How to bridle a neutrophil

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Abstract

Recent high-dimensional technologies have enabled the characterization of heterogeneity in the neutrophil compartment at an unprecedented resolution. In this review we discuss the emerging notion of heterogeneity within the neutrophil pool, and provide a detailed account of evolving concepts in the field. We place special focus on neutrophil differentiation in the bone marrow and plasticity in tissues, describe the limitations that arise when exploring neutrophil heterogeneity using single-cell analyses, and suggest state-of-the-art alternatives to improve their characterization. Finally, we propose strategies arising from these new concepts that may allow us to bridle neutrophil plasticity towards therapeutic benefit.

Highlights

-Recent identification of unipotent neutrophil progenitors in the mouse and human marrow

-Do neutrophils change in tissues?

-Definition of new neutrophil identities by single-cell multiomic approaches

-Can we reprogram neutrophils for therapeutic use?

Introduction

Diversity in the immune system is manifested by extensive cellular variation and recent high-dimensional technologies have enabled its characterization at an unprecedented resolution [1]. Indeed, deep profiling of individual cells identified unknown myeloid cell states in the central nervous system during health and disease [2,3], characterized new dendritic cell subsets in human blood [4] or redefined the differentiation states that occurs within the bone marrow progenitor pool during hematopoiesis [1-5].

Among myeloid cells, neutrophils are the most abundant (50–75%) leukocytes in human blood and form an essential part of the innate immune system. Importantly, however, their short lifespan has been perceived as a roadblock towards functional diversity. This property, in turn, has limited a thorough characterization of their true heterogeneity and the mechanisms underlying their potential diversity [5]. Nevertheless, neutrophils are growingly appreciated as a heterogeneous population of cells such that changes in quality, rather than mere changes in numbers, are now recognized culprits in chronic diseases such as cancer or atherosclerosis [6-8]. The nature of this heterogeneity, and their possible biological function, is still controversial; however, recent studies are uncovering its broad potential as a therapeutic, diagnostic or prognostic tool [7-15]. The challenge now resides in our ability to bridle neutrophil heterogeneity towards therapeutic benefit.

Neutrophil commitment in the bone marrow: a source of heterogeneity?

Neutrophils that reach the circulation are post-mitotic, non-diving cells. Following inflammation, increasing levels of systemic cytokines, such as granulocyte colony-stimulating (G-CSF), induce early release of neutrophils from the bone marrow to the blood [16]. These immature forms are also detected in mouse and human cancers and display a different functional capacity when compared to mature neutrophils, including reduced phagocytosis, enhanced immune-suppressive properties, reduced NETosis and reduced granularity [17]. However, identification of immature neutrophils has traditionally relied on morphology, surface marker expression, or physical separation in density gradients, which while simple and robust provide an incomplete picture of their true phenotypic diversity.

Refined profiling of the bone marrow myeloid pool by mass cytometry (CyTOF) has recently shed light on the molecular features of neutrophil maturation states, and characterized the phenotype of early progenitors already committed to the neutrophil lineage both in humans and mice [10,14]. A first study proposed a three-stage

compartmentalization of neutrophils in the murine bone marrow: a committed proliferative neutrophil precursor (referred to as *preNeu*, and defined as ckit+ CXCR4+ Gr1+ CXCR2^{NEG}) which differentiates into non-proliferating immature (Ly6G+ CXCR2^{NEG} CD101^{NEG}), and finally mature neutrophils (Ly6G+ CXCR2+ CD101+) [10]. A second study identified an early granulocytic progenitor (referred to as *Nep1*; ckit+ Gfi1^{low} Cebpa^{hi} Ly6G^{low}) with long-term unipotency *in vivo*, and a late-stage precursor (*Nep2*; ckit+ Gfi1^{hi} Cebpa^{low} Ly6G+) featuring a phenotype that resembles the *preNeu* state from the first study [14]. Importantly, quantitative changes in transcription factor (TFs) abundance in *preNeu* and *NeP*, including silencing of *Irf8* and activation of *Cebpe*, agree with regulatory processes previously identified during myeloid differentiation [10,14]. *PreNeu* and *NeP* expand upon systemic infection or tumoral stress [10,14], suggesting that these newly-defined early neutrophil progenitors could be used as biomarkers for early cancer discovery and even prognosis. Indeed, *NeP* and immature neutrophils that arise from proliferating *preNeu* correlate with tumor growth in mouse and human cancers (Fig. 1) [10,14]. These studies not only identified for the first time early unipotent, neutrophil-committed progenitors, but also suggest that these cells could be preferred targets of chronic disease states when mobilized into the periphery.

