The Neutrophil Life Cycle

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Abstract

Neutrophils are recognized as an essential part of the innate immune response, but an active debate still exists regarding the life-cycle of these cells. Neutrophils first differentiate in the bone-marrow through progenitor intermediaries before entering the blood, in a process that gauges the extramedullary pool size. Once believed to be directly eliminated in the marrow, liver and spleen after circulating for less than one day, neutrophils are now known to redistribute into multiple tissues with poorly understood kinetics. This review provides an update on the dynamic distribution of neutrophils across tissues in health and disease, and emphasizes differences between humans and model organisms. We further highlight issues to be addressed to exploit the neutrophil's unique features in the clinic.

The Neutrophil as a Double-Edged Sword in Health and Disease.

Neutrophils (also named polymorphonuclear leukocytes) are phagocytes that play an essential role in defending the host against invading pathogens, particularly bacteria and fungi [1,2]. The killing of these organisms in phagosomes is mediated by i) fusion with lysosomes (granules) liberating cytotoxic proteins, peptides and enzymes into the phagolysosome [3], and ii) activation of a membrane-bound NADPH-oxidase producing superoxide anions (O_2^-) that in turn are metabolized into hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) [4]. The cells employ these mechanisms both inside the phagolysosome as well as outside the cell. In the latter process the fusion of granules (degranulation) and activation of NADPH-oxidase localize at the plasma membrane [5,6]. The importance of these processes is illustrated by the severe immune deficiencies that in man are associated with impaired killing mechanisms in neutrophils, such as those found in **chronic granulomatous disease** (genetic defects in the multicomponent NADPH-oxidase) [7] and **Chédiak-Higashi syndrome** (granule deficiency) [8] .

Apart from their essential roles in immune homeostasis, neutrophils are involved in the pathogenesis of many inflammatory diseases ranging from acute lung injury following major trauma or sepsis, to chronic inflammation in diseases such as chronic obstructive pulmonary disease COPD and the many forms of inflammatory arthritis [9,10]. All these clinical conditions are associated with the dysregulated migration, activation and survival of neutrophils [11].

Despite the importance of neutrophils in innate immune responses and their well-recognized and deleterious role in inflammatory diseases, surprisingly little is known regarding their life span(s) both in time and place, and in health and disease [12,13]. Likewise, it is unclear whether heterogeneous neutrophil populations exist, with distinct temporal and anatomical properties [10]. However, It is clear, that on the one hand, targeting the full neutrophil compartment in inflammatory diseases will cause more problems than solutions. On the other hand, if neutrophils could be targeted via disease-specific mechanisms, while leaving their major immune and homeostatic functions intact, such interventions would hold significant promise in the treatment of a subset of inflammatory diseases, such as COPD, many of which represent a global epidemic in the aging population. To achieve this, it is mandatory to understand the basic rules guiding the life cycle of neutrophils in health and disease (Box 1). This review focuses on the recent new data in this area.

Neutrophil Formation and Release from the Bone Marrow

The Mammalian Mitotic Neutrophil Pool

The neutrophil originates from myeloid lineage progenitor cells (common myeloid progenitors) located within the bone marrow and extramedullary tissues including the spleen. During the initial differentiation steps the myeloid progenitors (myeloblasts) retain their propensity to differentiate into both the monocyte/macrophage lineage and the neutrophil lineage as well as the other myeloid cells, namely eosinophils and basophils. This common differentiation ends with the last progenitor that can differentiate into both lineages, the granulocyte macrophage progenitor or GMP [14–19]. Hereafter, the differentiation of neutrophils and monocytes (and other myeloid cells) bifurcates from a metastable bipotent progenitor (in mice) [20] and the first progenitor that is 'neutrophil-committed' is the neutrophil pro-myelocyte [21]. This cell-type in humans can be recognized by having a round nucleus and a relatively dark cytoplasm (**Figure 1**) and is able to divide; this is the first neutrophil maturation stage of the so-called mitotic neutrophil pool. The number of divisions occurring in these pro-myelocytes in humans is unclear as these cells can either proliferate or differentiate into the next stage, the myelocyte [18,21]. This cell can be recognized by having a round nucleus with an initial dent and less dark cytoplasm compared to the pro-myelocyte. It is the last neutrophil maturation stage in the neutrophil lineage that can proliferate, and represents the last cell in the mitotic pool (see **Figure 1**).

Several early studies indicated that human myelocytes [22,23] and possibly pro-myelocytes [23] might be heterogeneous in their propensity to divide. It was initially proposed that up to 50% of these cells proliferate albeit very slowly and are residing in a 'so-called' **lazy pool** [22,23]. It is thought that these cells do not contribute greatly to **granulopoiesis** in homeostasis yet provide a means to mobilize neutrophils quickly as/when required by accelerated or **'emergency' proliferation and differentiation** [24]. The presence of a putative lazy pool is of key importance for the interpretation of kinetic data obtained by cellular modeling as the number of progenitors in cell cycle is much lower than assumed in models (see below and [13]).

