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Regulation of neutrophil apoptosis: A role for protein kinase C and phosphatidylinositol-3-kinase

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Neutrophils play a central role in host defense and are recruited in vast numbers to sites of infection where they phagocytose and kill invading bacterial pathogens. Neutrophils have a short half-life that is extended at the inflamed site by pro-inflammatory cytokines and contact with bacterial cell walls. Normal resolution of inflammation involves the removal of neutrophils and other inflammatory cells by the induction of apoptosis. Spontaneous neutrophil apoptosis does not require Fas ligation, but is mediated by caspases 3, 8 and possibly caspase 9 and also involves activation of protein kinase C-δ. With chronic inflammatory disease, neutrophil apoptosis is delayed by pro-inflammatory cytokines, leading to persistence of neutrophils at the inflamed site and non-specific tissue damage. Here we discuss the evidence for inhibition of neutrophil apoptosis via signaling though PI-3-kinase and downstream pathways, including PDK-1 and PKB. Therapeutic strategies to resolve chronic inflammation could therefore usefully target neutrophil apoptosis and the PI-3-kinase or PKC-δ signaling pathways.

Keywords: apoptosis; inflammation; neutrophil; PI-3-kinase; PKC; T-cell.

Introduction

Neutrophils are short-lived, polymorphonuclear leukocytes that play a major role in the early stages of the inflammatory response to infection, phagocytosing and killing extracellular microbial pathogens. Human neutrophils are produced in the order of $1–2 \times 10^{11}$ cells per day and in the absence of infection they will survive in the circulation for only 24–36 hours before undergoing apoptosis.[1] During infection neutrophils leave the blood under the influence of chemotactic factors that include microbial products (fMLP), complement components (C5a) and chemokines (IL-8). They migrate into tissues and upon reaching the site of infection, begin to phagocytose and kill ingested pathogens, by a variety of mechanisms including release of lytic enzymes and generation of reactive oxygen and nitrogen species. The ingestion of microbes also induces the neutrophil to release pro-inflammatory cytokines that will attract additional inflammatory cells. After killing ingested microbes, neutrophils die by apoptosis and are themselves phagocytosed by macrophages, preventing loss of neutrophil contents and consequent tissue damage.[2] Once the pathogen has been eliminated the inflammatory response must be resolved by the elimination of residual inflammatory cells, including neutrophils, through the induction of apoptosis.[3] The correct regulation of the apoptotic programme is vital to ensure the maintenance of neutrophil numbers in the circulation, the efficient removal of invading pathogens and the rapid resolution of the inflammatory response.

Disregulation of apoptosis may lead to the persistence of immune cells at inflammatory sites and the development of chronic inflammatory disease.[4,5] Perturbation of neutrophil apoptosis has been proposed to contribute significantly to tissue damage associated with inflammatory diseases such as acute respiratory distress syndrome[5] and rheumatoid arthritis.[6] Novel anti-inflammatory therapies based on the restoration of neutrophil apoptosis have considerable promise, but to identify realistic targets it is important firstly to understand the precise pathways that regulate apoptosis in neutrophils, then identify survival factors for neutrophils at inflammatory sites and determine their mode of action. In this review we discuss the role of protein kinase C (PKC) and phosphoinositide-3-kinase (PI3K), signaling enzymes known to be major regulators of cell survival and apoptosis[7,8] in the control of spontaneous neutrophil apoptosis and their potential role in the inhibition of this process by pro-inflammatory cytokines.

