KEY TRANSCRIPTIONAL REGULATORS OF NEUTROPHIL FUNCTION IN INFLAMMATION

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DECLARATION

I declare that the present thesis is the result of my own work. All experimental data described in this thesis are original and have been performed by myself, unless stated below or indicated in the text. Some of the text and figures in the general introduction have been published with me as the first author in a literature review article and the co-first author in a research article (1).

Experiments and analyses described below were performed by or with help from a number of colleagues. Nicola Willemsen participated the initial round of CRISPR-mediate knockout in HoxB8 myeloid progenitor cells (section 4.2.1). mRNA sequencing was conducted by the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, Oxford. Tariq Khoyratty instructed me on the preparation of RNA samples for sequencing. Lihui Wang helped the establishment and optimisation of neutrophil functional assays (sections 5.2.2, 5.2.3, 5.2.4). In vivo model and adoptive transfer experiments were assisted by Hayley Eames (sections 4.2.4, 4.2.7). This work has been carried out at the Kennedy Institute of Rheumatology and was funded by China Scholarship Council.

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ABSTRACT

Neutrophils are important effector cells in innate immunity, possessing a wide range of effector functions, including reactive oxygen species (ROS) production, phagocytosis and chemotaxis. These properties enable neutrophils to rapidly respond to stimulation and orchestrate protective immunity. Currently, the transcriptional regulatory networks underlying neutrophil activation and function remain largely unexplored. Of note, there is partial understanding of transcription factors (TFs) that act as key regulators to control neutrophil development and inflammatory responses. Neutrophils undergo tightly controlled genomic and transcriptional changes while transitioning between bone marrow, blood, and tissue sites. However, the molecular mechanisms underlining neutrophil function during inflammation have yet to be fully elucidated. To determine the transcription factor networks that shape these responses, we have undertaken integrated transcriptional and chromatin analyses of neutrophils during acute inflammation and revealed distinct sets of putative transcription factors (TFs) associated with control of neutrophil differentiation and inflammatory responses.

To investigate the regulatory role of the selected TFs in neutrophils, I utilised HoxB8 myeloid progenitors as a model system for *in vitro* production of neutrophils and generated stable knockout lines for JUNB, RELB, IRF5, RFX2, KLF6, and RUNX1 in Hoxb8 myeloid progenitors, using the CRISPR/Cas9 mediated system. Additionally, I have demonstrated the importance of RUNX1, KLF6 in neutrophil differentiation. RUNX1, KLF6 deletion in HoxB8 progenitors caused a block in neutrophil differentiation and produced lower levels of mature neutrophils. KLF6 and RUNX1 deficient neutrophils also displayed impaired transmigration properties. Consistent with findings in the Hoxb8 *in vitro* model, conditional deletion of RUNX1 in myeloid populations produced lower mature neutrophils in the bone marrow.

To validate the functional role of the selected TFs in neutrophil activation, I examined the consequence of CEBPβ, RELB, IRF5, JUNB knockouts on neutrophil effector functions, such as phagocytosis, cytokine production, generation of ROS, formation of NETs and bacterial killing. I found that RELB, IRF5, JUNB knockout significantly affect the ability of neutrophils to produce inflammatory mediators. JUNB and RELB also contribute to neutrophil phagocytosis, ROS generation and NETosis.

1.0 General Introduction

1.1 Neutrophils and innate immunity

1.1.1 Innate immunity

Humans are exposed to potential pathogens constantly through direct contact, gastrointestinal digestion or inhalation. Our inherent ability to avoid infection depends largely on the innate immune system, which functions during the first critical minutes and hours of exposure to a new pathogen. Unlike the adaptive immune responses that are slow to develop before the responses are effective, innate immune responses depend on physical barriers to invading microbes (i.e. skin and mucus membranes of the respiratory, gastrointestinal and reproductive tracts), humoral fluids (i.e. secreted antibodies and complement proteins) and innate cellular components (i.e. monocytes. macrophage, dendritic cells and neutrophils) (2). These innate immune elements serve as the first line of the host immune defence and function to prevent pathogen invasion, as well as host-derived debris generated from unwanted tissue damage and inappropriate inflammatory responses.

Innate immune responses are antigen-nonspecific defence mechanisms that are initiated within minutes or hours of exposure to almost any pathogenic components (2). In vertebrates, the skins and mucus membranes, for instance, in the lung and gut, provide the physical barriers between the skin and interior of the body, effectively preventing us from exposure to infection. In response to invading pathogens that overcome the physical barriers, the innate immune system mades use of a group of proteins and phagocytic cells that rapidly recognise conserved features of pathogens and become quickly activated to help destroy invaders (2). Indeed, innate immune cells, such as neutrophils, macrophage and dendritic cells, express a variety of pattern-recognition receptors (PRRs), which not only sense the presence of external infectious antigens, termed pattern-associated molecular patterns (PAMPs), but also recognise endogenous molecules released from damaged cells, termed as damage-associated molecular patterns (DAMPs). The most well-known example of PRRs are toll-like receptors (TLRs), which specifically recognise bacterial lipopolysaccharide, peptidoglycan, bacterial DNA, double-stranded RNA, and lipids that are broadly expressed by large groups of microbes (3). Upon recognising substantiated PAMPs or DAMPs, PRRs rapidly activate intracel-

lular signalling pathways that eventually lead to the activation of transcriptional factors (TFs) and subsequent transcription of genes that encode pro-inflammatory chemokines, cytokines and proteins that are involved in modulation of inflammation (4).

1.1.2 Neutrophils in innate immunity

Neutrophils are the most abundant leukocytes in the innate immune system and have been recognised as the front-line soldiers in innate immunity, fighting against invading microbes and pathogens (5). They are produced in the bone marrow from hematopoietic stem cells (HSCs) and differentiate into mature neutrophils with full range of neutrophil effector functions. Under steady-state conditions, neutrophils circulate between the blood and tissue sites for immune surveillance, and gradually lose their ability to infiltrate into the inflammatory sites and become prepared for clearance into tissues, a process of neutrophil ageing (6). If pathogenic microbes successfully overcome the physical barriers provided by the skin and mucus membranes and have managed to invade into the tissues, signals produced by microbes and by resident macrophages that recognise microbes guide the recruitment of circulating neutrophils toward sites of infection, where infiltrating neutrophils rapidly become activated through direct interaction with activated endothelial cells and stimulation of pro-inflammatory chemokines and cytokines (7). Upon infiltration into inflammatory sites, neutrophils eliminate invading microbes efficiently through phagocytosis, cytokine production, release of reactive oxygen species (ROS) and neutrophil extracellular trap (NETs) formation (5). Quantitative and qualitative neutrophil defects contribute to various types of agranulocytosis, consequently resulting in a high risk of invasive infection (8). By contrast, excessive infiltration and activation of neutrophils at the inflammatory sites can cause tissue damage, leading to intense local and systematic inflammation (9). As a key player of the inflammatory responses, neutrophils can initiate and amplify immune responses through the release of cytokines, chemokines and other pro-inflammatory mediators, as well as promoting the recruitment and activation of additional neutrophils and adaptive immune cells, including both B cells and T cells. Therefore, when the infiltration and activation of neutrophils are poorly controlled, neutrophils contribute to the pathogenesis of a number of inflammatory and autoimmune diseases, including rheumatoid arthritis (RA), vasculitis and systemic lupus erythematous (SLE) (10).

Neutrophils are traditionally considered as a type of myeloid cells with conserved phenotype with defined surface markers and limited transcriptional plasticity. However, over the past

years, increasing evidence has supported the existence of distinct neutrophil subsets in physiological conditions, such as in the bone marrow (11, 12) and in healthy tissues (6, 13), as well as in pathogenic states, especially cancer (14), and infection (15). Neutrophils have been described to display distinct gene expression profiles at different developmental stages, functional states and micro-environments. In the bone marrow, differentiating neutrophils demonstrate a progressive upregulation of genes central to ROS production, phagocytosis and chemotaxis during maturation, such that mature neutrophils possess a full range of neutrophil effector functions (11). Similar heterogenous neutrophil subsets have also been observed in tissue sites as illustrated in the lungs, where neutrophils acquire featured transcriptional signature that support vascular homeostasis and hematopoietic recovery (13). Existing evidence has demonstrated that in response to signals in the tumour micro-environment, neutrophils exhibit different transcriptional profiles that results in diverse functions, favouring or inhibiting tumour survival and metastasis (14). These findings, together with others, collectively suggest that neutrophils are transcriptionally active cells. However, unlike other myeloid cells, in which diverse phenotype and function have been linked to the molecular mechanisms powered by specific transcriptional factors, the molecular mechanisms underlining neutrophil phenotype and function remain largely unexplored.

1.2 Transcriptional regulation of neutrophils

Cell development and fate decision are promoted through a complex and precise pattern of gene expression, which is largely controlled by the integrated action of the interconnected transcriptional regulatory network. Transcription factors (TFs) are master regulatory proteins that are central in regulating the transcription of genes (16). One major feature of TFs is the ability to recognise and bind to various cis-regulatory elements that are localised to the regulatory regions, which are distinct from transcription start sites (TSSs), such as core promoters and enhancers and, to regulate or initiate the transcription of the target genes (17). Enhancer regions, for instance, are small segments of DNA that typically contain clusters of different TF binding sites, and they serve as the operational platform for the binding of TFs to regulate transcription. As such, the interplay of multiple TFs, each with its own spatiotemporal window of expression, results in discrete and precise patterns of transcriptional activity (18, 19). Similarly, the cooperative action of several different TFs in response to the signalling cascade allows for the unique regulation of gene expression, thereby modulating many important functions and cellular processes (19).

TFs have been recognised as major regulators in the differentiation of hematopoietic progenitors into myeloid and erythroid lineages (20). Gata1, Klf1, and Gfi1b are key regulators in erythrocyte and megakaryocyte lineage commitment (21, 22), while CAAT/enhancer binding protein-α, -β, and -e (CEBPα, CEBPβ, and C/EBPe) play important roles in the commitment into myeloid lineages, primarily for granulocytes, monocytes and macrophages (23-25). The importance of TFs in hematopoiesis has been further appreciated by the aberrant expression of TFs in the mechanism of leukemogenesis (16). Runx1 is one of the most frequently mutated genes that associate with haematological malignancies. Somatic mutations and chromosomal rearrangements involving Runx1 results in chimeric RUNX1 fusion, such as AML1/ETO (26), which alters the normal differentiation of hematopoietic cells, eventually leading to leukemic transformation (27). Further evidence comes from studies using disrupted expression of TF in hematopoietic progenitor cells (28-30) and targeted deletion of genes encoding TFs in mice (31-33). Commitment into the early myeloid lineages essentially depends on PU.1, the protein encoded by the SPI1 gene. PU.1 deficiency in granulocyte-macrophage progenitors (GMPs) impair the production of macrophages, but not granulocytes (34). The absence of PU.1 expression in mice leads to deficient newborn hematopoiesis, in which there is a complete lack of B cells and macrophages (35). The functions of many of these lineage-specific TFs are highly conserved between mouse and human, and each of these TFs induces the activation of a selective set of lineage-specific genes as well as the silencing of lineage-foreign genes in a defined order, thereby controlling cell fate decisions and proper functions (16). Therefore, a key goal of future investigations is to explore the molecular mechanisms underlining normal and malignant haematological development processes.

1.2.1 Neutrophil differentiation

Neutrophils are differentiated in large numbers from HSCs in the bone marrow (BM). During, granulopoiesis, HSCs firstly differentiate into lymphoid-primed multi-potent progenitors (LMPPs) with the loss of their self-renewal capacity, and further develop into multi-potent progenitors (MPPs). Subsequent differentiation of MPPs gives rise to common myeloid progenitors (CMPs) and then GMPs, which possess only limited potential for proliferation with increased level of differentiation. GMPs have the potential to differentiate into neutrophils, monocytes, dendritic cells or macrophages as well as eosinophil lineage-committed progenitors or basophil/mast cell progenitors, which give rise to eosinophils, basophils and mast cells,

respectively (36). Under the control of granulocyte colony-stimulating factor (G-CSF), ongoing differentiation of GMPs initiates stepwise progression into myeloblast, promyelocytes, myelocytes, metamyelocytes, band neutrophils and finally segmented neutrophils released into the bloodstream (37).

Traditionally, the neutrophil differentiation process is classified into stages defined by the lobular shape of the neutrophil nucleus, cell size and expression of granular proteins. Neutrophils under different differentiation stages possess distinctive nuclear envelope protein profiles. The neutrophil nucleus envelope is made up of double nuclear lipid membranes rigidly embedded with membrane proteins and associated nuclear lamin proteins, such as lamin A/C protein and B-type lamins, thereby stabilising and segregating the nucleoplasm from the cytoplasm (36). Within the nucleus, nuclear lamin proteins are tethered to the nucleoplasm interface of the nuclear envelope, and the protein composition of neutrophil nucleus envelope differs across different differentiation stages and affects chromatin arrangement and nuclear biomechanics. Decreased levels of Lamin A/C in mature neutrophils reduce nuclear resistance and allows for higher nuclear plasticity during transmigration (38), Increased composition of B-type lamin protein also contributes to enhancing nuclear malleability and cellular plasticity (38). However, the role of B-type lamins in neutrophil cell motility and migration remains unknown. Indeed, mature neutrophils possess a superior capacity of migration and cell motility (11). Therefore, there is an under-explored functional importance of neutrophil-specific composition of the nuclear lamins.

Neutrophil maturation is accompanied with the sequential formation of distinct types of neutrophil granules, namely primary (azurophilic), secondary (specific), and tertiary (gelatinase) granules, as well as secretory vesicles. Each type of granules contains proteins and enzymes formed at specific stage of neutrophil differentiation: Primary granules expressed by pre-neutrophils contain myeloperoxidase (MPO) and hydrolytic elastase; Secondary granules expressed by pre-neutrophils and immature neutrophils consist of potent iron-binding protein lactoferrin; Tertiary granules expressed by mature neutrophils contain high levels of matrix metalloproteinases. As such, the protein composition of granules and vesicles is greatly different in neutrophils with distinct cell maturity and represent their differential ability to undergo exocytosis, a process of releasing pre-formed inflammatory mediators, and phagocytosis of invading pathogens (39). For instance, mature neutrophils highly expressing gelatinase gran-

ules and secretory vesicles execute exocytosis and phagocytosis more readily than neutrophil precursors (40).

Recent papers using a combined approach of transcriptomic, proteomic, and bioinformatic tools have demonstrated the sequential neutrophil development pathway, in which GMPs differentiate into neutrophil-committed progenitors, termed proNeu1 and proNeu2 populations, before developing into proliferative pre-neutrophils that subsequently maturate into post-mitotic immature and mature neutrophils , characterised by their associated surface marker profiles, transcriptional signature and functional potentials (11, 12). Multi-potent GMPs, which highly express stem cell surface markers c-Kit and CD34, possess high proliferation activity and retain the ability to differentiate into both monocytic and neutrophilic lineages. Neutrophil-committed progenitors proNeu1 and proNeu2 branch from GMPs and extend along the developmental trajectory of neutrophils. With lower proliferative and self-renewal capacities, proNeu2 populations act as the bridge between proNeu1 and pre-neutrophils, and are specified to differentiate into immature and mature neutrophils (12). In neutrophils beyond pre-neutrophil stage, the proliferative program is substituted by a gain of cell migratory and effector function, and high expression of neutrophil-lineage TFs, such as C/EBPe and Gfi1, promote the transition of pre-neutrophils into immature and mature neutrophils (11). Mature neutrophils, which are characterised with high Ly6G and CD101 expression, demonstrate a maximal expression of genes central to phagocytosis, ROS generation and chemotaxis, consistent with the full range of neutrophil effector functions in mature neutrophils (11).

The ability to delineate the developmental path of neutrophils allows for assessing the involvement of TFs in instructing neutrophil lineage specification and commitment. For instance, distinct expression of granular proteins in differentiating neutrophils coordinate the expression pattern of TFs from the C/EBP family, including CEBPα, CEBPδ, and C/EBPe, which govern the expression of granular enzymes and proteins (11). Additionally, differential expression of TFs that are known to control monocytic and granulocytic lineage commitment, such as Irf8, C/EBPe, and Per3, become evident between monocytic progenitors and proneutrophils committed toward the neutrophil lineage (12). These results collectively have supported the central role of lineage-specific TFs in myeloid lineage commitment (**Figure1.1**).

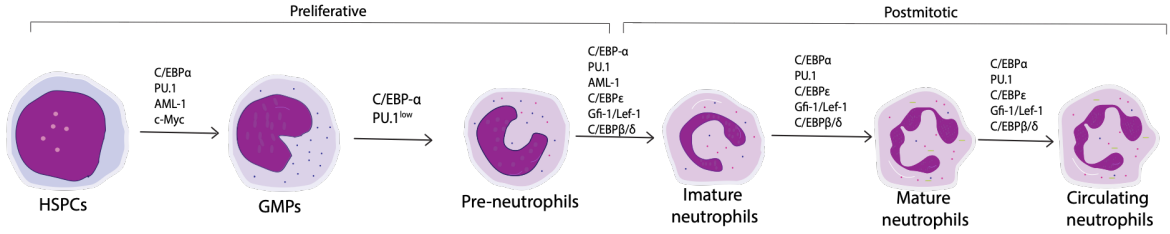
1.2.1.1 TFs that mediate myeloid lineage choice

Lineage commitment and subsequent differentiation during hematopoiesis involve the selective activation of lineage-favouring genes as wells the silencing of lineage-adverse genes, which are ultimately controlled by a number of TFs that collaboratively govern the lineage-determining commitment (41). In the downstream of HSCs and MPPs is the lineage choice between myeloid, erythroid, and lymphoid cell commitment, which depends on the the gene regulatory network of the TFs PU.1 (42), GATA-binding protein 1 (GATA-1) (21), early B cell factor (Ebf), and Notch (43). For lymphoid lineage commitment, a lower level of PU.1 expression cooperate with Ebf to instruct the commitment into B lymphoid lineage, but not for T lymphoid lineages, whereas Notch instructs T cell differentiation by antagonising B lymphoid development through repression of Ebf, B29, Pax5 and other B-cell-lineage genes (44). In contrast, a high expression of PU.1 is necessary for the myeloid lineage commitment. PU.1 interacts with other lineage-specific TFs, such as GATA-1, and block their transcriptional activities, representing one important mechanism by which PU.1 promotes the differentiation of MPPs into the myeloid lineage (45, 46). Several knockout models have supported the central role of PU.1 in the myeloid lineage commitment: a lack of PU.1 expression results in multi-lineage deficiency in the differentiation of B and lymphoid cells, and myeloid lineages such as monocytes, macrophages and neutrophils but has no influence on megakaryocyte/erythrocyte development (35, 47, 48). Enforced expression of PU.1 in multi-potent progenitors induces the commitment of multiple myeloid lineages (49), suggesting that PU.1 is a key regulator in the myeloid lineage commitment. PU.1 has also been shown to favour myeloid lineage development by promoting the expression of a number of myeloid-specific genes, such as granulocyte/macrophage colony-stimulating factor receptor (GM-CSFR) and granulocyte-colony-stimulating factor receptor (G-CSFR) (50).

1.2.1.2 TFs that promote neutrophil lineage commitment

Further differentiation of GMPs leads to the commitment into monocytic and neutrophilic lineages, in which the neutrophil-determining programme depends on several TFs, but extensive research has demonstrated a central role for C/EBPα in directing neutrophil generation. C/EBPα expression is restricted to the myeloid lineages, including monocytes, neutrophils, and eosinophils. Interferon-inducible deletion of C/EBPα in the BM cells blocks the transition between common myeloid cells and GMPs, resulting in decreased levels of monocytes and macrophages and a complete loss of mature neutrophils (51). Consistently, deficient expression C/EBPα in hematopoietic progenitor cells impairs the ability to generate GMPs and

downstream myeloid lineages, such as neutrophils, monocyte and macrophage populations (31). The need for C/EBPα in the transition between CMPs and GMPs has been further supported by enhanced granulopoiesis induced by exogenous expression of C/EBPα in myeloid cell lines (52). C/EBPα consists of three N-terminal transactivation domains and a C-terminal DNA-binding domain that mediates transcriptional activity of target genes (53, 54). It has been suggested that C/EBPα directly promotes myeloid lineage commitment through the transactivation of myeloid specific genes, such as genes encoding granular enzyme MPO (55) and G-CSF receptors (56), thereby promoting neutrophil development.



Bone marrow						Blood
		GMPs	Pre-neutrophils	Immature neutrophils	Mature neutrophils	Circulating neutrophils
Immuno-phenotype	Mouse	Linage- CD117$^+$ CD34$^+$ CD16$^+$ CD11b$^-$	Linage- CD115- Siglec-F- CD11b$^+$ CXCR4$^+$ Gr-1$^+$	Linage- Siglec-F- CD11b$^+$ Gr-1$^+$ Ly6G$^+$ CXCR2$^+$ CD101-	Linage- Siglec-F- CD11b$^+$ Gr-1$^+$ Ly6G$^+$ CXCR2$^+$ CD101$^+$	Fresh CXCR2$^+$ CXCR4$^-$ CD62L^{hi}
	Human	Linage- CD117$^+$ CD38$^+$ CD10- CD135-	Linage- CD66b$^+$ CD15$^+$ CD33^{inter} CD49d^{inter} CD101-	Linage- CD66b$^+$ CD15$^+$ CD10- CD33^{inter} CD49d$^-$ CD16^{inter} CD101^{inter}	Linage- CD66b+ CD15+ CD10- CD33^{inter} CD49d$^-$ CD16^{hi} CD101^{inter}	Aged CXCR2$^+$ CXCR4$^+$ CD62L^{low}
TFs		PU.1 C/EBPα AML-1 c-Myc	C/EBPα PU.1 Gfi-1 Lef-1 CEBP-ε	CEBPε Gfi-1 Lef-1 PU.1 C/EBPα C/EBPβ C/EBPδ	CEBP-ε Gfi-1 Lef-1 PU.1 C/EBPα C/EBPβ C/EBPδ	Bmal-1

Figure 1.1 Transcription factors (TFs) involved in neutrophil differentiation (57).
Neutrophil differentiation from hematopoietic stem cells (HPSCs) is regulated by a hierarchical network of TFs. CCAAT-enhancer binding protein α (C/EBPα), PU.1 and c-Myc are indispensable in the generation of granulocyte/monocyte progenitors (GMPs) from HPSCs. Subsequent C/EBPα upregulation and low PU.1 expression initiates the commitment towards neutrophil lineages. C/EBPe and Gfi-1 promote the terminal differentiation of neutrophils. Neutrophil differentiation is a hierarchical process and can be divided into three differential stages based on morphological features and the immune phenotype based on the surface markers. In normal granulopoiesis, GMPs first give rise to committed proliferative pre-neutrophils and subsequently produce post-mitotic immature neutrophils that finally develop into segmented mature neutrophils. In the steady state, only mature neutrophils are released into the blood. Under inflammatory conditions, immature neutrophils are also released into the circulation. Of note, a recent study has demonstrated that bacterial infection triggers a coherent drift in transcriptional regulatory networks and causes a shift in cellular resources towards defence responses, thereby accelerating neutrophil maturation.

The commitment choice of GMPs between monocytic and neutrophilic lineages primarily depends on the expression levels of the hematopoietic TFs C/EBPα and PU.1. Enforced expression of C/EBPα in myeloid progenitors rapidly induces the upregulation of C/EBPα and PU.1 expression, suggesting the transcriptional induction of PU.1 by C/EBPα in hematopoiesis (58). Indeed, C/EBPα has been demonstrated to bind and activate PU.1 distal enhancer to induce PU.1 expression, thereby promoting myeloid lineage commitment and further development in monocytes (59). Additionally, C/EBPα has the ability to antagonise the transcriptional activity of PU.1 by physically interacting and co-localising with PU.1 in a promoter region that contains multiple PU.1 binding sites, consequently increasing the threshold of PU.1 concentration and promoting monocytic and dendritic cell development (60). Consistent with the functional blocking of PU.1 by C/EBPα, G-CSF pretreatment directly upregulates C/EBPα expression in myeloid cell lines, consequently redirecting the monocyte lineage commitment toward granulocyte differentiation (52). PU.1 is essential for myeloid lineage commitment but a high PU.1 expression is detrimental for neutrophil development, because increased PU.1 activity promotes the transcriptional activation of many monocyte-favouring TFs, such as interferon regulatory factor 8 (Irf8) or activating protein-1 (AP-1) family TFs, thereby favouring monocyte development (52). In contrast, insufficient expression of PU.1 allows C/EBPα to support the development of neutrophils by promoting the neutrophil-specific transcriptional programme and suppressing monocyte development (24). Therefore, the ratio of C/EBPα to PU.1 expression is crucial for granulocytic and monocytic cell commitment.

C/EBPα also promotes myeloid lineage commitment through regulating the expression of many other granulocyte-specific TFs, for instance, the transcriptional repressor growth factor independent-1 (Gfi-1). C/EBPα is able to specifically recognise and activate the C/EBP binding site flanked in the Gfi-1 promoter region to enhance the transcription of Gfi-1, which supports the commitment into neutrophil lineages (61). In line with the need for C/EBPα in hematopoiesis is the association of inactivating C/EBPα mutation in leukemogenesis, such as acute myeloid leukaemia and myelodysplastic syndrome (62). C/EBPα promotes hematopoietic development by inducing the transcriptional activities of various genes, including but not limited to cell cycle regulator E2F (63) and myeloid-favouring gene Mef2c (64), thereby enforcing the arrest of cell proliferation and promoting subsequent differentiation.

Summarily, the influence of transcriptional network in deciding monocyte versus neutrophil development is dependent on C/EBPα and PU.1. C/EBPα is central to the transition between

CMPs and GMPs through the induction of PU.1expression, which cooperates in the development of myeloid lineages (41). PU.1 and C/EBPα synergistically participate in regulating the transcription of other myeloid-specific genes, such as G-CSF receptors and GM-CSF receptors, the major signals that guide myeloid development (56). By contrast, C/EBPα antagonises PU.1 to govern the neutrophil lineage commitment. High PU.1 activities silence the expression of neutrophil-specific TFs, such as Gfi-1, and promote monocyte development (65), whereas a low level of PU.1 expression activates a set of neutrophil-favouring genes (66), favouring the commitment of neutrophil lineages.

1.2.1.3 TFs that favour terminal differentiation

The terminal differentiation of neutrophils begins with a transition between GMP and pre-neutrophil stage, where the cellular state switches from proliferation into differentiation and involves the loss of ability to undergo cell division beyond the pre-neutrophil stage and the formation of neutrophil-specific primary granules. TFs from the C/EBP family promote the expression of granule- associated enzymes. Specifically, C/EBPα induces the transcription of genes that encoding primary granule enzymes, such as myeloperoxidase (Mpo), which are primarily expressed in early neutrophil precursors (67). As neutrophil maturation progresses, C/EBPe and C/EBP-δ regulate the expression of neutrophil secondary and tertiary granule enzymes. including but not limited to neutrophil gelatinase. collagenase, and cathelinlike peptides (68).

An important TF necessary for granulocytosis, Gfi-1, has additional effects on neutrophil terminal differentiation. Gfi-1 upregulation represses genes encoding monopoietic TFs Egr2 and Csf1, leading to the progression of granulocyte differentiation (69). Gfi-1 also suppresses the transcription of genes encoding HoxA9, Meis1, and Pbx1, thereby switching off the proliferative programme and promoting the progression of differentiation (70). The intrinsic requirement for Gfi-1 in neutrophil terminal differentiation has been demonstrated by multiple studies of several congenital and cyclic neutropenia, in which Gfi-1 mutations that interfere with its TF binding sites cause defective production of neutrophils (71-73). Neutrophil differentiation is blocked in mice with a N382S Gfi-1 mutation through unrestricted production of monopoietic cytokine CSF1 (72).

Lymphoid enhancer factor-1 (Lef-1) is another important TF that regulates neutrophil development. Lef-1 deficiency plays a critical role in the defective maturation program of myeloid progenitors in congenital neutropenia (CN) (74). Deficient expression of Lef-1 results in defective expression of proliferative and anti-apoptotic proteins cyclin D1 and c-Myc, thereby causing arrested neutrophil differentiation at the promyelocytic stage of granulopoiesis (75). Consistently, reconstitution of Lef-1 supports the differentiation of early hematopoietic progenitors from CN patients into mature neutrophils from CN patients, providing evidence that Lef-1 acts as a key TF for neutrophil differentiation (75). Lef-1 additionally promotes the granulocytic lineage commitment and subsequent differentiation through inducing the expression of C/EBPα, a key TF in granulopoiesis (76).

Terminal differentiation leads to a developmental transition into immature and mature neutrophils, which is characterised by increasing nuclear segmentation and formation of neutrophil secondary and tertiary granular enzymes that confer neutrophil effector functions (36). The most important TF that promote neutrophil terminal differentiation is the neutrophil-specific TF C/EBPe. C/EBPe expression is restricted to neutrophil differentiation and reach maximal level in neutrophils before terminal maturation (77). The importance of C/EBPe in myeloid differentiation has been supported by various *in vivo* murine models of C/EBPe deficiency, in which C/EBPe knockout commonly leads to abnormal granulopoiesis, generation of hypo-segmented morphologically atypical neutrophils and neutrophil-specific deficiency in phagocytosis, chemotaxis and cytokine production (11, 24, 76, 78). C/EBPe specifically involves the transition from GMPs to pre-neutrophils, as demonstrated in C/EBPe-deficient mice where deficient C/EBPe expression impairs the development of neutrophil downstream populations (11). Additionally, C/EBPe is essential in neutrophil terminal differentiation through promoting the formation of granular and secretory granular enzymes (68), as well as through direct interaction with the broad-spectrum proteins Rb and E2f1, thereby repressing their proliferative capacity and promoting the progression of terminal differentiation (78, 79). The absolute requirement for C/EBPe in neutrophil differentiation has also been supported by the finding that patients with neutrophil-specific granule deficiency (SGD), who have truncated production of C/EBPe, produced neutrophils with deficient formation of secondary granule proteins and compromised neutrophil function in chemotaxis and antimicrobial responses (80).

The importance of TFs in neutrophils has prompted researches to delineate the transcriptional landscape of neutrophil differentiation. Integrated epigenetic and transcriptome analysis with

human neutrophils has revealed the differential expression of TFs in neutrophils under different differentiation stages (81). Promyelocytes, myelocytes, and metamyelocytes highly express C/EBPα, C/EBPe, GFI1, and KLF5, which cooperate to promote the commitment into neutrophil lineages (31, 79, 82, 83), and several other TFs, such as Erg and Myb, which have been implicated in regulating neutrophil terminal maturation (84, 85). Notably, a wide range of hematopoietic TFs, including but not limited to BCL6, JUNB and KLF6, are constantly expressed with the progression of neutrophil differentiation and reach maximal expression in terminally differentiated neutrophils (81), reflecting their role in modulating in the inflammatory and cellular responses in neutrophils (86-88). In murine neutrophils, multi-parametric analysis has classified BM neutrophils into GMPs, pre-neutrophils, immature and mature neutrophils and has demonstrated that GMPs highly express TFs involving granulopoiesis initiation, such as C/EBPα (56) and Runx1 (33). Also, pre-neutrophils express high levels of C/EBPe and Gfi1, which is in the line with their role in neutrophil terminal differentiation (78, 89). Finally, mature neutrophils exhibit high expression of Cebpd and PU.1, two TFs crucial for normal neutrophil differentiation(11). These findings suggest a complex network of TFs involved in consecutive steps of neutrophil differentiation. This is consistent with the recent study based on transcriptional profiling of murine neutrophils via single-cell RNA sequencing (scRNA-seq), which indicated distinct activation patterns responsible for transiting between consecutive neutrophil differentiation stages and identified a number of novel TFs, including but not limited to Nfil3, Max, Mlx, and Xbp1(90), although further functional analysis are needed to elucidate the molecular mechanism underlying the TF-specific regulation of neutrophil differentiation.

1.2.1.4 TFs that mediate emergency granulopoiesis

Granulopoiesis gives rise to mature neutrophils with a full range of effector functions, which are required for the proper inflammatory response against invading pathogens (10). The ability of the haematopoietic system to replenish the circulating pool of neutrophils is crucial during pathological conditions, such as systemic infection and tissue injuries, in which the compensatory mechanisms of granulopoiesis are triggered mainly through the IL-23/IL-17/G-CSF axis to enhance *de novo* generation of neutrophils, a process termed as emergency granulopoiesis (91). Currently, it has been conceived that haematopoietic progenitors, such as GMPs, are capable of rapid adaption in granulocytic stress with selective expansion of the proNeu1 population and the cost of monocyte progenitors, to meet the immediate and enhanced requirement for neutrophils (12). However, in comparison to the temporal activation and function of TFs frequently observed in granulopoiesis under steady-state conditions, the intracellular sig-

nalling cascades and the transcriptional networks underlining emergency granulopoiesis are less well understood.

In contrast to the essential role of C/EBPα in steady-state granulopoiesis, C/EBPα expression is dispensable for granulocytic development under inflammatory conditions, as evidenced in C/EBPα-deficient haematopoietic progenitors, pre-incubation with granulopoietic growth factors, such as GM-CSF and IL-3, manages to recover the expression of G-CSFR, a target gene of C/EBPα, and rescue G-CSF-induced neutrophil differentiation, suggesting the existence of C/EBPα-independent pathway of granulopoiesis (92). C/EBPβ-deficient haematopoietic progenitors fails to generate more neutrophils in response to infection, although steady-state granulopoiesis remains unaffected, suggesting the involvement of C/EBPβ in emergency granulopoiesis (93, 94). It has been increasingly clear that in the process of emergency granulopoiesis, C/EBPβ is required to induce an emergency granulopoietic response whereas C/EBPα is dispensable (93).

C/EBPα and C/EBPβ exert specific but differential effects in promoting neutrophil generation under steady-state and inflammatory condition, respectively. C/EBPα functions as a master regulator of granulopoiesis by promoting the progression of differentiation and simultaneously switching off the proliferative programme by decreasing the expression of cell cycle regulators Cdk2 and Cdk4, and cell cycle accelerators, such as c-Myc, thereby limiting the rate of neutrophil generation under steady-state condition (95). C/EBPβ does not tend to arrest cell proliferation. Under inflammatory condition, increased levels of inflammatory mediators, mostly G-CSF and, to less extent, GM-CSF, induce rapid C/EBPβ activation through the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) signalling pathways, consequently accelerating the neutrophil differentiation process (94). C/EBPβ activation has additional effects on neutrophil generation through integrating with STAT3 to directly promote the expression of Cdk2, Cdk4 and c-Myc, and to indirectly inhibit C/EBPα binding to promoters of target genes, thereby accelerating the production and mobilisation of neutrophils under inflammatory conditions (96).

1.3 Neutrophil trafficking

Once neutrophils become mature, they are released from the bone marrow into the blood. The release of neutrophils is tightly regulated by the action of two chemokine receptors, C-X-C chemokine receptor type 2 (CXCR2) and CXCR4. In the bone marrow, C-X-C motif chemokine ligand 12 (CXCL12), the ligand for the surface C-X-C chemokine receptor type 4 (CXCR4), serves as the retention signal for differentiating neutrophils to adhere to endothelial and stromal cells and also antagonise the CXCR2-mediated signals that promote the release of neutrophils. As a consequence of neutrophil maturation, spontaneous CXCR4 downregulation and CXCR2 upregulation facilitates neutrophil mobilisation into the bloodstream. During inflammatory condition, the hematopoietic cytokine G-CSF induces the acute mobilisation of neutrophils through increased production of CXCR2 ligands by megakaryocytes (97), reduced expression of CXCR4 ligands in bone marrow stromal cells and the downregulation of CXCR4 in neutrophils themselves (98).

1.3.1 Neutrophil ageing

After release form the bone marrow, neutrophils circulate in the blood for only a short period of time (the half-life of murine neutrophils in circulation is estimated to be about 12 hours) (13), before they are marginated into tissue sites, such as liver, spleen, lung, and bone marrow itself. The dynamics of circulating and marginated neutrophils require a fine balance between neutrophil production and elimination, which are controlled through diurnal changes in the expression of chemokine receptors by neutrophils and the adhesiveness of endothelial cells that line the vasculature (7). In mice, diurnal fluctuations have been observed in the expression of P-selectin glycoprotein ligand-1 (PSGL1), CD62L and the chemokine receptors CXCR2 and CXCR4 (99). Specifically, neutrophils freshly released from the BM express a high level of CD62L, a surface marker whose expression is reduced upon ageing (**Figure1.2**). During the approximate 6-hour course of peripheral circulation, neutrophils gradually reduce CD62L expression and upregulate the CXCR4 expression, which mediates neutrophil migration back to the BM for final clearance, a process termed neutrophil ageing (6). Moreover, these phenotypic changes parallel diurnal changes in the transcriptional and migratory properties of circulating neutrophils. It has been revealed that diurnal change in cortical β-actin cause the loss of

surface microvilli, consequently resulting in reduced capacity of ageing neutrophils to migrate to inflamed tissues.

