Granulopoiesis and Neutrophil Homeostasis: A Metabolic, Daily, Balancing Act

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Abstract:

Granulopoiesis is part of the hematopoietic hierarchic architecture where hematopoietic stem cells give rise to highly proliferative multipotent and lineage-committed granulocytic progenitor cells that differentiate into unipotent neutrophil progenitors. Given their short lifespan, neutrophils are rapidly cleared from circulation through specialized efferocytic macrophages. Together with an intrinsic clock, these processes contribute to circadian fluctuations, preserving self-tolerance and protection against invading pathogens. However, metabolic perturbation of granulopoiesis and neutrophil homeostasis can result in low-grade chronic inflammation, as observed with aging. During acute pathogenic infections, hematopoiesis can also be switched into emergency mode, which has been recently associated with significant neutrophil functional heterogeneity. This review focuses on a new reassessment of regulatory mechanisms governing neutrophil production, life-cycle and diversity in health and disease.

A Day in the Life of a Neutrophil

Neutrophils are the most abundant white blood cells in mammals, representing a first line of defense from daily environmental insults. However, due to their short half-life, more than 10^{11} mammalian neutrophils need to be replaced every day to maintain their roles as sentinels [1–6]. Blood production is a highly regulated process that originates from both spatial and timely organization of hematopoietic stem and progenitor cell (HSPC) self-renewal, expansion and differentiation in the bone marrow (BM) [1–6]. Using different cellular tracking approaches, it has been debated whether **long-lived progenitors** or **short-term HSCs** (ST-HSCs), rather than classically defined **long-term HSCs** (LT-HSCs), are the main drivers of steady-state hematopoiesis in the adult mammal [7–9]. Nevertheless, blood development starts with HSPCs that tailors the output of committed progenitor cells based on the specific hematopoietic need of the organism. Although neutrophils are the predominant leukocyte population in blood and are released from the BM at a steady rate under homeostatic conditions, questions remain regarding how HSPCs specify lineage fate towards granulopoiesis.

In this review, we primarily focus on the sequence of events involved in granulopoiesis downstream of the myelopoietic lineage, as well as the emerging factors that regulate the commitment and differentiation of this myelopoietic branch. Given accumulating evidence of neutrophil diurnal oscillations being coupled to circadian metabolism and immune surveillance to maintain tissue homeostasis, we discuss recent advances in our understanding of these new aspects of granulopoiesis regulation, examining granulopoiesis in the context of health and sterile inflammation, as in cases of chronic inflammatory diseases, inflammaging and cancer.

Granulopoiesis: Neutrophil Production at Steady State.

Neutrophil Ontogeny

Granulopoiesis involves a series of maturational steps from multipotent HSCs to lineage-committed **Common Myeloid Progenitor** (CMP) cells, through differentiation, leading to the formation of granulocytes, including neutrophils. Seminal work identified and isolated CMP cells within a Lin[−]Sca-1[−]c-Kit⁺ fraction (cellular phenotype) that could give rise to either megakaryocytes and erythroid cells, or macrophages and granulocytes -- depending on the expression of Fcγ receptor-II/III (FcγR) and CD34 in mice (Key Figure, **Figure 1**) [10]. Even though these populations may still be heterogenous [11,12], they clearly represent two mutually exclusive intermediates in myelopoiesis; namely, the megakaryocyte/erythrocyte lineage-restricted progenitor (FcγR^{lo}CD34[−] MEP) or the granulocyte/macrophage lineage-restricted progenitor (FcγR^{hi}CD34⁺ GMP). Similarly, a myeloid-restricted progenitor population was identified in humans as being Lin[−]CD34⁺CD38⁺; with a IL3Rα[−]CD45RA[−] (double negative) fraction harboring an erythroid and megakaryocyte lineage potential, while the IL3Rα⁺CD45RA⁺ (double positive) fraction gave rise to cells of the granulocyte and macrophage lineages [13]-- with some heterogeneity within the two populations [14–16].

Stemming from a multi-omics integrative study [17], one report has elegantly characterized a specific **multipotent progenitor 3** (MPP3) subset present within the Lin[−]Sca-1[−]c-Kit⁺ (LSK) fraction of the mouse BM that is more efficient at producing GMPs with neutrophil-biased outputs than other MPPs (Key Figure, **Figure 1**) [18]. These mouse progenitors are produced by HSCs with specific kinetics depending on neutrophil demands [18]. Whether human Flk2⁺CD150⁺CD48⁺ MPP3 possess similar neutrophil-biased outputs remains to be clarified. At steady state, humans can produce up to 1 billion neutrophils per day per kg of body weight. Beyond its broad effect in producing active neutrophil proteases and transiently reorganizing the BM niche into a highly proteolytic environment, the granulocyte colony-