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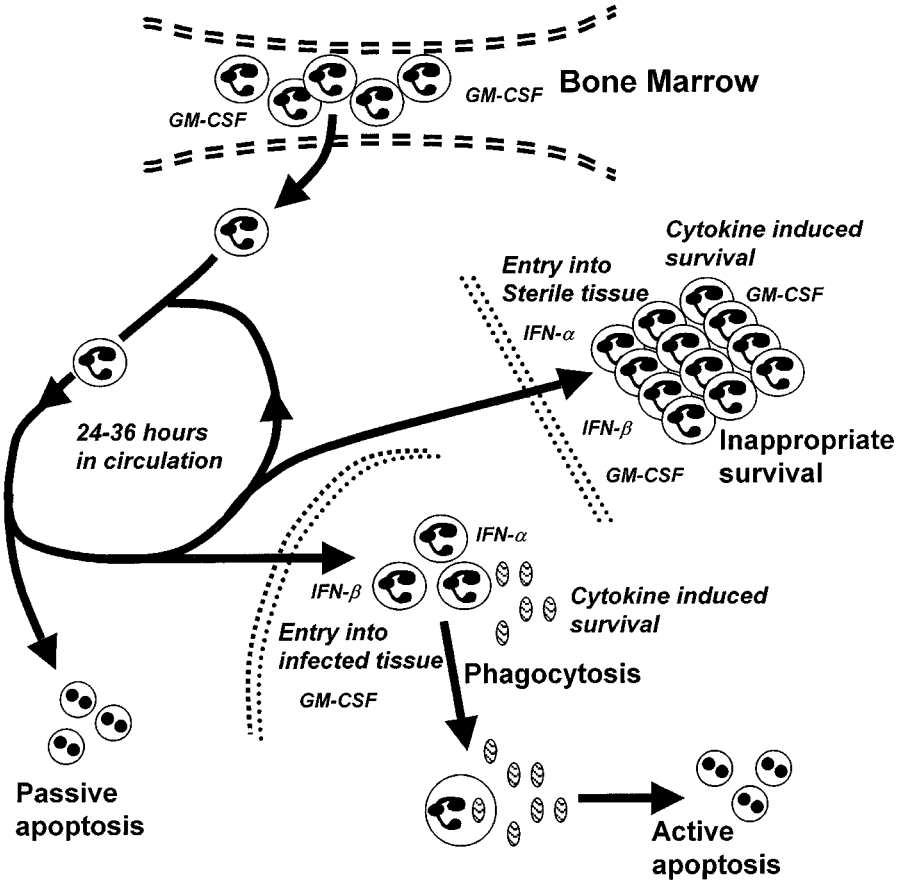
Figure 1. Neutrophils die by cytokine-deprivation induced apoptosis. In the bone marrow neutrophils receive survival signals from cytokines such as GM-CSF. Absence of these cytokines in the circulation limits the life-span of the neutrophil. Recruitment to sites of infection provides survival signals from pro-inflammatory cytokines and apoptosis is induced by neutrophil activation. Recruitment of neutrophils to sterile inflamed sites delays apoptosis inappropriately leading to neutrophil accumulation and tissue damage.



Neutrophil apoptosis

Neutrophils released from the cytokine rich environment of the bone marrow will circulate in the blood for approximately 1–2 days before dying by apoptosis. However, if neutrophils are recruited to a site of infection their life-span is extended by the actions of pro-inflammatory cytokines[5] and death is then induced as a consequence of phagocytosis of microbes and activation of microbicidal mechanisms.[9,10] If neutrophils are recruited to a sterile, chronically inflamed site apoptosis is also delayed by inflammatory cytokines.[4] Apoptosis can not be induced by microbes in this situation and neutrophils accumulate inappropriately leading to tissue damage.[6] We propose that as a normal homeostatic mechanism, effete neutrophils in the circulation die as a result of passive, cytokine deprivation-induced apoptosis rather than by an active induction pathway. Therefore cytokines produced at sites of inflammation can delay neutrophil apoptosis, which is beneficial for the elimination of pathogens, but will promote persistence of inflammation and tissue damage in the absence of infectious agents (Figure 1).

Induction of the apoptotic programme in effete neutrophils in the circulation and during the resolution of inflammation has been proposed to involve signalling through Fas/CD95/Apo-1,[11] reduced expression of anti-apoptotic members of the Bcl-2 family of proteins[12–14] and activation of pro-apoptotic members of the protein kinase C (PKC) isoenzyme family.[15,16] While neutrophils express both Fas and Fas ligand,[11] recent studies employing antagonistic anti-Fas antibodies have shown that signalling through Fas is not required for spontaneous neutrophil apoptosis.[17] Also neutrophils from Fas (*lpr*) or Fas ligand (*gld*) deficient, mice show a normal rate of spontaneous apoptosis.[18] Whether ligation of Fas operates during the resolution of acute inflammation, when a high concentration of neutrophils at the inflammatory site could promote neutrophil fratricide, remains to be established. In addition, the involvement of other death-inducing receptors, such as TNFRII or TRAIL

cannot be ruled out at this stage. However, neutrophil apoptosis is difficult to induce actively *in vitro* through either Fas[5,17] or TNF-α receptors,[5] arguing against a major role for active promotion of neutrophil apoptosis via membrane located death receptors.

