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**NANYANG
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SINGAPORE

**UNDERSTANDING NEUTROPHIL DEVELOPMENT
AND FUNCTION IN HEALTH AND DISEASE**

IMMANUEL KWOK WENG HAN

SCHOOL OF BIOLOGICAL SCIENCES

2019

**UNDERSTANDING NEUTROPHIL DEVELOPMENT
AND FUNCTION IN HEALTH AND DISEASE**

IMMANUEL KWOK WENG HAN

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang
Technological University in partial fulfilment of
the requirement for the degree of Doctor of
Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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Dr. Ng Lai Guan

Authorship Attribution Statement

This thesis contains material from one paper published in the following peer-reviewed journal(s) where I was the co-first author.

Chapter 3.1 is published as Evrard, M., Kwok, I. W. H., Chong, S. Z., Teng, K. W. W., Becht, E., Chen, J., et al. (2018). Developmental Analysis of Bone Marrow Neutrophils Reveals Populations Specialized in Expansion, Trafficking, and Effector Functions. *Immunity*, 48(2), 364–378.e9. <http://doi.org/10.1016/j.immuni.2018.02.002>

The contributions of the co-authors are as follows:

- Dr. Ng Lai Guan provided the initial project direction, conceptualized the project and edited the manuscript drafts.
- Dr. Maximilien Evrard and I prepared the manuscript drafts. The manuscript was revised by Dr. Chong and myself.
- I co-designed the study with Dr. Maximilien Evrard and performed all the laboratory work together at the Singapore Immunology Network. Dr. Maximilien Evrard and I also analyzed the data.
- Dr. Maximilien Evrard performed majority of the RNA-Sequencing analysis.
- All whole mount imaging, including sample preparation and analysis, was conducted by me.
- Ms. Karen Teng assisted in the preparation and execution of the mass cytometry experiments.
- Dr. Etienne Becht assisted in the analysis of the RNA-Sequencing data and provided scripts for analysis and graphing purposes.
- Dr. Chen Jinmiao assisted in the normalization and initial analysis of the RNA-sequencing data.
- Dr. Sieow Je Lin, Dr. Penny Leong and Dr. Wong Siew Cheng shared the murine pancreatic tumor model with us for our analysis.

- Dr. Goh Chi Ching conducted all intra-vital imaging experiments and analysed the data.
- Mr. Leonard Tan and Ms. Liong Ka Hang assisted with the experiments and the relevant mouse strains and models.
- Dr. Sapna Devi and Dr. Chua Jia Wang preliminary experiments which led to the conception of this project
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Date

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Videos are made available at the following url:

<https://www.dropbox.com/sh/lo8uzkfskv4dix8/AABchFfM6QPB47NtA0IpIEaVa?dl=0>

Abbreviations

B

BM: Bone marrow

BrdU: 5-bromo-2-deoxyuridine

Baso: Basophil

C

cDC: Conventional dendritic cell

CAR cells: Cxcl12-abundant reticular cells

CDP: Common dendritic cell progenitor

C/EBP: CCAAT/enhancer-binding protein

CLP: Caecum ligation and puncture

CFSE: 5,6-carboxyfluorescein diacetate succinimidyl ester

cMoP: Common monocyte progenitor

CPM: Count per million

CD: cluster of differentiation

CMP: Common myeloid progenitor

Cytof: Cytometry by time of flight

D

DC: Dendritic cell

dNTP: deoxy-nucleoside triphosphate

DEG: Differentially expressed gene

E

EDTA: Ethylenediaminetetraacetic acid

Eo: Eosinophil

F

FACS: Fluorescence-activated cell sorting

Fucci: Fluorescent ubiquitination-based cell-cycle indicator

FDR: False discovery rate

G

GMP: Granulocyte-Macrophage Progenitor

G-CSF: Granulocyte colony stimulating factor

G-CSFcx: Granulocyte colony stimulating factor antibody complex

GFP: Green fluorescent protein

GM-CSF: Granulocyte-macrophage colony stimulating factor

H

HSC: Hematopoietic stem cell

HPC: Hematopoietic progenitor cell

I

IdU: Iodouridine

IACUC: Institutional Animal Care and Use Committee

L

LT-HSC: Long-term hematopoietic stem cell

M

MDP: Monocyte-dendritic cell progenitor

MPP: Multi-potent progenitor

MDSCs: Myeloid-derived suppressor cells

N

NK: Natural Killer

P

preNeu: pre-neutrophil
PBS: Phosphate buffered saline
PFA: Para-formaldehyde
pDC: plasmacytoid dendritic cell

R

RBC: Red blood cell
RPA: reverse passive Arthus
RPM: Red Pulp Macrophage

S

SCF: Stem cell factor
SHG: Second harmonic generation
ST-HSC: Short-term hematopoietic stem cell
SGD: Specific granule deficiency

T

t-SNE: t-distributed stochastic neighbour embedding
TpMo: Transitional pre-monocytes

U

UMAP: Uniform manifold approximation and projection

W

WHIM: Warts, Hypogammaglobulinemia, Infections and Myelokathexis
WT: Wild-type

Y

YFP: Yellow fluorescence protein

5

5-FU: 5-Fluorouracil

Summary

Neutrophils are specialized innate cells that require constant replenishment from proliferative bone marrow (BM) precursors due to their short half-life. While it is established that neutrophils are derived from the granulocyte-macrophage progenitor (GMP), the differentiation pathways from GMP to functional mature neutrophils are poorly defined. Utilising several high-dimensional cytometric, transcriptomic and computational analyses, we characterised each neutrophil developmental stage and investigated their unique phenotypical and functional properties. Here, we present the identification of two distinct proliferative and committed neutrophil progenitors (proNeu1 and proNeu2) that give rise to a neutrophil precursor (preNeus), which sequentially differentiates into immature neutrophils and mature neutrophils. We showed the transcriptional programming of neutrophil commitment and maturation, as each subset progressively substitute their proliferative program and gain migratory and effector functions. Notably, the transcription factor C/EBP ϵ was critical for development, not only in governing the neutrophil lineage fate but also for the generation of preNeus. In summary, our study identifies specialized granulocytic populations in the BM that ensure supply under homeostasis and stress responses. We envision that these findings will help to unravel the complexity of neutrophil heterogeneity in both health disease conditions.

1. Introduction

1.1 Neutrophil Homeostasis

1.1.1 General Characteristics

Neutrophils are the most abundant innate immune cell type circulating in our bodies. Neutrophils patrol around the body to carry out their immune surveillance function, and they are known to be one of the first responders among leukocytes against many forms of inflammation. Responding to inflammatory cues, neutrophils are recruited to the inflammatory site and form large swarms around the site of injury or infection. These large 12-15µm granulocytes possess multi-lobed nuclei with cytoplasm-filled granules which are ammunition against bacterial and fungal invaders (Bamberg and Johnson, 2002). Alternatively, neutrophils can engulf pathogens into vesicles, called phagosomes, where highly potent reactive oxygen species (ROS) and granules are released to eliminate the microbial target (Winterbourn et al., 2016). With the advent of multi-photon intra-vital imaging technologies, we can observe these killers as they work together with other immune cells during the inflammatory process (Goh et al., 2015; Lammermann et al., 2013; Ng et al., 2011; Strydom and Rankin, 2013). Neutrophils elicit these effector functions in large numbers, accounting for at least 50 - 70 % of blood cells in humans, which increases during inflammation. This number is much lower in mice, but still forms a significant 10 - 25% of blood leukocytes. Large numbers are either found to marginate along blood vessels of tissues or stored in the bone marrow (BM) where over 90% of neutrophils are thought to be stored (Devi et al., 2013; Strydom and Rankin, 2013).

1.1.2 Production and Clearance of Neutrophils

Within the BM, large numbers of neutrophils are produced every day. In mice, these numbers can reach 10 million per day (Boxio et al., 2004) and over 100 billion in humans (Dancey et al., 1976). The high rate of production circumvents the short lifespan of neutrophils, with half-lives averaging 5-10 days in humans (Tak et al., 2013) and 8-12 hours in mice (Pillay et al., 2010). These numbers in humans are measured by administering deuterium (^2H)-labelled glucose, together with drinking water, to healthy volunteers over a period of time. The labelled glucose is then metabolised and incorporated into the DNA of dividing cells (Macallan et al., 2009; Macallan et al., 1998). Neutrophils can then be tracked across different time points to measure the fraction of ^2H -labelled neutrophils present in the blood (Tak et al., 2017). In a similar fashion, murine neutrophils have been tracked using nucleoside analogues like BrdU (5-bromo-2'-deoxyuridine) and EdU (5-ethynyl-2'-deoxyuridine) (Basu et al., 2002; Casanova-Acebes et al., 2013) by administering through drinking water.

As neutrophils circulate the body they acquire an aged phenotype, shedding CD62L selectin and up-regulating chemokine receptor CXCR4 (C-X-C chemokine receptor type 4) which allows them to migrate into tissues like the spleen and BM (Adrover et al., 2016). There, aged neutrophils are cleared by specialised efferocytic macrophages, inhibiting chemokine interleukin-23 (IL-23) production in these cells (Smith et al., 2007). Consequently, this leads to a feed forward loop involving IL17-producing Th17 helper T cells and their control of G-CSF (Granulocyte-colony stimulating factor) production that regulates

granulopoiesis and neutrophil egress from the BM (Stark et al., 2005). This production-clearance cycle oscillates in a circadian rhythm in both mice and humans (Adrover et al., 2019). Recent reports suggest that this rhythm is intrinsic in the neutrophil, with key genes like *Bmal1* and *CXCR4* driving this process. Interestingly, this process does not occur in neutrophils stored in BM, suggesting specific signals that prevent the initiation of the aging process (Adrover et al., 2019).

1.1.3 The CXCL12-CXCR4 axis

The interaction between *CXCR4*, a transmembrane G protein-coupled receptor, with its ligand *CXCL12* (C-X-C chemokine ligand 12) is known to be involved in many biological processes. Before *CXCL12-CXCR4* interaction was studied in neutrophil trafficking and recruitment, *CXCL12* was first shown to be a pre-B cell growth factor that can promote development together with *IL-7* (Nagasawa et al., 1994). Subsequently, interest grew as *CXCL12* was shown to be involved in blocking HIV entry (Bleul et al., 1996; Oberlin et al., 1996). Other pivotal roles are found requiring *CXCL12/CXCR4*, involving processes in immune responses and cellular organisation of organs (Nagasawa, 2014). Notably, *CXCL12/CXCR4* is a crucial component in embryogenesis as the absence of *CXCR4* or *CXCL12* results in the poor development of the heart, brain, and blood vessels (Nagasawa, 2014; Sierro et al., 2007; Wang et al., 2011).

The *CXCL12-CXCR4* axis is also critical for hematopoietic processes, including the engraftment and retention of HSCs (hematopoietic stem cells) in the BM (Ding and Morrison, 2013; Frenette et al., 2013) and the development of B cells

(Nagasawa et al., 1996), NK cells (Noda et al., 2011), and pDCs (plasmacytoid dendritic cells) (Kohara et al., 2007). CXCL12 is highly abundant in the BM, expressed by various types of stromal cells. These include endothelial cells, osteoblasts and a group of perivascular cells expressing high levels of CXCL12 identified with markers such as LepR and Nestin. Selective deletion of *Cxcl12* from these CXCL12-abundant reticular (CAR) cells highlighted their importance in forming stem cell niches for self-renewal, proliferation and expansion (Kunisaki et al., 2013; Omatsu et al., 2010). Recent advances in single-cell sequencing technology revealed, in detail, the heterogeneity of these stromal cell subsets in the BM and the pro-hematopoietic factors each subset can produce to exert their hematopoietic function (Baryawno et al., 2019; Tikhonova et al., 2019).

Besides the retention of HSPCs, CXCL12 and its interaction with CXCR4 mediates the retention and release of neutrophils within the BM (Eash et al., 2010; Martin et al., 2003; Suratt et al., 2004). Antibody blocking of CXCR4 displayed a rapid mobilisation of neutrophils into the periphery (Suratt et al., 2004). Specifically, mice with CXCR4-deficient neutrophils showed a redistribution of neutrophils from the BM to the circulation, further emphasizing the CXCL12-CXCR4 axis for neutrophil homeostasis (Eash et al., 2009). This interaction also allows for the homing of aged CXCR4^{hi} neutrophils back into the BM for clearance. Recent data suggested that CXCR4 expression intrinsically antagonises neutrophil aging and the loss of CXCR4 resulted in a constitutively aged phenotype with detrimental effects in vascular injury. Due to the CXCR4 deficiency, the homeostatic clearance and inflammatory recruitment of

neutrophils was impaired, emphasizing the requirement of CXCL12-CXCR4 interaction for homing and recruitment (Adrover et al., 2019).

The role of CXCL12-CXCR4 in retention of BM neutrophils is most apparent in patients who acquire the WHIM syndrome (for Warts, Hypogammaglobulinemia, Infections and Myelokathexis). A large proportion of these patients have a truncated C-terminal cytoplasmic tail CXCR4 protein. This results in a reduced recruitment of β -arrestin, leading to a gain-of-function in CXCR4 and accumulation of neutrophils in the BM, which results in neutropenia (Balabanian et al., 2005). Administration of CXCR4 inhibitor AMD3100 (plerixafor) can reverse this condition by releasing of neutrophils to the circulation.

CXCR4 is also responsible for the margination of neutrophils. Separate from the circulating pool of neutrophils, this intravascular population of neutrophils adhere closely to the endothelium of capillaries in the spleen, lung and the lungs (Athens et al., 1961). These neutrophils transit through these organs slowly, creating a reservoir of cells that do not contribute to the peripheral numbers. Importantly, the lung is thought to accumulate a large percentage of margined neutrophils, estimating around 10-30% of total blood volume (Dock et al., 1961). Studies have shown that the lung margination is dependent on the CXCR4-CXCL12 interaction as the inhibition of CXCR4 by plerixafor caused the rapid de-margination of neutrophils into the circulating pool (Devi et al., 2013). Together, it is clear that the role of CXCR4 and CXCL12 is central to neutrophil homeostatic functions.

1.1.4 Of Mice and Man

Neutrophils in mice are very different than their counterparts in humans. Besides the disparity in numbers in the periphery, human neutrophils possess receptors and granules not present in mice. For example, mice do not express FcRI which is known to be important in activating functions like oxidative burst, cytokine release and phagocytosis (Lublin et al., 1993; Pedotti et al., 2003). Murine neutrophils do not produce defensins, many granules like lysozyme and myeloperoxidase are also much lower as compared to human neutrophils (Yednock et al., 1992). Many chemokines are found in human neutrophils, including CXCL11, CXCL17, CCL14, CCL24 and CXCL8, are absent in mice (Pober et al., 2001). These differences affect neutrophils intrinsic function, suggesting extra caution should be taken into how studies in mice could be translated to human therapies and treatments. Still, much can be learnt from the many conserved features of neutrophils these two species share. One such feature is their developmental trajectory.

1.3 Neutrophil Development

1.3.1 General Haematopoiesis

Hematopoiesis is formation of all blood cells, a hierarchal and step-wise process with set molecular controls to instruct the various lineage choices each cell takes in order to mature fully as a functioning immune cell (Rieger and Schroeder, 2012). HSCs are found at the tip of the hierarchy comprising of both LT-HSCs (long-term hematopoietic stem cells) and ST-HSCs (short-term hematopoietic stem cells). LT-HSCs are the quiescent and dormant stem cells that rarely divide or self-renew, making them resistant to external stressors and influences. These adult stem cells self-renew, maintaining a pool of HSCs to repopulate the

immune system. ST-HSCs actively proliferate and differentiate into multi-potent progenitors (MPPs). At this stage, haematopoiesis splits into the lymphoid (MPP4) and myeloid lineages (MPP3), further giving rise to more proliferative progenitors such as the common lymphoid progenitors (CLPs) (Kondo et al., 1997) and common myeloid progenitors (CMPs) respectively (Akashi et al., 2000). During this process of differentiation, self-renewing properties are lost in favour of proliferative capabilities which allows them to populate the immune system with large numbers of mature leukocytes for effector functions. At the same time, these progenitors differentiate progressively in cell stages with declining multi-lineage potential, ultimately leading towards uni-lineage potential (Figure 1.1).

Figure 1.1 The hierarchical continuum of haematopoiesis

The understanding of haematopoiesis follows a stepwise differentiation process beginning from highly self-renewing haematopoietic stem cells (HSCs). These multi-potent, upon intrinsic or extrinsic programming, proliferate and differentiate into more mature progenitors that possess extensive proliferative potential while losing self-renewal properties. These haematopoietic progenitor cells (HPCs), with the help of transcriptional instruction, proliferate and

differentiate into mature leukocytes of the various myeloid and lymphoid lineages. Figure is taken from (Manz and Boettcher, 2014).

1.3.2 Transcriptional Control of Lineage Fate

CMPs are the oligo-potent progenitors that can either differentiate to the myeloid fate through granulocyte-macrophage progenitors (GMPs), or to the megakaryocytic-erythroid fate through megakaryocyte-erythroid progenitors (MEPs). Each lineage is thought to be directed by key transcription factors (TFs) that have been found to be highly up- or down-regulated in lineage-restricted cells. In this case, it is known that two key players, PU.1 and GATA1, are essential TFs for this binary fate decision of GMP or MEP lineage. Specifically, PU.1 and GATA1 binds to each other which cross-antagonise their ability to bind to their targets (Liew et al., 2006; Stopka et al., 2005; Zhang et al., 1999). PU.1 prevents GATA1 from binding to DNA while GATA1 interferes with PU.1 transactivation activity (Nishimura et al., 2000; Okuno et al., 2005). Using genetic knock-out models, researchers were able to show that a loss of PU.1 leads to a deficiency in neutrophils, macrophages and B cells. Similarly, GATA1 deficiency was shown to have detrimental effects on the development of the megakaryocyte and erythrocyte lineages (Iwasaki et al., 2006; Iwasaki et al., 2003).

However, the ability of TFs to drive the lineage decision does not equate to how the initial decision was made. It is still unclear what mechanisms lead to the up or down-regulations of these TFs that instruct lineage fate. One possibility is that cell-extrinsic factors like cytokines, and growth factors activate specific cell-signalling pathways to incite the initiation of cell fate. Another hypothesis is that TFs of multiple lineages are present and the dominance of one TF during random

cell-intrinsic fluctuations can lead towards positive regulation of that particular lineage. At the same time, this dominant TF can also repress other TF activity and production (Cantor and Orkin, 2001; Enver et al., 1998; Graf and Enver, 2009). Therefore, deeper analysis and measurements on the transcriptional regulatory state at each cell maturation stage is crucial to understand the differentiation and commitment of these progenitors.

