

Review

Apoptosis in Human Polymorphonuclear Leukocytes: Searching for a Genetic Roadmap

SCOTT D. KOBAYASHI and FRANK R. DELEO*

Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840, USA

Abstract. Polymorphonuclear leukocytes (PMNs or neutrophils) are essential components of the innate immune system in humans and function primarily to eliminate invading microorganisms. Neutrophil influx to sites of infection is desirable because it also initiates an inflammatory response. Paradoxically, PMNs are also intimately associated with inflammatory disease. As part of normal neutrophil turnover in humans and to limit inflammatory potential, PMNs undergo programmed cell death, or apoptosis. Several host factors, including cytokines and growth factors, are capable of extending neutrophil survival and, thus, capacity to fight infection. On the other hand, phagocytosis of bacterial pathogens generally accelerates PMN apoptosis. Due in part to the extensive complexity of programmed cell death, relatively little is known about the signaling pathways that govern these processes in PMNs. Recently, microarray strategies have been employed to gain an understanding of these processes in activated PMNs, and new evidence indicates that gene transcription is important in the regulation of neutrophil apoptosis and, thus, inflammation. A series of provocative discoveries led to the hypothesis that neutrophil programmed cell death is the result of an apoptosis-differentiation program, a final stage of transcriptionally regulated PMN maturation or hematopoietic differentiation. Further characterization of the apoptosis-differentiation program and associated biochemical pathways in mature PMNs will likely yield important insights into the resolution of inflammation and infection.

Key words: neutrophil; apoptosis; gene transcription; inflammation.

Introduction

Polymorphonuclear leukocytes (PMNs) are essential components of the host immune response and play a central role in the defense against invading microorganisms such as bacteria and fungi. This innate defense mechanism is dependent on the ability of PMNs to ingest and subsequently eliminate pathogens by oxidative

and non-oxidative processes⁴⁰. Importantly, PMNs have been implicated in the pathogenesis of tissue injury and trauma associated with inflammatory diseases. Thus, timely and effective removal of PMNs from affected sites is paramount to the resolution of inflammation.

PMNs have an intravascular half-life of 4–10 h and survive on the order of a few hours to several days in tissue⁴⁰. Approximately 10^{11} mature PMNs are pro-

Abbreviations used: PMN – polymorphonuclear leukocyte, FcR – Fc receptor, CR – complement receptor, ROI – reactive oxygen intermediates, HGE – human granulocytic ehrlichiosis.

* Correspondence to: Frank R. DeLeo, Ph.D., Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 South 4th Street, Hamilton, MT 59840, USA, tel.: +1 406 363 9448, fax: +1 406 363 9394, e-mail: fdeleo@niaid.nih.gov

duced daily in humans, which accounts for more than 60% of the leukocyte population produced in the bone marrow⁴⁰. Accordingly, this high production rate is coupled to rapid turnover in these short-lived terminal cells. The number of neutrophils at sites of infection and inflammation can be very high and there is a concomitant potential to cause severe tissue injury should they undergo necrotic lysis. Uncontrolled release of cytotoxic granule contents and reactive oxygen intermediates (ROI) resulting from cell lysis often leads to a persistent and acute inflammatory response²². Therefore, on the most basic level, the vigilant execution of a controlled cell death program in human PMNs is deemed necessary for the resolution of inflammation.

The phenomenon of apoptosis in immune cells is hardly a novel concept and its importance in homeostasis of the immune system has been widely studied over the past decade (reviewed in refs. ^{8, 10}). Morphologically and biochemically, apoptosis in mature PMNs is not unlike that in other cell types, although the implications of an alternative neutrophil necrotic death are potentially more hazardous to the host. It is commonly accepted that normal turnover of aging PMNs in tissues is accomplished by a constitutive or "spontaneous" apoptotic process which enables them to be recognized and cleared by macrophages, thereby limiting inflammatory potential⁴⁶. Numerous reports have evaluated factors that either enhance or inhibit PMN apoptosis *in vitro* and evidence has been provided for the occurrence of apoptosis *in vivo*^{21, 47}. However, the molecular signaling mechanisms responsible for the initiation and execution of programmed cell death in PMNs are incompletely characterized. Moreover, it is likely that the mechanism(s) for executing PMN apoptosis in an inflammatory milieu or at the site of infection differ from the "spontaneous" process responsible for normal PMN turnover. This review will focus primarily on activation or phagocytosis-induced apoptosis in human PMNs.