In contrast, the data suggesting loss of anti-apoptotic proteins as a primary mediator of spontaneous neutrophil apoptosis is substantial. Firstly, promyeloid cell lines such as HL60 express several anti-apoptotic members of the Bcl-2 family, including Bcl-2 itself. The level of these proteins declines as they differentiate towards the granulocyte lineage.[19] Secondly, a majority of authors have reported that freshly isolated human neutrophils do not express the anti-apoptotic proteins Bcl-2 or Bcl-x_L,[12–14] though they do have detectable levels of the cytosolic Bcl-2 homologues Mcl-1[14] and A-1[20] and the pro-apoptotic proteins Bak[21] and Bax.[14,22] While levels of Bax remained constant as neutrophils were aged in culture and entered apoptosis, Mcl-1 expression declined concomitant with the increasing level of apoptosis.[14] In addition, neutrophils from A-$1^{-/-}$ mice have an accelerated rate of spontaneous apoptosis.[23] Thus the selective loss of anti-apoptotic proteins expressed in neutrophils, in the presence of a high level of pro-apoptotic Bax and Bak proteins, is likely to be a key factor in the promotion of neutrophil apoptosis. Furthermore, loss of Mcl-1 can be maintained by pro-inflammatory cytokines such as GM-CSF,[14] supporting our contention that cytokine deprivation is the primary cause of spontaneous neutrophil apoptosis.

The lack of expression of mitochondrial proteins involved in the inhibition of apoptosis, such as Bcl-2, is intriguing and may reflect a lack of involvement of mitochondria in spontaneous neutrophil apoptosis, bearing in mind their relatively low numbers in neutrophils and their proposed vestigial function in these cells.[24] Alternatively, as a key role of Bcl-2 is to maintain mitochondrial membrane integrity, loss of Bcl-2 may represent a key event allowing mitochondrial permeability transition.[25] Loss of mitochondrial membrane potential ($\Delta\psi$m) occurs as an early event in apoptosis and can be induced downstream of death receptor ligation and caspase 8 activation[26] or as a result of increased intracellular calcium, ceramide or reactive oxygen species.[27] Release of cytochrome *c* resulting from the decrease in $\Delta\psi$m leads to activation of caspase 9 and caspase 3. Caspase 3 can also be activated directly by caspase 8 independent of mitochondrial events,[26,28] though the current literature does not support this mechanism in neutrophils. Neutrophils express only a limited number of the 13 known caspases: caspases 1, 3, 8 and 9.[29,30] Caspase 10 may also be present.[30] Caspase 3 activation has been reported during spontaneous[15,16] and UV irradiation-induced[31] neutrophil apoptosis and inhibition of either caspase 3 or caspase 8 significantly delays spontaneous neutrophil apoptosis.[15,16] There are also preliminary reports showing loss of mitochondrial membrane integrity[32] and activation of caspase 9[29] during spontaneous neutrophil apoptosis. Taken together these data suggest that the activation of caspase 3 seen during spontaneous neutrophil apoptosis occurs via the mitochondria route. However, the initial triggers for both activation of caspase 8 and loss of $\Delta\psi$m remain to be identified, but do not appear to involve Fas ligation.

Protein kinase C and neutrophil apoptosis

Protein kinase C (PKC) is a lipid activated serine/threonine kinase and consists of a catalytic domain and a hydrophobic regulatory domain separated by a protease sensitive hinge region, V3.[33] PKC comprises a multigene family of 11 isoenzymes that are regulated independently and have been sub-divided into three classes according to their requirements for co-factors: the classical PKCs (α, β_I, β_{II}, and γ); the novel PKCs (δ, ε, η, θ and μ); and the atypical PKCs (ζ and ι/λ). Several lines of evidence suggest that PKC isoenzymes are differentially involved in the regulation of apoptosis and the advent of isoenzyme specific inhibitors now means that PKC is a realistic target for the therapeutic modulation of apoptosis.