The contribution of *NeP* and *preNeu* to neutrophil heterogeneity, however, goes beyond their ability to be mobilized in response to systemic inflammation. Indeed, we currently know very little about their biology: Are these cells all equal? Are they amenable to the so-called immune regulatory processes? How could this impact on the phenotype and function of their progeny? This may be relevant, for example, in light of recent studies demonstrating that myeloid progenitors in the bone marrow are an integral component of the so-called “trained” immunity [18-20], an ability of innate immune cells to acquire “memory” of a challenge with pathogens. Indeed, the capacity of bone marrow progenitors to transmit this information to their progeny may be physiologically relevant as it leads to long-lasting alterations in myelopoiesis [18-20]. We speculate that discrete populations of unipotent neutrophil progenitors (e.g. *NeP* or *preNeu*) could transmit their epigenetic memory to the post-mitotic neutrophil pool. To unequivocally prove this, however, is challenging and can only be achieved by taking advantage of novel technical approaches. Recent efforts integrating genetic lineage tracing with single cell RNA sequencing yielded a detailed whole-genome state of hematopoietic cells that associated with their long-term dynamic behavior [21]. This or similar approaches may allow identifying discrete populations of committed progenitors that generate specific neutrophil descendants, including those harboring unique and long-lasting properties in phenotype and function.

Neutrophil reprogramming in tissues?

Neutrophils outside the bone marrow are present in at least two different pools: a free-flowing intravascular blood pool, and a pool residing within blood vessels of certain tissues but not in circulation, which is typically referred to as the "marginated pool" [22]. In addition to these intravascular pools, we have described widespread entry of neutrophils from blood into naïve tissues, including skin, intestine or bone marrow [23]. Intravital microscopy imaging of the murine lung microvasculature revealed a substantial number of neutrophils within the network of small capillary vessels that are rapidly mobilized by plerixafor, an antagonist of the chemokine receptor CXCR4 [24]. This finding demonstrated that neutrophils can enter healthy tissues, albeit with variable numbers depending on the tissue, implying that during their lifespan neutrophils do interact with a wide range of cell types and tissue components. This may be particularly relevant because environmental signals are known to induce epigenetic and transcriptional changes in myeloid cells, as shown for tissue resident macrophages [25-27]. A paradigm of where and how this genetic imprinting occurs has been recently defined, and shown to involve specific "niches" or areas within a tissue that provides the right signals to promote differentiation. For instance, specific niches orchestrate monocyte reprograming in the liver, lung [28-30] and, less efficiently, in the skin and brain [31,32]. These studies highlighted the remarkable plasticity of myeloid cells as they interact with the environment, and open important questions regarding the potential plasticity of neutrophils: Can neutrophils be reprogrammed by tissue signals? If so, does this affect their lifespan and function? Which are the molecular drivers that trigger, or limit, plasticity in mature neutrophils? And perhaps more importantly, what would the consequences of this reprogramming be for the normal physiology of the tissues?

In situ specification of mature neutrophils was first proposed to take place in the context of cancer [8]. This reprograming is now believed to be initiated by multiple factors in the tumor. For example, a recent study identified that granulocyte–macrophage colony-stimulating factor (GM-CSF) activates the transcription factor STAT5, which in turn promotes the expression of the fatty acid transport protein 2 (FATP2), a membrane protein involved in the uptake of arachidonic acid. The increase in intracellular arachidonic acid in turn enables synthesis of prostaglandin E2 (PGE2), whose immunosuppressive properties render these neutrophils pro-tumorigenic [33]. Although this study found $FATP2^{+}$ neutrophils in the blood of tumor bearing mice and cancer patients, recent single cell studies revealed that pro-tumoral neutrophil populations can be found in the tumor site of both human and mouse lung cancer, but not in blood [15], strongly suggesting that local signals are needed to reprogram tumor associated

neutrophil [15]. Similar evidence for locally-induced transcriptional reprogramming of neutrophils was found in the context of allergic asthma in the lung [13]. In both settings, neutrophil heterogeneity associates with specific transcriptional profiles. Transcriptional reprograming in asthma included responses associated with the formation and release of NETs [13]. On the other hand, the transcriptome of tumor-associated neutrophils differed in the expression of more than 700 transcripts when compared to those from blood. This included specific regulation of chemokines and cytokine receptors (i.e., CXCL8, IL17RA) and the upregulation of type I interferon response genes [15].