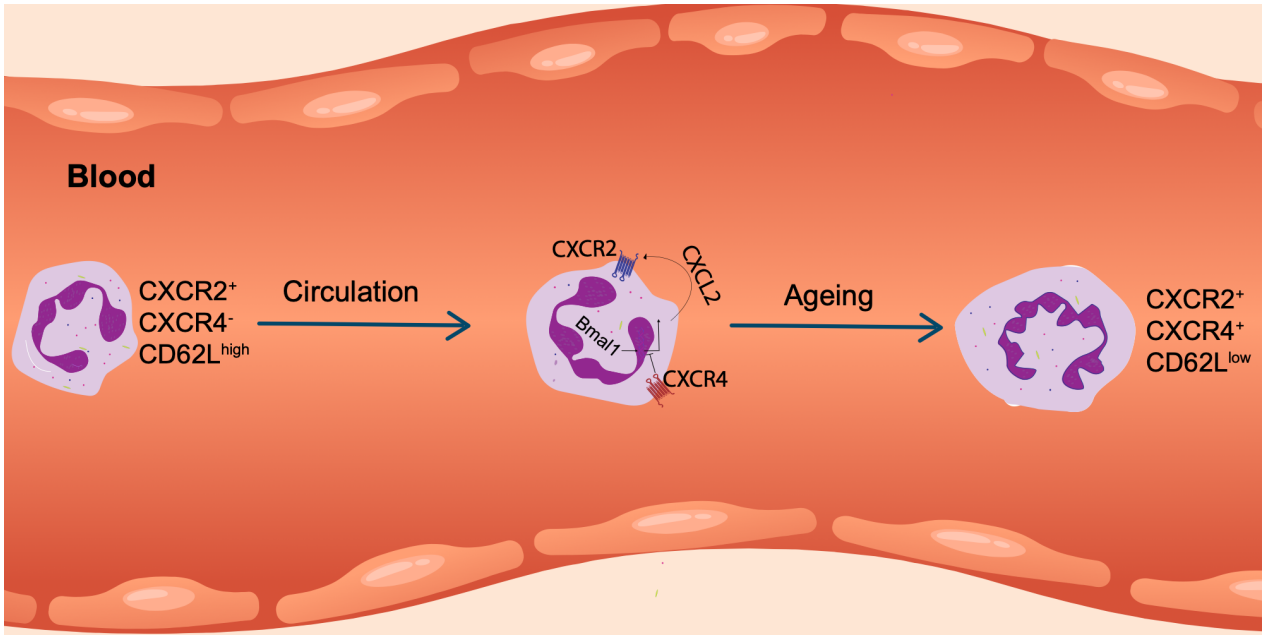


Figure 1.2 Transcriptional regulation of neutrophil ageing.
Neutrophils released from the bone marrow display a fresh phenotype featuring with high CD62L expression. In circulation, neutrophils gradually decrease CD62L expression and increase CXCR4 expression during ageing. It has been reported that Bmal1 intrinsically regulates neutrophil ageing by modulating the expression of CXCL2, a CXCR2 ligand that functions to promote neutrophil ageing in an autocrine manner. (6, 57)

1.3.1.1 External regulators of neutrophil ageing

Circadian production and clearance of neutrophils in the circulation is controlled by the sympathetic nerve system in humans(100), and external signals delivered by the microbiome in mice (101), In humans, adrenergic nerves regulate circadian oscillations in the expressions, thereby allowing for rhythmic recruitment of neutrophils into tissues. In mice, the microbiome drives the process of neutrophil ageing with microbiota-derived molecules that cross the intestinal barriers, which drive the process of neutrophil ageing through TLRs/MyD88-dependent signalling activation (101). Indeed, depletion of the gut microbiota abolishes this microbiota-driven neutrophil ageing and significantly lowers the number of aged neutrophils in the circulation, consequently resolving neutrophil-related inflammation and pathogenesis (101).

1.3.1.2 TFs that intrinsically control neutrophil ageing

Support for cell-intrinsic mechanisms of ageing has been found in studies showing that the clearance of neutrophils that have aged in the circulation is diurnally regulated by an intrinsic circadian-clock regulator, namely Brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (Bmal1) (6). Targeted deletion of Bmal1 or CXCR2 dramatically prevented neutrophil ageing, characterised by the loss of diurnal oscillations in the transcriptional and migratory properties of neutrophils in response to pathogenic infections (6). Mechanistically, Bmal1 intrinsically regulates the expression of CXCL12, a CXCR2 ligand that activates CXCR2 signalling in an autocrine manner, thereby controlling cell-intrinsic rhythms of diurnal neutrophil ageing (6). Diurnal alterations in clock gene expression have also been found in other hematopoietic lineages, including but not limited to Ly6C^{high} monocytes and Th17 T cells, supporting the existence of cell-intrinsic clock regulators in regulating leukocyte phenotype and function.

1.3.2 Neutrophil infiltration into tissues

Under healthy conditions, neutrophils are found in the vast majority of naive tissues where they execute normal homeostatic (or non-inflammatory) functions. For example, in the BM, phagocytosis of apoptotic neutrophils by resident macrophages triggers further neutrophil production in the bone marrow through the release of hematopoietic progenitor cells (HPCs) into the bloodstream (102). Neutrophils infiltrated into the spleen support the maturation of B cells and production of immunoglobulin in the marginal zone (103). Extra-medullary infiltration of neutrophils in the skin, muscle, and intestine support the process of hematopoiesis in the BM

(104). These and other studies (105, 106) have raised debate regarding the tissue-induced or tissue-restricted profiles that result in the generation neutrophils with alternative functions. Indeed, neutrophils have been shown to be transcriptionally active in different micro-environments. For instance, in cancers, neutrophils interact with cancer cells and cancer-produced mediators instruct different transcriptional profiles, which polarise neutrophil phenotypes to alter tumour behaviour (107). Similar neutrophil transcriptional plasticity has also been reported in healthy tissues, for instance, in the naive lung and intestine, CD49d^{high} CXCR4^{+} neutrophils accumulate in the vascular lumen and the interstitial space in large numbers. These neutrophils are kept in the tissue in a CXCR4-dependent manner and receive the signals needed for transcriptional reprogramming to acquire new properties and functions within tissues, including angiogenesis and hematopoietic homeostasis (13).

1.3.3 Neutrophil recruitment during inflammation

Circulating neutrophils are rapidly recruited into sites of inflammation, where they are exposed to inflammatory signals and acquire transcriptional profiles characteristic of neutrophil effector functions. The recruitment process of neutrophils is initiated by direct interactions between circulating neutrophils and endothelial cells that line the inflammatory sites. Upon activation, endothelial cells maximise the adhesiveness of neutrophils by transporting pre-stored P-selectin into the surface and upregulating the expression of E-selectin, two cell adhesion molecules that recognise the P-selectin glycoprotein ligand-1 (PSGL1), as well as CD44 and E-selectin ligands expressed by circulating neutrophils, leading to the tethering and adhesion of circulating neutrophils (108). Moreover, reverse interaction of L-selectin (CD62L) expressed by circulating neutrophils with P-selectin and E-selectin on endothelial cells allows for a secondary neutrophil tethering on already attached neutrophils, thereby augmenting neutrophil recruitment and accumulation during inflammation (108).

Having approached the endothelium, tethering neutrophils participate actively in a sequence of physical interactions: rolling, adhesion and crawling. Each step involves a distinct molecular mechanism. Initially, neutrophil rolling to endothelial cells is mediated by interaction between E-selectin and E-selectin ligands. Selectin engagement triggers intracellular signals in both selectin-bearing endothelial cells and ligand-expressing neutrophils (109). In rolling neutrophils, the cytoplasmic protein-tyrosine kinase spleen tyrosine kinase (SYK), signals downstream of PSGL1. Neutrophil rolling on E-selectin activates β2-integrin binding to intercellular

adhesion molecule (ICAM)-1 through a p38 mitogen-activated protein kinase (MAPK)-dependent pathway, facilitating neutrophil rolling and subsequent adhesion (110). Secondarily, the engagement of β2-integrins Mac-1 (αMβ2, CD11b/CD18) and LFA-1(αLβ2, CD11a/CD18) with their counter-receptors expressed by endothelial cells promotes neutrophil cell shape polarisation by inducing the activation of PIP5K1C kinase and downstream kinase RhoA, thereby increasing neutrophil cell contractility and eventually leading to neutrophil emigration out of the vasculature(111).

Neutrophil transmigration across the vascular endothelium facilitates chemokine-dependent activation of neutrophil integrins and subsequent transendothelial migration. Chemokines are usually produced from immune cells at inflammatory sites and transported to the endothelial surface via specific transcytosis systems, such as the Duffy-receptor-driven transcytosis for chemokines. For instance, IL-8 and soluble chemoattractants are produced by tissue-resident macrophages in response to inflammation and transcytosed by venular endothelial cells to the luminal surface (112). Integrin activation is induced by internal signals generated from G protein coupled chemokine receptors (GPCRs), Toll-like receptors and others. Merged in an environment full of chemokines, chemoattractants and pro-inflammatory cytokines, migrating neutrophils initially transmigrate toward a subliminal chemokine gradient formed by intermediate sites like the endothelium. Conformational changes in β2-integrins in induced by the chemokines lead to the activation of the PI3K and PTEN signalling pathways to promote the redistribution of Ras-related GTP-binding proteins, such as Arf and Rac, to the leading edge, thereby coordinating neutrophil orientation to the chemokine gradient. Neutrophil migration toward the inflammatory sites is then mediated by end-target chemoattractants, such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) and complement fragment 5a (C5a), which prioritise the signal of MAPK signalling activation over that of the PI3K pathway, thereby allowing for neutrophil migration preferentially toward the source of end-target chemoattractants, such as sites of inflammation.

1.3.3.1 TFs that involve neutrophil recruitment

Sequential steps of neutrophil recruitment through the vasculature are tightly regulated for an appropriate response to inflammation. Integrins are essential for mediating neutrophil adhesion and migration during inflammation. For instance, in the context of Staphylococcus aureus infection, a significant increase of neutrophil crawling is mediated by β2- and α4-integrins (113). TF serum response factor (Srf) has been shown to be crucial for integrin activation in

neutrophils (114). Specifically, in mice with hematopoietic-specific deletion of Srf expression, neutrophils fail to migrate toward inflammatory sites *in vivo* and along chemokine gradients *in vitro*. In response to fMLP stimulation, Srf-deficient neutrophils lost the ability of polymerising globular actin to form filamentous actin, which is important for neutrophil cytoskeletal remodelling (114). Another important TF that promotes neutrophil recruitment is activation transcription factor 3 (Atf3), which is known to act as a negative regulator in the TLR signalling pathways. Support for the requirement of Atf3 for neutrophil migration has been found in Atf3-deficient mice showing deficient recruitment of neutrophils into the lung after LPS challenge (115). Mechanistically, Atf3 functions to induce the expression of TIAM2, which in turn regulates cell attachment and motility through Rac1-dependent focal adhesion (115). Consistently, Atf3-deficient neutrophils, lacking TIAM2 expression, exhibited a profound deficiency in chemotaxis, along with dysregulated adhesion structure and cytoskeletal organisation, supporting a crucial role for Atf3 in neutrophil recruitment (115).

1.4 Neutrophil activation

Having infiltrated into inflammatory sites, neutrophils are able to modify their phenotypic and functional properties after being exposed to multiple inflammatory signals, through the process of neutrophils activation, a state where neutrophil effector functions are remarkably enhanced. The reinforcement of neutrophil functional responses is induced by signal-induced transcriptional activation and changes in the expression of surface markers and inflammatory mediators, such as chemokines and cytokines (116). During the recruitment, neutrophils upregulate the expression of stimulatory molecules and major histocompatibility complex (MHC) molecules, thereby influencing the immune function of other leukocyte types, such as T cells and B cells (7). Additionally, neutrophils are capable of recognising PAMPs or DAMPs, through specific PPRs, to induce a complex network of downstream signalling and subsequent activation of signal-dependent TFs (**Figure 1.3**), including but not limited to members of the NF-κB, IRFs and AP-1 family, thereby inducing the expression of genes that closely associate with neutrophil effectors functions (57).

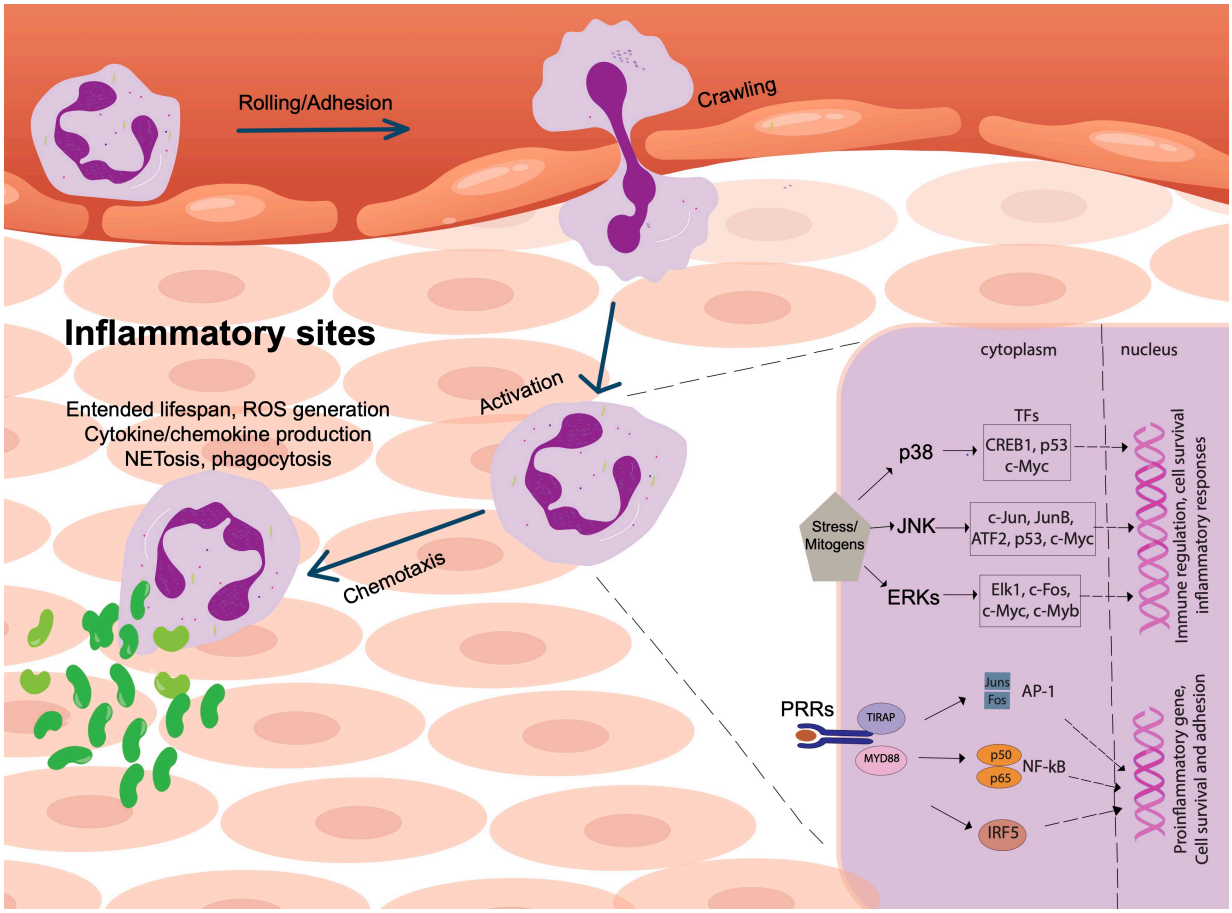


Figure 1.3 Signalling pathways and TFs involved in neutrophil activation.
Transcriptional regulation of neutrophil activation during inflammation (57). Upon infiltration into sites of inflammation, direct interaction between neutrophils and activated endothelial cells and subsequent exposure to pro-inflammatory stimulation promote neutrophil activation. Several TFs integrate extrinsic and intrinsic signals and act as nuclear effectors to induce the expression of defence-associated genes, thereby modulating neutrophil inflammatory responses and cell fate decision. Several studies have demonstrated that activated neutrophils significantly upregulate genes related to bactericidal functions, including synthesis of granule proteins, cytokines and chemokine production, NADPH oxidase complex and exhibit enhanced capacity of ROS generation, phagocytosis and chemotaxis (61, 90, 117).

1.4.1 TFs that mediate neutrophil function by NF-κB activation

TF NF-κB is essential in inflammatory and immune responses, regulating the expression of several genes that are closely related to acute inflammatory processes and cell survival. Resting neutrophils constitutively express a large amount of NF-κB inhibitors, IκBα and IκBβ, in the nucleus where they are sequestered together with other NF-κB subunits, such as p50/p65, c-Rel, and p52 (118). Following cell stimulation by inflammatory stimuli, such as tumour necrosis factor (TNF)-α and lipopolysaccharide (LPS), inhibitory IκBα is rapidly ubiquitinated, and selectively degraded in cytoplasm by the proteasome. Degradation of IκBα leads to the unmasking of the nuclear import signal, the nuclear localisation sequences (NLS), of NF-κB p50/p65 heterodimers, which induce the nuclear translocation of NF-κB and subsequent transcription of inflammatory and anti-apoptotic genes (119).

NF-κB activation in neutrophils induces the expression of genes that closely relate to the function of cytokine and chemokine production, cell adhesion and migration, and cell survival and proliferation. Activated neutrophils are able to produce a multitude of pro-inflammatory molecules. Specifically, NF-κB activation induces the upregulation of the cytokines, like TNF-α, IL-1, IL-6 (120), and chemokines, such as Cxcl-2, Cxcl-8 and Cxcl-10 (121), which function as important mediators to influence and regulate the inflammatory processes. Interestingly, NF-κB activation also promotes the release of micro-particles, which are small, intact micro-particles that are generated from neutrophils upon activation. These particles not only have an inflammatory effect on surrounding cells, such as endothelial cells (122), but also can be phagocytes by themselves, thereby forming a positive feedback loop of neutrophil activation that initiates and amplifies pro-inflammatory responses (123).

The target genes upregulated by NF-κB activation also involve the adhesion and migration of neutrophils through promoting the expression of adhesion molecules. For instance, neutrophils upon activation rapidly upregulate integrin αLβ2 (CD11b) and it is dependent on the activation of the NF-κB p50 and p65 subunits, as evidenced by p65 inhibition significantly abolishing the upregulation of CD11b by neutrophil stimulated by fLMP (124). In addition, migration of neutrophils occurs with the activation of CXCR2 signalling induced by CXC chemokines, which is a process that involves NF-κB-dependent induction of transcription factor C/EBPα

expression (125). In the context of Escherichia coli pneumonia, NF-κB p50 deficiency promotes the expression of genes that closely relate to neutrophil recruitment, along with enhanced neutrophil pulmonary infiltration (126). However, the molecular mechanisms underlining it remain yet to be explored.

Importantly, NF-κB activation supports neutrophil survival by shifting the balance between the pro-apoptotic and anti-apoptotic signalling. Specifically, TLR-induced NF-κB activation delays neutrophil apoptosis through the upregulation of anti-apoptotic proteins, such as Mcl-1 and Bcl2, promoting extended neutrophil survival and decreased apoptosis (127). NF-κB activation has additional effects to induce the expression of anti-apoptotic members of the TNF-α-associated complex, thereby preventing neutrophil apoptosis during inflammation (128).

Support for the central role of NF-κB in neutrophil activation has been additionally found in with NF-κB deficiencies causing impaired neutrophil generation and function. For instance, homozygous deletion of NF−κB subunit RelA/p65 is embryonically lethal (129), whereas no major phenotype is observed in mice with heterozygous RelA/p65 deficiency (123). Mice with hematopoietic-specific RelA/p65 deletion experience an impaired neutrophil generation, consequently resulting in an increased susceptibility to infection (130). On the contrary, deficiency in IKK-β deficiency leads to constitutive activation of neutrophils accompanied by enhanced granulopoiesis resulting from p50-dependent C/EBP-α upregulation(125, 131). These examples underline the importance of specific NF-κB subunits in neutrophil biology and explain the occurrence of recurrent and persistent infections in patients with deficient NF-κB function (132). However, the molecular mechanism of how specific NF-κB deficiency disrupts neutrophil phenotype and function remain largely unexplored.

1.4.2 TFs regulate neutrophil function through the MAPK pathways

Another prominent set of signalling pathways mediating neutrophil activation is the mitogen-activated protein kinase (MAPK) kinase cascade, which amplify and integrate signal from external stimuli, growth factors and play a central role in cell proliferation, differentiation and inflammatory responses. Neutrophils express all major types of MAPK cascade, including classical MAPK (ERK), p38 MAPK, ERK and c-Jun terminal kinase (JNK) (133). Each MAPK cascade is activated by dual phosphorylation of conserved threonine and tyrosine residues by

specific upstream MAPK kinases, such as MAPK/ERK (MEKK) and MAPK (MKK) 1, 3, 6 or 4/7. Upon activation, neutrophils adopt distinct MAPK subtype cascades, depending on the type of stimulatory agents that they are exposed to. For instance, G-CSF selectively activates the MEK-ERK cascade while GM-CSF preferentially induces activation of the MEK-ERK over the MKK3/6-p38 MAPK cascade to mediate signals modulating neutrophil activation (134). Downstream of p38, ERK and JNK cascades are a diversity of TFs, such as c-Jun, JunB, ATF2, p53 and c-Myc, which are largely regulated by JNKs (135), Sap1, CAMP responsive element binding protein 1 (CREB1), p53 and C/EBPβ, which are controlled by p38 MAPKs (136), Elk1, c-Fos, c-Myc and c-Myb under the control by ERKs (137). These TFs act as transcriptional regulators of several genes that control morphology determination, inflammatory responses, survival and apoptosis upon neutrophil activation(138-140). In the coming paragraphs, we will focus on the functional role of four classical TFs, C/EBPβ, CREB1, JunB and p53, whose activation promotes neutrophil function and may exemplify the mechanism underlying the activation of other TFs.

C/EBPβ belongs to the bZIP family of TFs that are characterised by a C-terminal dimerisation domain adjacent to a basic DNA-binding domain and a N-terminal regulatory domain. Several isoforms of C/EBPβ are generated through alternative splicing, different translation initiation codons from the same RNA template or by different patterns of proteolysis. The 32- and 35-kDa forms of C/EBPβ, with their N-terminal transactivation domain, function as transcriptional activators whereas the 20-kDa form of C/EBPβ with the truncated transactivation domain inhibits the transcription (141). As discussed above, in response to cytokine stimulation and/or infection, C/EBPβ-deficient mice show deficient emergency granulopoiesis (93). Moreover, neutrophils with C/EBPβ deficiency shows enhanced apoptosis(142), supporting the notion that C/EBPβ is required for neutrophil production and survival. In response to cell stimulation, C/EBPβ is rapidly phosphorylated and activate the promoter of genes encoding inflammatory mediators, such as IL-8 and IL-1B, promoting the production of pro-inflammatory cytokines (143).

CREB1 is a member of the CREB family of TFs that also include cAMP response element modulator (CREM), and activating transcription factor-1 (ATF-1). The CREM gene undergoes differential splicing to produce different isoforms, which functions as the activators of CREB1 target genes or negative regulators of CREB1 activation. The high homology between their bZIP domains allows CREB1, ATF-1 and CREM to dimers to regulate the transcription (144).

Upon cell stimulation, CREB proteins are rapidly phosphorylated on their signal domain by p38 MAPK kinases, which allows for increased transactivation potential(145). CREB1 is strongly expressed by resting human neutrophils and constitutively associated with the promoters of several chemoattractants, such as *Cxcl8* and *Ccl3* (144). Neutrophil activation leads to the phosphorylation of CREB-1/ATF-1 by p38 MAPKs and increased production of CXCL8, CCL3, CCL4 and TNF-α (144). CREB1 phosphorylation also enhances the expression of matrix metallopeptidase 9 (MMP9), which is highly expressed by tumour infiltrating neutrophils (146), further supporting the involvement of CREB1 in regulating neutrophil pro-inflammatory functions.

JunB is a member of the Jun family of TFs that regulate the inducible expression of many pro-inflammatory mediators, including cytokines, chemokines and growth factors in various cell types(147, 148). Jun proteins, including c-Jun, JunB and JunD, have the ability to form homo- or heterodimers with themselves or with Fos proteins to form multiple activator protein-1 (AP-1) variants (149). The heterodimeric AP-1 complexes are typically composed of a basic leucine zipper domain, which shares structural homology with different TFs families, such as NF-κB/Rel proteins, and CREB/ATF protein (150, 151) and allows them to associate with other TFs to modulate transcription. The ability of JunB to induce transactivation is greatly induced upon cell stimulation and phosphorylation by the upstream MAPK JNKs (152). Neutrophils constitutively express a number of the Jun family proteins. Among them, JunB is found to be mostly expressed upon activation (87, 153). Recent evidence suggests that JunB is an important positive regulator of neutrophil activation. JunB strongly enhances immune gene expression by binding to the promoter-proximal regions upstream of the genes that control infection and inflammation (87). PU.1 inhibits JunB binding by restricting the accessibility of enhancers via the recruitment of histone deacetylase 1 (HDAC1), and consequently restraining neutrophil activity (87).

1.4.3 Emerging TFs as potential regulators in neutrophils

P53 is another TF critical for the stress responses in neutrophils. Maintained at low expressional levels in resting cells through targeted degradation, p53 is stabilised and activated in response to cellular stress and and/or inflammatory agents, such as TNF-α(154) and ROS (155). It promotes the expression of anti-apoptotic protein Mcl-1(156). In fact, p53-deficient neutrophils demonstrate extended lifespans and enhanced capacity for phagocytosis in re-

sponse to inflammatory stimulation (157, 158), likely via induction of anti-apoptotic mechanisms. Recent studies demonstrated the mutual regulation of p53 and NF-κB in immune cells (159, 160). p53 inhibits NF-κB transcriptional activity and attenuates the NF-κB–dependent cytokine production by neutrophils (159). Loss of p53 expression results in enhanced NF-κB DNA-binding activity that induces the expression of many pro-inflammatory cytokines by neutrophils(159). Additionally, p53 regulates cell migration through the activity of the phosphatidylinositol 3-kinase/Rac1 pathway (161) and neutrophils with p53 deficiency demonstrated increased tissue infiltration and enhanced bacterial killing in response to infection(159), supporting the inhibitory role of p53 in neutrophil functions during inflammation.

Interferon regulatory factor-5 (Irf5) functions downstream of the TLR-MyD88 signalling pathway to induce targeted gene expression (162). Upregulation of Irf5 has been detected in neutrophils activated via the TLR signalling and cytosolic DNA-sensing pathways (61), as well as the pathways induced by bacterial infection (90). Upon TLR9 stimulation, Irf5-deficient neutrophils produced significantly lower levels of inflammatory cytokines, such as TNF-α (61), supporting the potential role of Irf5 as a novel regulator in neutrophil inflammatory responses, although more studies are needed for elucidating the underlying molecular mechanisms.

The importance of particular TFs in neutrophil activation has been established by multiple studies that utilised transcriptional profiling of neutrophils under exogenous stimulation (61, 163) or neutrophils under physiological and different disease states (90, 164). These studies indicated that activated neutrophils stimulated under different inflammatory conditions commonly upregulate the expression of genes enriched for cytokine production and bactericidal activities, including phagocytosis and synthesis of granular proteins (61, 90, 163) and that multiple signalling pathways activated during neutrophil activation eventually converge at common clusters of TFs, which share patterns specific to activating conditions (61, 90).

With the generation and adoption of novel genomic techniques, a wide range of novel TFs associated with neutrophil developmental stage and activation status are being discovered (**Figure 1.4**). scRNA-seq of murine neutrophils revealed a heterogenous and complex neutrophil population under steady-state and bacterial infection conditions and uncovered the transcriptional landscape driven by both known and uncharacterised TFs during the process of neutrophil differentiation and pathogen clearance. Under normal conditions, neutrophils undergoing differentiation gradually downregulate the expression of lineage-commitment associated TFs,

such as Gfi-1 and C/EBPα, which guide neutrophil development (89)(31), and upregulate inflammatory-response-related TFs, such as Irf5, C/EBPβ, which mediate neutrophil function (61)(143), consistent with their enhanced microbial clearance functions upon maturation (90). Bacterial infection accelerates neutrophil maturation by prioritising the upregulation of granulocytosis-favouring TFs, such as C/EBPβ, PU.1, which augment emergency granulopoiesis(93), and promoting the downregulation of metabolic TF networks that regulate biosynthesis and transcriptional regulation, thereby priming neutrophil functionality toward pathogen clearance responses(90). Notably, such studies have also identified numerous uncharacterised TFs involved the transition of each neutrophil subset and the regulation of neutrophil functions under steady and infection conditions, providing a basis for the further study of the molecular mechanisms underlying neutrophil differentiation and activation (90).

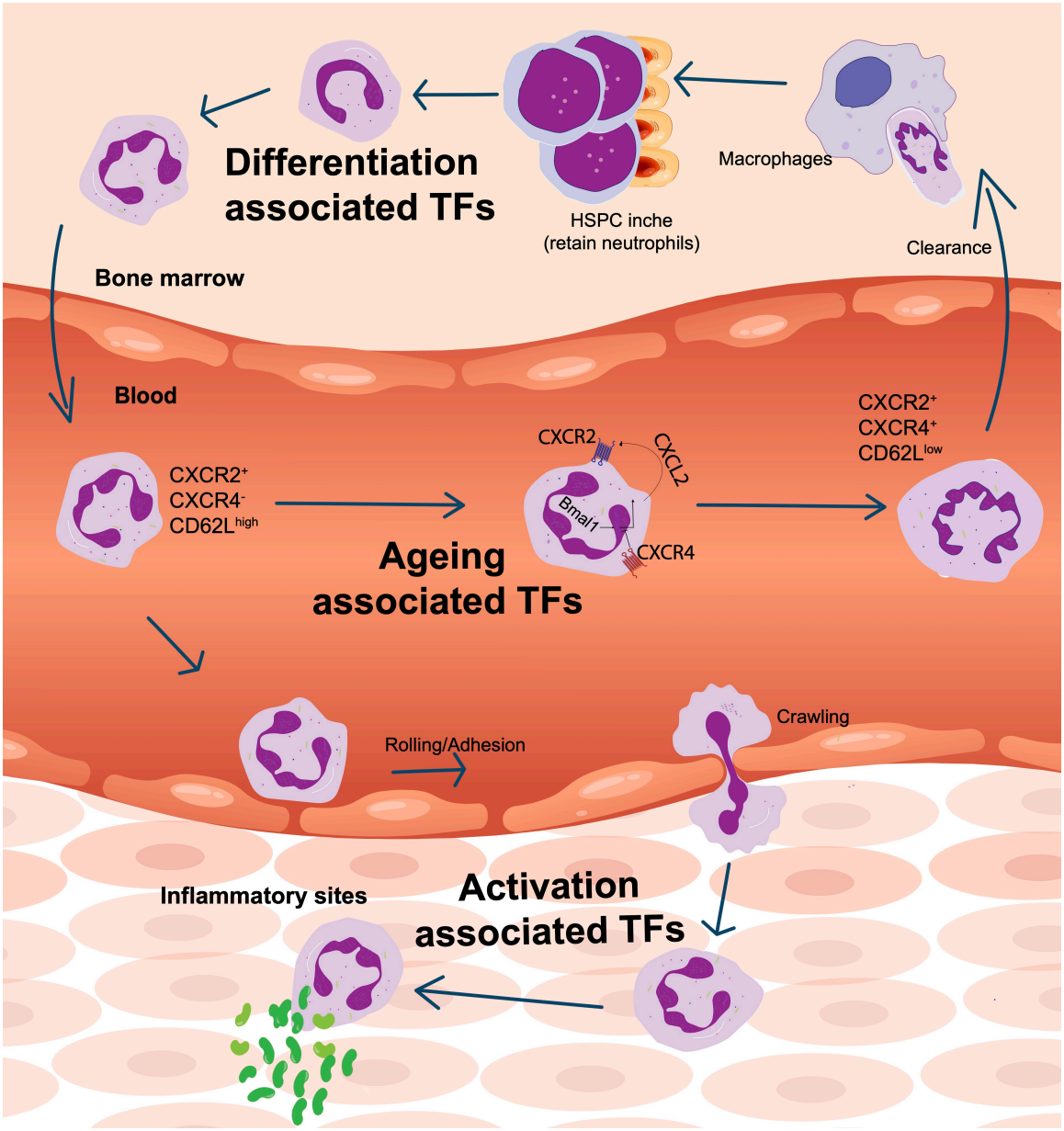


Figure 1.4 Transcriptional regulation of neutrophil during inflammation.
Neutrophil differentiation and activation have been considered as complex processes comprising of several changes at the morphological to transcriptional levels (57). Distinct sets of TFs operate both exclusively and cooperatively forming a transcriptional network that integrate several intrinsic and extrinsic signals to modulate the output of neutrophil differentiation and inflammatory responses.

1.5 Neutrophils as therapeutic targets

1.5.1 Neutrophils and pathogenesis

Despite playing diverse roles in inflammation, neutrophils are central in several disease processes, such as infection, inflammation disorders and cardiovascular diseases (9). Defective neutrophil generation or function results in severe immune deficiencies, such as recurrent infection commonly observed in inherited neutrophil defects and severe neutropenia. Dysregulated neutrophil activation leads to excessive inflammation and tissue damage and play an important role in the pathogenesis of autoimmune disorders, such as rheumatoid arthritis (RA), giant cell arteritis (GCA), and granulomatosis with polyangiitis (GPA).

1.5.1.1 Insufficient neutrophil generation

Insufficient neutrophil production results from genetic, drug induced and autoimmune neutropenia. Multiple genetic defects in the course of neutrophil differentiation leads to severe congenital neutropenia (SCN), which is characterised by an absolute count of neutrophils lower than 5x105/ml with an early onset of systemic bacterial infections in infancy. Neutrophils are major effector cells against both Gram-positive and Gram-negative bacteria, whereas insufficient neutrophil-mediated pathogen elimination causes increased susceptibility to infections, such as early onset of systematic infection found in Kostmann syndrome (165). In addition to infection, patients with severe congenital neutropenia suffer from a high risk of developing myelodysplastic syndrome and acute myeloid leukaemia, especially if co-expressing the mutated form of RUNX1 and G-CSFR. The cooperative effects of RUNX1 and G-CSFR mutations enhances G-CSF-induced neutrophil proliferation with decreased maturation, thereby contributing to leukemic transformation. and the progression of disease (166). Mutations in *ELA2*, the gene that encodes neutrophil elastase, is the major cause for autosomal-dominant SCN (167). Rare forms of SCNs are also attributable to mutations in the TF *GFI1*, which is an important regulator in neutrophil terminal differentiation (75).

1.5.1.2 Leukemogenesis

Acute myeloid leukaemia (AML) is featured with the accumulation of myeloid progenitors in the bone marrow, resulting from a block of neutrophil differentiation at the promyelocyte or myelocyte stage. One of the major mechanisms underlining leukemogenesis is the dysregula-

tion of TFs that are crucial for neutrophil differentiation and proliferation. For instance, the expression of AML1-ETO leukemic fusion protein is commonly found in patients with the t(8;21) translocation and it blocks the differentiation of myeloid lineages by suppressing C/EBPα mRNA expression (168). A predominant C/EBPα mutation pattern has been observed in AML patients, with a majority of them carrying heterozygous C/EBPα mutations, which generates two truncated isoforms of C/EBPα: p42 and p30. These mutated C/EBPα are able to bind with DNA and function as hematopoietic TF, thereby resulting in impaired myeloid differentiation and initiating AML (169).

1.5.1.3 Neutrophil functional deficiencies

Primary immune deficiency diseases with defects in neutrophil function include defective neutrophil adhesion and migration, impaired ROS generation and deficient formation of neutrophil granules. These disorders not only share an increased susceptibility to bacterial and viral infection but also have distinctive immunological and clinical features. For example, leukocyte adhesion deficiency 1 (LAD1) is a defect of surface adhesion molecule β2 integrin, which is necessary for neutrophil adhesion and migration. With absent or low β2 integrin expression, neutrophils from patients with LAD1 are incapable of binding to ICAM-1 expressed by the vascular endothelium and consequently they are unable to transmigrate towards inflammatory sites (170). Impaired neutrophil migration function leads to severe leukocytosis and recurrent infections, in which pus formation is impaired (170). Another exampled disease is neutrophil-specific granule deficiency (NSGD), which is a rare congenital disease primarily caused by *C/EBPe* mutation. Neutrophils from patients with NSGD are absent expression of all types of secondary and tertiary granule, consistent with an essential role for C/EBPe in neutrophil granular production (11). Multiple neutrophil effector functions are impaired in neutrophils from NSGD, including chemotaxis, cytokine production, ROS generation, and bactericidal activity. SGD patients suffer severe and recurrent pyogenic infections (171).