Factors that Influence PMN Apoptosis

The primary function of PMNs is to ingest and kill microorganisms. The binding and ingestion of pathogens by neutrophils elicits a robust production of ROI, one of the chief mechanisms employed by PMNs to kill ingested microorganisms⁴⁰. Recent studies suggest that ROI generated by the NADPH oxidase complex accelerate apoptosis¹¹. Blocking production of PMN ROI during phagocytosis with diphenylene iodonium, a compound which abolishes activity of flavoproteins without altering phagocytosis, inhibits induction of

apoptosis¹¹. Consistent with that finding, PMNs isolated from patients with chronic granulomatous disease, a hereditary disease resulting in the absence of PMN ROI production, have reduced apoptosis compared with cells from healthy individuals¹¹. Although these studies provide evidence for the involvement of ROI in programmed cell death, the exact role of ROI in the induction of apoptosis is still unclear. For example, phagocytosis of pathogens by PMNs is mediated, in part, by antibody (Fc receptor – FcR) and complement receptors (CR), and FcR-(but not CR-)mediated phagocytosis elicits robust production of ROI³¹. However, both FcR- and CR-mediated phagocytosis accelerate PMN apoptosis³¹. Tumor necrosis factor α (TNF- α) induces neutrophil apoptosis, but elicits relatively little ROI production^{13, 34}. It is likely that ligation of TNF- α receptors directly induces PMN apoptosis, abrogating the need for induction by ROI. Thus, it appears that production of reactive oxygen species (ROS) is sufficient, but not necessary to induce apoptosis in human PMNs.

Although the underlying mechanisms are incompletely characterized, neutrophils undergo apoptosis regardless of whether they are activated. Interestingly, PMN fate can be influenced by many external factors capable of either prolonging survival or inducing apoptosis. For example, inflammatory mediators such as interleukin (IL)-1⁹, IL-2⁴³, IL-4¹⁹, IL-15¹⁸, granulocyte colony-stimulating factor (G-CSF)⁹, granulocyte-macrophage colony-stimulating factor (GM-CSF)⁵, and interferon (IFN)- γ ³⁰ have been reported to delay PMN apoptosis, significantly extending the lifespan of the cell. The exact mechanism by which these cytokines prolong neutrophil survival is unknown; however, there are important *in vivo* implications for this phenomenon. It is possible that cytokine-mediated cell survival is a mechanism in the host to initiate the acute phase of inflammation, promoting a large influx and persistence of PMNs at the site of inflammation to resolve infection. Although the *in vivo* effects of cytokines on PMN persistence are unclear, the sequential release and compartmentalization of inflammatory mediators play significant roles in neutrophil extravasation and migration⁴⁰. There remains a fine line between resolution and persistence of infection²², and the question arises as to what actually induces programmed cell death in PMNs.

Dissecting the molecular mechanisms of apoptosis has become increasingly important over the past decade. TNF- α is a potent cytokine with a diversity of functions in host response to infection and is one of the earliest factors produced by activated cells⁵⁰. TNF- α modulates the systemic response of the host, including such processes as up-regulating adhesion molecules in

PMNs, recruiting peripheral blood mononuclear cells to sites of inflammation, and eliciting production of other cytokines⁵⁰. Importantly, TNF- α and the TNF- α receptor family are critical for modulating apoptosis in neutrophils and other cell types^{23, 33}. Treatment of PMNs with TNF- α both prolongs survival and induces apoptosis, depending on *in vitro* assay conditions^{2, 39, 44}. This phenomenon is likely a reflection of the pleiotropic nature of TNF- α and its overall importance in inflammation.

Neutrophils also express members of the Fas (CD95, APO-1)/Fas-ligand apoptosis-inducing pathway and several lines of evidence indicate that induction of this pathway in human PMNs leads to programmed cell death³³. Importantly, serum levels of Fas-ligand are 5-fold increased in patients with bacterial infections and are accompanied by Fas-ligand-dependent PMN apoptosis *in vitro*⁴¹. PMNs express a multitude of other apoptosis-inducing receptor systems, such as the Toll-like and transforming growth factor β (TGF- β) receptors, that may regulate PMN cell fate. These pathways have been recently reviewed elsewhere^{1, 48}.