CMPs differentiate and mature into more lineage-restricted progenitors, giving rise to characterised progenitor subsets like the common dendritic progenitor (CDP) (Shortman and Naik, 2007) and the GMP. GMPs were defined by Akashi and colleagues using key markers CD34 and CD16/32 (also known as Fc gamma receptor). Unlike CMPs, which are CD34⁺CD16/32^{lo}, GMPs are defined by their higher expression of CD16/32. Using this method of identification, they showed that GMPs were able to give rise to colonies representing granulocyte-macrophages (CFU-GM), macrophages (CFU-M) and granulocytes (CFU-G) (Akashi et al., 2000). It was shown later that the transcription factor C/EBP α is essential for the generation of GMPs (Paul et al., 2015; Zhang et al., 2004), showing that a transition is required before more restricted lineages can be produced.

1.3.2 History of Granulopoiesis

Neutrophils represent part of the group of granulocytes derived from the GMP. The developmental pathway towards functional blood neutrophils has been studied in detail, beginning over a century ago. German haematologist Viktor Schilling characterised four neutrophil developmental stages according to their

morphology (Schilling, 1950). These stages are in increasing maturity from left to right: myelocytes, meta-myelocytes (or young form), band cells (or stab cells) and segmented neutrophils. At that time, Schilling noticed a “left-shift” of higher proportions of immature myelocytes and meta-myelocytes in the periphery during infections (Schilling, 1957; Smith and Graham, 1985). In the bone marrow, two additional neutrophil precursor stages were identified, namely the myeloblasts and the promyelocytes. Myeloblasts were described as the first granulocytic precursor able to differentiate into basophils, eosinophils and neutrophils. They have high nuclear/cytoplasmic ratios and non-granular cytoplasms. Promyelocytes, on the other hand, contain azurophilic/primary granules (Glasser and Fiederlein, 1987). Through identifying all these subpopulations, researchers associated the relationships between each stage based on how similar they were to each other, thereby forming a developmental path from the myeloblasts to segmented neutrophils. However, it is still unclear how these subsets identified are developmentally related.

Figure 1.2 Classical nomenclature of neutrophil granulopoiesis.

Using differential staining of bone marrow and blood smears, like Giemsa, haematologists categorised neutrophils into six subpopulations using both granule content and nuclear morphology. This requires a level of technical training and experience to correctly quantify the subsets found in a given sample. Figure is adapted from (Ng et al., 2019).

1.3.3 Isolating neutrophil maturation stages

Since the identification of these granulocytic precursors were based upon morphological analyses, challenges remain in the isolating of pure populations of each stage for downstream analyses. A common method was the counterflow centrifugal elutriation separation of three BM granulocyte fractions: segmented

neutrophils, band cells and the remaining immature subsets (Glasser et al., 1994). With the introduction of monoclonal antibodies and flow cytometry, researchers were able to tag BM neutrophil subsets with a combination of markers like CD11b, CD15 and CD16 (Bjerregaard et al., 2003; Terstappen et al., 1990). These marker tests were confirmed by analysing their morphology after sorting with these markers. Since the delineation of each subset using the markers was only arbitrary, contamination was apparent and thus lowered the accuracy and precision of the conclusions made.

1.3.3 Transcriptional Control of Neutrophil Development

Using the available technologies and techniques, researchers studied the transcriptional programming of granulopoiesis and found the family of CCAAT/enhancer binding proteins to be critical factors that govern the neutrophil development and differentiation (Theilgaard-Monch et al., 2005). C/EBP α deficient mice displayed a developmental block from CMPs to promyelocytes. Blood from these mice showed no functional neutrophils or eosinophils but had normal monocyte and macrophage counts (Zhang et al., 1997). C/EBP α is known to trans-activate promoters of G-CSF, M-CSF and GM-CSF receptors; and the absence of C/EBP α resulted in the loss of G-CSF receptor expression, suggesting an interplay between both transcription factors and extrinsic signals from cytokines for normal granulopoiesis. It was later reported that interleukin 6 receptor (IL6R) was also not expressed in these knock-out (KO) mice and the retroviral transduction of IL6R or G-CSF receptor into these C/EBP α -deficient cells resulted in mature and functioning neutrophils (Zhang et al., 1998). Notably, C/EBP α was not required for subsequent development and

maturation of neutrophils as C/EBP α -deficient GMPs were able to give rise to similar distributions of GM, G and M colonies (Zhang et al., 2004).

C/EBP β is a non-essential TF at steady-state as C/EBP β -deficient mice had no abnormalities in granulopoiesis (Hirai et al., 2006). However, C/EBP β was instead critical during emergency granulopoiesis, where neutrophils are required in large quantities and in a short period of time. The authors showed, in a fungal infection model, mice deficient of C/EBP β had poor survival compared to wild-type controls. C/EBP β was also shown to be important for cytokine-driven proliferation and maturation as fewer colonies formed in the absence of C/EBP β (Hirai et al., 2006).

C/EBP ϵ is highly expressed in granulocytic cells, especially in the myelocytes and meta-myelocytes sub-populations (Yamanaka et al., 1997b). Mice deficient in C/EBP ϵ displayed non-functional granulocytes, resulting in lesion formations due to opportunistic bacterial infections (Yamanaka et al., 1997a). These granulocytes showed poor ROS activity and mostly resemble the morphology of myelocytes and meta-metamyelocytes. Additionally, an absence of eosinophils in both the bone marrow and blood was observed, suggesting a key fate decision point for eosinophil lineage determination through the activity of C/EBP ϵ . Besides fate determination, C/EBP ϵ is responsible for the production of secondary and tertiary granules such as lactoferrin, neutrophil gelatinase and murine cathelin-like protein (Gombart et al., 2003). Patients with specific granule deficiency (SGD), a rare disorder of abnormal neutrophils is caused by mutations in the CEPBE gene. Studies on these patients' neutrophils revealed an

upregulation of cytoskeletal proteins of the nucleus which controls nucleus shape. This change consequently prevents the nuclear condensation and segmentation that is commonly observed in mature neutrophils. Also, mutations in CEPBE prevented the nuclear localisation of C/EBP ϵ , preventing any activity of this TF (Serwas et al., 2018). However, it is still unclear how the production of granules is involved in the development and maturation of neutrophils.

Besides C/EBP ϵ , the transcription factor Gfi-1 (growth factor independent-1) is necessary for neutrophil development (Hock et al., 2003; Karsunky et al., 2002). Besides promoting granulocyte differentiation, Gfi-1 represses monocyte-promoting factors such as Egr2 and Csf1 (Laslo et al., 2006; Zarebski et al., 2008). Specifically, it was shown that a N382S mutation on Gfi-1 resulted in overexpression of Csf1, blocking granulopoiesis (Zarebski et al., 2008). Such mutations are observed in certain cases of severe congenital neutropenia, further suggesting the importance of the transcriptional machinery that are involved in generation of neutrophils (Person et al., 2003).

1.4 Neutrophil Diversity

1.4.1 The many faces of a neutrophil

Since their discovery, neutrophils were thought to function as phagocytic cells with powerful anti-microbial functions, including the secretion of granules and creating neutrophil extracellular traps (NETs) with their DNA. Due to their short half-lives, prolonged effector functions mediated by neutrophils were not highly considered. This view was challenged in the 1990s when it was shown that neutrophils can live longer than expected. Specifically, they have the potential to produce genes encoding for key inflammatory mediators such as cytokines,

chemokines and complement components (Cassatella, 1999; Colotta et al., 1992). Moreover, research from the past decade has revealed new functions and roles neutrophils play in homeostatic and inflammatory states. Notably, neutrophils have been shown to exhibit diverse phenotypic and functional plasticity, thus renewing the interest of scientists to better characterize neutrophil development and function. Using various methods, researchers have characterised several populations of circulating neutrophils defined by a variety of parameters such as cell-surface markers, cell density, morphology and localisation (Silvestre-Roig et al., 2019). Some subsets exert immune-regulatory responses while others have shown to interact with the adaptive immunity, such as the B cell helper neutrophil subset found in the spleen (Puga et al., 2011).

1.4.2 Neutrophil diversity exist at homeostasis.

As described earlier, circulating neutrophils progressively gain an aged phenotype, acquiring higher levels of CXCR4 while shedding CD62L expression, which are then cleared by CD169-expressing macrophages (Casanova-Acebes et al., 2013). Thus, there is already a level of heterogeneity of young and old neutrophils at any point in time. Recently, the differential functions of young and aged neutrophils have been revealed. Using specific mice models, Adrover and colleagues showed that aged neutrophils are much poorer in rolling, adhesion and extravasation under $\text{TNF}\alpha$ -induced inflammation (Adrover et al., 2019). Moreover, in a fungal infection model, constitutively aged neutrophils displayed lower fungal load and lower weight loss. However, aged neutrophils were shown to aggravate myocardial infarction with higher infarct sizes and lower survival rates (Adrover et al., 2019). This suggests that both fresh and aged neutrophils can play significantly varied roles during infection and inflammation. In addition

to these two subsets, a proangiogenic CD49d⁺CXCR4⁺VEGFR1⁺ subpopulation was identified in both mice and humans (Massena et al., 2015). These neutrophils were shown to accumulate at hypoxic sites, stimulating angiogenesis (Christoffersson et al., 2012).

In humans, more than half of the circulating neutrophils express CD177, a marker known to promote the interaction of neutrophils with endothelial cells and also the transmigration of neutrophils into tissues (Bai et al., 2017; Sachs et al., 2007). Studies on CD177-expressing neutrophils have suggested immune-regulatory functions (Zhou et al., 2018) and are associated with vascular injury (Deng et al., 2018; von Vietinghoff et al., 2007). In addition to the CD177 neutrophils, a small population of OLFM4 (glycoprotein olfactomedin 4) was found in healthy patients (Clemmensen et al., 2012). However, functions of this subset has yet to be defined. Nevertheless, with this existing heterogeneity at steady-state, it becomes a challenge to understand if the changes these neutrophils acquire stem from their innate differences or are a consequence of the environmental cues and factors.

1.4.3 Neutrophil heterogeneity during inflammation and cancer

As mentioned, the appearance of neutrophil precursors in the blood is a good sign of infection (Lawrence et al., 2018). This premature release can be elicited through G-CSF administration or through various inflammatory stimuli. Though developmentally juvenile, these neutrophils have been shown to perform various immune functions (Leliefeld et al., 2018; van Grinsven et al., 2019), and possess immunoregulatory functions against CD8 and CD4 T cells (Guerin et al., 2014;

Marini et al., 2017). Associated to these immature neutrophils are the appearance of granulocytic myeloid-derived suppressor cells (G-MDSCs) or low-density neutrophils (LDNs) in the blood of pregnant women and neonates (He et al., 2018; Scapini et al., 2016). These cells are thought to provide fetomaternal immune tolerance by suppressing CD4⁺ and CD8⁺ T cells and skew CD4⁺ cells to a Th₂ or T regulatory phenotype (Kostlin et al., 2016; Kostlin et al., 2017).

In cancer, G-MDSCs are consistently found in the blood of cancer patients and these cells are correlated to the severity of the tumour progression (Shaul and Fridlender, 2018). Some reports showed an immature neutrophil phenotype while others have a mixture of both mature and immature subpopulations. In addition, many of these studies used varying gating and identification strategies to characterise these subsets (Scapini et al., 2016). In addition to G-MDSCs, neutrophils have been shown to polarise towards divergent phenotypes based on specific factors within the tumour environment (Casbon et al., 2015; Waight et al., 2011). Specifically, the nomenclature of the N1 (tumour-inhibiting) and N2 (tumour-promoting) neutrophil polarisation has been suggested, as demonstrated by switch of neutrophil function using a TGFβ inhibitor. Like the MDSCs, the N2 immuno-suppressive neutrophils are reported to possess banded or ring-like nuclei, and are unlike the N1 hyper-segmented neutrophils (Fridlender et al., 2009). This morphological difference signifies their developmental immaturity, but could also be a consequence of neutrophil polarisation and activation (Coffelt et al., 2016). Therefore, morphological categorisation of neutrophils is insufficient in indicating maturity or function. This challenge was addressed, partially, with surface markers that can help mark the maturation status of cell.

Reports on metastatic breast cancer models have shown the presence of circulating neutrophils expressing cKit, which is a hematopoietic progenitor marker, signifying the presence of prematurely released neutrophils from the bone marrow (Coffelt et al., 2015b; Kuonen et al., 2012). These cKit⁺ neutrophils were found to also have undeveloped nuclear morphologies, similar to the phenotype of blood neutrophils in patients with breast or colorectal cancer (Sagiv et al., 2015; Wu et al., 2014). Thus, it is challenging to understand the heterogeneity of neutrophil function without a comprehensive understanding of the various changes that are present in the steady-state, and how these basal changes are then modulated during inflammatory insults and perturbations.

1.5 Multi-parameter cytometric analysis

Characterisation of cell subsets is based upon their effector functions and molecular phenotypes. T-cells, for example, contain a wide range of subsets that possess distinct roles in the immune response based on their cytokine production, surface marker expression levels and their localisation (Golubovskaya and Wu, 2016; Takata et al., 2012). Similarly, neutrophils are characterised based on their distinctive properties as well as their cell surface marker phenotype. Monoclonal antibodies are used to label specific receptors, ligands and adhesion molecules to define subsets of cells. Gr-1 and CD11b are well known markers used to define neutrophils. However, monocytes, eosinophils and some macrophages can express these markers, albeit at varying levels (Rose et al., 2012). In addition, myeloid cells can share multiple surface markers which can change in expression during inflammatory conditions. Therefore, the use of multiple and robust

parameters is critical to isolate and quantify pure populations of cells within heterogeneous samples like the bone marrow and spleen.

1.5.1 Flow Cytometry

Our study heavily utilises flow cytometric analyses and quantification to understand neutrophil development and heterogeneity. With its many advantages and uses, there are certain drawbacks and limitations that should be noted. This allows us to form better conclusions from the data generated. Flow cytometry is a brute force method, aimed at analysing hundreds of thousands of cell information at high speeds to generate statistical data from multiple parameters simultaneously. It works on the light scattering features of the cell, together with physical characteristics like size and granularity (Adan et al., 2017). Initially, flow cytometry was limited to one parameter to detect cell size. Now, technologies have enabled the simultaneous detection of over 25 parameters (Caielli et al., 2019). The staining intensities of each fluorophore-conjugated antibody that is bound onto the cell's surface is recorded and compiled together to generate single-cell resolution of cell identity information. Thus, we are able to define subpopulations of cells with a specific set of marker intensity expression levels at high reproducibility. This technology was instrumental in paving the way towards modern cellular immunology (Bendall et al., 2012), allowing the simultaneous measurement of cell frequencies, protein level expression and proliferation levels. One limitation is the pre-requisite knowledge of surface marker expression patterns of the cell types of interest. Various cell types share similar surface markers and can complicate the identification and analysis

strategies. Therefore, a well-planned panel of combinatorial surface markers is critical for successful flow cytometric analyses.

1.5.2 Mass Cytometry

The quest to better understand heterogeneity in samples and immune subsets, more parameters are required to delineate and distinguish these cells from each other and to probe relationships between each marker. Mass cytometry, also known as cytometry by time of flight (CyTOF), utilises the measurement of heavy metal-labelled antibodies bound onto cells instead of fluorophores in conventional flow cytometry (Spitzer and Nolan, 2016). Labelled cells are passed through a fluidic system where they are vaporised by plasma, releasing the metal isotopes on each cell. There, each metal isotope is measured using Time-Of-Flight (TOF) mass spectrometry. Unlike flow cytometry, each metal bears a defined atomic mass than can be measured and quantified without much crosstalk between each identifier (Ornatsky et al., 2010; Tanner et al., 2013). This allows for the number of channels to reach >100, only limiting to the availability and quality of these metal isotopes (Tanner et al., 2013). The downside is the much slower acquisition of cells and the lack of side/forward scatter information. Current practices typically measure 40 different parameters, enabling researchers to conduct deep phenotyping of immune subsets using a combination of cell surface markers, transcription factors, cytokine markers (Newell et al., 2012) and even antigen specific tetramer staining which allows for the measurement of T-cell specificity (Newell and Davis, 2014).

1.6 High-dimensional data analysis

During the analysis of cytometric data, a population is usually defined by drawing an arbitrary gate where the user believes a population exist. This is typically done through hierarchal sub-gating on biaxial dot plots with expression of each marker on each axis. This subjective and manual strategy relies on the training and knowledge of the user, which can vary the quantification and conclusion. Notably, with the introduction of multiple parameters and increased heterogeneity in the immune system, there is an increased amount of contamination in defined gates (Burkhard et al., 2014). With the introduction of higher number of parameters gained through high dimensional poly-chromatic flow cytometry or mass cytometry, analysing without subjective gating is critical. Both multi-parametric flow cytometry, mass cytometry, and even RNA-seq data contain a high level of complexity and spurred researchers to adopt high dimensional analysis methods to visualise and draw conclusions from the data (Bendall et al., 2012; Newell and Davis, 2014).

1.6.1 Principal Component Analysis (PCA)

This linear-dimensional reduction method is a widely used tool to transform high-dimensional cellular data into a set of linearly uncorrelated variables (called principal components). These components contain variances that allow for the segregation of populations based on their relatedness, and can then be plotted against each other to view the relationship between two principal components. Researchers routinely utilize PCA to interpret RNA-sequencing data, where the parameters are over 20000 genes, to understand similarities and differences between samples (Dulken et al., 2017; Kawamura et al., 2017).

1.6.2 t-distributed Stochastic Neighbour Embedding (t-SNE)

This widely used nonlinear transformation converts high-dimensional data into a matrix of pairwise similarities, giving similar points (or cells) high probability and dissimilar points low probability of being chosen, and projects the points with respect to the distances on a two-dimensional plot (van der Maaten and Hinton, 2008). Because of the nonlinearity, t-SNE is able to use the local relationships between points to create a non-linear structure which PCA cannot achieve. Thus, this method is frequently used to visualise mass cytometric data and enabled the identification of new subsets and rare populations (Amir el et al., 2013; Becher et al., 2014).

1.6.3 Uniform Manifold Approximation and Projection (UMAP)

t-SNE suffers in its limitations of losing large-scale information such as inter-cluster relationships, long run times and increased difficulty with bigger datasets. UMAP is a recent published dimensionality reduction method that is able to preserve both the local and global structure of the data set, allowing for relationship determinations between clusters near to each other. It also boasts a much faster computation time, even with larger datasets (Becht et al., 2018). These advantages spurred researchers to utilise UMAP to draw conclusions on the developmental relationships or maturation trajectory (Dress et al., 2019; Lewis et al., 2019; Saunders et al., 2019).

1.7 Objective of the study

Neutrophils are central players in innate immunity. Moreover, with the ever increasing reports of neutrophil subpopulations in health and disease, more

emphasis is required for proper characterisation and evaluation of each subset. This heterogeneity already begins within the bone marrow where they are produced. However, the developmental trajectory from GMPs and the subpopulations that form during this process is still poorly defined. Moreover, it is unclear how each subset is involved during inflammation. To address this, we utilised multi-parameter analytical techniques to fulfil the following objectives:

- 1) To establish a framework for the comprehensive analysis of the development of neutrophils and the transcriptional programming that governs this process.
- 2) To address the differential functional properties these neutrophil subsets at steady state and during inflammatory conditions.
- 3) To determine and characterize the heterogeneity of neutrophils in the human bone marrow.

