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REVIEW

Mechanisms regulating neutrophil survival and cell death

María Laura Gabelloni · Analía Silvina Trevani · Juan Sabatté · Jorge Geffner

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Abstract Neutrophils not only play a critical role as a first line of defense against bacteria and fungi infections but also contribute to tissue injury associated with autoimmune and inflammatory diseases. Neutrophils are rapidly and massively recruited from the circulation into injured tissues displaying an impressive arsenal of toxic weapons. Although effective in their ability to kill pathogens, these weapons were equally effective to induce tissue damage. Therefore, the inflammatory activity of neutrophils must be regulated with exquisite precision and timing, a task mainly achieved through a complex network of mechanisms, which regulate neutrophil survival. Neutrophils have the shortest lifespan among leukocytes and usually die via apoptosis although new forms of cell death have been characterized over the last few years. The lifespan of neutrophils can be dramatically modulated by a large variety of agents such as cytokines, pathogens, danger-associated molecular patterns as well as by pharmacological manipulation. Recent findings shed light about the complex mechanisms responsible for the regulation of neutrophil survival in different physiological, pathological, and pharmacological scenarios. Here, we provide an updated review on the current knowledge and new findings in this field and discuss novel strategies that could be used to drive the resolution of neutrophil-mediated inflammatory diseases.

Keywords Neutrophils · Apoptosis · Caspases · Bcl-2 family · ROS · NETosis

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M. L. Gabelloni · A. S. Trevani
Instituto de Medicina Experimental (IMEX), Academia Nacional de Medicina, Buenos Aires, Argentina

J. Sabatté · J. Geffner (✉)
Instituto de Investigaciones Médicas en Retrovirus y SIDA (INBIRS), Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155,
C1121ABG, Buenos Aires, Argentina
e-mail: geffnerj@fibertel.com.ar

J. Geffner
Instituto de Inmunología, Genética y Metabolismo, Hospital de Clínicas "José de San Martín", Universidad de Buenos Aires, Buenos Aires, Argentina

Introduction

Neutrophils are the most abundant circulating leukocytes in humans. They are produced in large numbers in the bone marrow under the control of growth stimulating factor such as granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-CSF (GM-CSF), and interleukin-3 (IL-3). Under steady state conditions, ~100 billion mature neutrophils are generated and released into the peripheral circulation per day in the healthy adult [1, 2]. In contrast with the cells of the adaptive immunity (i.e., B and T cells) that require their activation in secondary lymph organs to proliferate and acquire an effector phenotype, neutrophils are released into the blood as nondividing and fully competent effector cells [3].

Neutrophils play a major role in the immune response against bacteria and fungi infection [4]. This is illustrated by the increased susceptibility to infection and sepsis of patients with congenital or acquired neutropenia or defects of neutrophil functions [5]. In fact, all untreated patients with severe neutropenia (<100/mm^3) will develop a serious infection [6, 7]. Moreover, hereditary deficiencies in neutrophil function usually lead to overwhelming bacterial infection, which is fatal in the absence of specific treatment [4–7].

Neutrophils ingest and kill microbes through the action of a large array of antimicrobial weapons, which include

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proteolytic enzymes, antimicrobial proteins, and reactive oxygen species [4, 7]. These toxic weapons do not discriminate self from nonself. Hence, they induce host tissue damage in a variety of pathological conditions [8–10]. For this reason, it is very important that neutrophils be rapidly and efficiently removed from the circulation when the host is not challenged by noxious or dangerous stimuli. This might explain why neutrophils, under steady-state conditions circulating, show the shortest half-life of all the immune cells [11, 12]. This supports the notion that, in order to ensure neutrophil integrity and minimize the risk of host tissue injury, neutrophils must be quickly eliminated. This notion has been recently challenged by experiments performed using in vivo deuterioum-labeled water showing that the half-life of neutrophils under homeostatic conditions might reach 90 h [13], an observation that requires further investigation.

Over the last years, it has become clear that the function of neutrophils cannot be merely explained in terms of phagocytosis and killing of internalized pathogens. Neutrophils produce a variety of cytokines and chemokines such as IL-1, tumor necrosis factor alpha (TNF-α), interleukin-12p70 (IL-12), transforming growth factor beta (TGF-β), interleukin-8 (CXCL8), growth-related oncogene-alpha, beta and gamma (CXCL1, CXCL2, and CXCL3), macrophage inflammatory proteins 1 beta and 3 alpha (CCL4 and CCL20), and interferon-gamma-inducible protein 10 (CXCL10) [3, 14, 15]. In spite that, on a per-cell basis, neutrophils produce lower amounts of cytokines than monocytes and macrophages, the very high concentrations that neutrophils reach in areas of inflammation during the course of infectious and autoimmune processes suggest that neutrophils might play a role in the regulation of the local immune response [3, 15]. Moreover, there is a growing body of evidence suggesting that neutrophils are not only able to promote the course of inflammatory response but also to suppress the adaptive immune response and participate in wound healing and tissue repair mechanisms [16–18].

Apoptosis is the predominant cell death pathway in the neutrophil [19]. The term apoptosis was originally proposed by Kerr, Wyllie and Currie in 1972 [20] to describe a morphological pattern of cell death characterized by DNA fragmentation into nucleosome-length fragments and nuclear pyknosis, cytoplasmic condensation, membrane blebbing, exposure of phosphatidyl serine on the outer leaflet of the cell membrane, and the formation of apoptotic bodies that are efficiently phagocytosed by macrophages or neighboring cells [12].

Neutrophil apoptosis develops in two major scenarios in vivo. Under steady-state conditions, resting neutrophils remain in the blood for short periods (6–12 h). Then, they home to the spleen, liver, or back to the bone marrow, undergo apoptosis, and are phagocytosed by red pulp macrophages in the spleen, by Kupffer cells in the liver and by stromal macrophages in the bone marrow [11, 21]. The second scenario is the inflammatory focus. Under the influence of a variety of inflammatory mediators such as chemokines, cytokines, pathogen-associated molecular patterns (PAMPs), danger-associated molecular patterns (DAMPs), or inflammatory lipid mediators, neutrophils leave the circulation to infiltrate the challenged tissue, where their lifespan can be increased or decreased by a variety of inflammatory mediators [22, 23]. In this scenario, neutrophils phagocytose and kill invading pathogens, undergo apoptosis, and are cleared by tissue macrophages [12].

