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Regulation of neutrophil apoptosis by cytokines, pathogens and environmental stressors

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1. ABSTRACT

As a key component of the innate immune response, neutrophils play a major role in host protection against bacterial and fungi infections. Neutrophils are short-lived phagocytic cells and, as a first line of defense against host insult, they are rapidly and massively recruited from the circulation into inflammatory sites, where the expression of their apoptotic program can be regulated by a number of agents such as cytokines, pathogens and environmental stressors. Apoptosis of neutrophils is central to homoeostasis and the resolution of inflammation. Recent studies have highlighted the complex convergence of different pathways in the regulation of neutrophil survival. This review focuses on the mechanisms involved in the induction and regulation of neutrophil apoptosis.

2. INTRODUCTION

Neutrophils play an essential role in the early stages of the inflammatory response to infection by ingesting and killing invading pathogens. They are efficient phagocytes that engulf pathogens and rapidly kill them by means of proteolytic enzymes, antimicrobial proteins, and reactive oxygen species (1-3). Classically, neutrophils are viewed as terminally differentiated phagocytic cells which play a major role in the immune response against bacterial and fungal infections. Normal neutrophil counts are between 2,000 and 8,000/mm^3. The role of neutrophils in host defense is dramatically illustrated in neutropenic patients (4). In fact, all untreated patients with severe neutropenia (<100/mm^3) for 3 weeks or more will develop a serious infection (5). Moreover,

hereditary deficiencies in neutrophil function usually lead to overwhelming bacterial infection which is fatal in the absence of specific treatment (4-7).

Studies performed in the last ten years, however, have shown that the function of neutrophils cannot be merely explained in terms of phagocytosis, killing and degradation of internalized pathogens (8-10). Neutrophils are also able to produce a number of cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), interleukin-12 (IL-12), transforming growth factor beta (TGF-β), and several chemokines such as interleukin-8 (IL-8), growth related oncogene-alpha (Groα), macrophage inflammatory protein 1 alpha/beta (Mip-1α/β), Mip-3α/β, and interferon-gamma-inducible protein 10 (IP-10) (10, 11). On a per-cell basis, neutrophils produce lower amounts of cytokines than mononuclear leukocytes (10, 11). However, given that neutrophils consistently outnumber monocytes, macrophages and dendritic cells by one to two orders of magnitude in areas of acute inflammation, they likely play a role in the orchestration of the immune response at the inflammatory foci. Furthermore, there is a growing body of evidence supporting the view that neutrophils participate in wound healing and tissue repair mechanisms as well as in fine-tuning immune response (8-10).

Neutrophils are produced in the bone marrow at a rate of 10^{11} cells/day in response to a number of hematopoietic growth factors, most notably granulocyte colony-stimulating factor (G-CSF), a cytokine widely used in the clinical setting to treat or prevent neutropenia (1, 4, 12). They are released from the bone marrow to circulation in a regulated fashion. In fact, under steady state conditions only a small fraction of the bone marrow pool is released into blood (4, 12). Neutrophils are the most abundant cell type among circulating leukocytes and, as a first line of defense against host insult, they are rapidly recruited from the circulation to inflammatory sites, where the expression of their apoptotic program can be regulated by a number of agents providing a fine balance between their function as effector cells and a safe turnover of these potentially harmful cells (2, 12, 13-15).

Granulocyte neutrophils are short-lived cells. In the absence of appropriate stimuli, they rapidly (12-24 hs) undergo characteristic changes indicative of apoptosis, including cell shrinkage, nuclear chromatin condensation, DNA fragmentation into nucleosome-length fragments followed by chromatin condensation, and exposure of phosphatidylserine on the outer leaflet of the plasma membrane (13-15). All of these changes appear to be related to the activation of caspases, the central component of the apoptotic machinery that irreversibly commits a cell to die (13-16).

Apoptosis of neutrophils contributes to the resolution of acute inflammation. In apoptotic neutrophils the integrity of the cell membrane is retained, preventing both the loss of neutrophil cytotoxic content and the subsequent amplification of the inflammatory response (13-15, 17). On the other hand, even at early steps, apoptosis appears to be associated with a loss of neutrophil functions, such as chemotaxis, phagocytosis, stimulated shape change, degranulation, and respiratory burst (18, 19). Furthermore, apoptosis triggers specific cell surface changes that mark neutrophils for rapid uptake and disposal, thereby preventing the release of neutrophil cytotoxic content, as would happen if death occurred by necrosis (20-23).

In this review we focus on the multiplicity of signals able to modulate the apoptosis of neutrophils and analyze the major pathways involved in its regulation.

3. REGULATION OF NEUTROPHIL APOPTOSIS VIA DEATH RECEPTORS: THE EXTRINSIC APOPTOSIS PATHWAY

There are two main pathways responsible for the induction of apoptosis; a death-receptor-pathway and a mitochondrial pathway. Both pathways involve the sequential activation of caspases in two distinct but converging pathways. The death receptor-pathway is initiated by Fas, TNF receptors and TRAIL (TNF-related apoptosis-inducing ligand) receptors and occurs via the activation of caspase-8, as an initiator caspase. The mitochondrial pathway, also known as the intrinsic pathway, occurs via the activation of caspase-9 as an initiator caspase and involves the Bcl-2 family members (24-26).

