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# Role of granule proteases in the life and death of neutrophils

Charaf Benarafa [a, **], Hans-Uwe Simon [b, *]

[a] Theodor Kocher Institute, University of Bern, Bern, Switzerland
[b] Institute of Pharmacology, University of Bern, Bern, Switzerland

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ABSTRACT

Neutrophils constitute a crucial component of the innate immune defenses against microbes. Produced in the bone marrow and patrolling in blood vessels, neutrophils are recruited to injured tissues and are immediately active to contain pathogen invasion. Neutrophils undergo programmed cell death by multiple, context-specific pathways, which have consequences on immunopathology and disease outcome. Studies in the last decade indicate additional functions for neutrophils — or a subset of neutrophils — in modulating adaptive responses and tumor progression. Neutrophil granules contain abundant amounts of various proteases, which are directly implicated in protective and pathogenic functions of neutrophils. It now emerges that neutral serine proteases such as cathepsin G and proteinase-3 also contribute to the neutrophil life cycle, but do so via different pathways than that of the aspartate protease cathepsin D and that of mutants of the serine protease elastase. The aim of this review is to appraise the present knowledge of the function of neutrophil granule proteases and their inhibitors in neutrophil cell death, and to integrate these findings in the current understandings of neutrophil life cycle and programmed cell death pathways.



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* Corresponding author. Institute of Pharmacology, University of Bern, Inselspital, INO-F, CH-3010 Bern, Switzerland.
** Corresponding author. Theodor Kocher Institute, University of Bern, Freiestrasse 1, CH-3012 Bern, Switzerland.
*E-mail addresses:* charaf.benarafa@tki.unibe.ch (C. Benarafa), hus@pki.unibe.ch (H.-U. Simon).

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## 1. Specificities of neutrophil life and death

### 1.1. Granulopoiesis and neutrophil lifespan

The role of neutrophils in antimicrobial surveillance is undisputed as quantitative and qualitative defects in neutrophils lead to severe immunodeficiency [1]. Additional functions for neutrophils in immunomodulation have been described in certain disease contexts indicating that a small subset of neutrophils contribute to adaptive immune responses (see reviews [2–4]). These functions suggest that neutrophils are not homogenous. Seminal studies in the 1950s and 1960s in humans and various laboratory animals have shown that neutrophils labeled ex vivo and re-injected, or directly labeled in vivo have a short circulatory lifespan of less than a day [5–8]. These findings have remained unchallenged until an average lifespan of more than 5 days for human neutrophils was reported using a novel, non-toxic tracer [9]. The study received a lot of attention and, in the meantime, over 150 citations. The appeal of these findings is understandable: the experimental work is sound and the conclusions are provocative as they suggest that homeostatic neutrophil lifespan is sufficiently long to allow neutrophils to execute novel functions such as interaction with antigen-presenting cells and lymphocytes in the context of adaptive immune responses and cancer.

So was our textbook knowledge wrong regarding the neutrophil lifespan? Neutrophils derive from hematopoietic stem cells (HSCs) and develop in the bone marrow through a common myeloid progenitor shared with the monocyte/macrophage lineage. The process of granulopoiesis takes committed neutrophil progenitors from the stem cell pool and brings them through differentiation into mature neutrophils that then enter the blood circulation. These precursors first go through a proliferative phase called the mitotic pool followed by a non-proliferative phase named the post-mitotic pool, where cells further differentiate without dividing. Based on cellular size, granule content and nuclear morphology, several neutrophil differentiation stages were identified. The mitotic pool includes myeloblasts, promyelocytes and myelocytes, while metamyelocytes, band and mature neutrophils constitute the post-mitotic pool. To determine neutrophil lifespan in vivo, one can label neutrophils ex vivo and reinject them to measure the kinetics of cell disappearance from the blood circulation. This approach is straightforward but has important caveats including neutrophil activation during ex vivo manipulation and toxicity of the labeling method or compound. In vivo labeling has thus been performed with a pulse of tritiated ($^{3}$H)-thymidine to label the DNA of cells in the mitotic pool or by using $^{3}$H- or $^{32}$P-labeled diisopropyl fluorophosphate (DFP), which irreversibly reacts with the catalytic site of neutrophil serine proteases. These radioactive compounds are not available anymore for clinical research because of their inherent toxicity. Pillay et al. performed their study by labeling proliferating bone marrow cells in vivo by giving human subjects and mice deuterium labeled “heavy” water ($^{2}H_2O$), a safe and nonradioactive tracer that can later be detected in adenosine in the DNA of circulating neutrophils by gas chromatography coupled with mass spectrometry [9]. They first examined both bone marrow and blood neutrophil kinetics of mice and found that mouse neutrophils have a short lifespan of about 18 h as shown previously. They then calculated that circulating neutrophil lifespan is at least 5 days in human subjects following a 9-week labeling period with heavy water, a value perhaps 10-fold greater than that gleaned from long standing studies using toxic tracers and/or ex vivo labeling.