1.5.2 Therapeutic targeting strategies of neutrophils

As described above, neutrophils are involved in a diversity of immunological and inflammatory processes. A promising therapeutic approach has been proposed by specific modulation of neutrophils in pathological conditions. When neutrophil generation and function are insufficient to the combat invading pathogens, the overall output of neutrophil subsets needs to be enhanced. In certain diseases with excessive neutrophil activation, such as autoimmune dis-

eases and chronic inflammation, it is desired to limit the level of neutrophil activities without compromising host defence.

1.5.2.1 Enhancing neutrophil production

Neutropenia is an abnormally low concentration of neutrophils, resulting from deficient production of neutrophils in the bone marrow, or increased neutrophil destruction caused by autoimmune disease or chemotherapy. Treatment with hematopoietic growth factors, such as G-CSF (Filgrastim), GM-CSF, is recommended to increase the absolute amount of neutrophils in the circulation. Indeed, this treatment has been effective in enhancing the production of neutrophils in the bone marrow and recover circulating neutrophil levels following chemotherapy (172). G-CSF has additional effects to increase circulating neutrophil amount by interfering with the CXCL12/CXCR4 signalling as a retention signal for neutrophil release, thereby promoting the transition of neutrophils from bone marrow into the circulation. For neutropenia that is only temporary, such as neonatal neutropenia, the administration of recombinant G-CSF is encouraged, because of lower side effects than antibiotic prophylaxis (172).

1.5.2.2 Neutrophil functional enhancement

Insufficient neutrophil function can be reversed by enhancing the functional responsiveness of neutrophils by targeting inhibitory receptors on neutrophils. Neutrophils express various inhibitor receptors, which are used by tumour cells to escape host immune elimination. For instance, tumour cells express CD47, which specifically recognise CD172a inhibitory receptor expressed by neutrophils to decrease neutrophil-mediated cytotoxicity. One exciting potential has been proposed to enhance the cytotoxic effects of neutrophils through interference with CD47-CD172a interactions using blocking antibodies against CD47 or CD172a (173). In addition, NETosis by neutrophils facilitates tumour survival and progression (174), but whether NETosis can be therapeutically targeted remain yet to be validated.

Enhancement of neutrophil activation trigger more robust neutrophil effector function, as evidenced in *in vivo* study using animal model for clinical sepsis, in which pretreatment with recombinant IL-33 prevents mice from developing peritonitis signs (175). Mechanistically, IL-33 efficiently enhances neutrophil recruitment and bacterial clearance through reversing the TLR4-induced inhibition of CXCR2 expression. In addition to phagocytosis, neutrophils are also able to use ROS generation to regulate the inflammatory processes. Neutrophil-targeted ROS enhancement can be achieved by designing chemical compounds that binds to recep-

tors mediating neutrophil ROS generation, such as formyl peptide receptor (FPR) agonists (176), or redirecting the use of insulin-sensitising drugs, such as rotenone and metformin, which induce production of mitochondrial ROS in neutrophils, thereby increasing ROS production in normal neutrophils (176). It has been found in several animal models of human autoimmune and inflammatory diseases that controlled enhancement of neutrophil effector function is beneficial for pathological conditions with insufficient neutrophil function, such as chronic granulomatous disease (177) and RA (178).

1.5.2.3 Neutrophil function inhibition

Excessive neutrophil activation leads to tissue damages in various neutrophil-mediated pathological conditions, including sepsis, vasculitis as well as systemic lupus erythematous (SLE) (9). Therapeutic targeting of neutrophils to ensure balanced neutrophil function should aim to either decrease the number of neutrophils or limit the inflammatory responses of neutrophils infiltrated into affected tissue sites.

1.5.2.3.1 Interfering with neutrophil production

Therapeutic inhibition of neutrophil production has been developed in several inflammatory diseases and their effects are at least partially mediated by suppressing G-CSF expression and function. G-CSF receptor neutralising antibodies, which antagonise the binding of G-CSF and subsequent G-CSF receptor signalling, have been shown to suppress the progression of inflammatory joint disease in mice, without the concomitant decrease in circulating neutrophils (179, 180). Targeting upstream regulator of G-CSF expression, the interleukin (IL)-23/IL-17 axis, has been shown to effective in reducing neutrophil production, as evidenced by the use of anti-IL-17 neutralising antibodies, secukinumab and ixekizumab, for the treatment of inflammatory diseases (181). Additional clinical trials using anti-p40 blocking antibodies, which inhibit the IL-23 and IL-12 receptors, have been shown to be effective in the treatment of Crohn's disease, systemic lupus erythematosus (182). These findings support the role of G-CSF as a therapeutic target to inhibit neutrophil production in different inflammatory diseases.

1.5.2.3.2 Inhibiting neutrophil recruitment

Chemokine-dependent signalling pathways play important roles in neutrophil trafficking and activation. The prominent chemokine receptors expressed by neutrophils is CXCR2, which mediates both neutrophil release from the bone marrow and neutrophil infiltration into the inflammatory sites. This has led to the development of a number of CXCR2 antagonists, such

as SB-656933, which has been found to improve airway inflammation in patients with cystic fibrosis, along with decreased neutrophil inflammatory biomarkers (183). Another CXCR2 inhibitor, MK-7123, has also been shown to to inhibit neutrophil chemotaxis, thusly alleviating airway inflammation in chronic obstructive pulmonary disease (184). Moreover, blockade of CXCR1/CXCR2 using the antagonist SCH527123 diminishes neutrophil hepatic infiltration, along with decreased levels of ROS generation and cytokine production in neutrophils (185). Therefore, therapeutic CXCR2 inhibition seems to be beneficial to provide therapeutic benefits for a number of diseases.

1.5.2.3.3 Interfering with neutrophil activation

Excessive neutrophil activation contributes to tissue damages through diverse inflammatory processes, including but not limited to producing pro-inflammatory molecules, ROS, granule enzymes and forming NETosis. A better understanding on neutrophil-mediated pathogenesis leads to important advances in targeting neutrophil effector functions for neutrophil-mediated inflammatory diseases. Biological compounds such as monoclonal antibodies and recombinant proteins that target TNF-α, IL-6 receptor, IL-1 receptor have been shown to be effective in controlling the development and progression of inflammatory diseases like RA (186). For instance, TNF-α monoclonal antibodies, infliximab and adalimumab, have been reported to inhibit neutrophil *ex vivo* ROS generation, and decrease the infiltration of neutrophils into the inflammatory sites (187). Non-steroid anti-inflammatory drugs (NSAIDS, which prevents prevent the generation of inflammatory molecules of the prostaglandin family from arachidonic acid, have been shown to decrease neutrophil degranulation, ROS generation and also increase neutrophil apoptosis (188). One major mechanism of glucocorticoid-indued anti-inflammatory signals is the suppression of AP-1 and NF-κB, two key TFs in neutrophil activation (188). However, it is important to realise that these anti-inflammatory interventions do not specifically target neutrophil effector function and have considerable side effects.

Upon activation, neutrophils release granule proteins, such as elastase or MPO, which likely contribute to the pathogenesis of various inflammatory diseases. Their inhibition, however, has provided limited clinical benefits. Specifically, although neutrophil elastase has detrimental effects , such as inducing distal organ damage in mice (189), Sivelestat, a neutrophil elastase inhibitor, has been reported to have no real therapeutic benefit in the treatment of patients with acute respiratory diseases (190). Meanwhile, inhibition of neutrophil elastase by AZD9668, another neutrophil inhibitor, has been found to significantly improve lung function in patients

with bronchiectasis (191), therefore providing a potential approach to attenuate neutrophil-induced tissue injury.

NETosis, a process of neutrophils releasing discrete structural contents consisting of DNA and citrullinated histones, has been considered as causative factors for inflammatory diseases, including SLE, RA, and vascular thromboses (9). The enzyme arginine deiminase 4 (PAD4) participates in NET formation by promoting the citrullination of histones of chromatin and is considered to be critical for NETosis and a potential therapeutic target in neutrophil-mediated diseases. Unfortunately, despite the beneficial effects of PAD4 deficiency and PAD4 inhibitors, such as BB-Cl-amidine, no support has been found for the PAD4 inhibition in human patients. Additional approaches to inhibit NETosis are to target other enzymes that mediate NET-mediated pathology, such as DNA degrading enzyme DNase I (192), and Gasdermin D (193). Pharmacological inhibition of these enzymes might have therapeutic potential for neutrophil mediated diseases.

1.5.4 Future perspective on therapeutic targeting of neutrophils

Neutrophils are key immune cells that play a pathogenetic role in promoting the inflammatory process, degradation of extracellular matrix, causing unwanted tissue damage. The modulation of neutrophil differentiation and function can be considered as a potential target for pharmacological intervention for various inflammatory diseases. However, current controversies and uncertainties exist in the development of compounds specifically targeting neutrophils, due to their life-span and transcriptional activity. Additionally, a long-standing gap remains in understanding the molecular mechanism regulating neutrophil phenotype and function. Recent development in understanding novel functions in immunity and inflammation have provided some basis for the transcriptional regulation of neutrophils in different micro-environments. However, the transcriptional factor network underlining neutrophil phenotype and function is yet to be fully explored. An emerging and promising direction is the potential of TFs as key targets for therapeutic modulation of neutrophil phenotype and function.

1.6 Hypothesis of investigation

Preliminary work in the Udalova group has dissected neutrophil gene expression and chromatin accessibility during the transition from BM into the blood and then to sites of inflammation and identified putative TFs controlling neutrophil inflammatory responses. The hypothesis I investigated was that **the identified TFs, namely CEBPβ, Klf6, Rfx2, Runx1, RelB, Irf5, and JunB, will in fact play functional role in neutrophil differentiation and effector functions**. To address this hypothesis, I aimed to conduct the phenotypic and functional characterisation of neutrophils with knockout of target TFs, both *in vitro* and *in vivo*, in the air-pouch model of acute inflammation.

My main goals were:

1. To validate the applicability of HoxB8 neutrophils in studying neutrophil differentiation and function.

2. To generate specific TF knockouts on HoxB8 neutrophils and assess their impact on neutrophil differentiation *in vitro* and *in vivo*.

3. To investigate the functional contribution of TFs that do not alter neutrophil development in neutrophil effector functions and inflammatory response.

4. To examine the role of Zfp263 in neutrophil differentiation and activation.

These four goals formed the Results sections of this thesis.

2.0 Materials and Methods

2.1. Cell culture

2.1.1 Maintenance of cell lines

Cell lines were incubated in a 5% CO2 humidified atmosphere at 37°C. HoxB8 myeloid progenitors are immortalised by endogenous expression of Hoxb8 protein, which enables the arrest of myeloid differentiation resulting in an infinite myeloid progenitor expansion, which makes HoxB8 neutrophils an ideal model to study myeloid cell differentiation and investigate cellular functions of neutrophils under normal or inflammatory conditions. HoxB8 C57BL/6N myeloid progenitors and stem cell factor (SCF)-producing CHO cells were kindly provided by Professor Barbora Walzog (LMU Biomedizinsches Centrum). HoxB8 myeloid progenitors were routinely cultured in RMPI-1640 medium supplemented with 10% FCS, 30 mM β-mercaptoethanol (Life Technologies), 1% supernatant from SCF-producing CHO cells (provided by Hans Häcker, University of Utah, Salt Lake City, Utah, USA), and 1 μM β-estradiol (Sigma-Aldrich). Differentiation was induced by estrogen removal and culture in medium containing 1% SCF supernatant.

HEK293-FT cells were purchased from Invivogen USA and cultured in DMEM (Lonza) supplemented with 10 % v/v Foetal Bovine Serum (FBS; Gibco) and 1 % v/v Penicillin/Streptomycin (P/S; Lonza). For lentivirus production, HEK293-FT cells were cultured in 10 cm tissue culture dishes (Falcon, UK) up to a con-fluency of 80%, and passed for no more than 10 passages. Cells were regularly tested for Mycoplasma contamination. For cell passage, culture media was aspirated from the cells, and gently rinsed with Dulbecco's Phosphate Buffer Saline (PBS; Lonza). Trypsin-EDTA (Life Technologies) was then added to the dish and the cells were incubated for 5 minutes at 37°C to detach the adherent cells. Cells were resuspended with twice the volume of trypsin with complete media to stop the reaction, and centrifuged at 1,500 rpm for 5 minutes. The cell pellet was resuspended in fresh media and seeded onto 10 cm plates at a 1:5 dilution or cells were counted and seeded for experiments as necessary.

2.1.2 Cryopreservation of cell lines

To freeze cells, cells were washed with PBS and resuspended in FBS with 10% v/v dimethylsulfoxide (DMSO; Invitrogen) at a cell density of 20 million cells/ml. The cell suspension was

transferred as 1 ml aliquots into cryovials, and stored in an insulated freezing container at -80°C overnight to ensure slow freezing. The cryovials were then transferred to liquid nitrogen for long-term storage. To defrost cells, vials of frozen cells were thawed quickly by swirling in the 37°C water bath until just defrosted. The contents were transferred into pre-warmed media (10 ml total) and centrifuged at 1,500 rpm for 5 minutes to remove the DMSO. The cell pellet was resuspended in 10 ml media and seeded onto a 10 cm plate. Cells were passed for at least two passages before any experiments were carried out to allow recovery from the de-frozen/thawing stress.

2.1.3 Differentiation of HoxB8 neutrophils

Before differentiation, HoxB8 progenitor cells(25×10^5/per dish) were washed with 1%FBS PBS to remove the effect of β-estradiol and then resuspended with 12ml volume of complete RPMI 1640 medium supplemented with 30 μM β-mercaptoethanol, 4% SCF-containing supernatant and 20 ng/mL G-CSF to induce the differentiation on the day0, with no further addition of G-CSF for the 5-day period. Upon the day of cell collection, 12ml of HoxB8 neutrophils were transferred into a 50 ml tube, and centrifuged at 1,500 rpm for 5 minutes and the pellet resuspended in fresh complete RPMI-1640 culture medium before subsequent phenotypic and functional assessment.

2.2 Reagents and antibodies

2.2.1 Chemical reagents

HoxB8 neutrophils were stimulated with Zymosan A from Saccharomyces cerevisiae (Sigma-Aldrich) at indicated concentrations to induce the production of inflammatory cytokines and chemokines. Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) and ionomycin (Sigma-Aldrich) were made up to a 1 mg/ml stock in endotoxin-free water (Sigma-Aldrich) and used at indicated concentration to induce intracellular ROS generation and formation of neutrophil extracellular traps (NETosis). CRISPR lentiviral transductions was conducted with 8μg/ml polybrene (Sigma-Aldrich) and transduced HoxB8 myeloid progenitor cells were selected in 4 μg/ml puromycin (InvivoGen).

2.2.2 Detection antibodies

The combination of flow cytometry antibodies were used for surface marker staining of HoxB8 neutrophils as well as neutrophils from in vivo tissues to identify the population of pre-neu-

trophils, immature and mature neutrophils. cells were washed and pre-incubated with Fc Block reagent (BD) before surface staining with fluorophore-conjugated anti-mouse antibodies against CD11b (M1/70), cKit (2B8), Ly6C (HK1.4), Ly6G (1A8), CXCR2 (SA044G4), CXCR4 (2B11) and CD101 (Moushi101), together with exclusion ineage markers that include CD3e (145- 2C11), B220 (RA3-6B2), NK.1.1 (PK136), Sca-1 (D7), CD11c (N418) and Siglec-F (E50-2440). After exclusion of cell doublets and dead cells with Fixable Viability Dye eFluor®780 (ThermoFisher), pre-neutrophils were featured as Lineage- CD11b+ $Ly6C^{int}$ CXCR4+ cKit+ CXCR2-, and immature neutrophils were characterised as Lineage- CD11b+ $Ly6C^{int}$ CXCR2+ Ly6G+ CD101-, and mature neutrophils were characterised as Lineage- CD11b Ly6C CXCR4 cKit- CXCR2+$Ly6G^{high}$ $D101^{+}$. For intracellular cytokine staining, surface staining was followed by fixation and permeabilisation to allow for intracellular staining with pro–IL-1β (NJTEN3).

Primary antibodies diluted in PBS, 0.1 % v/v Tween, 2 % w/v BSA were used to detect cellular expression of C/EBPβ, Klf6, Runx1, Rfx2, RelB, Irf5, JunB, Lamin B2 and Mpo, with the supplementary use of accordingly secondary detection antibodies. Antibodies used to stain the cells are summarised in the **Table2.1** below.

Antibodies	SOURCE	IDENTIFIER
Anti-mouse B220 (Clone RA3-6B2)	Thermo Fisher	Cat# 103236; RRID: AB_893354
Anti-mouse CD3e (Clone 145-2C11)	Biolegend	Cat# 45-0031-82; RRID: AB_1107000
Anti-mouse CD11b (Clone M1/70)	Biolegend	Cat# 101245; RRID: AB_2561390
Anti-mouse CD11c (Clone N418)	Thermo Fisher	Cat# 45-0114-82; RRID: AB_925727
Anti-mouse CD19 (Clone 6D5)	Biolegend	Cat# 115534; RRID: AB_2072925
Anti-mouse cKit (Clone 2B8)	BD Biosciences	Cat# 105828; RRID: AB_11204256
Anti-mouse CXCR2 (Clone SA044G4)	Biolegend	Cat# 149304; RRID: AB_2565692
Anti-mouse CXCR4 (Clone 2B11)	Thermo Fisher	Cat# 17-9991-82; RRID: AB_10670878
Anti-mouse CD101 (Clone Moushi101)	Thermo Fisher	Cat# 25-1011-82' RRID: AB_2573378
Anti-mouse CD115 (Clone AFS598)	Thermo Fisher	Cat# 135526; RRID: AB_2566462
Anti-mouse Ly6C (Clone HK1.4)	Biolegend	Cat# 128041; RRID: AB_2565852
Anti-mouse Ly6G (Clone 1A8)	Biolegend	Cat# 127643; RRID: AB_2565971)
Anti-mouse NK1.1 (Clone PK136)	Thermo Fisher	Cat# 45-5941-82; RRID: AB_914361
Anti-mouse Siglec-F (Clone E50-2440)	BD Biosciences	Cat# 565526; RRID: AB_2739281
Anti-mouse TER119 (Clone TER-119)	Biolegend	Cat# 116202; RRID: AB_313703
Anti-mouse Sca-1 (Clone D7)	Biolegend	Cat# 108124; RRID: AB_893615
Anti-mouse pro-IL-1 beta (Clone NJTEN3)	Thermo Fisher	Cat# 25-7114-82; RRID: AB_2573526
Anti-mouse C/EBPe (Clone C-10)	Santa Cruz	Cat# sc-515192 AF647
Rabbit anti-C/EBPβ (Clone E299)	Abcam	Cat# ab32358; RRID: AB_726796

Mouse anti-KLF6 (Clone E-10)	Santa Cruz	Cat# sc-365633; RRID: AB_10841903
Mouse anti-RUNX1 (Clone 1C5B16)	Biolegend	Cat# 659302; RRID: AB_2563194
Rabbit anti-RFX2	Abcam	Cat# ab79241; RRID: AB_2042797
Mouse anti-RelB (Clone D-4)	Santa Cruz	Cat# sc-48366; RRID: AB_628212
Rabbit anti-IRF5	Abcam	Cat# ab21689; RRID: AB_446483
Rabbit anti-JunB/AP-1 (Clone SD081-08)	Novus	Cat# NBP2-67788
Rabbit anti-Lamin B2 (Clone EPR9701(B))	Abcam	Cat# ab151735; RRID: AB_2827514
Mouse anti-MPO (Clone 8F4)	Cell Bioscience	Cat# HM1051BT; RRID: AB_2146342
Rabbit anti-Histone H3 (citrulline R2 + R8 + R17)	Abcam	Cat# ab5103
Mouse anti-Caverolin-1 (Clone 7C8)	Origen	Cat# TA301445 RRID: AB_2072200
IRDye® 800CW Donkey anti-Rabbit IgG	LI-COR Biosciences	Cat# 926-32213 RRID: AB_621848
IRDye® 680RD Donkey anti-Mouse IgG	LI-COR Biosciences	Cat# 926-68072 RRID: AB_10953628
Goat anti-Mouse IgG Secondary Antibody, Alexa Fluor Plus 488	Thermo Fisher	Cat# A32723
Goat anti-Rabbit IgG Secondary Antibody, Alexa Fluor Plus 488	Thermo Fisher	Cat# A21244

Table 2.1 List of antibodies used for Flow Cytometry and Western Blotting examination.

2.2.3 Cell and tissue preparation

Isolation of whole bone marrow cells

C57BL/6 mice on wild-type or defined genotype background were sacrificed by CO2 inhalation and cervical dislocation, following the Schedule 1 procedure. Mice femurs and tibias were dissected and removed by dislocating the long bones at the ankle and pelvis and removing excess muscle tissue surrounding the long bones, followed by incubating the bones in RPMI1640 supplemented with 10% FBS before removing the remaining muscle using tissue scissors (Kimtech).

Cleaned bones were then cut at both ends, from which the bone marrow cells were flushed out into a 50 ml tube (Falcon) with 10 ml of RPMI1640 in a 10 ml syringe with a 27G needle until the bones appeared clear and white. Once both femurs from a single mouse were processed, the whole bone marrow cells were collected in the same tube and then centrifuged at 1,500 rpm for 5 minutes at room temperature. Centrifuged cell pellet was resuspended in 5 ml of bone marrow culture media consisting of RPMI-1640 medium with L-glutamine (Lonza) + 10 % v/v FBS (Gibco) + 1 % v/v P/S (Lonza) supplemented with 0.01 % beta-mercaptoethanol (Gibco). Cell density was counted using 10 µl aliquot of the cell suspension diluted in 10ml PBS by CasyTon and then depending on the cell density, the whole bone marrow cells were diluted to a concentration of 5 x 10^6 cells/ml in BM media for subsequent analysis.

Isolation of blood leukocytes

Blood was harvested by either tail vein bleed or cardiac puncture. Mice were culled by Schedule 1 method in accordance with the project licence. Prior to cardiac puncture a 1 mL syringe was coated with PBS + 2 mM EDTA. Cardiac puncture was performed with a 29G needle attached to the aforementioned 1 mL syringe. Collected blood was placed in 1 mL of sterile 2 mM EDTA/PBS solution to prevent coagulation in a 15 mL centrifuge tube (Greiner). Tubes were topped up with ice-cold PBS + 0.1% BSA. Cells were pelleted by centrifugation at 400 xg for 10 mins at 4 °C and the supernatant discarded. Erythrocytes were then lysed using 10-20x the blood sample volume of ACK lysis buffer (Gibco) for 3 mins. Tubes were then topped up to 15 mL with ice cold PBS + 0.1% BSA to quench the lysis buffer. Cells were washed in PBS/BSA, resuspended in PBS/BSA to the desired cell concentration, and stored at 4 °C until required.

Bone marrow isolation

Whole hind legs of mice were harvested and stored at 4 °C until processing. Processing was carried out in a class II lamina flow hood. Femurs and tibia muscle tissue was removed using scissors, followed by desiccation in 70% ethanol solution for 3 mins to sterilise bones and for ease of cleaning. Remaining muscle was cleaned using a lint-free tissue.

Scissors were used to cut the ends of bones. A 26G needle was inserted into the opening, and marrow was flushed into a 50 mL centrifuge tube using 10 mL of sterile, ice cold PBS. Cells were then filtered through a 70 µm cell strainer (Greiner Bio-One). Red blood cells were lysed as described above, using ACK. Cells were resuspended in PBS/BSA at 4 °C until required.

Splenocyte isolation

Spleens were harvested from mice and stored in RPMI + 2% FCS at 4 °C until required. Spleens were then mashed through a 70 µm and washed through with 10 mL ice cold PBS + 1 mM EDTA. Cells were pelleted by centrifugation for 10 mins at 400 xg at 4 °C and the supernatant was discarded. Erythrocytes were lysed as described above. Cells were resuspended in ice cold PBS/BSA until required.

2.3 Experimental methods

2.3.1 Isolation of blood and air pouch cells

Blood sample were obtained via cardiac puncture and then red blood cells were lysed in ACK lysis buffer (Thermo Fisher) before resuspended in RPMI-1640 medium with L-glutamine (Lonza) + 10 % v/v FBS (Gibco) + 1 % v/v P/S (Lonza) supplemented with 0.01 % beta-mercaptoethanol (Gibco). Air pouch membrane tissue was harvested from mice and digested with 20 mL RPMI + 10% FCS +1% PS + 2.5 U/mL Collagenase VIII (Sigma-Aldrich) + 2 U/mL DNAse I (Roche), and passed through a 70-mm nylon mesh sieve to achieve membrane cell suspension. Air pouch exudate was passed through a 70-mm nylon mesh sieve. Cells were pelleted by centrifugation at 400 rcf for 5minutes before counting.

2.3.2 Cell viability assay

Cell viability assessment was conducted using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega), a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present, an indicator of metabolically active cells. Briefly 5 x 10^4 cells were cultured in poly-lysine (Sigma-Aldrich) coated 96-well plates and incubated 4 hours before use. The kinase inhibitor was added at indicated concentrations and timepoints the following day. Plates were washed once with PBS, and then 75 µl PBS and 75 µl of CellTitre-Glo reagent was added to lyse the cells. After a 3-minute incubation at room temperature on a shaker, samples were transferred to a luminescent-plate (ThermoScientific), and the luminescence was recorded in a luminometer (BMG *FLUOstar*). The luminescence signals were normalised to the luminescence signal obtained from untreated cells.

2.3.3 Transfection of HEK-293FT cells for CRISPR-Cas9 transduction

All CRISPR-Cas9 expressing plasmids and lentiviral packaging plasmids were kindly provided by the Addgene. Specifically, CRISPR-Cas9 expressing plasmid lentiCRISPRv2-blast was a gift from Brett Stringer (Addgene plasmid # 98293; http://n2t.net/addgene:98293; RRID:Addgene_98293). CRISPR-Cas9 inducible expressing plasmid TLCV2 was a gift from Adam Karpf (Addgene plasmid # 87360; http://n2t.net/addgene:87360; RRID:Addgene_87360). Lentiviral vectors and envelope expressing plasmid psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260; http://n2t.net/addgene:12260 ; RRID:Addgene_12260). VSV-G envelope expressing plasmid pMD2.G was a gift from Didier Trono (Addgene plasmid #12259 ; http://n2t.net/addgene:12259; RRID:Addgene_12259). Prior to transfection, bacterial stab cul-

tures of these plasmids were plated on LB-amp plates and incubate at 37°C overnight. A single colony was picked up from the plate and seeded into a flask containing 50 mL LB liquid media with 60 µg/mL ampicillin before plasmid isolation using using QIAprep Mini-prep kit following the manufacturer's protocol.

For plasmid transfection, HEK293-FT cells were seeded into poly-lysine coated 10cm petri dishes (Sigma) at a density of 5 x 105 cells/dish in 12 ml DMEM containing 10%FBS without antibiotics and incubated overnight to allow for cell attachment. In order to transfect HEK293-FT cells for CRISPR-Cas9 transduction, the reagent quantities and protocol below were followed:

1. Prepare the transfection DNA mixture containing 8 µg CRISPR-Cas9 expressing plasmids, 4 µg VSV-G envelope expressing plasmid pMD2.G, and 4 µg lentiviral vectors and envelope expressing plasmid psPAX2.
2. Add 380 µl Optimem media supplemented with 20 µl Lipofectamine 2000 reagent (Invitrogen), and incubate for 30 minutes at room temperature.
3. After incubation, add the DNA-Lipofectamine mixture drop-wise into the HEK-293FT to transfect and Incubate cells at 37°C in a 5% CO2 humidified atmosphere.
4. After 18h-20h of transfection, replace half of HEK-293FT medium with 10%FBS DMEM. From here on, virus collection can be done every 24 hours for four days.

2.3.4 Lentiviral transduction of HoxB8 myeloid progenitor cells

The CRISPR-Cas9 system consists of a guide RNA (gRNA) and a CRISPR-Cas9 endonuclease. The gRNA is a short synthetic RNA composed of a scaffold sequence that binds with CRISPR-Cas9 endonuclease and a fragment of ~20-sequence nucleotide spacer that direct toward desired genomic target to generate a DNA double-strand break (DSB). The DSB is repaired by DNA repair processes such as non-homologous end joining (NHEJ) and the repair introduces mutations to generate a knockout. To generate specific knockouts, HoxB8 myeloid progenitor cells were transduced with lentiviral particles containing an all-in-one lenti-CRISPR-Cas9 vector (TLCV2, Addgene#), which confers the expression of gRNA, CRISPR-Cas9 endonuclease as well as cell selection markers, such as puromycin resistance and eGFP expression, for selecting positive transduced cells.

Lentiviral transfections were carried out on HoxB8 myeloid progenitor cells of low passage number (<10 passage).

For generating the knockout of *C/EBPβ, Klf6, Runx1, Rfx2, RelB, Irf5* or *JunB* CRISPR knockouts, gRNAs were designed using the CHOPCHOP web tool (194) (**table2.2** below). The specificity of designed gRNA was validated with the Benchling web tool, making sure that the targeting sequences were within the protein-encoding region and have no major off-target site. After the ligation of gRNA into lenti-CRISPR-Cas9 vector, CRPSIR-Cas9 lentiviruses were produced from HEK-293FT cells transfected with the lentiCas9-v2 plasmid mixture (as described in **2.3.3 section**). Lentivirus containing supernatants were harvested, filtered and ready for subsequent transduction.

Target Transcript	Gene	Sequence Designed (5'-3')	Exon
ENSMUSG00000056501	CEPBb	AGGCTCACGTAACCGT AGT	exon1
ENSMUSG00000000078	Klf6	TCGCTGTCGGGAAAAC AGGG	exon1
ENSMUSG00000022952	Runx1	TAGCGAGATTCAACGA CCTC	exon5
ENSMUSG00000024206	RelB	CTGCACGGACGGCGT CTGCA	exon4
ENSMUSG00000029771	Irf5	ACCCTGGCGCCATGC CACGAGG	exon2
ENSMUSG00000052837	JunB	GGAACCGCAGACCGT ACCGG	exon1

Table 2.2 List of gRNA designed for generating cells with CRISPR-Cas9 knockout

For transduction, 10,000 HoxB8 myeloid progenitor cells per well were seeded in at a volume of 500µl culture medium in 24-well plates and incubated at 37°C in a 5% CO2 humidified atmosphere for 2 hours, allowing cells to settle down. Post incubation, cells were transduced with supernatants that contain CRPSIR-Cas9 lentiviruses targeting *C/EBPβ, Klf6, Runx1,*

Rfx2, RelB, Irf5 or *JunB*, produced above. Cells were transduced with lentivirus particles in medium containing 8 µg/ml polybrene (Sigma) and undergo spin-infection at 1,500 x g for 90 minutes, using the formulas The plate is incubated at 37°C in a 5% CO2 humidified atmosphere overnight, and then the media was replaced with fresh media. 72 hours post transduction, cells were selected with puromycin (4 µg/ml) every 2-3 days for 2 weeks and then sorted by cell sorting to obtain pure population of GFP-positive cells. After sorting, sorted Cells were expanded further to several 10-cm plates and frozen down (as described in **Section 2.1.2**) for long-term storage or cultured for subsequent phenotypic analysis.

Target gene	Forward primer sequence (5'à3')	Reverse primer sequence (5'à3')
CEPBb	CACCGGCGGGGCTGGCG	CGGGCGTCCCGCCAGCC
Klf6	CTGCTCGGACTCCTGATCGT	AAGCACACACCATCTTGCCT
Runx1	AGCGGCGACCGCAGCATGG	CCATGCTGCGGTCGCCGCT
RelB	CAACAAGCCCATGTCTTCCT	AATCTCCATGAGCAGAGGTGAG
Irf5	GTTTCCTTCTCCCCTAACAGAACTC	GGATAGCCTTGATCCCTAGCACAAA
JunB	ACACCTCTCTTTCTAGTGACACTTC	ACTGGTGATCTCTATGCTGTCAAAGT

Table 2.3 List of primer sequences for PCR and Sanger sequencing.

2.3.5 DNA isolation for Sanger sequencing

To validate the consequence of CRISPR-Cas9-mediated genomic editing, the fragment of target genome was primarily isolated and amplified for validation by Sanger sequencing. Specifically, $1x10^6$ cells WT and KO HoxB8 myeloid progenitor cells were washed with PBS before lysis. Genomic DNA (gDNA) isolation was conducted with the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's protocols. Isolated gDNA from each sample was measured for the concentration and normalised with DNAse-free water into 100ng/ul for subsequent PCR amplification. Target gene of interest were amplified by designing primers approximately 300 bp from the CRISPR PAM sequence. All primer sequences can be found in table below. All primer sequences can be found in **Table 2.3**. PCR amplification products were

purified with QIAquick PCR purification kit (Qiagen) before sent for sequencing (Source Bioscience). SnapGene View software was used to analyse and align the sequencing results.

2.3.6 Protein-level examination validation

Western-Blot

Protein isolation from cultured cells

Western-Blot was used to confirm the deficient expression of proteins of interests. Specifically, cells were harvested from tissue culture plates by gently scraping with a cell lifter and pelleting in 1 ml of PBS in a micro-centrifuge machine by centrifugation at 1,500 rpm. Cell pellets were lysed in cold 1 % Tx-100 lysis buffer (1 % v/v TX-100, 10 % v/v glycerol, 1mM EDTA, 150 mM NaCl, 50 mM Tris pH 7.8) supplemented with protease and phosphatase inhibitors. Lysates were incubated on ice for 30 minutes and cleared by centrifugation at 13,000 rpm for 10 minutes at 4°C to remove cellular debris. Supernatants were transferred into new tubes and stored at -80°C for long term storage.

Protein concentration quantification

Protein quantification was performed with the Qubit assay (Thermo Fisher Scientific) as per the manufacturer's protocol. A working solution was made by diluting the Qubit Protein Reagent 1:200 in Qubit Protein Buffer. 10 µL of each Qubit standard and 1 µL of samples were added to 190 µL and 199 µL of Qubit working solution, respectively, then mixed by vortexing for 2-3 seconds. Tubes were incubated for 15 minutes at room temperature, after which protein concentrations were measured on a Qubit 2.0 Fluorometer.

SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis)

SDS-PAGE allows the separation of proteins according to their molecular weight. 10-20 µg of lysates were heated in 4 x loading buffer at 100°C for 5 minutes. Samples along with a full range rainbow molecular weight marker (RPN800; GE Healthcare) were resolved on a precast NUPAGE 4-12 % Bis-Tris gel (Invitrogen) in NuPAGE® MOPS SDS running buffer (Life technologies) for 1 hour at 160 V or until the blue dye ran to the bottom of the gel.

Membrane transfer

The gel was transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare) by wet western blotting. PVDF membranes were briefly activated in methanol before transfer. A western blot sandwich was made by placing the gel containing resolved proteins on top of two pieces of transfer buffer-soaked filter paper, followed by the PVDF membrane and two pieces of buffer-soaked filter paper. The assembled sandwich was placed between two transfer buffer-soaked sponges in a transfer cassette. Air bubbles were carefully removed by press-

ing the blot sandwich. The cassette was placed in a transfer tank filled with pre-cooled transfer buffer at 72 V for 2 hours, 4°C.

Antibody incubation

Following protein transfer, the membrane was incubated in blocking buffer (PBS, 0.1 % v/v Tween, 5 % w/v milk) for 1 hour at room temperature with gentle shaking. The block was removed and the membrane was incubated with primary antibodies (**Table 1.1**) diluted in PBS, 0.1 % v/v Tween, 2 % w/v BSA overnight with gentle shaking at 4°C. The next day, membranes were washed three times with PBS + 0.1 % Tween (PBS-T) with gentle shaking for 10 minutes between each wash step. Membranes were incubated with HRP-conjugated secondary antibody (**Table 1.1**) diluted in blocking solution for 1 hour at room temperature on a shaker. After three washes as described previous, the membrane was incubated with the chemiluminescent substrate solution ECL (GE Healthcare) according to the manufacturers protocols. Protein bands were visualised on X-ray film (Super RX; FujiFilm), and developed using an AGFA Cruis-60 automatic film processor (AGFA-Gaevert). When multiple protein detections of similar size were required on the same membrane, antibodies were stripped from the membrane using ReBlot Plus Strong Antibody Stripping Solution (Merck) as per the manufacturer's instructions.

2.3.6.2 Measurement of cytokine production

Cell culture supernatant preparation

HoxB8 neutrophils were seeded at 2 x 10^6 cells/2 mL on 6-well plates and left equivalent for 1 hour. After incubation, cells were stimulated with Zymosan at indicated concentration for indicated timepoints. Upon collection, cell supernatant were collected and filtered to remove cell debris and kept as input material.