2. Materials & Methods

2.1 Experimental model and subject details

2.1.1 Mice

Six to ten week-old C57BL/6 mice were bred and maintained under specific pathogen-free (SPF) conditions in the Biological Resource Centre (BRC) of A*STAR, Singapore. Both males and females were used for experiments, but animals were sex- and age-matched in each experiment as much as possible. *S100a8^{cre}* (B6.Cg-Tg(S100A8-cre,-EGFP)1Ilw/J), *Lyz2^{cre/cre}* (B6.129P2-Lyz2^{tm1(cre)lfo}/J), *Rosa26^{mT/mG}* (STOCK Gt(ROSA)^{26Sortm4}(ACTB-tdTomato,-EGFP)Luo/J, *Cxcl12^{DsRed/+}* (STOCK Cxcl12^{tm2.1Sjm}/J), *Rosa26^{LSL-YFP}* (B6.129X1-Gt(ROSA)^{26Sor^{tm1}(EYFP)^{Cos}}/J), Albino mice (B6(Cg)-Tyr^{c-2J}/J) and *Cxcr4^{fl/fl}* (B6.129P2-Cxcr4^{tm2Yzo}/J) mice were obtained from The Jackson Laboratory. For fate-mapping experiments, *S100a8^{cre}* and *Lyz2^{cre/cre}* mice were crossbred in-house with *Rosa26^{LSL-YFP}* and *Rosa26^{mT/mG}* mice respectively. Fucci-S/G2/M (#474) and the double transgenic Fucci-G1 (#639) mice were obtained from the RIKEN BioResource Center (Ibaraki, Japan;(Tomura et al., 2013)). *Lyz2^{gfp/+}* (*Lyz2^{tm1.1Graf}*) were provided by T. Graf (Centre for Genomic Regulation, Barcelona, Spain; (Faust et al., 2000)). Gain-of-function *Cxcr4^{1013/+}* (termed *Cxcr4^{WHIM}*) mice were provided by F. Bachelierie (INSERM 996, Clamart, France; (Balabanian et al., 2012)). *S100a8^{cre}* mice were crossbred in-house with *Cxcr4^{fl/fl}* to generate progeny with CXCR4-deficient neutrophils. For niche localization of neutrophil subsets, Fucci-S/G2/M (#474) mice were crossbred in-house with *Cxcl12^{DsRed/+}* mice. All transgenic mice were maintained on a C57BL/6 background and experiments were performed under the approval of the

Institutional Animal Care and Use Committee (IACUC), in accordance with the guidelines of the Agri-Food and Veterinary Authority (AVA) and the National Advisory Committee for Laboratory Animal Research (NACLAR) of Singapore.

2.1.2 Treatments

For 5-Fluorouracil (5-FU) myeloablative treatment, mice were injected once intraperitoneally with 150mg/kg 5-FU (Sigma-Aldrich) or PBS control. For G-CSF treatment, mice were injected once intraperitoneally with 1.5µg of G-CSF/anti-G-CSF antibody complex (G-CSFcx) as previously described (Rubinstein et al., 2013). Briefly, G-CSFcx were generated by incubating G-CSF (Neupogen) and anti-G-CSF (BVD11-37G10; Southern-Biotech) at 1:5 cytokine to antibody ratio for 20 min at 37°C, and were next diluted at least 10-fold in PBS before injection.

2.1.3 Human blood, bone marrow and cord blood samples

All samples were obtained in accordance with a favourable ethical opinion from SingHealth CIRB (2014/664/F) or A*STAR, the Singapore Immunology Network. Consent for bone marrow samples was sought from healthy donors who were already giving bone marrow for a different study or a medical cause. Cord blood units that do not meet clinical grade were obtained from the Singapore Cord Blood bank for research.

2.2 Tissue preparation for flow cytometry and cell sorting

2.2.1 Murine Blood & Human Umbilical Cord Blood

Murine blood was collected from the submandibular region using a 5mm single-use lancet. A volume of 200 µL was collected and transferred into an Eppendorf tube containing 20 µL of 0.5M Ethylenediaminetetraacetic acid (EDTA). For

both murine and human blood, samples were then lysed in 1 X RBC lysis buffer (eBioscience) for 4 min with occasional vortexing. Samples were quenched with FACS Buffer (1 X PBS containing 4mM EDTA and 3% fetal calf serum (FCS)) and spun down at 400g for 5 min. The supernatant was then discarded and RBC lysis was repeated for another 2 min. After discarding the supernatant, the blood samples are ready for staining with fluorophore-conjugated antibodies.

2.2.2 Murine and Human Bone marrow

Murine bone marrow was obtained by inserting a 19-gauge needle through the marrow cavity of the femur and then flushing the marrow out with 2 ml of FACS buffer using a 1ml syringe. For sorting purposes, where large numbers are required, both femur and tibia bones were used. Cells were then filtered and washed with FACS buffer, cells were spun down at 400g for 5min. After discarding the supernatant, an aliquot was used for staining and analysis. For human marrow samples, aspirates were spun down at 400g for 5min. The supernatant was removed and cells were counted using a haemocytometer. Cells are stained accordingly as described below after blocking with Fc block for 20min at 4°C.

2.2.3 Spleen

Spleens were excised from mice and single-cell suspensions were obtained by homogenising spleen samples through a 70-µm nylon mesh filter and washing with 10 ml FACS buffer. Samples were then spun down at 400g for 5 min before performing RBC lysis using 1 X RBC lysis buffer for 3min. Cells are quenched with FACS buffer and filtered through a 70-µm nylon mesh filter. Samples are spun down and an aliquot is then used for staining analysis.

2.2.4 Flow cytometry and cell sorting

Antibodies were purchased from BD, Biolegend, eBioscience or R&D. For the identification of mouse myeloid cells, cells were stained with fluorophore-conjugated anti-mouse antibodies against CCR2 (475301), CD11b (M1/70), CD11c (N418), CD16/32 (2.4G2), CD31 (390), CD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD49f (GoH3), CD62L (MEL-14), CD101 (Moushi101), CD115 (AFS598), cKit (2B8), CXCR2 (SA044G4), CXCR4 (2B11), CX3CR1 (SA011F11), F4/80 (BM8), Gr1 (RB6-8C5), I-A/I-E (M5/114.15.2), Ly6C (HK1.4), Ly6G (1A8) and Siglec-F (E50-2440), together with exclusion lineage markers that include CD3e (145-2C11), CD90.2 (53-2.1), B220 (RA3-6B2), NK.1.1 (PK136), and Sca-1 (D7). After exclusion of cell doublets and dead cells with DAPI, preNeu were identified as (Lin/CD115/Siglec-F)⁻Gr1⁺CD11b⁺CXCR4^{hi}cKit^{int}CXCR2⁻, immature Neu were identified as (Lin/CD115/Siglec-F)⁻Gr1⁺CD11b⁺CXCR4^{lo}cKit^{lo}CXCR2⁻ and mature Neu were identified as (Lin/CD115/Siglec-F)⁻Gr1⁺CD11b⁺CXCR4⁻cKit⁻Ly6G⁺CXCR2⁺.

For identification of HSCs and HPCs, cells were stained with CD16/32 (2.4G2), CD34 (RAM34), CD48 (HM48-1), CD150 (TC15-12F12.2), cKit (2B8), Flt3 (A2F10), Ly6C (HK1.4) and Sca-1 (D7), together with exclusion lineage markers that include CD3e (145-2C11), CD11b (M1/70), CD90.2 (53-2.1), B220 (RA3-6B2), Gr1 (RB6-8C5) and NK.1.1 (PK136). After exclusion of cell doublets and dead cells with DAPI, LT-HSC were identified as Lin⁻cKit⁺Sca-1⁺CD150⁺CD48⁺, ST-HSC were identified as Lin⁻cKit⁺Sca-1⁺CD150⁻CD48⁻, MPP were identified as Lin⁻cKit⁺Sca-1⁺CD150⁻CD48⁺, CMP were identified as Lin⁻cKit⁺Sca-1⁻CD16/32^{int}CD34^{int}, total GMP were identified as Lin⁻cKit⁺Sca-1⁻CD16/32^{hi}CD34^{hi}, MDP were identified as Lin⁻cKit⁺Sca-1⁻CD115⁺Flt3⁺Ly6C⁻

and cMoPs (common monocyte progenitors) were identified as Lin⁻cKit⁺Sca-1⁻CD115⁺Flt3⁻Ly6C⁺.

For identification of neutrophil progenitors (proNeus), cells were stained with CD106 (426), CD81 (Eat-2), CD205 (NLDC-145), Ly6C (HK1.4), CD34 (RAM34), CD115 (AFS598), CD11b (M1/70), cKit (2B8), Flt3 (A2F10) and CD16/32 (2.4G2), together with lineage markers CD90.2 (53-2.1), Ly6G (1A8) B220 (RA3-6B2), NK.1.1 (PK136), and Sca-1 (D7. proNeu1 were identified as Lin⁻cKit⁺Flt3⁻Ly6C⁺CD81⁺CD106⁻CD16/32⁺⁺CD34⁺, proNeu2 were identified as Lin⁻cKit⁺Flt3⁻Ly6C⁺CD81⁺CD106⁺CD16/32⁺⁺CD34^{lo/-}, Ly6C-GMPs were defined as Lin⁻cKit⁺Flt3⁻Ly6C⁻CD81⁺CD16/32⁺⁺CD34^{hi}.

Flow cytometry acquisition was performed on a a. Cell numbers were quantified with count beads (CountBright; Life Technologies) according to the manufacturer's instructions. Sorting of BM neutrophil subsets were performed using a BD ARIAll (BD) to achieve >98% purity.

2.3 Mass cytometry (CyTOF) sample preparation, acquisition and analysis

For mass cytometry analysis, purified antibodies were obtained from BD Biosciences, Biolegend, eBioscience, BioXCell, and conjugated using MAXPAR® DN3 antibody labelling kits (Fluidigm) according to manufacturer's instructions. Mice were injected once intraperitoneally with 2mg IdU (Sigma-Aldrich). Mice were euthanized 2h later, femurs were harvested, flushed in PBS and passed through a 70-µm nylon mesh sieve. BM cells (mouse or human) were plated in a 96-well round bottom plate at a density of 5 x 10⁶ cells per well. Cells were stained for viability with 100µL of 50µM of cisplatin (Sigma-Aldrich) for

5 minutes at 4°C. Cells were then washed with staining buffer (4% FBS, 0.05% sodium azide, 2mM EDTA in 1X PBS and incubated with anti-CCR2-APC, anti-CD34-FITC, anti-CD115-PE and anti-Flt3-biotin (mouse panel) or CXCR4-biotin, CXCR2-FITC, CD101-APC (human panel) in 50µL reaction volume for 90 minutes at 4°C. Red blood cells were lysed with 1X RBS lysis buffer (eBioscience) and cells were washed with staining buffer. Cells were stained with 50µL of metal isotope-labelled surface antibodies on ice. After 30 minutes, cells were washed twice with staining buffer, once with PBS, and then fixed in 2% paraformaldehyde (PFA) (Electron Microscopy Sciences) in PBS at 4°C overnight. The next day, cells were pelleted and re-suspended in 200µL 1X permeabilisation buffer (Biolegend) and allowed to stand for 5 minutes on ice. Cells were then washed once with PBS and incubated with cellular barcodes on ice for 30 minutes as previously described (Becher et al., 2014). Subsequently, cells were washed once with perm buffer and in staining buffer for 10 minutes on ice. Cellular DNA was labelled at room temperature with 250nM iridium intercalator (Fluidigm) in 2% PFA/PBS. After 20 minutes, cells were washed twice with staining buffer.

Prior to acquisition, cells were washed twice with water before final re-suspension in water. Cells were pooled from all samples, enumerated, filtered and diluted to a final concentration of 0.6×10^6 cells/mL. Mass-tag barcoding was used so that all samples could be acquired simultaneously. EQ Four Element Calibration Beads (Fluidigm) were added to the pooled samples at a final concentration of 1% prior to acquisition. Samples were acquired on a CyTOF2 (Fluidigm) equipped with a Super Sampler fluidic system (Victorian Airship & Scientific Apparatus LLC) at an event rate of <500 events per second. After mass

cytometry acquisition, data were exported in flow-cytometry (FCS) format, normalized and events with parameters having zero values were randomized using a uniform distribution of values between minus-one and zero. Each sample containing a unique combination of two metal barcodes was de-convoluted by Boolean gating using FlowJo software (Tree Star). Subsequently, manual gating was done to exclude residual beads, debris and dead cells. BM CD45⁺IdU⁺ (proliferative cells) and CD45⁺IdU⁻ (non proliferative cells) were gated using FlowJo, and exported as a FCS file. Random subsampling without replacement was performed to select 90000 events. Dimensional reduction of the CyTOF data was performed selecting the markers listed in Table 1 (mouse) or 2 (human) by t-distributed stochastic neighbour embedding (t-SNE) using the Cytokit R package (Chen et al., 2016; van der Maaten and Hinton, 2008). Clusters were generated using the FlowSOM implementation in Cytokit. Median intensity values per cluster for each marker were calculated and exported to produce heatmaps using R. The identity of each cluster was inferred based on the expression of each individual marker.

2.4 Deep tissue whole-mount tissue preparation and immunostaining

Three-dimensional (3D) mosaic images of femur sections were acquired using a LaVision TriM Scope II microscope (LaVision BioTec), equipped with a water dipping objective (20x magnification, 1.0 NA, 2mm WD; XLUMPLFLN20xW, Olympus) and a Chameleon-pulsed infrared laser (titanium sapphire; Coherent). Acquisitions were performed two excitation wavelengths: 990nm and 800nm. 990nm excitation was used for the simultaneous imaging of Fucci-S/G2/M

positive cells ($\lambda_{em} = 505nm$), second harmonic generation (SHG) ($\lambda_{em} = 495nm$), AF555 ($\lambda_{em} = 565nm$) and DsRed ($\lambda_{em} = 580nm$). Subsequently, imaging was performed at 800nm for the acquisition of AF647 ($\lambda_{em} = 670nm$). Filter used were: 494/41, 525/50, 565/40, 620/60 and 665/40 (Semrock). Dichroic mirrors used were a 495LP, 560LP, 620LP, 591sh (Semrock) and 640LP (Chroma Technology). Images were acquired with the following settings: $450\mu m \times 450\mu m$, 517×517 pixels, 600Hz line scan with 2 frames of line averaging, using a $2\mu m$ z-step size with a depth of $250\mu m$. The distal epiphysis was chosen as the area for imaging to maintain consistency between samples. 3D mosaic Z-stack images were stitched together using FIJI is just ImageJ (FIJI), and subsequently rendered and analysed using Imaris software (Bitplane). Spectral spillover between AF555 and DsRed was removed using Imaris with the channel arithmetic plugin. S100A9⁺Fucci-S/G₂/M⁺ and S100A9⁺Fucci-S/G₂/M⁻ cells were identified using the spots function tool in Imaris. Calculation of distance to the nearest vessels and CAR cell was performed using the Distance Transform Matlab-based XTension built in Imaris. Raw statistics were then exported for further analysis in Prism (Graphpad).

2.5 In-vitro functional assays

2.5.1 Oxidative burst assay

Sorted neutrophils (5×10^5 for each cell subset) were incubated with $2.5\mu g/mL$ Dihydrorhodamine 123 (DHR) (ThermoFisher) in RPMI, and subjected to 50nM Phorbol 12-Myristate 13-Actetate (PMA) (Sigma-Aldrich) for 20 min at 37°C. Cells were subsequently washed with PBS and the fluorescence intensities of each subset were measured by flow cytometry.

2.5.2 Phagocytosis Assay

DH5 α *Escherichia coli* (*E. coli*) expressing GFP (Chua and Wong, 2013) were grown in Lysogeny Broth (LB) medium overnight at 37°C to an Optical Density (OD) at 600 nm of 1.5–1.8, at which point the bacteria were diluted and grown for 1–2 hours to an OD₆₀₀ of ~ 0.5, and were finally washed twice with PBS. Sorted neutrophils (1×10^5 for each cell subset) were incubated with bacteria in a ratio of 1:100 for 2 hours at 37°C. After incubation, the cells were washed with PBS, fixed with 2% PFA and analysed by flow cytometry.

2.5.3 Proliferation assay

Sorted cells (3×10^4 for each neutrophil subset) were plated onto 96-well plates in triplicates and cultured at 37°C, 5% CO₂ in Iscove's Modified Dulbecco's Medium with 25mM HEPES and L-Glutamine (Chemtron) containing 10% (vol/vol) FBS, 1 mM sodium pyruvate, penicillin (100 U/ml) and streptomycin (100 ug/ml).

2.5.4 In vitro cell culture

Colony formation assays were performed as described before (Hettinger et al., 2013). Briefly, sorted cells (3×10^4 for each neutrophil subset) were cultured for in Iscove's modified Dulbecco's medium (Sigma) with the supplements mentioned above, 1% (wt/vol) methylcellulose (MethoCult M3134, Stem Cell Technologies) and a combination of cytokines (50ng/ml SCF, 20ng/ml LIF, 10ng/ml IL-3, 20ng/ml IL-6). Representative colony images were collected with an Olympus IX-81 microscope (Olympus). Image brightness was adjusted with Photoshop.

2.5.5 BrdU Pulsing

For in vivo assays, mice were injected intraperitoneally with 2mg 5-bromo-2'-deoxyuridine (BrdU; Sigma-Aldrich) at indicated time points. To detect BrdU

incorporation into neutrophil subsets, cells were stained with a fixable vitality dye (Zombie UV fixable viability kit; Biolegend), surface-stained, fixed, permeabilized, and subjected to intracellular staining with FITC-conjugated anti-BrdU antibody, according to the manufacturer's protocol (BrdU Flow kit; BD) before analysis by flow cytometry.

2.5.6 Cytospin and Wright-Giemsa staining

Sorted neutrophil subsets (1×10^5 cells each) were spun onto glass slides using Cytospin 4 Cytocentrifuge (Thermo scientific), dried for 20 minutes, fixed in methanol and stained with the Hema 3 manual staining system (Fisher Diagnostics) according to the manufacturer's protocol. Images were acquired with an Olympus BX43 equipped with a 100x oil immersion objective, and image brightness was adjusted with Photoshop (Adobe).