Death receptors comprise a subfamily of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily. They mediate the extrinsic cell death pathway in response to specific death ligands. Each death receptor contains a domain in its cytoplasmic portion called the death domain (DD), a protein-protein interaction motif allowing self-association, a critical event required for signaling and apoptosis induction (26).

3.1. Fas receptors

The ligand for the death receptor Fas is FasL, a homotrimeric molecule belonging to the TNF superfamily. The interaction with FasL triggers the cross-linking of Fas on the cell surface and the subsequent clustering of cytoplasmic DDs, which in turn recruit the adapter protein called Fas-associated death-domain-containing protein (FADD), via the C-terminal DD of FADD (26-28). FADD also contains a N-terminal death effector domain (DED) that homotypically interacts with the tandem DED in the prodomain of caspases-8 and -10. This interaction leads to the formation of a ternary death-inducing signaling complex (DISC), containing Fas, FADD, and the initiator caspases-8 or -10. Recruitment of procaspases-8 or -10 into the DISC triggers proteolytic activation of them, which in turn activate effector caspases such as caspases-3, -7, and -6 leading to apoptotic cell death (26-28). Caspase activation by DISC is inhibited by cFLIPs (cellular caspase-8–FLICE-like inhibitory protein), a family of tandem DED-containing proteins which contain a non-catalytic pseudo-caspase domain, able to interact with FADD (29).

A role for Fas/FasL interactions in the regulation of spontaneous neutrophil apoptosis has been proposed

mainly on the basis of two observations; a) the expression of Fas receptor and FasL in the neutrophil, and b) the fact that cross-linking of Fas receptors by agonistic antibodies directed to Fas results in a dramatic acceleration of apoptosis (30, 31). A possible contribution of the Fas/FasL system in the regulation of neutrophil apoptosis is also consistent with observations made in T cells showing that autocrine and paracrine Fas/FasL interactions seem to play a major role in the control of clonal expansion of activated T cells (32). However, experiments using antagonistic anti-Fas receptor antibodies or agents able to block FasL showed no increase in neutrophil survival. Moreover, neutrophils from Fas (*lpr*) or FasL (*gld*) deficient mice show a normal rate of apoptosis supporting the idea that spontaneous neutrophil apoptosis is not under the regulation of the Fas/FasL system (33). Could this system play a role in the regulation of the apoptotic death of activated neutrophils? Jonsson and coworkers (34) have shown in two models of neutrophilic inflammation (immune-complex-mediated inflammatory arthritis and thioglycollate-induced peritonitis) that the development of the inflammatory response requires the forkhead transcription factor Foxo3a. This requirement appears to be related to the ability of Foxo3a to bind and suppress the FasL promoter and, in this way, maintains neutrophil viability at the inflammatory focus through the inhibition of FasL expression. Moreover, the authors showed that FasL blockade renders Foxo3a-deficient mice susceptible to both, arthritis and peritonitis.

3.2. TNF receptors

The ligand for the death receptor TNFR1 is the trimeric molecule TNF-α, the prototypical member of a family of cytokines that includes TNF, lymphotoxin-α, FasL, CD40 ligand (CD40L), and TRAIL (35). TNFR1 signaling can activate the transcription of NF-κB, promoting cell survival, but can also induce apoptosis via the recruitment of TRADD (TNFR1-associated death domain protein) and caspase-8. The mechanism by which this decision between cell death and survival is taken was clarified by Micheau and Tschopp (36, 37). They showed that that activation of TNFR1 leads in a first step to the assembly of a membrane-bound complex (complex I) consisted in TNFR1, TRADD, RIP (receptor interacting protein), and TRAFs (TNF receptor-associated factors) and that this complex is able to trigger NF-κB activation. In a second step, TRADD dissociates from TNFR1 and associates with FADD and caspase-8, leading to the assembly of a cytoplasmic complex (complex II) which results in the activation of caspases and cell death. Thus, TNFR1 signaling involves a check point that might result in cell survival or cell death, depending on the regulated assembly of complex I and complex II (37). This mechanism might explain contrasting results regarding the action of TNF-α on neutrophil apoptosis. In fact, TNF-α has been variably reported to induce either increase, delay, or have no effect on neutrophil apoptosis, depending on the experimental setting (38-41). For example, Murray *et al.* (42) showed that although prolonged incubation of human neutrophils with TNF-α indeed causes a decrease in the extent of apoptosis, TNF-α actually induces apoptosis in a proportion of neutrophils at earlier times (4-8 h). These results could be explained on the basis of the model of Micheau and Tschopp, considering the possibility that the assembly and/or the activity of TNFR1 complex I and complex II may be differentially regulated in freshly isolated and *in vitro* aged neutrophils. On the other hand, studies performed in our laboratory have shown, in agreement with the results published by Murray (42), that neutrophils cultured with TNF-α alone for short periods undergo a low but significant increase in the number of apoptotic cells. More interestingly, when neutrophils were pretreated with TNF-α and then were exposed to a variety of stimuli such as IgG-coated erythrocytes, zymosan, PMA, *Escherichia coli*, or GM-CSF, a marked stimulation of apoptosis was observed (43). These results suggest that TNF-α might play a role in the control of neutrophil survival by virtue of its ability to induce an apoptotic death program which could be triggered by a variety of conventional agonists. The underlying mechanisms remain to be defined. It is possible to speculate that further activation of TNF-α-primed neutrophils by conventional agonists might favor the assembly of TNFR1 complex II and/or the activation of the mitochondrial patwhway of apoptosis. Supporting the participation of the intrinsic pathway of apoptosis in the above described results, are the observations from Daigle *et al.* (44), showing that the activation of death receptors Fas or TNFR1 blocks the capacity of GM-CSF to induce survival signals and that this effect is related to the inability of GM-CSF to suppress the expression of the proapoptotic Bax gene in neutrophils treated with either Fas or TNF-α.