Recently, several studies have used mass cytometry (**CyTOF**) and single-cell RNA sequencing (scRNA-seq) to re-examine the proliferation and differentiation of the neutrophil lineage and have identified (in both mouse and human bone marrow) the presence of neutrophil progenitor cells referred to as **neutrophil progenitors** (preNeu, NeuP or NEP);

these appear to be committed, unipotent and early-stage neutrophil progenitors as shown in adoptive transfer assays, IdU labeling and determination of cells in cell cycle in vivo applying Fucci-474 reporter mouse [16,17,19] . However, these studies did not 'bench-mark' these cells with the known pro-myelocyte and myelocyte stages as described above. Therefore, it remains uncertain whether these progenitors are new/unique or reflect all, or part of, the known (pro-) myelocyte pools.

The Post-mitotic Neutrophil Pool

Following the myelocyte stage, the neutrophil progenitors lose their capacity to divide and enter the so-called 'post-mitotic pool', particularly studied in human cells [25–28]. This represents the beginning of a true maturation program starting with meta-myelocytes, which in humans are recognized by a kidney shaped nucleus and clear cytoplasm. These cells in turn mature into banded cells with a horseshoe-shaped nuclei and again, clear cytoplasm (see **Figure 1**). These cells are not found in the peripheral blood in homeostasis, but can be identified in the circulation during periods of acute infection or inflammation, resulting in the so-called **'left-shift'** in the neutrophil population first described by Arneth [29]. The post-mitotic pool takes around 5-6 days from the last division of the myelocyte to the transition of the banded cell into the mature neutrophil in humans (see **Figure 1** and [13,25,27,28]). This time is significantly shorter (2-3 days) in rodents [30].

The Mature Neutrophil Pool

Most studies undertaken on neutrophil differentiation to-date consider that the mature neutrophil in the bone marrow represents the end of the post-mitotic stage. These studies imply that neutrophils undertake terminal differentiation in the bone marrow before being liberated into the peripheral blood (see [31]. Some authors suggest that part of the large number of human bone marrow neutrophils ($7x10^9$/kg body weight) are mobilized as a "**rapid mobilizable pool**" during periods of inflammatory stress [32–34] . However, it is uncertain what the fate of these cells is under homeostasis, given that overt apoptosis in the neutrophil pool in the bone marrow is a rare event. In support of this concept, a recent study analyzing the neutrophil transcriptome and epigenome [35] suggests that the largest differences arising during the differentiation of human neutrophils in the bone marrow occurrs during transition from the bone marrow mature neutrophil pool to neutrophils in the peripheral blood. However, an important caveat of this human study was that the bone marrow and blood cell samples were taken from different donors, and that the isolation procedures differed. This

leaves open the distinct possibility that the differences observed were in fact caused by inter-donor differences and/or *ex vivo* manipulation of these human cells.

There are further concerns regarding the concept that terminal differentiation of 'mature' neutrophils is completed in the bone marrow before mobilization to the peripheral blood, as mobilization of these cells during acute inflammation should then be associated by **'rejuvenation'** in the blood compartment [36]. However, this was not found in one study where metabolic labeling of neutrophils in humans *in vivo* showed that the neutrophils mobilized during acute experimental inflammation induced by lipopolysaccharide in human volunteer exhibited the same kinetics as mature cells before challenge implying the same age post labeling [36]. Hence it is possible that the mature neutrophil compartment behaves as a single compartment in full exchange between the blood, bone marrow, and possibly other tissue sites such as spleen. This would imply that neutrophilia and the 'left shift' seen during inflammation might be explained by a mere shift in relative sizes of the pools residing in bone marrow and peripheral blood. This shift in cell numbers can indeed quickly normalize by remigration of blood cells back to the bone marrow pool upon regaining homeostasis [37,38]. This interpretation, however, awaits experimental support.

Differentiation outside of the Bone Marrow

A fascinating issue when considering the life cycle of neutrophils is the possibility that these cells might undergo terminal differentiation outside the bone marrow. This concept is still hypothetical but is supported by several lines of evidence, including the presence of immature progenitors trafficking throughout multiple tissues in mice [39]. This concept provides a rationale as to why progenitors are mobilized into the peripheral blood to allow **alternative imprinting** by extramedullary sites. Not much is known about the putative underlying mechanisms but neutrophil progenitors have been found in the spleen [40]; it is tempting to speculate that tissue-induced, and possibly tissue-selective, alternative imprinting can result in the generation of neutrophil subsets with alternative functions, as discussed below for the mouse. Indeed, some studies have suggested that **neutrophil-myeloid derived suppressor cells** (granulocyte-MDSC) differentiate in the spleen and may contribute to cancer progression [41].

Cellular Markers of Different Neutrophil Differentiation Stages in Humans

The account of the myeloid lineage described above is descriptive and, until recently, based largely on direct microscopic analysis. A rather historic but landmark study [42] reported that the absolute number of promyelocytes/myelocytes, metamyelocytes, banded neutrophils and mature cells in the bone marrow is remarkably stable, suggesting a '**conveyor belt-like' model** [43] 1964). Another important finding from these early studies was that the total bone marrow pool of neutrophils is 6-8 times larger than the total peripheral blood pool [26,42,43]. Newer technologies based on flow cytometry have confirmed the heterogeneity of neutrophils within the bone marrow, but to date, this has not been aligned with the corresponding morphology of these populations [44]. A similar type of analysis can be seen in **Figure 1**, which shows that the entire differentiation pathway of human neutrophils can be captured using the expression of three cell surface receptors: Mac-1 (CD11b), L-selectin (CD62L) and FcγRIII (CD16). Flow-sorting of the different populations and subsequent analysis of the resulting **cytospin** preparations demonstrates that it is possible to identify and isolate the different maturing forms of neutrophils in the bone marrow and peripheral blood. Additional markers such as CD10, CD13, CD64 and CD87 can be used to facilitate the discrimination between mature and immature neutrophils [45–47].