2.6 Inflammation Models

2.6.1 Laser-induced sterile injury model

Neutrophil subsets were sorted from either *Lyz2gfp/+* (GFP) or *Rosa26mT/mG* (tdTomato) transgenic mice as indicated, and were mixed in a 1:1 ratio (each 2.5×10^5 cells). Cells were re-suspended at a concentration of 0.1×10^5 cells/ μ L. A 2.5 μ L volume of neutrophil suspension was injected intra-dermally in the ear with a Hamilton syringe (33-gauge, 62RN). B6(Cg)-Tyrc-2J/J (B6 albino) mice were used as recipient mice in all experiments. After two to three hours, mice were prepared for skin multiphoton imaging and laser focal injury was then performed as described previously (Li et al., 2012). Briefly, anesthetized mice were set up onto a custom ear imaging stage platform to stabilize the ear for intravital imaging. To induce a sterile injury, a chosen area (75 μ m²) close to the

injection site was briefly exposed to a focused laser pulse (850nm) for ~5s. For image acquisition, an excitation wavelength of 990 nm was used to collect GFP ($\lambda_{em} = 510\text{nm}$), tdTomato ($\lambda_{em} = 580\text{nm}$) and second harmonic generation (SHG) ($\lambda_{em} = 495\text{nm}$) simultaneously. Filters used were 494/41, 510/20 and 579/34 (Semrock). Dichroic mirrors used were a 495 LP (Semrock), 560 LP (Semrock) and 640 LP (Chroma Technology). A scan-field dimension of $500\mu\text{m} \times 500\mu\text{m}$, with a Z-step size of $4\mu\text{m}$ was used to acquire the 40–50 μm stacks, taken at every half-minute intervals for 1 hour. Mice body temperatures were kept at 37°C with a heating pad and mice ears were separately warmed at 35°C during imaging. After acquisition, data correction and analysis were conducted using Imaris (Bitplane). Where necessary, FIJI is just ImageJ (FIJI) was used to correct for drifts that occurred during acquisition. Cell tracking was done semi-automatically in Imaris using the “spots” function and the “auto-regressive motion” algorithm. Reconstructed images and videos were finally generated using Imaris.

2.6.2 Reverse Passive Arthus (RPA) reaction

RPA was conducted as described before (Li et al., 2016). Briefly, mice were intravenously injected with Evans blue dye (Sigma-Aldrich) at 8 $\mu\text{L/g}$ bodyweight, 10mg/ml in saline). RPA reaction is initiated by intradermal injection of 1.5 μL of 10mg/mL anti-BSA (Sigma-Aldrich), followed by intraperitoneal injection of 200 μL of 5mg/ml BSA (Sigma-Aldrich). For quantification of neutrophil numbers, mouse ears were subjected to tissue homogenization and enzymatic digestion as described (Li et al., 2016), followed by flow-cytometric analysis. For quantification of vascular leakage, readings were obtained through digital photographic analysis methods.

2.6.3 CLP-induced sepsis

Cecal ligation and puncture was performed as described previously (Rittirsch et al., 2009). Briefly, the peritoneal cavity was exposed under ketamine/xylazine anaesthesia and the cecum was exteriorized. 80% (high-grade) or 50% (mid-grade) of the cecum was ligated distal of the ileocecal valve using a non-absorbable 7-0 suture. A 26-gauge needle was used to perforate the distal end of the cecum, and a small drop of feces was extruded through the puncture before being relocated into the peritoneal cavity. The peritoneum was closed and mice were subsequently treated with saline and Buprenorphine (5-20 mg/kg) by subcutaneous injection. For sham-operated controls, the peritoneum was exposed and the cecum was exteriorized before closing the peritoneum as mentioned above. Mice were euthanized and harvested 2 weeks after the surgery or as indicated.

2.6.4 Orthotopic pancreas tumour model

Mice were administered intra-pancreatic injections of FC1242 tumour cells (kind gift from Dr. Dannielle D. Engle, Tuveson lab) derived from *Pdx1^{cre}; LsL-Kras^{G12D/+}; LsL-Trp53^{R172H/+}* (termed KPC) mice as previously described (Zambirinis et al., 2015). Briefly, mice were anesthetized with ketamine/xylazine, and had their abdomen shaved and swabbed with antiseptics. A 5mm vertical incision was made in the skin and abdominal layer at a point 1cm down from the xiphoid process of the sternum, and 1cm to the right of the midline. The pancreas was exposed, 1×10^5 tumour cells were re-suspended in 1X PBS and mixed with matrigel (BD) in a 1:1 ratio, and were injected as a volume of 50 μ L into the body of the pancreas to form a visible bolus using a 29-gauge insulin needle. The pancreas was then returned to the abdominal cavity. The abdominal layer was closed with absorbable 5/0 sutures, while the skin was closed with non-

absorbable 5/0 sutures. Superglue was applied over the sutures to ensure that they do not come undone after surgery. Mice were resuscitated with saline and were subcutaneously administered Buprenorphine (10mg/kg) and Enrofloxacin (Baytril, 1.5mg/kg) for the 2 days following surgery. Mice were euthanized at day 27-30 following surgery and tumour weights were recorded.

2.7 Transcriptomics

GMP, preNeu, immature Neu, mature Neu and blood Neu from three different mice were sorted based on the gating strategy depicted in Figure 3A. BM Transitional pre-monocytes (tpMo) and BM mature Ly6C^{hi} monocytes were sorted as Lin(CD3/CD90.2/B220/NK1.1/Ly6G)⁻CD115⁺Flt3⁻Ly6C⁺CXCR4^{hi}CD11b^{lo} and Lin(CD3/CD90.2/B220/NK1.1/Ly6G)⁻CD115⁺Flt3⁻Ly6C⁺CXCR4^{lo}CD11b^{hi} respectively from three different mice (see gating strategy in (Chong et al., 2016)). Total RNA isolation was subsequently performed using Arcturus PicoPure RNA Isolation kit according to the manufacturer's protocol. All mouse RNAs were analysed on Perkin Elmer Labchip GX system for quality assessment with RIN > 7.7. cDNA libraries were prepared using 2ng of total RNA and 1μL of a 1:50000 dilution of ERCC RNA Spike in Controls (Ambion) using SMARTSeq v2 protocol (Picelli et al., 2014), except for the following modifications: (1) use of 20μM TSO; and (2) use of 250pg of cDNA with 1/5 reaction of Illumina Nextera XT kit. The length distribution of the cDNA libraries was monitored using DNA High Sensitivity Reagent kit on the Perkin Elmer Labchip. All 18 samples were subjected to an indexed PE sequencing run of 2 × 51 cycles on an Illumina HiSeq 2500 Rapid mode.

RNA-Seq data in the form of FASTQ files were subsequently mapped to the mouse genome build mm10 using the STAR alignment software. The mapped reads were then counted using featureCounts (part of Subread package) based on the GENCODE M7 annotations. The raw counts were then used for a differentially expressed gene (DEG) analysis using edgeR (R version 3.1.2) with $FDR < 0.05$ and $\log_2FC > 2$ to identify genes differentially regulated in neutrophil subsets to generate volcano plots. Count per million reads (CPM) values were calculated from raw counts using edgeR (R version 3.1.2). The CPM values were then $\log_2$ -transformed in R ($x \rightarrow \log_2(1+x)$). For PCA, hierarchical clustering and correlation matrices, the gene expression matrix was first subsetted using the top 20% variable genes (as measured by standard deviation across samples) and then those that were significantly associated with a cell population (FDR-corrected ANOVA, $q\text{-value} < 0.05$) resulting in 4820 DEGs. For hierarchical clustering, we used Euclidean distance and the Ward aggregation criterion and the pheatmap package to plot the results as a heatmap. The correlation matrix was computed using Pearson's correlation coefficients. Gene ontology (GO) enrichment (GO Biological Process 2015) of DEGs was done using Enrichr (Chen et al., 2013). Single-cell RNA-seq was performed with SMART-Seq2 protocol. Cell subsets of interest were individually index-sorted into 96-well plates containing dNTPs (deoxy-nucleoside triphosphate) and 1% BSA. Plates were then processed for cDNA directly and tested for RNA quality before subjected to an indexed PE sequencing run of 2×51 cycles on an Illumina HiSeq 2500 Rapid mode.

2.8 Infinity Flow

Mouse bone marrow cells from femurs, tibias, pelvis, humeri and spines were pooled and gently crushed to extract single-cell suspensions. Cells were counted before staining live/dead ZombieUV (ThermoFisher Scientific) for 20min, 4°C. Cells were then stained with a backbone panel consisting of: FITC-Ly6G (1A8), FITC-NK1.1 (PK136), FITC-CD90.2 (53-2.1), FITC-B220 (RA3-6B2), Sca-1 (D7), CD48 (HM48-1), Flt3 (A2F10), cKit (2B8), CD16/32 (2.4G2), CD115 (AFS598), CD62L (MEL-14), CD11b (M1/70), Ly6C (HK1.4), CD34 (RAM34) and CD43 (S7). To enrich for early progenitor cells, FITC selection kit (Stemcell Technologies) was used to reduce the frequencies mature neutrophils, B cells, T cells and NK cells. LEGENDScreen™ (Biolegend) was then performed with the enriched cells according manufacturers' protocol. Samples were acquired with a 5-laser BD LSR II (BD) using FACSDiva software, and data was subsequently analyzed with FlowJo software (Tree Star). Compensated live singlets were exported from each file and used for Infinity Flow algorithm. Details of this method is in preparation for publication.

2.9 Computational and statistical analysis

2.9.1 Statistics

Statistical analyses were done using Prism software (Graphpad). Student's t-test or one-way analysis of variance (ANOVA) with Bonferroni correction were performed. For correlation analysis, linear regression was used to generate the best-fit line for graphical representation, and Pearson's correlation test was performed to generate p values. P values <0.05 were considered as statistically significant.

2.9.2 Computational inference of developmental path

Wanderlust was performed using cyt with MATLAB (R2015a). A total of 249 parameters using the following settings: nl = 20, dist = angular distance, ng = 20, k = 5, l = 30. Uncommitted GMPs (Ly6C-cKit+CD16/32++CD34+) were chosen to be early cells. The output trajectory was normalized to the [0, 1] range. Monocle2 (Qiu et al., 2017) development trajectory prediction was performed on the single-cell RNA-seq data of CMPs, Ly6C-GMPs, Ly6C+GMPs and Ly6C⁺CD34^{hi}CD11b^{lo} GMPs. Branched expression analysis modelling (BEAM) to generate all genes that differ between each branch.

3. Results and Discussion

3.1 Functional characterisation of BM neutrophil developmental subsets

The development of neutrophils is a critical process for the optimal resupply of these short-lived phagocytes. Currently, reported studies on neutrophil development have relied heavily on the morphological classification of these developmental stages, based on May-Grunewald Giemsa differential stainings and the characteristic granule contents of each stage. However, the reliance of cytological analysis and user-defined interpretation prevents further downstream analytical approaches. Therefore, much is not known of the developmental and functional properties of each subset and their involvement in inflammatory conditions. To address this, we utilized a combinatorial approach of both functional characteristics as well as transcriptomic profiles to clearly re-define and fully understand neutrophil granulopoiesis in both homeostatic and disease states.

3.1.1 Proliferative activity marks neutrophil precursor in the BM

Cell proliferation is a core function of progenitors, enabling them to quickly repopulate and replenish immune subsets for effector functions. Studies have provided tools to track and visualise this process. One such tool is the Fluorescence Ubiquitination-based Cell Cycle Indicator (Fucci) transgenic mice (Sakaue-Sawano et al., 2008) (Figure 3.1A). Sakaue-Sawano and his colleagues demonstrated that, by tagging the molecules Cdt1 and Geminin, they were able to distinguish cells in the non-proliferative G1/G0 phase (marked as red) and the

proliferating S/ G₂/M phase (marked as green) of the cell cycle. We analysed the blood, bone marrow and spleen tissues of these mice and measured the proliferation activity of various known subsets. As expected, we observed very low proliferation in HSCs but a steady increase in proliferation activity from MPPs to cMoPs (common monocyte progenitors) (Hettinger et al., 2013). This activity is ceased in most mature cells we observed (Figure 3.1B). Another tool is the use of thymidine analogues like 5-bromo-2-deoxyuridine (BrdU) and iodouridine (IdU). Cells undergoing DNA replication in the S phase take up these analogues, incorporating them into their DNA. These analogues can then be detected by antibody-specific antibodies.

parameters. Clusters were defined manually by lineage markers. Results are representative of three independent experiments. (D) Based on the clusters identified in (C), median intensities for each marker were calculated and plotted as heat maps to identify the respective immune cell population. (E) Surface marker expression levels (median intensity) for IdU⁺ and IdU⁻ neutrophils with differentially expressing markers as shown (black arrows).

To perform an unbiased phenotyping of BM cells neutrophils, we performed a 40-marker high-parametric analysis of total nucleated bone marrow cells with mass cytometry. To detect proliferating cells, we pulsed BM cells with IdU. Unlike BrdU, IdU is readily detected by cyTOF. Together, we gated on the proliferative IdU⁺ and the non-proliferative IdU⁻ cells and performed t-distributed stochastic neighbour embedding (t-SNE) dimensionality reduction to identify the different immune subsets in the BM with Cytokit (Chen et al., 2016) (Figure 3.1C). Next, we extracted the median intensities of each surface marker and generated heatmaps for each of the IdU⁺ and IdU⁻ cluster (Figure 3.1D). Using this method, we identified a large cluster of IdU⁺ BM neutrophils (right, in green) and found differential marker expression levels with the IdU⁻ neutrophils by performing a side-by-side comparison (Figure 3.1E). Such markers include: cKit, CXCR2, Ly6G, Gr1, CD62L and CXCR4 (black arrows).

To confirm our findings, we validated this set of markers with the Fucci-(S/G₂/M) transgenic mice model described earlier. We first excluded lineage-positive cells, early progenitors (cKit^{hi} cells), monocytes (SSC^{lo}CD115⁺), and eosinophils (SSC^{hi}SiglecF⁺), then gated on Gr1⁺CD11b⁺ neutrophils (Figure 3.2A). Dimensional reduction with t-SNE revealed two distinct clusters that were distinguishable on the basis of Fucci-(S/G₂/M) expression (Figure 3.2B). We next compared the expression of various markers between proliferating Fucci-

(S/G₂/M)⁺ and non-proliferating Fucci-(S/G₂/M)⁻ neutrophils, In agreement with the mass-cytometry data (Figure 3.1E), non-proliferating neutrophils highly expressed Ly6G and CXCR2, whereas proliferating neutrophils were Ly6G^{lo}CXCR2⁻ and were positive for cKit and CXCR4 (Figure 3.2C). Taken together, our cell-cycle-based approaches have identified heterogeneity among the neutrophil lineage and revealed a putative proliferative neutrophil precursor, which we term pre-neutrophils (preNeus).

Figure 3.2 Validation of the proliferation capacity of neutrophils in fucci mice

(A) BM Gr1⁺CD11b⁺ neutrophils of Fucci-S/G₂/M (#474) mice were gated accordingly and subjected to t-SNE dimensional reduction based on the expression of 11 markers. (B) Expression level plot of Fucci-S/G₂/M (#474) color mapped from blue (low expression) to red (high expression). (C) Differential expression levels of Fucci-S/G₂/M⁺ (green) and Fucci-S/G₂/M⁻ (grey) clusters (left) represented by overlaid histograms of indicated markers (middle) and plots (right). Data is representative of three independent experiments.

3.1.2 CXCR4 regulates the localization and mobilization of neutrophils in the BM

The development of neutrophils resides primarily in the bone marrow where specific niche cells like the CXCL12-abundant reticular (CAR) cells and endothelial cells which have been associated with hematopoietic stem cell

quiescence as well as their retention within the bone marrow (Ding and Morrison, 2013; Frenette et al., 2013).

To understand the spatial localisation of proliferating preNeus, thick 250µm femur sections were fixed, stained, clarified and imaged. This enabled us to visualise preNeus (S100A9⁺Fucci-S/G₂/M⁺, in yellow), non-proliferating neutrophils (S100A9⁺Fucci-S/G₂/M⁻, in red), blood vessels (Laminin⁺, in blue) and bone/collagen (SHG, in grey) (Figure 3.3A). Magnified areas of the section (Figure 3.3A, right) revealed a consistent clustering phenotype of preNeus throughout the section, reminiscent of colony forming properties of progenitors in vitro.

Figure 3.3 Identification of a proliferative neutrophil precursor that is found in clusters in close proximity with CAR cells.

(A) Snapshot of cleared distal epiphysis of BM femur (250µm thick vibratome section) showing Fucci-S/G₂/M⁺ cells (green), neutrophils (S100A9, red), collagen (second harmonic generation, grey) and blood vessels (laminin, blue) (scale bar = 300µm). A zoomed-in view of a cluster is shown (right) (scale bar = 20µm) (B) Representative images of Fucci-S/G₂/M⁺S100A9⁺ cells in close proximity to CXCL12⁺ stromal cells (top, white arrowheads) compared to Fucci-S/G₂/M⁻S100A9⁺ cells (bottom, yellow asterisk) (scale bar = 10µm). (C) The distance and mean distance to the nearest CAR cell (CXCL12⁺) or vessel (laminin⁺) ($n = 1893$ preNeu and $n = 1509$ Neu from the distal epiphyses of 3 BM femurs). Data reflect mean $\pm$ SEM from three independent experiments. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ (one-way ANOVA).

(D) Comparison of BM preNeu in wildtype mice, *S100a8^{cre}Cxcr4^{fl}* mice and *Cxcr4^{WHIM/+}* mice. Data are representative of at least two experiments. Results are expressed as a fold change in cell numbers $\pm$ SD ($n = 10$ mice per group). **, $p < 0.01$; ****, $p < 0.0001$.

As our data suggests a higher expression of both cKit and CXCR4 on preNeus, we hypothesised that these CAR cells (high producers of SCF and CXCL12) might be associated with these precursors at a specific niche within the bone

marrow. To address this, we crossed the Fucci-S/G₂/M mouse with CXCL12-DsRed mouse strain. By imaging and quantifying our observations, we showed that proliferative preNeus reside closely ($< 5\mu\text{m}$) to CAR cells as compared to non-proliferative neutrophils, suggesting a preferential niche of growth and differentiation (Figure 3.3B). Moreover, there was no significant difference in localisation of preNeus to endothelial cells (Figure 3.3C).

Since our data suggest a specific niche formation utilising the interaction of CXCL12 and CXCR4, we used a neutrophil-specific CXCR4 conditional knock-out mouse (termed S100a8^{cre}CXCR4^{fl}) to test if this loss of interaction loses the retention of preNeus in the bone marrow. We observed an approximate 50% reduction in preNeus numbers in the BM of S100a8^{cre}CXCR4^{fl} mice as compared to the wild-type controls. Inversely, we noticed a two-fold increase of BM preNeus when we analysed a gain-of-function model of CXCR4 (termed CXCR4^{WHIM/+}) (Figure 3.3D). Taken together, our data indicate that preNeus reside in close proximity to CAR cells and this CXCR4-dependent interaction is important for their retention in the BM.

