Heterogeneity of neutrophils

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Abstract

Structured models of ontogenic, phenotypic and functional diversity have been instrumental for a renewed understanding of the biology of immune cells, such as macrophages and lymphoid cells. There are, however, no established models that can be employed to define the diversity of neutrophils, the most abundant myeloid cells. This is largely due to their uniquely short lives, a consequence of their inability to divide once terminally differentiated, which have been perceived as roadblocks to functional diversity. This perception is rapidly evolving as multiple phenotypic and functional variants have been found among these cells, both in homeostatic and disease conditions. Here, we present an overview of neutrophil heterogeneity and discuss possible mechanisms of diversification, including genomic regulation. We suggest that neutrophil heterogeneity is an important feature of immune pathophysiology, such that co-option of the mechanisms of diversification by cancer or other disorders contributes to disease progression.

1. Introduction

Immunity functions through the concerted action of diverse cell types with specialized tasks. Hence, a central goal of modern immunology has been to catalogue cells in an effort to unify observations, generate hypotheses and propose basic biological principles. However, “naming” subsets creates rules and restrictions that typically prevent capturing the true biology of a cell. This is particularly evident for plastic immune cells, for which the capacity to adapt to environmental changes is a defining property. Recent, unbiased single-cell analyses are reshaping decades-old nomenclatures and models of ontology, and in fact challenge the possibility of defining discrete cell types and states in the immune system using simple and rigid rules. Instead, considering protein and transcript composition, functional properties and tissue distribution together with genomic organization may provide a more definitive way to classify immune cells, and to call for true heterogeneity (**Figure 1**).

Neutrophils are traditionally defined as a type of myeloid cell with a short half-life, specific nuclear morphology, defined granule content and surface expression of markers, such as the GPI-linked receptor Ly6G in mice, or CD66b in humans[1]. Over the last decade, however, neutrophils have been described in a variety of flavors: from immature cells that abound in the bone marrow and can be rapidly mobilized into the circulation, to cells with non-overlapping profiles and regulatory functions in physiological and pathological conditions, including infection, sterile injury, autoimmunity or cancer. Unlike other myeloid cells in which diverse functional properties have been linked to molecular programs driven by specific transcription factors (TF), the contribution of TF to neutrophil heterogeneity beyond developmental programs[2,3] remains unknown.

In this review we present a critical discussion of neutrophil heterogeneity and outline potential underlying mechanisms primarily based on experimental mouse models, unless otherwise specified. We first discuss recent high-resolution analyses of granulopoiesis that highlight how specific neutrophil differentiation stages may be prone to generating diversity. We then provide an overview of neutrophil heterogeneity in healthy and diseased tissues, focusing on examples that best illustrate their plasticity in phenotype and function. Finally, we describe how existing principles of genomic organization and cell identity may apply to neutrophil heterogeneity.

2. Neutrophil development and maturation

Neutrophils are the most abundant circulating leukocytes in the human. An estimate of 10^7 neutrophils in mice and 10^{11} in humans are produced each day, with transit times from the last cell division in the marrow to release into the circulation of about 3 and 6-7 days in mice and humans, respectively [4-6]. While it is generally accepted that the half-life of circulating neutrophils is shorter than one day[7], a recent study in humans calculated a lifespan of up to 5.4 days[8]. These studies highlight the need for a definitive and precise estimation of the neutrophil lifespan, and suggest that neutrophils may persist in the circulation for periods of time sufficient to translate environmental signals into specific molecular programs, a realization of conceptual importance for rationalizing neutrophil diversity in vivo.

Historically, granulocyte precursors in humans have been defined from density gradients followed by histological inspection of the different fractions upon Giemsa staining[9]. Classification of the different stages of granulopoiesis was assessed manually based on morphological features, such as cell size, nuclear condensation and granule content. According to current paradigms, neutrophil development starts from granulocyte-monocyte progenitors (GMP) and progresses through a continuum of maturation stages, ranging from a mitotic pool of granulocyte-committed precursors, comprising myeloblasts, promyelocytes and myelocytes, to a post-mitotic/transition pool of metamyelocytes, band cells and segmented neutrophils[10] (**Figure 2**). Although this model represents a valuable framework for defining granulopoiesis, it is generally acknowledged that morphological and histochemical observations are subjective, not indicative of developmental trajectories and functional properties, and incompatible with downstream analyses. Instead, transcriptomic advances at the single cell level have allowed analyses of the dynamics of hematopoiesis, and revealed the presence of early, intermediate and late human neutrophil precursors with distinct gene and TF

signatures[11]. Complementary to these studies, cell cycle-based and multiparametric flow analyses revealed three neutrophil subsets within the mouse bone marrow: a committed proliferative precursor, termed pre-neutrophil (preNeu), which sequentially differentiates into non-proliferating immature and mature neutrophils[12]. In mice, preNeu do not express markers for other leukocyte lineages but express CD117 (c-Kit), while differential expression of CXCR4, CXCR2 and CD101 allows discrimination of immature neutrophils and mature neutrophils (CXCR2+ CD101+). On the other hand, human marrow neutrophils comprise three major subsets based on the absent expression of differentiated lineage markers and of CD101, together with the presence of specific cell surface proteins, including CD66, CD15, CD33, CD10, CD16 and CD49d. Thus, a number of recent studies make it clear that early-stage neutrophil precursor populations exist in the human and mouse bone marrow[12-14] (see **Box 1**). This refined definition of the developmental hierarchy of neutrophils in the BM extends purely beyond taxonomical interest (**Figure 2**), since much of the heterogeneity of neutrophils in homeostasis and disease may partly arise from the neutrophil at different developmental stages in the bone marrow, although this still remains to be formally demonstrated.

3. **Diversity of neutrophils in health**

Once maturation has been completed in the BM and the pool of mature cells is released into the circulation, neutrophils circulate with a set of preformed adhesion and chemotactic receptors, and effector proteins to rapidly migrate and respond to multiple microbial and sterile challenges[15]. These defensive and inflammatory tasks constitute a primary function of neutrophils and are a source of phenotypic diversity that has been extensively investigated over the past decades[16-19]. Here we focus, however, in the phenotypic and functional diversity of neutrophils in the steady-state (see also **Figure 3**).