Bacteria and Induction of Apoptosis in PMNs

As mentioned above, the majority of host growth factors and cytokines influence the cell fate of mature PMNs by prolonging survival, which presumably benefits the host in the resolution of infection. Similarly, induction of PMN apoptosis likely promotes resolution of the inflammatory response. Thus, a delicate balance between signals for cell survival and death is crucial for the well-being of the host. It is then conceivable, if not probable that disruption of this balance would be unhealthy to the host. Several bacterial pathogens have evolved strategies that take advantage of the host immune system to promote their own survival. For example, the Gram-negative enteric pathogens *Salmonella* sp., *Shigella* sp., and *Yersinia* sp. have devised strategies to induce programmed cell death in macrophages³⁶. The diversity of mechanisms used by bacteria to induce host cell apoptosis varies greatly depending on the specific microorganism⁵⁵.

If the induction of apoptosis in macrophages indeed represents a bacterial virulence strategy, then one would expect a similar occurrence in neutrophils. PMNs are the first cells recruited to sites of infection and inflammation and are the most numerous of all leukocytes⁴⁰. Induction of PMN apoptosis by bacteria would then represent a strategy to undermine the initial onset of the acute inflammatory response and would

perhaps enable bacteria to establish infection. Enteric *Escherichia coli* was one of the first microbes identified as altering neutrophil survival and it induced PMN apoptosis in a dose-dependent manner⁵⁴. Since that time, several other studies have shown that many species of bacteria induce apoptosis in neutrophils. *Mycobacterium tuberculosis*²⁹, *Neisseria gonorrhoeae*⁷, and *Streptococcus pneumoniae*⁶¹ all accelerate PMN apoptosis. There are conflicting reports on the ability of *Staphylococcus aureus* to alter PMN fate^{3, 35}. These ambiguous findings could be attributed to several factors, including strain heterogeneity. The causative agent of human granulocytic ehrlichiosis (HGE) is one of the few agents identified thus far that prolongs neutrophil survival once ingested⁵⁸. HGE is an obligate intracellular pathogen that survives in normal human PMNs, and failure of HGE to promote neutrophil survival would result in the inability of these organisms to replicate.

Bacterial species are also capable of secreting products that alter neutrophil survival. For example, staphylococcal enterotoxins A and B, and toxic shock syndrome toxin 1 delay PMN apoptosis³⁸. On the other hand, *Burkholderia cepacia* produces a hemolysin that induces rapid apoptosis in neutrophils²⁶. *Shigella flexneri*¹⁴ and *Pseudomonas aeruginosa*^{12, 52} produce type III secretion system-dependent toxins that induce rapid PMN necrosis. Because this strategy is of no obvious benefit to the host, it has been postulated that the gross inflammation observed from massive necrotic lysis facilitates the ability of the pathogen to transcend physical barriers.

Taken together, the majority of studies indicate that bacteria accelerate neutrophil apoptosis. These findings are not a complete surprise, as the process of PMN phagocytosis *per se* accelerates apoptosis³¹. Although progress has been made toward understanding these processes, the mechanisms responsible for activation-induced PMN apoptosis remain to be elucidated.

Transcription and Translation in Mature PMNs

Historically, gene expression studies in human neutrophils have focused primarily on events in maturation and differentiation^{4, 15} or on cytokine production⁵¹. The fact that neutrophils are terminal cells has often led to the misconception that mature PMNs do not synthesize proteins or require new protein synthesis for function. This belief may stem from the ability of PMNs to initiate the processes of phagocytosis, degranulation and NADPH-dependent killing without new synthesis of proteins²⁴. However, mature PMNs do synthesize both

mRNA and proteins^{20, 28}, albeit at lower levels than bone marrow precursors. In fact, treatment of human PMNs with the transcription inhibitor actinomycin D for greater than 1 h decreases chemotaxis, phagocytosis, ROS production, and killing⁶. These effects of actinomycin D are likely attributed to the inability of PMNs to replenish mRNA and protein lost to normal turnover; however, blocking transcription or translation does not inhibit PMN phagocytosis or ROI production *per se*. Thus, it is clear from these and other early studies²⁰ that mature PMNs synthesize new mRNA and protein.