3.1.3 Transcriptome profiling reveals developmental program

The functional properties of preNeus, including proliferation and CXCR4-signaling, provide evidence that preNeus are a distinct subset of BM neutrophils with specific characteristics. To further understand preNeus and how they fit into neutrophil development, we analysed total BM neutrophils (Lin⁻Gr1⁺CD11b⁺) and excluded preNeus (cKit^{hi}CXCR4^{hi}). Although blood neutrophils were mostly Ly6G⁺ and CXCR2⁺, the non-proliferative pool of BM neutrophils contain a

heterogeneous mixture of cells with varying expressions of Ly6G and CXCR2 (Figure 3.4A). As CXCR2 is a critical chemokine receptor for neutrophil mobilisation and recruitment, we consider the CXCR2-negative population as immature and the CXCR2-positive fraction as mature based on their similar phenotype to blood neutrophils.

Figure 3.4 Identification strategy of three phenotypically distinct subsets of BM neutrophils

(A) Gating strategy of BM GMP, preNeu, immature Neu, mature Neu and blood Neu based on the markers identified with Cytof. (B) Wright-Giemsa staining of each sorted population (scale bar = 10µm). Data representative of 3 independent experiments.

To further characterise these populations, we sorted preNeus, immature Neus (CXCR2⁻), mature Neus (Ly6G⁺CXCR2⁺) and compared their morphology with GMPs and blood neutrophils. GMPs possess largely uncondensed nuclei and small cytoplasmic space with mainly primary granules. From preNeus to blood neutrophils, we observed a progressive condensation of nuclei and a reduction of primary granules in the cytoplasm (Figure 3.4B). Notably, BM mature Neus are morphologically similar to blood neutrophils with the signature poly-segmented nuclei of neutrophils.

Going beyond morphology, we used total mRNA-sequencing (RNA-Seq) to determine unique molecular signatures of each subset. Principal component analysis (PCA) of all transcripts revealed distinct expression profiles for each subset (Figure 3.5A). Though distinct, the Pearson correlation matrix showed a transcriptomic similarity between subsets (GMP with preNeus, preNeus with immature Neus, and immature with mature Neus) (Figure 3.5B). This suggests a developmental progression from GMPs to blood neutrophils. Interestingly, although immature Neus were only distinguished from mature Neus by CXCR2, these subsets showed vast transcriptomic differences with over 3,000 differentially expressed genes (DEGs) (Figure 3.5C).

Figure 3.5 Transcriptomic analysis reveal distinct expression signatures during development

RNA was extracted for each sorted population (according to Figure 3.4) for total mRNA-sequencing (n=3). (A) PCA of gene expression of subsets. (B) Correlation matrix generated using Pearson's correlation coefficients that represents similarities of gene expression between subsets (low similarity = red, high similarity = yellow). (C) Volcano plots of the various subsets. (D) Heat-map of differentially-expressed genes between subsets among the 20% most variable genes (4820 genes out of 24098 detected transcripts). Genes clusters (1 to 7) were defined following hierarchical clustering and exported for gene ontology (GO) biological process analysis. (E) GO biological process terms enriched in various indicated clusters. (F) Median gene expression ($\log_2$ CPM) of indicated cluster-associated genes across sorted populations.

We then plotted the top 20% variable genes in a heatmap, generating seven

distinct clusters of genes differentially regulated during granulopoiesis (Figure

3.5D). Clusters 1 and 2 are shown to be up-regulated during granulopoiesis,

consisting of chemotaxis-related functions (cluster 1) and response to bacterial stimuli (cluster 2) (Figure 3.5E & F). Conversely, we observe a progressive loss of genes related to cell cycle function (cluster 5) and RNA processing (cluster 6) (Figure 3.5E & F).

This change in cell cycle functions is clearly seen in the dual reporter Fucci 474 (S/G₂/M)/639 (G₀/G₁) mice (Tomura et al., 2013) (Figure 3.6A). In these mice, preNeus showed the highest proportion of cells in the S phase while immature Neus abruptly exits the cell cycle and progressively entered the G₀ phase upon maturation as mature Neus (Figure 3.6 B & C). We then investigated how these differences in cell cycle statuses could translate to biological function. To address this question, we performed in vitro cultures of preNeus, immature Neus, and mature Neus for two days. Although the numbers of immature and mature neutrophils rapidly declined in culture, we found that preNeus could expand in culture (Figure 3.6D). Colony-forming assays also revealed that, unlike GMPs, preNeus divided but did not form colonies, whereas immature and mature neutrophils did not divide at all (Figure 3.6E).

Figure 3.6 preNeus can proliferate but do not form colonies

(A) Gating strategy for identifying cell cycle stage using Fucci-(G₀-G₁) (#639) / Fucci-(S-G₂-M) (#474) BM cells and (B) the representative plots and proportion (C) of each stage in the indicated subsets. (D) Analysis of the in vitro proliferation assay of neutrophil subsets. Data are expressed as fold-change in numbers $\pm$ SD (n = 3) and is representative of four independent experiments. ****, p < 0.0001 (one-way ANOVA) (E) Colony forming assay of the sorted BM GMP and neutrophil subsets supplemented with the indicated cytokines. Results are representative of three independent experiments. Scale bar = 50μm.

3.1.4 Identification of the developmental relationship between neutrophil subsets

While our data suggest a maturation progression between neutrophil subsets (preNeus to immature Neus to mature Neus), it remains unclear if preNeus are fully committed to the neutrophil lineage. To address this, we first compared the transcriptomic signature of members of the neutrophil and monocyte lineages with GMPs (Figure 3.7A). PCA of all transcripts revealed that preNeus were more similar to members of the neutrophil lineage, such as immature Neus, than they were to members of the monocyte lineage (Figure 3.7A).

To further determine the developmental fate of preNeus, we used a fate-mapping model based on a *S100a8* neutrophil-specific cre recombinase. Through our RNA-Seq data, we verified that *S100a8* gene expression is selectively upregulated from the preNeu stage onwards and minimally expressed on GMPs and of the monocyte lineage (Figure 3.7B). This is consistent with previous published data (Passegue et al., 2004; Reber et al., 2017). We crossed the *S100a8*^{cre} mice together with Rosa26^{LsL-YFP} mice and determined the recombination rate of the various developmental stages. Although GMPs showed no detectable recombination, preNeus displayed a ~40% recombination rate that progressively increases in the immature, mature and blood neutrophil (~80%) subsets (Figure 3.7C). In contrast, other myeloid cells such as monocytes and eosinophils are minimally tagged (<10%).

We next validated the commitment and lineage-specificity of preNeus by performing *in vivo* transfer of sorted GFP⁺ preNeus into the BM of wild-type recipients. Our data showed that these GFP⁺ preNeus gave rise to immature Neus after one day and differentiated into mature neutrophils by day 2 (Figure 3.7D). Importantly, preNeus did not give rise to monocytes, eosinophils or other cell lineages. This further supports our fate-mapping analysis. To further establish the developmental timeline of preNeus *in vivo*, we utilised the unique proliferative ability of preNeus as a marker to track the progression of neutrophil development. We employed the use of BrdU, which will incorporate into all actively dividing cells like preNeus and enables the tracking of these cells as shown previously with monocytes (Yona et al., 2013). Expectedly, preNeus were the only BrdU⁺ neutrophil subset (Figure 3.7E). Through this method, we found that preNeus

progressively differentiated into immature neutrophils after 24 hr and mature neutrophils after 48 hr before egressing into the circulation after 72hr (Figure 3.7E). Furthermore, treatment with the chemotherapeutic drug 5-fluorouracil (5-FU) triggered a successive loss from GMPs to mature Neus by inhibiting thymidine synthesis and initiating apoptosis in dividing cells (Figure 3.7F).

Figure 3.7 preNeus are committed to the neutrophil lineage.

(A) PCA of gene expression data from GMP and neutrophil and monocyte subsets. (B) Relative expression of *S100a8* ($\log_2$ CPM) among GMPs and BM neutrophil subsets ($n = 3$). (C) Strategy for genetic cell fate-mapping (left) and recombination frequency in the indicated populations (right). Results are expressed as mean $\pm$ SD ($n = 3$) and are representative of two independent experiments. (D) Intra-BM transfer of sorted Lyz2-GFP+ preNeu into wild type recipients. Top-row: identification strategy of the different cell populations. Medium and bottom row: black dots represent transferred cells at day 1 (middle row) and day 2 (bottom row) after transfer. Data are representative of one out of five independent mice. (E) Kinetics of BrdU incorporation among neutrophil subsets after a single pulse of BrdU. Data are expressed as mean $\pm$ SD ($n \geq 3$ per time point) and are representative of two experiments. (F) Myelodepletion of BM cell populations using 5-FU. Data are expressed as mean $\pm$ SD ($n \geq 3$ per time point) and are representative of two experiments

3.1.5 Functional changes of neutrophil subsets with maturation and development

To this end, the data suggests a developmental process from preNeus to immature to mature neutrophils. To understand the underlying mechanisms in this process of maturation, we analysed the gene expression of various transcription factors (TFs) involved at different stages of myeloid cell development (Figure 3.8A). As anticipated, multipotent GMPs highly expressed *Cebpa*, which is necessary for granulopoiesis initiation, as well as TFs involved in the development of other myeloid lineages, such as *Irf8*, *Gata1*, and *Gata2* (Fiedler and Brunner, 2012; Yanez et al., 2015). In contrast, preNeus highly expressed *Gfi1* and *Cebpe*, two TFs crucial for early neutrophil differentiation (Hock et al., 2003; Yamanaka et al., 1997a). Finally, mature and circulating neutrophils showed high expression of *Cebpd* and *Spi1* (PU.1), which is in line with their role in terminal granulopoiesis (Borregaard, 2010) (Figure 3.8A).

Many TFs in the CCAAT-enhancer-binding protein (C/EBP) family promote the expression of granule associated enzymes. Specifically, C/EBP α induces the expression of primary granule enzymes (Ford et al., 1996) while C/EBP ϵ and C/EBP δ promote secondary and tertiary granule enzymes respectively (Gombart

et al., 2003). Our transcriptomic analysis showed a coordinated and sequential expression of these factors (Figure 3.8A) and allowed us to wonder if this pattern of expression coincides with other granule expression. Indeed, GMPs mainly expressed primary granule proteins, whereas preNeus and immature Neus expressed secondary granules. Tertiary granules are mostly expressed by immature and mature Neus (Figure 3.8B). This expression pattern matches the expression of C/EBPa, C/EBPε and C/EBPd.

Figure 3.8 Transcriptional regulation of development

(A-B) Expression of genes (z-score normalized) encoding (A) various myeloid development-related TFs and (B) granule production, assessed in GMPs and neutrophil subsets.

Apart from important granule production program for effector function, we also wanted to determine the functional differences that occur across the various stages of development. To do this, we looked at key genes involved in reactive oxygen species (ROS) production, phagocytosis and chemotaxis (Figure 3.9A, D and E). For all three functions, we found the highest expression among mature neutrophils. This finding was validated by *in vitro* assays which showed mature neutrophils having superior capacity to produce ROS upon stimulation (Figure 3.9B) as well as phagocytose bacteria (Figure 3.9C). Additionally, we found that while preNeus had minimal migratory capacity, mature neutrophils quickly

swarmed toward the inflamed site during sterile injury (Figure 3.9F and Movie 1). Together, we found a progressive functional maturation of neutrophils during development leading towards mature neutrophils having the full range of neutrophil effector functions.

Figure 3.9 Neutrophils functionally mature as they develop

(A) ROS biosynthetic process-related genes in GMPs and neutrophil subsets. (B) ROS production by neutrophils subsets assessed by flow cytometry using dihydrorhodamine 123 (DHR). Data are shown as a geometric mean $\pm$ SD ($n = 3$) and are representative of two independent experiments. **, $p < 0.01$ (one-way ANOVA). (C) Phagocytosis of GFP+ *E. coli*, expressed as a percentage of GFP+ cells $\pm$ SD ($n = 3$) and are representative of two independent experiments. *, $p < 0.05$; ***,

p < 0.001 (one-way ANOVA). (D) Phagocytosis-related genes expression in GMP and neutrophil subsets. (E) Chemotaxis-related genes and their corresponding expression in GMP and neutrophil subsets. (F) (top) Experimental set-up of the laser-induced sterile injury model. (bottom) Maximum z-projected snapshots from time-lapse showing neutrophil subsets migration towards the laser burn (grey square) (bottom left) with corresponding cell tracks (bottom right). Scale bar = 100µm. Time, h:min. Data are representative of three independent experiments (See also Movie 1).

3.1.6 Neutrophil development requires the transcription factor C/EBPε.

C/EBPε is a crucial TF for the production of secondary granules in mice and humans (Gombart et al., 2003; Yamanaka et al., 1997a). However, the precise involvement of C/EBPε in neutrophil development remains unclear. Because we had detected a strong upregulation of *Cebpe* expression in preNeus (Figure 3.8A), we hypothesized that C/EBPε could be involved in the transition from GMPs to preNeus. To examine this, we compared the cell number of GMPs and neutrophil subsets in the BM of *Cebpe*^{-/-} and wild-type (WT) mice (Figure A). We observed that preNeus and downstream populations were severely reduced but GMPs accumulated in *Cebpe*^{-/-} mice, supporting the conclusion that C/EBPε is important for the developmental transition from GMPs to preNeus (Figure 3.10A). In contrast, we found an increase in tpMo and Ly6Chi monocyte numbers in *Cebpe*^{-/-} mice (Figure 3.10B), which could indicate a compensatory differentiation of GMP to the monocyte fate. To confirm the role of C/EBPε in neutrophil development in a competitive setting, we generated BM chimeras with an equal mixture of CD45.1⁺ WT and CD45.2⁺ *Cebpe*^{-/-} BM cells (Figure 3.10C). In line with earlier results (Figure 3.10A), preNeus and downstream neutrophil populations were not derived from *Cebpe*^{-/-} cells but from WT cells.

Figure 3.10 C/EBP ϵ -deficiency impairs the development of preNeus and downstream neutrophil populations

(A-B) Absolute counts of BM myeloid cell subsets in WT and *Cebpe*^{-/-} mice expressed as mean $\pm$ SD (n = 5) and are representative of two independent experiments. (C) (top) Experimental set-up and (bottom) percentage contribution of various hematopoietic cells by WT CD45.1+ or *Cebpe*^{-/-} CD45.2+ cells expressed as mean $\pm$ SD (n = 5) and are representative of two independent experiments.

Because the development of preNeus is highly impacted in *C/EBP ϵ* -deficient animals, we next investigated how a lack of preNeus during inflammatory responses would result in functional consequences. We utilized a neutrophil-dependent model of immune complex-mediated inflammation (reverse passive Arthus [RPA] reaction) (Li et al., 2016). Here, we observed a severe reduction in neutrophil infiltration (Figure 3.11A) and vascular leakage (Figure 3.11B) at the site of the reaction. A mid-grade cecal ligation and puncture (CLP) sepsis model also revealed that *Cebpe*^{-/-} mice had poorer bacterial clearance in the blood and peritoneal cavity than did wild-type control mice (Figures 3.11C and D).

Figure 3.11 Functional consequence of preNeu deficiency

(A) Absolute counts of infiltrated skin neutrophils. (B) (left) Representative ($n = 3$) photographs showing RPA-induced leakage. Green insets, pixel classification: leakage, white; no leakage, black. (right) Measurement of total Evans blue dye in mouse ears. Results are pooled from two experiments, expressed as mean $\pm$ SEM ($n = 9$ –12 per group). **, $p < 0.01$ (Student's t test, Mann whitney). (C) Absolute counts of mature neutrophils in the blood and peritoneum of WT and *Cebpe*^{-/-} mice. (D) Bacteria CFU quantification of blood and peritoneal fluid 24 hours after mid-grade CLP. Results are expressed in mean $\pm$ SD ($n = 4$ –10 per group) and are representative of two experiments. ***, $p < 0.001$ (Student's t test).

3.1.7 PreNeus expand in the bone marrow and spleen during inflammation

Since preNeus were shown to have poor effector functions (Figure 3.9), we wondered if preNeus played any roles during inflammation. As shown earlier, the use of the *Cebpe*^{-/-} mice has indicated that the absence of these precursors results in a lack of neutrophil-mediated responses (See Figure 3.11). Since preNeus acted as a proliferative precursor in the steady state, we next sought to understand how preNeus were affected during diseases that require increased myelopoiesis. Using a model of sepsis, we found an increase in preNeu numbers in the BM and spleen upon sepsis (Figure 3.13A and B). Additionally, we observed a similar effect in an orthotopic tumor model of pancreatic carcinoma (Figure 3.13 C and D). Of note, while a 2- to 4-fold increase in BM preNeu

numbers was detected in both of these models, we noticed a significant and drastic >10-fold increase in spleen preNeu numbers (Figure 7B and D). Taken together, our results highlight the expansion of BM and spleen preNeu during inflammatory conditions, indicative of increased intra- and extramedullary granulopoiesis.

Figure 3.12 preNeus proliferate and expand extensively during inflammation (A-D) Representative plots (A & B) and absolute numbers (C & D) of preNeu in the bone marrow and spleen of CLP-induced septic mice. Data is expressed as mean $\pm$ SD (n=10 per group) and is representative of at least three independent experiments. (E-H) Representative plots and absolute numbers of preNeu in the bone and spleen of tumour-bearing mice. Data is expressed as mean $\pm$ SD (n=5 per group) and is representative of three independent experiments. *, $p < 0.05$, ***, $p < 0.001$, **** $p < 0.0001$ (Student's t test, Mann whitney).

3.1.8 CD101 as a marker for immature neutrophils during inflammation

Neutrophils are being increasingly recognized as important players in tumorigenesis. However, conflicting evidences indicate that neutrophils can carry both pro- and anti-tumoural properties (Coffelt et al., 2016; Nicolas-Avila et al., 2017). We speculated that these opposing observations might be explained by differing maturation status of neutrophils in tumours as recently suggested by others (Coffelt et al., 2016).

To address our hypothesis, we investigated whether immature and mature Neutrophils can be delineated in orthotopic pancreatic tumours using CXCR2 expression since this marker is differentially expressed between these two populations in the BM (Figure 3.14A). However, unlike in the BM and blood, we found a strong downregulation of CXCR2 in the tumour (Figure 3.14 A).

To overcome the challenge of segregating immature and mature Neutrophils in the tumour, we screened for differentially expressed genes (DEGs) to identify markers that could clearly distinguish these cells in the tumour. Among these DEGs, we identified *Cd101*, a surface marker that was significantly upregulated in BM mature and blood Neu (Figure 3.13B). To validate, we identified Gr1⁺CD11b⁺ neutrophils in BM, blood and spleen. Importantly, delineating neutrophils with CD101 allowed us to distinguish two populations of neutrophils that matched the expression pattern of CXCR2, such that immature Neu could be defined as Ly6G^{lo/+}CXCR2⁻CD101⁻ while mature Neu could be defined as Ly6G⁺CXCR2⁺CD101⁺ (Figure 3.13C). Notably, CD101⁻ immature Neu were nearly absent from the circulation at resting conditions (Figure 3.13C).

