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Promoting apoptosis of neutrophils and phagocytosis by macrophages: novel strategies in the resolution of inflammation

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Summary

Acute inflammation is the body's response to infection or injury, characterised by the rapid infiltration of polymorphonuclear neutrophils to the site of injury followed by monocytes, which differentiate locally into macrophages. The latter are essential for the removal of effete neutrophils and provided that the harmful agent is eliminated, removal of neutrophils will lead to the resolution of inflammation. Perturbations in this process result in the persistence of inflammation and close control of pathways associated with resolution are necessary to avoid chronic inflammation, autoimmunity, or both. As our understanding of these processes increase, drugs able to trigger pro-resolution pathways may represent an effective strategy for treating chronic inflammatory diseases.

Key words: *inflammation; neutrophil; macrophage; apoptosis; efferocytosis; PCNA; vasculitis; arthritis; systemic inflammatory disease*

The resolution of inflammation: an active process

Neutrophils play an essential role in the acute phase of inflammation, they are the most abundant leucocyte in the blood and typically the first mobilised to the site of injury or infection [1, 2]. They are essential in the clearance of pathogens and function through a number of intra- and extra-cellular mechanisms (refer to fig. 1 for a summary of these actions). During inflammation, neutrophils are activated and mobilised by cytokines, growth factors and even components of pathogens. Their importance in immune responses is highlighted by the fact that absence or decreased neutrophil numbers results in immunodeficiencies [3]. In addition to their capacity to eliminate pathogens, recent research suggests that neutrophils also play a significant role in the inflammatory process associated with cancers through their ability to interact with the tumour microenvironment [4]. While neutrophils are necessary for mounting an appropriate immune response to pathogens, injury and even tumours, they also play a key role in the initiation of various pathologies including chronic inflammatory diseases and autoimmunity. In fact in some cases, an exaggerated influx of neutrophils is more deleterious than the infection or injury itself [2]. In various diseases including rheumatoid arthritis, cardiac infarction, acute graft rejection and acute respiratory distress syndrome, neutrophils are the most potent mediator of tissue damage and facilitate the inflammatory process. That being said, neutrophils are essential in the maintenance of homeostasis and recent research suggests that they have an ability to interact not only with other immune cells but also non-immune cells (epithelial, endothelial or stromal cells) in different pathophysiological conditions to regulate biological processes [5]. Indeed, neutrophils possess a wide variety of mediators with microbicidal activity as well as mediators which contribute to resolution of inflammation [6]. For example, neutrophils play an important anti-inflammatory role during both acute and chronic microbial infections, through the secretion of immunoregulatory cytokines such

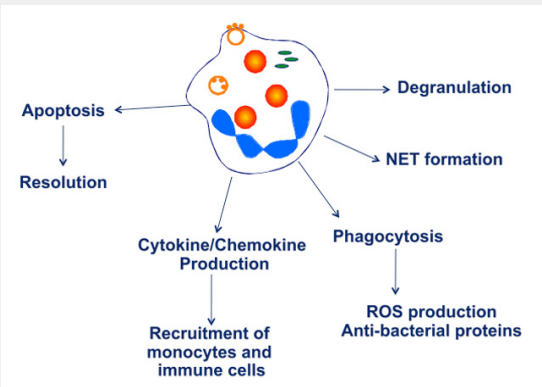

Figure 1
A summary of the function of neutrophils during immune responses.

as IL-10, IL-1 receptor antagonist and TGF-beta [7–10]. Radical oxygen species produced by the NADPH oxidase system are not only essential for killing bacteria but also appear to be pivotal in terminating the inflammatory process. The importance of radical oxygen species (ROS) in the resolution of inflammation is highlighted by the presence of granuloma in patients with chronic granulomatous disease, a disease characterised by a genetic defect in one component of the NADPH oxidase complex. This concept is also illustrated in intestinal inflammation [11].

While neutrophils can be pro-resolving, too much of a good thing is deleterious as neutrophils are also loaded with extremely powerful molecules. Therefore a key to mounting an effective immune response while avoiding unnecessary damage to surrounding tissue is by controlling neutrophil activation and survival. Mechanisms used to control neutrophil survival and promote the resolution of inflammation will be the focus of this review as well as potential therapeutic strategies aimed at exploiting the pro-resolving power of apoptotic neutrophils for the treatment of chronic inflammatory diseases.