Most physiological processes are ultimately regulated at the level of gene transcription. The observation that mature PMNs are capable of synthesizing mRNA and new protein invokes the question of whether or not transcription is involved in apoptosis. Treatment of neutrophils with actinomycin D or cycloheximide accelerates PMN apoptosis⁵⁷. The balance between survival and apoptotic cell death is often manifested as an interplay between pro- and anti-apoptotic factors. Therefore, one can imagine that accelerated apoptosis in neutrophils might be the result of a paucity of anti-apoptotic effectors in the absence of new transcription or translation. Neutrophils express abundant levels of the apoptosis-inducing factors Bax and Bad, and apoptosis-inhibiting factors such as A1, Mcl-1 and Bcl-XL^{37, 42}. However, the interplay between these molecules during PMN apoptosis is undefined.

Toward a Genome-Scale Analysis of PMN Function

Understanding apoptosis at the molecular genetic level is a seemingly impossible task due to the exquisite complexity of programmed death in mammalian systems. Conventional attempts at dissecting apoptotic gene expression in PMNs using reverse transcriptase-polymerase chain reaction (RT-PCR) examined only a limited number of different genes (up to 20)^{25, 45}. Fortunately, recent technological advances have made genome-scale expression analysis of human cells possible. The first demonstration of global gene expression in human neutrophils was obtained from sequence analysis of 3'-directed cDNA libraries²⁷. Although capable of detecting relative transcript levels of highly expressed RNA species, this method is limited by both the ability to detect rare mRNA moieties and the understandably modest sample throughput capability. Nevertheless, this study gave an initial appreciation for the potentially large number of genes that are

expressed by neutrophils isolated from venous blood (748 different mRNA species)²⁷.

More recently, SUBRAHMANYAM et al.⁴⁹ used a modified differential display analysis to detect changes in neutrophil gene expression when exposed to the bacterial pathogen *Yersinia pestis* and the commensal *E. coli* strain, K12. This study revealed that 350 known genes and 292 EST sequences were differentially expressed during exposure to bacteria⁴⁹. They identified several differentially regulated apoptosis genes, including *GADD34*, *GADD45B*, *BCL2A-1*, *MCL1*, *TNFAIP3*, *PPIF*, *IER3*, and *CFLAR*⁴⁹. This finding indicates that apoptosis-related genes are regulated by PMNs within 2 h after incubation with bacteria and likely facilitate accelerated apoptosis. Although neutrophil apoptosis was not evaluated in that study, the data suggest that genome-scale analysis of gene expression is a tool useful for obtaining information pertaining to PMN programmed cell death.

Perhaps the most desirable attribute of genome level transcription analysis is the ability to detect changes that occur in mRNA species in regard to both time and treatment schemes. In addition, statistical confirmation can be performed on replicate samples. To this end, oligonucleotide microarrays have proven to be of significant value based on their reproducibility and accuracy⁶⁰, low background noise, relatively high throughput capabilities, and rapid identification of genes without requiring sequence analysis. Recently, we used oligonucleotide microarrays to determine the downstream effects of receptor-mediated phagocytosis in human neutrophils³¹. Two hundred seventy-nine differentially expressed genes were induced or repressed 90 min after receptor-mediated phagocytosis in human PMNs, including 38 genes encoding proteins that are involved in at least three distinct apoptosis signaling pathways (Toll-like receptor, TNF- α receptor, and glycosphingolipid) including: *RAIDD*, *GADD34*, *GADD45B*, *TRAF2*, *DEDD*, *BCL-XL*, *PKR*, *CASP2*, *CASP3*, *FAN*, *TEGT*, *CAS*, and *PSEN1*³¹. In addition, genes encoding four nuclear orphan receptors, TR3, NOR1, NURR1, and NR1D2, were up-regulated after FcR- and CR-mediated phagocytosis³¹. These findings are significant in that the orphan receptors TR3 and NOR1 participate in T cell apoptosis⁵⁹, but have never been described in neutrophils. Furthermore, several genes encoding downstream effectors of apoptosis were expressed but not differentially regulated, such as caspases-4 and -10, DAXX, FADD, PUMA, and Apaf-3³¹.