Figure 3.13 Identification of CD101 distinguishes immature Neus from mature Neus during inflammation

(A) Representative histogram of total neutrophil expression level of CXCR2 in the indicated organs. (B) mRNA expression level of Cd101 in various neutrophil subsets represented as Log2CPM. Results is expressed as mean $\pm$ SD (n=3). ****, $p < 0.0001$ (Mann Whitney's student t-test). (C) Representative plots of CD101 expression in indicated organs as well as the corresponding CXCR2 expression in each subset. Data is representative of at least three experiments.

Using CD101 expression to distinguish immature and mature Neus, we first determined if this approach would allow us to detect them in the circulation. We administered mice with G-CSF, a strong neutrophil mobilizer that is similarly upregulated during cancer (Kowanetz et al., 2010). We used a stable complex version of G-CSF bound to anti-G-CSF (called G-CSFcx) to stimulate a milder but prolonged effect of G-CSF. This effect simulates sustained inflammatory stimulus, instead of creating a transient spike of G-CSF level in the blood. Using this stimulus, we detected immature Neus in the blood and their numbers were maintained in the circulation for up to four days after treatment (Figure 3.14A & B). Since immature Neus are able to enter the circulation, we next addressed whether these cells had the capacity to migrate into tissues. We sorted BM GFP+

immature neutrophils and BM RFP⁺ mature neutrophils and co-transferred them intra-dermally before inducing sterile inflammation. Both immature and mature Neus could swarm equally towards the injury core in a sterile laser injury model (Figure 3.14 C and Movie 2). This is in sharp contrast to preNeus that displayed poor interstitial motility (Figure 3.9F and Movie 1), and suggests that immature Neus already possess functional migratory machinery.

Figure 3.14 G-CSF induces mobilisation of CD101⁻ immature Neus into the periphery.

(A) Representative plots of G-CSF-complex mobilised blood neutrophils 1 day after administration. (B) Absolute numbers of blood neutrophil subsets after G-CSFcx administration at the indicated time points. Data is reported as mean $\pm$ SD (n=3 per time point) and is representative of at three independent experiments. (C) Laser injury-induced sterile inflammation response by co-transferred immature or mature neutrophils as indicated (top). Snapshots of various timepoints are shown at the indicated time (min) (bottom). Tracks representing migration path of immature neu (in green) or mature neu (in red) (bottom, right). Data is representative of three independent experiments

3.1.9 CD101- immature neutrophils are associated with severity of tumour progression

Having established that CD101 distinguishes immature Neus from mature Neus during G-CSF stimulation, we next validated this strategy in the tumour setting. Compared to naïve mice, mice bearing pancreatic tumours showed increased numbers of immature Neus in the blood and pancreas (Figure 3.15A and B). Furthermore, a positive correlation between the number of immature Neus in the blood and the pancreas suggested that these cells were actively recruited to the tumour site from the circulation (Figure 3.15C). To test whether infiltration of immature Neus may be related to tumour progression, we separated tumour-bearing mice into two groups according to their tumour weight, and found that mice with a higher tumour burden showed higher infiltration of immature Neus into the pancreas (Figure 3.15D & E). Additionally, mice with a higher tumour burden had significantly more immature Neus, but no significant differences in mature Neus in the circulation (Figure 3.15F & G). Notably, the number of immature Neus in the blood was highly correlated with the weight of the pancreas (Figure 3.15H). In contrast, circulating mature Neus and Ly6Chi monocytes poorly correlated with the pancreas weight, which suggests that the presence of immature Neus in the blood may serve as a biomarker of disease progression.

Figure 3.15. Immature Neutrophils can be distinguished from mature Neutrophils through CD101 expression and are associated with tumour progression.

Absolute counts of blood (A) and intra-pancreas immature or mature neutrophils. Data is pooled from three independent experiments and reported as mean $\pm$ SEM (n=15 per group). ***, $p < 0.001$, ****, $p < 0.0001$. (Mann Whitney, student t-test). (C) Pearson correlation test of blood immature Neu and intra-tumour immature Neu. Data is pooled from three experiments. (D) Blood and (E) tumour immature Neu frequencies of mice with low and high tumor-burden. (F) Pancreas tumour weight of tumor-bearing mice. The top 50% was defined as high tumor burden while the bottom 50% was considered low tumor burden. (G) Comparative neutrophil cell numbers between low and high tumor burden. Data is expressed as mean $\pm$ SEM (n=7-8 per group). n.s., not significant, **, $p < 0.01$ (Mann Whitney, Student t-test). (H) Pearson correlation test of blood immature neutrophils with weight of pancreas. Data is pooled from three independent experiments.

In summary, we have identified a strategy to distinguish immature from mature Neu in cancer, and reveal that circulating immature Neu numbers are associated with increased tumour burden.

3.1.10 Discussion

While neutrophil heterogeneity is increasingly appreciated, their developmental path and functional properties from multipotent GMP to mature neutrophils

remains elusive (Silvestre-Roig et al., 2016). Here, we report the identification of a committed neutrophil precursor population, which we termed pre-neutrophils (preNeus). We also discovered that preNeus undergo a distinct developmental step into immature Neus before differentiating into mature Neus. Our study hence fills a long-standing gap in the model of neutrophil development pathway by identifying the presence of preNeus as a committed neutrophil precursor downstream of GMP and its subsequent developmental steps into mature Neus.

Neutrophil development in the BM has been characterized historically through a density gradient separation technique, followed by their identification with Giemsa stain (Bjerregaard et al., 2003; Borregaard et al., 1995). While this approach delivered useful insights about neutrophil development and maturation, it lacked precision in delineating neutrophil heterogeneity at the single cell level. Furthermore, this method does not allow downstream functional and molecular characterization. To solve this gap and identify a putative neutrophil precursor, we combined CyTOF-based multi-parameter analytical approach and cell cycle reporters followed by unbiased computational dimensionality reduction analysis. Surface markers derived from the CyTOF datasets were then adopted for subsequent flow cytometric analysis of Fucci-S/G2/M mouse, followed by cell cycle- and functional based (i.e. CXCR2 expression) stratification, which led us to the identification of three BM neutrophil subsets, namely: preNeus, immature Neus and mature Neus.

Differentiation of multipotent precursors to cell-restricted precursors is known to involve a complex and dynamic balance between the upregulation and silencing of lineage-specific and lineage-irrelevant genes respectively (Fiedler and Brunner, 2012). Specifically, our transcriptomic analysis of BM neutrophil subsets revealed a silencing of TFs such as *Irf8* and *Gata1/2*, as well as the activation of *Gfi1* and *C/EBPε* in preNeus. These findings establish a molecular developmental pathway and cell commitment of preNeus toward the neutrophil lineage as these factors are hallmark molecular signatures of the loss of potential towards the monocyte, eosinophils and basophil lineages (Fiedler and Brunner, 2012; Kurotaki et al., 2014). More importantly, *Gfi1* is critical for multipotent precursor commitment to the granulocytic lineage as deficiency in *Gfi1* expression results in a block of neutrophil development at the early differentiation stage (Hock et al., 2003; Karsunky et al., 2002). Thus, a specific upregulation of *Gfi1* expression in preNeus further suggests that these cells are the first precursors committed towards the neutrophil lineage.

Besides the identification of lineage-specific TFs in the neutrophil developmental pathway, granule proteins have also been traditionally used to define neutrophil differentiation and maturation (Cowland and Borregaard, 1999). To further understand the functional development of our identified neutrophil subsets, we profiled granule gene expression and found a hierarchical clustering of granule-associated genes with specific neutrophil subsets. In particular, we found an enrichment of primary and secondary granule genes in preNeu, secondary and tertiary granules genes in immature Neus, and tertiary granules genes mature BM Neus. This development hierarchy was further confirmed using a combination of

fate-mapping, trajectory prediction algorithm and *in vivo* assays, thereby corroborating the notion that proliferative preNeu differentiate into immature Neus before giving rise to functionally mature Neus.

The proliferative nature of preNeus suggest that these cells may require the input of cytokines or growth factors to sustain their proliferative activity. Indeed, emerging evidence suggests that HSCs and lineage-committed precursors reside in specialized BM niches to receive instructive signals for their maintenance and differentiation. We found that preNeus formed clusters that are closely associated with CAR cells in BM, and that the CXCR4 signalling pathway is crucial for preNeu retention in the BM. However, CXCR4-mediated interaction with CAR cell does not seem critical for preNeu differentiation as conditional deletion of CXCR4 in preNeus did not result in neutropenia (data not shown). This is in stark contrast to the essential role of CXCR4 in promoting the positioning of common lymphoid progenitors and pre-pro B cells near the IL7-expressing CAR cells for efficient differentiation to mature B cells in the BM (Cordeiro Gomes et al., 2016; Tokoyoda et al., 2004). Hence, our current findings suggest that preNeus can differentiate and mature into neutrophils independently of BM niche signals. Based on our analysis of differentially regulated genes assigned to cell cycle and cellular functions, supported by results from various functional assays, we believe that preNeus are proliferative but functionally immature. Thus, it is tempting to speculate that preNeus possess a cell intrinsic program, which directs them to fulfil their role as proliferative precursors for neutrophils independent of specialized niche signals. Further studies are required to test this hypothesis. When taken together, these findings suggest that neutrophil progenitors

sequentially acquired distinct molecular profiles and functions during their maturation.

The cellular expansion of myeloid precursors during emergency granulopoiesis is fundamentally necessary to meet the demand for functionally mature neutrophils (Manz and Boettcher, 2014). In line with this notion, our experimental models of sepsis and tumour demonstrated an expansion of preNeus in the BM during inflammation. Interestingly, elevated splenic preNeu numbers was also detected in these models. Two possibilities may account for this phenomenon: (1) an activation of extramedullary granulopoiesis in the spleen; (2) deployment of BM preNeus to the extramedullary sites in response to inflammatory stimuli. Our current findings rule out the latter possibility as preNeus were absent in the circulation during inflammation. This finding suggests that cytoskeleton machineries are unable to cater to the needs of proliferation and migration simultaneously, which is in line with the “go or grow” hypothesis in cancer biology (Garay et al., 2013; Giese et al., 1996). Therefore, we believe that the expansion of splenic preNeus is most likely attributed to heightened extramedullary granulopoiesis through increased production of GM-CSF and IL-3 in the spleen microenvironment (Robbins et al., 2012; Weber et al., 2015).

In contrast to preNeus, immature Neus are non-proliferative but migratory active. During the resting state, immature Neus are absent in the circulation as they are retained in the BM where they undergo further maturation. However, these cells can enter into the bloodstream under inflammatory conditions. Importantly, our

imaging data revealed that immature Neus can migrate actively towards the local site of injury/inflammation as efficiently as mature Neus. These data hence suggest that while preNeus are proliferative precursors that can fine-tune the output of neutrophils; immature Neus, on the other hand, serve as a reservoir that can be deployed to sites of inflammation. It is currently unclear what the implications of this “premature” mobilization of immature Neus to the circulation and local sites of inflammation are. We believe that this is an important question to address, and that future research should focus on: (1) understanding if immature Neus can undergo further maturation at sites of inflammation, and (2) determining the plasticity and function of immature Neus.

As short-lived cells, neutrophils were classically regarded to play an insignificant role in chronic pathologies such as cancer. In contrast to this view, accumulating evidences suggest the presence of neutrophils with immature/aberrant nuclear morphology in tumour-bearing mice (Coffelt et al., 2015a; Fridlender et al., 2009; Youn et al., 2012) that have been negatively associated with disease outcome (Sagiv et al., 2015; Yang et al., 2011). However, it has been difficult to delineate the specific contribution of each subset due to the challenges of distinguishing neutrophils of such immature morphology from mature neutrophils. To address this challenge, we believe that our “CD101-based” identification stratification presents a major advancement in the methodology for neutrophil characterization as it precisely delineates immature and mature Neus in both steady and inflammatory conditions. More importantly, our CD101-based strategy allowed us to observe a strong correlation between the numbers of circulating immature Neus and tumour burden, suggesting that the number of blood immature Neus

could be used as an indicator of tumour burden. Future work will include the determination of CD101 in distinguishing immature Neus from mature Neus in humans. Since neutrophils generally promote tumour progression, the neutrophil-to-lymphocyte ratio (NLR) has also been proposed as a prognostic indicator for solid tumours (Templeton et al., 2014). However, this measurement is limited by the circulating neutrophil counts that varies in individuals. Patients with a higher basal level of neutrophil numbers can seem to have a worse prognosis as compared to patients with lower circulating numbers at physiological conditions. In contrast, immature Neus are not present in the circulation under baseline conditions, and may serve as a better predictor of disease progression for clinical studies.

In summary, our study here has provided further insight into the essential stages in the developmental pathway of neutrophils. More importantly, our current model may also serve as a fundamental platform for the re-examination of granulopoiesis under physiological and disease states, as well as provide the basis for new therapeutic interventions for neutrophil-related diseases.

3.2 Identification and characterisation of a committed neutrophil progenitor within the GMP

Neutrophils are produced in large numbers every day. Yet, a common neutrophil progenitor, responsible for this production, is largely unknown. We have shown that, unlike GMPs, preNeus could not form colonies like typical myeloid progenitors, only dividing once or twice in colony assays (See Figure 3.6E). This led us to believe an upstream progenitor exist, and this progenitor is responsible for the large production of neutrophils in the BM at steady-state and during inflammation. Here, we employed multi-dimensional single-cell transcriptomic and proteomic approaches to trace the origins of neutrophil commitment and production.

3.2.1 GMP population contains a heterogeneous group of lineage-committed progenitors

Granulocyte-macrophage progenitors are known to give rise to neutrophils (Akashi et al., 2000). Recent studies have suggested that these progenitors are perhaps not a truly multi-potent progenitor, but instead contain a heterogeneous mixture of lineage-committed precursors (Giladi et al., 2018; Paul et al., 2015; Yanez et al., 2015). To verify this presence of heterogeneity, we utilized the available published single-cell transcriptomic datasets and analysed the GMP population from these datasets. By extracting the annotated GMP cells (based on Lin⁻cKit⁺Sca-1⁻CD34⁺CD16/32⁺⁺), we performed high-dimensional reduction t-SNE analysis to visualise this heterogeneity. As expected, in both datasets, we observed multiple clusters pertaining to the different granulocyte and monocyte lineages (Figure 3.16A and B). Importantly, we observed a neutrophil-like cluster

containing high expression of neutrophil elastase (*Elane*) and ficollin (*Fcnb*) which are known genes for neutrophil function (Lawrence et al., 2018; Tidwell et al., 2014). While these analyses provide insights into the heterogeneity of progenitors and prediction of cell lineage commitment, they do not offer definitive information for protein markers for downstream molecular analysis.

Figure 3.16 GMP heterogeneity is present at the single-cell level

(A) MAR-seq data from Paul et al, 2015 clustered through iouvain clustering method. Colors represent distinct subsets identified through Phenograph. (B) Dataset from Giladi et al, 2018. Extracted GMP cells from dataset were used for t-SNE dimensional analysis. Phenograph clustering was performed and key signature genes were used to identify clusters as shown.

To overcome this challenge, we utilized a proteomic approach by evaluating the protein expression of over 261 surface markers (LEGENDScreen™) on total mouse BM cells. We then used Infinity Flow: a predictive algorithm, which enables the concatenation of the expression information of each marker into a single analysis file. This enabled us to view every possible co-expression of each screened marker used (Figure 3.17A). By gating on GMPs, we visualised 81 predicted marker expressions in a t-SNE plot and clustered these cells unbiasedly using PhenoGraph (Levine et al., 2015) (Figure 3.17B). To identify the best markers for validation, we took the top 30 most variable surface-marker-related genes among each cluster identified in Giladi's dataset (Figure 3.16B) and matched them against the 81 predicted markers. By using both transcriptomic and proteomic information, we plotted the 23 common markers from both

approaches in a heat map (Figure 3.17C). Out of these 23 markers, we found that commonly used myeloid markers like CD11b and CD34 are able to help distinguish the various GMP subsets (Figure 3.17C and D). Together, our data validates the existence of heterogeneity within the current definition of the GMP and provides a means to isolate these populations for further studies and validation.

Figure 3.17 Combinatorial use of transcriptomic and proteomic data reveals GMP heterogeneity.

(A) Infinity Flow workflow. Whole BM cells are stained with a base panel of lineage markers (see methods) to allow separation of each lineage for UMAP generation. Cells are then aliquoted into wells containing PE-conjugated antibodies. Each well is then recorded by flow cytometry and processed using Infinity flow algorithm. (B) (left) Gating Strategy of total GMPs used for (right) t-SNE dimensional reduction analysis of Infinity Flow data. (C) Using the top 30 variable membrane genes among the clusters in Figure 3.16B, 23 overlapping markers were identified to be expressed on GMP clusters in (B). These markers are represented as a Heat map. Cluster number corresponds to clusters found in (B). Common myeloid markers shown in purple (D) Surface marker expression level of common myeloid cell markers (in purple) from high (red) to low (blue) expression.

3.2.2 UMAP reveals the developmental continuity from GMPs to preNeus

We observed that GMPs are heterogeneous in transcriptomic signatures, cell lineage profiles and surface marker expression levels. This suggests that GMP may consist of a committed neutrophil progenitor population. However, due to a lack of markers for identification, we were unable to determine if such a population exist. Monocytes, on the other hand, have a clear macrophage colony stimulating factor (M-CSF) receptor expression (CD115), indicating their commitment towards the monocyte fate.

To circumvent the requirement of a lineage-restricted marker, we performed Uniform Manifold Approximation and Projection (UMAP) on total BM cells to better understand the developmental relationships between clusters (Becht et al., 2018). UMAP dimensional analysis on total BM cells, together with Infinity Flow, distinguished the various cell lineages within the BM (Figure 3.18A). Each cell type was identified and annotated through the co-localisation of each marker on the UMAP space (Figure 3.18B). This allowed us to identify GMPs and the three BM neutrophil subsets previously characterised, namely: preNeus, immature Neus and Mature Neus (Evrard et al., 2018). We also observed a clear developmental continuum of cells from early progenitors (in red) to mature cells of the various lineages. Notably, a key feature of UMAP was its ability to outline

the developmental paths of basophils, eosinophils, monocytes and neutrophils from the GMPs. Importantly, we observed a branch connecting GMPs and preNeus (Figure 3.18A). Closer inspection of this branch revealed both CD34 and CD11b as critical markers which differentiate them from GMPs (Figure 3.18C). These markers were also predicted to be good discriminating markers for revealing GMP heterogeneity (See Figure 3.17C and D). Using the deep phenotypic information of Infinity Flow, we identified several key markers at this branch point, which can potentially serve as markers in identifying a putative neutrophil progenitor (Figure 3.18D).