Heterogeneity in the bone marrow

Neutrophils are the most abundant cells in the BM, and this organ contains the largest pool of neutrophils in the body. Indeed, classical studies in human and animal models investigating the kinetics of neutrophil mobilization and distribution in different body compartments found that marrow reserves of granulocytes are much more abundant than those in the circulation [20-22]. Besides providing an immune reservoir for deployment in situations of alarm, the BM is a primary site of HSC maintenance, a function that relies on a dense network of vascular structures such as sinusoids and arterioles[23]. Interestingly, a recent study found that marrow-resident, but not circulating, neutrophils

exert important regenerative support for the medullary sinusoids after a genotoxic insult through the production of TNFα[24]. Mature neutrophils within the marrow also produce prostaglandin E2 in response to adrenergic stimulation, and this lipid enhances HSC retention by activating osteolineage niche cells[25]. A key feature of hematopoietic niches is the capacity to maintain HSC in a quiescent state, which is fundamental in preventing proliferative exhaustion or DNA damage of the stem cell pool. Notably, immature neutrophils that express histidine decarboxylase (Hdc), a histamine-synthesizing enzyme, were shown to support the quiescence and repopulating capacity of a subset of myeloid-biased HSC[26]. Overall, these recent studies in mice reveal specialized niche- and HSC-supportive functions and functional diversity of neutrophils within the BM, suggesting that neutrophils can adapt their tissue of residence.

Besides the various types of immature neutrophils at different stages of maturation, the BM is also a site for recycling of circulating neutrophils at least in mice. Indeed, a large fraction of mature neutrophils that have aged in the circulation are recruited back to the marrow with circadian frequency[27,28]. While a major purpose of this return may simply be elimination of dysfunctional cells, these aged neutrophils display niche-inhibitory functions leading to the circadian release of hematopoietic precursors into the circulation[27,29]. Thus, the BM provides an illustrative example of neutrophil diversity within a single organ, with cells at different stages of maturation fulfilling specialized roles. It will be important to validate whether such functional diversity also exist in humans.

Neutrophils in blood, time-induced heterogeneity?

Under steady-state conditions, neutrophils and other leukocyte subsets are released from the bone marrow and circulate for about half-day before infiltrating tissues and being removed from blood[27,30,31]. Both processes occur with circadian frequency and, in the case of neutrophils, their time in blood is thought to represent their full extramedullary life while for lymphocytes, for example, it normally represents a transit between lymphoid organs[32]. Although the relatively short time of neutrophils in blood would suggest homogeneous properties of these cells, marked diurnal changes in phenotype do occur, a phenomenon referred to as neutrophil *aging*[33]. Indeed, mouse and human neutrophils lose CD62L (L-selectin) and gain CD11b and CXCR4 expression over about 6 hours[27,34], their nuclei become hypersegmented and, at least in inflamed murine venules, aged-like neutrophils appeared to display enhanced integrin activation and capacity to form DNA-based extracellular traps (or NETs) [27,35]. Notably, these features are significantly blunted in the early morning in mice, when neutrophils are freshly released from the marrow. This

suggests that neutrophils adjust their functions to the changing demands of the day, for example to protect from microbial invasions during the animal's active phase (when the exposure to pathogens is highest) or to exert reparative functions during the resting phase[33,36]. Similar phenotypic oscillations have been found in neutrophils from healthy human volunteers, and correlated with diurnal oscillations in ROS production and phagocytosis[37]. The changing properties of neutrophils during the day align with studies in mice showing that aged neutrophils (i.e., those present at daytime) are more prone to damage the vasculature in a mouse model of sickle cell disease[35]. These findings are also consistent with the observed circadianicity of many forms of vascular disease in mammals[38]. Interestingly, these diurnal changes in neutrophil function correlate with transcriptional changes associated with toll-like receptor and CXCR2-signaling, adhesion and cell death[35], and with dramatic changes in their migratory properties during the day[34]. One interpretation of these observations is that the lifetime of neutrophils in blood allows for synchronous diversification over time.

Of particular interest are the underlying mechanisms of circadian diversification of neutrophils. Glucocorticoid signaling in humans[37], or bacterial-derived metabolites in mice [35,39] have been proposed to drive diurnal aging in neutrophils. Alternatively, we have found that circadian clock genes also regulate the diurnal variations in phenotype and function in a cell-intrinsic manner[34], through a process similar to that reported for inflammatory monocytes [40]. Indeed this would be consistent with diurnal patterns of clock gene expression in human and mouse neutrophils[34,37]. Defining the exact mechanisms underlying neutrophil diversification in blood may hold the key for therapeutic intervention against the detrimental activity of specific subsets, particularly those prone to damage the cardiovascular system [41,42].

Do tissue-specific neutrophils exist?

Tissues provide instructive signals for immune cell activation, differentiation and functional diversity. For example, macrophages are highly responsive to their microenvironment and adopt diverse phenotypes, transcriptional profiles and functions tailored to the demands of each tissue[43,44]. Indeed, despite their "immune" denomination, it is now clear that macrophages perform specialized tissue-supportive functions that are unrelated to immunity: from neuronal maturation in the brain to electrical conduction in the heart[45]. While compelling evidence suggests that, like macrophages, neutrophils are also present in many unperturbed tissues at least in the mouse, they are typically found in low numbers, with the exception of the bone marrow, spleen and lungs[46]. Thus, the prevailing assumption has been that tissue-borne neutrophils reflect technical

contaminations from blood. Challenging this assumption, however, mass cytometry coupled with dimensionality reduction analyses uncovered several clusters of mouse neutrophils in different tissues based on the expression of over 30 markers, hinting for the first time towards true phenotypic diversity in healthy tissues[47]. Consistent with this, we have found that most tissues are actively infiltrated by neutrophils in the steady-state, with the conspicuous exception of the brain and gonads[46]. While the potential functions for these homeostatic populations remain uncertain, it is noteworthy that neutrophils present in the intact skin display scout-like behavior that may allow for early detection of damage, and facilitate secondary recruitment of other neutrophils from the vicinity or from the circulation [48,49]. In the lower female reproductive tract, homeostatic infiltration is regulated by chemokine gradients that form during the ovarian cycle, thereby conferring protection against pathogens that could potentially breach the vaginal lumen [50,51]. In the lungs, a large population of neutrophils is marginated in the pulmonary microcirculation through CXCR4-mediated signaling [52], thereby enabling rapid responses to microbial challenges [53]. These findings suggest that neutrophils in naïve tissues may generally serve as immune sentinels, yet the acquisition of tissue-specific phenotypes suggests that neutrophils may be differentially primed by tissue-derived signals. It is important to remark that these features of neutrophil diversity in mouse tissues are yet to be confirmed in humans.