Such flow technology-based approaches to cell phenotyping can also allow for a more accurate quantification of the cell numbers within various differentiation stages in the bone marrow. However, the existing studies do not completely align [42,44]. This might reflect the fact that bone marrow aspirates are poorly representative of the total cell content of the bone marrow consequent upon hemodilution [48] and/or a more sturdy association of progenitors with the bone marrow **stromal niche**, which may serve to trap the more immature dividing cells in the stroma. It is of utmost importance to obtain experimental data to determine the absolute numbers of neutrophils and neutrophil precursors in bone marrow as this is the basis for future models describing differentiation and kinetics of the neutrophil compartment (see below). Work in the last decade has demonstrated that the peripheral neutrophil compartment is heterogeneous and different populations of cells are characterized by distinct nuclear morphologies [11,45,49]. Particularly, human neutrophils with an hyper-segmented nucleus have gained interest as they have been shown to have the ability to suppress T-cells [45,49], but have lost the capability to intracellularly contain bacteria [50]. The issue of neutrophil heterogeneity is beyond the scope of this review and has been addressed by a number of recent reviews [2,10,46] and Silvestre-Roig et al. 2019 this series.

The Kinetics of Circulating Neutrophils

The Intravascular Neutrophil Pools

Mature neutrophils are present in the vasculature in two pools: a free-flowing intravascular blood pool and a blood pool residing in certain tissues. This latter pool is generally referred to as the '**marginated pool'**. Early studies suggested that marginated neutrophils were in complete equilibrium with the free-flowing cells and, therefore neutrophils from either pool were indistinguishable (Athens et al., 1961). The major sites for marginated neutrophils in humans are the liver, spleen and bone marrow itself [51], with debate existing as to their presence in the lungs, which may be specific only for certain species such as primates, mice and dogs [52–54].

The kinetics of circulating neutrophils and neutrophil precursors remains somewhat uncertain. In 1929, Weisskotten carried out experiments to determine the kinetics of neutrophils in the peripheral blood of rabbits using a toxin, benzol, which specifically targets cycling cells [55]. This work concluded that the half-life (t½) of circulating neutrophils in the rabbit was 3-4 days. However, this study fell into oblivion as more recent labeling studies did not support this relatively long half-life (see below).

Kinetics of Neutrophils in the Peripheral Blood Determined by Reinfusion of *Ex Vivo* Labeled Neutrophils

Important experiments performed in the fifties and sixties, and confirmed recently, have led to the commonly cited belief that circulating human neutrophils are short-lived cells with a t½ of 7-9 hours [26,43]. In these experiments *ex vivo* labeled autologous neutrophils were infused into volunteers and the disappearance rate of label in the blood was interpreted as representative of the circulatory half-life of these cells. This conclusion was supported by metabolic labeling in mice with D_2O showing t½ of around 9 -18 hr [12,56]. The interpretation of the human data has been challenged by authors who have argued that *ex vivo* manipulation of neutrophils might have changed the homing characteristics of these cells and affect their behavior in peripheral blood (see [31]). While deliberate priming of neutrophils both *ex vivo* and *in vivo* clearly impacts on the distribution of these cells within the vasculature, with most neutrophils homing to the lung [57,58], the above data have been consistently reproduced using newer techniques that induce minimal-to-no detectable cell **priming** (e.g. [59]). Hence, while priming undoubtedly has a profound impact on the behavior

of neutrophils *in vivo* [60–63], the effect of priming and activation *per se* on the intravascular (circulating, marginated and intra-vascular entrapment e.g. in the pulmonary capillary network) half-life of neutrophils has yet to be determined, and much of the above data still stands.

Kinetics of Neutrophils in the Peripheral Blood Determined by *In Vivo* Labeling with Radioactive or Stable Isotopes