3.3. TRAIL receptors

Although Fas and TNFR1 are the best-characterized death receptors expressed by neutrophils, the expression of TRAIL receptors has also been demonstrated. TRAIL is a homotrimer that, unlike other cytokines of the TNF family, is able to interact with a complex system of receptors including two proapoptotic death receptors, TRAIL-R1 and TRAIL-R2, and three decoy receptors devoid of functional DDs, which appear to act as anti-apoptotic receptors (45). It is well documented that under appropriate stimulation neutrophils produce TRAIL. In fact, Cassatella *et al.* (46, 47) have shown that the intracellular pool of TRAIL present in interferon-α or interferon-γ-pretreated neutrophils is secreted following exposure to proinflammatory mediators such as TNF-α, LPS, fMLP, IL-8, insoluble immune complexes, and heat shock protein Gp96. These results support the notion that TRAIL does not play a role in the regulation of apoptosis of unstimulated neutrophils but it might be involved in the regulation of apoptosis of activated cells. Interestingly, Lum *et al.* (48) have reported that TRAIL plays an important role in the elimination of senescent neutrophils. They showed that the interaction of stromal cell-derived factor 1 (SDF-1) with the chemokine receptor CXCR4, that is preferentially expressed on senescent neutrophils, increases the expression of TRAIL and TRAIL receptors in the neutrophil, leading to TRAIL-dependent apoptosis.

4. REGULATION OF APOPTOSIS VIA THE MITOCHONDRIA: THE INTRINSIC APOPTOSIS PATHWAY

The mitochondrial pathway, also known as intrinsic or stress pathway, leads to the activation of caspase-9 on the scaffold protein Apaf-1 (apoptotic protease-activating factor 1), when cytochrome c and other proapoptotic proteins such as Smac/DIABLO (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis [IAP]-binding protein with low pI), apoptosis-inducing factor (AIF), and Omi/HtrA2 (Omi/high temperature requirement protein A2) are released from the space between the outer and inner mitochondrial membrane to the cytosol (49, 50). This occurs as a consequence of the permeabilization of the mitochondrial outer membrane, a critical event that is usually considered to be the "point of no return" in the induction of apoptosis. This event can be triggered by a variety of stressors such as DNA damage, growth factor deprivation, cytoskeleton damage, endoplasmic reticulum stress, detachment from the cell matrix (anoikis), inhibition of macromolecular synthesis, chemotherapy drugs and gamma-irradiation (49-51). Cytochrome c released from the mitochondria interacts with the WD repeat domain of APAF-1. This interaction appears to open up the Apaf-1 structure, leading to the oligomerization of Apaf-1 and the formation of the apoptosome, which then recruits caspase-9 via caspase recruitment domain (CARD) interactions between Apaf-1 and caspase-9, enabling caspase-9 to activate effector caspases-3, -6, and -7 inducing apoptotic cell death (49-51).

The intrinsic pathway is under the control of the Bcl-2 family of proteins, which act as prominent gatekeepers controlling the release of cytochrome c and other proapoptotic proteins from the mitochondria to the cytosol (52, 53). They are classified into three subfamilies on the basis of function and sequence similarity. The anti-apoptotic subfamily comprises Bcl-2 itself, Bcl-x_L, Bcl-w, Mcl-1, A1, and Bcl-B. All of them contain four conserved BCL-2 homology domains called BH4, BH3, BH1, and BH2. The pro-apoptotic proteins include two subfamilies, the multidomain subfamily represented by Bax and Bak, which contain the BCL-2 homology domains BH3, BH1, and BH2, and the "BH3-only" subfamily represented by Bim, Bad, Bid, Bik, Bmf, Puma, Noxa, and Hrk, which only bear a single BCL-2 homology domain (BH3) (52, 53).

A first step in the induction of the intrinsic pathway of apoptosis is the activation of BH3-only proteins. Different BH3-only proteins appear to be activated by distinct stress signals. BID is activated by caspase-8 and granzyme B. Bim is sequestered to microtubule complexes and Bmf to the myosin V motor complex by distinct dynein light chains. Both are released by UV-irradiation through a process that appears to involve their phosphorylation. BAD appears to be inactivated by phosphorylation, while Noxa and PUMA are transcriptionally upregulated in response to distinct proapoptotic stimuli (52, 53). BH3-only proteins do not usually display intrinsic cell destructive properties, instead, they seem to function mainly as inhibitors of the antiapoptotic Bcl-2 subfamily of proteins. In fact, after activation, BH3-only proteins interact with the members of the antiapoptosis Bcl-2 subfamily of proteins. In these proteins, the BH1, BH2 and BH3 domains, although not contiguous in the sequence, form a surface-exposed hydrophobic groove which functions as the binding site for the BH3 domain of the pro-apoptotic BH3-only proteins. Recent studies have shown that these proteins are selective for their pro-survival counterparts. Bim, Puma, and tBid (the activated, truncated form of Bid) targ*et* all pro-survival proteins, and thus are potent inducers of apoptosis. Noxa engages only Mcl-1 and A1, and Bad engages only Bcl-2, Bcl-x_L, and Bcl-w (54-56).