stimulating factor (G-CSF) remains the main growth factor driving granulopoiesis across mammalian species [19–22]. Accordingly, three populations of unipotent neutrophil progenitors that respond to G-CSF in mice and humans, have been recently identified downstream of GMPs [23–25]. A hierarchical tree with these advanced committed neutrophil progenitors has also been recently proposed in mice [26]. Similar observations exist in humans, and a heterogeneous c-Kit⁺CD66b⁺CD34⁺ cell population has been identified as an early neutrophil progenitor subset [24] -- presumably preceding the c-Kit⁺CD66b⁺CD34⁺ population [25] (Key Figure, **Figure 1**). One major characteristic of these proliferative neutrophil precursors across species is that they rapidly differentiate into mature Ly6G⁺CXCR2⁺ neutrophils, and this process involves several known transcription factors downstream of G-CSF, such as PU.1, C/EBP α or GFI1 [27]. Indeed, a recent study identified C/EBP ϵ as a novel master transcriptional factor dictating neutrophil-exclusive commitment under homeostatic conditions in both mice and humans [25]. Collectively, these recent findings have helped demonstrate developmental pathways in granulopoiesis downstream of the myelopoietic lineage, and may provide a better understanding of how dysregulated granulopoiesis can impact immune homeostasis.

C/EBPs Dominate Transcriptional Networks during Granulopoiesis

The process of specification and differentiation of hematopoietic progenitors towards the myeloid lineage during resting state requires the coordinated activation of gene expression programs and myeloid transcription factors [28]. It is well established that transcription factors such as PU.1, RUNX1 and GFI1 play a fundamental role in granulopoiesis across species (see review [29]). Here, we focus specifically on the role of transcription factors belonging to the CCAAT enhancer-binding protein (C/EBP) family comprising six members (C/EBP α , β , δ , γ , ϵ and ζ). Studies of CEBP family proteins have revealed that these are involved in numerous biological functions during homeostatic maintenance, as well as under pathological conditions [30]. Notably, each individual isoform has a specific role in granulopoiesis. Original analyses of genetically-modified mice lacking specific CEBP members have revealed a requirement for distinct C/EBP transcription factors during different developmental stages of granulopoiesis. The study of *c/ebp α* ^{-/-} mice initially revealed neutrophil loss, and this deficiency was attributed to the failure of C/EBP α to regulate the G-CSF receptor (G-CSFR) promoter [31]. These findings are consistent with studies of *csf3*^{-/-} mice (i.e, G-CSF deficient mice) and *csf3r*^{-/-} mice (i.e, G-CSFR deficient mice) that are severely neutropenic [32,33]. Subsequent studies of genetically-modified animals and cell lines revealed that the lack of G-CSFR expression in C/EBP α deficiency could be restored by exogenous factors such as the mixture of granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3 and IL-6, to initiate C/EBP α -independent granulopoiesis [34–36]. Given that GM-CSF, IL-3 and IL-6 serum concentrations are increased during inflammatory responses, it is reasonable to suggest that C/EBP α might be dispensable for granulopoiesis under inflammatory conditions such as acute infections. In contrast, a study has shown that *in vitro* cultures of C/EBP β -deficient hematopoietic cells have impaired responsiveness to GM-CSF [37]. This in turn suggested that C/EBP β could contribute to C/EBP α -independent granulopoiesis. Consistent with these findings, an elegant study illustrated the requirement of C/EBP β in emergency granulopoiesis using both *in vitro* cultures and *in vivo* murine models of fungal infection or cytokine treatment [38]. Independent studies have also shown that *c/ebp ϵ* ^{-/-} mice are devoid of functionally mature neutrophils and have “atypical” granulocytes that lack secondary and tertiary granules [39,40]. Recent in-depth analysis using mass cytometry (CyTOF), cell-cycle-based analysis and transcriptomic profiling showed that neutrophil development is blocked at the pre-neutrophil stage in C/EBP ϵ deficient mice, rendering these mice unable to either

clear bacteria or mount immune complex-mediated inflammation [25]. While classical studies of knockout animals of different C/EBP members have provided insights into the role of C/EBP proteins in granulopoiesis, this approach can be cell- and marker-biased, thereby lacking a global perspective of the transcriptional networks involved. Using single-cell RNA sequencing technology to map bone marrow myeloid progenitor subpopulations, several laboratories have established workflows to study transcriptional programs in myelopoiesis in an unbiased manner. These studies have provided a detailed view of HSC commitment into different leukocyte lineages with unprecedented resolution. Notably, one key concept arising from these transcriptomic and functional data at single-cell resolution is that mouse and human hematopoietic differentiation does not occur through a “rigid” step-wise manner, as previously thought, but rather, through a continuum pathway in which progenitor cells exist in transitional states with dynamic lineage potentials [41–43]. Moreover, recent single-cell studies combined with CRISP-seq (CRISPR-Cas9 sequencing) perturbation analyses showed that C/EBP α was required for progenitors to enter the myeloid lineage in mice, and furthermore, that subsequent differentiation and maturation towards the neutrophil lineage was dependent on the cooperation between C/EBP ϵ and PU.1 [41,44]. In summary, we posit that these studies have formed an initial transcriptional framework for studying granulopoiesis under healthy or perturbed conditions.