To circumvent the difficulties of *ex vivo* cell manipulation, several studies have applied *in vivo* labeling methodologies to track and trace the kinetics of neutrophils. Several labels have been used including ^{3}H-thymidine [25], ^{3}H-DFP and ^{32}P-DFP [43]. These studies have produced a slightly more finessed dataset that support both a short as well as a relatively long neutrophil lifespan. A short life span in humans is supported by the quick disappearance of label in a logarithmic fashion [43]; however, this assumes that the majority of the neutrophil compartment is present in the peripheral blood. As discussed above [26,42] the majority of the neutrophil compartment resides outside the bloodstream and is likely to be in **complete exchange**, although this remains to be determined. Therefore, the disappearance rate of the label might also be explained by a redistribution of the cells into the whole neutrophil compartment that could take several hours. Indeed, in a rat model where labeled neutrophils were re-infused, it took several hours for neutrophils to end up in the bone marrow [37]. Of note, this population of cells that can 'disappear' from the circulating bloodstream can be mobilized again, as evidenced from bovine experimental models where calves were challenged with corticosteroids [64], . These results implied that bone marrow margination from the bloodstream seems to occur, rather than the formal uptake of neutrophils within a tissue compartment [64]. However, this hypothesis awaits further experimental support. In an early study, pulse labeling with ^{32}P-DFP in vivo led to stable labeling of blood neutrophils for 11 days, whereas the post-mitotic time was around 5-6 days ([43] and see below); thus, these data might also be interpreted as supporting a longer half-life for neutrophils than 7-9 hrs This would support other neutrophil lifespan results indicating that these cells can live up to 3-5 days ([12] and below). Nevertheless, it should be noted thate experiments applying in vivo ^{32}P-DFP labeling are difficult to repeat/reproduce because of ethical constraints.

Fortunately, new technology can re-evaluate these studies by *in vivo* labeling with the stable isotope deuterium (^{2}H) in the form of ^2H_2O or [2]H-6,6-glucose [65]. Under these conditions, deuterium is built into the ribose moiety of the DNA of cycling cells including the cells of the

mitotic pool of neutrophils progenitors. This technology allows a '**pulse-chase'** type of experiment by following the enrichment of ^{2}H in the DNA, and was only recently applied for the analysis of the kinetics of neutrophils in peripheral blood. It concluded that the lifespan of human neutrophils in peripheral blood is around 5 days and around 18 hr for murine neutrophils [12]. However, the finding for human cells has been challenged by several authors [13,66] arguing that the slowness in disappearance of label from the blood could be explained by a 'slow neutrophil compartment' in the bone marrow and a 'fast compartment' in the peripheral blood. Indeed, This latter view has been supported by studies using short term labeling with ^{2}H-glucose, which also concluded that human neutrophils have a short half-life in peripheral blood [13]. However, these conclusions were based on the assumptions that all (pro-) myelocytes divide equally, that neutrophils in the bone marrow and blood do not belong to a homogenous pool, that neutrophils do not return to the bone marrow, and that there is no lazy-pool of myelocytes [13]. These assumptions are in contrast with data showing that rat and human (pro) myelocytes that are dividing keep dividing with a cycle time of around 14 hr [67,68], that neutrophils can migrate back to the bone marrow [37,38], and by the presence of a so-called 'lazy neutrophil pool' [22,23]. In addition, there are kinetic data applying metabolic labeling that support the concept that human neutrophils in bone marrow and blood belong to the same kinetic pool [69].

In fact, even these published data can be used to support a significantly longer half-life of human neutrophils in the peripheral blood as the **R-value** of 5.5 that leads to an equally good fit of the data, supports a t½ of 2.6 days (lifespan of 3.7 days) [13]. It is, however, debatable that a model based on ordinary differential equations is the best choice for describing neutrophil kinetics as the experimental data are not supportive of a model where all (pro-) myelocytes have an equal chance to divide. It seems more likely that only a part of the (pro-) myelocyte pool is dividing with a narrow division time supporting a 'conveyor belt' type of differentiation as suggested before [43], rather than a model based on probabilities.

From another angle, data obtained in vitro support the hypothesis of prolonged neutrophil survival in vivo under conditions of inflammation and argue against a short half life in vivo. Multiple mechanisms have been implicated in contributing to prolonged neutrophil survival, including hypoxia, the release by inflammatory cells of cytokines and growth factors (e.g. GM-CSF and TNF), of microbe associated molecular patterns (e.g. lipopolysaccharide), as well as an outcome of viral infections such as with cytomegaly virus and respiratory syncytial virus [70–74]. Unfortunately, direct in vivo data in humans are lacking.