Heterodimerization with and inactivation of pro-survival members of the Bcl-2 family by BH3-only proteins cannot explain per se the induction of cell death. The BH3-only proteins act upstream of Bax and Bak. In fact, they can not induce apoptosis in cells lacking both Bax and Bak, suggesting that a major role of the pro-survival members of the Bcl-2 family is to bind and inactivate Bax and Bak. Two models have been proposed to explain how BH3-only proteins promote the activation of Bax and Bak, enabling these proteins to form a channel for the efflux of cytochrome c across the outer mitochondrial membrane. The direct activation model supports that certain BH3-only proteins such as Bim, tBid, and Puma are able to directly bind to Bax and Bak promoting their activation. By contrast, the indirect activation model proposes that all BH3-only proteins bind to the pro-survival members of the Bcl-2 family, thereby preventing them to interact and to inhibit Bax and Bak. Recent data support this latter possibility (54-56).

A further level of complexity in the regulation of the intrinsic pathway of apoptosis relates to the fact that, in addition to Bax/Bak-dependent permeabilization of the outer membrane of the mitochondria, a complex remodeling of the inner membrane is triggered during the activation of the intrinsic pathway of apoptosis (57, 58). This process appears to be required to allow the release of high amounts of cytochrome c to the cytosol, since almost 90% of the mitochondrial cytochrome c content appears to be sequestered within the cristae folds of the mitochondrial inner membrane. These structures function as relatively closed compartments limiting free passage of their content out of the mitochondria even if the outer membrane is breached (57, 58).

4.1. The intrinsic pathway of apoptosis in the neutrophil

It has long been assumed that mature human neutrophils have few functional mitochondria. This assumption was largely based on studies performed by electron microscopy showing that neutrophils have very low numbers of mitochondria, and that these organelles usually show poorly defined cristae and inner mitochondrial membranes (59, 60). This point of view was reinforced by the fact that mitochondrial poisons like cyanides do not inhibit neutrophil function, supporting that mitochondria hardly participate in ATP synthesis (61). These observations are in line with the traditional view of the neutrophil as cells that can rely on glycolysis for their

energy requirements, allowing them to function at inflammatory sites were oxygen tensions are usually low. The dramatic advances in the field of apoptosis and the critical role that the mitochondria plays in its induction, lead to different laboratories to reinvestigate the nature and the function of the mitochondrial network in the neutrophil. As a consequence, the traditional view of the neutrophil as a cell which has neither the capacity nor the requirement for active mitochondrial functions was challenged.

Studies performed by Fossati *et al.* (62) and by Maiansky *et al.* (63, 64), using fluorescent indicators of mitochondrial function, confocal microscopy, flow cytometry, and Western blotting, clearly demonstrated that neutrophils actually possess a highly developed mitochondrial network. Even though this network contains very low amounts of cytochrome c and it is markedly deficient in electron transport, it plays a major role in the control of neutrophil survival (63-68). It has been proposed that the intrinsic pathway of apoptosis in the neutrophil has a lowered threshold requirement for cytochrome c which is compensated by an increased expression of Apaf-1 and/or by other proapoptotic proteins such as Smac/DIABLO and HtrA2/Omi, that are massively released from the mitochondria into the cytosol (69).

4.2. Expression and function of proapoptotic proteins of the Bcl-2 family in the neutrophil

A prominent feature of neutrophils is their very short-half life. Consistent with this short-life, pro-apoptotic proteins of the Bcl-2 family such as Bax, Bak, Bad, Bid, and Bik are constitutively expressed in the neutrophil (68). Bax and Bak appear to play a major role in the apoptosis of both, resting and activated neutrophils. Bax $^{-/-}$ and Bak $^{-/-}$ mice have neutrophil counts similar to those observed in control mice, while Bak and Bax double deficient mice show, not only lymphoproliferative complications resulting in splenomegaly, lymphoadenopathy, and lymphoid infiltration of peripheral organs, but also a marked increase in the number of blood neutrophils (70). While these observations suggest that both, Bax and Bak, display some overlapping functions, reports from the laboratory of Peter Henson show that, when cultured *in vitro* for 12 h, neutrophils from Bax-null mice displayed 50% less spontaneous apoptosis compared with their littermate controls, indicating a non-redundant role for Bax in the control of neutrophil apoptosis (71). Reinforcing a role for Bax in the apoptosis of human neutrophils, Dibbert and coworkers (72) showed that the delayed neutrophil apoptosis observed in several inflammatory diseases is associated with markedly reduced levels of Bax, and also that this phenotype can be induced *in vitro* upon stimulation of normal neutrophils with anti-apoptotic cytokines usually found at sites of neutrophilic inflammation, such as G-CSF and GM-CSF. Moreover, they showed that Bax-deficient human neutrophils generated *in vitro* by using antisense oligodeoxynucleotides undergo apoptosis at a delayed rate compared with controls, providing a direct evidence for the proapoptotic role for Bax (72). Consistent with these results, other studies have shown that prevention of human neutrophil apoptosis by GM-CSF leads to a down-regulation of the expression of Bax (73, 74), while the induction of apoptosis by either TNF-α or *Mycobacterium tuberculosis* results in an increased Bax/Bcl-X_L ratio (73, 75). Spontaneous apoptosis of neutrophils, on the other hand, has been shown to be associated with translocation of Bid and Bax to the mitochondria and truncation of Bid, with subsequent release of Smac/DIABLO and Omi/HtrA2 into the cytosol. This process is accompanied by the activation of caspases-8, -9, and -3, being all these events prevented by G-CSF (64, 76). Interestingly, the activity of Bax in the neutrophil appears to be regulated by phosphorylation. In neutrophils cultured in the presence of anti-apoptotic cytokines Bax is phosphorylated at Ser^{184} by the Akt/protein kinase B. Phosphorylated Bax remains in the cytoplasm, heterodimerized with anti-apoptotic members of the Bcl-2 family of proteins such as Mcl-1, A1, and Bcl-x_L. Upon induction of apoptosis, Bax seems to be dephosphorylated, loses its ability to heterodimerize with the Bcl-2 subfamily of anti-apoptotic proteins, and translocates to the mitochondrial membrane promoting apoptosis (71). Bak is also constitutively expressed by human neutrophils (77, 78), however, its participation in the apoptotic process of human neutrophils has not been rigorously analyzed yet.