To fully understand the influence of local environments on neutrophil plasticity, we will first need to identify whether tissue "niches" that reprogram neutrophil states indeed exist, and the signals that control such phenotypic switch. For example, in the liver, coordinated interactions of monocytes with hepatocytes induces the expression of the specific Kupffer cell regulator *Id3*, whereas endothelial cells and stellate cells induces expression of LXRα, a TF needed for differentiation to a Kupffer-like program [29]. We propose that neutrophils could be instructed in similar ways (Fig. 1). Thus, physical characterization of neutrophil niches within tissues will be key to identify possible instructing signals, and to begin answering the questions raised above. Another emerging issue, discussed below, is how to identify *bona fide* neutrophil subsets.

Single cell analysis and the neutrophil conundrum

Given the success of single cell RNA-sequencing (scRNA-seq) in uncovering cell subpopulations, as best illustrated for macrophages [34-37], it is not surprising that this has been the technique of choice to gain insights on neutrophil heterogeneity in tissues (**Table 1**). High throughput single cell RNA sequencing platforms, including drop-based and microwell-based methods have been recently used to generate single cell atlases [38,39], which offered cell profiling from a wide range of mouse tissues. Our own efforts, however, make it clear that even the mere identification of neutrophils in these databases is challenging. First, because the number of neutrophils found in most tissues is low (with the exception of the bone marrow and blood); and second, because the transcriptional activity of neutrophils rapidly decreases once they leave the marrow, the RNA yield is low when compared to other cells [15,22]. Given that one of the first steps in single cell analyses is filtering out cells with low transcript counts, it is likely that most neutrophils are eliminated during single cell analyses in tissues, especially if they are not the primary cells under study. This, together with the relatively low depth of scRNA-seq techniques and the unbiased nature of these approaches complicate accurate identification of neutrophil in most tissues.

As described above, however, droplet-based single cell sequencing studies have provided evidence of neutrophil heterogeneity in the lungs [13,15]. In one of these studies, neutrophils were annotated using datasets from IMMGEN as a reference [15]. However, using pre-annotated profiles as a reference is not a reliable strategy to classify cells that feature non-canonical signatures, as may be the case for neutrophils in many tissues. In this regard, it is important to note that common neutrophil markers such as *Ly6g* in mice, or *Ceacam8* (CD66b*)* in humans, are usually undetectable in the single cell transcriptomes and cannot be used for their identification. When automated classification is not sufficient, others have used manual inspection of the principal markers in the clusters obtained after an unbiased clustering in order to identify neutrophil subsets in the lung [13]. This step adds further complexity and creates a "neutrophil conundrum", because the most widely-used clustering methods require an *a priori* knowledge of the level of complexity they should be looking for. In essence, some sort of complexity parameter must be specified which represents the estimate of the overall heterogeneity. This is a major limitation when searching for heterogeneity itself; if the complexity parameter is set too low it may lead to loss of subtle but relevant differences, which is likely the case for most neutrophil subsets. On the other hand, setting the complexity too high can produce spurious heterogeneity to arise. CITE-seq (Cellular Indexing of Transcriptomes and Epitopes by Sequencing) and MARS-seq (Massively parallel single-cell RNA-sequencing) technologies could provide acceptable solutions to this problem [40,41]. The first combines single-cell antibody-derived tagging with RNA sequencing, while the second allows RNA sequencing of cells previously indexed during sorting. In both cases, the end result is that for each cell we know its surface marker and transcriptional profiles, thus allowing accurate analyses of heterogeneity within the population of interest.