Neutrophil Fate and Function within Tissue Compartments

Dynamics of Neutrophils in Blood and Tissues

Mouse and human neutrophils newly released into the bloodstream are endowed with distinct phenotypic properties in that they gradually change over time following circadian oscillations (Adrover, J.M. et al, 2019) and, at least in the mouse, these phenotypic changes parallel changes in their transcriptional and migratory properties of neutrophils (Adrover et al., 2019; Adrover et al., 2016) (**figure 2**). A major functional pathway affected by (circadian) time is the rearrangement of the actin cytoskeleton over time, leading to the loss of surface microvilli and the subsequent reduction in the capacity of murine neutrophils to roll on **endothelial selectins** at daytime. Ultimately, these types of changes can result in a reduced ability to migrate to inflamed tissues over time (Adrover et al., 2019). While the mechanisms of migration are discussed in more detail in other reviews from this collection (e.g. Margraf et al 2019, and Phillipson et al 2019) , it is important to note that **rolling-defective neutrophils** can still adhere to unstimulated vessels in the dermal microcirculation (and possibly in other tissues) in a selectin-independent manner [76],. The mechanisms involved might be potentially similar to those identified for patrolling monocytes [77]. These features might explain the efficient entry of neutrophils from blood into naïve tissues, including skin, liver, intestine or bone marrow, while sparing tissues generally considered to be immune-privileged, such as the brain and gonads [78]. However, this warrants further investigation. Although similar patterns of recruitment of neutrophils into human tissues have not yet been evaluated, similar circadian properties and kinetics of neutrophils have been reported in cells from human blood [75]. This suggests that multi-organ infiltration in the steady-state might be a conserved feature across species. In addition, indirect demonstration that neutrophils infiltrate tissues as part of their natural life cycle comes from studies in mice defective in genes needed for neutrophil adhesion and elimination, including integrins, selectins or the phagocytic receptor *Mertk*; these mice have bene found to develop severe alterations in **granulopoiesis** and trafficking of **hematopoietic stem and progenitor cells** (HSPC), even under homeostatic conditions [79,80]. Similar hematopoietic alterations have been found in patients bearing **leukocyte adhesion deficiency** (LAD) mutations [81]; this suggests that a similar regulatory loop involving neutrophil migration into naïve tissues might also operate in humans, although this remains to be further investigated. Mechanistically, this type of regulation in mice appears to be mediated by transcriptional repression of IL-23 in tissue-resident phagocytes as they take up senescent neutrophils, as shown in vitro studies and mice lacking the indicated genes in vivo [79,80,82]. These murine studies have thus prompted a renewed interest in defining the dynamics and fate of neutrophils in healthy tissues, beyond their lifetime in circulation.

Retention and Function of Neutrophils in the Lung Microvascular Bed

While the dynamics of neutrophil entry into naïve or inflamed tissues is increasingly well-appreciated in the mouse [78], whether and how this occurs in human tissues remains largely unknown. Current knowledge in humans largely relies on comparative studies, and only in certain organs, such as the lung spleen. For instance, Intravital microscopy studies in the murine lung microvasculature have revealed a substantial number of neutrophils within the network of small capillary vessels that are rapidly mobilized by the chemokine receptor CXCR4 antagonist plerixafor in both mice and primates [52], or which are actively crawling on small pulmonary capillaries in mice [83]. Nonetheless, conflicting studies in humans and mice debate whether CXCR4 is indeed a retention signal for neutrophils in the lungs [84] and further work is required. This margination of circulating neutrophils and **intra-vascular crawling** in murine lungs dramatically increase upon exposure to endotoxin or live bacteria relative to naïve mice [83]. Although the size of the intra-vascular marginated neutrophil pool in humans is thought to be much smaller than the one shown in mice [85], similar changes in surface markers and in the number of circulating human neutrophils have been observed after in vivo treatment with endotoxin [83] or platelet-activating factor [57]. This, together with the rapid *in vitro* adhesion of LPS-stimulated human neutrophils to primary pulmonary endothelial cells [83], suggests that hypothetically, similar dynamics and intravascular behavior might take place in the human lung. In keeping with this notion, humanized sickle-cell disease (SCD) mice display frequent interactions of neutrophils with platelets within the lungs, with formation of microemboli that trigger vaso-occlusive crises and which can recapitulate those occurring in SCD patients [86]. These series of findings suggest the presence of an abundant population of neutrophils in the resting murine pulmonary microcirculation that might potentially serve as a pool for rapid mobilization[52] . The studies mentioned above additionally suggest potential roles in local anti-microbial responses, but also that they can elicit acute pulmonary injury. Additional studies in the mouse have shown that the lung may also provide a site of neutrophil re-programming that enables their return to the BM for final elimination [87], while in turn pulmonary neutrophils can instruct transcriptional programs in the mouse lungs that influence metastatic invasion [78].

Retention and Function of Neutrophils in the Spleen

The spleen also represents a tissue in which the function of neutrophils has been studied in considerable detail both in resting and diseased scenarios. Studies of human spleens

demonstrated the presence of at least two populations of neutrophils in the perifollicular zone which induce IgM secretion and **Ig-class switch** in **marginal zone** B cells through the secretion of factors BAFF, APRIL, IL-21 and pentraxin 3 [88,89]. Notably, these neutrophil populations in the human spleen were found to be induced post-natally by local signals such as IL-10 and GM-CSF, and involved in inducing anti-microbial immunoglobulin production in a T cell-independent manner [89]. However, these presumed specialized functions of neutrophils in the human spleen could not be reproduced in an independent study [90]. Furthermore,a B-helper phenotype similar to that found in the naïve human spleen has been reported only in the context of chronic lymphocytic leukemia in the mouse [91], which may promote leukemic cell expansion. A recent report demonstrated the presence of additional neutrophil populations in the red pulp of the mouse spleen that cleared *S. pneumoniae* from the surface of macrophages, and an additional immature splenic population of neutrophils that expanded upon infection with this bacteria [92]. Thus, resident (and possibly recruited and reprogrammed) neutrophil populations appear to be endowed with distinct antimicrobial functions in the spleen. Of note, while there is clear evidence from transfer experiments for neutrophil accumulation and destruction in the spleen in humans, where approximately 30% of all circulating neutrophils end up [93], evidence for the spleen acting as a major site of destruction of endogenous neutrophils in mice is lacking.