Neutrophils constitutively express BAD, a proapoptotic member of the Bcl-2 family of proteins that is tightly regulated by survival factors via PI3K-dependent phosphorylation (79-82). Unphosphorylated BAD promotes apoptosis by interacting with antiapoptotic members of the Bcl-2 family forming a heterodimer. Phosphorylation of BAD induces the liberation of the anti-apoptotic protein from the heterodimer, resulting in the promotion of cell survival. Anti-apoptotic stimuli such as GM-CSF, IL-8, C5a, and toll-like receptor agonists (TLR) such as LPS (TLR4), peptidoglycan (TLR2), R-848 (TLR7/8), and CpG-DNA (TLR9) have shown to inhibit the pro-apoptotic activity of Bad by inducing phosphorylation of Bad at Ser^{112} and Ser^{136}, leading to the accumulation of Bad in the cytosol (80-83). By contrast, nicotinic acid, a pro-apoptotic stimulus, induces dephosphorylation of BAD enabling its interaction with anti-apoptotic members of the Bcl-2 family (84). Neutrophils also express the pro-apoptotic protein Bim. Bim null mice show neutrophil counts higher than control mice (85), while *in vitro* assays indicated that Bim deficiency renders neutrophils resistant to cytokine withdrawal and cytotoxic drugs (86).

Bid, BH3-interacting domain death agonist, first reported in 1996, is also expressed in the neutrophil (68). Bid is cleaved and activated by caspase-8 after death-receptor activation, and the truncated Bid can then promote apoptosis through different mechanisms; i.e., by engaging their prosurvival Bcl-2 like relatives, by directly binding to the essential cell death mediators Bak and Bax, and/or by directly promoting permeabilization of the mitochondrial outer membrane (87). The efficient activation of Bid by caspase-8 supports the notion that the intrinsic pathway plays a role in the induction of apoptosis triggered by death receptors. Full length Bid can also induce this response, but not so efficiently (87). Bid can be also cleaved and activated by the effector caspase-3. This event appears to

occur downstream of the mitochondria activation, supporting the notion that Bid might also play a role in the amplification of proapoptotic signals not related to the activation of death receptors (87). Interestingly, recent studies show that Bid is also a target of other proteases such as granzyme B (88), calpains (89), and cathepsins (90), which are usually activated in response to a number of pro-apoptotic stimuli. This suggests that Bid serves as a major sentinel to protease-mediated death signals. Although recent findings reinforce the notion that Bid is a major linker bridging distinct peripheral death pathways to the mitochondria (87), little is known about the role of Bid in the control of neutrophil apoptosis. Maiansky and coworkers (76) showed that spontaneous apoptosis of neutrophils (after ~ 20 h of culture) is associated to the appearance of tBid. However, in apoptotic cells, tBid mainly remains in the cytosol while the full-length Bid translocates together with Bax to the mitochondria. All these changes, as well as the activation of caspases-8, -9, and -3 are prevented by G-CSF. Other anti-apoptotic factors such as leptin (91), CD134 ligands (92), and macrophage migration inhibitory factor (MIF) (93), have also shown to prevent Bid truncation, supporting the notion that Bid actually plays a role in the induction of neutrophil apoptosis. Blomgran and coworkers (94), on the other hand, describe a novel pathway for the induction of apoptosis in activated neutrophils in which Bid appears to be involved, and intracellular reactive oxygen species (ROS) and lysosomal proteases function as upstream initiators of apoptosis. They found that Type 1-fimbriated *Escherichia coli* stimulates an intrinsic mode of apoptosis in human neutrophils via the induction of lysosomal membrane permeabilization (LMP) by oxidative-stress. This process results in the permeabilization of azurophilic granules, release of cathepsins, cleavage of Bid, permeabilization of the mitochondrial outer membrane, and induction of caspase activation.