In addition to indexed sequencing, obtaining single cell transcriptomics and epigenomics of the same cell coupled with surface marking can provide a solid ground for basic and clinical determination of heterogeneity. For instance, a recent study has taken advantage of CITE-seq and single cell ATAC sequencing data to effectively profile neutropenia-associated human polymorphisms introduced in mouse models. Use of Seurat, a popular single cell analysis suite, to combine both datasets proved useful to demonstrate that human mutations alter neutrophil development, thus causing neutropenia. [42].

Even with improved single cell multiomics, an important piece of the puzzle to understand neutrophil heterogeneity, namely spatial information, is still missing. Most single cell

protocols require that tissues be digested in order to isolate the cells, making it impossible to define where the cell was originally located within the tissue. Recent approaches have tackled this particular issue by using a grid to label the cells from tissue sections before proceeding with sequencing [43]. Given previous reports suggesting that neutrophils may have defined niches in some tissues [23], spatial transcriptomics would be an extremely useful resource to corroborate this possibility. Further, this technique may provide a better understanding of myeloid cell heterogeneity and how their interactions with localized niches in tissues modulate their plasticity.

Bridling neutrophils for therapy

Several therapeutic interventions targeting neutrophil recruitment, production or activation have been proposed (For review see [44]). A poorly explored alternative, however, is the possibility to target cell reprogramming and to exploit neutrophil plasticity for therapeutic purposes. In particular, targeting neutrophils in the context of cancer is an attractive candidate for these approaches. This interest emanates from the realization that they constitute a relevant part of the tumor microenvironment, that they are actively involved in disease progression and metastasis, and that they can be reprogramed in vivo towards pro-tumoral or anti-tumoral phenotypes. In some tumor models, reprogramming has been shown to involve TGF-β and type-1 interferons (IFN) (Fig. 1) [8]. Blockade of TGF-β, or IFN administration enhanced the anti-tumoral capacity of neutrophils [45], but unfortunately caused significant side effects in cancer patients including fatigue, skin alterations, flu-like symptoms or psychiatric sequelae [46,47]. However, their therapeutic potential in combination with immunotherapy or other strategies is promising [48,49]. In this regard, TGF-β antagonists, such as *galunisertib* [50] or IFN pathway activators such as DMXAA (5,6-dimethylxanthenone-4-acetic acid) [51], could be delivered to neutrophils *in vivo* by using targeted immunotherapy, e.g. by aiming at relatively neutrophil-specific receptors such as CD15, CD66b or CD16.

Other potential strategies may co-opt natural changes occurring in blood. When neutrophils are released to the circulation, they undergo phenotypic shifts that adjust to light and dark (i.e., circadian) cycles. These natural changes can have major impact on the neutrophil's immune and inflammatory properties (for rev. see [52]). For example, the so-called "aged" neutrophils that enter tissues during the behavioral active phase of a mouse (night), display enhanced anti-microbial responses but can cause severe thrombo-inflammatory reactions if forced to stay within blood vessels [9]. In contrast, "fresh" neutrophils, which are abundant in blood in the early morning, spare infarcted tissues from inflammation, but have weaker anti-microbial properties [9]. Because the

molecular mechanism underlying this phenomenon of neutrophil “aging” has been identified [9,53], it should be feasible to manipulate this natural reprogramming phenomenon therapeutically. Specifically, blocking neutrophil aging in patients at risk of cardiovascular events might be beneficial, whereas immunocompromised patients susceptible to infections might benefit from drugs that promote neutrophil aging.

In a broader context, neutrophil subsets with pro-angiogenic, immunosuppressive or antitumoral programs have been identified [33,54,55]. Refined characterization of these subsets using single cell technologies alone or in combination with approaches to determine their spatial, epigenetic or signaling profiles, could illuminate key upstream regulators that ultimately orchestrate neutrophil heterogeneity, and could be the basis for efficient reprograming of neutrophil subsets in many pathologies.