Protein ID		
CCL11/Eotaxin	CCL6/C10	CXCL11/I-TAC
CCL12/MCP-5	CCL8/MCP-2	CXCL12/SDF-1
CCL2/JE/MCP-1	CCL9/10/MIP-1 gamma	CXCL13/BLC/BCA-1
CCL21/6Ckine	Chemerin	CXCL16
CCL22/MDC	Complement Component C5/C5a	CXCL2/MIP-2
CCL27/CTACK	CX3CL1/Fractalkine	CXCL9/MIG
CCL28	CXCL1/KC	IL-16
CCL3/CCL4	CXCL10/IP-10/CRG-2	LIX
CCL5/RANTES		

Table 2.4 List of cytokines and chemokines in the Mouse Chemokine Array

ELISA detection

For detecting the secretion of cytokines and chemokines and from HoxB8 neutrophils, cytokine concentrations were analysed by ELISA (Mouse CCL2, #DY479-05; Mouse TNF-α, DY410-05), and Mouse Chemokine Antibody Array (ARY020, R&D Biosciences) as per manufacturer's instructions. Mouse Chemokine Array Kit is a membrane-based sandwich immunoassay that simultaneously capture multiple cytokines and chemokines, as listed in the **Table 2.4**. All cytokine detection was performed according to manufacturer's instructions.

For CCL2/TNF-α ELISA, ELISAs 96-well plates (Nunc) were coated with 50 µl capture antibody diluted in PBS (final concentration of 4.00 µg/ml), and incubated overnight at 4°C. The following day, plates were washed three times with Wash buffer (PBS + 0.05 % Tween-20). Remaining wash buffer was removed by inverting the plate and blotting against clean paper towels. Plates were blocked with 100 µl PBS + 1 % BSA for 1 hour at room temperature, before three washes with wash buffer. Standards ranging from 39.1-2500pg/ml were prepared in PBS + 1 % BSA. 50 µl of standards/samples in triplicates were applied to the capture antibody-coated plates and incubated overnight at 4°C.

After three washes with wash buffer, 50 µl detection antibody to a final concentration of 400 ng/ml (in PBS + 1 % BSA) were added to each well, and incubated for 2 hours at room temperature. Following three washes, 50 µl of Streptavidin-HRP in 1 % BSA in PBS were added to the wells. The plates were incubated for 20 minutes at room temperature in the dark, before a final three washes. The HRP substrate TMB (ThermoFisher Scientific) was added at a volume of 50 µl per well. Plates were monitored for a blue colour change, before 50 µl of Stop Solution (1 M H_2S0_4) were added to the wells to terminate the enzymatic reaction. The Stop Solution resulted in a colour change to yellow. Absorbance was read at 450 nm by a SPECTROstar Omega microplate reader (BMG Labtech). For analysis a standard curve was generated by plotting the mean absorbance for each standard against the concentration. The sample concentrations were interpolated from the standard curve.

Mouse cytokine and chemokine array

For the Mouse Chemokine Antibody Array, cell culture supernates were spited down to remove remove particulates by centrifugation and then incubated with pre-labelled membranes for 1 hour before blocking. Following three washes, 15 µL of reconstituted Detection Antibody Cocktail to each prepared sampl, and then incubate for another hour before washing steps at RT. After primary antibody incubation. Following primary antibody incubation, the membrane was incubated in blocking buffer (PBS, 0.1 % v/v Tween, 5 % w/v milk) for 1 hour at room temperature with gentle shaking. The block was removed and the membrane was incubated with primary antibodies diluted in PBS, 0.1 % v/v Tween, 2 % w/v BSA overnight with gentle shaking at 4°C. The next day, membranes were washed three times with PBS + 0.1 % Tween (PBS-T) with gentle shaking for 10 minutes between each wash step. Membranes were incubated with diluted Streptavidin-HRP diluted in blocking solution for 1 hour at room temper-

ature on a shaker. After three washes as described previous, the membrane was incubated with the chemiluminescent substrate solution ECL (GE Healthcare) according to the manufacturers protocols. Protein bands were visualised on X-ray film (Super RX; FujiFilm), and developed using an AGFA Cruis-60 automatic film processor (AGFA-Gaevert). When multiple protein detections of similar size were required on the same membrane, antibodies were stripped from the membrane using ReBlot Plus Strong Antibody Stripping Solution (Merck) as per the manufacturer's instructions.

2.3.7 mRNA-level examination

Reverse-transcript real-time quantitive PCR (RT-qPCR) determination

RT-qPCR is a combination of three steps: (i) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA, (ii) the amplification of the cDNA using the PCR and (iii) the detection and quantification of amplification products in real time. Total RNA from HoxB8 neutrophils, stimulated with Zymosan or vehicle control, were isolated from cells, and transcribed into complementary DNA (cDNA) with MultiScribe Reverse Transcriptase Kit (4311235, Life Technologies Ltd) . The cDNA is then used as template in qPCR reactions to detect and quantitate gene expression.

Total RNA isolation

Total RNA was extracted with the RNeasy Mini kit according to the manufacturer's instructions (Qiagen). RNA was quantified using the Nanodrop 1000 (Thermo Scientific), normalised to 100 ng/µl or the lowest sample concentration for for HoxB8 neutrophils and stored at -80°C.

First-strand cDNA synthesis

The High Capacity cDNA Reverse Transcription kit (Life Technologies) was used to synthesise cDNA from total RNA. The following reaction mix was prepared as T**able 2.5** below:

Volume	Components
2µl	10x RT buffer
0.8µl	10 x RT random primers
4.2µl	RNase-free water
1µl	Reverse Transcriptase
10µl	total

Table 2.5 First-strand cDNA synthesis reaction

Before the reverse transcriptase enzyme was added to the master mix, 9 µl of Master-mix was taken and added to 3 x 3 µl random RNA samples and 2 µl nuclease-free water as a 'no reverse-transcriptase' control to monitor genomic DNA contamination. 10 µl of the complete master mix was added to 10 µl RNA to give a total cDNA reaction volume of 20 µL. Reverse transcription was performed in a thermal cycler using the conditions as described in **table2.6** below:

Temperature	Duration	Procedure
25°C	10min	primer anealing
37°C	120min	DNA polymerization
85°C	5min	enzyme deactivation.

Table 2.6 Steps of reverse transcription for RT-qPCR.

Samples were stored at -20°C until needed for quantitative PCR (qPCR).

qPCR analysis

Taqman qPCR probes

To determine the comparative levels of target gene mRNA expression levels, we designed and ordered the Taqman probes that allow for the sensitive, specific detection and quantification of nucleic acid sequences of target genes, as listed as in the table, qPCR master-mix was prepared as T**able 2.7** below with the addition of diluted cDNA for each qPCR reaction:

Volume	Components
3µl	Fast Blue qPCR Master Mix + dTTP (Eurogentec)
0.3µl	10 x Taqman probe
0.3µl	RNase-free water
2.4µl	diluted cDNA
6µl	total

Table 2.7 TaqMan qPCR reaction system

Samples were run in duplicates on a 384 well plate (Life Technologies) with a 6 µl total reaction volume. The qPCR reaction was performed on a ViiA7 system (Life Technologies), using the following cycling program (**Table2.8**).

Table 2.8 qPCR themocycling program

Temperature	Duration	Procedure
95°C	3min	Denaturation
95°C	3s	Annealing
60°C	5s	Extension
Repeated for 45 cycles		

For determining the comparable levels of target genes (as in **Table 2.9**), we quantified their mRNA levels by measuring the accumulation of a fluorescence signal at the exponential phase. The cycle threshold value (Ct) is the number of cycles at which the fluorescence signal crosses the threshold, as in exceed background levels. Gene expression was analysed using the comparative Ct (ΔΔCt) method where the Ct values of the samples are compared to those of a control. Here, control samples used were WT unstimulated cells. All Ct values were normalised to the endogenous housekeeping gene *Hprt*.

Table 2.9 List of Taqman probes used for qPCR analysis

Gene	Product code
CCL2	Mm00441242_m1
CCL4	Mm00443111_m1
CCL5	Mm01302427_m1
CXCL1	Mm04207460_m1
CXCL3	Mm01701838_m1
HPRT	Mm03024075_m1
IL-1a	Mm00439620_m1
IL-1b	Mm00434228_m1
IL-6	Mm00446190_m1
IL-10	Mm00439614_m1
IL-12b	Mm01288989_m1
IL-23a	Mm00518984_m1
TNF-α	Mm00443258_m1

RNA sequencing analysis

To determine how specific TFs affect neutrophil gene expression, RNA sequencing was used to reveal the presence and quantity of the whole transcriptome of WT or KO HoxB8 neutrophils under the stimulation of Zymosan or vehicle control PBS for indicated duration, analysing the continuously changing cellular transcriptome. $2x10^6$/2ml WT and KO neutrophils were either unstimulated, zymosan treated for 2hours. After treatment, total RNA samples were isolation using the RNeasy Mini kits (Qiagen), including an optional DNaseI digestion with RNase-Free DNase kit (Qiagen) as described above. Library preparation and sequencing was conducted by the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, Oxford. mRNA-seq data were analysed using custom scripts by Dr Tariq Khoyratty (*Udalova* lab). Specifically, RNA libraries were prepared using Nextera XT kits (Illumina) followed by Illumina sequencing was carried out on a Hiseq2000v4 sequencer (Illumina). This work was carried out by the Bowden High-Throughput Genomics group at the Wellcome Trust Centre for Human Genetics (University of Oxford).

Before in depth analysis of sequencing data was conducted, stringent quality control (QC) parameters were applied using FastQC package*. FastQC: a quality control tool for high throughput sequence data.* Available online at:http://www.bioinformatics.babraham.ac.uk/projects/fastqc). FastQC was able to generate per-base read quality (Phred score), per-sequence quality score, per base sequence content (GC, N), and analyse sequence length distribution, sequence duplication, over-representation of sequences, adapter content and Kmer content. Upon analysis, data were mapped to the mm10 genome using hisat2 using a two-pass strategy (195). featureCounts was used to generate read counts used for downstream statistical analysis (196). Cufflinks generated per-gene length-normalised frequency per kilobase million mapped reads (FPKMs) for direct comparison of gene expression levels between samples (197).

Post-mapping QC was performed using the Picard tools developed at the Broad Institute (Broad Institute, http://broadinstitute.github.io/picard). Sequencing data were mapped to the mm10 genome using STAR (*136*) with the options: "--runMode alignReads --outFilterMismatchNmax 2." Uniquely mapped read pairs were counted over annotated genes using featureCounts with the options: "-T 18-s 2-Q 255." Differential expression was then analysed with DESeq2 and genes with fold changes > 2 and false discovery rates (FDRs) < 0.05 were deemed to be differentially expressed. Variance stabilised (VST) counts for all DESeq2 differ-

entially expressed genes were used for dimensionality reduction. For direct comparisons genes with fold changes >2 and FDR< 0.05 were deemed to be differentially expressed. Gene set enrichment analysis was performed using one-sided Fisher's exact tests (as implemented in the 'gsfisher' R package https://github.com/sansomlab/gsfisher/).

2.3.8 Flow cytometry analysis

Flow cytometry was used to assess the surface marker phenotype of neutrophils. For the identification of different neutrophil subsets, cells were washed and pre-incubated with Fc Block (BD) before surface staining with fluorophore- conjugated anti-mouse antibodies against CD11b (M1/70), cKit (2B8), Ly6C (HK1.4), Ly6G (1A8), CXCR2 (SA044G4), CXCR4 (2B11) and CD101 (Moushi101), together with exclusion lineage markers that include CD3e (145-2C11), B220 (RA3-6B2), NK.1.1 (PK136), Sca-1 (D7), CD11c (N418) and Siglec-F (E50-2440). After exclusion of cell doublets and dead cells with Fixable Viability Dye eFluor®780 (ThermoFisher), pre-neutrophils were identified as Lineage- CD11b+ Ly6Cint CXCR4+ cKit+ CXCR2-, and immature neutrophils were identified as Lineage- CD11b+ Ly6Cint CXCR4- cKit- CXCR2+ Ly6G+ CD101-, and mature neutrophils were identified as Lineage- CD11b+ Ly6Cint CXCR4- cKit- CXCR2+ Ly6Ghi CD101+.

Surface marker staining

For surface marker staining, $5x10^5$ - $2x10^6$ cells were plated on either V-bottomed or U-bottomed 96 well plates. The cells were washed twice with 150 µL FACS buffer (PBS + 0.1 % BSA + 1 mM EDTA + 0.01% Sodium Azide) at 400 xg for 3 min 4°C. Cells were then Fc blocked for 10 min with αCD16/CD32 (BD) 1/100 in 20 µL FACS buffer at room temperature (RT) followed by washing once in 150 µL FACS buffer. Fixable Viability Dye eFluor®780 (ThermoFisher) and primary extracellular antibodies (Supplementary Table.2) were added at indicated concentrations for 20 min at 4 °C in 20 µL FACS buffer in the dark. Labelled cells were then washed twice with 150 µL FACS buffer. Cells were then fixed for 30 mins in 50 µL Cytofix (BD), washed twice with 150 µL FACS buffer, and resuspended in 200 µL FACS buffer before acquisition.

Intracellular cytokine staining

For intracellular labelling of cytokines, cell surface markers were firstly labelled as described above, and then for intracellular labelling, cells in each well were fixed in 50 µL Cytofix/Cytoperm (BD), washed twice in 150 µL Perm/Wash buffer (BD) at 600 xg, 4 °C. Intracellular antibodies were incubated in 20 µL Perm/Wash at the optimised concentrations for 30 mins at 4 °C in the dark, after which samples were washed twice in 150 µL Perm/Wash, and once in 150 µL FACS buffer before resuspension in 200 µL FACS buffer for acquisition.

Foxp3 transcription factor staining

For intracellular labelling of transcriptional factors, cell surface markers were first labelled as described above. For nuclear staining, cells were fixed in 50 µL Fix/Perm (eBioscience), following the manufacturer's instructions, washed twice in 150 µL Perm buffer (eBioscience) at 600 xg, 4 °C. Intracellular antibodies were incubated in 20 µL Perm at the indicated concentrations (Table.4) for 20 mins at 4 °C, protected from light. Next, samples were washed twice in 150 µL Perm, and once in 150 µL FACS buffer before resuspension in 200 µL FACS buffer for acquisition.

Flow cytometry acquisition

Following staining, cells were then acquired to determine the expressional levels of target surface markers or proteins. For making fluorescent compensation, CompBeads (BD) were used to prepare single stained controls as per manufacter's instructions to set up fluorophore compensation. Fluorescence minus one (FMO) controls were used to set gates for cell populations. Labelled cells were acquired using either an LSR II (BD), or Fortessa X20 (BD) flow cytometer using FACSDiva (BD). Data were analysed using Flowjo (Treestar, Inc.) software.

Cell sorting

Cells were stained with extracellular cytokines as described above, except no fixation step was performed. Cells were then sorted using a FACSAria™ II (BD) at the Kennedy Institute of Rheumatology FACS facility by Jonathan Webber (University of Oxford). Flow cytometry acquisition were performed with an BD LSR II or Fortessa X20, respectively (BD Biosciences). Sorting of BM, blood, AP membrane and exudate neutrophils were performed using a BD ARIAII (BD) to achieve > 98% purity. Data analysis was performed using FlowJo v10 (Tree Star, Inc., Ashland OR).

2.3.9 Cytospin

To determine the morphology of neutrophils, sorted neutrophils or differentiated HoxB8 neutrophils were prewahsed in DPBS to remove cellular debris and then resuspended in DPBS at a density of 5x105/ml. Then, 100ul of re-suspended cells were spun onto glass slides by centrifugal forces using a Cytospin 3 Cytocentrifuge (Shandon, ThermoScientific) at 400 g for 5 minutes. The slides were then air dried and fixed with methanol for 10 minutes before being stained with ready to use modified Wight-Giemsa stain from Sigma-Aldrich (Cat no: WG16), following the manufacturer's protocol. After staining, cells were obtained from stained slides under bright field using an Olympus BX51 Ostometric fluorescence microscope (Olympus).

2.3.10 Neutrophil functional assays

Boyden chamber migration assay

Differentiated HoxB8 neutrophils were differentiated as described above. Post differentiation, cells were seeded at 20,000 cells/well in 80 uL on, Wells of a 96 well ChemoTX disposable chemotaxis system (Neuro Probe) were filled with 300 µL Boyden Media (RPMI + 0.1% BSA + 25 mM HEPES) with or without chemotaxis stimuli CCL3 at desired concentrations. A 96 well, 8 µm pore size mesh was placed on top of filled wells. 80 µL of enriched monocytes at a concentration of 250 cells/µL in Boyden Media was overlain on each membrane well. Cells were incubated for 90 mins at 37 °C, 5% CO_2 in a humidified HeraCell 150 incubator (ThermoFisher).

After incubation, the media were discarded, and the membrane was rinsed briefly with PBS + 0.1% BSA to remove loosely, and non-adherent monocytes. The membrane was then fixed for 3 hours - overnight in 4% paraformaldehyde/PBS solution, rinsed with PBS, and again incubated for 3 hrs – overnight in 30% sucrose/PBS. The membrane was rinsed in PBS one final time, before air drying. Air-dried membranes were excised from the frame, and experimental groups were mounted on positively charged polysine slides in Glycerol Mounting Medium with DAPI + DABCO (ab188804, Abcam). Slides were coverslipped, and covers were painted around the edges with nail polish to seal. Slides were kept overnight in the dark at room temperature to air dry.

Slides were then imaged using a BX51 fluorescent microscope (Olympus). Four images/well were acquired at 10 x magnification and resolution of 1240 x 1024 pixels. Numbers of cells/field were counted using imageJ (198). For counting, images were converted into 16 bit format. Cells were defined as having a circularity coefficient ≥ 0.8 and between 25 and 400 pixels in diameter. These parameters gave greatest concordance vs counting by eye.

Adoptive transfer of HoxB8 neutrophils

HoxB8 neutrophils were differentiation as described above. For the differential labelling of HoxB8 neutrophils, 2 x 10^7 WT and KO HoxB8 neutrophils were stained with CellTraceTM Far Red (Life Technologies) and CellTraceTM CFSE (Life Technologies), respectively, at a final concentration of 5 µM following the manufacturer's procedure.

Before adoptive transfer, WT and KO cells were mixed in a ratio of 1:1, confirmed by flow cytometry. HoxB8 neutrophils were were transferred intravenously into mice subjected to air pouch model of in vivo inflammation. Four hours post-adoptive transfer, mice were culled and tissues, including bone marrow, blood, air pouch membrane and exudate were harvested for analysis by flow cytometry. Percentage for WT and KO HoxB8 were calculated in total neutrophils isolated from blood, air pouch membrane and exudate.

NETosis

To induce NETosis, HoxB8 neutrophils were seeded into 8 well lab-Tek II chamber slide (VWR international) coated with 2% poly-lysine (Sigma-Aldrich) at a volume of 100 uL at the density of 1x106/mL. Neutrophils were stimulated with 5μM ionomycin and PMA (Sigma- Aldrich) overnight at 37°C in 5% CO2 tissue culture incubator and were subsequently fixed with 4% paraformaldehyde (Sigma-Aldrich) in DPBS for 30 minutes at RT. Afterwards, cells were treated with 1:2000 SYTOX (Invitrogen) in DPBS for 5 minutes, washed three times with DPBS, and incubated with blocking buffer (2%BSA TBST) for 20minutes. Following blocking, primary antibodies: rabbit anti-citrullinated histone 4 (ab5103, Abcam) and mouse anti-mouse MPO (HM1051BT, Hycult) at 1:100 dilution with blocking buffer (2%BSA TBST), were added and incubated for 2 hours at RT or overnight at 4°C. Cells were washed with DPBS before adding secondary antibodies: mouse anti rabbit DyLight 647 conjugated secondary antibody (ThermoFisher Scientific) and rabbit anti mouse IgG secondary antibody conjugated with Alexa fluro-488 (ThermoFisher Scientific) at 1:300 dilution for an 1h incubation at RT in the dark and washed again with DPBS. Images were obtained by an Olympus BX51 Ostometric fluorescence microscope with excitation at 480 nm; emission 525 nm for green fluorescence and excitation at 594 nm; emission at 610 nm for red fluorescence). Neutrophils with a clear formation of fibres stained by citrullinated-histone 3, together with a diffuse nucleus stained by SYTOX and colocalization with MPO, were counted as neutrophils under NETosis. For each condition, at least 200 cells have been counted from different fields and independent replicates.

Estimation of mitochondrial transmembrane potential

Routinely, HoxB8 neutrophils were differentiated in medium 199 containing 100 nM tetramethyl rhodamine methyl ester (TMRM; Invitrogen) for 25 min at 378C. After loading, cells were washed with PBS, and coverslips were mounted in a tem- perature-controlled chamber attached to the stage of an inverted microscope (Axiovert 200 M, Carl Zeiss, Jena, Ger- many)

equipped with a 363, 1.25 NA Plan NeoFluar oil- immersion objective. Experiments were performed at 378C during which cells were maintained in a HEPES-Tris (HT) solution (132 mM NaCl, 4.2 mM KCl, 1 mM MgCl2, 5.5 mM D-glucose, 10 mM HEPES, 1 mM CaCl2, pH 7.4). TMRM was excited at 540 nm with an acquisition/illumination time of 100 ms using a monochromator (Polychrome IV, TILL Photo- nics, Gr€afelfing, Germany), and fluorescence light was directed by a 560DRLP dichroic mirror (Omega Optical, Brattleboro, VT, USA) through a 565ALP emission filter (Omega) onto a CoolSNAP HQ monochrome CCD-camera (Roper Scientific Photometrics, Vianen, The Netherlands). All hardware were controlled using Metafluor 6.0 software (Molecular Devices Corporation, Downingtown, PA, USA).

Mitochondrial membrane potential was monitored with the voltage-sensitive fluorescent indicator tetramethyl rhodamine methyl ester (TMRM; Invitrogen). Experiments were performed at 378C during which cells were maintained in a HEPES-Tris (HT) solution (132 mM NaCl, 4.2 mM KCl, 1 mM MgCl2, 5.5 mM D-glucose, 10 mM HEPES, 1 mM CaCl2, pH 7.4). For TMRM staining, HoxB8 neutrophils (2×10^6 cells/mL) were loaded with 200 nM TMRM for 20 min for 10 min. The TMRM fluorescence was estimated using a BD LSR II flow cytometer.

Metabolic flux analysis

The real-time extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using a XF 96 extracellular flux analyzer (Seahorse Bioscience). For the Seahorse in vitro mitochondrial respiration studies, 2×10^6 HoxB8 neutrophils were washed twice in RPMI 1640 without sodium bicarbonate, 20 mM glucose, 1% FCS, 2mM pyruvate and seeded in corresponding assay medium in an XF plate coated with poly-L-lysine (Sigma-Aldrich). Neutrophils were rested at 37°C for one hour before analysis. Three independent experiments were performed with four independent replicates per group. Mitochondrial function parameters were evaluated by basal respiration, ATP turnover rate, proton leak and maximal and spare respiratory capacity measurements.

The cellular bioenergetic profiles were measured by serial injections of oligomycin (1 μM final concentration, which blocks ATP synthase to assess respiration required for ATP turnover), FCCP (carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone, 1 μM final concentration, a proton ionophore which induces chemical uncoupling and maximal respiration), and rotenone plus antimycin A (1 μM final concentration of each, which completely inhibits electron transport

to measure non-mitochondrial respiration). The data were normalized to the total protein in each well. The OCR and ECAR values were determined from four wells per sample and experiments were replicated.

Intracellular ROS measurement

Intra-cellular ROS was measured by a FACS based method. HoxB8 neutrophils were incubated with 2.5 µg/mL (7 µM) Dihydrorhodamine 123 (DHR123) (ThermoFisher Scientific) in complete RPMI 1640 medium, and stimulated by 50nM Phorbol 12-Myristate 13-Actetate (PMA) (Sigma-Aldrich) for 20 minutes at 37°C. Cells were subsequently washed with PBS and the fluorescence intensities of each subset/cells were measured by flow cytometry. 4°C incubation was used as controls.

Phagocytosis

Phagocytosis capacity of HoxB8 neutrophils was measured by FACS based method using fluorescent E. coli (25922GFP, ATCC). Briefly, HoxB8 neutrophils were incubated with fluorescent E. coli at a multiplicity of infection (MOI) of 10 in complete RPMI 1640 medium for 15 minutes in at 37°C. Neutrophils were subsequently washed with PBS and the fluorescence intensities of each subset/cells were measured by flow cytometry. 4°C incubation was used as controls.

Bacterial killing assay

Bacterial killing assay was performed with S. aureus (NCTC 6571), which was used at a MOI of 10. For the bacterial killing assay, HoxB8 neutrophils were infected for 2 hours with S. aureus and then lysed in 1% triton buffer and the lysate was plated on agar plates. Bacterial culture plates were incubated at 37 °C overnight, and the colony number on each plate was counted the following morning as an absolute CFU count.

3.0 Phenotypic characterisation of HoxB8 neutrophils

3.1 Introduction

Neutrophils are constantly produced by myeloid committed progenitors in the bone marrow, a process that is regulated by the growth factor GCSF. During an inflammatory episode or in response to infection, circulating neutrophils quickly exit blood circulation to accumulate at sites of inflammation in adequate numbers. Genetically modified mice represent the most widely used model system to study neutrophils, because they permit functional *in vivo* characterisation and a certain degree of in vitro analysis of neutrophils with genetic modification of interests. However, distinct obstacles also exist in the use of primary mouse neutrophils for *in vitro* experimentation. In particular, while only limited number of neutrophils are available as circulating mature neutrophils, bone marrow neutrophils are considered as a heterogenous cell population and purification of a specific population of neutrophils easily leads to the differentiation or activation of neutrophils. In addition, as terminally differentiated cells, mature neutrophils are non-proliferative and rapidly undergo apoptosis, with a short lifespan, about 6-12 hours in culture, therefore unsuitable for the long-term assays. Moreover, genetic modification of primary neutrophils is not possible and is obstructed by their short lifespan and low transduction efficiency. Therefore, a reliable and stable platform allowing for analysis of gene function in neutrophils in a physiological context is lacking.

Many *in vitro* myeloid models have been established to investigate the process of neutrophil differentiation and function as an alternative to using primary neutrophils. For instance, human promyelocytic NB-4 cells and myeloblastic HL-60 cells are immortalised leukemic cell lines that possess the potential of differentiation through the metabolism of all-*trans* retinoic acid (199, 200). However, the differentiation induction of these cell lines is followed by bilineage maturation block and progressive signalling defects and fail to share the feature of primary neutrophils (201). Additionally, there are some murine immortalised myeloid cells lines, such as committed myeloid progenitor EML/EPRO cells, and 32D clone 3 (32Dcl3) cells, which possess the potential of fully differentiating into mature neutrophils in presence of growth factors or retinoic acid and morphologically share the features of primary neutrophils (202, 203). However, neutrophils generated from EML/EPRO cells are deficient in expressing Gr-1, which is a featured surface marker for primary neutrophils (202). Furthermore, differentiated

32Dcl3 neutrophils exhibit deficient formation of superoxides and secondary granular proteins (203).

These drawbacks mentioned above can be overcome by the application of HoxB8 murine myeloid progenitors, which is conditionally immortalised with the estrogen-driven expression of homeobox oncoprotein HoxB8 (ER-HoxB8) (204). In the presence of estrogen, HoxB8 murine myeloid progenitors are similar to GMP cells, which retain the capacity to self-renewal by cell dividing. Upon estrogen deprivation, under the control of G-CSF, these HoxB8 progenitors progressively differentiate into mature neutrophils with full range of neutrophil effector functions. Terminally differentiated HoxB8 neutrophils are morphologically indistinguishable from bone marrow mature neutrophils (204). Functional *in vivo* analyses conducted by several different laboratories have demonstrated that HoxB8 myeloid progenitors highly recapitulate neutrophil differentiation and function *in vivo* (205-207). Upon adoptive transfer, HoxB8 neutrophils are efficiently recruited into sites of inflammation, where they execute full range of neutrophil effector mechanisms, such as phagocytosis, cytokine production (205) as well as ROS generation and NETosis (207). HoxB8 neutrophils have been also reported to recapitulate the phenotype and function of human neutrophils with different differentiation phenotype. Similar to pathological immature neutrophil subsets found in patients with GCA and GPA, HoxB8 immature neutrophils respond to stimulation with excessive extracellular ROS generation, possibly due to the lack of antioxidant system, consequently causing irreversible damage to neighbouring cells (208). Importantly, these HoxB8 progenitors are highly amenable to genetic modification by viral transduction, thereby making it possible to generate genetically modified HoxB8 neutrophils (204, 205). Therefore, the functional investigation of genetic modification in HoxB8 neutrophils permits high-throughput screening of neutrophil gene function both *in vivo* and *in vitro*.

The ability of HoxB8 myeloid progenitor cells to differentiate into fully functional neutrophils allows for further investigations into the differentiation, signalling and effector mechanisms of neutrophils in physiologically relevant context. In current section, we will conduct the phenotypic and functional assessment on HoxB8 neutrophils, with the following aims: (1) to determine the phenotypic changes of HoxB8 neutrophils under differentiation, specifically in cell surface maker expression, morphology, cell proliferation and viability, expressions of granular proteins and nuclear envelope proteins, and (2) to determine the functional maturation of

HoxB8 neutrophils under differentiation, specifically in NETosis formation, phagocytosis, ROS generation, and cell migration.

3.2 Results

3.2.1 Surface marker change of HoxB8 neutrophils under differentiation

Before differentiation, HoxB8 myeloid progenitors are routinely cultured in the medium supplemented with estradiol. In the presence of estradiol, HoxB8 expression is induced to maintain HoxB8 myeloid progenitors in the phenotype of GMPs, which is featured with a high expression of the canonical stem-cell marker c-Kit, and negative expressions of myeloid committed surface markers, such as CD11b, CD11C, CD64, Ly6C and Ly6G. Upon estrogen deprivation, HoxB8 myeloid progenitors progressively differentiate into mature neutrophils. Resuspended HoxB8 myeloid progenitor cells are then seeded at a low cell density in the medium supplemented with growth factor G-CSF, which promotes neutrophil lineage commitment. Differentiation of HoxB8 neutrophils is monitored by flow cytometry daily to assess the expression of neutrophil surface differentiation markers (**Figure 3.2.1**). For instance, c-Kit expression is significantly reduced upon day 2 of differentiation and onward (after day 2) becomes completely negative. Starting from day 2, differentiating HoxB8 neutrophils gradually increase the expression of myeloid-specific surface markers, including CD11b, Ly6C, Ly6G, and upon day 5 after initiation of differentiation, HoxB8 neutrophils express maximal levels of CD11b, Ly6C and Ly6G, suggesting the generation of fully mature neutrophils. Additionally, differentiation of HoxB8 neutrophils also recapitulates the expression of the chemokine receptors CXCR4 and CXCR2, which antagonistically regulate neutrophil retention and release in the bone marrow (209). Specifically, HoxB8 neutrophils on day 1 and day 2 of differentiation upregulate CXCR4, which mediate the retention of neutrophils in the BM, while HoxB8 neutrophils upon maturation (day 4 and day 5) highly express CXCR2, which promotes the mobilisation of neutrophils into the circulation. Interestingly, there was a low expression of immature myeloid cell marker CD64 in HoxB8 neutrophils on day 2 of differentiation, supporting the immature phenotype of Hoxb8 neutrophils. To summarise, at the earlier stages of differentiation (day 1 and day 2) Hoxb8 neutrophils highly express immature cell surface markers (c-Kit, CD64 and CXCR4), and during the intermediate stage of differentiation, they gradually downregulate these immature markers and upregulate neutrophil markers, such as CD11b Ly6C, Ly6G and CXCR2, supporting a developmental continuum of neutrophil differentiation.

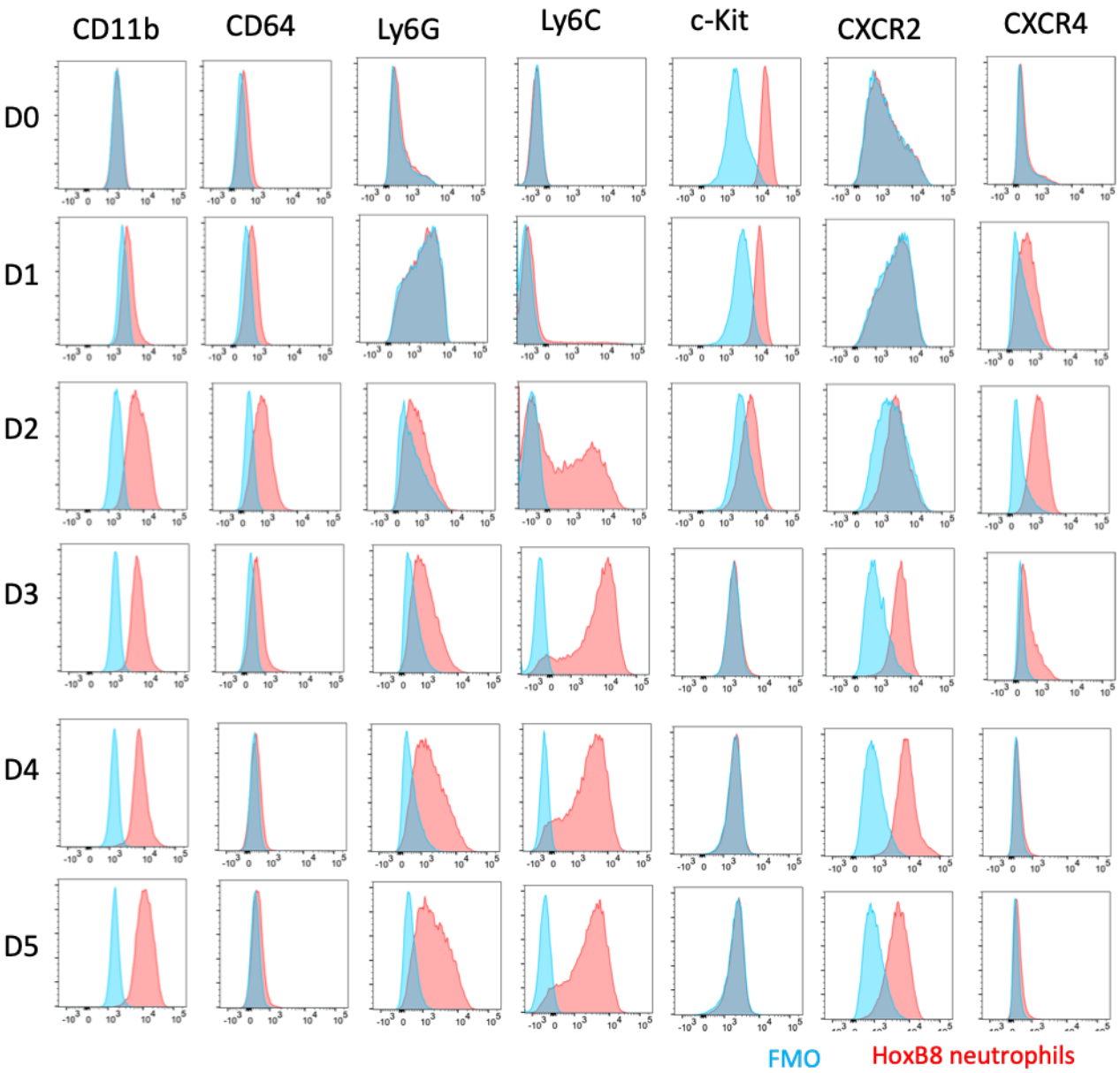


Figure 3.2.1 Surface marker phenotype of HoxB8 neutrophils.
HoxB8 myeloid progenitors were seeded into the medium containing G-CSF to induce differentiation. Following the differentiation, cell surface markers were measured by flow cytometry on day 0-5 of differentiation Representative histograms of cell marker expression with fluorescence intensity on the x-axis and the percentage of expressing cells on the y-axis (% of max) are shown from three independent experiments . D0 (undifferentiated progenitors), D2-5 (day 0-5 of Hoxb8 differentiation).

3.2.2 Morphological change of HoxB8 neutrophils under differentiation

Neutrophils undergo major morphological changes during differentiation, including cytoplasmic granule formation and changes in nuclear morphology. Next, we determined the morphological change of HoxB8 neutrophils between day1 and day5 of G-CSF-induced differentiation and compared to primary neutrophils isolated from the bone marrow (**Figure 3.2.2**). Initially on day1 of differentiation, HoxB8 neutrophils have large, round and fine nucleus, basophilic cytoplasm, and a high ratio of nuclear volume to cytoplasmic volume. By day 2 of differentiation, the nucleus of HoxB8 neutrophils shrink into the bean shape with slightly condensed chromatin, and primary granules are obvious in moderate bluish pink cytoplasm. Subsequent differentiation beyond day 2 is characterised with increasing nucleus segmentation with blue-purple clumped granular chromatin. HoxB8 neutrophils differentiated for 5 days are distinguished by the multi-lobulated nucleus and the transparent cytoplasm, characteristic of segmented mature neutrophils. Morphological quantification of neutrophils with different maturity, based on nuclear size and shape, staining density of chromatin, presence of granules, supported the stage-wise differentiation of HoxB8 neutrophils, which progress through myelocytes, metamyelocytes, and band neutrophil, and eventually differentiate into segmented mature neutrophils. In particular, HoxB8 neutrophils on day 5 of differentiation, which consist of 80.33% ± 6.11% (mean ± SD) segmented mature neutrophils, were morphologically more mature in comparison to bone marrow neutrophils that contain 61.8% ± 4.24% (mean ± SD) segmented mature neutrophils.