Neutrophil Circadian Oscillations

Circadian rhythms are controlled by what are termed ‘circadian clocks’, present in every organ and every cell, and which synchronize an organism to anticipate daily environmental changes according to the day-night cycle. Leukocytes continuously circulate in the bloodstream under steady-state conditions and their circadian fluctuations have recently emerged as a critical mechanism aligning the body to the usual recurring cycles of the environment [45–49] (**Figure 2**). Although circulating immune cells display an opposite rhythm between mice and humans [50][51], peripheral neutrophils display daily oscillations in both species [50][51]. Most of the underlying mechanisms have been identified in mice (Box 1). One study revealed that circulating neutrophils exhibited robust circadian fluctuations in mice peaking a few hours after the initiation of light [52], paralleling earlier observations made for HSCs [53], or monocytes [54]. The mechanisms controlling the rhythmicity of mouse peripheral HSCs and monocytes involved oscillations in their BM retention by modulation of chemokines such as C-X-C motif chemokine 12 (CXCL12) within the BM microenvironment, or cell-autonomous control of chemokine receptors such as C-C chemokine receptor type 2 (CCR2) in a clock-dependent manner [53,54][55]. An additional layer of regulation for neutrophil rhythmicity involves their unique pattern of aging in the blood and re-entry in the BM for clearance [52]. Specifically, murine neutrophil aging of in the circulation is characterized by reduced L-selectin (CD62L) expression and increased CXCR4 expression, mediating their homing back to the BM within 8-12 hours [52]. Although a recent report has questioned the estimated lifespan of neutrophils in circulation in mice [56–58], these findings are consistent with the expected short lifespan (8-12 hours) of neutrophils in both mice and humans [59,60]. Apart from their re-entry in the BM, aged murine neutrophils can also be cleared by specialized tissue-resident, as observed in the spleen [61,62]. Using a parabiosis approach of neutrophil reporter mice (conjoined with shared circulation), in addition to whole-mount imaging of cleared large intestine, a role of intestinal macrophages in clearing infiltrated neutrophils was also recently proposed [63]. Additionally, a slower rate of neutrophil aging was noted in germ-free mice, antibiotic-treated animals, or in mice with a specific myeloid deletion of the **Toll-like receptor** adaptor protein Myd88 (LysM-cre/Myd88^{fl/fl} mice) indicated this process was driven by the activation of Toll-like receptor by the microbiota, relative to controls [64]. Recently, the functional importance of oscillatory expression of the endothelial intracellular cell adhesion molecule (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1), was established for the rhythmicity of neutrophils and other

leukocytes through their emigration from blood [65]. Specifically, the authors showed that neutrophils had a unique capacity to exhibit rhythmic homing to different tissues in mice; chronopharmacological blockade approaches revealed that L-selectin was required for neutrophil homing to the lymph node and spleen, while in the liver, a predominant role was attributed to VCAM-1 [65]. These findings extend existing knowledge of integrin and selectin-dependent specificity of neutrophil recruitment to various inflamed tissues (reviewed in [3]). Additionally, an intrinsic clock mechanism in neutrophils was identified by targeting the clock in mice with specific *Bmal1* deficiency in myeloid cells, which displayed a lack of oscillations in the migration behavior of neutrophils to the spleen, along with altered expression of the nuclear receptor *Nr1d1* (also known as Rev-Erb α) or the P-selectin glycoprotein ligand-1 (*Psgl-1*) relative to controls [65]. *Bmal1* also controlled the expression of the chemokine CXCL2, as evidenced from chromatin immunoprecipitation (ChIP) of *Bmal1* binding to E-box-containing promoter regions of *Cxcl2*. The relevance of this regulation was highlighted by the aged neutrophil phenotype and impaired diurnal recruitment to multiple tissues with myeloid-specific deficiency of *Bmal1* and *Cxcr2* (LysM-cre/*Bmal1*^{fl/fl} and *Cxcr2*^{fl/fl} mice) relative to controls [66]. Furthermore, mice subjected to chronic sleep fragmentation developed myeloid expansion during the light period, reflecting an exacerbated production of the growth factor CSF1 relative to controls; this was prevented in mice with pre-neutrophil-specific deficiency in the hypocretin-receptor [67]. Human peripheral neutrophils also display daily oscillations paralleling time-dependent changes in superoxide production and phagocytic capacity [68]. These daily oscillations have been further correlated with synchronous fluctuations in plasma CXCL12 [68] but not cell surface expression of CXCR4 [65], suggesting that cell surface markers of aged neutrophils may differ between mice and humans. Although currently uninvestigated, the ratio of young vs. aged neutrophils might be expected to follow circadian oscillations in humans. Most neutrophils present in blood in the evening are likely to be young neutrophils mobilized from the BM, whereas in the morning, this subset may have already aged significantly and have returned to the BM or migrated to peripheral tissues; however, this remains to be investigated. It is reasonable to speculate that neutrophil circadian oscillations are most likely a part of the immune surveillance mechanisms set in place to maintain mammalian homeostatic tissue function (i.e., anti-microbial activity or elimination).