4.3. Expression and function of anti-apoptotic proteins of the Bcl-2 familiy in the neutrophil

Neutrophils express the anti-apoptotic proteins Mcl-1 and A1/Bfl-1 (66-68, 81). While there are some contradictory results regarding the expression of Bcl-X_L at the protein level, Bcl-2 has not been detected either in resting nor in activated neutrophils (68, 81). A role for A1 in the control of neutrophil survival is suggested by observations made in A1-deficient mice indicating that although neutrophils develop normally, they show an acceleration of spontaneous apoptosis when cultured *in vitro* (95). Moreover, neutrophils from A1 null mice were shown to be refractory to the anti-apoptotic effects induced by either LPS-treatment or transendothelial migration (95). On the other hand, an increased expression of A1 was observed in neutrophils during the course of systemic inflammation triggered by LPS in a murine model (96). In human neutrophils it was found that A1 mRNA is up-regulated by survival factors such as LPS and G-CSF, and that inhibition of mRNA synthesis by actinomycin D results in a rapid loss of A1 transcripts suggesting that A1 protein is subject to rapid turnover (81, 83).

Mcl-1 was originally cloned in 1993 (97). Mcl-1 is expressed in a variety of cell types and its expression is rapidly stimulated by increases in transcription. Post-transcriptional control of Mcl-1 activity involves phosphorylation at Ser^{121} and Thr^{163}, which results in the inactivation of its anti-apoptotic function (67). Mcl-1 appears to play a major role in the control of neutrophil survival (67). Recent observations made by Dzhagalov and coworkers (98) in mice conditionally lacking Mcl-1 expression in neutrophils and macrophages show that they have a severe defect in neutrophil survival, but not in macrophage survival. The authors found that mature neutrophils were reduced by 80% to 90% in the blood, spleen, and peritoneal exudates of Mcl-1 deficient mice, and that all these changes appear to be related to the decreased survival of neutrophils after the excision of Mcl-1. Numerous studies, on the other hand, have demonstrated that cellular levels of Mcl-1 decline as neutrophils undergo spontaneous apoptosis (99) and that the reduction of Mcl-1 is faster in the presence of proapoptotic agents such as TNF-α (99), nicotinic acid (84), *Trichomonas vaginalis* (100), viscum album agglutinin-I (101), sodium salicylate (102), and cyclin-dependent kinase inhibitors (103). By contrast, treatment of neutrophils with anti-apoptotic agents such as GM-CSF (99, 104, 105), LB4 (106), IL-15 (107), cyclic AMP (108), respiratory syncytial virus (109), hypoxia (110), as well as neutrophil transmigration through an epithelial monolayer (111), results in an increased expression of Mcl-1.

5. CASPASES AND IAPs

Caspases (cysteinyl aspartate proteases) are the executioners in both extrinsic and intrinsic pathways of apoptosis. They are the central component of the apoptotic machinery that irreversibly commits a cell to die. All caspases are synthesized as inactive zymogens (procaspases). When apoptosis is triggered, caspases undergo a maturation process which requires intramolecular cleavage to separate p20 and p10 subunits. Initiator caspases (caspases-2, -8, -9, and -10) and effector caspases are activated by distinct mechanisms (112, 113). Activation of initiator caspases such as caspase-8 (extrinsic pathway), and caspase-9 (intrinsic pathway), is triggered by oligomerization, enabling protein-protein-interactions mediated by DEDs or CARDs, respectively, which are responsible for their activation. Initiator caspases are the apical caspases, which are activated by apoptotic stimuli; their most relevant function is to activate the downstream effector caspases by catalyzing a specific intra-chain cleavage. Effector caspases such as caspases-3, -7, and -6, kill cells by cleaving a variety of cellular components (112, 113).

The proteolytic activity of caspases is controlled by inhibitors of apoptosis proteins (IAPs), a family of negative regulators of apoptosis that protect cells from intracellular damage due to premature or aberrant caspase activation. The major function of IAPs is to bind and to inhibit caspases, in particular caspases-3, -9, and -7 (112-114). Molecules such as Smac/DIABLO and Omi/HtrA2 that are released by the mitochondria at the onset of apoptosis, are able to interact with IAPs, preventing their inhibitory activity. Eight IAPs have been described in

humans; c-IAP-1, c-IAP-2, XIAP (X-linked IAP), ILP-2, NAIP, ML-IAP, apollon and survivin (112, 114). Little information is currently available on the expression and function of IAPs in neutrophils. Hasegawa and coworkers (115) have shown that human neutrophils express c-IAP-1, c-IAP-2, and XIAP. They found that cIAP2 was selectively up-regulated by G-CSF, but not by GM-CSF. Interestingly, elevated levels of cIAP2 were also detected in peripheral blood neutrophils from healthy donors receiving G-CSF administration (115). It was also shown that *in vitro* aging of human neutrophils is associated with the loss of c-IAP-1 expression, and that this decreased expression is prevented by both, the pancaspase inhibitor zVAD-FMK and LPS (116). Moreover, the delayed apoptotic rate of neutrophils from septic patients was shown to be associated to increased levels of XIAP, with no changes in the expression of cIAP-1 and c-IAP-2 (116). Results from the Simon Hu laboratory shed new light on the role of IAPs in neutrophil function. These results show that survivin expression is high in immature neutrophils, while mature neutrophils contain only little or no survivin protein. Strikingly, the authors reported that mature neutrophils re-express survivin after stimulation by G-CSF or GM-CSF as well as in active inflammatory diseases, such as acute appendicitis, ulcerative colitis, and cystic fibrosis (117, 118). Of note, anti-sense-mediated inhibition of survivin expression in mature neutrophils prevented the anti-apoptotic effect mediated by both, GM-CSF and G-CSF, and a similar blockade of the anti-apoptotic effect mediated by these cytokines was observed in neutrophils from survivin null mice (117). As indicated by the authors, these data suggest that, although survival cytokines are able to increase the ratio between anti- and proapoptotic members of the Bcl-2 family of proteins, the inhibition of neutrophil apoptosis induced by these cytokines is strongly-dependent on the enhancement in survivin levels.

