Neutrophil Apoptosis During Viral Infections

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Abstract: Apoptosis, or programmed cell death, is a highly conserved cellular suicide mechanism. Apoptosis is critical to the effective resolution of inflammation, particularly in regulating the lifespan of neutrophils. Neutrophils are key components of the first line of defense against microorganisms. Thus, subversion of this critical host defense mechanism by pathogens can contribute to susceptibility to severe and recurrent infections. In this review, we describe the molecular mechanisms involved in PMN death in relationship with viral infections.

INTRODUCTION

Polymorphonuclear neutrophils (PMN) contribute to the early innate response by rapidly migrating to inflamed tissues, where their activation triggers microbicidal mechanisms such as the release of proteolytic enzymes and antimicrobial peptides, and rapid production of reactive oxygen species (ROS), in the so-called oxidative burst.

PMN are terminally differentiated cells with a short life span in the circulation (8-20 h); aged PMN undergo spontaneous apoptosis (programmed cell death), in the absence of cytokines or other proinflammatory agents, prior to their removal by macrophages [1]. This phagocytic removal of intact, apoptotic neutrophils prevents them from releasing their cytotoxic content into the extracellular environment that would occur if the cells died by necrosis. Apoptosis is an intrinsic cellular process that can be regulated by external factors. In particular, PMN activation by circulating bacterial products, endogenous cytokines and other pro- inflammatory mediators can affect the rate of PMN apoptosis [2-4]. The prolongation of PMN life span is critical in their efficiency against pathogens [5]. Inappropriate PMN survival and persistence at sites of inflammation are thought to contribute to the pathology of chronic inflammatory diseases [6]. In contrast, shortened PMN survival due to apoptosis may contribute to susceptibility to severe and recurrent infections in some pathological situations [7, 8]. Thus, the death program in PMN needs to be well controlled to provide appropriate balance between their immune functions and their safe clearance. Finally, PMN death contributes to PMN's pathogen killing capability. It is an essential step for the generation and the release of PMN extracellular traps (NETs), extracellular structures composed of chromatin, and granules proteins that bind and kill invading microorganisms. This mechanism allows PMN to fulfil their antimicrobial function even beyond their life span [9].

Pathogens, have evolved diverse mechanisms to evade the innate immune response and escape clearance by immune cells. Modulation of PMN apoptosis is recognized as a key mode of immune evasion, altering the timing of PMN death. This review point to some examples of viral infections associated with modulation of PMN apoptosis which may be involved at least in part in the pathophysiology of the disease.

NEUTROPHIL APOPTOSIS

PMN have a short life-span [10]. Apoptosis is one of the main types of cell death characterized so far [11]. PMN death display features of classical apoptosis, such as cell body shrinkage, cellular crenation, vacuolated cytoplasm, mitochondria depolarization, nuclear condensation, internucleosomal DNA fragmentation, and externalization of phosphatidylserine residues from the inner to the outer leaflet of the plasma membrane [12, 13]. This externalisation facilitates the recognition of apoptotic neutrophils by macrophages [14-16]. Some of the features of classical apoptosis, including those observed in constitutive neutrophil death, are also shared by autophagic cell death [17].

Dying PMN show molecular alterations on their cell surface and is accompanied by the down-regulation of the immunoglobulin superfamily members (e.g. CD31, CD50, CD66acde, CD66b, CD63 and CD87) and cell surface receptors (e.g. CD15, CD16, CD32, CD35, CD88, CD120b) [18].

MOLECULAR MECHANISMS OF NEUTROPHIL APOPTOSIS (FIG. 1)

PMN apoptosis involves the activation of a family of cysteine proteases, called caspases, which cleave cellular substrates at an obligatory aspartic acid within a preferred sequence [19]. Caspase activation is a central event in apoptosis and results in the proteolytic degradation of multiple substrate proteins that contribute to the apoptotic