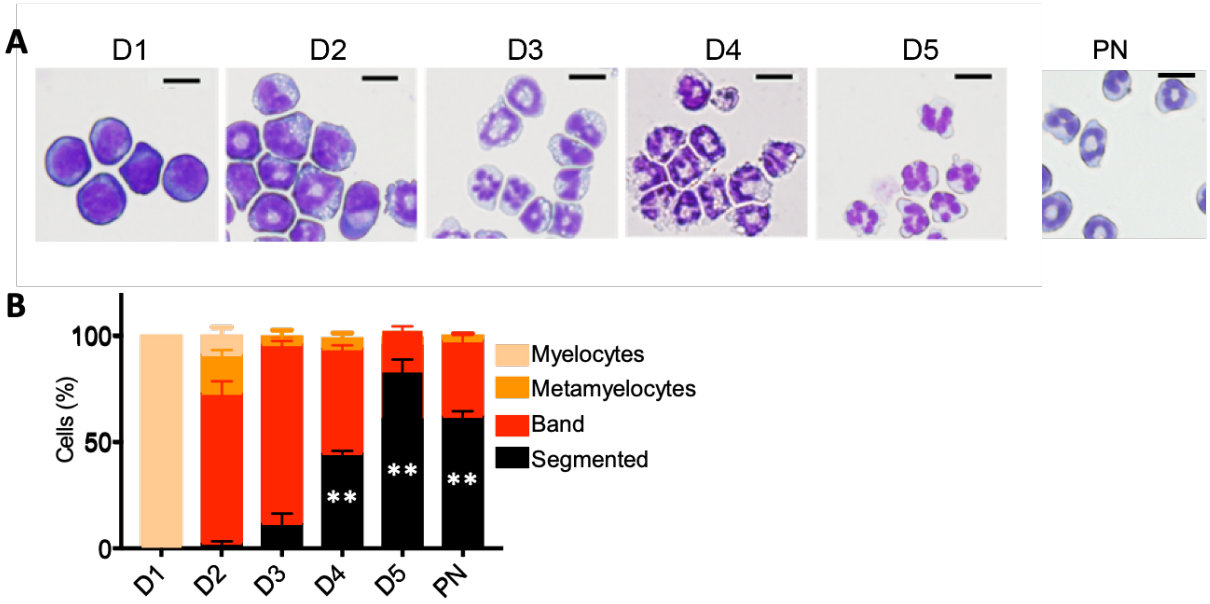


Figure 3.2.2 Morphological change of HoxB8 neutrophils.
HoxB8 myeloid progenitors were seeded into the medium containing G-CSF to induce differentiation. Cytospin was prepared using HoxB8 neutrophils under different differentiation day or primary neutrophils isolated from the bone marrow and then followed by Wright-Giemsa staining. Imaging was made on a brightfieqld light microscope at x 60 magnification. (A) One representative image on each group from three independent experiments is shown. Scale bars indicate 10 μm. (B) Morphological quantification of HoxB8 neutrophil under different days after G-CSF treatment and primary neutrophils to measure the proportion of myelocytes, metamyelocyte, banded neutrophils and segmented neutrophils. The data are expressed as mean ± SD percentages of neutrophils with different maturity, out of at least 200 cells counted from different fields and independent replicates. D0 (undifferentiated progenitors), D2-5 (day 0-5 of Hoxb8 differentiation), PN (primary neutrophils isolated from the bone marrow).

3.2.3 The developmental path of HoxB8 neutrophils

The surface marker expression and morphology analysis of HoxB8 neutrophils under differentiation demonstrates that G-CSF-induced differentiation of HoxB8 myeloid progenitors results in the generation of HoxB8 neutrophils with neutrophil-specific surface markers and morphology. However, the developmental continuum of HoxB8 neutrophils remains poorly defined. To address this question, we utilised the flow-cytometry based approach to determine the phenotypic differences that occur in HoxB8 neutrophils across the different stages of differentiations. Previous study using the multi-parameter analytical techniques has discovered a developmental order from GMPs to pre-neutrophils, immature and mature neutrophils released into the blood, and identified the surface markers that are differentially expressed by neutrophils under different differentiation stages, including but not limited to c-Kit, CXCR4, CXCR2, Ly6G, and CD101 (11). We made use of these surface markers to monitor the differentiation process of HoxB8 neutrophils. Specifically, As shown in **Figure 3.2.3A**, during the early stages of differentiation (day 1 and day 2), HoxB8 neutrophils express c-Kit and CXCR4, two surface markers that are expressed in pre-neutrophils (11). In subsequent differentiation, HoxB8 neutrophils gradually differentiate into mature neutrophils, as evidenced by the loss of c-Kit and CXCR4 expression on day 3 and the increasing expression of neutrophil mature surface markers Ly6C, Ly6G, and CXCR2. HoxB8 neutrophils, upon day 5 of differentiation, express high levels of Ly6G and CXCR2, and highly express CD101, the surface marker that segregates immature neutrophils from mature neutrophils (11). Similar to the bone marrow compartments, day 1 and day 2 HoxB8 neutrophils are characterised to be $CD11b^{+}$ $c\text{-}Kit^{+}$ $CXCR4^{+}$ pre-neutrophils, and day 3 HoxB8 neutrophils are phenotypically identical to $CD11b^{+}$ $Ly6C^{+}$ $Ly6G^{+}$ $CXCR2^{+}$ $CD101^{-}$ immature neutrophils. The increasing expression of CD101 in day 4 HoxB8 neutrophils defines their developmental transition into $CD11b^{+}$ $Ly6C^{+}$ $Ly6G^{+}$ $CXCR2^{+}$ $CD101^{+}$ mature neutrophils. Day 5 HoxB8 neutrophils, which consist of 61.8% ± 4.24% (mean ± SD) mature neutrophils, represent the phenotype of terminally differentiated mature neutrophils. Consistent to the differentiation process of neutrophils in the bone marrow (11), differentiation of Hoxb8 neutrophils follows a developmental path from pre-neutrophils into immature neutrophils and finally mature neutrophils.

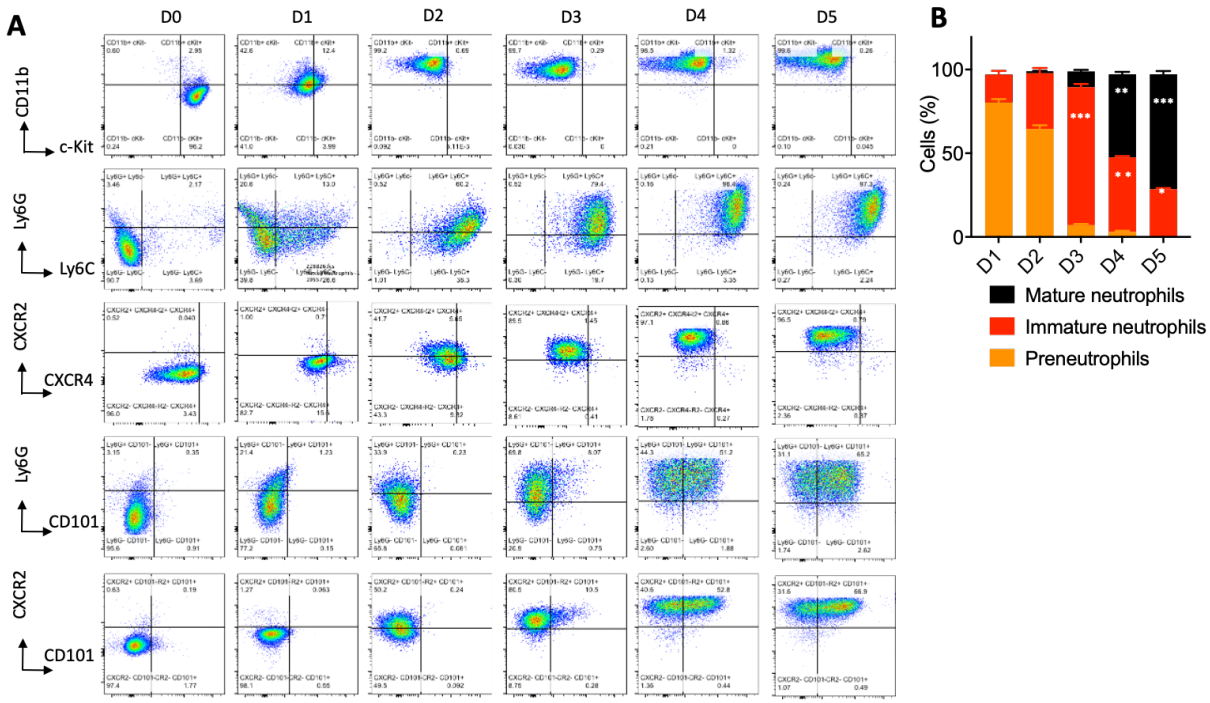


Figure 3.2.3 The developmental path of HoxB8 neutrophils.
HoxB8 myeloid progenitors were seeded into the medium containing G-CSF to induce differentiation. Following the differentiation, neutrophil-specific surface markers, such as c-Kit, CD11b, Ly6C, Ly6G, CXCR2, CXCR4, CD101, were measured in differentiating neutrophils by flow cytometry on day 0-5 of differentiation. (**A**) Representative flow cytometry plots of HoxB8 neutrophils under under different differentiation days after G-CSF treatment. One example in each group from three independent experiments is shown. (**B**) Quantification of CD101+ HoxB8 neutrophils differentiated for 5 days, as shown in (A). Data are shown as means and SD and are representative of three independent experiments. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.

3.2.4 Stage-wise expression of granular protein and envelope protein

So far, our findings demonstrate that HoxB8 myeloid progenitor cells recapitulate the differentiation process of neutrophils. To gain further insight into the cellular properties of HoxB8 neutrophils under differentiation, I subsequently analysed the gene expression of neutrophil granular proteins that relate to different stages of neutrophil differentiation as well as nuclear envelope proteins that involve the process of neutrophil nuclear segmentation, a major feature of increasing neutrophil maturity. As described above, distinct stage of neutrophil differentiation is closely related to specific expression of neutrophil granule enzymes. For instance, myeloperoxidase (Mpo) is primarily expressed by neutrophils under early differentiation stage and becomes downregulated with the progress of differentiation (11). We next investigated Mpo expression pattern in HoxB8 neutrophils under differentiation by Western Blotting. HoxB8 myeloid progenitor cells were differentiated in medium containing G-CSF for 1 to 5 days, and cytoplasmic protein were extracted on the different days of differentiation. In this experiment, Mpo protein levels were normalised to the housekeeping protein β-actin, which is constitutively expressed by HoxB8 neutrophils. As expected, Mpo is more abundant in HoxB8 neutrophils on day 1 and day 2 of differentiation and levels are reduced as differentiation progresses (**Figure 3.2.4 A**). It matches the downregulation of common promyelocytic genes, including *Mpo*, during the maturation of HoxB8 myeloid progenitor cells (204). Consistently, increased expression of Mpo has been also observed in the population of pre-neutrophils from the bone marrow (11). It suggests that differentiating HoxB8 neutrophils maintain all tested characteristics of granular formation during normal neutrophil differentiation.

A hallmark of neutrophil terminal maturation is increasing nuclear segmentation, which is at least partially conferred by increased composition of B-type lamins, particularly Lamin B2 (LB2), contributes to enhancing neutrophil nuclear malleability and increasing overall cellular plasticity (210). However, the functional roles of B-type lamins in neutrophil migration and recruitment remain largely unexplored. Therefore, I determined whether differentiation of HoxB8 neutrophils correlates the expression pattern of B-type lamins by analysing the expression of LB2 in HoxB8 neutrophils with different maturity by Western Blot technique. HoxB8 myeloid progenitor cells were differentiated in medium supplemented with G-CSF, and total cellular proteins were extracted from HoxB8 neutrophils with different days of differentiation. β-actin was used as house keeping control to calculate the protein abundance of LB2 in HoxB8 neutrophils with different maturity. Consistent with the increasing nucleus segmentation observed during maturation of HoxB8 neutrophils (**Figure 3.2.2**), the continuous increase of LB2 ex-

pression was observed with the differentiation of HoxB8 neutrophils. Maximal LB2 expression was detected in HoxB8 neutrophils differentiated for 5 days, terminally differentiated neutrophils (**Figure 3.2.4 A**). It is consistent with the increased composition of LB2 found in myeloblastic HL-60 cells after differentiation (211). Therefore, increasing nuclear segmentation observed in HoxB8 neutrophils under differentiation closely correlates the continuous upregulation of nuclear envelope protein LB2.

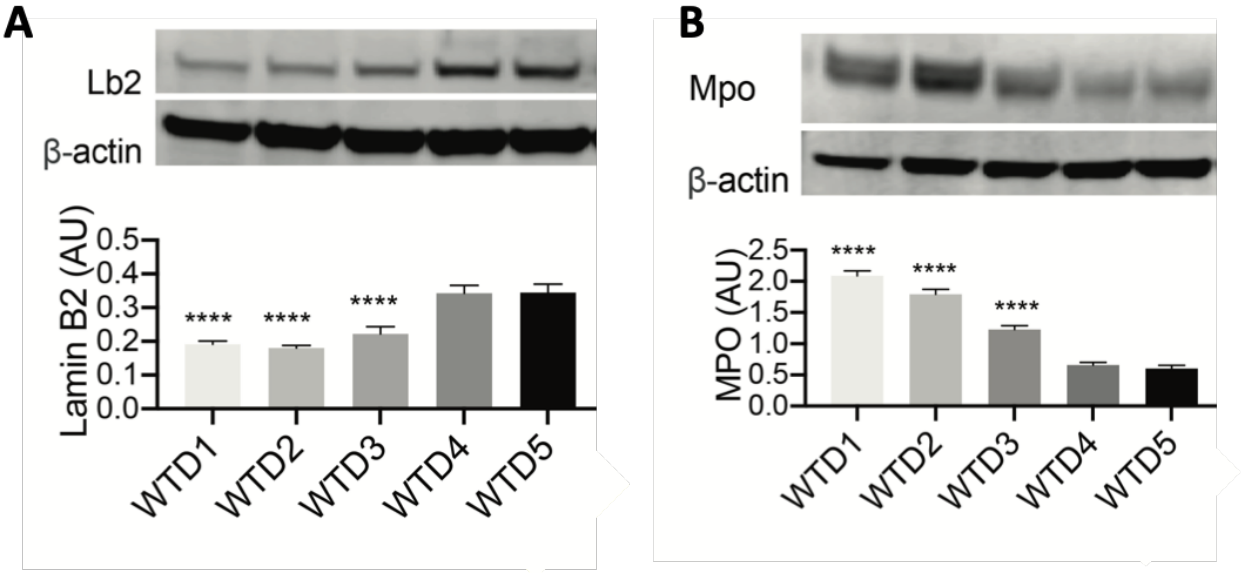


Figure 3.2.4 Mpo and Lb2 expression in HoxB8 neutrophils.
HoxB8 myeloid progenitors were differentiated into the medium containing G-CSF to induce differentiation for different days. Cytoplasmic proteins were extracted to analyse the expression of neutrophil granular protein MPO, and total cellar proteins were prepared to determine the expression of nuclear envelope protein LB2 in HoxB8 neutrophils with different maturity by Western Blotting. Relative expression of Mpo and were analysed in normalisation to β-actin and shown as arbitrary units (AU). Data are shown as means and SD from three independent experiments. Statistical comparison was made between HoxB8 neutrophils with day 1-4 of differentiation and HoxB8 neutrophils with day5 of differentiation. Statistical comparison was analysed by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001. WTD1-5 (day 1-5 of wild-type Hoxb8 differentiation).

3.2.5 Mitochondrial change of differentiating HoxB8 neutrophils

Sequential differentiation of GMPs into myeloblasts, promyelocytes, metamyelocytes, band neutrophils and finally segmented mature neutrophils occurs with active metabolic change to meet their energy demand. For instance, myeloblasts and promyelocytes shift their metabolism from anaerobic glycolysis into mitochondrial-dependent respiration to enhance the autophagy-mediated fatty acid oxidation (212), whereas neutrophils near terminal differentiation decrease their mitochondrial function, as evidenced by the low abundance of mitochondria observed in neutrophils (213). It was further found that neutrophils have restricted function in the initiation of apoptosis and do not contribute to neutrophil energy production (214). However, the mechanisms of how mitochondrial function is modulated during neutrophil maturation are still unclear. Because we observed that HoxB8 myeloid progenitors recapitulate the process of normal neutrophil differentiation, we next determined the mitochondrial changes that occurs across different differentiation stages by measuring the mitochondrial membrane potential in HoxB8 neutrophils with different maturity. HoxB8 myeloid progenitor cells were differentiated in medium supplemented with G-CSF for 1 to 5 days, and the mitochondrial membrane potential of HoxB8 neutrophils was measured by flow cytometry with the use of the fluorescent dye Tetramethylrhodamine, methyl ester (TMRM), which binds to and accumulates in active mitochondria with intact membrane potential. Using this analysis, we found that day 2 and day 3 HoxB8 neutrophils have higher level of mitochondrial membrane potential, suggesting a higher mitochondrial potential during the early stage of differentiation (**Figure 3.2.5**). It is consistent with the more mitochondrial-dependent metabolism in early-stage neutrophils. Additionally, in agreement with the observations that mature neutrophils only have a limited amount of mitochondria (215, 216), HoxB8 neutrophils beyond day 3 of differentiation exhibit a continuous decrease of mitochondrial membrane potential, supporting lower mitochondrial membrane potential in mature neutrophils.

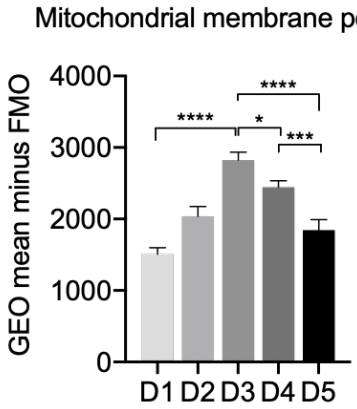


Figure 3.2.5 Mitochondrial membrane potential of HoxB8 neutrophils under differentiation.
HoxB8 myeloid progenitors were differentiated in the medium containing G-CSF to induce differentiation for 1-5 days. Mitochondrial memorable potential was assessed by flow cytometry with the fluorescent dye TMRM, and then analysed by normalising fluorescence signals of stained cells to unstained cells. Data are shown as means and SD from three independent experiments, each with two replicates. Statistical comparison was made between HoxB8 neutrophils with different differentiation days. Statistical comparison was analysed by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001. D0 (undifferentiated progenitors), D2-5 (day 0-5 of Hoxb8 differentiation).

3.2.6 Mitochondrial respiration of differentiating HoxB8 neutrophils

To further characterise the metabolic properties of HoxB8 neutrophils with different maturity, we performed the metabolic flux analysis using a XF 96 extracellular flux analyser, which continuously measure the oxygen consumption rate (OCR) in the cell supernatant over time and allows for direct quantification of mitochondrial respiration. HoxB8 myeloid progenitor cells were differentiated in medium supplemented with G-CSF to induce differentiation for 1-5 days before seeding into 96-well tissue culture plates. Two million cells per well have been optimised to generate the signals beyond the threshold of XF 96 extracellular flux analyser. We evaluated the key parameters of mitochondrial respiration by consecutively exposing cells to sequentially added mitochondrial perturbing reagents oligomycin, FCCP, and finally Antimycin A. As **Figure 3.2.6 A** shown, before the addition of Oligomycin, OCR were measured under basal condition to reflect basal mitochondrial respiration. Then, Oligomycin inhibits ATP synthase to reduces OCR for measuring ATP-linked respiration. Then, FCCP uncouples oxygen consumption from ATP production and allow for maximal mitochondrial respiration. Finally, Antimycin A blocks the electron transport chain and reduces OCR to a minimal mitochondrial activity. Therefore, such an analysis provides readouts on basal mitochondrial respiration, ATP turnover, maximal respiration capacity, and spare mitochondrial capacity of cells.

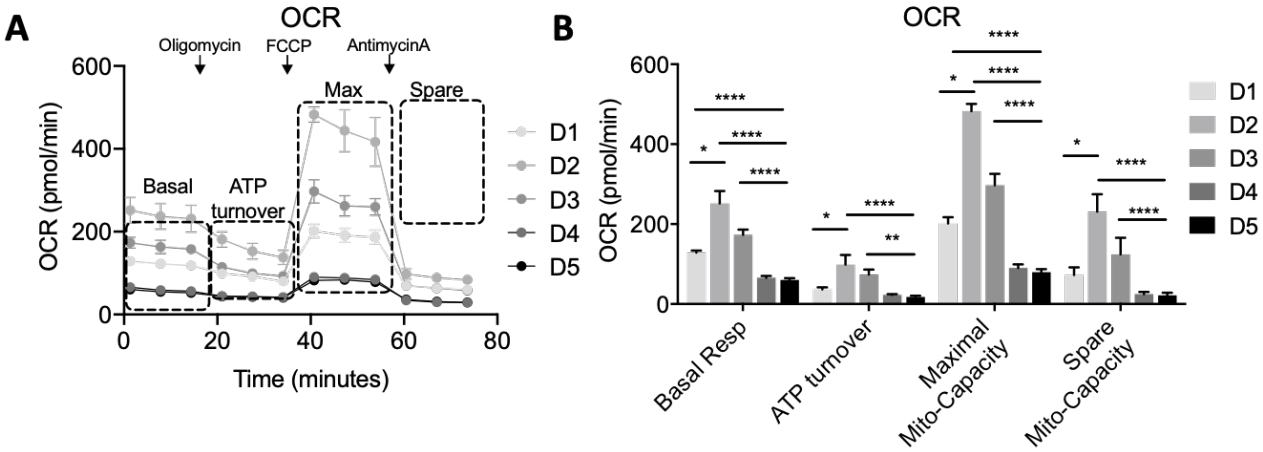


Figure 3.2.6 Mitochondrial respiration by differentiating HoxB8 neutrophils.
HoxB8 myeloid progenitors differentiated for 1-5 days were loaded into XF 96 extracellular flux analyser for the real-time OCR measurement. Sequential exposure of HoxB8 neutrophils to oligomycin, FCCP, and finally Antimycin A allows for generating the readouts on basal mitochondrial respiration, ATP turnover, maximal respiration capacity, and spare mitochondrial capacity of cells. Data are shown as means and SD from three independent experiments, each with two replicates. Statistical comparison was made between HoxB8 neutrophils with different differentiation days. Statistical comparison was analysed by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001. D0 (undifferentiated progenitors), D2-5 (day 0-5 of Hoxb8 differentiation).

With this analysis, we found that day 2 and day 3 HoxB8 neutrophils have maximal levels of basal mitochondrial respiration ATP turnover, maximal respiration capacity, and spare mitochondrial capacity (**Figure 3.2.6 B**), suggesting an active mitochondrial respiration during the early stage of differentiation. This is consistent with the finding that the initial stages of granulopoiesis depends on ATP-dependent mitochondrial activity (217). Additionally, in agreement of the observations that neutrophil maturation is accompanied with the decrease of mitochondrial activity (216, 218), HoxB8 neutrophils differentiated for 5 days have the lowest level of basal mitochondrial respiration ATP turnover, maximal respiration capacity, and spare mitochondrial capacity (**Figure 3.2.6 B**), suggesting an inactive mitochondrial respiration in terminally differentiated mature neutrophils. Overall, these results highlight the more mitochondria-dependent respiration in neutrophils under early stage of differentiation and the decreased level of mitochondrial activities in neutrophils upon terminal differentiation.

3.2.7 Functional analysis of HoxB8 neutrophils under differentiation

Our data suggests a developmental process whereby HoxB8 neutrophils differentiate into mature neutrophils with changes in morphology, surface marker phenotype, composition of neutrophil granules and nuclear envelope proteins, and mitochondrial respiration. To gain further insight into the functional difference that occurs across the different differentiation stages, we next looked into key neutrophil effector functions, for instance, NET formation, phagocytosis, ROS production, and *in vitro* migration. It is known that the developmental process of neutrophils is accompanied with a progressive functional maturation, consequently producing mature neutrophils with the full range of neutrophil effector responses (11). For testing the functional maturity of HoxB8 neutrophils, HoxB8 myeloid progenitors were differentiated in medium supplemented with G-CSF to induce the differentiation for 1-5 days. Subsequently, we determined the capacity of HoxB8 neutrophils differentiated with different maturity to produce NETosis under the stimulation of PMA and ionomycin, to phagocytose fluorescent *E.coli* , and to induce intracellular ROS in response to PMA stimulation. It was found that HoxB8 neutrophils differentiated for 5 days possess the superior capacities to produce NETosis potently, with a higher rate of cells undergoing NETosis (**Figure 3.2.7 A**), and to phagocyte fluorescent *E.coli* (**Figure 3.2.7 B**), and to produce intracellular ROS (**Figure 3.2.7 C**). It is consistent with the recently reported analysis of human and murine neutrophils supporting the acquisition of neutrophil effector functions during differentiation (11, 81). In addition, using Boyden chamber assay, we determined the migration capacity of HoxB8 neutrophils differentiated for different days. Under the chemotactic effect of Ccl3, HoxB8 neutrophils with 5 days of differentiation migrate rapidly, whereas no significant migration was found in day 1 and day 3 HoxB8 neutrophils (**Figure 3.2.7 D**), consistent with the reduced migration capacity observed in neutrophil precursors from the bone marrow (11). Therefore, the differentiation process of HoxB8 neutrophils occurs with the functional maturation that confers enhanced neutrophil functions.

Apart from intracellular ROS generation that activates granule proteases and regulates NETosis, neutrophils are able to produce extracellular traps to attack invading pathogens, as well as detrimentally affect neighbouring other types of cells (9). We then assessed the capacity of HoxB8 differentiated neutrophils with different maturity to produce extracellular ROS. HoxB8 neutrophils with 1-5 days of G-CSF-induced differentiation were treated either with PBS or with 1 μM fMLP and the release of extracellular ROS was measured by the fluorescence-based method using OxyBURST® Green H_2HFF BSA, a sensitive fluorogenic protein conjugate, which allows for a continuous detection of extracellular oxidative species (**Figure 3.2.7**

E). HoxB8 neutrophils with 5 days of differentiation released a higher level of extracellular ROS in response to fMLP stimulation (**Figure 3.2.7 E&F**), consistent with the abundant NADPH oxidase enzyme complex formed during the terminal differentiation stage (11) . Interestingly, in response to fMLP stimulation, day 3 HoxB8 neutrophils are also able to continuously produce ROS into the extracellular space (**Figure 3.2.7 E**), suggesting immature neutrophils as another important player in generating extracellular ROS.

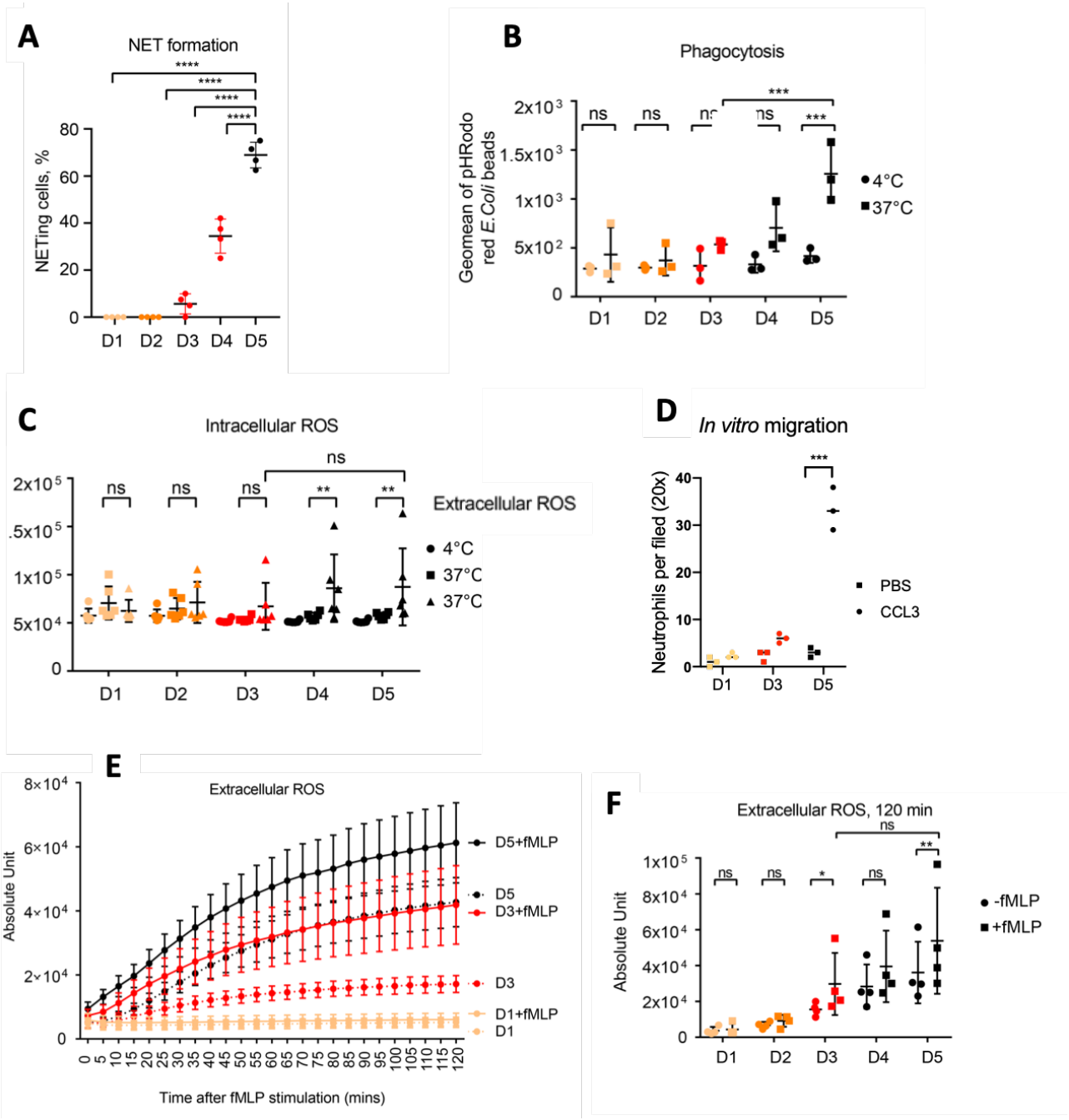


Figure 3.2.7. Functional analysis of HoxB8 neutrophils under differentiation.
(A) NETOsis quantification in differentiating HoxB8 neutrophils. HoxB8 myeloid progenitors differentiated in medium supplemented with G-CSF to induce differentiation for 1-5 days, and are NET formation in response to PMA and ionomycin stimulation were assessed by staining with fluorescently labelled anti-citrullinated-His3 and anti-Mpo antibodies and SYTOX DNA staining. The data are expressed as percentages of neutrophils undergoing NETosis out of at least 200 cells counted from different fields and independent replicates. Data are shown as means and SD derived from three independent experiments. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001. (B) Phagocytosis of fluorescein conjugated *E. coli* by were measured by flow cytometry, and then analysed by normalising fluorescence signals of stained cells to untreated cells. (C) PMA induced intracellular ROS production by HoxB8 neutrophils differentiated for different days were assessed by flow cytometry with dihydrorhodamine 123 (DHR123)., and then analysed by normalising fluorescence signals of stained cells to unstained cells. Data are shown as means and SD from three independent experiments with replicates. (D) Cell migration of HoxB8 neutrophils was measured by Boyden chamber migration assay. Migrated cell numbers were representative of three independent experiments, each assessed by average cell number within different fields and independent replicates. Data are shown as means and SD derived from three independent

experiments. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001. (E) Extracellular ROS release by Hoxb8 neutrophils were measured over a period of 120 minutes. Five-minute intervals were used to plot each time point. (F) Extracellular ROS production at 120 minutes was compared across different stages of differentiation. Data are shown as means and SD from three independent experiments with replicates. (B&C&F) Statistical comparison was made between HoxB8 neutrophils with different differentiation days. Statistical comparison was analysed by two-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001. D0 (undifferentiated progenitors), D2-5 (day 0-5 of Hoxb8 differentiation).

3.2.8 Transcriptional profiling of HoxB8 neutrophils under differentiation

Although less efficient than mature neutrophils, immature neutrophils have been shown to produce ROS in the context of cancers (219) as well as inflammatory diseases (220). Subsequently, to explore the molecular mechanisms underlining early-onset extracellular ROS production in immature neutrophils, we compared the transcriptome of HoxB8 neutrophils differentiated for 1-5 days by bulk RNA-sequencing (RNA-seq), with the assistance by Tariq Khoyratty from the Udalova lab. Principle component analysis (PCA) of differentially expressed genes (adjusted p < 0.01) generated a map with clearly separated clusters of different differentiation stages (**Figure 3.2.8 A**), supporting the distinct transcriptional signature in HoxB8 neutrophils differentiated under different stages. As expected, clearly-separated PCA clusters of differentiating HoxB8 neutrophils support consecutive stages of HoxB8 neutrophil differentiation. Gene ontology (GO) analysis for 11,412 differentially expressed genes (adjusted P < 0.01) across the different differentiation stages revealed that they are predominantly enriched in a number of key neutrophil processes (**Figure 3.2.8 B**). For instance, genes upregulated during early differentiation stage are more relevant to DNA replication and biogenesis while genes upregulated during neutrophil terminal differentiation are more enriched in phagocytosis, cytokine production, and regulation of inflammatory responses, consistent with the enhanced capacity of HoxB8 neutrophils with 5 days of differentiation. Of note, GO terms that relate to biosynthesis of oxidative organelle peroxisome are highly enriched in day1 and day3 HoxB8 neutrophils (**Figure 3.2.8 B**). In phagocytes, including neutrophils, ROS production can be induced by activation of NADPH oxidase complex 2 (NOX2), which is a membrane-bound enzyme complex that faces the extracellular space (221). Genes that encode the catalytic components of *NOX2*, such as cytochrome b α (*Cyba*) and β (*Cybb*) chains, are highly upregulated in in the transition of day 1 and day 3 HoxB8 neutrophils (**Figure 3.2.8 C**), consistent with the high abundance of NADPH oxidase components in immature neutrophils from the bone marrow (11, 218). Activated ROS generation is tightly regulated by antioxidant system enzymes, including but not limited to superoxide dismutase (Sod) and peroxiredoxin (Prdx), which convert ROS into water to limit dams to the host (221). However, in contrast to

biosynthesis of oxidative organelle peroxisome, antioxidant system enzymes, such as *Sod2*, *Prdx1*, *Hmox1*, and *Txnrd1*, are not highly expressed before the stage of day 5 of differentiation (**Figure 3.2.8 D**), in agreement with the previously reported observation that s consistent with antioxidant system enzymes only upregulate in the stage of neutrophil terminal differentiation (218). Consistently, lower expressions of antioxidant system enzymes, including *SOD2*, *HMOX2*, *SRXN1*, and *TXNRD1*, were observed in immature neutrophils observed in patients with chronic inflammatory diseases, such as SLE (222) and GCA (208).