6. NON-CONVENTIONAL PATHWAYS INVOLVED IN THE INDUCTION OF NEUTROPHIL CELL DEATH

6.1. Induction of neutrophil cell death by ROS

A large amount of ROS is produced by the enzyme NADPH oxidase during the activation of the respiratory burst (1). ROS can also be generated in the neutrophil by the mitochondria, through a NADPH oxidase-independent mechanism, an event that appears to be related to the very low levels of cytochrome c found in the neutrophil mitochondrial network, leading to the accumulation of electrons in the respiratory chain and the subsequent production of ROS (68). Numerous reports indicate that ROS are able to induce the death of neutrophils. Chronic granulomatous disease (CGD) is an inherited disorder of phagocytic cells, characterized by the inability of phagocytes to activate the respiratory burst, due to a defect in the enzyme NADPH oxidase (1). Neutrophils from CGD patients show a delayed rate of spontaneous apoptosis, supporting that ROS actually stimulates apoptosis (119). Also supporting a role for ROS in the induction of neutrophil death are the observations indicating that hydrogen peroxide scavengers such as glutathione and catalase (120), the pharmacologic inhibition of NADPH oxidase (120) and hypoxic conditions (110), delay spontaneous neutrophil apoptosis. On the other hand, several reports have shown that ROS are involved in the induction of neutrophil cell death triggered by a variety of stimuli such as *Escherichia coli* (121), *Mycobacterium tuberculosis*) (75), *Entamoeba histolytica* (122), immune complexes (123), among others. The mechanisms through which ROS interact and/or influence neutrophil death pathways are not well defined. It has been proposed that ROS limit neutrophil life span by inducing ligand-independent death receptor signaling via clustering of preformed DISC components in lipid rafts (124). Alternatively, ROS may also promote apoptosis by interfering the activation of survival pathways such as those mediated by NF-κB and MAPKs (125). Interestingly, ROS can also induce a caspase-independent death pathway in the neutrophil, perhaps reflecting the ability of ROS to inhibit the activation of caspases (126).

6.2. Induction of neutrophil cell death by sialic binding immunoglobulin (Ig)-like lectin 9 (Siglec-9)

Sialic acid-binding immunoglobulin-like lectins (Siglecs) are characterized by the presence of an N-terminal V-set domain that binds sialic acid. In humans 11 members of the Siglec family have been identified. Siglecs are expressed predominantly on the surface of hematopoietic cells. Whereas some members of the Siglec family are broadly expressed, others are restricted to specific cell types. Siglec-9 is expressed predominantly on neutrophils and monocytes (127). Von Gunten and coworkers (128, 129), have shown that Siglec-9 ligation induces apoptosis of resting neutrophils. Strikingly, death induction by Siglec-9 is enhanced when neutrophils were exposed to proinflammatory anti-apoptotic cytokines, such as GM-CSF, interferon-α, or interferon-γ and also in neutrophils obtained from patients with acute septic shock or rheumatoid arthritis. On the other hand, while the death induced by Siglec-9 ligation in both resting and inflammatory neutrophil was similarly dependent on the generation of ROS, major differences were observed in the mechanisms responsible for the induction of cell death. Death induced by Siglec-9 in inflammatory neutrophils, but not in resting cells, was shown to be largely caspase-independent, and it was characterized by cytoplasmic vacuolization and other nonapoptotic morphologic features. Interestingly, similar caspase-independent death pathways were described for neutrophils stimulated by TNF-α in the presence of the caspase inhibitor z-VAD-fmk (126) as well as in cell lines upon induction of cell death by TNF-α or Fas ligation in the presence of caspase inhibitors (130, 131), thus suggesting a distinct pathway of cell death.

6.3. A novel cell death pathway that leads to neutrophil extracellular traps

Zychlinsky and coworkers have recently shown that upon activation by IL-8, LPS, or phorbol myristic acid (PMA), the nuclei of neutrophils display dramatic changes including lose of their shape and homogenization of the eu- and heterochromatin, followed by desintegration of the nuclear envelope and the granule membranes. This process allows the mixing of chromatin and granule proteins, forming neutrophil extracellular traps (NETs), which are