Granulopoiesis during Inflammation and Disease

Neutrophil Heterogeneity

The field of neutrophil plasticity is still in its infancy and most of the concept of neutrophil heterogeneity has emerged from the cancer field [69–71][72]. By contrast, The plasticity of macrophages has now been largely accepted and is reflected by their ability to sense, respond and rapidly adapt to their local microenvironment [73–77]. Nevertheless, Similar to macrophage studies, a helpful and simplified way for understanding neutrophil function is to arbitrarily divide these cells into two extreme phenotypes. These phenotypes consist of a pro-inflammatory N1 phenotype induced by IFN γ (alone or in combination with LPS) and an alternative N2 phenotype induced after TGF β stimulation, across species [78–80]. However, this classification certainly limits our ability to clearly define a boundary and categorize these cells during homeostasis, as neutrophils are likely to respond to steady-state environmental cues in a single or combinatorial fashion. Thus, it is important to note that such approaches cannot fully recapitulate the precise mechanisms and metabolic flows that govern neutrophil basic cellular functions and their heterogeneity *in vivo* (Box 1). Consistent with this notion, various subsets of neutrophils have been described in mice -- not only under steady-state [81,82], but also under inflammatory conditions, such as CD49d⁺VEGFR1^{hi}CXCR4^{hi} pro-angiogenic neutrophils [83,84] or CD11b⁺CD15^{hi}CD10⁻CD16^{lo} 'hybrid' antigen-presenting neutrophils [85,86], among others [87–90]. Given the constant identification of infiltrated tissue neutrophil populations with novel functions, it is unclear whether these neutrophil

subsets belong to separate granulopoiesis lineages or are simply a reflection of their propensity to sense environmental cues and adopt a wide array of phenotypes.

Effects of Metabolism and Aging on Granulopoiesis

Despite the original notion that HSCs are immortal and exempt from aging, it is now well-recognized that the functional capacity and quantity of blood cells changes dramatically with age in both mice and humans (Box 1); this is accompanied by a decrease in the regenerative capacity of these cells, and a bias towards the myeloid lineage [5,91–93]. Over the last few years, several studies have identified an increased prevalence of somatic mutations that occurs with age in the blood and BM HSPCs of normal individuals, even in the absence of hematologic abnormalities. While this phenomenon does not typically give rise to noticeable symptoms, it does lead to increased risk for hematological cancers, infectious diseases and atherosclerotic cardiovascular diseases [94–99]. This common aging-related phenomenon has been named '**clonal hematopoiesis of indeterminate potential**' (CHIP) [100]. While the origin of these mutations in the initiation of cancers remains unknown, [101], these might arise from a '**smoldering inflammation**' known to impact hematopoietic stem cell fate in health and disease [102]; or, they might depend on telomere length dynamics [103]. Alternatively, numerous studies have shown that mitochondrial dysfunction, altered **autophagy, replicative stress**, and accrual of DNA damage all contribute to stem cell aging, at least in mice [104–107]. CHIP is linked to mutations in multiple genes encoding proteins such as methylcytosine dioxygenase 2 (TET2), DNMT3A and ASXL1 (reviewed elsewhere [108–111]). *Dnmt3a* and *Tet2* deficient mice, or *Dnmt3a*^{-/-} and *Tet2*^{-/-} bone marrow transplantation experiments were initially shown to promote HSPC expansion and a bias in lineage fate [112–115]. It is now well-recognized that epigenetic regulation is a driver of aging-associated HSC decline [116]. Together, these studies identify a potential mechanism for the origin of the bias towards the myeloid lineage with age, though it is yet unclear what underwrites granulopoiesis specificity, or how the latter may be related to altered neutrophil function.

The metabolic flexibility of myeloid progenitors is an integral component of many inflammatory diseases [117]. Studies initially showed that mouse models of hypercholesterolemia, defective cholesterol efflux pathways, or hyperglycemia, exhibited myelopoiesis, while bone marrow transplantation experiments revealed that deficiency in the main glucose transporter *Glut1* limited glycolysis-dependent HSPC differentiation towards the myeloid lineage [118–121]. Cholesterol metabolism could also impact the osteoblastic and vascular endothelium niches to instruct HSPC fate [122,123]. Recently, intrinsic metabolic modulation of myelopoiesis progenitors (i.e. dual increase in glucose and cholesterol metabolism) was also revealed as an integral component of **trained immunity** [124]. Long-term epigenetic reprogramming of myelopoiesis progenitors could be recapitulated by feeding mice a Western type diet (WD), known to trigger myelopoiesis [125]. WD-induced myelopoiesis seemed to occur via direct and indirect mechanisms, such as cell extrinsic IL-1 β signaling subsequent to peripheral macrophage toll-like receptor 4 (TLR4) activation, or cell-autonomous metabolic regulation of myeloid progenitors [126][127]. These findings raise questions regarding the interplay between 'hematometabolism' and epigenetics, as putative underlying mechanisms of **accelerated myelopoiesis** in many chronic inflammatory diseases, including those accompanying aging [128–130]. Of note, perturbation of the cellular Krebs cycle has been observed in many inflammatory diseases and Krebs cycle metabolites can act as central epigenetic modifiers -- with succinate and fumarate acting as antagonists of histone and DNA demethylases; alpha-ketoglutarate (α KG) as a cofactor of epigenomic dioxygenases such as TET2 and JmJc domain-containing demethylases [117]. In certain hematological malignancies, there is evidence that a shunt between bone marrow monocyte and neutrophil production can exist [131,132]; this suggests suggesting that dedicated signaling pathways for myeloid lineage specification may exist. Of relevance, during granulopoiesis and neutrophil differentiation, there are reports suggesting