The life and death of a neutrophil

Mechanisms of neutrophil apoptosis

The life span of a neutrophil must be tightly regulated, as extended survival is essential for the effective elimination of pathogens and cell death necessary to prevent the release of the highly cytotoxic contents of activated neutrophils and subsequent tissue damage. It is also a process that may be induced, delayed or enhanced by the microorganisms depending on their ability to evade host defences or to be affected by the health status of the host [2]. A key feature in the regulation of neutrophil fate is their ability to undergo spontaneous apoptosis through the up regulation of death signalling and down regulation of survival signalling [12]. There are many different mechanisms responsible for the balance between neutrophil apoptosis and survival and disruptions in these mechanisms has the potential to lead to uncontrolled inflammation. In fact, there is now an established field of research aimed at understanding the regulation of neutrophil apoptosis and its potential as a target for therapeutic intervention during the acute phases of inflammatory and infectious diseases [13–15].

Pro-apoptotic

One well-characterised mechanism involved in neutrophil apoptosis is the caspase signalling pathway. Both intrinsic pathways involving the mitochondria and extrinsic mechanisms involving death receptors are able to activate caspases. Extrinsic death factors include FasL and TNF-alpha, although it should be noted that while neutrophils do express both FasL and FasR, it does not appear to be the principal pathway involved in neutrophil death as mice deficient in both proteins display normal levels of apoptosis [16]. Intrinsic signal pathways include ROS and oxidative stress, Bcl-2 family members and cytochrome C [17, 18]. Interestingly, while neutrophils contain barely detectable levels of cytochrome c, the trace amounts of this protein found within the cytoplasm are still able to facilitate apoptosis by promoting high levels of procaspase 9 and the assembly of the Apaf-1 apoptosome [10]. Although less characterised than caspases, lysosomal enzymes also promote apoptosis in neutrophils and triggering “lysosomal cell death” [19].

We employed a proteomic approach to identify cytosolic proteins cleaved or modified during neutrophil apoptosis. This study identified a number of cytoskeleton-associated proteins cleaved during apoptosis, including gelsolin, moesin and coronin-1A. Coronin-1A is a protein associated with phagosome formation and NADPH-oxidase components and our results suggest it may inhibit neutrophil apoptosis. When coronin-1A is overexpressed in the myeloid cell line PLB985, which can differentiate into neutrophils, this protein affected the activity of NADPH oxidase and was able to inhibit apoptosis in neutrophils. We have also observed an increase in coronin-1A expression and associated decrease in apoptosis in cystic fibrosis patients compared to healthy controls [20]. Others have found that coronin-1A may also be involved in the survival of T lymphocytes and mutation in this gene can participate in the development of autoimmunity [21, 22]. Taken together, these observations indicate that coronin-1A, a well-characterised structural protein, has the unexpected ability to regulate neutrophil activation and apoptosis. Further studies examining the expression of coronin1A and its role in apoptosis may provide an insight into inflammatory disease and targeting this pathway may lead to novel therapeutics for the treatment of chronic inflammation.

Pro-survival

In addition to pro-apoptotic signalling pathways regulating neutrophil death, there are also a number of pro-survival factors that can prolong the lifespan of neutrophils. Upon migration to the site of inflammation, the lifespan of a neutrophil increases due to the presence of pro-survival cytokines and chemokines such G-CSF and GM-CSF, which are present in the inflammatory microenvironment [23]. Neutrophil survival also increases following the adhesion of cells to the microvasculature. Indeed, IL-8 is a well-characterised cytokine involved in neutrophil adhesion and increases in this cytokine delays neutrophil apoptosis. While the mechanisms responsible for this increased survival of neutrophils is poorly understood, it likely acts to promote neutrophil function, facilitate the recruitment of monocytes and differentiation of macrophages and ensures a rapid and robust immune response.

There are a number of transcription factors important in neutrophil survival including hypoxia-inducible factor (HIF) and Forkhead box O3A (FOXO3A). HIF-1α and HIF-2α are upregulated in response to hypoxia and both enhance neutrophil survival while FOXO3A is able to promote neutrophils survival by suppressing the transcription of FasL [24–26]. Another protein known to be involved in the balance between apoptosis and survival is the Bcl-2 homolog Myeloid Cell Leukaemia 1 (Mcl-1). Studies have shown that continual expression of this normally short-lived protein was able to delay neutrophil apoptosis and mice deficient in Mcl-1 suffer from neutropenia due to reduced neutrophil survival [27]. While the mechanisms responsible for this increased survival are not fully under-

stood, it appears to be a result of an interaction and inhibition of the pro-apoptotic proteins BIM and Bak [28].