Intra-tissular heterogeneity can also occur in defined microenvironments, as suggested by studies showing that immature neutrophils in the spleen are immotile while mature neutrophils actively patrol the red pulp, suggesting specialized roles during bacterial infection [54]. In addition, marginal zone neutrophils in the human spleen adopt unique B-cell stimulating properties through the secretion of cytokines, chemoattractants, and the pattern recognition receptor Pentraxin 3 that stimulate class switch and immunoglobulin production by B cells residing in this region [55,56]. These neutrophils, which represent the best characterized pool of tissue neutrophils in humans, acquire their distinctive low levels of CD15 and CD16 and transcriptional signature postnatally, through microbiota-dependent IL-10 and JAK2/STAT3 signaling[56]. A more thorough characterization of neutrophils in other mouse and human tissues will allow defining whether, like macrophages, neutrophils adopt functions tailored to their tissue of residence (**Figure 3**).

4. **Heterogeneity of neutrophils in disease**

While the recognition that neutrophils are phenotypically heterogeneous in healthy tissues is recent, their diversity in conditions of inflammation, infection and chronic disease has been appreciated for decades. Various phenotypic and functional properties of neutrophils rapidly change under conditions of sterile or infectious inflammation. For example, they can adopt different forms of migration across vascular walls [57], express an array of pattern-recognition receptors and secrete different types of cytokines during infections [58], or be endowed or not with the ability to impair T cell activation [59]. In the context of vascular repair and hypoxia, a distinct population of VGEFR1+ CXCR4+ neutrophils was found that displayed tropism for angiogenic foci, produces Bv8, MMP9 and VEGF-A, and cooperates with macrophages for vascular growth[60,61]. Heterogeneity under all these scenarios has been reviewed recently [45,62,63] and will not be further addressed here. Instead, we focus our discussion on chronic inflammatory disease and cancer, as they represent prime examples of disease-induced heterogeneity among neutrophils.

Neutrophil heterogeneity in cancer

Tumors are endowed with a functionally-important immune component. This "immune stroma" plays varying and even opposing roles in disease progression [64]. For example, macrophages can be anti-tumoral at early stages of disease and later adopt pro-tumoral functions as signals from the tumor instruct reparative, immune-suppressive, and pro-angiogenic properties [65]. Only recently neutrophils have emerged as similarly important players and contributors of tumoral stromal, and are found at highly variable numbers within the tumor, depending on the type of cancer [66]. Importantly, a large pan-cancer meta-analysis in thousands of human tumors found a neutrophil signature associated with poor disease outcome despite relative low numbers compared with other leukocyte subsets [67]. In keeping with this notion, the frequency of circulating neutrophils and the ratio between neutrophils and lymphocytes are being evaluated as prognostic biomarkers of cancer progression [68].

Like macrophages, neutrophils appear to undergo a reprogramming process in the spleen to favor tumor growth as shown in an experimental mouse model of K-ras driven lung adenocarcinoma [69]. Consistent with the notion of an immune switch during the course of disease, depletion of neutrophils is detrimental at early disease stages and becomes protective at late stages [70-72]. An outstanding question therefore is how tumor-derived signals reprogram neutrophils to allow this functional switch and heterogeneous behavior. Is it at the BM level whereby decisions on cell differentiation and mobilization are taken? In support of this view, an ACKR2-dependent program in hematopoietic

progenitors was shown to elicit pro-metastatic functions[73]. Additionally, factors produced by the primary tumor (e.g., IL-1β, G-CSF or GM-CSF) can induce granulopoiesis through Rorc1 and C/EBPβ expression and release of immature neutrophils (including the so-called granulocytic myeloid-derived suppressor cell, or G-MDSC) to the circulation and into the tumor [74]. This mobilizing axis appears to be critical for the recruitment of tumor- and metastasis-supportive neutrophils in several settings, including obese mice and in humans, in which GM-CSF critically favors pulmonary metastasis of breast cancer cells [75]. The premature release of neutrophils due to inflammation and cancer can result in the presence of circulating immature cells with incomplete nuclear condensation and lesser granule content, which may contribute to the presence of neutrophils with low buoyant density in patients with cancer or chronic inflammatory disease. Consistent with these elevations, preNeu expansion is observed in the spleen of tumor-bearing mice [12] and splenic immature neutrophils with immunosuppressive properties have also been reported [76]. Thus, cancer triggers a type of "emergency" granulopoiesis similar to that elicited by infection[77] that contributes to neutrophil heterogeneity and disease progression.

Early evidence revealed TGF-β as a central regulator of tumor-associated neutrophil responses, as its blockade induced a functional switch in neutrophils from pro-tumoral to anti-tumoral [78]. More recently, at least three distinct populations of neutrophils were reported in the circulation of tumor-bearing mice and human patients [79] on the basis of density properties. Those with lower density (low density neutrophils, or LDN) increased during disease progression, while high-density neutrophils (HDN), which predominate in healthy individuals, differentiated into LDN through a mechanism dependent on TGF-β that rendered them less cytotoxic against malignant cells [79]. In the context of lung adenocarcinoma, a subset of tumor-infiltrating Siglec-F+ neutrophils with pro-tumoral properties presented a TGF-β signaling signature [80]. Although the pro-tumoral profile was instructed by BM osteoblasts and the Siglec F+ signature already appeared in blood, full reprograming required entry into the lung and correlated with disease outcome in a human cohort [80]. Thus, TGF-β is of particular interest as a neutrophil reprogramming factor given its broad links with tumor progression[81]. Opposing the actions of TGF-β, growing evidence suggests that IFN signaling instruct anti-tumoral properties in neutrophils [82,83]. Collectively, the observations made in the context of cancer highlight the plasticity of neutrophils, and reveal that instructive signals from different tissues (BM, spleen, blood and tumor) can contribute to the phenotypic and functional heterogeneity of neutrophils.

Neutrophil heterogeneity in chronic inflammation

Neutrophils play dominant roles in early stages of inflammation, but can additionally perpetuate damage to organs if the instigating stimulus persists[84]. Like in cancer, acute insults elicit rapid activation of BM niches, resulting in remodeling of stromal elements and activation of myelopoiesis[85,86]. Acute inflammatory insults, including infection or ischemia, induce the production of G-CSF, GM-CSF or other myelopoietic factors that favor granulocyte production [77,85,87]. In humans treated with low doses of endotoxin at least three populations with distinct phenotypic and proteomic properties appear in blood, of which only $CD16^{bright}$ $CD62L^{dim}$ cells have T cell-suppressing activity[59]. This population, however, does not display features of immaturity, suggesting that newly produced neutrophils and those recruited from other sources contribute to generating phenotypic and functional heterogeneity during inflammation. Similarly, G-CSF can also recruit CD10+ mature neutrophils with immunosuppressive functions into the human blood, though interestingly, it additionally mobilizes $CD10^{Neg}$ immature, immunostimulatory neutrophils that promote T cell survival and proliferation[88].