that mitochondrial respiration must be repressed; indeed, the phagocytic function of neutrophils and their relative rapid death compared to other immune cells have been linked to high glycolytic rate and low mitochondrial respiration *in vitro* [133,134]. However, a recent study using autophagy protein Atg7-deficient neutrophil precursors *in vivo* questioned this hypothesis because autophagy-dependent generation of free fatty acids (i.e. lipophagy) was shown to be critical for supporting fatty acid-dependent oxidative phosphorylation and normal neutrophil differentiation from neutrophil precursors [135]. Similarly, mice with neutrophil-specific deficiency in Atg5 exhibited increased neutrophil maturation relative to control littermates [136]; moreover, human HL-60 promyelocytic cell lines treated with the ATP-synthase inhibitor oligomycin to reduce the electron transport chain flow, exhibited limited neutrophil differentiation *in vitro* relative to controls [137]. However, most of these studies have focused on neutrophil maturation without offering deep insights into the myelomonocytic branch; thus, dedicated metabolic pathways may emerge as central rheostat for granulopoiesis specification, especially during chronic inflammatory diseases.

Aged and Metabolically-Perturbed Macrophages Can Impact Granulopoiesis

As for HSC decline, there is emerging evidence of impaired innate and adaptive immune responses in the elderly -- a phenomenon referred to as 'inflammaging' [138–143]. Impaired macrophage effector function, along with hyperactivation and dysfunctional signaling, is a culprit of inflammaging that can be initiated by intrinsic and extrinsic effects [138–143]. This includes low-grade inflammation from acquisition of a **senescence-associated secretory phenotype**, or from chronic exposure to bacteria and bacterial products (e.g., endotoxin), e.g. secondary to increased intestinal permeability in mice (and perhaps in humans) [143,144]. One main effector function of macrophages is the clearance of apoptotic cells to support tissue integrity by destroying billions of dying cells everyday [145–148]. Of relevance, an *in vivo* study showed that homeostatic regulation of murine neutrophils relied on the clearance of dying neutrophils by specialized **efferocytic macrophages** located in peripheral tissues, such as the lung, bone marrow and spleen (following their entry via adhesion molecules e.g. integrins or selectins) [61]. Subsequently, when efferocytic cells ingested apoptotic neutrophils, IL-23 production was suppressed in these cells, leading to a feedback loop controlling G-CSF and granulopoiesis, and thus, regulating overall neutrophil numbers in blood [61,62]. Moreover, efferocytosis of secondary necrotic neutrophils was attenuated in peritoneal macrophages of aged mice relative to young mice, thus leading to prolonged peritoneal inflammation [149]. Of note, in certain chronic inflammatory diseases such as atherosclerosis, an inefficient clearance capacity of macrophages has also been observed when macrophages are metabolically perturbed, for example in mice with myeloid-specific deficiency of Glut1, cholesterol efflux transporters, Ucp2, or mitochondrial complex III [150–155]. Furthermore, we and others have recently observed that impaired clearance of apoptotic cells is linked to reduced lysosomal and mitochondrial functions [156,157] -- two processes that govern lipid handling by macrophages during efferocytosis [158]. Moreover, research into the resolution of inflammation has also identified new lipid signaling molecules secreted by macrophages and altered in aged mice [159], or with defective efferocytosis [160], currently termed '**metabokines**' (such as lactate) [153] or '**pro-resolving lipid mediators**' (e.g. resolvins). However, whether we can improve the efferocytic capacity of macrophages by resetting their metabolic efficiency, or by exploiting the pro-resolving activities of SPMs, remains to be determined; such putative manipulations are of interest, as if successful, they might enable mitigating the perturbed neutrophil homeostasis observed with age, and/or to overcome age-dependent effects on granulopoiesis.

Emergency and Extramedullary Granulopoiesis during Inflammation

While circulating neutrophil numbers are maintained at a constant number in the resting state, the hematopoietic system can rapidly switch to an '**emergency hematopoiesis**' mode

to increase neutrophil production to meet the demand for these cells during stress conditions (e.g. infection) (**Figure 3**). While the BM is the primary site for hematopoiesis at steady-state in adults (medullary), the spleen and liver can support **extramedullary hematopoiesis** (EMH) across species during immune challenges, such as infection [128–130]. Specifically, the spleen has been shown to be a unique reservoir, able to supply additional myeloid cells continuously during sepsis or conditions of stress [161]. Emergency and extramedullary granulopoiesis involve a series of shared cascading events including: i) the sensing of pathogens through recognition of **pathogen associated molecular patterns (PAMPs)** derived from microorganisms by **pattern recognition receptor** (i.e. TLRs); ii) the release of granulopoietic cytokines and growth factors; and iii) the mobilization of neutrophils to effector sites as part of the ‘fight-or-flight’ reflex [162,163]. To support EMH, hematopoietic stem/progenitor cells (HSPCs) can circulate in the bloodstream for immunosurveillance [164] and can seed in extramedullary tissues under acute and chronic stress conditions (i.e., infection, myelofibrosis, myeloablation, pregnancy, anemia, myocardial infarction or cancer) [165]. A seminal study showed that HSPCs were localized in close proximity to **Tcf21⁺ stromal cells** within murine splenic red pulp and these cells could secrete niche factors such as CXCL12 and stem cell factor (SCF) to support splenic EMH [166]. Additionally, a novel transcription factor Tlx1 has been identified that supports splenic EMH after LPS challenge in mice [167]. Indeed, conditions such as elevated G-CSF, GM-CSF, IL-3, FMS-like tyrosine kinase 3 (FLT3) ligand and IL-6 in mouse and human sera in response to infection, or induced IL-1 β production upon adjuvant administration, suggests that these soluble factors can serve as ‘relay signals’ to potentiate increased neutrophil production after challenge, in both experimental mouse models and humans [163]. Other considerations regarding emergency granulopoiesis have been recently reviewed [162,163].