Given that neutrophils are terminally differentiated and have no proliferative capacity, it was assumed that they would also lack cell-cycle regulatory proteins. This is not the case, as an expression of a number of cell cycle proteins including cyclin-dependent kinases (CDK) and proliferating cell nuclear antigen (PCNA) have been observed in mature neutrophils. In fact an emerging concept in neutrophil apoptosis and resolution of inflammation is the involvement of these proteins. For example, while the expression of survivin is generally restricted to proliferating cells where it is able to control cell cycle, recent studies have described the re-expression of this protein in neutrophils during inflammation and increased expression is associated with improved neutrophil survival [29]. Other studies have demonstrated that neutrophils contain functionally active cyclin-dependent kinases (CDK) and inhibition of these kinases by roscovitin triggers apoptosis [13].

Proliferating cell nuclear antigen (PCNA): novel anti-apoptotic role promoting neutrophil survival and chronic inflammation

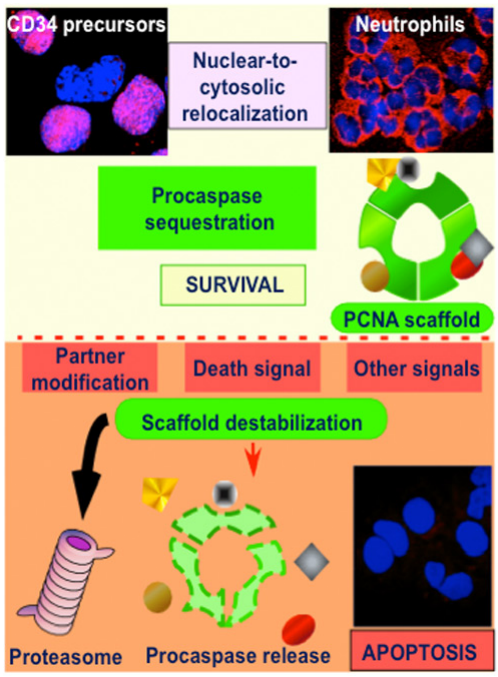

Figure 2

Proliferating cell nuclear antigen (PCNA) is localised to the cytosol of myeloid progenitors and possesses a proliferative capacity. During the late phases of granulocytic differentiation, PCNA is exported from the nucleus and become exclusively cytosolic. In mature neutrophils, PCNA controls neutrophil survival through its interaction with other cytosolic proteins, such as procaspases, to prevent their activation. Upon apoptosis induced by death signals or during the physiologic turnover of neutrophils and potentially following the modification of a partner protein, PCNA is targeted to the proteasome to induce neutrophil death. As a result, cytosolic PCNA is considered to be a scaffolding protein capable of controlling neutrophil survival.

Continuing this search for cell-cycle regulatory proteins expressed in neutrophils, our group has identified an unexpected anti-apoptotic role for PCNA. PCNA is a critical factor in DNA synthesis and repair, initially characterised as the auxiliary protein for DNA polymerases delta and epsilon [30]. To date, most of the function attributed to PCNA related to its nuclear localisation and ability to control cell proliferation. It is able to interact with a number of proteins within the nucleus including various enzymes and regulatory proteins like CDKs as well as CDK-inhibitor p21/waf1 [31]. As a result of the broad array of interacting partners, PCNA can be described as a "cellular communicator", capable of linking numerous important cellular processes [32]. PCNA is also involved in other cellular processes unrelated to replication, which include nucleotide-excision repair, mismatch repair and cell cycle.

In addition to its role in the nucleus, we have demonstrated that mature neutrophils express high levels of PCNA exclusively within the cytosol (fig. 2). This cytosolic PCNA is degraded by the proteasome during physiological, Fas- or gliotoxin-induced apoptosis and was found to increase following G-CSF-induced survival *in vitro* without transcriptional regulation and *in vivo* in G-CSF-treated patients [18]. In a mouse model of LPS-induced lung inflammation, airway neutrophils displayed increased survival associated with increased PCNA levels and its expression decreased in apoptotic cells during the resolution phase. During inflammation in cystic fibrosis and vasculitis, isolated neutrophils exhibit a delay in apoptosis and have increased levels of PCNA. This same observation was made in neutrophils isolated from the synovial fluid of rheumatoid arthritis patients and again this correlated with increased neutrophil survival [33].