When inflammation is chronified by a persistent stimulus, e.g. elevated cholesterol in atherosclerosis (the main cause of cardiovascular disease (CVD), the sustained production of neutrophils can create a vicious cycle of inflammation and tissue damage [89]. In CVD models, like in cancer, monocytes undergo maturation in the spleen before migrating to the injured tissues[90], suggesting that this may also be an organ of further functional specification for neutrophils during chronic inflammation. Neutrophils have also been associated with long-term neurodegenerative disorders [91,92] and with acute brain damage after stroke[93]. Paradoxically, in a model of stroke neutrophils were essential to reduce brain injury in the presence of rosiglitazone, a PPARγ agonist [94]. These beneficial neutrophils exhibited features of M2-like macrophages (Ym1 and CD206 expression) and could be already detected in the BM and blood of infarcted mice[94]. These observations suggest that neutrophils can be rapidly reprogramed in the BM towards phenotypes that antagonize inflammation, yet the signals and the exact cell populations targeted (mature or immature) remain undefined.

Besides CVD, neutrophils have been prominently associated with autoimmune disease. A prime example is systemic lupus erythematosus (SLE), a disease characterized by the presence of autoantibodies against dsRNA and ribonucleoproteins that deposit in various organs and cause progressive damage[95]. Early studies showed that lupus patients display interferon and neutrophil signatures in blood[96], a finding that was later extended to show that neutrophils incited activation of plasmacytoid dendritic cells and

IFNα production through the release of NETs[97,98]. These DNA-protein structures contain danger-associated molecular patterns (DAMPs) and autoantigens that can additionally elicit antibody production, and have in fact been associated with other forms of autoimmunity including vasculitis, rheumatoid arthritis, antiphosphiolipid syndrome and even type 1 diabetes[99]. In most instances, cytokines, antibodies or metabolites associated with each of these disorders can trigger NET formation, thereby perpetuating disease.

Relevant for our discussion is whether specific populations of neutrophils exist that are prone to produce NETs and trigger disease. Indeed, only a fraction of neutrophils from the blood of healthy individuals form NETs even when challenged with strong agonists. Likewise, there is a marked variability in the capacity of neutrophils across species and even among different mouse strains to form NETs[100]. In the case of SLE, LDN with density features similar to those described in cancer are markedly elevated in the circulation of patients but they are pro-inflammatory as they actively produce inflammatory cytokines and kill endothelial cells in vitro[101]. LDN in lupus patients also display enhanced NET formation, suggesting that the presence of this type of neutrophils may underlie other forms of autoimmune disease[99,102]. As proposed in the context of cancer, LDN could represent populations of immature neutrophils prematurely mobilized from the BM that co-exist with fully mature cells in the blood of lupus patients. The presence of mobilizing cytokines and IFN in these patients may release immature neutrophils filled with granule proteins and partially-condensed DNA, which makes them prone to form NETs. While this remains speculative, it could explain the elevated presence of granule transcripts in lupus-associated LDN and the strong association of SLE with atherosclerosis and CVD[103]. Alternative origins for immune-suppressive neutrophils are nonetheless possible since, for example, activation with potent agonists (LPS, fMLP and PMA) can generate neutrophils of low density but mature morphology with T suppressive activity[59,104]. Intriguingly, epigenetic marks in the neutrophil genome have been associated with different types of autoimmune inflammation in human patients[105], suggesting specific programming of neutrophils under this environment.

5. **Mechanisms of heterogeneity**

While the existence of heterogeneity among neutrophils is now recognized, the underlying mechanism(s) and biological relevance of this diversity remain under debate. To what extent heterogeneity represents bona-fide cell programming rather than activation? Are there unifying mechanisms of diversity? And how do they adapt to

specific pathophysiological contexts? In our earlier discussion we have hinted to two potential and non-mutually exclusive mechanisms: intrinsically-driven heterogeneity of neutrophils in the BM and blood, and exposure to local or systemic extrinsic factors that modify neutrophil properties. Accumulating evidence indicate that both processes impinge on highly coordinated transcriptional and epigenomic dynamics, a feature often overlooked in neutrophils. Below, we highlight recent examples of genomic plasticity in neutrophils, and speculate how they may provide a mechanistic framework to rationalize heterogeneity.

Structure of the neutrophil genome and transcriptional plasticity

The neutrophil nucleus is organized into a peculiar structure with 3-5 lobes, each comprising physically interacting regions located at large distances (> 3 Mb) on the linear DNA[106]. This compacted architecture may provide physical flexibility during crawling or phagocytosis[107], support the formation and release of NETs[108], but may also limit transcriptional dynamics[106]. Indeed, the low RNA content of mature neutrophils as compared to other myeloid cells may be viewed as a constraint to plasticity. Recent analyses are challenging this notion, as broad and selective genomic remodeling occurs throughout the neutrophil life cycle.

Neutrophil maturation is linked to progressive silencing of hundreds of genes controlling biosynthetic and proliferative processes, while granule, antimicrobial and immune response genes are induced[109]. Notably, genes involved in effector functions such as antiviral defense are selectively expressed in human circulating neutrophils as compared to immediate bone marrow precursors[12,110], showing that even terminal neutrophil maturation is linked to active gene transcription. Dynamic changes of the epigenome, namely the repertoire of gene regulatory elements and associated epigenetic, histone and nucleosome marks also occur during neutrophil development[110,111].

At steady-state, mature neutrophils sense and adapt to subtle environmental changes. Recent analyses found that the basal neutrophil transcriptome is highly variable among human donors[112] to a higher extent than monocytes or lymphocytes[113], and that genes with hypervariable expression in neutrophils were enriched in immune functions such as inflammasome activation and antiviral responses. These and other studies[114-117] showed that, while genetic factors dictate most of the inter-individual variability in neutrophil gene expression[114,117], hundreds of high-variance genes might be linked to epigenetic or chromatin control[112,113]. Accordingly, human neutrophils display inter-individual variability in DNA methylation profiles[112,113,115], reinforcing the notion that epigenomic mechanisms may fine-tune neutrophil gene expression.