The apoptotic death of neutrophils contributes to the resolution of acute inflammation. The major secretory pathways are shut down in apoptotic neutrophils. Cell membranes remain intact, thus preventing the extracellular release of cytotoxic agents and DAMPs and the subsequent amplification of the inflammatory response. Early in the course of apoptosis neutrophils show “eat me” signals leading to their phagocytosis by surrounding macrophages [7, 12]. Phagocytosis of apoptotic neutrophils by macrophages not only prevents the secondary necrosis of apoptotic neutrophils and the release of injurious contents but also turns macrophages into an alternative or anti-inflammatory profile by promoting the production of cytokines IL-10 and TGF-β while suppressing the production of inflammatory cytokines [12, 24]. Interestingly, the systemic administration of apoptotic cells in murine models of septic shock have shown to protect mice from endotoxin lethality, suggesting that passive administration of apoptotic neutrophils could represent a useful tool for the treatment of inflammatory diseases [25].

While cell death is usually discussed dichotomously in terms of apoptosis or necrosis, new forms of cell death have been characterized over the last years. They include NETosis, autophagic cell death, pyroptosis, necroptosis, and oncosis [26]. It is important to note that the analysis of the mechanisms regulating neutrophil survival and death is not easy to be performed since the standard tools of molecular biology such as exogenous gene expression (transfection) or gene-silencing strategies are very challenging. Moreover, the use of neutrophil-like human cell lines hardly reflects the physiology of primary neutrophils while the extrapolation from in vivo murine models should be taken with care since major differences have been observed when the function of mouse and human neutrophils were compared. Here, we provide an updated review of the current knowledge and emerging issues concerning the mechanisms involved in the regulation of neutrophil survival under physiological and pathological conditions and also discuss whether these mechanisms might be exploited for the development of novel therapeutic approaches in infectious and inflammatory diseases.

The players of neutrophil apoptosis: caspases, death receptors, and Bcl-2 family

Caspases and IAPs

Caspases are crucial for the initiation, propagation, and execution of apoptosis. They are activated through two main pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic pathway monitors the extracellular microenvironment, and it is induced by proapoptotic stimuli recognized through specific cell surface receptors. The intrinsic pathway monitors the intracellular microenvironment, and it is induced by proapoptotic stimuli such as damaged DNA or oxidative stress resulting in the permeabilization of the outer mitochondrial membrane [26, 27].

Caspases are cysteine proteases synthesized as inactive zymogens (procaspases). After activation, caspases cleave substrates at sites next to the aspartic acid residues. To date, 11 different caspases have been characterized in humans: caspase-1 to caspase-10 and caspase-14. In the mouse, 10 different caspases were identified: caspase-1, caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, caspase-9, caspase-11, caspase-12, and caspase-14. The human caspase-4 and caspase-5 are functional orthologs of mouse caspase-11 and caspase-12, whereas caspase-10 is not found in the mouse [28–30].

According to their functions, caspases are usually classified as proapoptotic and proinflammatory caspases. Proapoptotic caspases include caspase-2, caspase-3, caspase-6, caspase-7, caspase-8, caspase-9, and caspase-10. They are mainly involved in mediating cell death signaling transduction. Proinflammatory caspases include caspase-1, caspase-4, caspase-5, caspase-11, and caspase-12. They are responsible for the activation of a set of inflammatory cytokines, which includes IL-1, IL-18, and IL-33 [28, 30]. We focus our attention on the family of proapoptotic caspases expressed by the neutrophil (caspase-3, caspase-6, caspase-7, caspase-8, caspase-9, and caspase-10), which is divided into two groups, according to their role in the induction of apoptosis: initiator caspases (caspase-8, caspase-9, and caspase-10) and effector caspases (caspase-3, caspase-6, and caspase-7) [28].

The structure of proapoptotic caspases is illustrated in Fig. 1. All caspases contain an N-terminal prodomain followed by a large catalytic subunit of about 20 kDa (p20) and a small catalytic subunit of about 10 kDa (p10). Initiator caspases contain large prodomains with protein–protein interaction motifs that belong to the so-called death domain superfamily: the death effector domain (DED) and the caspase recruitment domain (CARD). Procaspase-8 and procaspase10 possess two tandem DEDs in their prodomain, while CARD is expressed in caspase-9. These death domains are 80- to 100-residue-long motifs and are responsible for the recruitment of initiator caspases into death-inducing signaling complexes (DISCs), resulting in proteolytic autoactivation of caspases and the subsequent induction of apoptosis [28, 31, 32]. The effector caspases contain short prodomains and are responsible for the execution step of apoptosis. They cleave a large variety of cellular substrates leading to the demolition phase of apoptosis. The substrates of these caspases include enzymes involved in DNA metabolism, cytoskeletal scaffold proteins, cell cycle regulators, repair and housekeeping enzymes, signaling molecules, transcription factors, among others [28, 29]. All caspases are synthesized as catalytically inactive zymogens and require proteolytic processing to be activated. The activation of caspases involves their intramolecular cleavage at the specific Asp-X bonds leading to the release of the N-terminal prodomain and the formation of the mature caspase, which involves the assembly of the heterotetramer $p20_2$–$p10_2$ [28].

The proteolytic activity of caspases is regulated by IAPs (inhibitors of apoptosis), a family of proteins that includes eight members in mammalian: c-IAP-1, c-IAP-2, XIAP (X-linked IAP), ILP-2, NAIP, ML-IAP, Apollon, and Survivin [33, 34]. All IAPs express in their amino-terminal region one to three baculovirus IAP repeat motifs of about 70 amino acids that mediate protein–protein interactions. IAPs mainly inhibit the initiator caspase-9 and the effector caspase-3 and caspase-7 [33, 34]. In turn, the activity of IAPs is inhibited by proapoptotic molecules released by the mitochondria during the activation of the intrinsic pathway of apoptosis such as the second-mitochondria-derived activator of caspases (Smac/Diablo) and the high-temperature requirement A2 (HtrA2/Omi) [33, 35].