Given that PCNA has no intrinsic enzymatic activity, its biological function relies on its ability to mediate the associations between different interacting partners. Most PCNA interactions occur at the interdomain-connecting loop. In the cytosol of a neutrophil, PCNA is able to bind procaspase-3, 8, 9 and 10, which in turn precludes their activation (fig. 2) [18]. Our group demonstrated that purified PCNA protected procaspase-9 from cytochrome c-induced activation which in turn had a strong anti-apoptotic effect on neutrophils. PCNA overexpression in DMF-differentiated PLB985 also significantly decreased TRAIL- or gliotoxin-induced apoptosis and when the interdomain-connecting loop of PCNA was mutated, this anti-apoptotic effect was abolished [34]. Another well-characterised binding partner is the CDK inhibitor p21/waf1, which also binds PCNA at the interdomain-connecting loop. A small peptide corresponding to the residues 141–160 of p21/waf1 known as carboxyp21 was found to interfere with PCNA-protein interactions and in turn had a strong antiproliferative effect [31]. Treating neutrophils with carboxyp21 peptide triggered PCNA degradation and apoptosis, while treatment with a mutated version of this peptide incapable of binding to PCNA, had no effect on apoptosis. Moreover, carboxyp21 peptide significantly inhibited G-CSF–induced neutrophil survival. We believe that PCNA is an important mediator in the survival of mature neutrophils and that the interdomain-connecting loop is essential for this function.

Efferocytosis: macrophages eating apoptotic neutrophils holds the key to inflammation resolution

As discussed above neutrophil survival and apoptosis are important in ensuring an appropriate immune response while at the same time limiting the destructive capacity of neutrophil on the surrounding tissue [35]. Another essential step in this process is the subsequent recognition and elimination of apoptotic neutrophils by phagocytic cells such as macrophages, a process known as efferocytosis [36]. In fact, this process is now viewed as being central to the successful resolution of inflammation and the initiation of tissue repair where dying neutrophils themselves actually exert an anti-inflammatory effect by modulating the response of surrounding cells towards an anti-inflammatory phenotype (fig. 3) [37].

Phagocytic leucocytes, predominantly macrophages, not only ingest and destroy invading pathogens, but are charged with clearing dead and dying neutrophils. The process of efferocytosis involves a number of steps, which ultimately result in the removal of apoptotic cells. When a neutrophil undergoes apoptosis, it releases a "find me" signal that can be detected by a macrophage and attracts it towards the site of the dying cell. "Find me" signals include nucleotides (ATP, UTP), chemokines (CX_3CL1) and lipid lysophosphytidlycholine (LPC). Once the macrophage reaches the apoptotic neutrophil, it is able to detect an "eat me" signal on the surface of the dying cells through receptors which signal to the macrophage to engulf the target. To date a number of "eat me" signals have been characterised including the exposure of phosphatidylserine (PtdSER), changes in charge and glycosation patterns on the cell surface, changes in ICAM-1 epitopes and exposure of calreticulin, an endoplasmic reticulum protein [38]. It is worth nothing that apoptotic neutrophils can also express "don't eat me" signals on their surface, such as proteinase 3 or PAI-1, which delays their removal by macrophages [39, 40]. Once the apoptotic neutrophil has been ingested, it is processed by the phagolysosomal pathway, which degrades and reprocesses the material of the apoptotic neutrophil [41].

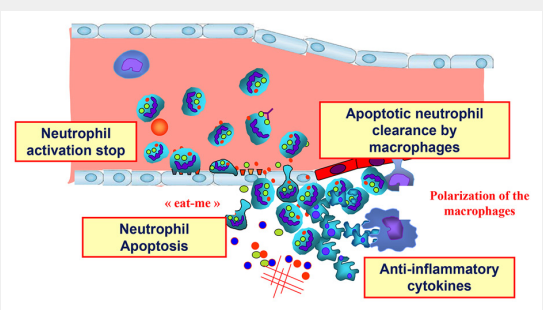

Figure 3

During sterile inflammation, neutrophils first adhere to the endothelium and undergo diapedesis. Following a gradient of chemotactic compounds, they migrate to the site of inflammation and become activated, where they secrete oxidant and release proteinases. During the normal progression of inflammation, neutrophils undergo apoptosis and express "eat-me-signals" which are recognised by macrophages. These macrophages phagocytose the apoptotic neutrophils and are reprogrammed towards an anti-inflammatory and pro-resolving state. This figure demonstrated the various steps in the neutrophil cycle, which may be targeted by an anti-inflammatory intervention.