PKC-α and the atypical PKCs appear to be predominantly anti-apoptotic, whereas novel PKC-δ and PKC-θ are pro-apoptotic.[7,34–36] Thus PKC-α is overexpressed in a variety of tumours[7,37] and its down-regulation or removal by anti-sense RNA,[37,38] both lead to increased apoptosis and tumour regression. Substrates for PKC-α include Bcl-2, with phosphorylation increasing the anti-apoptotic actions of this molecule.[39] In addition, PKC-α is inactivated by ceramide during stress-induced apoptosis[36] and atypical PKC-ζ is cleaved and inactivated by caspase 3 during apoptosis in HeLa cells.[40] In contrast, novel PKC-δ has been implicated in the promotion of apoptosis, initially by the work of Emoto *et al.*[41] who showed that PKC-δ was cleaved by caspase 3 in the V3 hinge region. In the case of PKC-δ, caspase 3 cleavage was not inhibitory as it resulted in the release of the active catalytic 40 kDa kinase domain fragment. Moreover, transfection of HeLa and NIH 3T3 cells with the caspase generated PKC-δ fragment was sufficient to induce an apoptotic morphology.[42] PKC-θ, which has a very high homology to PKC-δ, is also cleaved and activated by caspase 3 during apoptosis.[43] Thus altered expression or changes to the activation status of specific PKC isoenzymes could play a role in neutrophil apoptosis.

Neutrophils express a range of PKC isoenzymes, including pro- and anti-apoptotic members, namely PKCs-α, -β_I, -β_{II}, -δ and -ζ.[15,16,44–46] The classical PKCs are known to be involved in neutrophil activation during the immune response[47] and it is now clear that distinct PKC isoenzymes are involved in the regulation of neutrophil

apoptosis. Analysis of the activation status of PKC isoenzymes in healthy and apoptotic neutrophils revealed that association of PKC-β and δ with the cell membrane, an indicator of PKC activation, was increased in apoptotic cells.[15] In addition the caspase 3-generated catalytic fragment of PKC-δ was also detected in apoptotic neutrophils.[15,16] However, inhibition of PKC-β, using either Go6976[16] or LY379196,[15] did not reduce the rate of spontaneous neutrophil apoptosis,[15] suggesting that this isoenzyme is not directly involved in neutrophil apoptosis. However, neutrophil apoptosis was inhibited by the PKC-δ inhibitor Rottlerin.[15] Furthermore, in a cell free system comprised of healthy nuclei and apoptotic cytosol, nuclear DNA fragmentation was reduced when PKC-δ was depleted from the cytosol using antibody, whereas removal of the other PKC isoenzymes expressed in neutrophils had no effect.[15] Therefore activation of PKC-δ, rather than reduced activity of anti-apoptotic PKC-α, is involved in the promotion of spontaneous neutrophil apoptosis.

Exactly how activation of PKC-δ contributes to apoptosis in human neutrophils is not known. In proliferating cells PKC-δ has been shown to translocate to the nucleus[48–50] and mitochondria[51] during apoptosis. Whilst the significance of mitochondrial translocation is not known, nuclear targets for PKC-δ include proteins involved in DNA replication and repair, DNA-PK,[49] and maintenance of nuclear structure, lamin B.[50] Phosphorylation of lamin B by PKC-δ is required for disassembly of the nuclear lamina during apoptosis.[50] In the non-proliferating neutrophil, PKC-δ also localises to the nucleus during apoptosis[15] and as DNA replication and repair are not operational, it is possible that nuclear PKC-δ primarily regulates the disassembly of the nuclear lamina.

Neutrophils in the circulation are therefore committed to death by apoptosis, achieved by activation of caspases, including caspase 3, caspase 8 and possibly caspase 9 and activation of pro-apoptotic PKC-δ. Although neutrophils are committed to apoptosis, their death can be delayed at sites of inflammation by external factors including pro-inflammatory cytokines,[52] bacterial membrane components such as lipopolysaccharide,[52] and pro-granulocyte differentiation factors such as GM-CSF.[52,53] These mechanisms are important during acute inflammation responses when extension of neutrophil lifespan will contribute to accumulation of neutrophils at sites of infection and benefit microbicidal efficiency. However, inappropriate retention or recruitment of neutrophils to sterile sites (Figure 1) can lead to chronic inflammatory disease.[5,6] Knowledge of the signalling pathways employed by cytokines to delay neutrophil apoptosis will identify novel therapeutic targets for inflammatory diseases such as rheumatoid arthritis.