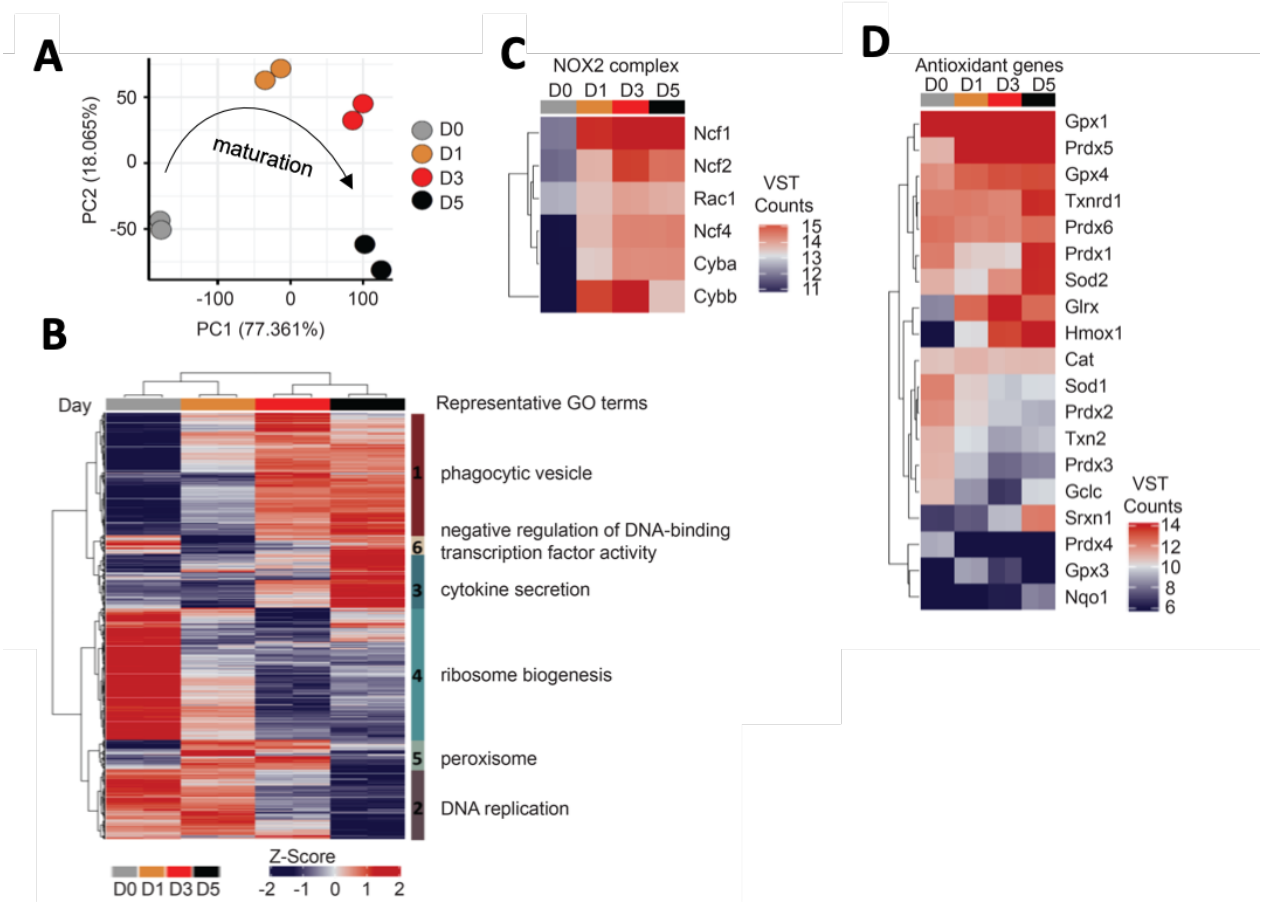


Figure 3.2.8 Transcriptional profiling of HoxB8 neutrophils under differentiation.
(A) HoxB8 neutrophils were differentiated in medium supplemented with G-CSF to induced the differentiation for 1-5 days, and total RNA were extracted for bulk RNA-seq. PCA analysis of of 11,412 differentially expressed (adjusted $p < 0.01$) are shown across different stages of differentiation. (H) Z-scores of differentially expressed genes (as in G). Hierarchical clustering of Euclidean distances reveals 6 distinct clusters, which differ in their pattern of expression across differentiation. Differentially expressed genes from each cluster were used for gene ontology (GO) enrichment analysis. Representative GO terms are shown for each cluster. (C and D) Heatmaps of variance stabilised counts (VST) for differentially expressed genes comprising the NADPH oxidase 2 (NOX2) complex (C) and selected antioxidant genes (D). D0 (undifferentiated progenitors), D2-5 (day 0-5 of Hoxb8 differentiation).

3.3 Conclusion

Quantitative and qualitative neutrophil defects increase the risk of invasive infection, while excessive neutrophil function leads to tissue damage during inflammation. Better understanding the molecular mechanisms underlining the differentiation and activation processes provides novel therapeutic strategies to specifically target neutrophils engaged in the pathogenesis of several inflammatory diseases like arthritis and vasculitis. In the current study, we have investigated the suitability of using HoxB8 myeloid progenitors, an *in vitro* platform of generating murine neutrophils, for the analysis of neutrophil differentiation and function. HoxB8 myeloid progenitors can be differentiated within 5 days into mature neutrophils *in vitro*, which are morphologically and phenotypically comparable from isolated mouse primary neutrophils. HoxB8 neutrophils faithfully recapitulate normal neutrophil maturation, including changes in granular formation, nuclear segmentation and mitochondrial respiration. Functional analysis of HoxB8 neutrophils allows for us to evaluate the efficiency of ROS generation, NETosis, phagocytosis in neutrophils.

To validate the suitability of HoxB8 myeloid progenitors, a direct comparison of cell morphology and surface marker phenotype was made between *in vitro* differentiated HoxB8 neutrophils with primary neutrophils isolated from bone marrow. In our analysis, HoxB8 neutrophils were indistinguishable to primary neutrophils from the bone marrow, in terms of cell morphology, nuclear segmentation. The use of surface markers that segregate pre-neutrophils, immature and mature neutrophils is important to distinguish the developmental process of neutrophils (11). By employing these surface markers, we demonstrated that HoxB8 myeloid progenitors progressively differentiate into pre-neutrophils after 1 day, and then immature neutrophils after 3 days, and they eventually maturate into matter neutrophils after 5 days of differentiation, a process that replicate normal neutrophil differentiation process. The development of HoxB8 neutrophils was further validated with the gradual downregulation of neutrophil primary enzyme Mpo, and the increasing composition of B-type lamin LB2, which corroborate the progressive maturation into mature neutrophils. These findings support that HoxB8 myeloid progenitors may present a physiologically relevant platform for studying neutrophil differentiation.

Cellular metabolism plays a crucial role in the differentiation and function of various cell types, including neutrophils. The differentiation process of neutrophils requires a metabolic shift toward mitochondrial oxidative phosphorylation, which is powered by autophagy to provide suf-

ficient energy supply (212). Measurement of mitochondrial function in HoxB8 neutrophils under differentiation supported enhanced mitochondrial activities during the early stage of neutrophil differentiation, as evidenced by the significantly higher levels of mitochondrial membrane potential and mitochondrial oxidative phosphorylation in HoxB8 neutrophils differentiated for 2 and 3 days, consistent with the essential role of mitochondrial function in the initial stage of neutrophil differentiation. With the progress of neutrophil maturation, mitochondria no longer contribute to energy production, but still control neutrophil survival and programmed cell death (218). By assessing the changes of mitochondrial membrane potential and mitochondrial oxidative phosphorylation, we confirmed the lower level of mitochondrial activities in HoxB8 neutrophils with terminal differentiation, in agreement with the low abundance of mitochondria found in circulating mature neutrophils (218).

Granulopoiesis ends up with a consequence of producing a large amount of functional mature neutrophils. Differentiation of HoxB8 neutrophils is accompanied with functional maturation in neutrophil effector mechanisms, as supported by the superior capacities of HoxB8 neutrophils differentiated for 5 days to produce NETosis, phagocytosis, intracellular ROS generation and chemotaxis, consistent with the full range of neutrophil effector functions observed in mature neutrophils from bone marrow (11). It matched the upregulation of key genes that involve phagocytosis, ROS production and chemotaxis during maturation of HoxB8 neutrophils. Interestingly, using HoxB8 neutrophils, we also demonstrated an early-onset ROS production in immature neutrophils, as evidenced by the increased release of extracellular ROS by HoxB8 neutrophils differentiated for 3 days. Previously, immature neutrophils have been shown to be linked with the progression of cancer diseases. Additionally, augmented release of immature neutrophils correlates with the pathogenesis of inflammatory diseases, such RA (222). However, the molecular mechanisms underlining it remain largely unexplored. Using HoxB8 neutrophils, we screened for differentially expressed genes across the different differentiation stages and identified a list of genes, such as *Sod2*, *Prdx1*, *Hmox1*, and *Txnrd1*, which encode the antioxidant system enzymes and are significantly upregulated in neutrophil terminal differentiation stage. It suggests an underdeveloped antioxidant system in immature neutrophils and could at least partially explain the excessive ROS production by immature neutrophils observed in various diseases.

Through the current section, we have demonstrated the usefulness of HoxB8 myeloid progenitor cells as an *in vitro* platform of studying neutrophil differentiation and function, and it could

be useful for future experiments investigating the role of TFs in neutrophils. We have confirmed that HoxB8 neutrophils phenotypically recapitulate normal neutrophil differentiation, as evidenced by distinct morphological change, differential expression of surface markers, granule enzymes and nuclear envelope composition as well as mitochondrial reprogramming in different differentiation stages. Importantly, segregating neutrophils with these markers will allow us to distinguish the differentiation stage of neutrophils with different maturity, such that neutrophils with terminal differentiation could be defined as having multi-lobulated nucleus, high expressional levels of Ly6G and CD101, and decreased mitochondrial abundance and activities. Our functional analysis of HoxB8 neutrophils revealed the functional maturation in neutrophil effector functions, including NETosis, phagocytosis, and ROS generation. Further phenotypic and functional assessment of HoxB8 neutrophils with specific TF knockout will be necessary for determining how specific TFs affect neutrophil differentiation and function. Such studies are described in subsequent sections of this thesis.

4.0 TFs that mediate neutrophil differentiation

4.1 Introduction

Neutrophils are produced in large numbers in the bone marrow (BM) through stepwise differentiation of myeloid progenitors, featuring distinct morphological changes and stage-specific expression pattern of surface markers, granular enzymes. When released into the circulation, neutrophils patrol between blood and tissue sites for immune surveillance. Increasing evidence have been supporting the notion that neutrophils are transcriptionally active cells. They display distinct gene expression pattern and exhibit phenotypic and functional heterogeneity in different neutrophil developmental stage, activation state, and micro-environment. For instance, recent single cell RNA-seq (scRNA-seq) of murine neutrophils revealed a heterogeneous and complex neutrophil population proposed neutrophil-specific networks, including previously reported TFs and new regulons that are closely related to the functional transition between the steady-state and bacterial infection conditions (15). Additionally, multi-parameter analysis of murine neutrophils in different tissues revealed a tissue-specific neutrophil programming, in which differential expression of TFs is impinged in the functional adaption of neutrophils within tissues (13). However, the transcription factor network underlining neutrophil heterogeneity remains still largely unexplored.

TF networks are consisted of several TFs that share joint features, such as lineage-specific commitment via mutual regulation of transcriptional effects by antagonism and the lineage-commitment function through selectively activating lineage-favourable genes and silencing lineage-hostile genes. For instance, TFs Cebpa and Gata1, which participate in stem cell maintenance and early linage commitment (223), are highly expressed in GMPs and are gradually downregulated during maturation of neutrophils (11). Terminal differentiation TFs, such as C/EBPe and Gfi1 that promote secondary and tertiary enzymes and stop cell proliferation (70, 80), are highly expressed in pre-neutrophils, the intermediate stage of differentiation (11). The selective expression of lineage-specific TFs in distinct differentiation stage corroborates the notion that neutrophil differentiation process is tightly regulated by a complex TF network. However, there is only partial understanding on the TFs that promote neutrophil differentiation. In comparison, TFs involvement in neutrophil function during inflammation is supported with only very limited evidence, which majorly focus on the interaction between STAT proteins and NF-kB isoforms (224). It has been recently reported that augmented functionality of neut-

rophils during inflammation is powered by the upregulation of the TF networks that regulate immune effector processes and ROS metabolism, such as *Irf7*, a crucial TF for type 1 interferon production (225), and the downregulation of the TF networks that promote biosynthesis and transcriptional regulation, such as *Foxp1* and *Ctcf*, suggesting an active transcriptional reprogramming of neutrophils during inflammation (15). However, the molecular mechanisms by which TFs mediate neutrophil inflammatory responses requires more work to be fully elucidated, and the functional relevance of specific TFs in neutrophil function remain to be established.

To reveal the TF networks that regulate neutrophil differentiation and function, we have conducted integrated transcriptional and chromatin accessibility analysis of neutrophils *en route* to sites of inflammation Specifically, we use the air pouch model of zymosan-induced acute inflammation to provide a robust and reliable method to replicate the process of neutrophil release from bone marrow into the blood and subsequent recruitment into air pouch inflammatory sites (226). Transcriptional profiling of neutrophils transiting from bone marrow into the blood and then into the inflammatory sites demonstrated the upregulation of genes that relate to ROS generation, cytokine production and chemotaxis and the downregulation of genes that associate with mitochondrial oxidative phosphorylation (57). It matches the acquisition of core neutrophil effector functions and the decrease of metabolic and biosynthesis potential during neutrophil differentiation and extravasation (218). To fully characterise chromatin accessible regions, we looked for enriched TF binding sites, which were then integrated with the differentially expressed genes to highlight CEBPβ, Klf6, Runx1, RelB, Irf5 and JunB, which potentially mediate myeloid development and play a central role in control of neutrophil inflammatory responses. For instance, Runx1 belongs to the Runt-related transcription factor (Runx) family and participate actively in various hematopoietic processes (227). Consistent in our analysis, Runx1 is highly expressed and accessible in differentiating neutrophils from bone marrow. JunB is a TF of the activator protein 1 (AP-1) family with a key role in mediating the inflammatory activation (87). It was found in our analysis that JunB is highly activated in neutrophils transiting from the blood to the inflammatory sites. RelB is a TF of the NF-κB family that prime the inflammatory functions (228). In our analysis, RelB upregulation was observed to be linked with increased chromatin opening in both circulating and activated neutrophils. However, little is known about the functional contribution of these TFs in modulating neutrophil phenotype and function during inflammation.

Genetic modification of HoxB8 has been described for analysis of gene function in neutrophil effector functions. Firstly, HoxB8 myeloid progenitors cells are amenable to genetic modification with lentiviral transduction, therefore enabling the generation of terminally differentiated neutrophils with target knockout of gene of interests. One interesting sample is the genetic modification of HoxB8 myeloid progenitor cells to allow for alternative differentiation into osteoclasts (229). Secondly, once differentiated, HoxB8 neutrophils exhibit characteristic morphologic and functional features of primary mature neutrophils. Indeed, HoxB8 neutrophils have been reported to resemble primary neutrophils in many effector functions, such as phagocytosis, ROS generation and chemotaxis, while in absence of integrin alpha expression, HoxB8 neutrophils exhibited a marked impairment in their ability to recognise zymosan *in vivo* (205). Therefore, in combination with CRISPR-Cas9-mediated genetic modification, HoxB8 myeloid progenitor cells represent a physiologically relevant platform for investigating the process of neutrophil differentiation and inflammatory responses.

4.2 Results

4.2.1 CRISPR/CAS9 knockouts of specific TF in HoxB8 myeloid progenitors

Due to the fact that HoxB8 myeloid progenitors faithfully recapitulate neutrophil differentiation and inflammatory responses, using HoxB8 myeloid progenitors, we have used a loss-of-function approach to investigate the functional role of putative TFs shortlisted from *in vivo* transcriptional and chromatin profiling. Stable cell lines were generated in which endogenous expression of specific TFs had been ablated. To do this, genetic modification of HoxB8 myeloid progenitor cells was performed with the CRISPR/Cas9 genome editing system. Candidate TFs were shortlisted by checking the TF expression pattern across major immune cell populations from the ImmGen datasets (230), in search of neutrophil-specific TFs that are also highly expressed in neutrophils. For instance, Klf6 was identified as a TF that is maximally expressed in neutrophils while Runx1 is the only TF out of the RUNX family expressed in neutrophils. This led us to identify TF C/EBPb, JunB, RelB, Irf5, Rfx2, Klf6, and Runx1 for further functional validation in HoxB8 neutrophils.

Genetic modification of HoxB8 myeloid progenitor cells was conducted through a lentivirus-mediated transfection system Lenti-CRISPR-Cas9-TLCV2 (Addgene Plasmid 87360), which allows for constitutive sgRNA expression and doxycycline-inducible Cas9 nuclease to generate double stranded DNA breaks. The Lenti-CRISPR-Cas9-TLCV2 construct contains puromycin resistance and green fluorescent protein (GFP) that serves as two selection markers for enriching cells with positive transduction (**Figure 4.2.1A**). Following puromycin selection and cell sorting enrichment, the knockout efficiency of target TF was measured at the protein level by Western Blotting. As shown in **Figures 4.2.1 B**, the lentiviral CRISPR knockout in HoxB8 myeloid progenitor cells infected with gRNAs targeting the exon region of genes *CEPBb*, *Klf6*, *Runx1*, *Rfx2*, *RelB*, *Irf5* and *JunB* successfully generated cells with deficient expression of these TFs.

To eliminate any false positives of target knockout, HoxB8 myeloid progenitor cells with specific TF knockout were validated by Sanger sequencing. Primer pairs were designed to sequence roughly 200 basepairs (bps) around the protospacer adjacent motif (PAM) sequence of the gRNA. Sequencing results were aligned between wild type HoxB8 myeloid progenitor cells and cells with specific TF knockout (**Figure 4.2.1C**). Consistently, all the knockout lines displayed deletions at various length preceding the PAM sequence, which could lead to result

in amino acid deletions, insertions, or frameshift mutations that lead to premature stop codons within the open reading frame of target genes, eventually causing a loss-of-function mutation within the targeted gene. Overall, these results demonstrate that genetic modification with the lentivirus-mediated CRISPR-Cas9 system successfully generated knockouts of CEPBb, Klf6, Runx1, Rfx2, RelB, Irf5 and JunB in HoxB8 myeloid progenitor cells.

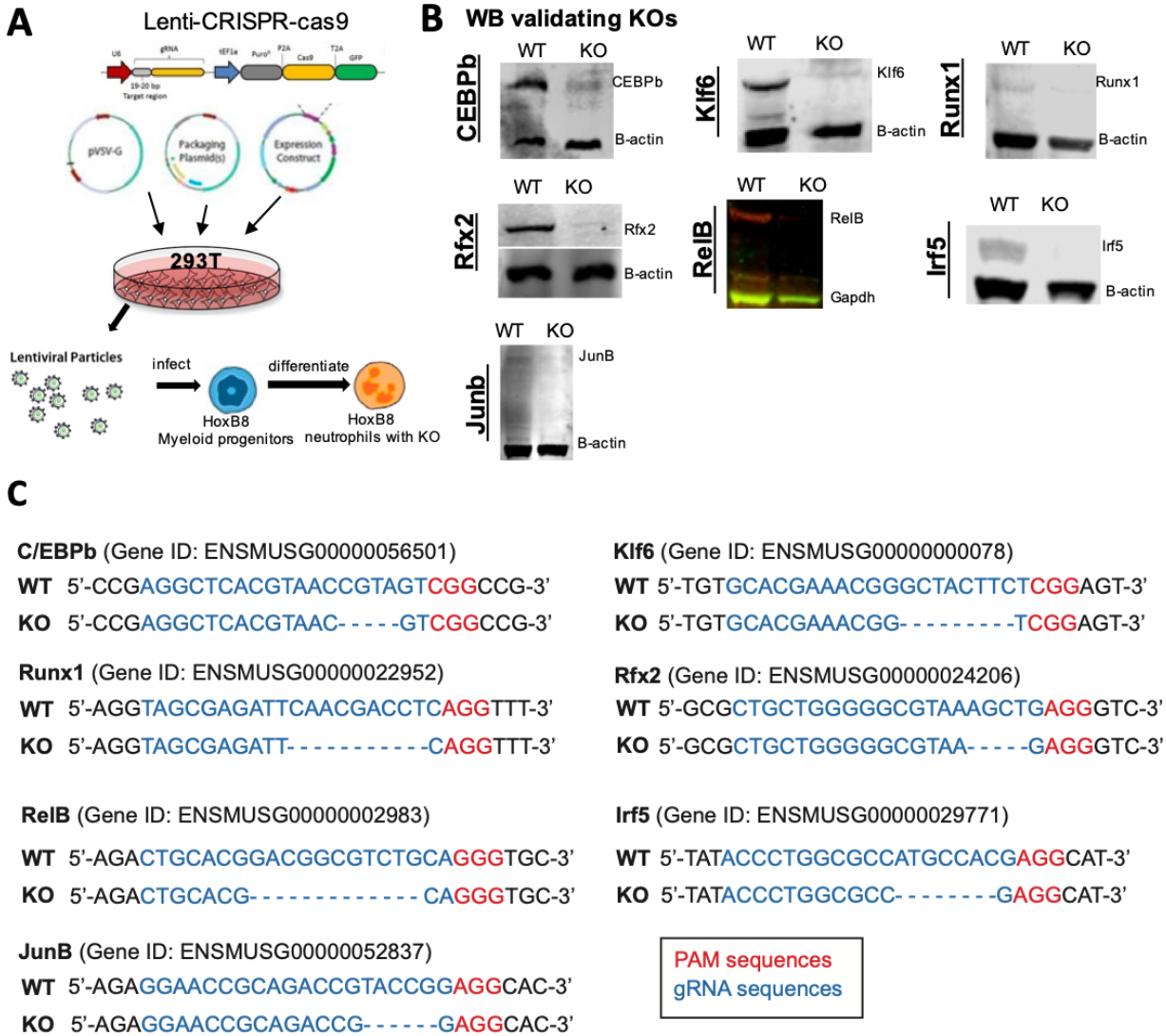


Figure 4.2.1 CRISPR/Cas9 knockouts of specific TF from HoxB8 myeloid progenitor cells
(A) Overview of lentiviral CRISPR/Cas9 system. Lentivirus packing cells 293T cells were transfected with lentiCas9-v2 plasmid mixed at a 2:1:1 DNA ratio of the lentiviral packaging plasmids pVSVG (Addgene plasmid #8454) and psPAX2 (Addgene plasmid #12260). 48 hours post transfection, the lentivirus containing supernatants were harvested, filtered. For virus transduction, HoxB8 myeloid progenitor cells were infected with lentiviral particles containing the CRISPR/Cas9 construct and selected for deletion by puromycin and GFP-mediated cell sorting. Western Blot were conducted for for validating the targeted knockout. (**B**) Western Blotting validation for the targeted knockout of C/EBPβ, Klf6, Runx1, Rfx2, RelB, Irf5 and JunB. For the experiments, 40 µg of total protein was collected from both CRISPR-edited cells and unedited cells by diluting cell lysates in Laemmli sample buffer and boiling at 100°C for 10 minutes. Samples were then loaded onto a 4-20% precast gel followed by SDS-PAGE. Verification of gene knockout was performed by immunoblotting for target proteins and β-actin

or Gapdh as the loading control. Cells infected with a non-targeting construct were used as wild type (WT) control. (**C**) Sanger sequencing validation of CRISPR/Cas9 mediated gene editing. HoxB8 myeloid progenitors cells with/without genetic modification were harvested for genomic DNA isolation and then isolated genomic DNA were used for PCR amplification for amplify the targeted exon 1 of *C/EPBb*, exon1 of *Klf6*, exon3 of *Runx1*, exon5 of *Rfx2*, exon4 of *RelB*, exon2 of *Irf5* and exon1 of *JunB*. Then, the forward PCR primers were also used for Sanger sequencing. Gene ID was annotated next to the gene name.

4.2.2 Morphological assessment of HoxB8 neutrophils with specific TF knockout

To investigate how specific TFs affect neutrophil differentiation, we firstly investigated the morphological change of wild type and knockout HoxB8 neutrophils. HoxB8 myeloid progenitor cells replicate normal neutrophil differentiation and differentiate into segmented mature neutrophils after 5 days. After 5 days of differentiation with G-CSF, the morphology of HoxB8 neutrophils was assessed by cytospin, followed by morphological quantification based on nuclear size and shape, staining density of chromatin, and presence of granules to define metamyelocyte, banded neutrophils and segmented neutrophils. HoxB8 myeloid progenitors with Irf5, JunB or C/EBPβ knockout gave rise to morphologically fully mature neutrophils, comparable to wild-type HoxB8 neutrophils (**Figure 4.2.2A**), while morphological quantification of these neutrophils also demonstrated C/EBPβ, Irf5, JunB deficient HoxB8 neutrophils were comparable to wild-type HoxB8 neutrophils in the proportions of metamyelocyte, banded neutrophils and segmented neutrophils (**Figure 4.2.2B**). In contrast, after 5 days of differentiation, HoxB8 neutrophils with Klf6 or Runx1 deficiency displayed features of immature neutrophils such as low level of nucleus segmentation with a high cytoplasm-nucleus ratio. Morphological quantification demonstrated that significantly lower proportion of segmented mature neutrophils was found in neutrophils generated from Klf6 and Runx1 deficiency, 9.33 ± 2.52% or 31 ± 15.52% (mean ± SD), respectively, in comparison to wild-type neutrophils, 79.67 ± 2.08% (mean ± SD). Consistently, there were higher levels of metamyelocytes (69.33 ± 10.50% and 43.67 ± 10.07%, mean ± SD, respectively) and banded neutrophils (21.33 ± 9.71 and 25.67 ± 5.03, mean ± SD, respectively) in Klf6- or Runx1-deficient HoxB8 neutrophils, in comparison to wild-type HoxB8 neutrophils (20.33 ± 2.082% and 0 ± 0%, mean ± SD, respectively), indicating that neutrophils with Klf6 or Runx1 deficiency are morphologically immature. Notably, Rfx2-deficient neutrophils were also demonstrated to be with lower level of segmented mature neutrophils and higher level of banded neutrophils, which were later shown to result from higher apoptosis of Rfx2-deficient neutrophils under differentiation.

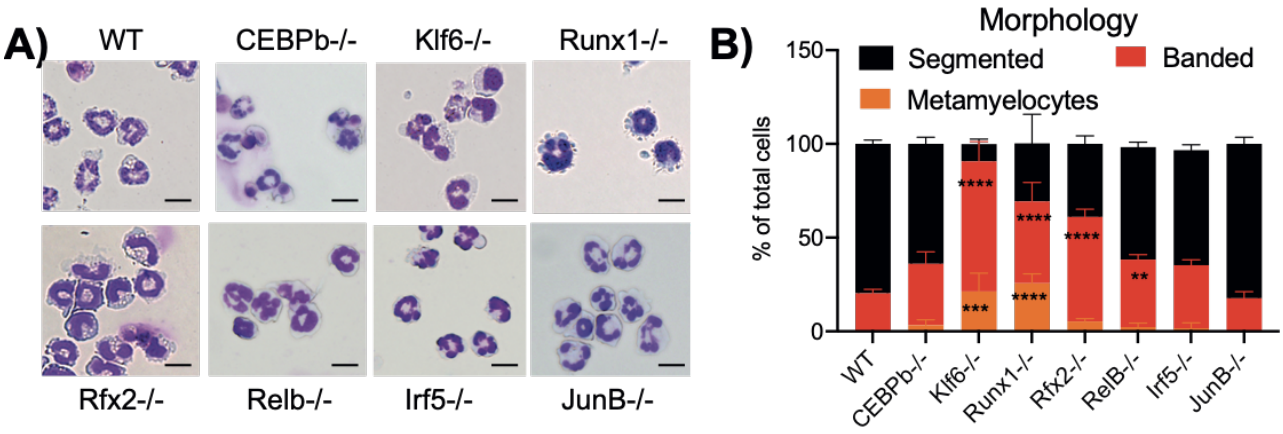


Figure 4.2.2 Morphological assessment of wild-type and knockout HoxB8 neutrophils. Wild-type and knockout HoxB8 myeloid progenitors were seeded into the medium containing G-CSF to induce the differentiation. Cytospin was prepared using HoxB8 neutrophils differentiated for 5 days and then followed by Wright-Giemsa staining. Imaging was made on a bright-fieqld light microscope at x 60 magnification. (A) One representative image on each group from three independent experiments is shown. Scale bars indicate 10 μm. (B) Morphological quantification was made on HoxB8 neutrophils to measure the proportion of metamyelocyte, banded neutrophils and segmented neutrophils. The data are expressed as mean ± SD percentages of neutrophils with different maturity, out of at least 200 cells counted from different fields and independent replicates.

4.2.3 Cell proliferation and apoptosis of wild-type and knockout HoxB8 neutrophils

A maturation arrest probably results from increased apoptosis during the differentiation process, as it occurs in the bone marrow of severe congenital neutropenia (206). To eliminate the apoptotic effect, I examined the rate of cell apoptosis in wild-type and knockout HoxB8 neutrophils under G-CSF induced differentiation by the Annexin V staining, which is a flow-cytometry-based method to detect apoptotic cells. Throughout the process of differentiation, wild-type HoxB8 neutrophils maintained a low rate of apoptosis, 18.73 ± 4.92% (mean ± SD), consistent with the fact that mature neutrophils are programmed to undergo constitutive apoptosis in circulation. In comparison to wild-type HoxB8 neutrophils, HoxB8 neutrophils with Rfx2 deficiency had an early onset of apoptosis during the differentiation process, 13.90 ± 0.4 % on day 4 of differentiation, and reach a significantly higher rate of apoptosis, reaching 74.33 ± 9.78% (mean ± SD) upon day 5 of differentiation. Meanwhile, most TF knockouts did not alter the apoptotic rate of neutrophils under steady condition, which are close to 20% upon day 5 of differentiation. Notably, RelB deficiency also resulted in an increased level of neutrophil apoptosis, and is consistent with the already known function of NF-κB in supporting neutrophil survival and inhibiting spontaneous apoptosis (41).

To assess the effect of RFX2 and RELB on neutrophil survival upon inflammation *in vivo*, we performed adoptive transfer experiments, in which the equivalent mixtures of wild-type neutrophils and either RFX2-deficient or RELB-deficient HoxB8 neutrophils were labelled with the red fluorescent marker CellTracker™ Red, to help monitor cell movement or migration, while knockout HoxB8 neutrophils were labeled with the green fluorescence marker CellTracker™ CFSE, to distinguish fluorescently labeled knockout cells from the wild-type cells during flow cytometry acquisition. Upon adoptive transfer, an equal mix of CellTracker™ Red-labelled WT and CellTracker™ CFSE-labelled knockout HoxB8 neutrophils was intravenously injected 10 minutes before injection of zymosan into the pouch cavity. The mice were then subjected to zymosan challenge. 4 hours post zymosan challenge, the apoptotic rate and recruitment of adoptively transferred neutrophils were assessed by flow cytometry (**Figure 4.2.3 C**). RELB-deficient neutrophils infiltrated the site of inflammation as efficiently as WT cells (**Figure 4.2.3 D**), but showed an increased level of apoptosis at the site of inflammation (**Figure 4.2.3 E**). In contrast, RFX2-deficient neutrophils were more apoptotic than WT cells already in the blood, and their rate of apoptosis increased further at the site of inflammation (**Figure 4.2.3 F**). Consequently, a significant reduction in recovery of RFX2-deficient cells was observed in all the compartments (**Figure 4.2.3 G**), supporting the intrinsic requirement of RFX2 and RELb in maintaining neutrophil survival *in vivo*. Collectively, these results suggest that Rfx2 deficiency causes increased neutrophil apoptosis and negatively affect neutrophil differentiation.

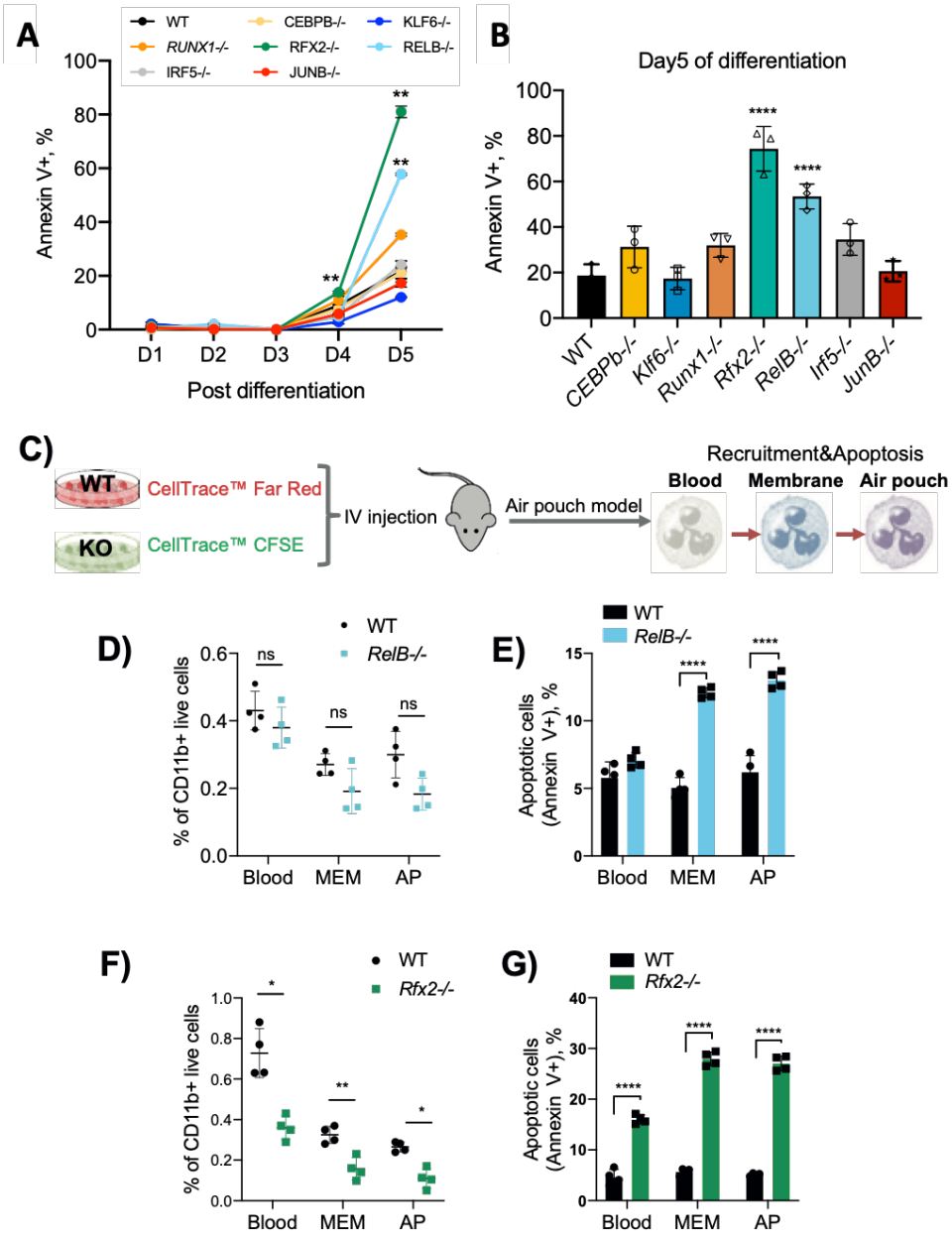


Figure 4.2.3 Apoptosis assessment of wild-type and knockout HoxB8 neutrophils. Wild-type and knockout HoxB8 myeloid progenitors were seeded into the medium containing G-CSF to induce the differentiation. Annexin V staining was performed on HoxB8 neutrophils differentiated for various days. (A) Apoptotic quantification was made on HoxB8 neutrophils differentiated for different days to measure the proportion of apoptotic cells. The data are expressed as mean ± SD percentages of Annexin V positive neutrophils. Statistical comparison was made between knockout and wild-type HoxB8 neutrophils using two-way ANOVAt, *P<0.05, **<0.01. (B) Apoptotic cell rate was comparison between of wild-type and knockout HoxB8 neutrophils upon day 5 of differentiation. Statistical comparison was made between knockout and wild-type HoxB8 neutrophils using one-way ANOVAt, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.D0 (undifferentiated progenitors), D2-5 (day 0-5 differentiation of Hoxb8 myeloid progenitor cells). (**C**) Experimental set-up of adoptive transfer experiments, and the recruitment and apoptosis of transferred WT and KO HoxB8 neutrophils were assessed by flow cytometry. Percentages of wild-type and RELB-deficient (**D**) or RFX2-deficient (**E**) HoxB8 neutrophils in the blood, air pouch membrane and exudate. Percentages of apoptotic wild-type and RELB-deficient (**F**) or RFX2-deficient (**G**) neutrophils in the same compartments.

4.2.4 Mitochondrial function of HoxB8 neutrophils with specific TF knockout

Neutrophil differentiation occurs with a shift of mitochondrial respiration. To this end, we observed the immature phenotype of Klf6-deficient and Runx1-deficient HoxB8 neutrophils. However, whether specific TF knockout affects neutrophil mitochondrial function remain yet to be explored. We next determined the change of mitochondrial membrane potential in wild-type and knockout HoxB8 neutrophils. Wild-type and knockout HoxB8 myeloid progenitor cells were differentiated in medium supplemented with G-CSF for 5 days, and the mitochondrial membrane potential of HoxB8 neutrophils were measured by flow cytometry with TMRM staining. Using this analysis, we found that Klf6 and Runx1 deficiency resulted in an increase of neutrophil mitochondrial activities, as evidenced by the higher level of mitochondrial membrane potential observed in HoxB8 neutrophils deficient in Klf6 or Runx1, whereas other TF knockouts did not exhibit any difference in comparison to wild-type HoxB8 neutrophils (**Figure 4.2.4 A**).

To provide more insight into the metabolic status of HoxB8 neutrophils, we conducted the metabolic influx analysis, in which the basal, compensatory and maximal levels of mitochondrial respiration were measured in wild-type and knockout HoxB8 neutrophils. HoxB8 myeloid progenitor cells were differentiated in medium supplemented with G-CSF for induce differentiation for 5 days before seeding into 96-well tissue culture plate. With this analysis, we found that Klf6 or Runx1 deficiency resulted in a significantly higher level of basal mitochondrial respiration, ATP turnover, maximal and spare mitochondrial capacity (**Figure 4.2.4 B**), which corroborates the notion that Klf6-deficient and Runx1-deficient neutrophils are more mitochondrially active under steady condition (**Figure 4.2.4 C&D)**. Additionally, we observed a lower level of basal mitochondrial basal respiration, maximal and spare mitochondrial capacity in Rfx2-deficient HoxB8 neutrophils (**Figure 4.2.4 E**). Mitochondria is crucial for inducing cell apoptosis, and the lower level of mitochondrial respiration could at least partially explain the increased apoptosis events observed in Rfx2-deficient cells.