The extent of transcriptional plasticity of neutrophils is evident upon exposure to stimuli such as microbial components, cytokines and growth factors. Hundreds of genes are modulated under these conditions[116,118,119] in a manner reflecting diverse chromatin-based control[120]. Some proinflammatory genes are induced with very fast kinetics, reaching maximal expression minutes after stimulation. This behavior, exemplified by *CXCL8* (encoding for IL-8), is indicative of a pre-poised local chromatin organization able to support immediate transcription. Conversely, induction of genes such as IL6[121] requires previous chromatin remodeling and deposition of histone marks at regulatory elements in order to permit recruitment and licensing of the transcriptional machinery. Chromatin-dependent mechanisms are also in place to prevent gene induction at specific loci, such as *IL10* in human neutrophils[122]. Thus, both pre-existing and stimulus-induced locus accessibility and chromatin modifications enable dynamic responses to micro-environmental signals, overall contributing to the plastic phenotype of neutrophils.

Genomic mechanisms of neutrophil plasticity: nature or nurture?

Accumulating evidence indicate that neutrophils are capable of functional, phenotypic and molecular adaptations to context-specific cues. While these features are incorporated into mechanistic models for plastic immune cells such as macrophages [123], analogous frameworks are not available for neutrophils. We suggest that, at least to some extent, available principles of genomic organization may also apply to neutrophils and help to rationalize their plasticity and context-dependent heterogeneity.

In macrophages, few lineage-determining TFs (LDTFs) like PU.1 and C/EBPα/β collaborate with a heterogeneous set of TFs with tissue-restricted[43,124,125] and/or stimulus-dependent activity[126,127,128,129] to specify the repertoire of active promoters and enhancers and ensuing gene expression programs. Myeloid LDTFs can access their target sequences and modify the surrounding chromatin even when ectopically expressed in unrelated cell types (a property of 'pioneer TFs')[130]. Because neutrophils express PU.1 and C/EBPα/β at high levels and require them for proper maturation and stimulus-induced gene expression[109], it is plausible that myeloid LDTFs may also establish the epigenome of these cells during granulopoiesis, likely in coordination with other TFs[131] (**Box 2**). Whether tissue-restricted TFs also act in neutrophils as they migrate to tissues and are exposed to local homeostatic signals, such as heme in the spleen, remains to be determined.

Understanding how the neutrophil epigenome is established during differentiation is relevant, since pre-existing epigenomic differences between neutrophil subsets in the

BM or blood may contribute to neutrophil heterogeneity in tissues or disease (**Figure 4**). Upon stress, neutrophils at different stages of maturation, with diverse chromatin and transcriptional landscapes[113] are mobilized from the BM or recruited from the blood to target sites. This is evident in tumors, where immature and mature neutrophils often co-exist, are exposed to a common milieu but display heterogeneous activation states [132]. One possible explanation for this diversity could be that the neutrophil subsets recruited to tumors may mount different transcriptional responses to shared extrinsic signals. While this remains speculative at the moment, it is well-known that the binding sites of most stimulus-activated TFs is largely cell type-specific and is dictated by the pre-existing chromatin landscape. For instance, TGF-β stimulation of myeloid, muscle or embryonic stem cells resulted in binding of SMAD TFs to different sites, previously made accessible by LDTFs[133]. Analogously, other families of stimulus-activated TF, including NF-κB and STATs, bind to the genome in a cell type-specific fashion and lead to diverse transcriptional outputs[129,134]. An attractive hypothesis is therefore that cytokines or other stimuli present in the tumor microenvironment or different tissues may trigger different biological outputs in recruited neutrophil subsets, at least partly because of differences in the chromatin landscape. Recent and future developments linking high-resolution single-cell genomics, lineage tracing and imaging technologies are poised to address these issues and uncover the rules of neutrophil diversity.

6. Final remarks and insights into the future

With the increased appreciation that neutrophils are far more heterogeneous that initially thought, and the characterization of new populations under health and disease, it is becoming clear that these cells are in functional terms far more than mere effectors of inflammation. High-end analytical technologies including genomic and epigenomic sequencing at single cell resolution, advanced imaging and mass cytometry will expand the palette of neutrophil subsets and discover new functions. This knowledge will in turn open up the possibility to harness the therapeutic potential of neutrophil subsets. For instance, the proliferative neutrophil precursors could be used as a bridging treatment when transferred in combination with HSC to accelerate the recovery of hematopoiesis, and to enhance the immune competence of patients undergoing BM transplantation. On the other hand, dissection of the mechanisms underlying heterogeneity will offer new avenues for therapeutic intervention in diseases driven by neutrophils, for example by promoting effector functions during neutropenia or suppressive properties in autoimmune disorders. Likewise, manipulation of circadian aging may provide benefit by

promoting clearance of neutrophils from blood into tissues, thereby improving immune surveillance while at the same time protecting the vasculature from their toxic action. Finally, development of new anti-tumoral strategies will enormously benefit from proper comprehension of the origin and programing mechanisms of tumor-supportive neutrophils. The exponential growth that we are witnessing in this emerging area of research should place neutrophils –in its many flavors- in a prominent position among immune cells.

Acknowledgements

We are grateful to members of our labs for continued enthusiasm and discussions, which are reflected in many parts of this text, and J.M. Adrover for art. We apologize to the many colleagues whose contributions could not be discussed in this review. This review paper is supported by Singapore Immunology Network (A*STAR) core funding to L.G.Ng. This paper is also supported in part by SAF2015-65607-R and Fondo Europeo de Desarrollo Regional (FEDER) to A.H. The CNIC is supported by the Ministerio de Ciencia, Innovacion y Universidades (MCIU) and the Pro CNIC Foundation, and is a Severo Ochoa Center of Excellence (MCIU award SEV-2015-0505). Research in the R.O. lab is supported by grants from the European Research Council (ERC Starting Grant # 759532, X-TAM), Italian Telethon Foundation (SR-Tiget grant award F04), Italian Ministry of Health (GR-2016-02362156), Associazione Italiana per la Ricerca sul Cancro (AIRC MFAG, # 20247), Cariplo Foundation (2015-0990) and the EU (Infect-ERA #126).

The authors declare no conflicts of interest.