Efferocytosis is not just important because it removed dying neutrophils before they cause unnecessary tissue damage, these apoptotic neutrophils also act as a powerful anti-inflammatory stimulus for other cells involved in the resolution of inflammation. When a macrophage engulfs an apoptotic neutrophil, it results in the release mediators capable of suppressing inflammation. Studies performed *in vitro* and *in vivo* demonstrate that following efferocytosis, macrophages produce high levels of IL-10, TGF-β and PGE2 and secrete factors involved in tissue repair including VEGF. At the same time, apoptotic neutrophils suppress the production of TLR-dependant cytokine including IL-6, IL-8 and TNF by macrophages [36]. In essence, macrophages in an activated state are switched to a pro-resolving phenotype that is able to facilitate the resolution of inflammation and promote tissue repair. Any defect in this "eat me" process has the potential to perpetuate inflammation and may eventually lead to chronic inflammatory diseases.

Harnessing pro-resolving pathways for therapeutic purposes?

Treatment of inflammatory diseases today is largely based on interrupting the synthesis or action of mediators that drive the host response to infection and injury. In fact, non-steroidal anti-inflammatories, steroids and antihistamines were all developed on this basis. While small-molecule inhibitors have provided the main treatment for chronic inflammatory disease such as arthritis and asthma, they are not without their shortcomings including undesirable side effects and increased susceptibility to infection. An alternative approach being explored for the development of novel therapeutics is now looking towards endogenous mediators and exploring mechanisms able to switch off acute inflammation and bring about resolution. Research into activating pro-resolving pathways will likely reveal new avenues for the management of inflammatory diseases in the future [42].

One group of inflammatory disorders which may benefit from promoting the resolution of inflammation are autoimmune diseases. One theory regarding the induction of autoimmune diseases hypothesises is that they result from a defect in apoptosis or removal of apoptotic cells. These defects lead to the immune system being exposed to fragments of the dying cell, which are able to induce a sustained humoral response to protein found on those fragments [43–45]. Given the reliance of a number of autoimmune diseases on neutrophils, promoting neutrophil apoptosis and the subsequent resolution of inflammation, represents an attractive therapeutic option that could ameliorate disease symptoms while leaving host defence intact [46].

Neutrophils are believed to play a critical role in the pathogenesis of autoimmune vasculitides including Granulomatosis with polyangiitis (GPA, formally known as Wegener's), Microscopic polyangiitis (MPA) and Churg-Strauss syndrome (CSS) [47]. In these diseases, neutrophils are not

only the target of the autoantibodies but also the effector cell responsible for mediating damage to the endothelium and potentiating inflammation [48]. The autoantibodies found in these diseases, anti-neutrophil cytoplasmic antibodies (ANCA), target two neutrophil granule proteins, proteinase 3 (PR3) and myeloperoxidase (MPO). Anti-PR3 ANCAs are associated with GPA, a disease characterised by granulomatous inflammation of the upper and/or lower respiratory tract, while anti-MPO ANCAs are present in patients with MPA, an idiopathic necrotising crescentic glomerulonephritis, and less frequently in Churg-Strauss syndrome, a small vessel vasculitis associated hypereosinophilia and asthma.

A number of studies have found that patients with ANCA-associated vasculitis (AAV) display a defect in neutrophil apoptosis compared to healthy controls [49, 50]. Additionally, apoptotic neutrophils expressing ANCA antigens on their surface can be opsonised by ANCA which enhances recognition and subsequent uptake by macrophage via Fc-receptor interaction. This leads to an increased synthesis of pro-inflammatory mediators such as IL-8 and IL-1 by macrophages, which results in the recruitment of more neutrophils and enhances the progression of inflammation [51]. One treatment currently used in the management of these diseases is endoxan, an immunosuppressive drug able to trigger severe neutropenia [52]. While combinations of endoxan and corticosteroids are beneficial in reducing the symptoms of AAV, they are often poorly tolerated and have serious side effects including the development of secondary immunodeficiency. While these side effects are highly undesirable, this drug demonstrates that reducing neutrophils may be beneficial and provides further support for the development of drugs able to enhance neutrophil apoptosis, increase elimination by macrophages and limit the damage caused by prolonged activation of these cells.