Very little is known about the function of IAPs in neutrophils. They express c-IAP-1, c-IAP-2, XIAP, and Survivin. Administration of G-CSF to healthy donors has shown to enhance cIAP2 levels in peripheral blood neutrophils [36]. The delayed apoptotic rate of neutrophils in septic patients, on the other hand, was shown to be associated to increased levels of XIAP [37]. Survivin is the smallest member of the IAPs family [38]. Simon and coworkers reported that the expression of Survivin is high in immature neutrophils and markedly decreases in mature neutrophils [39]. Moreover, they reported that mature neutrophils re-express Survivin after stimulation by G-CSF or GM-CSF as well as during the course of inflammatory diseases, such as acute appendicitis, ulcerative colitis, or cystic fibrosis [39, 40]. Of note, inhibition of Survivin expression in mature neutrophils has shown to prevent the antiapoptotic effect induced by either GM-CSF or G-CSF. A similar effect was observed in neutrophils from Survivin null mice [39]. These observations suggest that beyond the ability of GM-CSF and G-CSF to increase the ratio between anti- and proapoptotic

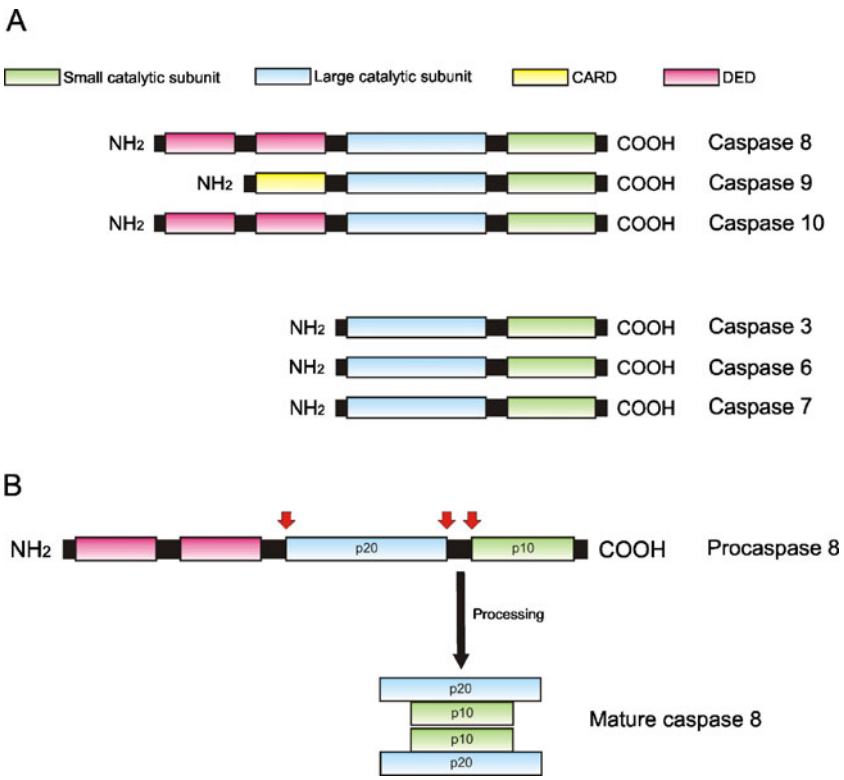

Fig. 1 Caspases. **a** Structure of initiator and effector apoptotic caspases. *DED* Death effector domain. *CARD* caspase recruitment domain. **b** Activation of procaspase-8 leading to the assembly of the heterotetramer $p20_2$–$p10_2$

members of the Bcl-2 family, the inhibition of neutrophil apoptosis induced by these growth factors is strongly dependent on the expression of Survivin. Interestingly, like most other IAP family proteins, Survivin does not directly interact with or inhibit caspase activation. The antiapoptotic function of Survivin appears to depend on its association with XIAP [38].

IAPs were first thought to be solely involved in the inhibition of apoptosis. However, a large body of evidence proved that IAPs also display a variety of functions mainly attributed to the E3 ubiquitin ligase activity of some IAPs such as XIAP, cIAP1, and cIAP2. They control MAPK and NF-kB signaling pathways, regulate signal transduction downstream of several pattern recognition receptors, and contribute to the maturation of pro-IL-1β and pro-IL-18 by regulating inflammasome activation [41–43].

Death receptors and the extrinsic pathway of apoptosis

Death receptors are members of the tumor necrosis factor receptor superfamily characterized by the presence of a cytoplasmic region of ~ 80 amino acids called death domain (DD). This is a protein–protein interaction motif allowing self-association and induction of apoptosis [44–46]. The death domain subfamily together with the DED, CARD, and pyrin domain subfamilies define the DD superfamily, one of the largest and most studied domain superfamilies [44].

The death receptors best characterized are Fas (CD95/APO-1), TNF-receptor 1 (TNF-R1), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1), and receptor 2 (TRAIL-R2). Death receptors are activated by their cognate ligands, which belong to the TNF protein family. These ligands are mostly trimeric and can be either membrane attached or soluble [44]. Once thought to be primarily responsible for the induction of apoptosis, it is now clear that death receptors are also able to promote the activation of survival pathways in many cell types and tissues and to induce a variety of functions not related to apoptosis. These functions include regulation of cell proliferation and differentiation, production of inflammatory cytokines and chemokines and modulation of the immune response. In this section, we focus on the mechanisms through which death receptors trigger the activation of initiator caspases 8 and 10 [45–47].