Uptake and Function of Neutrophils in the Liver

The liver is an organ of active accumulation and destruction of neutrophils. Studies in humans have demonstrated rapid accumulation of infused, radio-labeled neutrophils in the liver (the '**hepatic marginated pool'**) followed by intense phagocytic uptake, suggested that this organ may also be an important site for the homeostatic destruction of neutrophils [93,94]. In the rat liver, apoptotic (**TUNEL+)** neutrophils can be detectable at steady-state, but their number escalates dramatically following LPS treatment. Moreover, **Kupffer cells** that line the hepatic sinusoids actively phagocytose phosphatidyl serine (PS)-positive neutrophils, while depletion of Kupffer cells re-routes neutrophils to other tissues, such as spleen and lungs [95]. These findings agree with the observed accumulation of exogenously-infused mouse neutrophils mostly in the bone marrow and liver. In this study, immature neutrophils preferentially homed back to the bone marrow and these could be re-mobilized to sites of infection to participate in anti-microbial defense [96]. Of note, the liver is also a key tissue for the elimination of circulating bacteria [97], and neutrophils that have engulfed bacteria and die are taken up by Kupffer cells, in a process that blunts production of inflammatory cytokines by macrophages and favors resolution of inflammation once the

pathogens have been cleared [98]. Thus, the liver represents a primary site of neutrophil elimination, however other possible functions for neutrophils within this organ remain to be explored.

Migration and Function of Neutrophils in the Bone Marrow

In addition to being the main site of production, the bone marrow is also a site of active neutrophil clearance. Studies using mice in **parabiosis** demonstrated that senescent or aged neutrophils could return to the marrow upon completion of their life cycle in blood; this was deemed to occur mainly via a CXCR4-dependent mechanism as shown by impaired migration of CXCR4 mutant cells to the parabiont's marrow [38,87,99],. Furthermore, there is evidence for active recycling of radiolabeled neutrophils in the human marrow as well [94]. Whether this scenario maps equally to humans is yet to be determined as circulating human neutrophils express trace amounts of cell surface CXCR4, and studies using *ex vivo* radiolabeled neutrophils suggested that these cells are removed randomly rather than in an age-dependent manner [93]. However, various roles of neutrophils in the marrow are beginning to emerge, at least in the mouse. For example, aged neutrophils that return to the BM after circulating in blood are phagocytosed by medullary macrophages [100], thus suppressing CXCL12-producing niche cells, temporally inhibiting the niche's retention activity leading to the circadian release of HSPC into the circulation [99]. Because similar variations in circulating HSPC numbers occur in the bone marrow of humans [101], it is reasonable to speculate that they might perform similar suppressive functions, although this remains to be investigated.

An important but still poorly-defined issue when considering the fate of neutrophils is their actual lifetime within different tissues before final destruction, as this has not been yet rigorously measured. Our own preliminary evidence in the mouse suggest dwell times of less than one day in the steady-state in certain tissues such as spleen and skin, which may still be sufficient to influence many aspects of tissue physiology through the release of cytokines, **granule proteins**, or even **NETs** as reported in the human spleen [89], but this awaits full validation. In other contexts, for example the murine lymph node, our preliminary work, yet to be confirmed, suggests much longer tissue residency times than 1 day. Of note, these lifetimes can be markedly extended in the context of inflammation, as multiple cytokines and bacterial products extend the survival of human and murine neutrophils, at least in part, through regulation of the anti-apoptotic factor Mcl-1 [102,103]. Thus, essential features of the

neutrophil life cycle, namely their lifespan in different tissues and under inflammatory states, remain to date poorly defined and this should be an important task for the coming years.

The Yin and Yang of Neutrophil Targeted Therapies.

It is noteworthy that several important clinical conditions are associated with either hyper- or hypo-activation of the neutrophil compartment. On the one hand, Chronic inflammatory diseases such as chronic obstructive pulmonary disease [104] as well as acute inflammatory conditions such as systemic inflammatory response syndrome (**SIRS**) [105,106] are typically associated with hyper-activation of neutrophils . Under these conditions, it is possible that inhibition of neutrophils may be beneficial for the patient to prevent clinical complications brought about by a hyperactive immune system. A similar inhibition of neutrophils might be suited for certain cancer patients where activated neutrophils can play a role in suppressing anti-tumoral immunity ex vivo such as found in head and neck cancer [107,108].

On the other hand, insufficient activation of neutrophils in clinical conditions such as in the compensatory anti-inflammatory response syndrome (**CARS**) in patients with multiple injuries or following major surgeries can result in the activation of these cells to prevent the development of severe infectious complications such as sepsis [109,110]. Nowadays, the term CARS is used less often as the hypo-inflammation appears to be initiated alongside the SIRS response [111].   The hypothesized rationale for the occurrence of hypo-inflammation is that it might limit tissue injury and facilitate healing [112]. However, this remains to be directly demonstrated.

It is clear that the timing of therapies modulating immune responses is of essence as both hypo- and hyper-activation of neutrophils co-exist in patients suffering from acute inflammatory conditions [109,113]. A clear example are multi-trauma patients where SIRS is dominant during the first days after trauma and a dominant hypo-inflammation becomes prominent after several days [114]. Apart from the modulation of activation or inhibition of neutrophil responses under these conditions, novel therapies might also be targeted at the level of survival/apoptosis [115] of neutrophils; examples may include targeting the pro-survival factor mcl-1 [116] and/or PI-3 kinase [117].