Other diseases that may benefit from therapies targeting neutrophil apoptosis are various types of non-infectious human arthritis such as rheumatoid arthritis, where the presence of neutrophils is deleterious. Importantly, neutrophils are present in the acute phase of these diseases and are believed to play a significant role in tissue damage through the release of toxic products such as oxidants, proteinases and cytokines [53–55]. The presence of neutrophils perpetuates the inflammatory process and enhances tissue degradation and destruction, leading to disability and eventual surgical joint replacement. In line with this idea, neutrophils isolated from the synovial fluids of arthritis patients display increased survival compared to control [54]. Given the aforementioned ability of neutrophils to modulate the inflammatory process and immune responses, it is possible that modulating neutrophil survival and apoptosis in arthritis may present an exciting therapeutic target for reducing joint inflammation and damage [56]. Preliminary studies exploring the use of apoptotic cells in the treatment of arthritis has found that mice injected with apoptotic cells prior to the onset of collagen-induced arthritis were protected from severe joint inflammation and bone destruction [57]. This protection appears to be the result of macrophage reprogramming towards an anti-inflammatory and pro-resolving state and subsequent modulation of other important cells including T and B lymphocytes, which also act to secrete pro-resolving factors, promote tolerance and inhibit chronic inflammation [58].

Conclusion

As our understanding of inflammation increases, there is evidence to suggest that chronic inflammatory diseases involve a functional defect in the resolution of inflammation, which may act to maintain activation of the immune system. There is strong evidence to suggest that neutrophils, their apoptosis and subsequent elimination are able to act as a strong pro-resolving force [24]. By understanding how neutrophils live and the mechanisms that control their death, it may lead to highly effective therapies for the treatment of non-resolving and chronic inflammatory diseases.

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Figures (large format)