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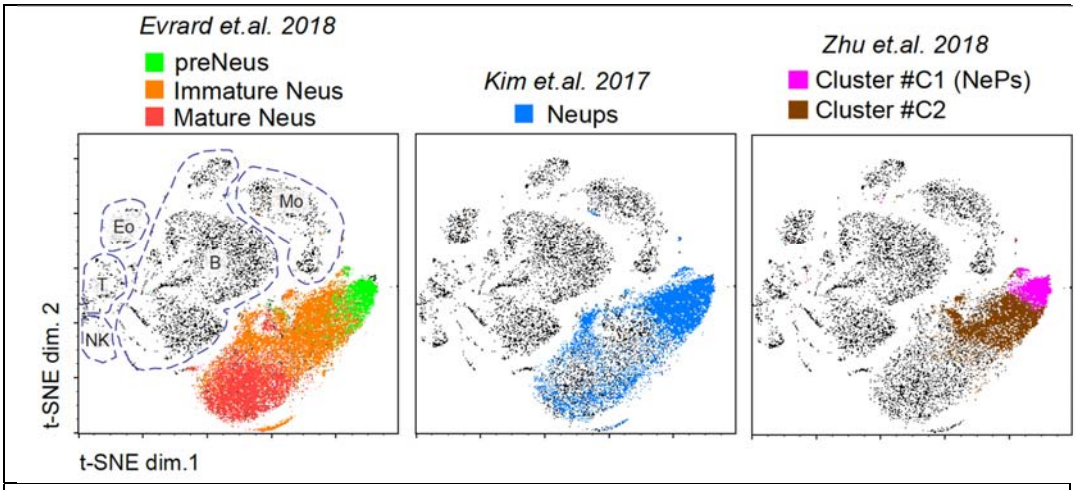
Box1: Neutrophil heterogeneity in the bone marrow

Within the bone marrow, stromal, vascular and perivascular cells constitute the hematopoietic niche that provides instructive signals for the maintenance and differentiation of hematopoietic precursors[23]. Various studies have shown that hematopoietic stem cells and progenitor cells, including preNeu, are in close contact CXCL12-expressing stromal cells in the bone marrow[12]. While the CXCL12-CXCR4 signaling pathway is crucial for preNeu retention in the bone marrow, CXCR4-mediated signals are dispensable for their differentiation into mature neutrophils. Unlike mature neutrophils, immature neutrophils do not express CXCR2 and are normally absent from the circulation. Because CXCR2 signaling is essential for neutrophil mobilization from the BM[52,135,136], this observation may suggest that immature neutrophils are programmed to remain in this organ. However, in response to inflammatory stimuli, these cells can be mobilized into the circulation and recruited to sites of inflammation much like mature neutrophils. In contrast, preNeu are not mobilized to the circulation or affected tissues during inflammatory responses[12]. These migratory and other characteristics observed in the BM are indicative of significant heterogeneity of neutrophils during maturation. This raises the fundamental question of whether heterogeneity originates only from the release into blood of medullary neutrophils at different stages of maturation, and/or through "priming" of homogeneous circulating neutrophils by local signals in the extramedullary milieu. Here, we propose a model whereby this pre-neutrophil (preNeu) population serves as proliferative pool that can rapidly amplify neutrophil numbers on demand; in contrast, non-proliferative immature neutrophils represent a reservoir of neutrophils that can be rapidly deployed to the circulation, and mature neutrophils are important for effector functions. Additional subsets of recently identified unipotent neutrophil precursors further add to resolving the stages of neutrophil specification in the marrow. One study delineates these proliferative precursors into neutrophil precursors (NeP) and late-stage precursors[14], while another study identified a late-lineage murine neutrophil precursor in the bone marrow (NeuP)[13]. Interestingly, NeP were shown to have pro-tumoral function[14]. Figure A presents a comparative overview of the analyses defining these various committed precursors and possible overlaps at the single cell level. While the nomenclature differs among these studies, the overarching concept is that medullary neutrophil subsets can be defined by specific phenotypic, proliferative, transcriptional and functional properties.



Box 1 Figure A: t-SNE analysis of neutrophil precursors. Aggregate t-SNE analysis of mouse bone marrow cells with the mapping of different neutrophil precursors. The leukocyte subsets, as well as the BM neutrophil subsets are annotated and color-coded on the basis of published studies (Refs [12-14]).

Box 2: Maturation TFs and the organization of neutrophil epigenomes

As the myeloid lineage-determining TF PU.1 and C/EBPα/β are generally expressed at high levels throughout neutrophil development, it is likely that additional TF control stage-specific epigenomic organization. These TF are also expected to be required for proper neutrophil differentiation, so that their absence or dysfunction is linked to congenital neutropenias [137]. One example is C/EBPε, which is expressed in lineage-committed granulocyte precursors and its deletion leads to neutrophil progenitor arrest, defective expression of genes encoding for granule proteins and neutropenia [3,9,12,138]. KLF5 is also active during early stages of granulocyte differentiation, where it controls neutrophil production at the expense of eosinophils [139]. LEF1 is expressed in myeloid progenitors and its inactivation leads to a differentiation block at the promyelocyte stage, as revealed by studies in individuals with congenital neutropenia [140]. In addition to positive regulators of neutrophil maturation, it is likely that repressive mechanisms play a role in establishing the neutrophil epigenome. The transcriptional repressor GFI1 is expressed during early stages of monocyte-neutrophil commitment and is essential for neutrophil development [141]. Indeed, GFI1 is co-expressed with IRF8 in rare populations of hematopoietic progenitors with bivalent monocyte-neutrophil potential, and counteracts the formation and maintenance of IRF8-induced enhancers[142]. The antagonistic circuit involving GFI1 and IRF8 is likely to be a critical component of neutrophil maturation, as IRF8 is a major driver of monocyte lineage commitment and expansion at the expense of neutrophils [143-145], and it actively controls the formation of the enhancer landscape in these cells [146,147]. As more high-resolution genomic analyses of neutrophil maturation are performed [12,14,110], we expect more candidates to be added to this list.