G-CSF-dependent and independent emergency granulopoiesis can occur under conditions of acute inflammation. While it is well accepted that G-CSF is a prototypic neutrophil growth factor, there is a large body of literature showing that G-CSF is also a strong neutrophil mobilizer, especially under systemic inflammatory responses that trigger a profound increase in serum G-CSF. This is best exemplified by various experimental animal models and in humans, where a single dose of G-CSF injection is sufficient to increase neutrophil counts in peripheral blood, relative to controls [163]. The mobilization of neutrophils from BM into circulation has been studied best in experimental mouse models. These studies show that mobilization can occur through synergistic and/or distinct mechanisms, including i) CXCR2-mediated mobilization [168]; ii) the release of thrombopoietin to potentiate neutrophil motility leading to neutrophil egress [169]; and iii) inhibition of CXCR4/CXCL12-mediated BM retention and lung margination of neutrophils [170–173]. However, under specific bacterial infections, G-CSF can also act as a negative regulator of neutrophil mobilization, as blocking G-CSF can elevate circulating neutrophil numbers through the inhibition of CXCR2-mediated mobilization relative to controls [174]. Paradoxically, results derived from experimental mouse models have also demonstrated that *csf3^{-/-}* mice can still mount profound neutrophilia following *Candida albicans* infection, but yet, these animals remain more susceptible to candidiasis compared to wildtype (WT) infected mice [175]. Thus, this indicated that although G-CSF deficiency did not impact the development of neutrophilia after infection, neutrophil effector function could be impaired due to the lack of G-CSF production [175]. Of interest, G-CSF has also been recently shown to induce fever through the modulation of prostaglandin E2 released from neutrophils, further supported by the observation that fever can be ameliorated in prostaglandin E synthase (*mPGES-1^{-/-}*)-deficient mice following G-CSF treatment relative to WT [176]. Collectively, these findings suggest that emergency granulopoiesis can occur in a G-CSF-independent manner in response to certain pathogens,

and may require the adequate combination of different factors to efficiently produce, mobilize, or lead to 'fight-or-flight' neutrophils. Overall, it is clear that G-CSF plays multifaceted biological roles in the generation/maintenance of neutrophils, which highlights the need to reassess the mechanisms of action of G-CSF in neutrophil development as well as its functions in these cells. In particular, it will be important to re-examine the outcomes of granulopoiesis under different types of infection in G-CSF deficient mice, relative to controls [25,177].

Granulopoiesis and Cancer

In various mouse models of cancer, the spleen has been identified as a unique reservoir for generating tumor-associated neutrophils that can migrate towards tumors [178]. A study using a hepatoma mouse model showed that cancer-induced EMH could reprogram the function of hematopoietic stem/progenitor cells in the spleen to generate immunosuppressive neutrophils. This was based on the observation that splenectomy synergistically improved the efficacy of anti-(programmed death ligand 1) PDL-1 treatment in the tumor-bearing mice, relative to controls [179]. Although the BM niche and splenic stroma are quite different, neutrophil development in the spleen during EMH still follows the same hierarchical developmental order as in the mouse BM [25]. Future studies should therefore determine what and how these factors lead to neutrophil differentiation when generated from EMH in the spleen versus the BM. G-CSF can also modulate granulopoiesis during malignancy (Box 1). For instance, in one study, metastatic breast cancer cell-derived G-CSF was also shown to reprogram myeloid precursors into retinoblastoma Rb^{low} immunosuppressive neutrophils in the mouse BM; furthermore, neutralization of G-CSF restored Rb expression in this model [180] (**Figure 3**). Apart from G-CSF, another key pro-inflammatory cytokine is soluble IL-1 β , which has been shown to induce neutrophil accumulation in the stomach tissue of a gastric cancer mouse model relative to controls [181]. Moreover, a recent study in mice provided evidence that distal lung cancer cells could modulate the BM niche, generating pro-tumoral Siglec-F⁺ neutrophils, relative to controls [182]. Apart from such findings where tumor cells can modulate the subtype of neutrophils being generated, a recent report has also indicated that ovarian cancer cells can trigger early neutrophil recruitment to the **omentum** to establish the pre-metastatic niche [183]. Collectively, these studies demonstrate that cancer cells can produce local and systemic factors that not only modulate neutrophil production at intra- and extramedullary sites but also drive neutrophil recruitment and function within tumor sites. It will be interesting in the future to examine how neutrophil production and function are influenced by tumor types, and how neutrophils that are recruited to establish a pre-metastatic niche may impact tumor metastatic processes. We posit that with such an increased understanding, it may be possible to contribute to devising putative neutrophil-based therapeutic interventions to treat certain cancer patients.