Inhibition of neutrophil apoptosis

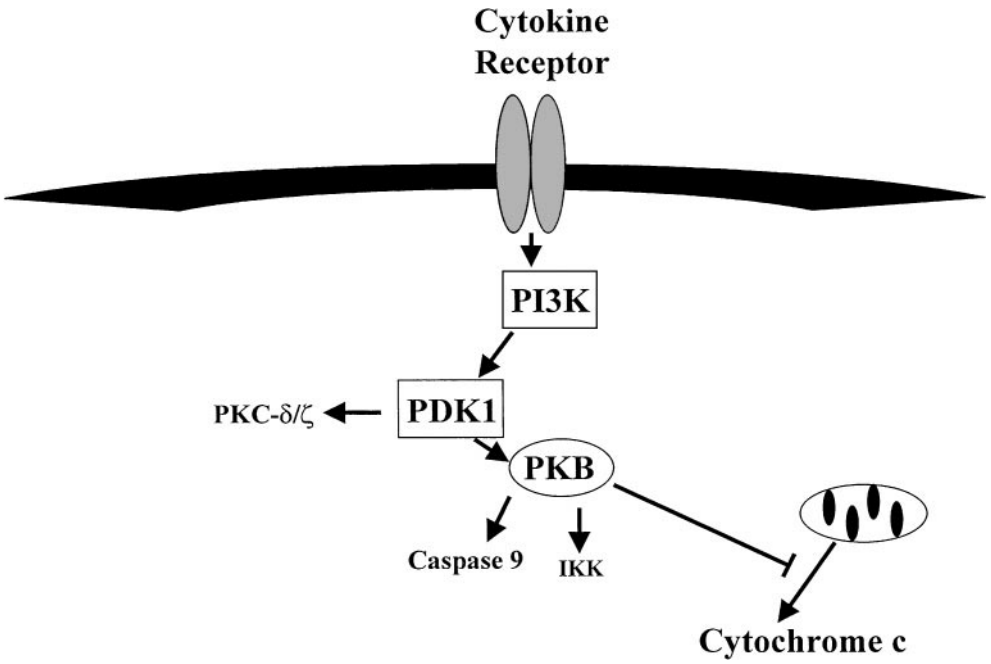
As stated above, several pro-inflammatory cytokines are able to delay neutrophil apoptosis.[52–55] In the normal immune response to infection many of these agents function to prime the neutrophil. Neutrophils in the circulation are relatively unresponsive to bacterial compounds such as fMLP and require prior contact with priming agents to induce optimal activation. Priming also influences neutrophil survival, which can be beneficial during infection, but can have pathological consequences if the primed state is inappropriate.[5] Neutrophils then persist inappropriately at inflamed sites and induce tissue damage.

Priming agents include the chemokine IL-8[56] that binds to G-protein linked receptors, as well as cytokines whose receptors signal through tyrosine kinase activation, e.g. GM-CSF[57] and G-CSF.[52] Neutrophil apoptosis can also be delayed by pro-inflammatory cytokines that are not involved in neutrophil priming. Our own studies have shown that Type-1 interferon, which delays T cell apoptosis in the synovial fluid of patients with rheumatoid arthritis,[58] also delays neutrophil apoptosis (Wang and Scheel-Toellner, unpublished observations). As neutrophils comprise a significant proportion of the cellular infiltrate in the rheumatoid synovium, Type-1 interferon may mediate their increased survival and promote chronic inflammation and neutrophil mediated joint damage. Whether there is a common link between the downstream signaling pathways activated by these agents that delay neutrophil apoptosis remains to be established, though we propose here that one possibility is the activation of the phosphatidylinositol-3-kinase (PI3K)–3-phosphoinositide dependent kinase-1 (PDK-1)–Protein kinase B (PKB) pathway.

PI3K is activated by a variety of growth and survival factor receptors[8,59] and phosphorylates inositol lipids at the 3′-OH position to generate the second messengers phosphatidylinositol 3,4-bisphosphate (PI3,4P_2) and phosphatidylinositol 3,4,5-trisphosphate (PI3,4,5P_3). PI3,4,5P_3 binds to PKB inducing its translocation to the cell membrane and also activates PDK-1, which then phosphorylates and activates PKB (Figure 2). PDK-1 has a variety of downstream targets in addition to PKB, including PKC, cyclic AMP dependent protein kinase (PKA) and p70 S6 kinase.[60] PKB has been identified as playing a key role in transduction of survival signals by cytokine and growth factor receptors in many cell types[8,59] and may therefore also be involved in the delay of neutrophil apoptosis by pro-inflammatory cytokines.