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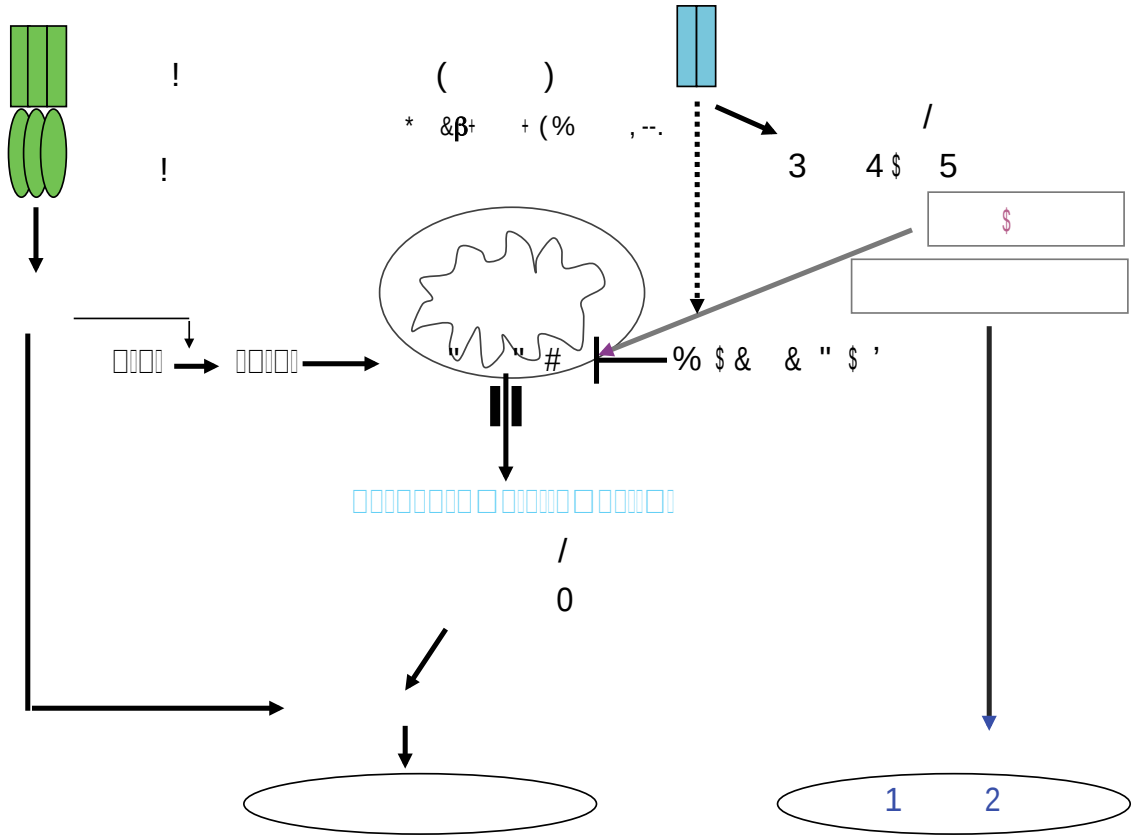

Fig. (1). Pathways leading to neutrophil death.

phenotype. Two major pathways that regulate apoptosis have been defined in a number of different cell types, including PMN. The first pathway depends on death receptors such as TNFRs and Fas (CD95) that can directly activate a caspase cascade *via* the activation of the caspase-8, an initiator caspase [20]. Bid, a pro-apoptotic members of the Bcl-2 family is cleaved [21]. Activated Bid is then translocated to mitochondria and can induce the release of cytochrome *c* and other proapoptotic factors.

The second pathway termed intrinsic apoptosis pathway involves mitochondria and the Bcl-2 family members, and results in activation of caspase cascade *via* the activation of caspase-9 as an initiator caspase [22]. The release of mitochondrial cytochrome *c* is under the control of the relative levels of pro- and anti-apoptotic members of the Bcl-2 family [22].

PMN have been reported to express a variety of regulatory and effector caspases, including caspases-1, -3 and -8 [23, 24]. PMN contain barely detectable levels of cytochrome *c*; however, the trace of amount of cytochrome *c* present in PMN is both necessary and sufficient for caspase activation [25].