Figure 3.18 UMAP identifies developmental link between GMPs and preNeus with variable CD34 and CD11b expression

(A) UMAP projection of total mouse BM cells showing cKit expression from high (red) to low (blue) levels. Neu = neutrophil, preNeu= pre-neutrophil, Eo= Eosinophil, T,B = T and B cells, Mo = monocytes, Baso = Basophil. (B) Surface marker expression of various lineages demarcating clusters for annotation. (C) Zoomed area from (A) showing the marker expression levels for GMPs. (D) Identified screened hits from LEGENDScreen™. Expression intensities (Log₂MFI) are shown from high (red) to low (blue) levels

Using the available 266 phenotypic markers identified from the infinity flow and the wanderlust algorithm (Bendall et al., 2014), we further validated the chronological developmental order of neutrophils. We first excluded other cell

lineages with the help of UMAP before subjecting the cells to align them onto a development trajectory (Figure 3.19A). Each trajectory line represents the expression pattern of the indicated surface marker along development. We noted that mapping of neutrophil developmental trajectory was robust, and was able to correctly place the chronological developmental order from preNeus (CXCR4^{hi}cKit^{hi}) to immature Neus (Ly6G⁺/CXCR2⁻) to mature Neus (Ly6G⁺CXCR2⁺) (Figure 3.19A). Importantly, with this approach, it revealed a Ly6C⁺CD34^{hi}CD11b^{lo} and a Ly6C⁺CD34^{lo}CD11b^{hi} region during development, supporting our findings with UMAP (Figure 3.18C). Together with markers identified with Infinity Flow, we validated this observation with flow cytometric analysis and was able to recapitulate a Ly6C⁺CD34^{hi}CD11b^{lo}CD106^{lo} and a Ly6C⁺CD34^{lo}CD11b^{hi}CD106^{hi} population (Figure 3.19 B and C). Collectively, our data suggest that the neutrophil lineage begins with a Ly6C⁺CD34^{hi}CD11b^{lo} progenitor population within the GMP.

Figure 3.19 Wanderlust predicts $CD34^{hi}CD11b^{lo}$ and $CD34^{lo}CD11b^{hi}$ developmental intermediates during neutrophil development.

(A) Developmental trajectory prediction by wanderlust. Each trajectory line represents the indicated surface marker expression across pseudotime in neutrophil development. (B) Flow cytometric gating strategy of $CD34^{hi}CD11b^{lo}$ and $CD34^{lo}CD11b^{hi}$ populations utilising CD106 and CD81. (C) Marker expression level comparison between indicated populations. Data is representative of 3 independent experiments.

3.2.3 $CD34^{hi}CD11b^{lo}$ neutrophil progenitor shows initial specification towards neutrophil lineage

To determine if the neutrophil lineage originates from the $Ly6C^{+}CD34^{hi}CD11b^{lo}$ GMP population, we performed single-cell RNA-Seq analysis on CMPs and GMPs to capture the various GMP developmental states (Figure 3.20A). We then used Monocle (Qiu et al., 2017) to predict the trajectory and bifurcation points of myeloid development. Monocle was able to predict, in pseudotime, the developmental path from CMPs, to $Ly6C^{-}$ GMPs and finally to $Ly6C^{+}$ GMPs (Figure 3.20B). From this prediction, we noted the first bifurcating point which

branches off the monocytic lineage from the granulocytic lineage (Figure 3.20B). Deeper analysis of the bifurcating point revealed monocyte branch-determining genes like *Ly86*, *Csf1r* and *Irf8* and granulocyte branch-determining genes like *Fcnb*, *Cd63* and *Mgl2* (Figure 3.20C & D). We also noted a second bifurcating point which splits the neutrophil lineage from the eosinophil lineage as seen from their branch-determining genes (Figure 3.20E & F). Importantly, we identified a $\text{Ly6C}^+\text{CD115}^-\text{CD34}^{\text{hi}}\text{CD11b}^{\text{lo}}$ GMP subset that are the initial progenitors programmed towards the neutrophil lineage as indicated by the neutrophil-biased transcriptomic profile. We term these putative neutrophil progenitors cells pro-neutrophils (proNeus). Specifically, we term the initial $\text{Ly6C}^+\text{CD115}^-\text{CD34}^{\text{hi}}\text{CD11b}^{\text{lo}}$ population proNeu1 and the $\text{Ly6C}^+\text{CD115}^-\text{CD34}^{\text{lo}}\text{CD11b}^{\text{hi}}$ population proNeu2.

Figure 3.20 The neutrophil fate is specified at the CD34^{hi}CD11b^{lo} proNeu1 progenitor stage

SMART-Seq2 Single-cell RNA-Seq was performed on 219 cells containing CMPs, Ly6C⁺GMPs, Ly6C⁺GMPs and Ly6C⁺CD34^{hi}CD11b^{lo} GMP populations. (A) Gating strategy and index information of each cell sorted for sequencing. (B) Monocle2 trajectory prediction of myeloid progenitors. Cells are aligned according to pseudotime from right to left. Bifurcation points indicated as shown. (C) Key bifurcation determining genes for both granulocytic and monocytic lineages. (D) Heat map of all branch-determining genes for bifurcation point 1. (E) Key bifurcation determining genes for eosinophil and neutrophil lineages. (F) Heat map of all branch-determining genes for bifurcation point 2.

3.2.4 proNeus are highly proliferative, colony-forming progenitors

To better characterise these putative neutrophil progenitors, we gated and sorted these subsets to better examine their phenotypic and functional differences

(Figure 3.21A). The putative neutrophil progenitors can be separated into a CD106⁻ proNeu1 subset and a CD106⁺ proNeu2 subset. Morphological analysis revealed an initial hollowing of the nucleus in the proNeu1 stage, which increases in size in the proNeu2 stage (Figure 3.21B). *In vitro* cultures showed higher proliferative capabilities of proNeu1 compared to proNeu2. Expectedly, preNeus showed minor proliferation (Figure 3.21C). Moreover, proNeus (1&2) form granulocytic colonies, although proNeu1 produced more (Figure 3.21D & E). Our data suggests a developmental progression of proNeu1 to proNeu2, at the expense of lower proliferative potential.

Figure 3.21 Differential proliferative potential of proNeu1 and proNeu2

(A) Gating and sorting strategy of indicated CMP and GMP subsets. (B) Cytological analysis of sorted populations in (A). (C) *in-vitro* proliferation assay of indicated subsets. Results are expressed as mean (n=3 per subset) $\pm$ SD and is representative of at least 3 experiments. (D) Representative colonies of the indicated subsets at indicated time points. Data is representative of at 4 independent experiments. Scale bars = 50 μ m. (E) Colony morphology and counts in (D) at day 3. *, p<0.05 (Mann Whitney Student t-test).

3.2.5 proNeu2 are committed towards the neutrophil lineage

To determine the *in vivo* differentiation capacity of proNeus, we transferred sorted proNeus (1&2) into wild-type recipients and tracked their development after three days. Our results showed that proNeu2, like preNeus, are fully committed to the neutrophil lineage. proNeu1 conversely showed a partial commitment towards the monocyte fate (Figure 3.22). This suggests that, although both proNeu1 and proNeu2 can produce neutrophils, proNeu2 has acquired certain transcriptional programming which prevents alternative differentiation paths.

Figure 3.22 In vivo differentiation assay of neutrophil progenitors.

Sorted populations of proNeu1, proNeu2 and preNeus are mixed in an equal proportion and transferred intra-BM into wild-type recipients. Black dots represent transferred subset after 3 days. Data is representative of 3 independent experiments.

3.2.6 BM skewing of myeloid progenitors is associated with sepsis severity

We have shown and characterised two neutrophil progenitors with distinct proliferative and differentiation potential. However, it is still unclear what are the roles for these two populations during inflammatory conditions, which require the increased demand of neutrophils. To address this question, we analysed the BM of high-grade (80% ligation) septic mice and found higher frequencies of proNeu (1&2) compared to the monocyte progenitors (cMoPs) after one day. This skewing effect was evident two days after sepsis onset (Figure 3.23A and B). Since mice do not survive more than three days of high-grade sepsis (Rittirsch et al., 2009), we used a mid-grade (50% ligation) model to further understand the kinetics of this neutrophilic skewing (Figure 3.23C). Our results showed a continued skewing after three days but a sharp recovery by day 6 onwards (Figure 3.23D). Cytokine levels from the various time points showed high levels of G-CSF and IL-6 at day 3, suggesting potential roles these cytokines might have in skewing the myeloid decision fate (Figure 3.23E). Interestingly, we noticed a high variability in the skewing of BM progenitors at day 6 and observed a good correlation of this skewing with the size of the spleen. Mice with small spleens usually continued to show neutrophil skewedness with higher levels of G-CSF and IL-6 (Figure 3.23F). Taken together, our data suggests a cytokine-related skewing effect of proNeu commitment in the early phase of the sepsis inflammatory phase.

Figure 3.23 Skewed differentiation potential of myeloid precursors is associated with cytokine profile of sepsis.

(A) Cell frequencies and (B) representative plots of Ly6C⁺GMP subsets after high grade CLP-induced sepsis. Data is shown as mean $\pm$ SD (n=5 per time point) and is representative of three independent experiments. **, p<0.01 (Mann Whitney, Student t-test). (C) Experimental setup of mid-grade CLP. (D) Cell frequencies of Ly6C⁺GMP subsets after mid-grade CLP-induced sepsis. Data is reported as mean $\pm$ SD (n=6-8 per time point) and is representative of three independent experiments. **, p<0.01 (Mann Whitney, Student t-test). (E) (F) Serum concentrations of G-CSF and IL-6 at the indicated time points. Pearson correlation test of cMoP frequencies and spleen weight. IL-6 levels are reported from low (black) to high (blue) levels. G-CSF serum concentrations are expressed from low (small circle) to high (large circle) levels.

3.2.7 Discussion

The abundance of neutrophils is continuously supplied by highly proliferative GMPs and its progeny. These progenitors were once thought to be oligo-potent progenitors, capable of producing a variety of myeloid cells including monocytes, eosinophils, basophils and neutrophils. However, it is increasingly accepted that GMPs contain a mixture of lineage-committed progenitors. In our study, we utilized a combinatorial approach of transcriptomic and proteomic analysis to

visualise the heterogeneity within the GMPs. Using this workflow, we were able to identify 10 clusters of progenitor subsets with differential surface protein expression levels. Moreover, by utilizing the unique attributes of UMAP, we were able to project the developmental relationship of GMPs and identify putative early progenitors of neutrophils expressing differential levels of CD34 and CD11b (proNeus). Thus, our study clearly demarcates the developmental origin of neutrophils and the source of their abundance.

The heterogeneity of GMPs has been probed in the past few years by the introduction of single cell RNA sequencing, enabling the determination of the transcriptional activity at the single-cell level. This has allowed for the identification of cells primed to various lineages as indicated by their transcription profile (Giladi et al., 2018; Paul et al., 2015). Using flow cytometric analyses, previous studies have shown the existence of GMP heterogeneity using Ly6C and CD115 to identify Ly6C⁺CD115⁺ monocytic progenitors (MPs) and Ly6C⁺CD115⁻ granulocytic progenitors (GPs) within the uncommitted GMPs that are Ly6C⁻ (Yanez et al., 2017; Yanez et al., 2015). Moreover, the phenotype of both cMoPs (Hettinger et al., 2013) and MDPs (Fogg et al., 2006; Waskow et al., 2008) have largely overlapping phenotype with the GMP population (Akashi et al., 2000). Single-cell analysis of total GMPs confirmed a heterogeneous pool of progenitors, with the identification of cMoPs as one of the major populations within the GMP. Moreover, while CMPs were found to be similar to Ly6C⁻ GMPs, Ly6C⁺ GMPs could be separated into two clusters and are transcriptomically distinct from Ly6C⁻ GMPs. Moreover, through monocle developmental prediction, we were able to identify a CD34^{hi}CD11b^{lo}CD115⁻

neutrophil progenitor within the Ly6C⁺ fraction of the GMPs, thus suggesting Ly6C as a maturity marker for both the monocyte (cMoP) and neutrophil (proNeu) lineage. This suggests a re-definition of the GMP nomenclature to exclude lineage-committed progenitors, using Flt3 to exclude MDPs and Ly6C to exclude proNeus, cMoPs and possibly eosinophil progenitors.

While it is well established that the neutrophil lineage originates at the GMP stage, the exact mechanism for this specification remains unclear. The interplay of the transcription factors Irf8 and Gfi1 have been studied as key components counteracting myeloid-gene-regulatory networks (Laslo et al., 2006). Irf8 plays significant roles in macrophage and dendritic cell differentiation, as Irf8-deficient mice displayed low numbers of macrophages and CD8a⁺ dendritic cells, consequently leading to poor immune functions (Aliberti et al., 2003; Tamura and Ozato, 2002). This effect is clearly seen in human subjects possessing specific mutations in the IRF8 gene (Hambleton et al., 2011). Absence of Irf8 led to an increased expansion of mature neutrophils and associated precursors in the bone marrow and severe neutrophil infiltration in the liver and lymph nodes leading to a Chronic Myeloid Leukaemia (CML)-like syndrome in Irf8-deficient mice. Conversely, the loss of Gfi1 resulted in severe neutropenia in mice and the overexpression of Gfi1 increased neutrophil differentiation and impeded monocyte/macrophage fate specification (Hock et al., 2003). Through single-cell analysis of transcriptional states, we showed that Irf8 was important for monocyte specification but Gfi1 was not detected to participate in granulocyte lineage specification. Instead, Gfi1 was found to be specifically important for the bifurcation point between the neutrophil and eosinophil lineage. Gfi1 was shown

previously as a repressor of monocyte fate by inhibiting the expression of TFs such as Klf4, Zeb2 and Irf5 through the repression of the transcriptional activity of PU.1 and Irf8 (Olsson et al., 2016). As our data shows the gradual increase of *Gfi1* expression on cells leading to proNeu1, it suggests that the specification of the neutrophil lineage is dependent on the repressive effect of factors such as Gfi1. This partially explains both the observed bipotent differentiation potential of proNeu1 and exclusive commitment of proNeu2 and preNeus to the neutrophil lineage.

Our *in vivo* differentiation experiments suggested that proNeu1 can give rise to both monocyte and neutrophil progeny. The presence of monocyte progeny through proNeu1 suggests two plausible explanations. The first explanation is the initial presence of contaminating cMoPs or monocyte-primed progenitors. These small numbers can then expand rapidly in large numbers and result in the apparent monocytic differentiation potential. This is limited to the staining efficiency of surface marker CD115 to exclude possible monocytic progenitors. Thus, cMoPs expressing low levels of CD115 may have contaminated proNeu1. The second and more interesting possibility is the ability of proNeu1 to ‘switch’ its transcriptional programming from a granulocytic potential towards a monocytic fate. As haematopoietic cytokines were shown to instruct lineage specification in GMPs (Rieger and Schroeder, 2009), it is possible that proNeu1 can be influenced by local environmental cues for developmental fate decisions. Isolating these cells from their native environment and re-introducing them back into recipient tibias may have triggered specification changes. However, preliminary evidence suggests that neither proNeu1 and proNeu2 can give rise to

higher numbers of monocytes through M-CSF administration, suggesting the action of haematopoietic cytokines directly influence the GMPs and before transcriptomic fate is specified. The use of bulk RNA-Seq of GMPs, proNeu1 and proNeu2 can help reveal the transcriptional status of these populations, and the monocytic/granulocytic transcriptional activators or repressors that govern the specification of each lineage. ATAC-Seq (Assay for Transposase-Accessible Chromatin using Sequencing) can further reveal the accessible binding domains for TFs, activators and repressors to define the propensity of lineage specification.

Through our study, we have showed the existence of two neutrophil progenitors possessing distinct proliferative potential. Notably, the low expression of CD34 on proNeu2 suggested that these cells might not be progenitor cells but instead are late precursors like preNeus. CD34, in both mice and human, is the most commonly used marker for HSPC identification and it is involved in roles such as cytoadhesion and regulation of cell differentiation and proliferation (Healy et al., 1995; Nielsen and McNagny, 2009). Interestingly, CD34 is known to bind to adhesion molecule L-Selectin (CD62L), which might be important for the localisation of HSPCs in specific niches. This loss of CD34 is accompanied by the gain of VCAM-1 (CD106), which is an adhesion molecule that binds to VLA-4 (very late antigen-4) expressed by a variety of HSPCs including proNeus and preNeus. This change of adhesion molecule expression signifies a switch in niche localisation or a change in cell-cell interactions for differentiation and maturation factors. This could also suggest the formation of GMP clusters within the bone marrow observed previously (Herault et al., 2017). It would be interesting if the inhibition of this adhesion interaction will affect the proliferative potential of

proNeus or its ability to expand during emergency granulopoiesis. Studies can also implement high parametric multiplex staining protocols to capture the localisation of neutrophil progenitors and their specific niches and organisation structures that allow for the high output of neutrophils required.

Sepsis is a serious clinical condition leading to high morbidity and mortality worldwide. Few effective treatments are available and many clinical trials that are based on mice studies failed to show promising results (Brown et al., 2016). These treatments attempt at reducing the inflammatory cytokine levels present in septic patients to increase survival outcomes. Here, our study showed that the balance of granulocyte and monocyte differentiation is altered during the early stages of sepsis onset with an increased bias towards the neutrophil lineage. Moreover, the neutrophil-biased differentiation is correlated with the high levels of G-CSF and IL-6. This finding brings a new perspective on the consequences of these increased inflammatory cytokines by how they not only affect organ dysfunction but also affect the myeloid cell type output. This preferential differentiation phenomenon is also observed in the balance between myelopoiesis and lymphopoiesis during sepsis. One study showed that sepsis reduced the osteoblast number in the bone marrow, leading to lower production of IL-7 which is critical for lymphopoiesis (Terashima et al., 2016). This change was attributed also to G-CSF, as inhibiting G-CSF reversed the loss of osteoblasts and lymphoid progenitors. We hypothesize that this same treatment may also reverse the neutrophil skewing effect and increase the production of monocytes which have been shown to be important for the resolution of sepsis (Shalova et al., 2015).

3.3 Characterisation of analogous human neutrophil subsets

Our study has led us to the characterisation of a proliferative yet committed neutrophil precursor in the mouse bone marrow. These cells acquire functional properties of effector neutrophils as they mature and egress into the circulation. However, it is unclear if such a population exist in humans.

3.3.1 Unsupervised identification of proliferative BM neutrophil precursor

To determine this, we employed a similar workflow performed in our mouse study (see Figure 3.1C). Using mass cytometry and a panel of 40 surface markers, we analysed total bone marrow cells and characterised each cluster according to their surface marker expression levels (Figure 3.24A). To detect a putative neutrophil precursor in human BM, we manually gated CD15⁺CD66b⁺ total neutrophils (Figure 3.24B) and identified differentially expressed markers between proliferative (IdU⁺) and non-proliferative (IdU⁻) neutrophils including CD10, CD16, CD49d and CD101 (Figure 3.24C, black arrows). Using this strategy, we identified the human equivalents of preNeus, immature and mature Neus (Figure 3.24D). Akin to mice, preNeus and immature Neus were virtually absent from the blood thereby validating our workflow (Figure 3.24E). Also, similar to mouse neutrophils, CXCR2 was only expressed on human mature Neus (Figure 3.24F). Together, we show that the development of neutrophils is also comprised of developmentally distinct stages that can be identified through proliferation activity.