Concluding Remarks

As discussed in this review, granulopoiesis represents one of the most important biological processes for generating neutrophils as a first line of defense from daily environmental insults. Here, we have provided an overview of the immune contexture of granulopoiesis (Key Figure, Figure 1), which is determined by many factors including immune status, circadian rhythms and metabolic processes. Numerous recent studies have helped to increase our understanding of these vital regulatory mechanisms. Based on these findings, a complex picture is emerging, comprising a continuum-based hierarchical program of

granulopoiesis and of neutrophil functional heterogeneity; the ultimate appropriate outcome is a transcriptionally- and metabolically-controlled protective granulopoietic response.

However, there are still many unanswered questions about these processes (see outstanding questions). This is in part due to our incomplete understanding of neutrophil ontogeny, differentiation and phenotypic/functional heterogeneity. With the recent identification of neutrophil precursors and advanced knowledge of neutrophil heterogeneity, coupled to improved animal models, genomics, bioinformatics and imaging technologies, new lines of research in the field of neutrophil biology may emerge. These approaches may help to dissect neutrophil roles in immunodeficiencies and immuno-dysregulated conditions, contributing to the development of potential therapeutic interventions for neutrophil-related diseases. Accordingly, future research will need to comprehensively tease apart the relative 'hematometabolic' and circadian perturbations affecting granulopoiesis in acute and chronic inflammatory diseases, inflammaging, or cancer. Indeed, it might be possible to develop novel chronopharmacological approaches to regulating granulopoiesis. A special emphasis should be placed on determining the most appropriate experimental biomarkers to be used in measuring –if at all possible -- inefficient neutrophil immunosurveillance. Why is this important? Most of our knowledge in the clinic is based on neutrophil counts, yet we are now truly appreciating the complexity of granulopoiesis in health and disease. Indeed, many different inflammatory conditions or pathologies might converge in their molecular underpinnings, perhaps sharing common transcriptional and metabolic pathways that lead to inefficient neutrophil responses. These in turn, might or might not be related to perturbations in emergency or extramedullary granulopoiesis. Indeed, this concept has just been recently illustrated by the demonstration of metabolic alterations and over-expression of fatty acid transporter protein 2 in neutrophils from certain cancers [184]. Altogether, we envision new discoveries emerging from studies such as these, where the neutrophil no longer represents a 'forgotten cell' within the myeloid cell family.

Box 1. Clinician's corner

- The maintenance of constant circulating neutrophil numbers requires an exquisite balance between granulopoiesis and neutrophil mobilization into the circulation as well as efficient efferocytosis. This represents key intervention points for potentially treating perturbations in neutrophil homeostasis such as neutropenia or neutrophilia.
- Hematopoietic stem cell transplantation patients often remain neutropenic for weeks. Recently-identified proliferative neutrophil precursors may serve as a source for generating neutrophils, which might help bridge treatments by aiming to avoid prolonged periods of neutropenia.
- The immune response weakens during aging and chronic inflammatory diseases, and can be accompanied by a bias towards myeloid lineage differentiation; there is a wide array of phenotypes that neutrophils can adopt under these circumstances. Strategies aimed at limiting myelopoiesis or reprogramming efficient neutrophil functions might constitute a new way to enable fighting against certain diseases.
- Neutrophils are the most abundant leukocytes in the blood. An improved knowledge of neutrophil phenotypic heterogeneity might lead to the development of blood-based candidate biomarkers for certain inflammatory diseases, including cancer.
- Protein factors within tumor microenvironments have been shown to modulate granulocyte properties. Conceivably, the modulation of systemic factor(s) in cancer patients might allow the reprogramming of pro-tumoral neutrophils into anti-tumoral neutrophils.

Glossary

Accelerated myelopoiesis: event causing increased (above normal) production of bone marrow myeloid leukocytes (i.e, white blood cells), including neutrophils.

Autophagy: cellular detoxification process that disassembles unnecessary or dysfunctional cellular components for recycling.

Common Myeloid Progenitor (CMP): hematopoietic progenitor that gives rise to myeloid leukocytes.

Clonal hematopoiesis of indeterminate potential (CHIP): common age-related phenomenon where hematopoietic stem and progenitor cells acquire a non-tumor mutation contributing to the clonal formation of a genetically-distinct subpopulation of leukocytes.

Efferocytic macrophages: Macrophages are professional phagocytic cells that daily remove billions of apoptotic and necrotic (dying/dead) cells in our body.

Emergency hematopoiesis: Rapid increased production of hematopoietic cells (i.e, white blood cells or leukocytes) due to, for example, infection.

Extramedullary hematopoiesis (EMH): Production of hematopoietic cells (i.e, white blood cells or leukocytes) occurring outside of the bone marrow under physiopathologic conditions.