It is now generally agreed that human PMN do not express the anti-apoptotic protein Bcl-2 but they express mRNA for the anti-apoptotic proteins, Mcl-1, A1 and Bcl-X_L [4, 26]. Mcl-1 and A1 proteins are expressed in bloodstream neutrophils and their levels decrease prior to the onset of apoptosis [4, 26]. Mcl-1 and A1 proteins have very short half lives (approximately 2-3 h) whereas the half lives of the pro-apoptotic proteins such as Bax, Bak and Bad, are relatively long. Hence, PMN apoptosis may be governed by the cellular levels of the relatively short-lived survival proteins, Mcl-1 and A1. In the absence of *de novo* synthesis of Mcl-1 and A1, the activity of the longer lived pro-apoptotic proteins prevails and tips the balance towards apoptosis. However, in the presence of survival signals (e.g. cytokines) the enhanced expression of the anti-apoptotic proteins, *via* pathways involving activated transcription, will ensure survival. It has been demonstrated that separate transcriptional signalling pathways leading to NF-κB and MAPKs activation regulate the expression of these proteins.

Calpains are a family of noncaspase cysteine proteases present in isolated PMN [27]. The ubiquitous calpain isoforms calpain-1 and calpain-2 are distinguished by their *in vitro* calcium requirements. During PMN spontaneous death, levels of calpastatin, a highly specific calpain inhibitor, decreases, leading to a drastic enhancement of the calpain-1 activity. Activated calpain-1 cleaves Bax into an active fragment [28]. Selective reduction of calpastatin by antisense oligodeoxynucleotides has been shown to accelerate spontaneous PMN apoptosis [29]. Further support for the importance of the calpastatin-calpain system was obtained by analysing PMN from patients with cystic fibrosis that exhibited delayed apoptosis. PMN display markedly increased calpastatin and decreased calpain-1 protein levels compared with PMN from healthy individuals [28]. More recently, it has been proposed that cathepsin D, a serine protease localized in the azurophilic granules, mediates caspase-8 activity. Pharmacological or genetic inhibition of

cathepsin D resulted in delayed caspase activation and reduced PMN apoptosis [30]. Furthermore, it has been reported that calpain mediates the cleavage of Atg5, an autophagy-related gene require for the formation of autophagosomes, swithching autophagy to apoptosis [31].

MODULATION OF NEUTROPHIL APOPTOSIS

The expression of constitutive PMN death programme can be delayed or accelerated by environmental factors.

Antiapoptotic Factors

PMN life-span increases significantly once they migrate out of the circulation and into the sites of inflammation, where they encounter various pro-inflammatory mediators. In particular, it has been reported that pro-inflammatory mediators including cytokines (IL-1β, IL-2, TNF-α, IL-15, IFN-γ, G-CSF, GM-CSF) can prolong PMN survival [2]. The reported effects of IL-6 on PMN apoptosis are controversial [32, 33]. IL-8, a chemokine, has also been shown to delay PMN apoptosis mediated by Fas and TNF-α receptors [34, 35].

Toll Like Receptors (TLR) agonists such as Malp2 (TLR2), LPS (TLR4), R-848 (TLR7/8), and CpG-DNA (TLR9) can also prolong PMN survival [4]. Treatment of PMN with such agents, that delay apoptosis, either increases or maintains Mcl-1 and A1 levels, providing a mechanism to explain cytokine-mediated increased survival *via* enhanced expression of anti-apoptotic proteins.

Similarly, a role of endothelial transmigration in the regulation of PMN apoptosis has been reported again indicating that adhesion molecules can generate intracellular signals that trigger enhanced survival of neutrophils as they migrate from the bloodstream into tissues [36, 37]. In this context, soluble ligand of $\alpha_M\beta 2$ integrin, including fibrinogen, delay PMN apoptosis through Akt activation [38].

Proapototic Stimuli

Fas signaling can mediate PMN apoptosis, and overrides the antiapoptotic effect of GM-CSF and TNF [39, 40]. An interaction between Fas and its counterpart, Fas ligand (FasL), was originally suggested to represent a mechanism to explain constitutive PMN apoptosis [41]. PMN express significant levels of Fas and whilst early reports indicated that they could also express FasL [41], this finding was not confirmed in later reports [42, 43]. PMN from CD95 deficient mice (lacking CD95) undergo apoptosis at the same rate as control mice, arguing against a role for the Fas system in constitutive apoptosis [44, 45]. Interestingly, it has been shown that membrane-bound form of Fas-L induce PMN infiltration *in vivo* which may be related to the release of IL-1β from inflammatory cells [46]. Hence, the role of the Fas/FasL system in regulation of PMN apoptosis in inflammation and disease is still controversial.