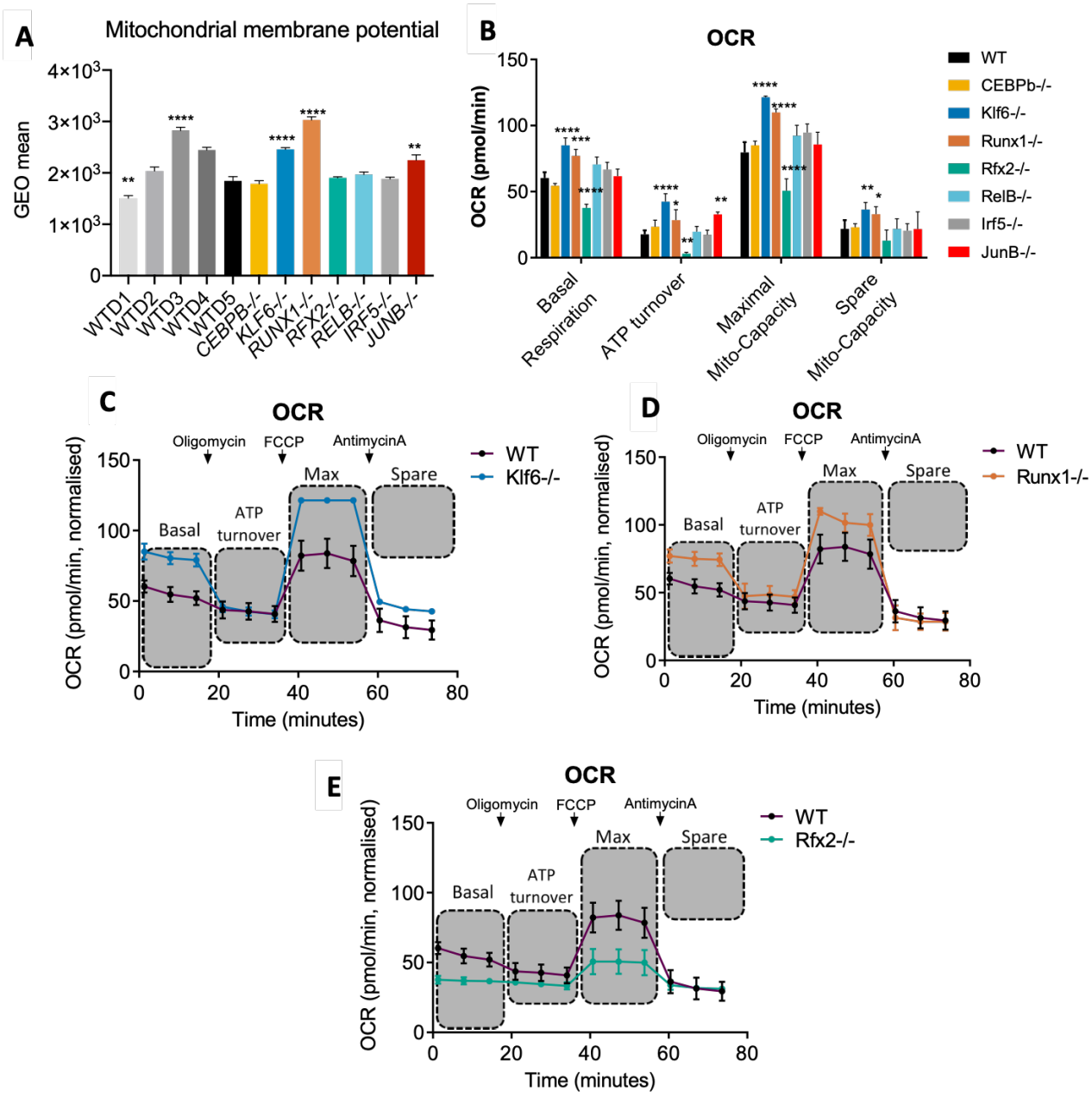


Figure 4.2.4 Mitochondrial function of HoxB8 neutrophils with specific TF knockout. HoxB8 myeloid progenitors were differentiated in the medium containing G-CSF to induce differentiation. (**A**) Mitochondrial memorable potential was assessed by flow cytometry with the fluorescent dye TMRM, and then analysed by normalising fluorescence signals of stained cells to unstained cells. Data are shown as means and SD from three independent experiments, each with two replicates. Statistical comparison was made between HoxB8 neutrophils within different differentiation days and between wild-type and knockout cells. Statistical comparison was analysed by one-way ANOVA. (**B**) Basal respiration rate, ATP turnover, maximal and spare mitochondrial capacities of WT and indicated KO HoxB8 neutrophils under basal conditions measured using Seahorse assay. Data are shown as means and SD from three independent experiments, each with two replicates. Statistical comparison was made between wild-type and knockout HoxB8 neutrophils. Statistical comparison was analysed by one-way ANOVA. (**C, D, E**) OCRs were measured over 80 minutes for WT, Klf6-/-, Runx1-/-, Rfx2-/- HoxB8 neutrophils. Data are shown as means and SD from three independent experiments, each with four replicates. HoxB8 myeloid progenitors differentiated for 1-5 days were loaded into XF 96 extracellular flux analyser for the real-time OCR measurement. Sequential exposure of HoxB8 neutrophils to oligomycin, FCCP, and finally Antimycin A allows for generating the readouts on basal mitochondrial respiration, ATP turnover, maximal respiration capacity, and spare mitochondrial capacity of cells. (**A**,**B**) *P<0.05, **<0.01, ***<0.001, ****P<0.0001. WTD1-5 (day 0-5 of wild-type Hoxb8 differentiation).

4.2.5 Surface marker phenotype of HoxB8 neutrophils with specific TF knockout

HoxB8 neutrophils recapitulate normal neutrophil differentiation and follow a developmental path into mature neutrophils. To further investigate how TF knockout affects neutrophil maturation, we used flow-cytometry to assess the expression of surface markers Ly6C, CXCR2, Ly6G, and CD101, which segregate the stage of preneutrophils, immature and mature neutrophils (11). After 5 days of differentiation, wild-type HoxB8 neutrophils express high levels of neutrophil surface markers Ly6C and Ly6G and become positive in expression of CD101 (**Figure 4.2.5)**, the surface marker that segregate immature and mature neutrophils. Deficiency of most TFs, such as CEBPβ, RelB, Irf5 or JunB, did not significantly alter the expression of Ly6G, Ly6C and CD101 in HoxB8 neutrophils (**Figure 4.2.5 A**), except Klf6, Runx1 or Rfx2 deficiency. Quantification of Ly6G+ CD101+ mature neutrophils demonstrated that HoxB8 neutrophils with deficient expression of Klf6, Runx1 or Rfx2 produced significantly lower levels of Ly6G+ CD101 mature neutrophils, 27.2 ± 5.15%, 46.6 ± 3.76%, 58.7 ± 3.89%, (mean ± SD), respectively, in comparison to wild-type HoxB8 neutrophils 75.1 ± 3.21%, (mean ± SD), while deficiency of CEBPβ, RelB, Irf5 or JunB in HoxB8 neutrophils did not significantly affect neutrophil maturation (**Figure 4.2.5 B&C**). Summarily, these results suggest that Klf6 and Runx1 knockouts inhibit the differentiation process of neutrophils.

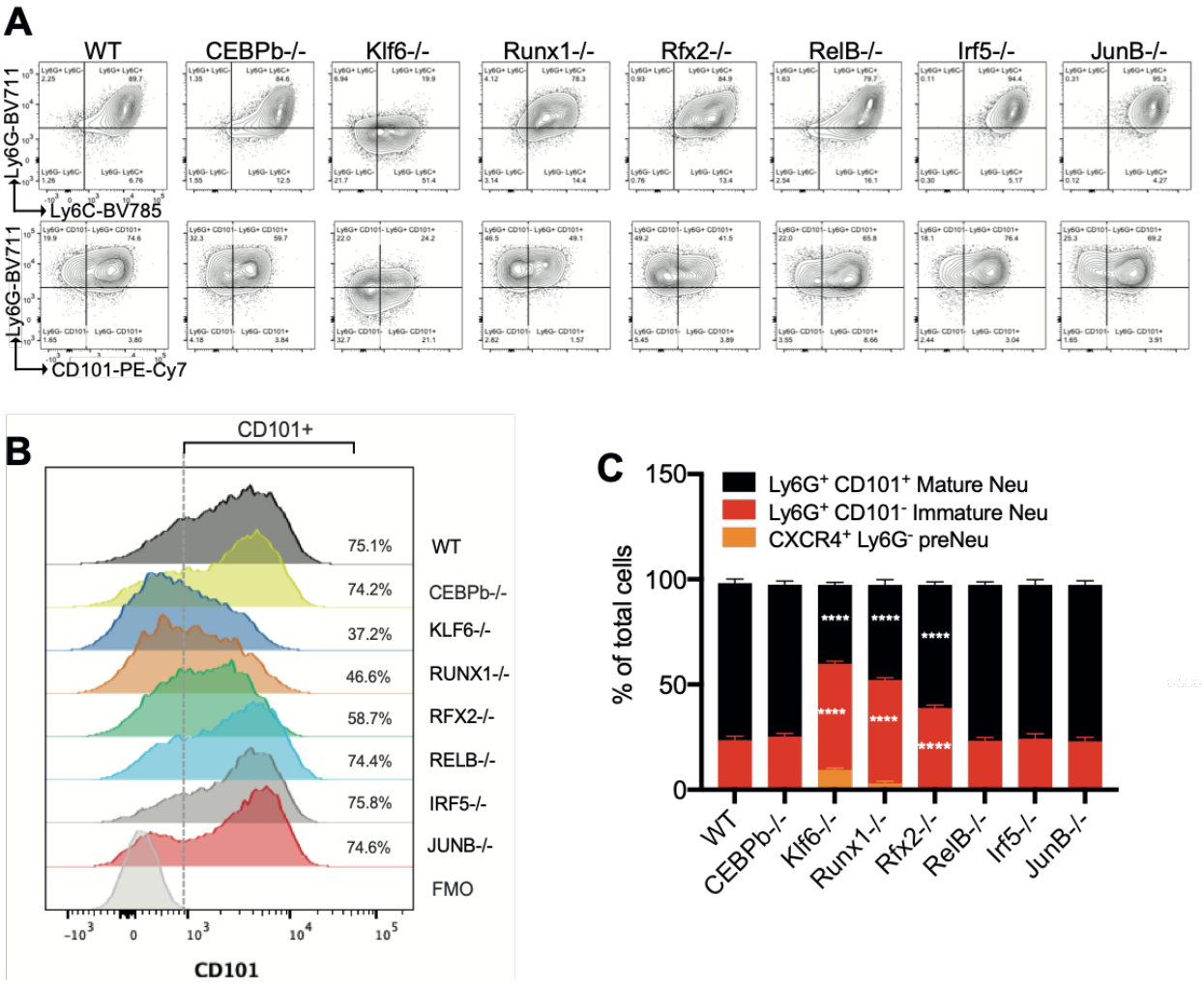


Figure 4.2.5 Surface marker phenotype of HoxB8 neutrophils with specific TF knockout. HoxB8 myeloid progenitors were seeded into the medium containing G-CSF to induce differentiation. HoxB8 neutrophils differentiated for 5 days were stained for assessing the expression of Ly6C, CXCR2, Ly6G, and CD101 and then followed by the acquisition by flow cytometry. (**A**) Representative flow cytometry plots of WT or CEBPβ-deficient, Klf6-deficient Runx1-deficient, Rfx2-deficient, RelB-deficient, Irf5-deficient or JunB-deficient HoxB8 neutrophils co-labelled with Ly6C, Ly6G and CD101. One example in each group from three independent experiments is shown. (**B**) Differential expression of CD101 in HoxB8 neutrophils upon G-CSF-induced differentiation for 5 days, measured by flow cytometry. Data are shown as a representative of three independent experiments. The data are expressed as percentages of CD101+ neutrophils. (**C**) Quantification of CD101+ HoxB8 neutrophils differentiated for 5 days, as shown in (A&B). Data are shown as means and SD and are representative of three independent experiments. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.

4.2.6 Granular proteins and nuclear envelope composition of HoxB8 neutrophils

To provide further insight into the functional role of specific TFs in neutrophil differentiation, we subsequently analysed the gene expression of neutrophil granular proteins, which closely associates the differentiation process of neutrophils. As previous section shown, neutrophil primary enzyme Mpo was enriched in the early stage differentiation of wild-type HoxB8 neutrophils and then downregulated with the progress of maturation. To determine how specific TF knockout affects the expression of neutrophil granular enzymes, wild-type and knockout HoxB8 myeloid progenitor cells were differentiated in the medium containing G-CSF for 5 days, and cytoplasmic protein were extracted for Western Blotting examination. The protein abundance of Mpo in HoxB8 neutrophils was measured and normalised to the housekeeping protein β-actin. Comparison between wild-type and knockout HoxB8 neutrophils differentiated for 5 days demonstrated that primary granule protein Mpo is more abundant in HoxB8 neutrophils with deficiency of Kl6 and Runx1, whereas no significant differences were observed in HoxB8 neutrophils deficient in CEBPβ, Rfx2, RelB, Irf5 and JunB (**Figure 4.2.6A**).

To further confirm the effect of specific TF knockout on neutrophil maturation, we compared the expressional level of LB2, the most abundant nuclear envelope protein in mature neutrophils (38). Total cellular proteins were extracted from HoxB8 neutrophils after 5 days of differentiation to check for LB2 levels by Western Blotting. As shown in **Figure 4.2.6 B**, in wild-type HoxB8 neutrophils, LB2 was gradually upregulated during differentiation and reaches maximal expression during terminal differentiation. Deficiency of Klf6 or Runx1 suppressed the upregulation of LB2 and consequently resulted in lower LB2 abundance in HoxB8 neutrophils differentiated for 5 days (**Figure 4.2.6 B**), suggesting the immature phenotype of Klf6-deficient and Runx1-deficient neutrophils, whereas other TF deficiencies did not significantly affect LB2 expression. It matches the lower levels of nuclear segmentation resulting from Klf6 and Runx1 deficiency. Overall, these results suggest Klf6 and Runx1 deficiency inhibits neutrophil maturation in granular protein formation and nuclear segmentation.

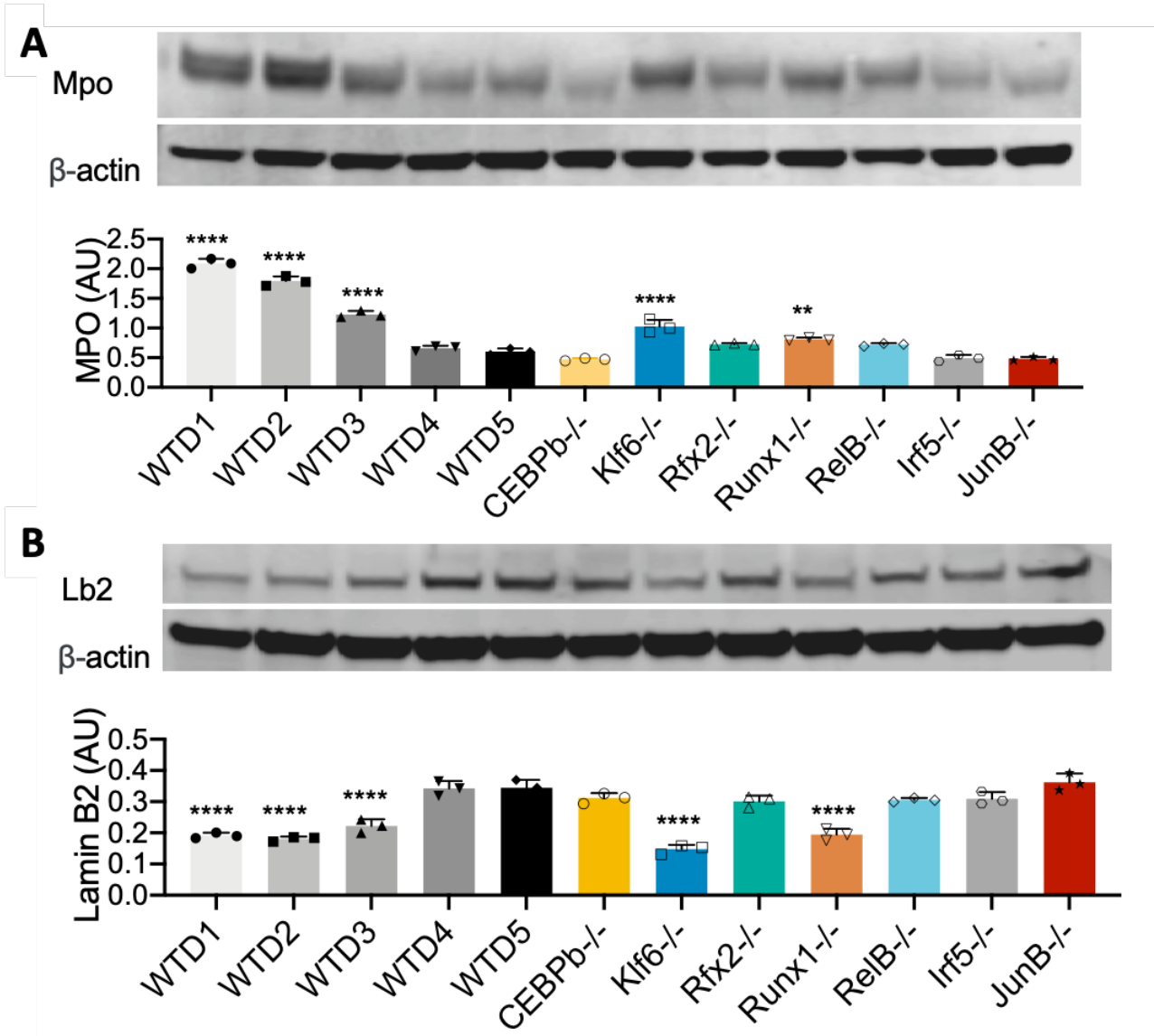


Figure 4.2.6 Granular proteins and nuclear envelope composition in HoxB8 neutrophils. Wild-type and knockout HoxB8 myeloid progenitors were differentiated into the medium containing G-CSF to induce differentiation. Cytoplasmic proteins were extracted to analyse the expression of neutrophil granular protein MPO, and total cellar proteins were prepared to determine the expression of nuclear envelope protein LB2 in HoxB8 neutrophils by Western Blotting. Relative expression of Mpo and were analysed in normalisation to β-actin and shown as arbitrary units (AU). (A) MPO expression in HoxB8 neutrophils. Top: one representative Western blot probed with antibodies specific for Lb2 and β –actin is shown. Bottom: statistical analysis of MPO expression normalised against β-actin amount in the lysates. (B) LB2 expression in HoxB8 neutrophils. Top: one representative Western blot probed with antibodies specific for Lb2 and β –actin is shown. Bottom: statistical analysis of LB2 expression normalised against β-actin amount in the lysates. Data are shown as means and SD from three independent experiments. Statistical comparisons were made within wild-type HoxB8 neutrophils differentiated for various days and between wild-type and knockout HoxB8 neutrophils differentiated for 5 days. Statistical comparison was analysed by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001. WTD1-5 (day 1-5 of wild-type Hoxb8 differentiation).

4.2.7 Conditional knockout of Runx1 in neutrophil differentiation

To this end, we have demonstrated the functional role of Klf6 and Runx1 in neutrophil differentiation, including changes in cell morphology, expression of surface markers and granular enzymes, nuclear segmentation and mitochondrial respiration. Runx1 represents a crucial regulator for emergent hematopoiesis and its mutation is the most frequent cause of leukemogenesis. However, the functional role of Runx1 in neutrophil differentiation is yet to be established. To address this, we next characterised naïve *LysM*$^{cre/cre}$*Runx1*$^{fl/fl}$ mice, in which Runx1 expression is conditionally deleted from myeloid populations, with a focus on the phenotype of bone marrow neutrophils. Naïve *Runx1*$^{fl/fl}$ mice was used as the negative control. Whole bone marrow cells were obtained from *LysM*$^{cre/cre}$*Runx1*$^{fl/fl}$ mice and *Runx1*$^{fl/fl}$ mice and then stained for surface markers that segregate pre-neutrophils, immature and mature neutrophils. For distinguishing different neutrophil subsets, pre-neutrophils were featured with positive CXCR4 and c-Kit expression, and immature neutrophils were characterised with high levels of Ly6G and CD101 expression. It was found that conditional Runx1 knockout mice exhibited a significantly lower percentage of mature neutrophils in the bone marrow, 15.29 ± 0.99% (mean ± SD), in comparison to *Runx1*$^{fl/fl}$ mice, 24.24 ± 0.92% (mean ± SD) (**Figure4.2.7 A**). Additionally, the percentages of pre-neutrophils and immature neutrophils were observed to be higher in *LysM*$^{cre/cre}$ *Runx1*$^{fl/fl}$ mice, 13.38 ± 2.37%, 57.18 ± 2.09% (mean ± SD), respectively, in comparison to *Runx1*$^{fl/fl}$ mice, 18.03 ± 1.94%, 64.33 ± 1.55% (mean ± SD), respectively (**Figure4.2.7 A)**. It is consistent with decreased production of mature neutrophils differentiated from Runx1-deficient HoxB8 neutrophils.

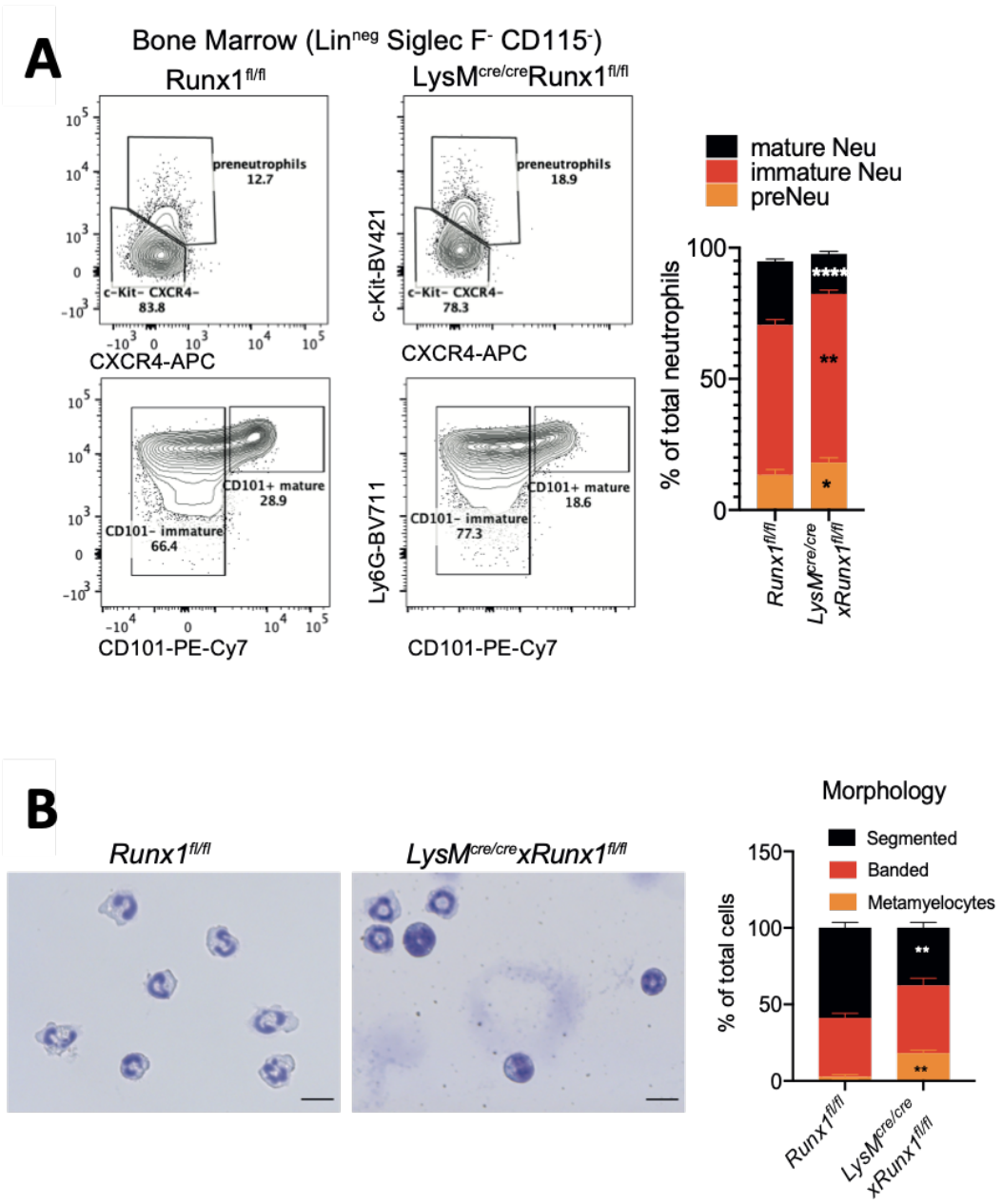


Figure 4.2.7 TF Conditional knockout of Runx1 in neutrophil differentiation.
Whole bone marrow cells were obtained from naïve *LysM*$^{cre/cre}$*Runx1*$^{fl/fl}$ mice and naïve *Runx1*$^{fl/fl}$ mice and flow cytometry was used to the phenotype of bone marrow neutrophils.(**A**) Left panel: representative scatter plot of neutrophil subsets (pre-neutrophils, immature and mature neutrophils) from *Runx1*$^{fl/fl}$ and *Lysm*$^{Cre/cre}$ *Runx1*$^{fl/fl}$ mice. After exclusion of cell doublets and dead cells, pre-neutrophils were identified as Lineage$^-$ Siglec-F$^-$ CD115$^-$ CD11b$^+$ Ly6C^{int} CXCR4$^+$ cKit$^+$ CXCR2$^-$, and immature neutrophils were identified as Lineage$^-$ Siglec-F$^-$ CD115$^-$ CD11b$^+$ Ly6C^{int} CXCR4$^-$ cKit$^-$ CXCR2$^+$ Ly6G$^+$ CD101$^-$, and mature neutrophils were identified as Lineage$^-$ Siglec-F$^-$ CD115$^-$ CD11b$^+$ Ly6C^{int} CXCR4$^-$ cKit$^-$ CXCR2$^+$ Ly6G$^+$ CD101$^+$. Right panel: Statistical analysis of percentages of indicated neutrophil subsets. Data are shown as means and SD from four mice from each group within one experiment. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001. (B) Morphology assessment of CD11b+Ly6G+ neutrophils sorted from *Runx1*$^{fl/fl}$ and *Lysm*$^{Cre/cre}$ *Runx1*$^{fl/fl}$ mice. Left: one representative image of neutrophils using Wright-Giemsa staining. Scale bar represents 10μm. Right: quantification of neutrophils under different maturation stage. The results are expressed as percentages of segmented, banded neutrophils and metamyelocyte out of at least 200 cells counted from different fields and independent replicates. Data are shown as means and SD derived from three mice from each group within one experiment. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.

The importance of Runx1 was further supported by subsequent morphological analysis of neutrophils sorted from *Lysm$^{Cre/cre}$Runx1$^{fl/fl}$* and *Runx1$^{fl/fl}$* mice. Morphological quantification of neutrophils was based on neutrophil nuclear size and shape, staining density of chromatin, presence of granules to define metamyelocytes, banded neutrophils and segmented mature neutrophils. In comparison to *Runx1$^{fl/fl}$* neutrophils that appear banded or multi-lobulated nucleus with the transparent cytoplasm, neutrophils sorted from *Lysm$^{Cre/cre}$Runx1$^{fl/fl}$* mice have a small population of metamyelocytes, which are featured with a kidney bean nucleus within the blue-purple cytoplasm (**Figure4.2.7 B)**. Morphological quantification also demonstrated lower percentage of segmented mature neutrophils and higher percentage of metamyelocytes in neutrophil sorted from *Lysm$^{Cre/cre}$Runx1$^{fl/fl}$* mice (**Figure4.2.7 B)**. It matches the lower proportion of segmented mature neutrophils observed in Runx1-deficient HoxB8 neutrophils.

4.2.8 Runx1 regulates C/EBPe expression to induce neutrophil differentiation

Runx1 promotes myeloid commitment by inducing lineage-specific gene expression patterns during hematopoietic development. We next investigated the effect of Runx1 deficiency on gene expression of important TFs in neutrophil differentiation. PU.1, CEBPa and C/EBPe are central to promote neutrophil lineage commitment and maturation (57). CEBPβ is essential for emergent granulocytosis (93). Klf6 deficiency inhibit neutrophil maturation and it mutation is linked to the development of acute myeloid leukaemia (83). Therefore, mRNA expressions of *PU.1*, *CEBPa*, *C/EBPe*, *CEBPβ* and *Klf6* were determined in neutrophils sorted from *Lysm$^{Cre/cre}$Runx1$^{fl/fl}$* and *Runx1$^{fl/fl}$* mice. Runx1 deficiency resulted in significantly lower level of C/EBPe, a crucial TF that promotes neutrophil terminal differentiation, and no significant difference was observed in the expression of PU.1, CEBPa, CEBPβ and Klf6 (**Figure4.2.8 A**). Additionally, downregulation of C/EBPe in Runx1-deficient neutrophils was further supported in intracellular staining analysis for C/EBPe, in which C/EBPe expression were determined in CD11b+ Ly6G neutrophils. Quantification of C/EBPe fluorescent signals demonstrated a lower level of C/EBPe in neutrophils from the bone marrow of *Lysm$^{Cre/cre}$Runx1$^{fl/fl}$* mice (**Figure4.2.8 B**), supporting that Runx1 deficiency inhibits neutrophil differentiation through blocking C/EBPe expression.

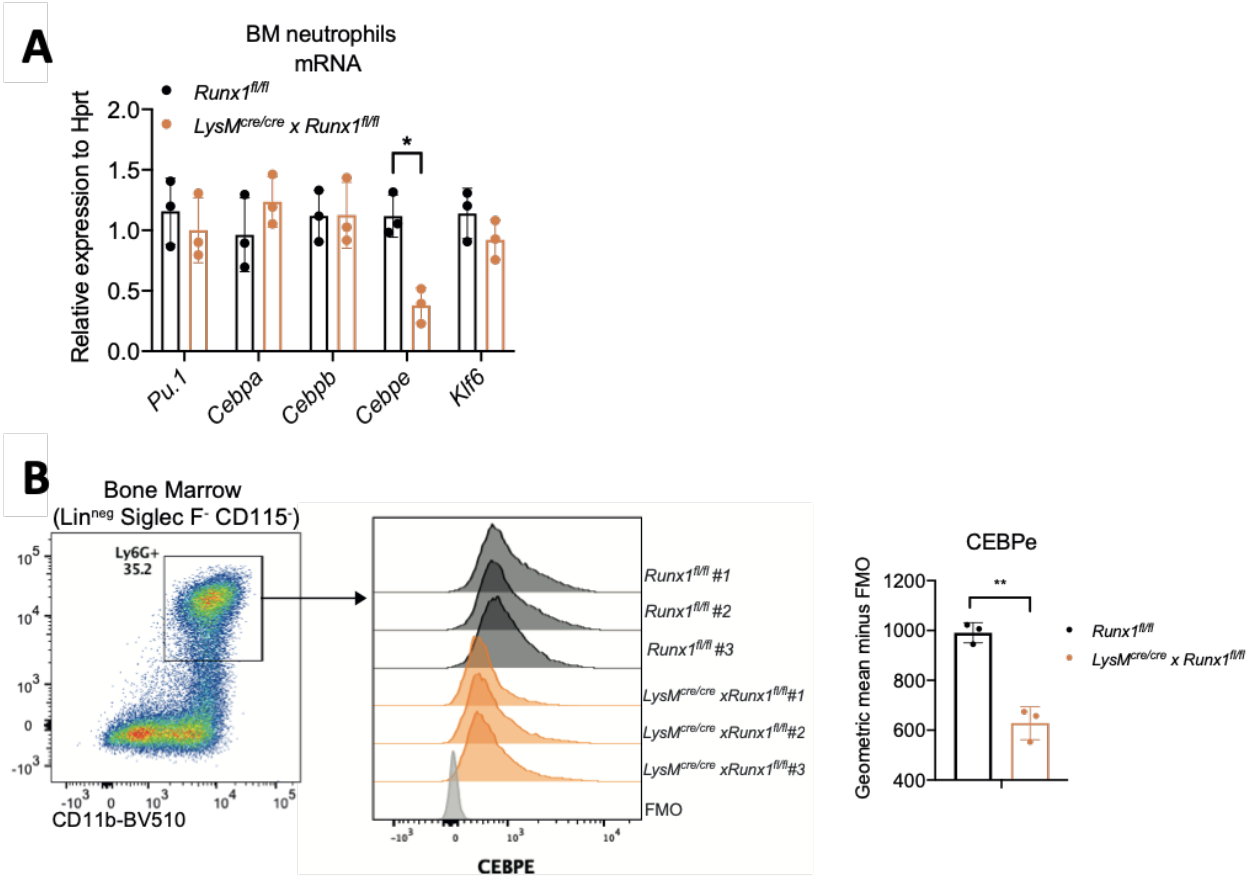


Figure 4.2.8 Runx1 regulates C/EBPe expression to neutrophil differentiation.
(A) *Pu.1, Cebpa, CEBPβ, C/EBPe* and *Klf6* mRNA expression in CD11b+ Ly6G+ neutrophils sorted from *Runx1*$^{fl/fl}$ and *Lysm*$^{Cre/cre}$*Runx1*$^{fl/fl}$ mice. Gene expression was measured by qPCR with the use of Hrpt as housekeeping gene. Data are shown as means and SD derived from three mice from each group within one experiment. Statistical comparison was made by one-way ANOVA, *P<0.05 (**B**) Differential expression of C/EBPe in CD11b^{+}Ly6G^{+} neutrophils sorted from *Runx1*$^{fl/fl}$ and *Lysm*$^{Cre/cre}$*xRunx1*$^{fl/fl}$ mice, measured by flow cytometry. Left: Gating strategy of neutrophils for identifying CD11b^{+}Ly6G^{+} neutrophils. Fluorescence histogram (middle) and geometric mean of Cepbe expression (left) in CD11b^{+}Ly6G^{+} neutrophils. Data are shown as means and SD derived from three mice from each group within one experiment. Statistical comparison was made by Student's t-test, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.

4.2.9 In vitro migration of HoxB8 neutrophils

Based on the phenotypic assessment of wild-type and knockout HoxB8 neutrophils, Klf6 and Runx1 came up as key regulators in the process of neutrophil differentiation. Mature neutrophils migrate rapidly towards the sites of inflammation, whereas neutrophil precursors have a reduced migratory capacity in response to sterile inflammation (11). Thus, we hypothesised that Klf6-deficient and Runx1-deficient neutrophils are immotile in response to chemotactic signals. To address this, we made use of the Boyden chamber migration assay, in which HoxB8 neutrophils were added to the upper chamber of a 96-well Boyden chamber and they migrated through the 5 mm porous membrane towards chemoattractants loaded in the lower chamber (**Figure4.2.9 A**). Wild-type HoxB8 myeloid progenitor cells were differentiated with G-CSF for 1-5 days and knockout myeloid progenitor cells were differentiated for 5 days before seeding into the 96-well Boyden chamber, with C-C motif chemokine CCL3 loaded as the chemoattractant, as CCL3 exclusively mediates the regulation of intravascular adherence and transmigration of neutrophils during inflammation (231). Upon incubation for 90 minutes, HoxB8 neutrophils that have migrated through the pores into the lower chamber are detected and quantified by confocal microscopy. Using this assay, we observed that wild-type HoxB8 neutrophils differentiated for 5 days migrated rapidly under the chemotactic effect of CCL3, reaching 33.33 ± 4.51 (mean ± SD) cells/field on the porous membrane, whereas HoxB8 neutrophils differentiated for 1 and 3 days, with lower maturity, had lower numbers of neutrophils migrated through porous membrane, 2.33 ± 0.58 and 6.24 ± 1.51 (mean ± SD) cells/field on the pore membrane, respectively (**Figure4.2.9 B**). This is consistent with a reduced capacity of migration observed in neutrophil precursors from bone marrow (11). In comparison to wild-type HoxB8 neutrophils, Klf6-deficient and Runx1-deficient HoxB8 neutrophils differentiated for 5 days demonstrated a significantly reduction in trans-migration in response to the CCL3 chemotactic signal, 5.33 ± 1.25 and 8.33 ± 2.75 (mean ± SD) cells/field on the pore membrane, which are comparable to the level of wild-type HoxB8 neutrophils at day 3 of differentiation (**Figure4.2.9 B**). Overall, these results suggested that Klf6 and Runx1 deficiency cause impaired neutrophil migration.