Concluding Remarks

The variety of kinetics and functions described for neutrophils are consistent with the emerging view that these cells are multifaceted. At least part of the neutrophil pool is essential in host defense against invading micro-organisms and is crucial for a successful immune response. On the negative side, neutrophils are involved in the pathogenesis of a plethora of inflammatory diseases, and can additionally, in certain instances, suppress anti-tumor responses. Thus, it is now clear that neutrophils may become important targets of future therapies to suppress hyper-inflammation as well as hypo-inflammation dependent on the nature of the disease. Elucidation of the precise mechanisms underlying these antagonistic functions might allow the development of new putative therapies that target one arm of neutrophil functions while sparing the other arm of their beneficial functions (see Outstanding questions). In particular, lessons learned from understanding the lifecycle of neutrophils in different states and tissues may translate into clinical benefit; for example, manipulation of the life-cycle of neutrophils might potentially allow extending the duration of specific beneficial functions, while blocking detrimental functions under pathological scenarios. As we continue to better understand the dynamics and physiological facets of neutrophils, we hope that new therapeutic strategies will appear that harness the unique features of these cells.

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Box 1. Clinician's Corner

- Neutrophils are among the principal effectors of the innate immune response and are instrumental in the first line of defense against invading microbes

- Whilst there may be important differences between human neutrophils and those of other mammalian species, much has been learned from studies in transgenic animal models

- The production, circulation and clearance of neutrophils is altered by inflammatory stimuli such as those encountered in acute conditions such as bacteremia, and chronic conditions such as chronic obstructive pulmonary disease

- Therapies precisely targeted at the deleterious effects of neutrophils, whilst leaving beneficial ones intact, will be required for successful manipulation of these cells in the clinic

Glossary

CGD, chronic granulomatous disease is an immunodeficiency characterized by mutations in genes needed for the generation of reactive oxygen species in granulocytes;

Chédiak-Higashi syndrome is caused by deficiency in a gene required for lysosomal trafficking and phagocytosis that results in immune-deficiency and albinism;

CMP, or common myeloid progenitors are a type of hematopoietic progenitors that give rise to all myeloid-lineage cells in adult hematopoiesis;

Compensatory anti-inflammatory response syndrome (CARS) is period of systemic immune suppression induced by extensive tissue damage or uncontrolled infections. SIRS and CARS can co-exist.

Conveyor belt-like model, a model of granulopoiesis in which immature, but not proliferative, neutrophils give rise to a progressively mature neutrophil;

CyTOF or mass cytometry is a technique that combines mass spectrometry and flow cytometry thus allowing multiparametric (>30) assessment of cell markers;

Cytospin a preparation of cells that have been centrifuged on a slide for staining and morphological evaluation;

Endothelial selectins, are two receptors (E- and P-selectins) present on endothelial cells that enable leukocyte rolling under flow conditions **Granulocyte-MDSC**, or granulocytic myeloid-derived suppressor cells is a subset of neutrophils with T cell-suppressive activity;

Granule proteins is a group of enzymes and anti-microbial proteins found within cytoplasmic granules;

Granulopoiesis, refers to the process of proliferation and differentiation of granulocyte progenitors into mature cells;

Hematopoietic stem and progenitor cells (HPSC) are a rare population of hematopoietic cells that can give rise to all blood lineages;

Hepatic marginated pool, is the group of leukocytes found within the liver microvasculature;

Intravascular crawling, is a type of cell migration on the endothelial surface;

Kupffer cells, macrophages of the liver;

Lazy pool, is a subset of neutrophil progenitors that rarely divide in the steady-state but activate upon acute demand for granulopoiesis;

Left-shift, refers to the process of mobilization of immature neutrophils in the peripheral blood;

Leukocyte adhesion deficiency, a group of genetic disorders that affect the capacity of leukocytes to roll or adhere on the vascular endothelium;

Marginal zone of the spleen is regions at the interface between the lymphoid white pulp and the non-lymphoid, macrophage-rich red pulp.

Marginated pool, a population of intravascular neutrophils that is adhered to the endothelial lining and is not free flowing in the bloodstream;

NETs, or neutrophil extracellular traps are DNA-based structures that are released by activated neutrophils and have microbicidal and pro-thrombotic properties.

Neutrophil progenitors, are a group of hematopoietic cells that are already committed to the neutrophil lineage by successive proliferation and/or differentiation;

Neutrophil rejuvenation refers to the appearance of immature forms of neutrophils in the blood after release in the bloodstream;

Neutrophil re-programming, is a theoretical phenomenon whereby neutrophils change their phenotype and function;

Priming, an intermediate activation state of neutrophils that involves active intracellular signaling;

Pulse-chase experiment, an approach typically based on metabolic labeling of cells to follow their dynamics in live animals

Rapid mobilizable pool, is a population of neutrophils in the bone marrow that are rapidly mobilized into the circulation during stress conditions;;

Rolling-defective neutrophils, are neutrophils that lack the glycoprotein ligands that engage selectins during the rolling process;

R-value is the ratio of blood neutrophils to mitotic precursors in the bone marrow;

Systemic inflammatory response syndrome (SIRS) is a period characterized by enhanced levels of systemic inflammatory signals caused by extensive tissue damage or uncontrolled infections;

TUNEL, orTerminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling assay is a method that detects early apoptosis in cells that undergo extensive DNA degradation

Parabiosis is an experimental system that allows the study of shared exchange of circulatory molecules or cells between organisms, and normally achieved by surgical conjoining of the circulatory systems.