Figures and figure legends

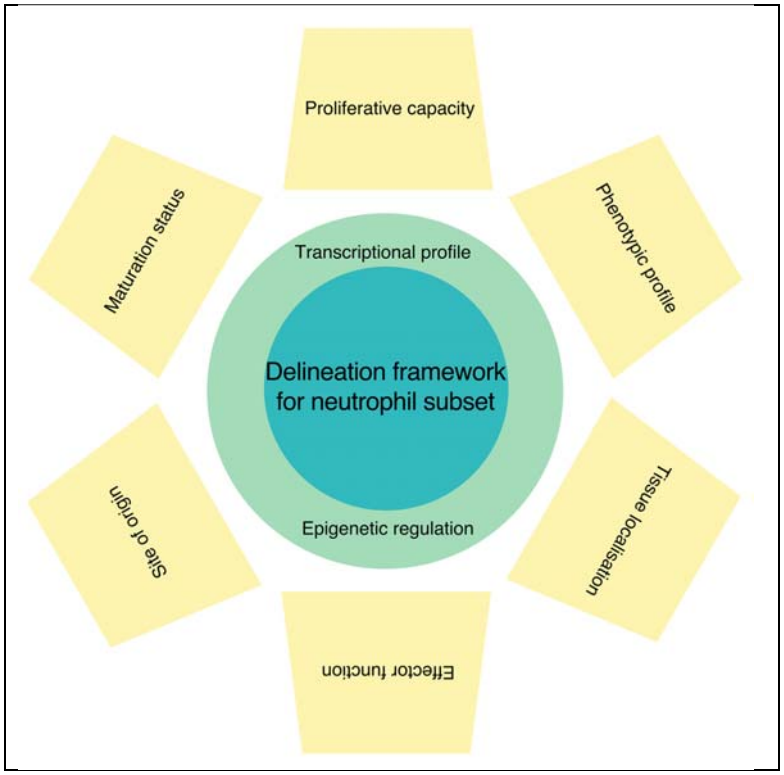


Figure 1: A framework for subset identification. A systematic and integrated framework for assessing neutrophil subsets based on their proliferative capacity, maturation status, phenotypic profile, site of origin, tissue localization and effector function, which can change rapidly. In contrast, transcriptional and epigenetic properties represent core characteristics for longer-term marking of true subsets.

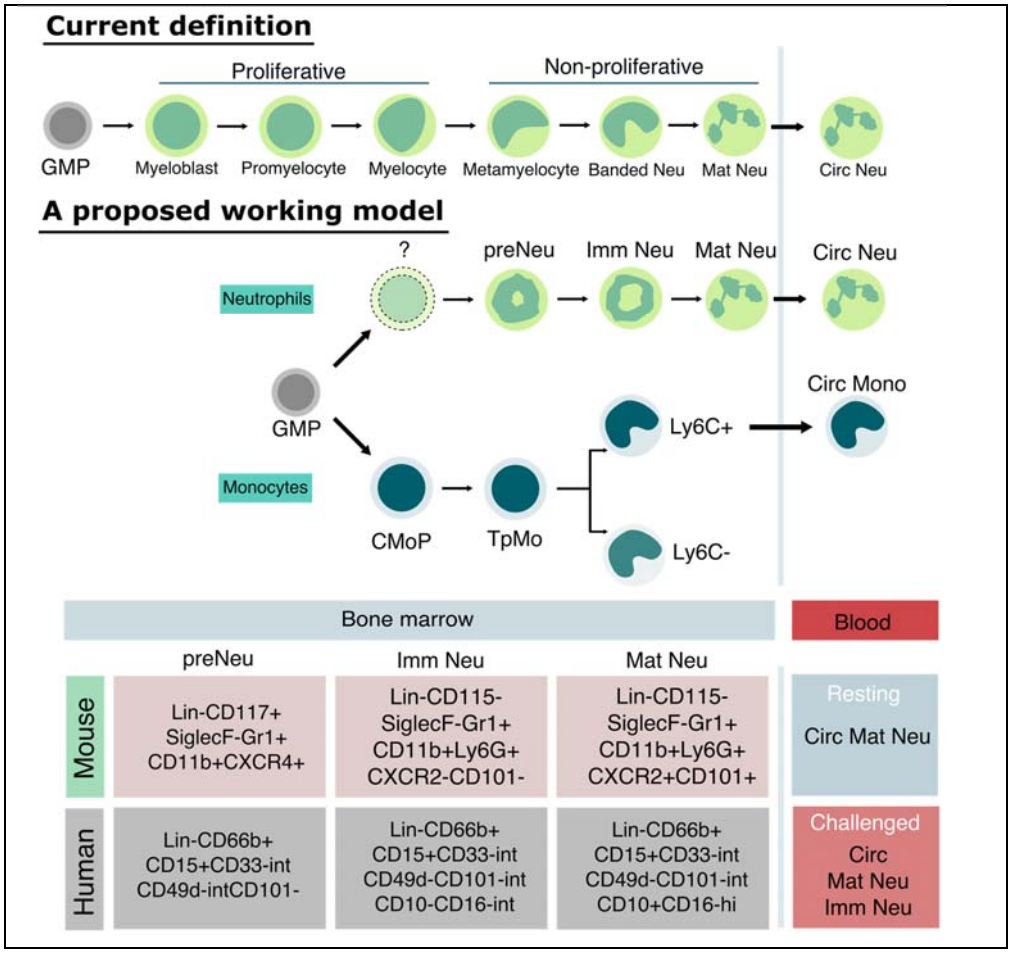


Figure 2: The neutrophil differentiation pathway. Neutrophils are derived from granulocyte-monocyte progenitors (GMP). Current characterization of neutrophil development primarily divides into two major phases, a proliferative stage whereby GMP differentiates to myeloblasts, promyelocytes and myelocytes. This is followed by a non-proliferative stage in which myelocytes give rise to non-proliferating metamyelocyte, band cells and finally mature into neutrophil. Here, we proposed a working model in which bone marrow neutrophils in mouse and human can be divided into three subsets: a committed proliferative pre-neutrophil (preNeu) that sequentially differentiates into non-proliferating immature neutrophils (Imm Neu) and mature neutrophils (Mat Neu). Comparing this pathway to the developmental hierarchy of monocytes, preNeus have the functional attributes of transitional pre-monocytes (TpMo), suggesting that there could be a "common neutrophil progenitor" that is equivalent to the common monocyte progenitor (cMoP). Of note, a recent study identified a heterogeneous early neutrophil progenitor that is likely upstream of preNeu[14]. It will be interesting to further define the earliest steps of neutrophil progenitor specification and their subsequent commitment during granulopoiesis. In the steady-state, only Mat Neu are detected in the circulation. In response to inflammatory stimuli, Imm Neu are also released into the circulation[12]. This proposed working model may provide a basis for the re-examination of granulopoiesis within the broader context of myeloid cell development, paving the way toward better alignment of neutrophil functional heterogeneity in mouse and human. Notably,