The initial proapoptotic signaling cascade induced by FasL has been analyzed in several cell types. The binding of FasL stabilizes Fas trimers at the plasma membrane while inducing a conformational change enabling the assembly of a multiprotein complex at the cytosolic tail of the death receptor, which leads to the activation of the initiator caspase-8 and caspase-10. In a first step, the DD of Fas recruit the adaptor protein called Fas-associated death-domain-containing protein (FADD) via its C-terminal DD. This adaptor protein also contain a N-terminal death effector domain (DED), which interacts with the tandem DED in the

prodomain of caspase-8 and caspase-10, resulting in the formation of a ternary DISC, containing Fas, FADD, and the initiator caspase-8 or caspase-10. Recruitment of procaspase-8 or procaspase-10 into the DISC triggers the autoproteolytic cleavage and activation of caspase-8 and caspase-10, which are released into the cytosol as activated intiator caspases [26, 45]. Caspase activation by the DISC can be inhibited by cFLIPs (cellular caspase-8–FLICE-like inhibitory protein), a family of tandem DED-containing proteins, which expresses a noncatalytic pseudo-caspase domain, able to interact with FADD [48].

The amount of active caspase 8, and perhaps caspase 10, released into the cytosol seems to determine apoptosis signaling pathways induced downstream DISC. In cells able to produce large amounts of active caspase-8, it can efficiently activate effector caspase-3, caspase-6, and caspase-7, thereby triggering apoptosis directly without the activation of the intrinsic pathway of apoptosis mediated by truncated Bid. These cells have been termed type I cells. In contrast, in cells producing low amounts of active caspase-8, termed type II cells, the induction of apoptosis triggered by Fas requires an amplification loop for apoptosis mediated by truncated Bid, which translocates to the mitochondria and activates the intrinsic pathway of apoptosis [26, 45]. Neutrophils appear to represent type II cells [49]. In fact, the induction of Fas-mediated neutrophil apoptosis has shown to accelerate the cleavage of Bid and the release of cytochrome c and Smac/Diablo by the mitochondria [50, 51]. The interplay between the extrinsic and intrinsic pathways of apoptosis is illustrated in Fig. 2.

A role for Fas in neutrophil apoptosis has been mainly proposed on the basis of two observations: (a) the expression of Fas receptor and FasL in neutrophils and (b) the marked acceleration of apoptosis induced by agonistic antibodies directed to Fas [52, 53]. However, blockade of either Fas or FasL does not increase neutrophil survival. Moreover, neutrophils from Fas (*lpr*) or FasL (*gld*) deficient mice have shown a normal rate of apoptosis [54]. Together, these observations suggest that apoptosis of resting neutrophils is not under the regulation of the Fas/FasL system. However, suggesting a role for the Fas/FasL system in the apoptosis of activated neutrophils, Jonsson and coworkers [55] showed that the course of neutrophilic inflammation in murine experimental models is regulated by FasL.

The death receptor TNFR1 can not only induce apoptosis via the recruitment of TRADD and the subsequent activation of caspase-8, but it can also activate the transcription of NF-κB, promoting cell survival [26]. The mechanism that underlies the ability of TNF-α to induce either cell survival or cell death was clarified by Legler et al. and Micheau and Tschopp [56, 57]. They reported that activation of TNFR1 by TNF-α leads to the assembly of a membrane-bound complex containing TNFR1, TRADD, receptor interacting protein, and TNF receptor-associated factors. This complex, termed complex I, triggers the activation of NF-κB promoting cell survival. In a second step, TRADD dissociates from TNFR1 and associates with FADD and caspase-8, to form a cytoplasmic complex II, which results in the activation of caspases and cell death. Thus, TNFR1 signaling can results in cell survival or cell death, depending on the regulated assembly of complexes I and II [56, 57].

The model described above has been characterized in many cell types and clearly establish that the proapoptotic signaling induced by TNFR1 requires a DISC containing TRADD, FADD, and caspase-8 or caspase-10. Interestingly, in a recent study, Simon and coworkers have shown that the induction of apoptosis by TNFR1 in the neutrophil actually involves a distinct and novel mitochondria-independent apoptotic pathway, which does not involve the participation of caspase-8. The authors reported that TNFR1 ligation does not induce the activation of caspase 8 or Bid but effectively stimulates the sequential activation of p38 and class IA PI3Ks leading to the production of reactive oxygen species (ROS) and the subsequent activation of effector caspases [58].

Neutrophils produce TRAIL and express TRAIL receptors [59, 60]. TRAIL is able to interact not only with the two death receptors TRAIL-R1 and TRAIL-R2 but also with three decoy receptors devoid of functional DDs, which modulate the interaction of TRAIL with signaling receptors [61]. Cassatella and coworkers [62, 63] have shown that under the influence of interferon-α or interferon-γ neutrophils synthesize and store TRAIL. Moreover, upon stimulation by a variety of inflammatory mediators such as TNF-α, LPS, fMLP, IL-8, and insoluble immune complexes, neutrophils release the stored TRAIL. The role of TRAIL in the regulation of neutrophil survival, however, has not been clearly defined. TRAIL appears to be involved in the elimination of senescent neutrophils [64]. It has been shown that the interaction of stromal cell-derived factor 1 with the chemokine receptor CXCR4, which is preferentially expressed on senescent neutrophils, increases the expression of TRAIL and TRAIL receptors in the neutrophil, leading to TRAIL-dependent apoptosis [64]. Moreover, TRAIL appears to accelerate the apoptotic rate of inflammatory neutrophils (but not resting neutrophils) promoting the resolution of inflammatory reactions [65].

Bcl-2 family and the intrinsic pathway of apoptosis

The intrinsic pathway of apoptosis is regulated by the Bcl-2 family, which controls the integrity of the outer membrane of the mitochondria [66]. On the basis of both their pro- or antiapoptotic actions and the BCL-2 Homology (BH) domains they express, the BCL-2 family of proteins is classified in three groups. The group of antiapoptotic