Based on the preponderance of apoptosis-linked genes, it was hypothesized that induction of cell fate-related genes would be accentuated at later time points

coinciding with apoptosis³¹. Of the 869 unique genes that are differentially transcribed between 3 and 6 h after phagocytosis in human PMNs, 94 are directly related to cell fate³¹. Genes encoding key mediators of apoptosis, such as TNF- α , TNF- α receptor, TGF- β -inducible early growth response protein (TIEG), caspase-1, modulator of apoptosis (MAP-1), Toll-like receptor 2 (TLR2), interleukin receptor-associated kinase-1 (IRAK-1), MAP3K4 (MTK1), tumor-suppressing sub-transferable candidate 3 (TSSC3), and BAX, are up-regulated, whereas inhibitors of apoptosis, such as TGF- β -induced anti-apoptotic factor 1(TIAF1), tumor up-regulated CARD-containing antagonist of caspase-9 (TUCAN), API5L1, Bcl-2-related protein A1 (Bcl2A-1/BFL-1), and AKT1, are down-regulated³¹. In those studies, apoptosis in PMNs was confirmed by FACS-based programmed cell death assays. Changes in gene expression correlate precisely with PMN apoptosis³¹ and, importantly, these findings suggest that gene tran-

scription regulates programmed cell death in activated PMNs.

Cell fate-related genes account for 18.3% of differentially expressed PMN genes with known function up to 6 h after phagocytosis³¹. The question then arises as to the function of the remaining ~82% of differentially transcribed genes in activated neutrophils, which is especially intriguing considering that the cells are embarking on the final stage of differentiation, namely programmed cell death. Mature neutrophils demonstrate impaired function in direct association with apoptosis⁵⁶, including impaired chemotaxis, phagocytosis, degranulation, and respiratory burst within 24 h of *in vitro* culture⁵⁶. Presumably, these important processes are intrinsically regulated by an overall decrease in gene transcription and protein degradation in apoptotic PMNs. However, there is provocative evidence that neutrophils actively up-regulate numerous cellular processes during the initiation of apoptosis³². For example,