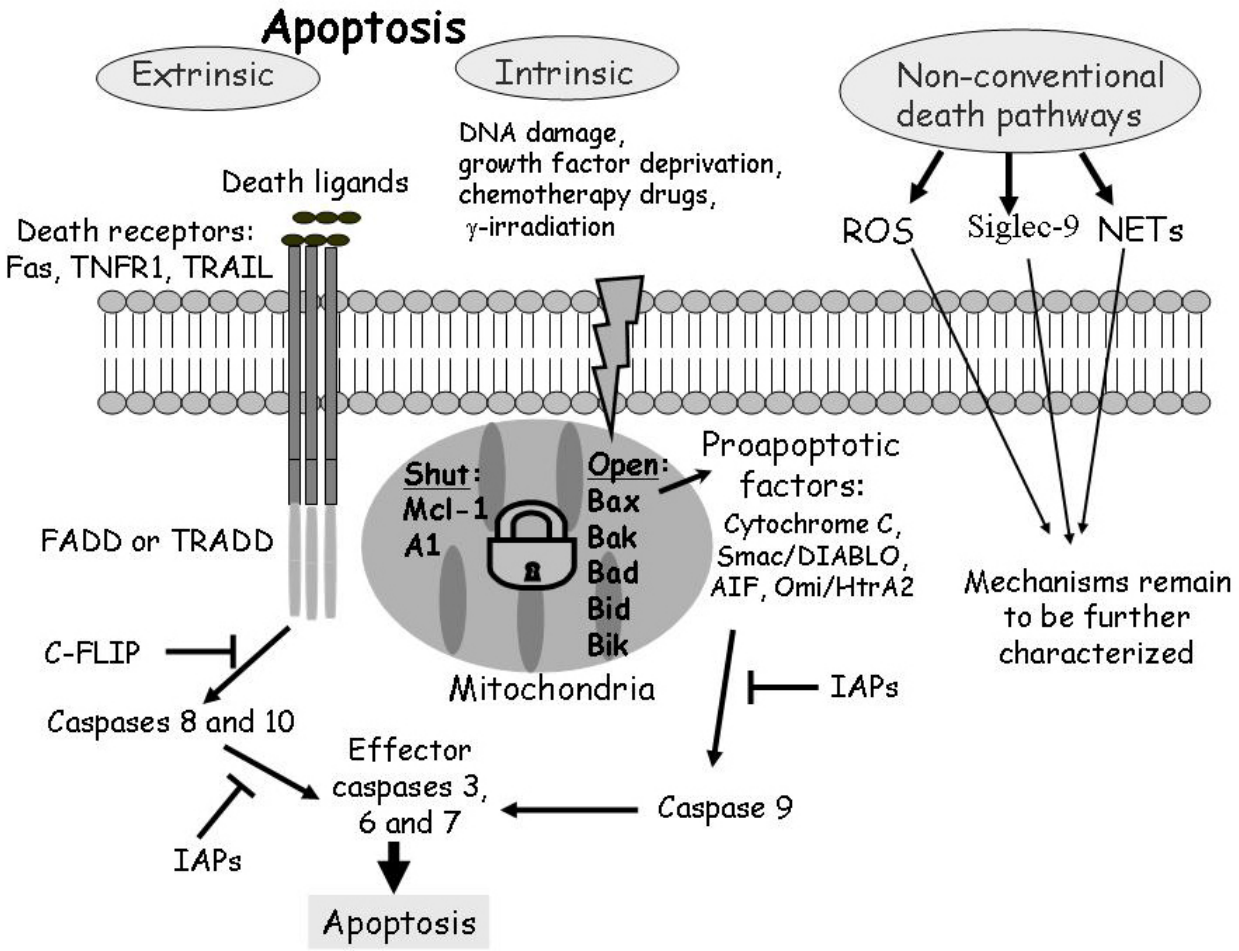

Figure 1. Major pathways leading to neutrophil death. Pro-apoptotic and anti-apoptotic activities are indicated by → and ⊤ respectively. See text for details.

released in the extracellular space and kill a variety of bacteria and fungi (132, 133). This mechanism appears to play an important role in host response during streptococcal necrotizing fasciitis and pneumococcal pneumonia (134). While recent observations support the notion that activated platelets are able to trigger the production of NETs by neutrophils in septic patients (135), it has been described that bacteria can display virulence factors in order to counteract the antimicrobial actions mediated by NETs (134). This pathway of neutrophil death, which appears to be dependent on ROS production, is associated to a number of specific morphological changes different from those observed in apoptotic and necrotic neutrophils, such as disintegration of the nuclear envelope, mixing of cytoplasmic and nuclear materials, and loss of internal membranes and cytoplasmic organelles, thus defining a novel cell death pathway distinct from apoptosis and necrosis.

7. CONCLUSIONS

Figure 1 illustrates the major pathways leading to neutrophil death. Many questions remain about the mechanisms involved in the regulation of neutrophil survival and apoptosis. Chief among them are how the signals able to modulate neutrophil survival and apoptosis are integrated in an inflammatory context, usually characterized by the presence of a variety of cytokines, microbial components and other environmental stressors. Understanding the circuits that control neutrophil survival at the inflammatory focus holds therapeutic promise in diverse settings including a variety of infectious and inflammatory diseases.

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Abbreviations: TRAIL: TNF-related apoptosis-inducing ligand, DD: death domain, FADD: Fas-associated death-domain-containing protein, cFLIP: cellular caspase-8–FLICE-like inhibitory protein, TRDD: TNFR1-associated death domain protein, Apaf-1: apoptotic protease-activating factor 1, Smac/DIABLO: (second mitochondria-derived activator of caspase/direct inhibitor of apoptosis [IAP]-binding protein with low pI), AIF: apoptosis-inducing factor, Omi/HtrA2: Omi/high temperature requirement protein A2, ROS: reactive oxygen species, Siglecs: Sialic acid-binding immunoglobulin-like lectins, NETs: neutrophil extracellular traps.

Key Words: Apoptosis, Neutrophils, Fas, Extrinsic Pathway, Intrinsic Pathway, Mitochondria, Review

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