DYNAMICS OF NEUTROPHIL APOPTOSIS DURING VIRAL INFECTIONS

Several lines of evidence suggest a key role of PMN, at least through defensin expression, in controlling viruses such as adenovirus, influenza virus and herpes simplex virus [47-49]. In addition, human neutrophil α-defensins 1-4 have been reported to inhibit HIV-1 replication *in vitro* [50-52], and activated PMN have been demonstrated to exert cytotoxic activity against HIV-infected cells [53]. PMN also attract and stimulate other immune cells through the release of proinflammatory chemokines and cytokines [54] and through direct interactions with immune cells such as dendritic cells [55], implying that PMN have the potential to orchestrate adaptive immune responses and play a key role during viral infections. Therefore, modulation of PMN survival during viral infections may have important consequences in disease evolution.

Inhibition of PMN Apoptosis During Viral Infections (Table 1)

1. Respiratory Syncytial Virus (RSV) Inhibit PMN Apoptosis

RSV, a ssRNA virus, is the major cause of lower respiratory tract disease (LRTD) in infants. In addition to virus-induced pathology, the immunological response to viral infection is thought to be responsible for disease pathogenesis, ultimately leading to respiratory distress. One of the characteristic phenomena of RSV LRTD is the presence of large numbers of neutrophils in the lower airways [56]. This is more profound than in any other viral respiratory condition in childhood in which mostly alveolar macrophages and lymphocytes prevail. It has been suggested that PMN may even contribute to the pathology observed in the airway. In this context, IL-9 production by PMN, which regulates mucus production by goblet cells, was found to be associated with severe RSV infection. In addition, it has been reported that RSV leads to an inhibition of the constitutive apoptotic program. This process was found to be dependent on both PI3K activity and NF-κB activation. The antiapoptotic effect was abrogated by preincubation with the lysomotropic agent chloroquine, indicating the requirement for endolysosomal internalization. Furthermore, addition of ssRNA, a ligand for the intracellular TLR7/TLR8, also inhibited PMN apoptosis, suggesting that intracellular TLRs could be involved in induction of the antiapoptotic effect. IL-6, reported to inhibit neutrophil apoptosis, was present in supernatants from RSV-exposed PMN, suggesting that there is an autocrine or paracrine antiapoptotic role for IL-6. Finally, RSV treatment of PMN resulted in increased expression of the antiapoptotic Mcl-1 protein [57]. Whether or not an increased neutrophil life span is to the benefit of the host is unclear. It also may be a non specific immune evasion technique evolved by viruses that may even be harmful to the host when occuring in neutrophils by increasing pathology to the airway.

2. Human Cytomegalovirus (HCMV) Inhibit PMN Apoptosis

HCMV is one of the eight viruses that belong to the herpes virus family. As with other herpes viruses, a primary HCMV infection is followed by life-long persistence of the virus in a latent state, and reactivation may occur repeatedly throughout life. Generally, HCMV infection is subclinically in immunocompetent individuals, but the virus can cause fatal disease in immunocompromised patients, for example after transplantation in AIDS patients and in the fetus. Patients undergoing active HCMV infection also have an increased risk of developing severe secondary clinical

Table 1. Inhibition of PMN Apoptosis during Viral Infections

	Respiratory Syncitial Virus (RSV)	Human Cytomegalovirus (HCMV)
Virus-induced Pathology	- Lower respiratory tract disease in infants - Associated with respiratory distress	- Belong to the Herpes family: persistence of the virus in a latent state and repeated reactivation - Subclinically in immunocompetent individuals but fatal disease for immunocompromized patients
Characteristics	- Accumulation of PMN in lower airways - PMN production of IL-9 which increases mucus production by Goblet cells	- Presence of activated-PMN
Mechanisms	- Presence of the anti-apoptotic cytokine IL-6 - Interaction of ssRNA from RSV with TLR7/8 ? - Activation of PI3K and NFκB signaling pathways - Increased expression of the anti-apoptotic protein Mcl1	- Interaction of the viral protein UL36 with apoptotic pathways - Blockade of caspase 8 activation - Inhibition of Fas ligand-mediated apoptosis
Consequences	- Immune evasion	-Exacerbation of inflammatory responses e.g. in intestinal tissue

bacterial infections. This higher incidence of bacterial infections in HCMV patients may not be due directly to a dysfunction of PMN. Instead, HCMV-infected PMN become more activated, in terms of adhesion molecules expression and ROS production, than uninfected cells. Moreover, PMN apoptosis was significantly inhibited in HCMV-infected PMN [58]. This may be related to binding of the viral protein UL36 to caspase 8, blocking its activation and in this way inhibiting Fas-ligand-mediated apoptosis [59]. An additional anti-apoptotic effect may be mediated by the shedding of IL-8 from the endothelial cells at the time of infection as suggested [60].