Table I: Relevant differences between murine and human immune systems

Property	Murine neutrophils	Human neutrophils	Reference
Percentage of neutrophils in peripheral blood	10-25%	50-70%	
Neutrophil size	8.64 ± 0.14 μM	10.39 ± 0.19 μM	[118]
Nuclear morphology	Ring-like	Segmented	[119]
Neutrophil granule contents	Defensins absent; low expression of BPI, MPO, β-Glucuronidase, lysozyme, alkaline phosphatase, and Arginase-1	Defensins present; high expression of BPI, MPO, β-Glucaronidase, lysozyme, alkaline phosphatase, and Arginase-1	[120]
Chemokine and chemokine receptor expression	CCL6, CCL9, CXCL15, CCL12 found in mice, but not humans	CXCR1, CXCL8, CXCL7, CXCL11, CCL13. CCL14, CCL15, CCL18, CCL23, CCL24/CCL26 found in humans, but not mice	[121]
Neutrophil antigen expression	Express Gr-1 and Ly-6G	Absent	[122]
Different Fc receptor expression	Do not express FcαRI Do not express FcγRI	Express FcαRI Inducible expression of FcγRI	[123–125]
Affinity of fMLF receptor	Low	High	[126]

Figure Legends

Figure 1: Differentiation of the neutrophil compartment in the human bone marrow

A. Model for neutrophil differentiation in the bone marrow. In this model differentiation of neutrophil and its progenitors behaves like a conveyor belt as firstly suggested by Cartwright et al. [43]*. Neutrophil committed differentiation starts with the neutrophilic promyelocyte and myelocyte that both have the propensity to divide and as such are part of the mitotic pool. Hereafter, the cells stop dividing and mature into metamyelocytes and band neutrophils both of which are not found in the peripheral blood (i.e. post-mitotic pool). Hereafter, the cells enter the mature state in which they are in exchange between bone marrow, blood and other tissues.*
B. Differences in marker expression of differentiating neutrophils in bone marrow. The neutrophil lineage in the bone marrow can be visualized flow cytometry and sorted on scatter characteristics and the expression of three markers: Mac-1 (CD11b), FcγRIII (CD16) and L-selectin (CD62L). Examples of cytometry contour plots are shown. The dump gate was designed by applying next to forward scatter (FSC) and side scatter (SSC) co-staining with CD193, CD56 and HLA-DR antibodies. Cytospins are shown of the cells sorted from the indicated gates.

Key Figure, Figure 2: Neutrophil fates within tissue compartments

Neutrophils produced in the BM and released into the circulation can enter multiple tissues after a process of diurnal aging (oscillations in phenotype), even in the absence of inflammatory stimuli. In these tissues they play prominent roles related to anti-microbial defense, immune cell maturation, regulation of stem cell niches, or are ultimately eliminated. For example, while in the BM granulopoiesis and vascular regeneration may represent relevant functions before they are released into blood, neutrophils acquire the capacity to regulate the circadian release of hematopoietic stem and progenitor cells (HSPC) when they return to the marrow for elimination. Similarly, specialized functions in the spleen for B cell maturation or in the lungs for immune surveillance suggest a wealth of functions tailored to each tissue as detailed in the text. HSPC, hematopoietic stem and progenitor cell; Ig, Immunoglobulin; BM, bone marrow.

Highlights box

- Neutrophils are produced by committed progenitors in the bone marrow and some extramedullary tissues
- The lifetime of mammalian neutrophils remains controversial
- Neutrophils are found in most healthy tissues at varying numbers
- Manipulation of the neutrophil life-cycle may be a promising strategy for the treatment of inflammatory diseases
- Differences among species may reflect the remarkable sensitivity of neutrophils to environmental conditions.

Outstanding questions box

- What are the real transit times of neutrophils in blood, bone marrow and tissues? These times may provide insights into non-immune roles and the pathogenic potential of neutrophils in tissues.
- What is the evolutionary basis for the diurnal behavior of neutrophils? Gating anti-microbial functions or protecting the host's tissues are possible benefits of this behavior.
- What are the mechanisms of neutrophil clearance in mice and humans? In which tissues does clearance occur? Understanding such mechanisms may enable extension or reduction of the number of neutrophils depending on clinical needs.
- Can the life cycle of neutrophils be reprogrammed or manipulated for the clinic? In what ways? Rewiring epigenetic and transcriptional programs in neutrophils may confer protection from derailed inflammation or against unwanted immune suppression in cancer.
- What is the role of the large marginated pools of neutrophils that reside under physiological conditions in the liver and the spleen? Responding this question may provide a rational to induce the mobilization of these physiological stores.
- How do viruses and bacteria subvert the life cycle of neutrophils? This is a critical question to define how pathogens may co-opt neutrophils for propagation.