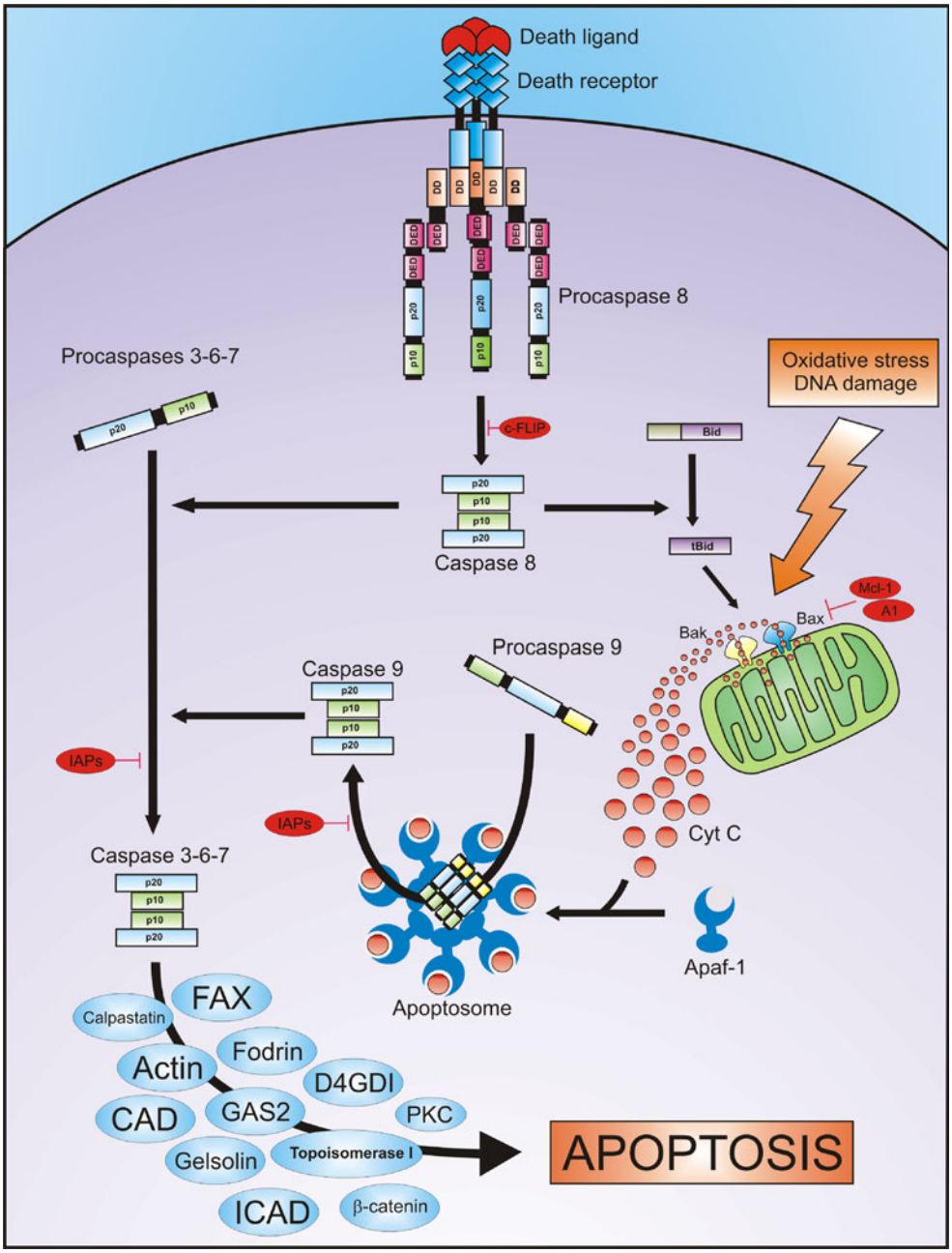

Fig. 2 The extrinsic (death receptor) and intrinsic (mitochondrial) pathways of apoptosis. *DD* death domain, *DED* death effector domain. Cellular caspase-8–FLICE-like inhibitory protein (c-FLIP) prevents caspase-8 activation. Inhibitors of apoptosis (*IAPs*) prevent the activation of the initiator caspase-9 and the effector caspase-3 and caspase-7. The two pathways of apoptosis are interconnected by truncated Bid (*tBid*) produced when Bid is cleaved and activated by caspase-8. The activation of the proapoptotic members of the Bcl-2 family Bax and Bak involves their conformational change and homo-oligomerization on the outer membrane of the mitochondria. Cytochrome c is released from the mitochondria and together with apoptotic-protease-activating factor-1 (Apaf-1) form the apoptosome, which recruits and activates caspase-9. The demolition phase of apoptosis is mediated by effector caspase-3, caspase-6, and caspase-7

Bcl-2 like proteins includes Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1/Bfl-1. All of them express four BH domains. The group of proapoptotic Bax-like proteins includes Bax and Bak, which also express four BH domains. A second group of proapoptotic proteins, the BH3-only proteins, displays a unique short motif called the BH3 domain and include Bid, Bim, Bad, Bmf, Puma, Noxa, Bik, Blk, and Hrk/DP5 [66–68].

The intrinsic pathway leads to the activation of caspase-9 on the scaffold protein apoptotic protease-activating factor 1 (Apaf-1) when cytochrome c and other proapoptotic proteins are released from the space between the outer and inner mitochondrial membrane to the cytosol, due to the loss of integrity of the outer mitochondrial membrane, a process called mitochondrial outer membrane permeabilization (MOMP). In the presence of modest levels of dATP or ATP, cytochrome c released from the mitochondria interacts with the WD 40 repeat domain of APAF-1. This interaction opens up the Apaf-1 structure leading to the oligomerization of Apaf-1 into a large heptameric complex called apoptosome, which then recruits procaspase-9 via CARD-mediated interactions between Apaf-1 and caspase-9, resulting in the cleavage and activation of caspase-9. The intrinsic pathway 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 [26, 66–68].

The induction of MOMP is mediated by the activation of the proapoptotic members of the Bcl-2 family Bax and Bak, which involves their conformational change and homo-oligomerisation on the outer membrane of the mitochondria leading to its permeabilization. The induction of MOMP is antagonized by the antiapoptotic Bcl-2 proteins, which inhibits the function of Bax and Bak. The antiapoptotic Bcl-2 proteins are in turn regulated by BH3-only proteins, which bind to the antiapoptotic Bcl-2 proteins preventing their interaction with Bax and Bak. Heterodimerization with and inactivation of prosurvival members of the Bcl-2 family by BH3-only proteins do not fully explain the proapoptotic function of these proteins. Some of the BH3-only proteins appear to be also able to directly bind to Bax and Bak promoting their activation [66–69].