Conflicts of interest statement

The authors declare no conflicts of interest

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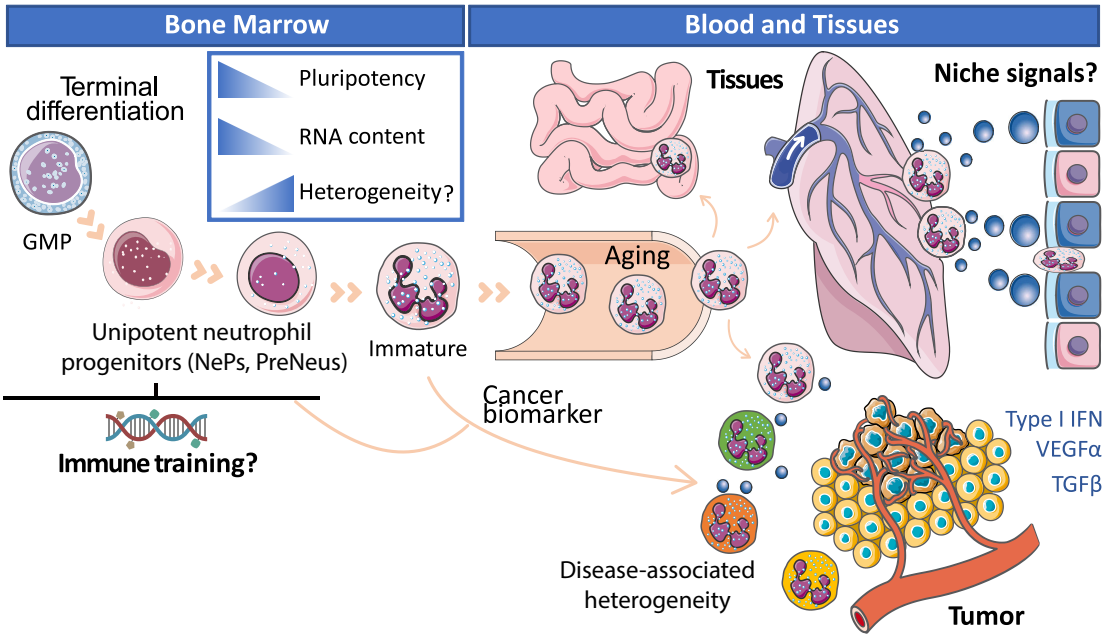

Figure 1. Neutrophil differentiation and specification in tissues. Progenitors that show unipotency for neutrophils within the committed neutrophil pool (*PreNeu* and *NeP*) were recently identified in human and mouse bone marrow. These progenitors expand upon tumoral stress and can be used as cancer biomarkers. Whether neutrophil progenitors are amenable to training or other regulatory processes is currently unknow. When neutrophils are released to the circulation they undergo a natural phenotypic shift called *neutrophil aging* that critically affects their immune function, a phenomenon that may be potentially manipulated for therapeutic purposes. In addition, neutrophils can be found in several tissues during homeostasis, with variable numbers depending on the tissue. How local microenvironments imprint neutrophil heterogeneity is currently not well understood but there is evidence for their locally-induced reprogramming and heterogeneity in tissues under pathological conditions. In tumors, factors such as TGFβ, or type I IFN have been proposed to promote neutrophil polarization *in situ*. We propose the existence of tissue "niches" that reprogram neutrophil fates similar to those previously described for other myeloid subsets.

Table 1 Available single cell transcriptomics public datasets containing neutrophils in tissues

Dataset	Type	Aprox. cell count	Technology	Classification method	Reference	Accession
Radermecker	Specific research	6300	Chromium	Manual	Radermecker et. al., 2019	E-MTAB-6902
Zilionis	Specific research	16000	inDrop	Automated classifier	Zillionis et. al., 2019	GSE127465
Tabula Muris	Atlas	100000	SMART-Seq2/Chromium	Manual	Schaum et. al., 2018	GSE109774
MCA	Atlas	400000	Microwell-seq	Manual	Han et. al., 2018	GSE108097

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Importance (*High; **Very high)

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Figure 1. Neutrophil differentiation and specification in tissues