The fact that apoptosis is inhibited by HCMV may cause overreactive PMN creating inflammation. In this context, active HCMV infection in inflammatory cells, including PMN, has been reported in intestinal tissue sections from approximately 90% of patients with active inflammatory bowel disease (IBD) [61]. This may result in further aggravation of disease such as IBD and possibly also in other chronic inflammatory diseases.

Exacerbation of PMN Apoptosis During Viral Infections (Table 2)

1. Exacerbation of PMN Apoptosis During HIV and SIV Infection

The use of non-human primate models, particularly SIV-infected Asian Rhesus macaques (RMs), has allowed the detailed and sequential investigation of the events of SIV infection in terms of virus dynamics, immune response, and changes in the pool of $CD4^+$ T cells [62]. Thus, SIVmac infection of RMs has proved an invaluable animal model for studies of AIDS pathogenesis, therapeutics, and vaccines. In particular, we and others have demonstrated that RMs of Chinese origin is a particularly relevant model to study human diseases [63-69].

Recently, we showed that PMN death increased early during the acute phase of SIV infection in RMs, and coincided with the peak of viral replication on day 14 post-inoculation. The level of PMN death was significantly more severe in RMs that progress rapidly to AIDS and coincided with neutropenia. In contrast, no changes in the levels of PMN death and PMN counts were observed in the non-pathogenic model of SIV infection of natural African Green Monkeys (AGMs) despite similar high viral replication. Consequently, the early increase in PMN death that we identified may account for the decline in PMN numbers that occurs during primary SIV infection and may, thus, have important implications for subsequent viral replication and disease progression. PMN death was a Bax and Bak-independent mitochondrial insult, which is prevented by inhibiting calpain activation but not caspase activation. Accelerated PMN death was not related to PMN infection, but we found that SIV particle binding to the cell surface is sufficient to prime PMN for death [70].

During the chronic phase, we also demonstrated that PMN from RM chronically infected with the virulent strain SIVmac251 display increased susceptibility to undergo apoptosis. PMN apoptosis was significantly increased in RMs progressing faster to AIDS as compared to non progressors RMs. Furthermore, the percentage of apoptotic cells correlated with PMN activation state reflected by increased CD11b expression and reactive oxygen species production. Interestingly, inflammatory cytokines IL-8 and IL-1β that prevent *in vitro* PMN death, were lower in RMs progressing towards AIDS. Thus, this decrease of inflammatory cytokines might lead to an abnormal tendency of PMN to die during the chronic phase. However, this is not reflected by an apparent decline of PMN counts. This result contrasts with the data observed during the acute phase demonstrating that PMN death is associated with neutropenia [71]. Increased PMN apoptosis has been also observed in HIV-infected patients having less than 200 $CD4^+$ cells/mm^3 [72-78]; the introduction of HAART reducing spontaneous PMN apoptosis. This increased PMN apoptosis found in both HIV infection and pathogenic SIV models may also be due to increased translocation of gut bacterial products into the bloodstream that results from HIV/SIV associated enteropathy [79].

Increased emigration from the bone marrow of mature PMN could be an explanation for the absence of apparent depletion compensating cell death in chronically infected macaques. The consequences of PMN activation generating

Table 2. Exacerbation of PMN Apoptosis During Viral Infections

	HIV/SIV	Influenza Virus A
Virus-induced Pathology	- Immune deficiency	- Bacterial superinfections which constitute the major cause of morbidity and mortality
Characteristics	- Neutropenia during primary SIV infection - No changes in the levels of PMN death and PMN counts in the non-pathogenic model of SIV infection (AGM)	- PMN infiltration of the airway - Increased recruitment into the lung related to chemokines
Mechanisms	- Increased expression of BOB/GPR5 - Priming of PMN for death by particle binding to cell surface - Calpain-dependent PMN death - Bax and Bak-independent mitochondrial insult - Lower expression of the anti-apoptotic cytokines IL-8 and IL-1B during the chronic phase.	- ROS-dependent PMN apoptosis - Activation of PMN oxidative burst by the virus ? - Increased expression of Fas antigen and Fas ligand
Consequences	- Correlation with disease severity	- Increased susceptibility to bacterial infections