Calculating neutrophil lifespan in humans is not as easy as in mice because sampling the bone marrow compartment is highly invasive and is not available to investigators for repeated sampling. Moreover, modeling human neutrophil lifespan has to be calculated based on several assumptions regarding proliferation rate of the mitotic pool, transit time from the last proliferation to entry in the blood circulation, apoptosis within the bone marrow and neutrophil numbers determined from ratios of labeled vs non-labeled adenosine. Indeed, using the same data but assuming that the mitotic pool is the rate-limiting factor, Dale and colleagues suggested that the data was actually compatible with a neutrophil lifespan of less than a day [10]. A new study now appears to settle the issue using $^{2}$H-labeled glucose in parallel with a limited set using double-labeling with heavy water [11]. $^{2}$H-glucose is more efficiently and rapidly metabolized in adenosine to be incorporated into the DNA of the mitotic pool, allowing labeling over a few hours or in a single injected bolus instead of weeks for heavy water and therefore more precise estimates of neutrophil half-life. Using a new model, they found that different assumptions of the ratio (R) of circulating cells to the mitotic pool is the key factor that can predict neutrophil lifespan of 1 or 5 days. The numbers suggest that the mitotic pool is five times larger than the circulating pool with $R = 0.2$. The model of Koenderman and colleagues [9] leading to a long neutrophil lifespan implies a large value of R that is not compatible with historical measurements of the various neutrophil compartments. So it seems that the old studies had it right regarding the short lifespan of neutrophils, at least until a new method, tracer or model will again naturally challenge our brains and dogmas.

### 1.2. Cell death in neutrophils

Programmed cell death (PCD) can be executed by various cell autonomous mechanisms that are highly intertwined and triggered by both internal and external signals (Fig. 1) [12]. Apoptosis is the best molecularly characterized mode of cell death and it culminates in the activation of a subset of cysteine proteases with aspartate specificity, known as caspases, which irreversibly execute cell death through cleavage of multiple targets in the nucleus, cytoplasm and cytoskeleton. Apoptosis has been recognized as the main PCD pathway that is triggered upon isolation of blood neutrophils and is often referred to as “spontaneous” or “constitutive” apoptosis. PCD was until recently synonymous to apoptosis, while necrosis did not require any active mechanistic input from the dying cell and resulted from external mechanical, physical, chemical and osmotic stresses causing the rupture of the plasma membrane and release of the cytosolic contents. It is now accepted that necrosis can also result from distinct molecular pathways that have been defined as necroptosis and pyroptosis, which, unlike apoptosis, lead to cell membrane lysis [13]. Necroptosis generally proceeds when caspases (particularly caspase-8) are inhibited or inactive and is triggered by specific protein kinases, principally receptor-interacting protein kinase−3 (RIPK3). Ultimately, necroptosis was shown to be dependent on the pseudokinase mixed lineage kinase domain−like protein (MLKL), which is thought to have pore-forming capacity [14]. Necroptosis has recently been shown to occur in neutrophils following phagocytosis of *Staphylococcus aureus* [15] and ligation of adhesion receptors [16]. Pyroptosis depends on the cleavage of the cytoplasmic gasdermin D by caspase-1, -4, -5 and -11 following inflammasome activation [17–19]. However, pyroptosis appears so far not to be evoked in neutrophils [20]. Secondary necrosis is readily observed in neutrophils in vitro when isolated neutrophils are left to undergo spontaneous apoptosis and, after some time, their plasma membrane becomes permeable and they release their cytoplasmic content. Secondary necrosis also occurs in vivo when phagocytic removal of apoptotic cells is impaired or overwhelmed [21].

PCD pathways associated with neutrophil granule functions are incompletely defined. Neutrophils are exquisitely sensitive to cell