BH3-only proteins play a critical role in the activation of the intrinsic pathway of apoptosis by neutralizing prosurvival proteins. They are regulated by diverse transcriptional and posttranslational mechanisms. Full-length BID is inactive until cleaved proteolitically. Bid is cleaved and activated by caspase-8 after death-receptor activation, and the truncated Bid can then promote apoptosis by engaging their prosurvival Bcl-2 like relatives or by binding to the effector death proteins Bak and Bax [69]. Interestingly, it has been shown that Bid can also be activated by other proteases such as granzyme B [70], calpains [71], and cathepsins [72]. Transcription of Bim is induced in response to different stimuli such as cytokine withdrawal or during endoplasmic reticulum stress. Bim is also regulated posttranslationally by sequestration to the microtubule-associated dynein motor complex from where it is released by UV irradiation. Noxa and PUMA are transcriptionally upregulated in response to distinct proapoptotic stimuli [69, 73].

Studies conducted 40 years ago reported that neutrophils have the particularity to contain few mitochondria with poorly defined cristae and inner membranes [74, 75]. On the other hand, it is well known that poisons like cyanides do not inhibit neutrophil function, suggesting that mitochondria hardly participate in ATP synthesis [76]. These observations are consistent with the traditional view of neutrophil as cells that depend on glycolysis for their energy requirements, allowing them to act as immune effector cells at inflammatory sites, where oxygen tensions are low. Nevertheless, more recent studies directed to further analyze the function of mitochondria in neutrophils showed that these cells contain a developed network of mitochondria, which express a transmembrane potential and contain proapoptotic proteins such as cytochrome c, second-mitochondria-derived activator of caspases (Smac/Diablo) and high-temperature requirement A2 (HtrA2/Omi), which can be released to the cytosol [22, 77, 78]. Even though this mitochondrial network contains reduced levels of cytochrome c, it appears to play a major role in the control of neutrophil survival. The intrinsic pathway of apoptosis in the neutrophil seems to express a low threshold requirement for cytochrome c, which is compensated by the high cytosolic expression of Apaf-1 and the large amounts of mitochondrial proapoptotic proteins Smac/Diablo and HtrA2/Omi, which can be massively released from the mitochondria into the cytosol upon activation of the intrinsic pathway [79, 80].

Neutrophils constitutively express the proapoptotic proteins of the Bcl-2 family Bax, Bak, Bad, Bid, and Bik [81, 82]. Bax plays an important role in neutrophil apoptosis. In spite of the fact that $Bax^{-/-}$ and $Bak^{-/-}$ mice have neutrophil counts similar to control mice, Bak and Bax double-deficient mice show a marked neutrophilia [83]. These observations suggest that Bax and Bak display a redundant function on neutrophil survival. However, Henson and coworkers showed that neutrophils from Bax-null mice display a delayed spontaneous apoptosis compared with their littermate controls, suggesting a non-redundant role for Bax in the control of neutrophil survival [84]. Consistent with this view, Dibbert and coworkers [85] reported that the delayed neutrophil apoptosis usually found in inflammatory diseases is associated to a reduced expression of Bax. Moreover, they reported that antiapoptotic colony-stimulating factors G-CSF and GM-CSF reduce the expression of Bax in the neutrophil and also that Bax-deficient neutrophils produced in vitro by antisense oligodeoxynucleotides display a delayed rate of apoptosis, thus providing a direct evidence for the proapoptotic role for Bax [85]. In agreement with these observations, it was reported that the induction of apoptosis by either TNF-α or *Mycobacterium tuberculosis* results in an increased Bax/Bcl-X_L ratio [86, 87]. On the other hand, spontaneous apoptosis of neutrophils has shown to be associated with translocation of Bax and Bid to the mitochondria and truncation of Bid, with subsequent release of Smac/Diablo and Omi/HtrA2 into the cytosol, being all these changes prevented by G-CSF [78, 88].

The proapoptotic protein Bad plays an important role in the control of neutrophil survival [89, 90]. The function of Bad is inhibited via PI3K-dependent phosphorylation and stimuli able to delay apoptosis such as GM-CSF, IL-8, C5a, LPS, and CpG-DNA have shown to inhibit the proapoptotic activity of Bad by inducing phosphorylation of Bad at Ser^{112} and Ser^{136}, leading to the accumulation of Bad in the cytosol [89, 91]. In contrast, the proapoptotic stimulus nicotinic acid induced dephosphorylation of Bad enabling its interaction with the antiapoptotic members of the Bcl-2 family [92]. On the other hand, supporting a role for Bim in the control of neutrophil survival, it has been reported that Bim null mice have higher neutrophil counts compared with control mice [93]. Moreover, in vitro assays indicated that Bim deficiency renders neutrophils resistant to cytokine withdrawal and cytotoxic drugs [94].

The antiapoptotic proteins Mcl-1 and A1/Bfl-1 are expressed in the neutrophil [80, 95–97]. Bcl-2 has been detected neither in resting nor in activated neutrophils, while the expression of Bcl-X_L at the protein level is a matter of debate [80, 95]. The antiapoptotic protein Mcl-1 plays a major role in the control of neutrophil survival [97]. Compared with other Bcl-2 family members Mcl-1 exhibits an extremely high turnover rate, which is well suited for the effective control of neutrophil survival. Promotion of neutrophil survival by Mcl-1 appears to involve heterodimerisation with and inhibition of proapoptotic proteins Bim and Bak in the mitochondrial outer membrane [98]. Mice conditionally lacking Mcl-1 expression in neutrophils display a severe defect in neutrophil survival; in fact, neutrophil counts were reduced by more than 80 % in blood, spleen, and peritoneal exudates [99]. Moreover, a large number of studies have shown that Mcl-1 levels decline as neutrophils undergo apoptosis [100] and also that the reduction of Mcl-1 is faster in the presence of proapoptotic agents such as TNF-α [100], nicotinic acid [92], *Trichomonas vaginalis* [101], *Viscum album* agglutinin-I [102], sodium salicylate [103], and cyclin-dependent kinase inhibitors [104]. In contrast, neutrophil treatment with antiapoptotic agents such as GM-CSF [100, 105, 106], LB4 [107], IL-15 [108], cyclic AMP [109], respiratory syncytial virus [110], and hypoxia [111] results in the enhancement of Mcl-1 expression. With regards to the role of A1/Bfl-1, it was reported that neutrophils develop normally in A1-deficient mice; however, they are refractory to the antiapoptotic effect induced by LPS or transendothelial migration and show an acceleration rate of spontaneous apoptosis when cultured in vitro [112]. A role for A1/Bfl-1 in the regulation of neutrophil apoptosis is also supported by observations made in human neutrophils showing that the mRNA for A1/Bfl-1 is upregulated by survival factors such as LPS and G-CSF [95].