general oxidative stress molecules might include an increased PMN susceptibility to apoptotic death during the chronic phase of infection [80]. In addition, these results support the idea that increased granulopoiesis in bone marrow leads to a compensatory release of mature PMN. Interestingly, PMN activation has been reported in the bone marrow of chronically SIV-infected macaques [81], contrasting with a defect in bone marrow lymphopoiesis [82, 83]. In fact, reciprocal dynamics of the bone marrow lymphocyte and neutrophil populations lead to cellular competition within a developmental niche. In particular, blocking bone marrow lymphopoiesis results in the specific and reciprocal expansion of the granulocytic compartment of bone marrow [84].

2. Influenza A Viruses Increased PMN Apoptosis

PMN have been reported to be involved in the initial host response to influenza A virus (IAV). Early after IAV infection, neutrophils infiltrate the airway probably due to release of chemokines that attract PMN [85]. Clearly, severe IAV infection is characterized by increased neutrophil influx into the lung or upper respiratory tract [86]. However, IAV also causes neutrophil dysfuction and accelerated neutrophil apoptosis [87, 88]. The latter effect is most pronounced when neutrophils are coincubated with IAV and bacteria, such as *Escherichia Coli, Streptococcus pneumoniae* [88-92]. These effects appear to contribute to the predisposition of IAV-infected individuals to suffer bacterial superinfections. In particular, IAV infection appears to increase susceptibility to bacterial pneumonia, otitis and meningitis [93]. The reduced PMN recruitment in lungs post influenza virus infection clearly impairs protection against secondary bacterial infections. Although TLR desensitization may be a contributing factor for reduced recruitment [94], PMN apoptosis occurring during influenza virus infection may be a more likely explanation as these bacterial infections occur quickly after or during influenza virus infection. Such bacterial superinfections constitute a major cause of morbidity and mortality during IAV epidemics [93]. The ability of IAV to accelerate neutrophil apoptosis on exposure to bacteria may be related, at least in part, to the virus-induced respiratory burst response [89, 90]. In addition, IAV increased neutrophil expression of Fas antigen and Fas ligand into the cell supernatant [88].

CONSEQUENCES OF PMN DEATH ON IMMUNE RESPONSE AND FUTURE PERSPECTIVES

One consequence of such abnormal PMN apoptosis could be to facilitate the dissemination of viruses *in vivo* by modulating immune responses. Apoptotic cells are sources of biologically active oxidized phospholipids which serve as recognition signals on apoptotic cells, facilitating phagocytosis by macrophages [95]. Engulfment of apoptotic PMN has been shown to inhibit the production of pro-inflammatory mediators by macrophages, by secretion of anti-inflammatory cytokines such as TGF-β [96, 97]. In this context, we recently demonstrated that TGF-β is increased in the tissues of SIV-infected RMs [67]. Such anti-inflammatory events can inhibit antigen presentation and promote microbial growth within macrophages [97], HIV replication [98] as well as the expansion of IL-17-producing cells [99, 100].

Altogether these data lead us to discuss the use of anti-apoptotic PMN drugs in HIV-infected patients. It has been previously reported that G-CSF and GM-CSF exert potent stimulatory effect on PMN functions in HIV-infected patients at the late stage of the disease [101, 102]. In addition, IL-15 significantly enhanced PMN functional activity and decreased the percentage of apoptotic PMN from untreated advanced HIV-infected patients [103]. Finally, Lichtner *et al*. have recently reported that HIV protease inhibitor (PI) therapy reverses *in vitro* PMN apoptosis of AIDS patients by direct calpain inhibition [104]. Further studies are necessary to evaluate the *in vivo* effect of such anti-apoptotic factors in non-human primates models. Indeed, it remains to provide the proof of concept that inhibiting PMN death early after infection is beneficial for patients preventing further disease evolution to Aids.

Overall, PMN apoptosis or survival during viral infections can have important consequences in promoting or impairing the ability of the host to clear infection but also contribute or reduce infection-associated pathology.

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