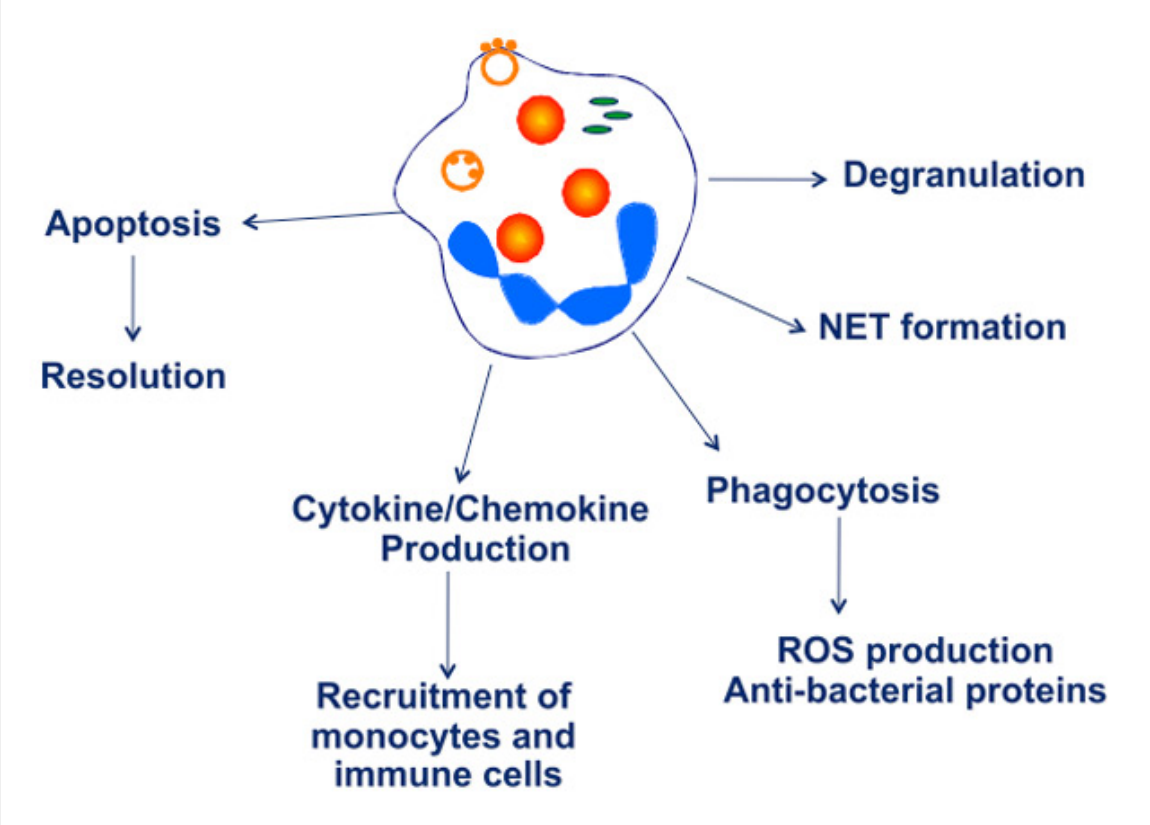

Figure 1

A summary of the function of neutrophils during immune responses.