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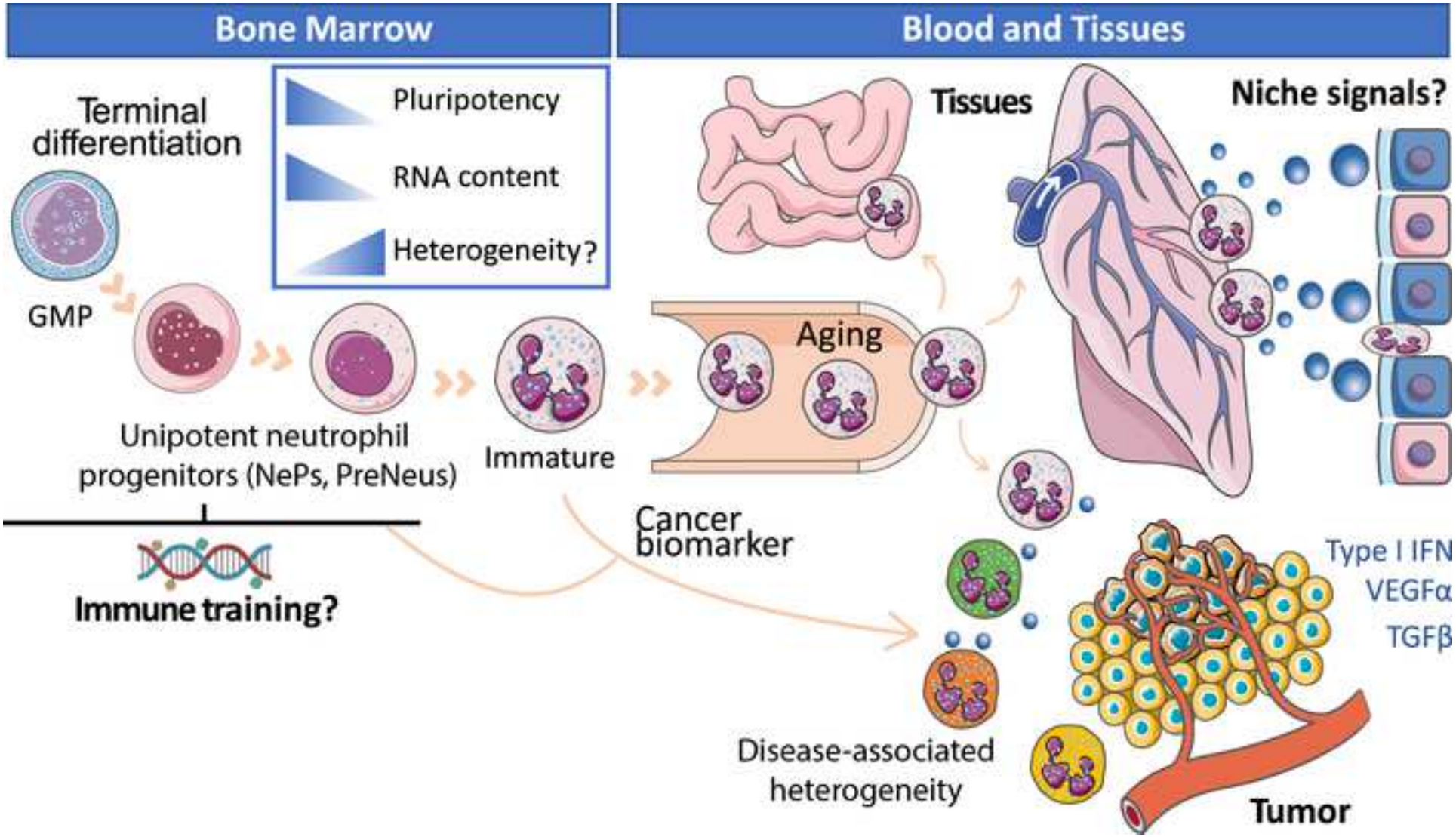

Available single cell transcriptomics public datasets containing neutrophils in tissues

Dataset	Type	Aprox. cell count	Technology	Classification method	Reference	Accession
Radermecker	Specific research	6300	Chromium	Manual	Radermecker et. al., 2019	E-MTAB-6902
Zilionis	Specific research	16000	inDrop	Automated classifier	Zillionis et. al., 2019	GSE127465
Tabula Muris	Atlas	100000	SMART-Seq2/Chromium	Manual	Schaum et. al., 2018	GSE109774
MCA	Atlas	400000	Microwell-seq	Manual	Han et. al., 2018	GSE108097