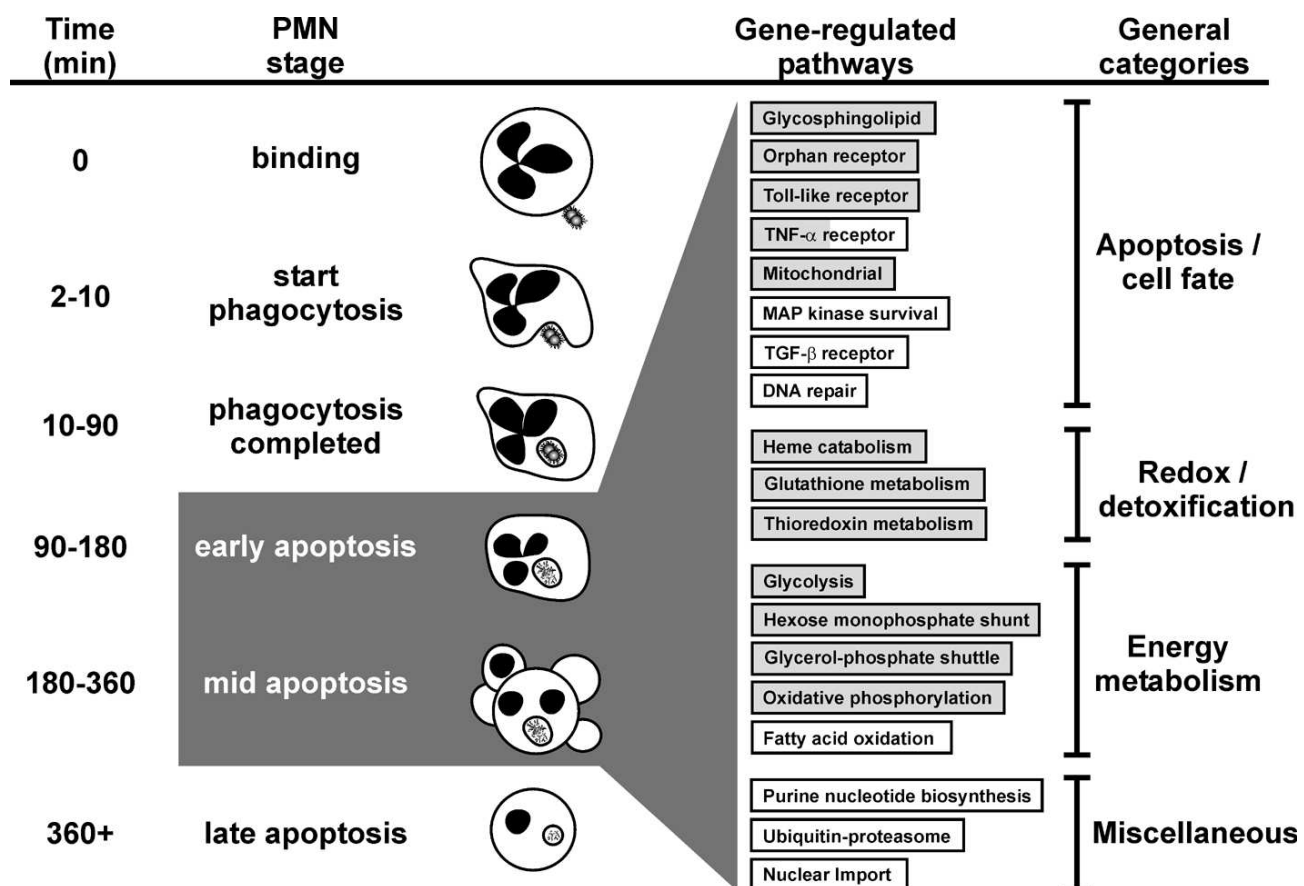


Fig. 1. Apoptosis-differentiation program in human polymorphonuclear leukocytes. Phagocytosis-induced PMN apoptosis is accompanied by differential expression of hundreds of genes that can be assigned to general categories (far right). Gene-regulated pathways are further identified as the basis of the known function for each gene (boxes). Gray shaded boxes indicate up-regulated pathways, white boxes indicate down-regulated pathways, and the TNF- α pathway contained both up- and down-regulated genes. The corresponding time after phagocytosis and stage of apoptosis is indicated at left and the dark gray shading indicates the time period in which the gene-regulated pathways were identified

several key genes participating in complex cellular pathways involving glutathione and thioredoxin detoxification systems, heme catabolism, ubiquitin-proteasome degradation, purine nucleotide metabolism, and nuclear import are regulated at the level of gene expression during the onset of PMN apoptosis³². The findings on the regulation of detoxification systems are especially important in the context of apoptosis in activated PMNs, which produce ROI that might influence the mechanism for induction of programmed cell death. Genes encoding key regulators of glycolysis, the hexose monophosphate shunt, the glycerol-phosphate shuttle, and oxidative phosphorylation are also induced in apoptotic PMNs³². These data are supported by the findings that glycolysis is increased during PMN apoptosis and that activation-induced apoptosis is partially inhibited in the absence of glucose³². ATP generated through glycolysis provides the energy for apoptosis in other cell types as well¹⁷.

As mentioned previously, apoptosis in PMNs represents a mechanism for promoting resolution of inflammation. The ability of neutrophils to regulate both cell fate and important, related biochemical pathways such as detoxification systems and energy metabolism can be regarded as the final stage of differentiation in these terminal cells. Therefore, an apoptosis-differentiation program represents a well-orchestrated series of transcriptionally regulated events that promote healthy removal of PMNs from inflammatory sites (Fig. 1). Although the specific mechanisms of apoptosis in neutrophils remain to be elucidated, it is becoming clear that transcription plays a direct role in determining cell fate in PMNs, and future genomic and proteomic-based studies will be of significant value in dissecting the apoptotic circuitry and ultimately inflammation.

Conclusions

Inflammation is a process that requires a delicate balance between pro- and anti-inflammatory mediators. Paradoxically, PMN-mediated inflammation is both beneficial to the host in aiding the resolution of infection and harmful in cases where excessive accumulation of neutrophils facilitates pathogenesis of inflammatory diseases. Apoptosis in PMNs has emerged as a key mechanism for the resolution of inflammation. PMN apoptosis is a constitutive process responsible for normal neutrophil turnover in humans; however, the process can be influenced by both host and pathogen factors. To prolong life or accelerate death in PMNs implies either modifications of pre-existing factors or

the need for newly synthesized proteins or both. In support of the latter hypothesis, apoptosis in neutrophils is accompanied by increases in differential expression of cell fate-related genes. Recent evidence suggests that the majority of gene transcription in mature PMNs is directed toward regulation of cell-fate and resolution of inflammation. Genomic-based approaches have enabled researchers to begin to elucidate and subsequently confirm individual biochemical pathways and demonstrate their importance in neutrophil biology and the human innate immune system. A thorough understanding of the molecular pathways that determine cell fate in PMNs will provide insight into the pathogenesis of inflammatory diseases and infection processes, resulting in potential therapeutic strategies.

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