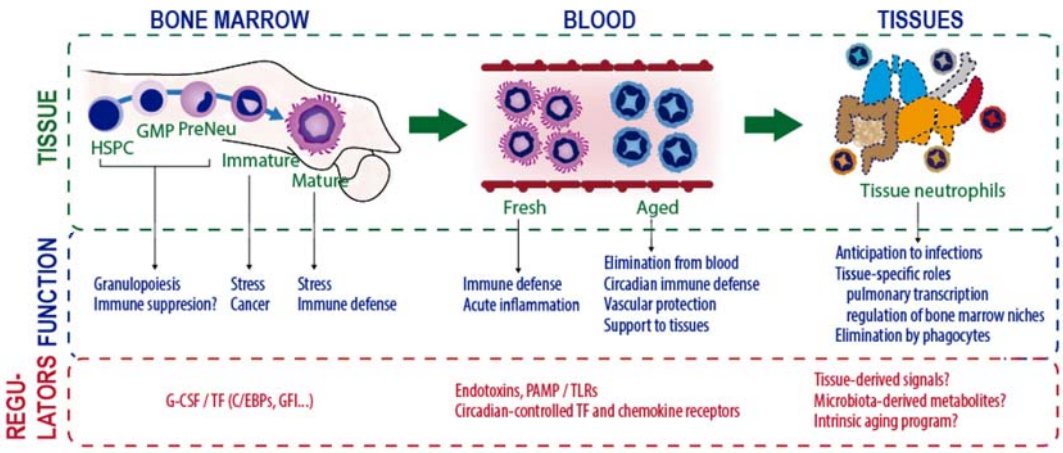


Figure 3. Stages of neutrophil heterogeneity in the steady-state. Progressively mature neutrophils in the bone marrow can be discriminated by defined sets of markers and morphological phenotypes, from GMP to mature neutrophils, and display distinct functions: basic granulopoiesis (GMP and preNeu), roles under stress (including cancer) which mobilize immature cells, and finally mature neutrophils which enter the bloodstream in the steady-state or during stress situations for immune defense. Once in blood, neutrophils undergo circadian aging, a process that instructs additional heterogeneity during the day, and induces a functional switch from merely defensive (fresh) to homeostatic clearance from blood and infiltration of tissues (aged). Circadian alterations in blood and entry into tissues possibly confer vascular protection against excessive inflammation, and anticipates potential infections in tissues. Once in tissues, neutrophils may undergo further phenotypic and functional diversification, display support roles in at least certain tissues (lungs and bone marrow), and are ultimately eliminated by phagocytosis. Various mechanisms could mediate the homeostatic changes of neutrophils during their life cycle, including cell-intrinsic myeloid- and circadian-related transcription factors (TF), or environmental cues derived from the microbiota or from tissues.

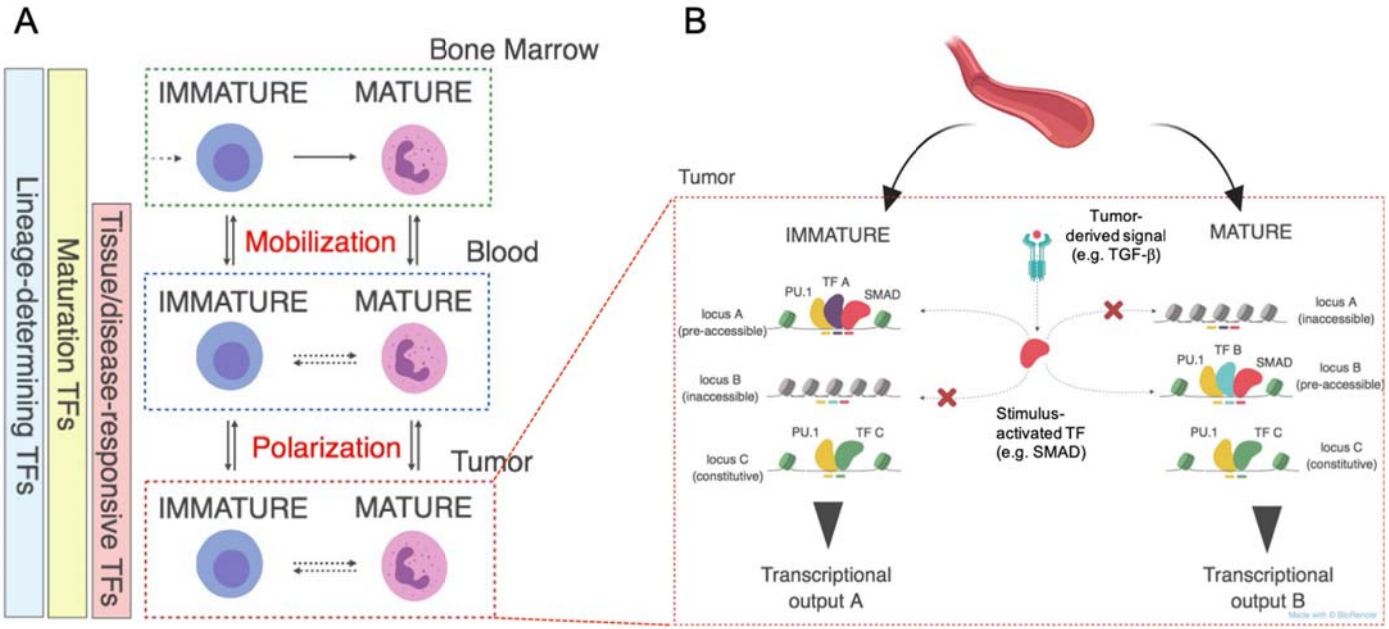


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Figure 4. A model for genomic control of neutrophil heterogeneity in cancer. A) During homeostasis, neutrophil differentiation in the bone marrow is controlled by myeloid lineage-determining TF (*e.g.* PU.1, C/EBPα/β) as well as by TFs (see **Box 2**) with stage-specific expression or activity. We hypothesize that the combinatorial actions of these TF may shape intrinsic epigenomic diversity of neutrophil subsets. In this model, upon systemic inflammatory stress elicited by growing tumors (*e.g.* G-CSF), neutrophil populations with diverse stages of maturation are mobilized to the blood and to the tumor tissue, where both immature and mature neutrophils are exposed to tumor-derived factors that further activate tissue/disease-associated TF. **B**) Genomic model describing how pre-existing differences in the epigenomic landscape of neutrophil subsets (e.g. immature versus mature) may influence transcriptional response to shared tumor-derived signals. Representative loci are shown to exemplify a constitutively active region (locus C), and two loci that are selectively accessible in immature (locus A) or mature (locus B) neutrophils as a consequence of the genomic activity of different maturation TFs (TF A and TF B, respectively). Upon exposure to tumor-derived factors (e.g. TGF-β), activated TFs (represented in the Figure by SMAD TFs) bind to already accessible sites, result in diverse TF occupancy genome-wide and in distinct transcriptional outputs. While the model depicted here is an over-simplification and does require experimental validation, we propose that it may provide a framework to rationalize neutrophil diversity.