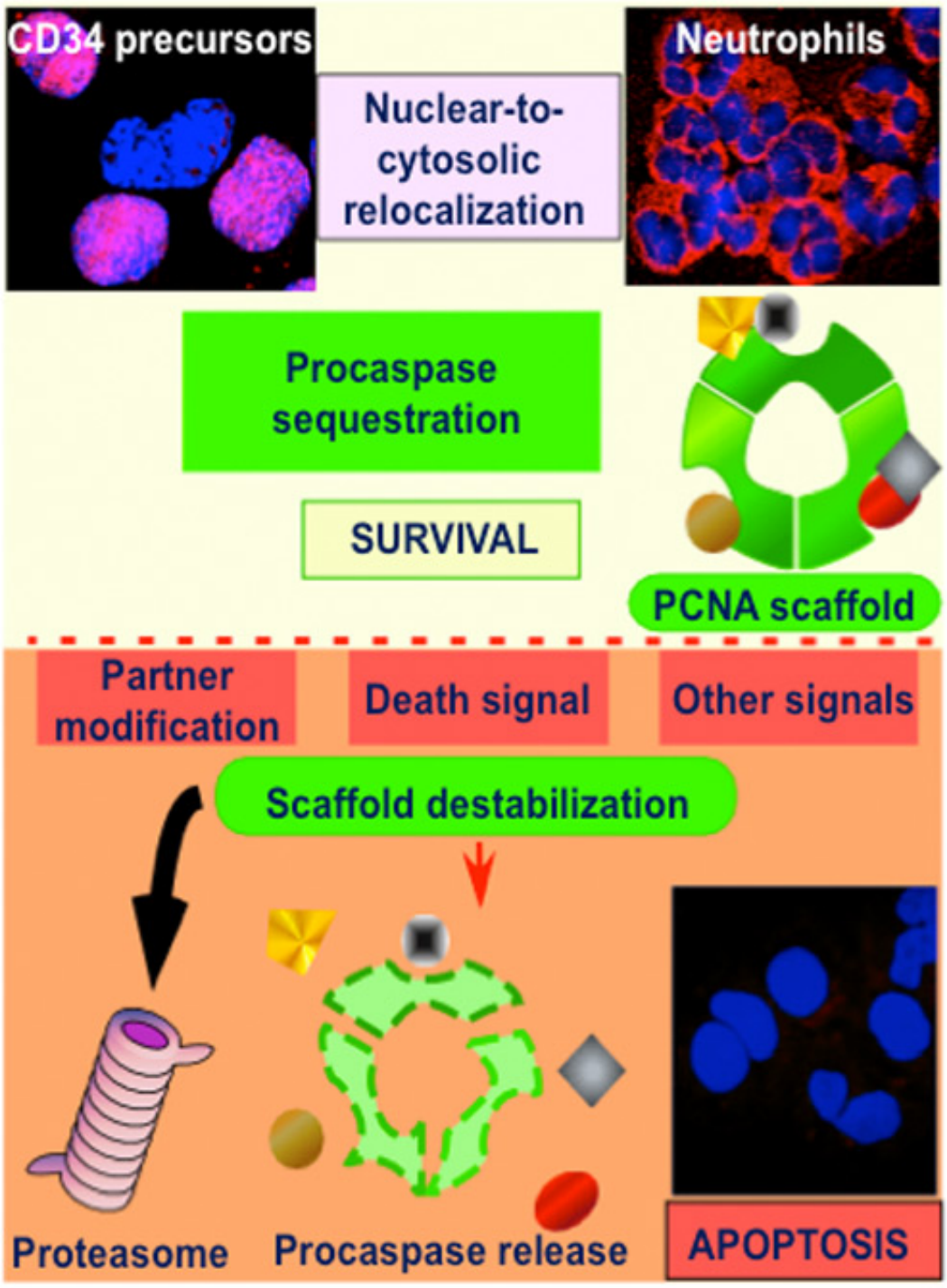

Figure 2

Proliferating cell nuclear antigen (PCNA) is localised to the cytosol of myeloid progenitors and possesses a proliferative capacity. During the late phases of granulocytic differentiation, PCNA is exported from the nucleus and become exclusively cytosolic. In mature neutrophils, PCNA controls neutrophil survival through its interaction with other cytosolic proteins, such as procaspases, to prevent their activation. Upon apoptosis induced by death signals or during the physiologic turnover of neutrophils and potentially following the modification of a partner protein, PCNA is targeted to the proteasome to induce neutrophil death. As a result, cytosolic PCNA is considered to be a scaffolding protein capable of controlling neutrophil survival.

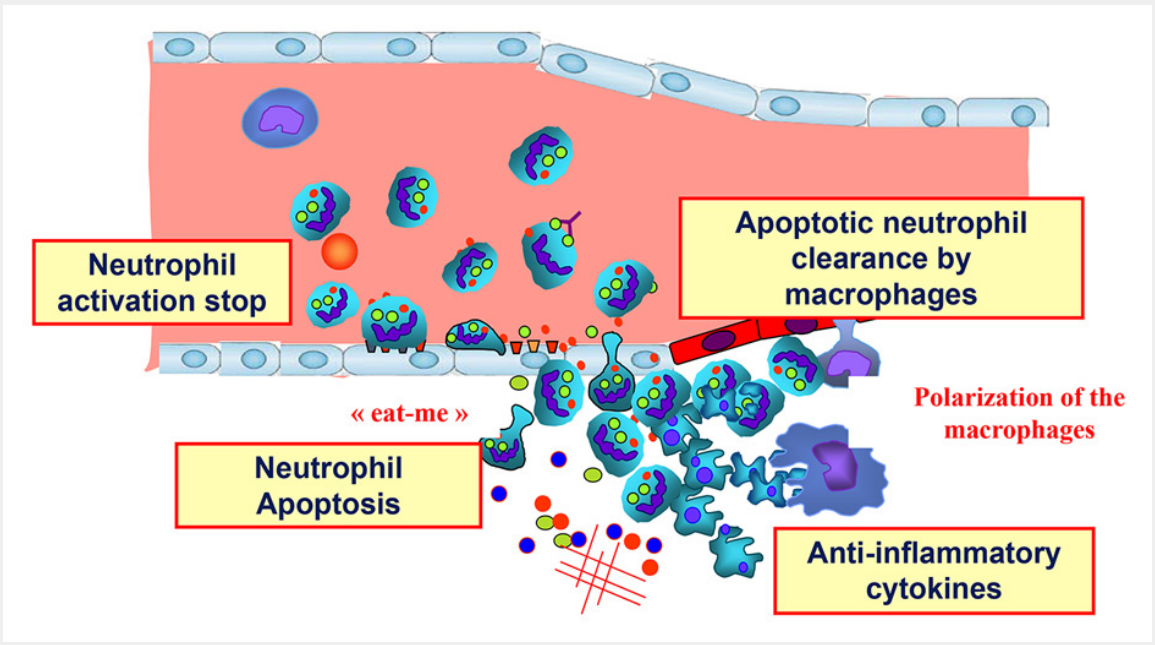

Figure 3

During sterile inflammation, neutrophils first adhere to the endothelium and undergo diapedesis. Following a gradient of chemotactic compounds, they migrate to the site of inflammation and become activated, where they secrete oxidant and release proteinases. During the normal progression of inflammation, neutrophils undergo apoptosis and express “eat-me-signals” which are recognised by macrophages. These macrophages phagocytose the apoptotic neutrophils and are reprogrammed towards an anti-inflammatory and pro-resolving state. This figure demonstrated the various steps in the neutrophil cycle, which may be targeted by an anti-inflammatory intervention.