Figure 3.24 CyTOF reveals proliferative human equivalents of preNeus, Immature Neus and Mature Neus

(A-F) Unsupervised analysis of healthy human bone marrow. (A) t-SNE visualization of human BM showing the various identified immune subsets in the sample. (B) Representative plot of the IdU incorporation in total neutrophils and (C) the differentially expressed markers (indicated by black arrows) between IdU⁺ and IdU⁻ neutrophils. (D) Gating Strategy of human BM neutrophil subsets using the markers found in (C). (E) Representative plot of the neutrophil subsets in healthy human whole blood. (F) Surface marker expression levels of human BM neutrophil subsets. Data are represented as median intensity. Data (A-F) are representative of three independent experiments and donor samples.

3.3.2 Human cord blood neutrophils contain a heterogeneous pool of neutrophil subsets

The umbilical cord blood is a rich source of CD34⁺ stem cells that is widely used for therapeutic purposes to treat various blood disorders. Since the cord blood contains proliferative and regenerative CD34⁺ stem cells, we wondered if proliferative neutrophil precursors could also be identified. To answer this question, we performed flow cytometric analysis on samples of whole cord blood. Using the same markers identified previously (Figure 3.24C), we characterised preNeus, immature Neus and mature Neus within the cord blood (Figure 3.25A). We noted the lowered expression of CD101 on immature Neus, which may suggest certain differences between cord blood and bone marrow neutrophil subsets. Cytospin analysis revealed that preNeus were similar in morphology to myelocytes, while immature Neus are like band cells and mature Neus are segmented neutrophils (Figure 3.25B). This suggests that bone marrow neutrophil subsets share similar morphologies. The number of preNeus are considerable (Figure 3.25C and D), indicating a potential untapped resource of proliferative neutrophil precursors that might be used for treatments where neutrophils are required in large numbers. Together, our study presents the classification and characterisation of neutrophil subsets in both the human bone marrow and cord blood, allowing for a clearer understanding of neutrophil heterogeneity in physiological conditions and in various diseases and infections.

Figure 3.25 Identification of neutrophil subsets in human umbilical cord blood

(A) Representative gating Strategy of cord blood neutrophil subsets. (B) Cytopsin micrographs of sorted neutrophil subsets. scale bar = 10 μ M. (C) Frequencies of pre-neutrophils (preNeus) in cord blood. (D) Absolute counts of total preNeus per cord blood unit (left) and per mL of cord blood sample (right).

(C-D) Data are expressed as mean $\pm$ SD, pooled from 13 donor samples.

3.3.3 Discussion

Although human granulopoiesis is well characterised by haematologists a long time ago, the functional understanding of the various maturation stages remained largely unknown due to the lack of studies that isolate pure populations of these subsets for functional tests. Moreover, characterisation of neutrophils in disease states is becoming increasingly challenging. Here, we present a comprehensive analysis of human bone marrow immune cells and identified the analogous populations of preNeus, immature Neus and mature Neus in both the bone marrow and cord blood.

Previously studies have characterised neutrophil maturation status by performing differential staining on blood and bone marrow smears. By analysing the morphological differences between each subset, scientists were able to associate the morphology of the subsets with the developmental progression of these neutrophil subsets. However, the functional properties of each subset and their roles during inflammatory conditions remain largely unknown. From our characterisation of murine neutrophil subsets, we showed that each subset possesses distinctive roles and functions. Using various models, we showed how each subset respond towards inflammation and the functional features of each subset. This allows for further research to deepen the understanding of the heterogeneity of neutrophils in diseases. To translate these findings in mice, we performed an unbiased characterisation of human bone marrow neutrophils and identified the corresponding neutrophil subsets found in mice. Thus, we are able to better understand the functions of each human neutrophil subset by inferring from the analogous subsets observed in mice.

Like in mice, the absence of human preNeus and immature Neus in the peripheral blood suggests similar functions these subsets share with the subsets found in mice. This suggests that these immature subsets do not have chemotactic responses or participate in immune-surveillance like the mature neutrophils in the circulation. Similar to mature neutrophils in mice, only human mature Neus subset expressed high levels of CXCR2 (Figure 3.23F), suggesting similar attributes for chemotaxis, migration and effector functions. The use of these markers to identify these specific developmental stages of neutrophils allow for

a deeper understanding of the increasing heterogeneity reported in the literature (Ng et al., 2019; Scapini et al., 2016; Silvestre-Roig et al., 2019). Through this functional characterisation of neutrophil subsets, we hope to pave the way for a better classification strategy of neutrophils present in various pathological conditions.

The identification of preNeus in the cord blood indicates a new source of therapeutic cells that can be harvested in large quantities. Previously, large numbers of peripheral neutrophils are transfused into neutropenic patients to support immune functions for these patients (Hubel et al., 2001). As neutrophils are short-lived and do not self-renew, this transfusion is performed frequently to maintain an immunocompetent level of neutrophils. Transfusing proliferative preNeus can potentially provide an additional source of functional neutrophils to these patients. Sources of these cord blood preNeus can be easily obtained at cord blood banks where CD34⁺ stem cells are extracted and stored while non-CD34⁺ cells are disposed of. These harvested preNeus can possibly be expanded ex vivo, as some reports have done so for neutrophil, erythrocyte and platelet generation (Jie et al., 2017; Matsunaga et al., 2006; Zhang et al., 2017). Further studies can demonstrate the regenerative potential of preNeus in humanised mice, whereby irradiated humanised mice can be engrafted with cord blood preNeus to demonstrate the proliferative generation of functional mature neutrophils in these mice which have poor neutrophil-forming haematopoiesis (Shultz et al., 2012).

4. Conclusion

The study of neutrophil development and function had led to an increased appreciation and understanding of the heterogeneity present at both homeostatic and inflammatory conditions. Collectively, our study provides a thorough characterisation of the various developmental intermediates of neutrophil development and presents the various roles and implications each distinct subpopulation plays during various forms of inflammation.

Specifically, we provide a firm baseline of neutrophil biology from a developmental perspective, which will help resolve the increasingly reported heterogeneity of neutrophil phenotypes and functions. Here, we presented a developmental heterogeneity that exist within the bone marrow among the typically gated Gr-1⁺CD11b⁺ neutrophils. These subsets were shown to possess unique transcriptomic signatures and functional properties that have specific consequences during inflammation. We then further showed the existence of common neutrophil progenitors that highly proliferative, having the ability to generate the large numbers of neutrophils present in the circulation. These findings and identification strategies have led to the observation of similar subsets in the human marrow, identifying developmental heterogeneity that can serve as a baseline to understand the diverse neutrophil subsets increasingly reported in the literature. Hence, we envision that our study will give a framework for understanding neutrophil heterogeneity and lead to the engineering of neutrophil subset-specific targeting strategies for therapeutic purposes.

5. Future Work

This study encompasses the entire spectrum of neutrophil heterogeneity during development, from common myeloid progenitors to fully functional circulating neutrophils. Each distinct subset of neutrophils possesses signature gene profiles that distinguish them from each other. However, although our study provides a clear characterisation of neutrophil subsets at homeostatic conditions, there is still much to learn about the neutrophil developmental pathways and molecular functions during inflammatory stimuli.

Importantly, it is still unclear if these early precursors of neutrophils (proNeus and preNeus) can be mobilised into the periphery for effector functions during inflammation. It is possible that these precursor populations seed extramedullary sites such as the spleen or liver for increased neutrophil production with their proliferative potential. Maturation in these distinct niches can result in tissue-specific imprinting and alterations, allowing for a modulated neutrophil response appropriate to the inflammatory threat and demand. Future studies should be answering two major questions: (1) Can these precursors mobilise prematurely in certain inflammatory diseases? If so, what are the functions and consequences of these cells in the periphery? (2) What are the characteristics of neutrophils generated at extramedullary sites? Answering these questions can reveal a possible origin of neutrophil diversity through developmental heterogeneity.

Another important issue to address is the reconciliation of our identified neutrophil subset characterisation with the currently accepted haematological nomenclature. Routinely, haematologists perform smears of bone marrow or blood samples to perform counts of the differentially stained stages of neutrophils. This tedious process of visual inspection and interpretation can be substituted by unbiased flow cytometric analyses of patient samples with the differential phenotypic markers we have present here in our study. By unifying the characterisation of neutrophil maturation stages, we can then better study haematological disorders and diseases relating to neutrophil insufficiency and developmental aberrations.

6. References

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7. Appendices

Table 1: Mouse CyTOF panel

Metal	Antibody	Clone	Cat number	Company
89	CD45	30-F11	3089005B	Fluidigm
112/114	CD19	6D5	Q10379	Invitrogen
115	CD90	T24/31	BE0212	BioXCell
127	IdU		I7125	Sigma-Aldrich
141	CD43	S7	553268	BD Biosciences
142	MHCII	Y-3P	BE0178	BioXCell
143	B220	RA3.3A1/6.1	BE0067	BioXCell
144	CD11a	FD441.8	BE0005-1	BioXCell
145	Gr-1	RB6-8C5	108402	Biolegend
146	CD88	20/70	135802	Biolegend
147	Ly6G	1A8	127602	Biolegend
148	Ly6c	HK1.4	128002	Biolegend
149	CD31	MEC13.3	102502	Biolegend
150	CX3CR1	SA011F11	149002	Biolegend
151	CD62L	MEL-14	104402	Biolegend
152	CD11c	N418	117302	Biolegend
153	CD11b	M1/70	101202	Biolegend
154	CD49b	DX5	108902	Biolegend
155	cKit	2B8	105829	Biolegend
156	BST2	120G8	N/A	Purified in house
157	CXCR2	SA044G4	149302	Biolegend
158	TER119	TER-119	116202	Biolegend
159	F4/80	Cl:A3-1	MCA497GA	Bio-Rad
160	Flt3 Biotin (Primary)	A2F10	135308	Biolegend
	Streptavidin (Secondary)			Purified in house
161	CD34 FITC (Primary)	RAM34	553733	BD Biosciences
	anti-FITC (Secondary)	FIT-22	408302	Biolegend
162	PD-L1	10F.9G2	124302	Biolegend
163	CD150	TC15-12F12.2	115933	Biolegend
164	NK1.1	PK136	108743	Biolegend
165	Ly6B.2	7/4	NBP2- 13077AF488	Novus Biologicals
166	CD48	HM48-1	103402	Biolegend
167	CXCR4	L276F12	146502	Biolegend
168	CCR2 APC (Primary)	475301	FAB5538A	R&D Systems
	anti-APC (Secondary)	APC003	408002	Biolegend
169	CD115 PE (Primary)	AFS598	61-1152-82	Thermo Fisher
	anti-PE (Secondary)	PE001	408102	Biolegend
170	CD49f	GoH3	313602	Biolegend
171	FceR1	MAR-1	14-5898-82	Thermo Fisher
172	Sca-1	D7	108102	Biolegend
173	CD49d	R1-2	553154	BD Biosciences

174	CD24	M1/69	101802	Biolegend
175	Siglec-F	E50-2440	552125	BD Biosciences
176	CD16/32	2.4G2	553140	BD Biosciences

Table 2: Human CyTOF panel

Meta I	Antibody	Clone	Cat number	Company
89	CD45	HI30	3089003B	Fluidigm
113	CD15	HI98	301902	Biolegend
115	CD57	HCD57	322302	Biolegend
140	CD2	RPA-2.10	300202	Biolegend
141	CD13	WM15	301702	Biolegend
142	CD5	UCHT2	300602	Biolegend
143	CD62L	DREG-56	555541	BD Biosciences
144	CD38	HIT2	303502	Biolegend
145	CD45RA	HI100	304102	Biolegend
146	CD3	UCHT1	300402	Biolegend
147	HLA-DR	L243	307602	Biolegend
148	CD66b	G10F5	555723	BD Biosciences
149	CD10	HI10a	312202	Biolegend
150	CD235ab	HIR2	306602	Biolegend
151	CD7	M-T701	555359	BD Biosciences
152	Siglec 8	7C9	347102	Biolegend
153	FcER1	AER-37 (CRA1)	16-5899-82	eBioscience
154	CCR3	5E8	310702	Biolegend
155*	CD123	6H6	306002	Biolegend
156	CD14	M5E2	301802	Biolegend
157*	CD31	WM59	303102	Biolegend
158	CD56	NCAM16.2	559043	BD Biosciences
159	CD33	WM53	303402	Biolegend
160	CXCR4 Biotin (Primary)	12G5	306504	Biolegend
	Streptavidin (Secondary)			Synthesized in house
161*	CXCR2 FITC (Primary)	5E8/CXCR2	320704	Biolegend
	anti-FITC (Secondary)	FIT-22	408302	Biolegend
162*	CD88	S5/1	344302	Biolegend
163*	CD66a/c/e	ASL-32	342302	Biolegend
164	CD116	4H1	305902	Biolegend
165	CD303	201A	354202	Biolegend
166	CD117	104D2	313202	Biolegend
167	CD49d	9F10	304302	Biolegend

168	CD101 APC (Primary)	BB27	331007	Biolegend
	anti-APC (Secondary)	APC003	408002	Biolegend
169	CD49f	GoH3	313602	Biolegend
170	CD64	10.1	305002	Biolegend
171	CD34	581	343502	Biolegend
172	CD44	IM7	103002	Biolegend
173*	CD19	HIB19	302202	Biolegend
	CD20	2H7	302302	Biolegend
174	CX3CR1	K0124E1	355702	Biolegend
175	CD11c	B-ly6	555390	BD Biosciences
176	CD11b	ICRF44	301312	Biolegend
209	CD16	3G8	3209002B	Fluidigm

Table 3: List of Antibodies used for Flow Cytometry

Antibody Name	SOURCE	IDENTIFIER
B220 (Clone RA3-6B2)	Thermo Fisher	Cat# 25-0452; RRID: AB_2341160
CD11b (Clone M1/70)	Biolegend	Cat# 101259; RRID: AB_2566568
CD11c (Clone N418)	Thermo Fisher	Cat# 48-0114; RRID: AB_1548665
CD150 (Clone TC15-12F12.2)	Biolegend	Cat# 115033
CD16/32 (Clone 2.4G2)	BD Biosciences	Cat# 565272
CD24 (Clone M1/69)	Biolegend	Cat# 101802
CD31 (Clone 390)	Biolegend	Cat# 102427; RRID: AB_2563982
CD34 (Clone RAM34)-FITC	BD Biosciences	Cat# 553733; RRID: AB_395017
CD3e (Clone 145-2C11)	Biolegend	Cat# 25-0031; RRID: AB_469571
CD43 (Clone S7)	BD Biosciences	Cat# 553268
CD48 (Clone HM48.1)	Biolegend	Cat# 103402
CD49d (Clone R1-2)	BD Biosciences	Cat# 553154
CD62L (Clone MEL-14)	Thermo Fisher	Cat# 63-0621; RRID: AB_2637416
CD90.2 (Clone 53-2.1)	Biolegend	Cat# 140310; RRID: AB_10643586
cKit (Clone 2B8)	BD Biosciences	Cat# 562417; RRID: AB_11154233
CX3CR1 (Clone SA011F11)	Biolegend	Cat# 149027; RRID: AB_2565937
CXCR2 (Clone SA044G4)	Biolegend	Cat# 149306; RRID: AB_2565694
CXCR4 (Clone 2B11)	Thermo Fisher	Cat# 13-9991-82; RRID: AB_10609202
CD101 (Clone Moushi101)	Thermo Fisher	Cat# 12-1011-82; RRID: AB_1210728
CD115 (Clone AFS598)	Thermo Fisher	Cat# 61-1152-82; RRID: AB_1937254

Gr1 (Clone RB6-8C5)	Thermo Fisher	Cat# 45-5931-80; RRID: AB_906247
Ly6C (Clone HK1.4)	Biolegend	Cat# 128026; RRID: AB_10640120
Ly6G (Clone 1A8)	Biolegend	Cat# 127618; RRID: AB_1877261
MHCII (Clone M5/114.15.2)	BD Biosciences	Cat# 563414
NK1.1 (Clone PK136)	Thermo Fisher	Cat# 25-5941; RRID: AB_469664
Sca-1 (Clone D7)	Biolegend	Cat# 108114; RRID: AB_493596
Siglec-F (Clone E50-2440)	BD Biosciences	Cat# 562757; RRID: AB_394341
TER119 (Clone TER-119)	Biolegend	Cat# 116202
F4/80 (clone BM8)	Biolegend	Cat# 123106; RRID: AB_893501
FceR1a (Clone MAR-1)	Thermo Fisher	Cat# 14-5898-82
Flt3 (Clone A2F10)	Thermo Fisher	Cat# 12-1351; RRID: AB_465859
Donkey anti-rabbit Alexa-Fluor® 647	Life Technologies	Cat# A31571; RRID: AB_162542
Goat anti-rat Alexa-Fluor® 555	Life Technologies	Cat# A21434; RRID: AB_141733
Rabbit polyclonal laminin 1 + 2	Abcam	Cat# 7363
S100A9 (Clone 2B10)	Abcam	Cat# ab105472; RRID: AB_10862594
CD81 (Clone Eat-2)	Biolegend	Cat# 104906, RRID:AB_2076266
CD106 (Clone 429 (MVCAM.A))	Biolegend	Cat# 105716, RRID:AB_1595489
CD45.1 (Clone A20)	Biolegend	Cat#110707; RRID: AB_313496
CD45.2 (Clone 104)	Biolegend	Cat#109807; RRID: AB_313444

8. Posters, Invited Talks

SgSI 5th Advanced Singaporean Immunology PhD Student Retreat (2016). Mobilization of neutrophils by G-CSF is a two-step process involving a CXCR2-independent pathway (Poster)

1st Cold Spring Harbor Asia Conference on Inflammation: Basic Mechanisms & Relevant Diseases (2017) Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking and effector functions (Invited Speaker substitute for supervisor)

iFREC-SIgN Winter School on Advanced Immunology (2018). Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking and effector functions (Poster and oral presentation)

SgSI 10th Anniversary Symposium (2018). Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking and effector functions (Invited Speaker)

The 6th Neutrophil International Symposium (2018). Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking and effector functions (Poster and oral presentation)

SgSI 6th Advanced Singaporean Immunology PhD Student Retreat (2018). Reshaping the paradigm of the Granulocyte-Monocyte Progenitor (Poster and oral presentation)

CyTOF seminar with Fluidigm (2019). Fantastic Progenitors and where to find them...with cyTOF! (Invited Speaker)

SgSI 11th Anniversary Symposium (2019). Reshaping the paradigm of the Granulocyte-Monocyte Progenitor (Poster)