Protein kinase B (Akt), is a 57 kDa serine/threonine kinase with a high degree of homology to PKA and PKC, that was first identified as the cellular homologue of the oncogene, *v-akt*, transduced by the retrovirus AKT8.[61] PKB comprises three isoforms in mammals, namely

Figure 2. Signaling pathways potentially involved in the prevention of neutrophil apoptosis by pro-inflammatory cytokines. Ligation of cytokine receptors recruits PI3K to the receptor, via G proteins or receptor associated tyrosine kinases. PI3K generates $PI3,4,5P_3$ which leads to the activation of PDK-1 and PKB. PKB targets include anti-apoptotic proteins caspase 9 and IκB kinase (IKK) and mitochondrial proteins. Downstream targets of PDK-1 include PKC and PKA.



PKBα/Akt1, PKBβ/Akt2, PKBγ/Akt3 (reviewed in [60]). Any differential functions of the three PKB isoforms have not yet been identified. Recent studies have identified several potential anti-apoptotic targets of PKB, including: the pro-apoptotic Bcl-2 family protein BAD, whose activity is reduced by phosphorylation at Ser^{112} or Ser^{136};[62] human caspase-9, which has reduced ability to cleave and activate caspase-3 in a phosphorylated state;[63] and IκB kinase, which is activated upon association with PKB,[64,65] leading to activation of NF-κB and transcription of anti-apoptotic genes.[66] More recently, PKB has been shown to inhibit upstream events in the apoptotic programme, most notably the loss of mitochondrial membrane potential.[67] Several of these survival mechanisms are potentially relevant to neutrophil apoptosis, which may involve loss of mitochondrial membrane integrity,[32] activation of caspase 9 [29] and loss of NF-κB activity.[68]

Despite the current interest in PKB-mediated cell survival, the majority of studies in neutrophils have concerned the role of PKB in neutrophil activation rather than apoptosis. Activation of neutrophils by fMLP and ligation of Fcγ-receptors results in PKB activation in a PI3K-dependent manner, indicating a role for PKB in respiratory burst and phagocytosis.[69] However, GM-CSF, which delays neutrophil apoptosis, has been shown to activate PI3K via recruitment and activation of the tyrosine kinase Jak 2.[70] Our own studies have shown that the inhibition of neutrophil apoptosis by Type-1 interferon is also PI3K-dependent (Wang and Scheel-Toellner, unpublished observations) and it is likely that PI3K is activated via association of STAT3 with the interferon receptor as reported by others.[71] However, the activation of PKB downstream of PI3K by either of these survival promoting cytokines has not yet been demonstrated and it is possible that PKB is involved primarily in neutrophil activation. If this is true then survival may be mediated directly by the lipid products of PI3K, independent of PKB. For example, PDK-1 is activated by PI3K-generated lipids and has substrates in addition to PKB that are also implicated in the prevention of apoptosis, for example PKC-ζ.[72] Also, in our studies in T cells, Type-1 interferon mediated inhibition of apoptosis was associated with a rapid reversal of the nuclear translocation of PKC-δ that occurred early in the apoptotic process.[48] As PKC-δ can also be phosphorylated by PDK-1,[72] it is possible that movement away from the nucleus is mediated by this kinase. Whether Type-1 interferon prevents nuclear PKC-δ translocation in neutrophils is currently under investigation.

Conclusions

Effete neutrophils die by apoptosis and are removed from the circulation by macrophages. Their spontaneous death does not require signaling through Fas, but does involve activation of caspase 8, caspase 3, PKC-δ and possibly

caspase 9. The initial stimulus for the constitutive apoptotic programme in neutrophils remains to be established. However, as neutrophil apoptosis can be delayed by factors such as GM-CSF, which are present at high levels in the bone marrow, we speculate that release of neutrophils from the marrow essentially removes survival factor signals. Spontaneous neutrophil apoptosis thus results from a constitutive growth/survival factor deprivation. Furthermore, the lack of expression of anti-apoptotic proteins such as Bcl-2, ensures that the cell dies rapidly unless death is delayed at sites of inflammation by pro-inflammatory cytokines.

Neutrophils are present in high numbers at inflammatory foci in chronic inflammatory diseases such as rheumatoid arthritis (RA) and their presence at these sites has been linked to tissue damage.[73] These inflammatory sites also contain high levels of pro-inflammatory cytokines able to delay the apoptosis of leucocytes,[6,58] including neutrophils,[6,52] by signaling through the PI3K pathway. The continued presence of pro-inflammatory cytokines at inflammatory foci in diseases such as RA ensures that inflammatory cell apoptosis is delayed and hypercellularity persists. Future therapeutic approaches to the treatment of chronic inflammatory disease may thus be usefully targeted at pathways regulating neutrophil apoptosis, such as PI3K and PKC-δ.

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