in understanding their physiology and will contribute in the search for novel approaches for the management of pathologies such as, cystic fibrosis, asthma, COPD, ARDS, sepsis, IBD, systemic lupus erythematosus, myelokathexis, COVID-19, thrombosis, cutaneous vasculitis, ulcerative colitis, and psoriasis, all of them related with alterations in the death mechanisms of these cells. It will also increase our understanding of inflammation in general, allowing us to identify new factors, molecules, and mechanisms that could have possible medical applications. Therefore, their study and their effects are maintained as a broad field of research.

AUTHOR CONTRIBUTIONS

EP-F and PÁ-C reviewed the literature and prepared drafts of the manuscript. EO and CM-B assisted in evaluation of the literature and finalizing manuscript for submission. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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