Some special features of neutrophil apoptosis

Although the mechanisms involved in the regulation and execution of apoptosis in the neutrophil have many similarities with those described in other cell types, the apoptotic death of neutrophils show some peculiarities. Upon stimulation, neutrophils produce huge amounts of ROS due to the activation of the enzyme NADPH oxidase [7]. A large body of evidence suggests that ROS promote neutrophil apoptosis; however, the interplay between ROS and the traditional pathways of apoptosis is not clearly defined.

Chronic granulomatous disease (CGD) is an inherited immunodeficiency characterized by the inability of phagocytes to activate the respiratory burst due to a defect in the enzyme NADPH oxidase [113]. Neutrophils from CGD patients display a delayed rate of spontaneous apoptosis, suggesting that ROS stimulates the apoptosis of resting neutrophils [114]. Consistent with this notion, it has been shown that hypoxia, hydrogen peroxide scavengers, and the pharmacological inhibition of NADPH oxidase delay the spontaneous rate of neutrophil apoptosis [115]. Not only resting but also activated neutrophils appear to be sensitive to the proapoptotic action of ROS. Activation of neutrophils by *Escherichia coli* [116], *M. tuberculosis* [87], *Entamoeba histolytica* [117], and immune complexes also resulted in the acceleration of apoptosis [118].

The mechanisms through which ROS stimulate neutrophil apoptosis remain poorly defined. ROS might stimulate apoptosis by inducing ligand-independent death receptor signaling via clustering of preformed DISC components in lipid rafts [119]. Alternatively, ROS might also promote apoptosis by interfering with the activation of survival pathways mediated by NF-κB and MAPKs [120]. On the other hand, and uncovering a new pathway in the regulation of neutrophil function, Honda and coworkers [121] have recently reported that the kinase Btk negatively regulates both the production of ROS and apoptosis in human neutrophils.

Recently, Rossi and coworkers and Leitch et al. [104, 122, 123] have shown that inhibitors of cyclin-dependent kinases (CDK) such as R-roscovitine effectively trigger caspase-dependent neutrophil apoptosis, antagonize survival effects induced by several antiapoptotic agents, and markedly improve the resolution of inflammatory diseases in different experimental models. These observations indicate that CDK activity is necessary and fundamental to neutrophil survival and identify a new potential target for the treatment of a variety of inflammatory diseases. These results are very interesting and unexpected because neutrophils are terminally differentiated cells and the main function of CDK is the regulation of the cell cycle. Although the mechanisms through which CDK inhibitors stimulate neutrophil apoptosis remain to be clearly defined, the proapoptotic activity of CDK inhibitors appears to be related to their ability to reduce the expression of the antiapoptotic protein Mcl-1. In fact, providing insight into the mechanisms by which these compounds downregulate Mcl-expression, Rossi and coworkers [124] have recently shown that CDK induce the phosphorylation of RNA polimerase II in the neutrophil and that the inhibition of this process by CDK inhibitors compromise neutrophil-transcriptional activity resulting in a diminished expression of Mcl-1.

Cassatella and coworkers [125] have recently shown that the cell cycle regulatory protein called proliferating cell nuclear antigen (PCNA) is expressed by neutrophils and plays an important role in the control of neutrophil survival. They showed that neutrophils, despite their inability to proliferate, express high levels of PCNA exclusively in the cytosol. Interestingly, PCNA was shown to be constitutively associated with procaspase-3, procaspase-8, and procaspase-9

presumably to prevent their activation. In fact, peptides derived from the cyclin-dependent kinase inhibitor p21, which compete with procaspases to interact with PCNA, effectively induced neutrophil apoptosis. The authors also reported that the expression of PCNA diminished during apoptosis and increased after in vitro and in vivo exposure to the survival factor G-CSF. Moreover, providing a direct evidence for a prosurvival effect of PCNA, using neutrophils differentiated from PLB985 cells, they showed that PCNA overexpression rendered neutrophils more resistant to proapoptotic stimuli while the inhibition of PCNA expression by small-interfering RNA sensitized neutrophils to apoptosis [125]. Together, these novel and unexpected observations identify PCNA as a key regulator of neutrophil survival and suggest that it might represent an interesting target for therapeutic interventions directed to ameliorate the course of inflammatory diseases [23].

The ability to phagocyte bacteria and fungi represents one of the most prominent features of neutrophils. Phagocytosis has been shown to modulate neutrophil survival. The uptake of target cells opsonized by either complement or IgG results in the induction of neutrophil apoptosis. A similar effect was observed after phagocytosis of a variety of pathogens such as *Staphylococcus aureus*, *E. coli*, *M. tuberculosis*, and *Streptococcus pyogenes* [82, 126]. The link between phagocytosis and apoptosis appears to involve the production of ROS. In fact, blocking of ROS generation prevented the induction of apoptosis [87]. Moreover, neutrophils isolated from CGD patients showed a reduced increase in the apoptotic rate after phagocytosis [126, 127]. The mechanisms through which ROS promote apoptosis have not been well defined; however, they seem to act upstream of initiator caspases because ROS inhibition resulted in the inhibition of caspase-3 activation [87]. Interestingly, phagocytosis of pathogens not always leads to neutrophil apoptosis. For certain pathogens able to survival within neutrophils such as *Neisseria gonorrhoeae* [128], *Francisella tularensis* [129], and *Paracoccidioides brasiliensis* [130], it was shown that phagocytosis actually delays apoptosis.