Long-term HSCs (LT-HSCs): Quiescent hematopoietic stem cells, capable of self-renewal; can give rise to all hematopoietic lineages at least four months after transplantation.

short-term HSCs (ST-HSCs): Proliferative hematopoietic stem cells that can differentiate into all types of blood cells within four weeks.

Metabokines: Signaling molecules derived from metabolic processes; bearing paracrine and autocrine effects.

Multipotent progenitors (MPP): Hematopoietic progenitor cells that maintain a limited capacity for self-renewal compared with LT-HSC and ST-HSC but are capable of differentiating into all cell types of the hematopoietic system.

Pathogen associated molecular patterns (PAMPs): highly conserved structural components of microbes; recognized by pattern-recognition receptors.

Pattern recognition receptor: detect microbial pathogens via the recognition of characteristic microbial molecular motifs.

Pro-resolving lipid mediators: large, growing class of cell signaling molecules formed in cells via the metabolism of polyunsaturated fatty acids (PUFA).

Smoldering inflammation: Chronic exposure to irritant particles or inherited gene mutations causing exacerbated inflammatory macrophage responses

Replicative stress: event during DNA replication when the genome is exposed to various types of stress, and can result in replication stress (i.e, activation of checkpoint signaling).

Senescence-associated secretory phenotype: Cellular senescence is a phenomenon by which normal cells cease to divide and acquire an inflammatory secretome characterized by inflammatory cytokines, growth factors and proteases.

Tcf21⁺ stromal cells: primarily found around sinusoids in the red pulp of the bone marrow.

Trained immunity: the ability of an innate immune cell (in the absence of antigen receptor recognition) to develop an increased and protective immunological (trained) response against second inflammatory challenge such as infection.

Toll-like receptor: recognizes conserved microbial pathogens via recognition of characteristic microbial lipopolysaccharide (LPS) motifs

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Conflicts of interest. *None*

Figure Legends

Key Figure, Fig. 1 Hierarchy of Granulopoietic Development in Mice and Humans.

Schematic illustration of how neutrophils are generated from committed progenitors that originate from hematopoietic stem and progenitor cells (HSPCs) in the bone marrow. LT-HSC, long-term hematopoietic stem cells, reconstituting, self-renewing; ST-HSC, short-term hematopoietic stem cell; MPP, multipotent progenitors with limited self-renewal leading to transient but multilineage reconstitution; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor. Surface markers used for isolation are indicated in the white boxes for mouse, and yellow boxes/green font for human.

Fig. 2 Circadian Rhythmic Production and Clearance of Neutrophils in Mice.

Peripheral neutrophils exhibit robust circadian fluctuations peaking a few hours after the initiation of light. Three mechanisms have been implicated in this rhythmicity: i) Approximately 10^{11} neutrophils per day are produced in the bone marrow and their release is controlled by circadian oscillations of chemokines such as C-X-C motif chemokine 12 (Cxcl12), under G-CSF regulation and the Bmal1-dependent intrinsic clock (also dependent on other factors, as indicated); ii) Short-lived (8-12 h) neutrophils also dictate their fluctuations and (as opposed to young ($CD62L^+CXCR4^-$)), when neutrophils age ($CD62L^-CXCR4^+$), they are cleared by professional efferocytic cells (i.e, mainly macrophages) in the spleen, and potentially, in the intestine. Iii) When efferocytic cells ingest apoptotic neutrophils, IL-23 production is suppressed, leading to a feedback loop involving Th17 helper T cells (pink cells), controlling G-CSF and the subsequent production and release of neutrophils into the bloodstream. Black cell: phagocytosed neutrophil. Blue cell: macrophage. The process of neutrophil aging or their removal from the circulation might be driven by the activation of Toll-like receptor signaling by the microbiota [64]. Additionally, oscillatory expression of endothelial ICAM-1 (and other cell adhesion molecules) accompanies the circadian rhythmicity (white boxes with waves) of neutrophil extravasation or homing (i.e, L-selectin is required for neutrophil homing to the spleen, while in the liver, a predominant role has been attributed to VCAM-1) [65]. HSPCs: hematopoietic stem and progenitor cells. White boxes with single lines: cleared neutrophils.

Fig. 3 Beyond Homeostatic Granulopoiesis in Mice and Humans.

While circulating neutrophil numbers are maintained at a constant level during resting state, the hematopoietic system can rapidly switch into an 'emergency' mode to increase neutrophil production and meet the demand for these cells during acute pathogenic infections. This 'fight-or-flight' reflex process involves a series of cascading events beyond the prototypic G-CSF growth factor. Combined serum elevation of GM-CSF, IL-3 or FLT3 in response to infection is required to dictate bone marrow and extra-medullary granulopoiesis. In the case of tumorigenesis, G-CSF and potentially other inflammatory cytokines derived from cancer cells can also reprogram myeloid precursors in the bone marrow and spleen into neutrophils that are immunosuppressive (e.g. retinoblastoma-1 (Rb)^{low} neutrophils). Notably, C/EBP master transcription factor family members play a central role in dictating neutrophil-exclusive lineage commitment under both homeostatic and emergency conditions [185].

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Figure 1

Figure 2

Figure 3