Nonconventional pathways of neutrophil death

NETosis

Zychlinsky and coworkers reported in 2004 that activated neutrophils produce neutrophil extracellular traps (NETs) [131]. These structures containing highly decondensed chromatin associated with nuclear histones, and many granular proteins are released into the extracellular space where they can trap and kill bacteria and fungi [131–134]. The production of NETs results from a unique form of cell death called NETosis characterized by the loss of intracellular membranes before the integrity of the plasma membrane is compromised [132]. In vitro studies directed to determine the morphological changes during the course of NETosis showed that, during the first hour postactivation, the neutrophil nucleus loses their lobules, the chromatin decondenses, and the inner and outer nuclear membranes detach from each other. After 1 h, the cell lost its nuclear envelope, and simultaneously, the granules found in the cytosol lost their membranes leading to the nucleoplasm and cytoplasm to form a homogeneous mass. This pathway of neutrophil death is thus characterized by 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 [133, 134], hence defining a novel cell death pathway. Interestingly, Simon and coworkers [135] reported that neutrophils can also produce NETs using mitochondrial DNA instead of nucleus DNA.

NETosis is activated by a variety of proinflammatory stimuli (LPS, IL-8, and TNF-α) and pathogens [134, 136]; however, the mechanisms underlying the induction of NETosis remain unclear. Reactive oxygen species appear to be important mediators for NET production. Neutrophils from patients with CGD, who lack NADPH activity, are unable to release NETs in response to inflammatory stimuli, while pharmacological inhibition of NADPH oxidase in neutrophils isolated from healthy donors also results in the inhibition of NET release [133, 134, 136] .

A significant advance in the field of NETosis has been the appreciation that histone hypercitrullination mediates chromatin decondensation and NET formation [137]. Neutrophils express high levels of peptidylarginine deiminase 4 (PAD4), which catalyzes histone citrullination. Wand and coworkers have shown that hypercitrullination of histones by PAD4 mediates chromatin decondensation in the neutrophil, a prominent hallmark of NETosis. Chemical inhibition of PAD4 impairs chromatin decondensation and NET production in response to inflammatory stimuli, while neutrophils isolated from PAD4-deficient mice fail to citrullinate histones, decondense chromatin, and generate NETs [137–139]. Together, these observations indicate a critical role for PAD4 in the induction of NETosis.

The production of NETs has been mainly investigated in studies performed in vitro. As mentioned above, these studies revealed that NETs are released as neutrophils die through a process requiring hours [133, 134]. Contrasting with this view and using live-imaging microscope techniques, Yipp et al. [140] have recently reported that during *S. aureus* skin infection neutrophils rapidly produce NETs. Moreover, they showed that neutrophils undergoing NET formation remain alive and were able to crawl through the

infected tissue and phagocytose bacteria. In spite that NETosing neutrophils adopt unusual cell morphology these observations indicate that they remain functional. Intriguingly, even after loss of their own DNA, anuclear neutrophils were still able to chase and phagocytose bacteria [140, 141].

Autophagic cell death

The term autophagic cell death is usually employed to indicate instances of cell death associated to a massive cytoplasmic vacuolization [26]. However, it should be emphasized that this morphological signature does not imply that autophagy would actually be responsible for the induction of cell death. In fact, autophagy represents an evolutionary conserved catabolic process involved in the clearance of damaged organelles and proteins enabling cells to recycle intracellular components. Despite that autophagy is actually able in some circumstances to induce cell death, it usually represents a cytoprotective response activated by stressed cells in the attempt to prevent or delay cell death [26, 142, 143].

Few studies have focused on the autophagic processes in neutrophils [82]. Phagocytosis of *E. coli*, zymosan or IgG-coated particles has shown to result in the recruitment of LC3, an E3 ubiquitin ligase-like enzyme required for autophagy, at neutrophil phagosomes [144, 145]. This suggests a link between phagocytosis and the autophagic machinery. Not only phagocytosis but also activation of neutrophils by PMA or Toll-like receptor ligands results in vacuole formation and LC3 recruitment [144, 145]. Moreover, death induced by Sialic acid-binding immunoglobulin-like lectins 9 (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, suggesting that cell death might be induced by autophagy [146]. Interestingly, and highlighting the complexity of the mechanisms responsible for the induction of neutrophil death, Simon and coworkers have recently reported that neutrophils exposed to inflammatory cytokines undergo an authopagic-related form of programmed necrosis, after ligation of CD44, the receptor for hyaluronan [147].

In the last few years, other forms of cell death have been characterized [26]. Necroptosis represents a type of programmed necrosis induced by classic apoptotic stimuli, such as the death receptor ligands Fas and TNF-α, when apoptosis is inhibited by caspase inhibitors or through mutations in caspase-8 or FADD. The morphological features of necroptosis—organelle swelling, permeabilization of the plasma membrane, mitochondrial dysfunction, and lack of nuclear fragmentation—are clearly different from those shown by apoptotic cells [26, 148]. Pyroptosis represents a form of cell death induced by caspase-1 activation. Pyroptotic cells can exhibit apoptotic and/or necrotic morphological features [149]. Other types of cell death recently characterized include anoikis, entosis, parthanatos, among others [26]. However, no studies have yet analyzed whether neutrophils or neutrophil precursors undergo these forms of cell death.

Conclusions

Recent findings shed light on the complex mechanisms responsible for the regulation of neutrophil survival in different physiologic and pathologic scenarios. These findings have significant implications for the development of new therapeutic approaches applied to the treatment of inflammatory and infectious diseases. In fact, in the last few years, new therapeutic targets have been defined such as Bcl-2 family members, cyclin-dependent kinases, the cell cycle regulatory protein, and different signaling pathways. Based on the identification and characterization of these new targets, promising strategies in the fight against inflammatory diseases have been successfully tested in experimental models. Moving to clinical trials require a better understanding of the molecular networks involved in the regulation of neutrophil survival in order to fully exploit the plasticity of neutrophil death programs for therapeutic proposals.

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