Neutrophils are rapidly recruited into sites of inflammation during inflammatory conditions (9). We then determined how specific TF knockout affects neutrophil recruitment *in vivo* by adoptive transfer of Hoxb8 neutrophils into mice subjected to the air pouch model and zymosan challenge. Wild-type and knockout HoxB8 myeloid progenitor cells were differentiated in me-

dium supplemented with G-CSF to induce the differentiation for 5 days. Before adoptive transfer, wild-type HoxB8 neutrophils were labelled with the red fluorescent marker CellTracker™ Red, while knockout HoxB8 neutrophils were labeled with the green fluorescent CellTracker™ CFSE, to monitor cell movement or migration and distinguish wild-type cells from knockout cells. Upon adoptive transfer, an equal mixture of CellTracker™ Red-labelled WT and CellTracker™ CFSE-labelled knockout HoxB8 neutrophils was intravenously injected 10 minutes before injection of zymosan into the pouch cavity. Wild-type and knockout HoxB8 neutrophils competitively migrate toward sites of inflammation. 4 hours post zymosan challenge, the percentages of adoptively transferred HoxB8 neutrophils recruited into the blood, air pouch membrane and exudate were measured by flow cytometry (**Figure4.2.9 C**). It has been demonstrated that HoxB8 neutrophils transferred into mice can be easily detected in bone marrow, peripheral blood as well as multiple tissue sites (205). Consistently, upon adoptive transfer into air pouch model of acute inflammation, both wild-type and knockout HoxB8 neutrophils were obviously detected in the blood, air pouch membrane and exudate. In comparison wild-type HoxB8 neutrophils recovered from the air pouch membrane and exudate, occupying 0.136 ± 0.027 % and 0.167 ± 0.034% (mean ± SD), respectively, Klf6-deficient neutrophils were less frequently infiltrated into the air pouch membrane and exudate, 0.047 ± 0.022 % and 0.041 ± 0.028% (mean ± SD), respectively. We also observed a significantly reduced infiltration of Runx1-deficient HoxB8 neutrophils into the air pouch membrane and exudate, 0.037 ± 0.026 % and 0.017 ± 0.011% (mean ± SD), respectively, in comparison to the levels of wild-type HoxB8 neutrophils, 0.192 ± 0.081 % and 0.184 ± 0.024% (mean ± SD) in the air pouch membrane and exudate, respectively (**Figure4.2.9 D**). In contrast, the recruitments of C/EBPβ-deficient, RelB-deficient, Irf5-deficient and JunB-deficient HoxB8 neutrophils were comparable to that of wild-type HoxB8 neutrophils (**Figure4.2.9 D**). Overall, these results suggested that the poor migratory machinery in neutrophils with Klf6 or Runx1 deficiency, consistent with the lower migratory capacity observed in neutrophil precursors from the bone marrow (11).

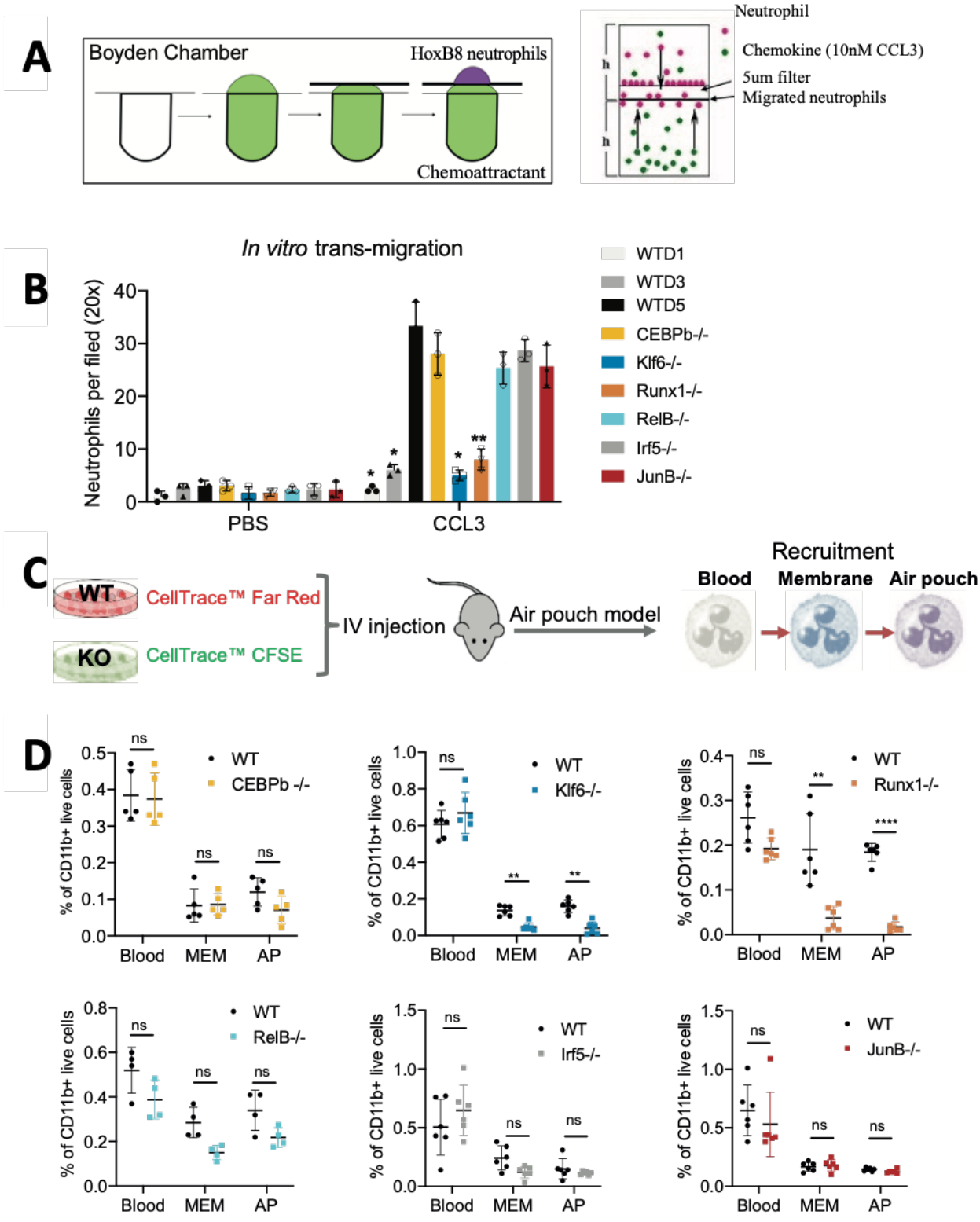


Figure 4.2.9 In vitro migration and in vivo recruitment of HoxB8 neutrophils.
(A) Schematic of the Boyden chamber migration assay with HoxB8 neutrophils. Wild-type HoxB8 myeloid progenitors cells were differentiated for 1-5 days and knockout cells were differentiated for 5 days before loading into 96-well plates. Neutrophils were placed in the upper compartment and are allowed to migrate through the 5mm porous membrane of the membrane into the lower compartment where CCL3 was added to induce neutrophils migration. Neutrophils that migrate through the pore membrane were imaged on the bottom of the pore membrane and quantified by confocal microscopy after DAPI staining. (**B**) Cell migration of HoxB8 neutrophils was measured by Boyden chamber migration assay. Migrated cell num-

bers were counted as means and SD and are representative of three independent experiments, each assessed by average cell number within different fields and independent replicates. Statistical comparison was made within wild-type HoxB8 neutrophils differentiated for different days and between wild-type and knockout HoxB8 neutrophils differentiated for 5 days. Statistical analysis was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001. (**C**) Experimental set-up of differentially labelled wild-type and knockout HoxB8 neutrophils adoptively transferred into mice subject to air pouch model of acute inflammation. Four hours post zymosan challenge, the recruitment of neutrophils was assessed by the percentage of differentially labeled HoxB8 neutrophils recovered from the blood, air-pouch membrane and air pouch exudate in mice subjected to the air pouch model and zymosan challenge. (**D**) Percentages of wild-type and knockout HoxB8 neutrophils recovered in the bone marrow, blood, air pouch membrane and air pouch exudate recovered from mice subjected to the air pouch model and zymosan challenge. All results are shown as means and SD derived from at least three mice within one experiment. Statistical comparison was made between wild-type and knockout HoxB8 neutrophils. Statistical analysis was made by one-way ANOVA, n.s., not statistically significant; *P<0.05; **<0.01; ***<0.001; ****P<0.0001. WTD1-5 (day 1-5 differentiation of wild -type Hoxb8 myeloid progenitor cells).

4.2.10 KLF6 and RUNX regulation in neutrophil recruitment

To determine the molecular mechanism underling the impact of RUNX1 and KLF6 in neutrophil maturation and migration, we performed mRNA-seq analysis of WT Hoxb8 neutrophils differentiated for 0, 1, 3, 5 days and *Runx1-/-* and *Klf6-/-* neutrophils differentiated for 5 days. Under the threshold of the threshold of adjusted $p < 0.05$ and $\log_2FC > 1$, there were 3,306 and 2,611 genes that were differentially expressed in Runx1-deficient and Klf6-deficient neutrophils compared to D5 WT cells, as defined by the number of differentially expressed genes. Unsupervised hierarchical clustering of the DEGs identified five clusters, which encompassed genes downregulated (clusters 1 and 4) or upregulated with the progress of maturation (cluster 2, 3 and 5). Genes in clusters 2 and 5 were downregulated in Runx1-deficient or Klf6-deficient neutrophils, respectively (**Figure4.2.10 A**). GO annotation analysis revealed that these two clusters encompassed transcriptional programs of immune responses, cytokine production and leukocyte migration (**Figure4.2.10 B**). Genes in clusters 4 and 3 were upregulated in Runx1-deficient or Klf6-deficient neutrophils, respectively, with programs capturing metabolic and biosynthetic processes (**Figure4.2.10 B**). RUNX1 and KLF6 controlled a significant number of genes involved in leukocyte migration, such as important cell adhesion and chemotaxis molecules including Cxcr2, Sell, S100a8 or Itgal for RUNX1; and Vcam1, Cd9, C3ar1, Cx3cr1 and Icam1 for KLF6 (**Figure4.2.10 C**).

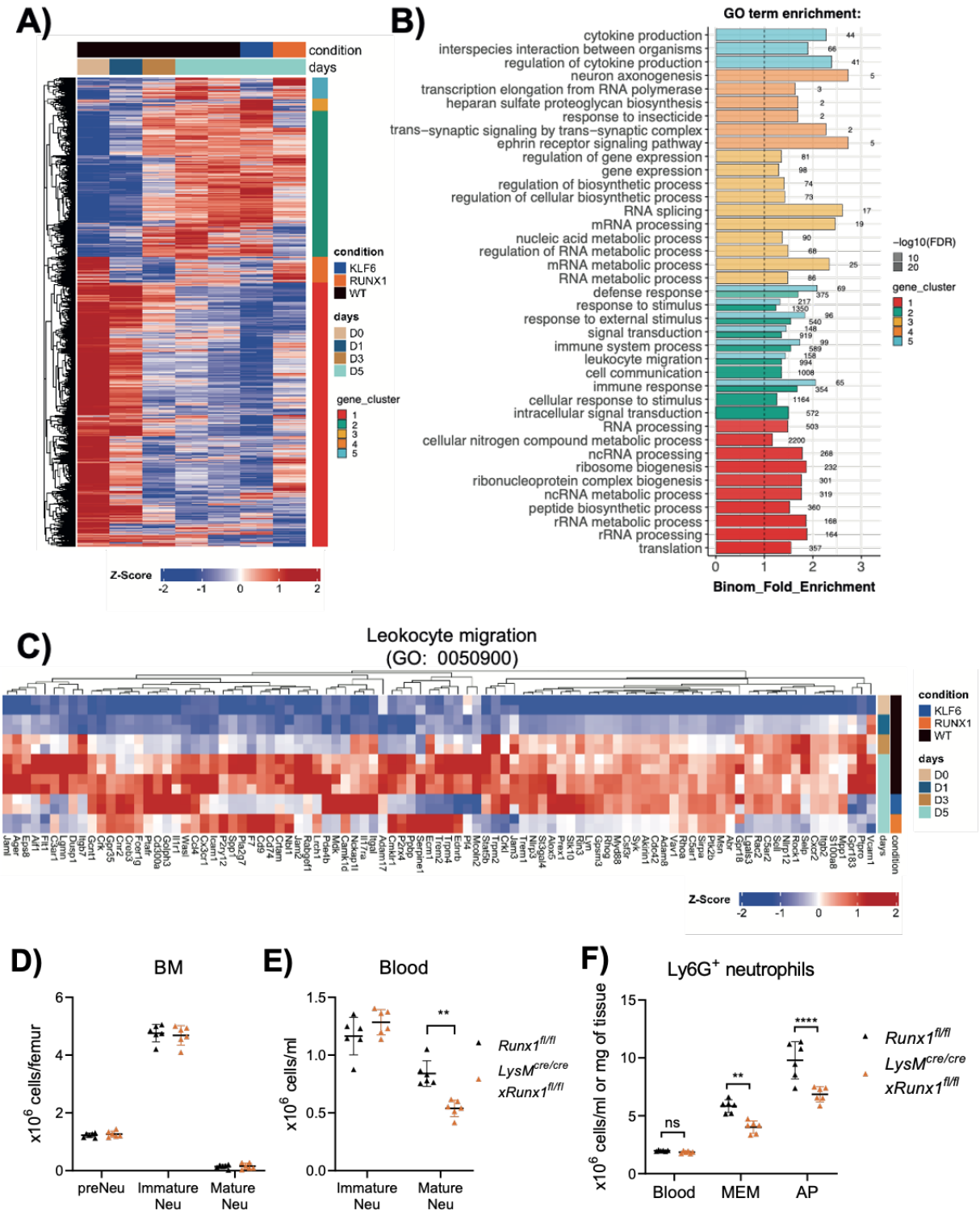


Figure 4.2.10 KLF6 and RUNX1 regulation in neutrophil recruitment.
(A) Unsupervised hierarchical clustering of all DEGs (padj < 0.05, |log2FC|>1). Data are presented as heatmap normalized to the minimum and maximum of each row. B) Gene ontology (GO) analysis showing the log2 odds ratio of genes regulated by specific TF knockout with the indicated GO annotation. Differentially expressed genes from each cluster in **Figure4.2.10A** were used for gene ontology (GO) enrichment analysis. Representative GO terms are shown for each cluster. c) Representative immunofluorescence images of NETosis from WT, RelB–/– and JunB–/– HoxB8 neutrophils stimulated with 5μM ionomycin and PMA overnight, stained for DNA (blue), MPO (green) and citrullinated histone3 (red). Representative images from three independent experiments are shown. Scale bar indicates 10μm. (D,E) Quantification of

indicated neutrophil subsets in the bone marrow (D) and blood (E) of LysM$^{cre/cre}$ xRunx1$^{fl/fl}$ and Runx1$^{fl/fl}$ control mice subjected to the air pouch model of acute inflammation. (F) Quantification of total neutrophil numbers in the blood, air pouch membrane and exudate from mice subjected to the air pouch model and zymosan challenge. Data are shown as means and SD from four to six mice. Statistical comparison was made by two-way ANOVA, *P<0.05; **<0.01; ***<0.001; ****P<0.0001.

Neutrophil infiltration into tissues are exaggerated in the setting of inflammatory diseases, such as arthritis (9). Therefore, we next examined to investigate whether specific TF knockout affects neutrophil *in vivo* recruitment, using *Lyz2*$^{cre/cre}$*xRunx1*$^{fl/fl}$ and control *Runx1*$^{fl/fl}$ mice subjected to the air pouch model and zymosan stimulation, which leads to depletion of mature neutrophils from bone marrow into the circulation and subsequent infiltration into sites of inflammation. 4 hours post zymosan challenge, the percentage and absolute quantity of neutrophils were quantified in the bone marrow, blood, air pouch membrane, and air pouch exudate in mice subjected to air pouch model and zymosan challenge with flow cytometry. We confirmed that zymosan induction leads to depletion of mature neutrophils in the BM in both *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ and control *Runx1*$^{fl/fl}$ mice and stimulates release of a significant number of mature and immature neutrophils from bone marrow into the circulation (**Figure4.2.10 D**). We observed a shift towards a lower number of mature neutrophil in the blood of *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ mice, but the total number of neutrophils in circulation was comparable between *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ and control *Runx1*$^{fl/fl}$ mice (**Figure4.2.10 E**). Of importance, the recruitment of neutrophils to the air pouch membrane and exudate was significantly lower in *LysM*$^{cre/cre}$ *xRunx1*$^{fl/fl}$ mice (**Figure4.2.10 F**). Collectively, these results have further supported our data on the reduced capacity of RUNX1-deficient neutrophils to trans-migrate *in vitro* and infiltrate the tissue *in vivo* (**Fig 4.2.9**).

4.3 Conclusion

TFs have been recognised as major regulators in the commitment and functional regulation of several hematopoietic lineages. The majority of studies have focused on the role of TFs in the cell fate and polarisation of T cells (232), monocytes and macrophages (233). However, several fundamental questions on the TF network in neutrophils remain yet to be answered. Following integrated chromatin accessibility and transcriptomic profiling of neutrophils in an *in vivo* model of inflammation, we have functionally validated a list of TFs that potentially mediate neutrophil differentiation and inflammatory responses. We have demonstrated the requirement of Klf6 and Runx1 in neutrophil differentiation and the functional relevance of Rfx2 in neutrophil survival. Firstly, we made use of Hoxb8 neutrophils and demonstrated that Klf6 and Runx1 deficiency inhibit neutrophil maturation, including nuclear segmentation, expression of granular enzymes and mitochondrial function, and that Rfx2 knockout negatively neutrophil survival. Furthermore, using mice with conditional Runx1 deficiency, we have confirmed the importance of Runx1 in neutrophil differentiation and additionally demonstrated the regulation of Runx1 in CEPBe expression, thereby regulating neutrophil differentiation. Further functional investigation *in vitro* and *in vivo* demonstrated a lower migration capacity of Klf6-deficient and Runx1-deficient HoxB8 neutrophils.

Current study favours the use of HoxB8 myeloid progenitors as an physiologically relevant platform for in vitro neutrophil generation, as it has been evidenced in previous section that HoxB8 myeloid progenitors recapitulate normal neutrophil differentiation process and differentiate into mature neutrophil with full range of neutrophil effector functions (204, 208). We further explored the potential of HoxB8 myeloid progenitors for the functional analysis of TFs in neutrophils. With the use of the lentivirus-mediated CRISPR-Cas9 transduction, HoxB8 myeloid progenitor cells were efficiently transduced to generate cells with targeted TF knockout. Generated knockout could be easily validated by the absence of target protein expression as well as the genomic sequencing. Importantly, after CRISPR-Cas9 lentiviral transduction, HoxB8 myeloid progenitor cells maintain the potential of robust expansion *in vitro* and allow for the generation of neutrophils with specific TF knockout, therefore providing a genetically tractable model for investigating neutrophil phenotype and function through *in vitro* and *in vivo* experiments.

Neutrophil differentiation represents a continuous and highly coordinated process, which has been characterised by distinct morphology change, differential expression of surface markers, sequential formation of granular enzymes, increasing nuclear segmentation with the progress of differentiation (9). As described in previous chapter, phenotypic assessment of HoxB8 neutrophils has delivered useful insights about the immunophenotype of neutrophils under different differentiation stages, and it also provided a basis for downstream phenotypic characterisation of neutrophils with specific TF knockout. Phenotypical comparison between wild-type and knockout HoxB8 neutrophils suggested that deficiency of Klf6 and Runx1 suppressed neutrophil maturation and generated neutrophils with neutrophil precursor morphology, expression of immature surface makers and primary granular enzymes Mpo, and decreased nuclear segmentation, which help define the immature phenotype of Klf6-deficient and Runx1-deficient Hoxb8 neutrophils. Additionally, terminal neutrophil differentiation is featured with the decrease in their mitochondrial activity, and mature neutrophils possess a limited number of mitochondria and are defective in ATP production (218). Consistent with being with the immature phenotype, Klf6-deficient and Runx1-deficient HoxB8 neutrophils displayed higher levels of mitochondrial membrane potential and mitochondrial reparation, in comparison to wild-type cells. Unexpectedly, phenotypic assessment of HoxB8 neutrophils with specific TF knockout also revealed the role of Rfx2 in sustaining neutrophil survival, as evidenced by the increased rate of apoptosis in Rfx2-deficient neutrophils upon terminal differentiation and elevated apoptosis during inflammation in vivo. Hence, one might speculate that during different differentiation stages, TFs exert different regulator mechanisms to control neutrophil cell fate decision and development.

One major finding in current section is the intrinsic requirement of Klf6 and Runx1 for neutrophil maturation. Dysregulated expression of Klf6 closely associates with the development of acute myeloid leukaemia (30). Here we also demonstrated that Klf6 promotes the process of neutrophil maturation, as evidenced that deficient Klf6 expression in HoxB8 myeloid progenitors significantly attenuated neutrophil maturation. However, the molecular mechanism of how Klf6 regulates neutrophil maturation is yet to be investigated. Runx1 is crucial for emergent hematopoiesis and participate in neutrophil lineage commitment. Current results confirmed the importance of Runx1 in neutrophil maturation, as evidenced that Runx1 deficient Hoxb8 myeloid progenitors failed to differentiate into mature neutrophil. It is in agreement with dysregulated function of Runx1 found in the development of acute myeloid leukaemia (27).

Current study also made use loss-of-function animal model, in addition to in vitro system of HoxB8 neutrophils, to understanding how specific TFs regulate neutrophil differentiation. For instance, the importance of Runx1 in granulocytosis was further analysed in *Lysm*$^{Cre/cre}$*Runx1*$^{fl/fl}$ mice, in which both alleles of *Runx1* were disrupted in myeloid cell populations to generate myeloid conditional Runx1 knockout. The absence of Runx1 significantly inhibited neutrophil maturation, as evidenced by a decreased proportion of mature neutrophils in the bone marrow of mice with Runx1 conditional knockout. Runx1 regulates hematopoietic processes through direct regulation of its partner TFs, such as C/EBPe (234). We also observed a decreased level of C/EBPe expression in neutrophils from mice with Runx1 condition knockout, consolidating the notion that Runx1 regulates neutrophil differentiation by modulating the expression of C/EBPe. It is consistent with other studies supporting that the loss of Runx1 caused accumulation of neutrophil progenitor compartment in bone marrow (227, 235).

One important consequence of neutrophil maturation is to produce mature neutrophils with a full migratory machinery that enables efficient migration toward sites of inflammation (218). Indeed, mature neutrophils have the highest expression of gene that relate to cell migration and chemotaxis such that they are able to swarm rapidly toward the inflammatory sites (11). Importantly, HoxB8 neutrophils have been shown by multiple laboratories that they can be adoptively transferred into recipient mice and are recruited to sites of inflammation, from where they can be recovered and assessed for immune function (204, 205). Consistently, adoptive transfer of HoxB8 neutrophils were successfully used in current study to investigate the recruitment of neutrophils in response to inflammatory challenge. Our adaptive neutrophil transfer model demonstrated that neutrophils deficient in Runx1 or Klf6 could not efficiently reach the site of inflammation.

Overall, in current chapter, our work have managed to established HoxB8 myeloid progenitors with specific knockout of CEBPβ, Klf6, Runx1, Rfx2, RelB, Irf5 and JunB. Phenotypic assessment of HoxB8 neutrophils with specific TF knockout have supported that Klf6 and Runx1 promote neutrophil maturation, as evidenced that deficiency of Klf6 or Runx1 significantly attenuated neutrophil maturation, including granule proteins, nuclear envelope proteins, neutrophil segmentation, migration and mitochondrial metabolism. We additionally discovered that Runx1 regulates neutrophil differentiation in a C/EBPe-dependent manner, as evidenced by the finding that the absence of Runx1 suppressed C/EBPe expression, consequently causing impair production of mature neutrophils in the bone marrow. The importance of Klf6 in neutro-

phil maturation has been established known, however, the molecular mechanism of how Klf6 affect neutrophil maturation is yet to be determined.

5.0 TFs that mediate neutrophil activation

5.1 Introduction

Following the identification of Klf6 and Runx1 as TFs important in neutrophil differentiation, we subsequently investigated the functional relevance of other TFs that did not alter neutrophil development, including CEBPβ, RelB, Irf5 and JunB. Our integrated epigenetic and transcriptional profiling suggested that these TFs are associated with chromatin opening and expressed highly in neutrophils transitioning from blood into inflammatory sites, suggesting their involvement in neutrophil activation. To address this hypothesis, we investigated the effect of CEBPβ, RelB, Irf5 and JunB knockout on neutrophil transcriptome and effector functions, with a focus on the role for specific TFs in neutrophil inflammatory response.

Neutrophils have been shown to be transcriptionally active cells that alter their gene expression profiles as they migrate from the circulation into different tissue sites. Infection, chronic inflammation, and cancer differentially prime neutrophils to undergo prominent phenotypic changes with different gene expression patterns. For instance, bacterial infection activates neutrophils for augmented effector functions, which are powered by enhanced transcriptional activities in genes related to bactericidal activities including synthesis of granular proteins, ROS generation, phagocytosis, and chemotaxis (15). Tumour micro-environment primes neutrophils to favour tumour survival and progression, with the expression signature capturing downregulation of genes related to cytotoxicity, antigen presentation and phagocytosis (236). Thus, neutrophils are able to acquire novel functional properties tailored to the needs of different states, different differentiation stages and different micro-environment. However, the cell-intrinsic molecular regulators orchestrating the neutrophil transcriptional plasticity remain largely unexplored.

It has been recently proposed that neutrophils reshape the transcriptional landscape, powered by both well-known and uncharacterised TFs, to adopt alternative phenotypes and functions. Moreover, when infiltrated into different tissues, neutrophils acquire alternative phenotype and functions, which might be powered by the coordination between master regulator, such as PU.1, and different TFs to enable regional gene transcription in a tissue-specific manner, as evidenced by the tissue-restricted activation of TFs (13), which corroborate the notion that TFs might act as key regulators in neutrophil phenotype and inflammatory responses.

Upon inflammation, activated neutrophils exhibit prolonged lifespan and enhanced effector functions, such as phagocytosis of pathogens, intracellular killing in phagosomes with reactive oxygen species (ROS), and antimicrobial granule components (9). An outstanding question is how neutrophils gain augmented cell functionality during inflammation. One possible answer is that neutrophils adopt the TF networks that are ultimately activated the inflammatory signals to enable the transcriptional and functional activities, as hinted from single cell transcriptomic analyses of neutrophils under bacterial challenge (15).

To further understand the TF networks that mediate neutrophil inflammatory responses, we looked into the gene expression and chromatin accessibility in neutrophils *en route* to inflammatory sites and found an active transcriptional and epigenetic remodelling, featured with the selective activation of distinct sets of putative TFs during different transition stages (chapter 3). For instance, hematopoietic TFs, such as Runx1 and Klf6, were highly expressed and accessible in differentiating neutrophils and become downregulated during the bone-marrow-to-blood transition and signal-regulated TFs JunB, Irf5 and RelB are expressed more highly and accessibly by neutrophils infiltrating toward the inflamed tissue where they become activated, suggesting an incremental transcriptional programme of neutrophil activation during inflammation.

Using the CRISPR/Cas9 mediated system, we generated stable knockout cells for JunB, RelB, Irf5, Rfx2, Klf6, and Runx1 in Hoxb8 myeloid progenitor cells. Following 5 days of differentiation, Klf6 and Runx1 deficiency in progenitor cells blocked neutrophil differentiation, producing a mix of metamyelocytes and banded neutrophils, suggesting the importance of Klf6 and Runx1 in neutrophil maturation. In contrast, CEBPβ, RelB, Irf5 and JunB knockout did not alter the process of neutrophil differentiation and give rise to segmented mature neutrophils, which are phenotypically comparable to wild-type HoxB8 mature neutrophils, including surface marker phenotype, expression of granular enzymes, nuclear segmentation and mitochondrial function (chapter 4). Our integrated transcriptomic and chromatin accessibility analysis suggested that CEBPβ, RelB, Irf5 and JunB are progressively upregulated in neutrophils transiting from the blood into inflammatory sites. Therefore, the effects of CEBPβ, RelB, Irf5 and JunB knockout on neutrophil function and inflammatory responses remain to be determined.

5.2 Results

5.2.1 CEBPβ, RelB, Irf5 and JunB specifically regulates neutrophil gene transcripts

Neutrophil activation is a multifaceted process that involves several biological pathways and it is difficult to pre-decide a list of genes based on the current knowledge. Therefore, to better understand neutrophil functions and their regulation, we next sought to make use of transcriptional profiling by mRNA sequencing to analyse the global gene expression of unstimulated and Zymosan-stimulated wild-type and TF (RelB, Irf5, JunB) knockout neutrophils. Total RNA samples were collected from wild-type HoxB8 neutrophils differentiated for 0, 1, 3, 5 days and TF-knockout HoxB8 neutrophils differentiated for 5 days, followed by zymosan stimulation for a further 2 hours or left unstimulated for mRNA sequencing. Principal component analysis (PCA) of differentially expressed genes (adjusted $p<0.05$) created a map of the clusters of cells (**Figure 5.2.1 A**). As expected, PCA clearly separated wild-type and TF (CEBPβ, RelB, Irf5, JunB)-knockout HoxB8 neutrophils differentiated for 5 days from those wild-type cells with lower maturity (differentiated for day 0, 1, 3) (**Figure 5.2.1A**). Consistent with previous results, wild-type HoxB8 myeloid progenitor cells progressive differentiate into mature neutrophils and knockout of CEBPβ, RelB, Irf5, JunB did not significantly affect neutrophil maturation. Additionally, Zymosan-stimulated HoxB8 neutrophils were clearly separated from unstimulated cells, indicating a process of neutrophil activation in response to zymosan stimulation. Notably, TF-knockout cells were grouping distantly to wild-type cells, in both stimulated and unstimulated conditions, suggesting overall distinct transcriptional profiles between wild-type and TF-knockout HoxB8 neutrophils. This was reflected in the number of differentially expressed genes, which were defined by the threshold of adjusted $p < 0.05$ and $\log_2$fold change (FC) > 1. Under zymosan stimulation, CEBPβ-deficient, RelB-deficient, Irf5-deficient and JunB-deficient HoxB8 neutrophils had 2914, 3047, 3691 and 6292 differentially expressed genes in comparison to wild-type HoxB8 neutrophils, respectively (**Figure 5.2.1 C-F**). Notably, 2188, 3056, 2618, 4671 genes were significantly downregulated in CEBPβ-deficient, RelB-deficient, Irf5-deficient and JunB-deficient HoxB8 neutrophils, respectively, compared to wild-type cells under zymosan stimulation (**Figure 5.2.1 B**).

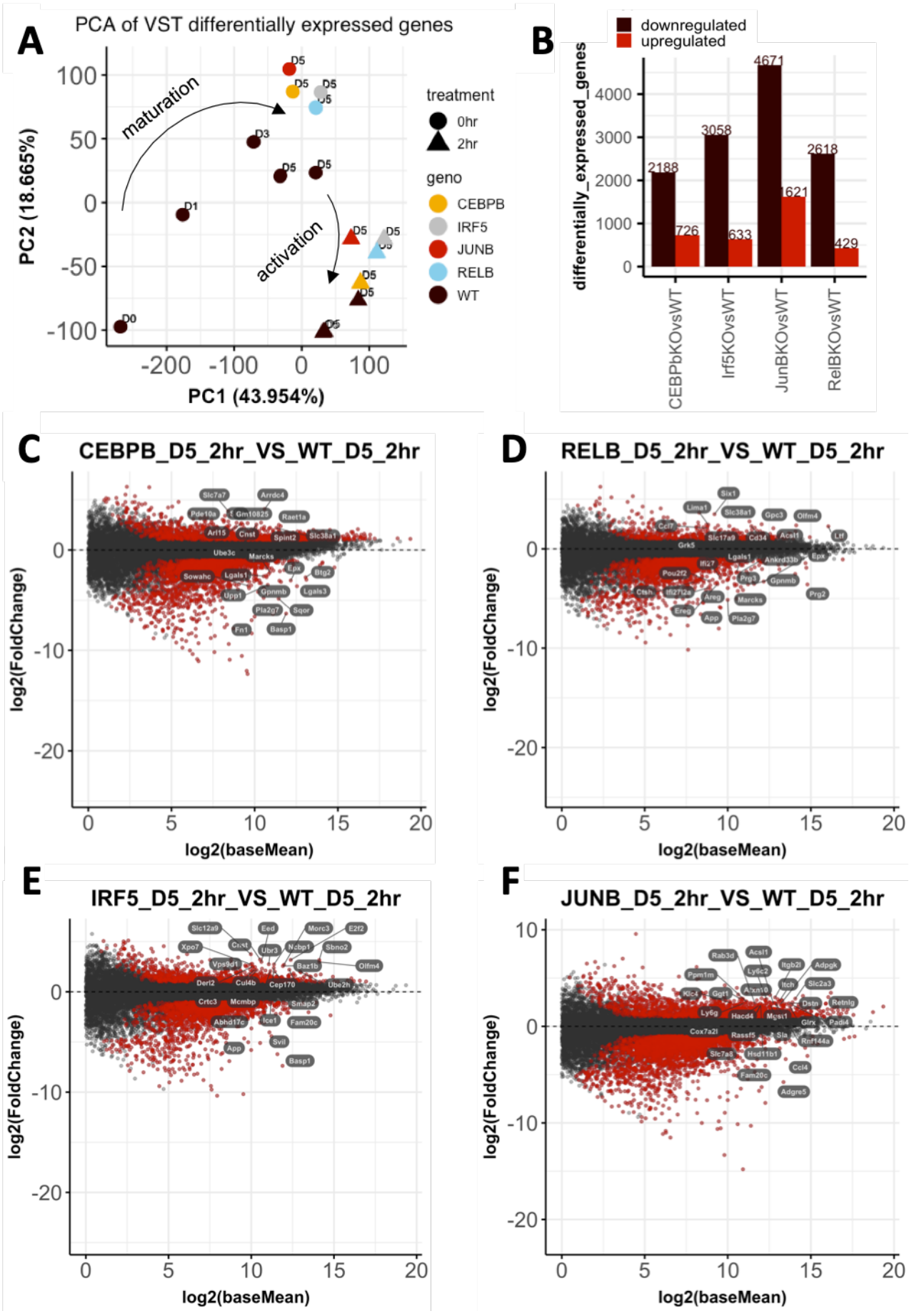

A
PCA of VST differentially expressed genes
PC2 (18.665%)
PC1 (43.954%)
maturation
activation
treatment
0hr
2hr
geno
CEBPB
IRF5
JUNB
RELB
WT
B
downregulated
upregulated
differentially_expressed_genes
2188
726
3058
633
4671
1621
2618
429
CEBPbKOvsWT
Irf5KOvsWT
JunBKOvsWT
RelBKOvsWT
C
CEBPB_D5_2hr_VS_WT_D5_2hr
D
RELB_D5_2hr_VS_WT_D5_2hr
E
IRF5_D5_2hr_VS_WT_D5_2hr
F
JUNB_D5_2hr_VS_WT_D5_2hr
log2(FoldChange)
log2(baseMean)

Figure 5.2.1 CEBPβ, RelB, Irf5 and JunB specifically regulates neutrophil gene transcripts.
(A) PCA analysis of differentially expressed genes from wild-type and TF-deficient HoxB8 neutrophils (n=2) stimulated with 25 μg/ml Zymosan (triangle) or left unstimulated (circle) for 2 hours. Wild-type and TF-deficient cells were incubated int the medium supplemented with G-CSF to induce the differentiation for different days, and it is followed by 25 μg/ml Zymosan stimulation for 2 hours or left unstimulated. Total RNA samples were collected from wild-type and TF-deficient HoxB8 neutrophils differentiation for 0, 1, 3, 5 days and with or without zymosan stimulation for mRNA sequencing. Line arrows indicate the the process of neutrophil maturation for different days and activation under zymosan stimulation. Feature counts were made with the assistance from Dr Tariq Khoyratty. **(B)** Summary plot of the number of upregulated (red) and downregulated (black) differentially expressed genes from wild-type and TF-deficient HoxB8 neutrophils differentiated for different days and with or without zymosan stimulation (**C-F**) MA plots depicting the effect of C/EBPβ knockout (**C**), RelB knockout (**D**), Irf5 knockout (**E**), and JunB knockout (**E**) on Zymosan stimulated HoxB8 neutrophils differentiated for 5 days. Differentially expressed genes (TF knockout vs WT, fold change >1 and padj < 0.01) are highlighted in red. WTD1-5 (day 1-5 differentiation of wild -type Hoxb8 myeloid progenitor cells).

5.2.2 RelB, Irf5 and JunB regulate genes that relate to neutrophil inflammatory responses

Through our chromatin accessibility analysis of neutrophils from mice subject to air pouch model of acute inflammation, we searched for consensus binding motifs for the TF CEBPβ, RelB, Irf5 and JunB, and identified the genes that were proximal to these motifs. Then, we identified the putative target genes for each TF by intersecting those genes with the differentially expressed genes in activated CEBPβ-deficient, RelB-deficient, Irf5-deficient and JunB-deficient HoxB8 neutrophils. By this analysis, we found that JunB deficiency had the maximal number of putative target genes, 240 of which were significantly down-regulated compared in JunB-deficient HoxB8 neutrophils, while RelB, Irf5 and CEBPβ had relatively more limited number of putative target genes, 156, 130 and 170 of which are significantly down-regulated, respectively, in comparison to wild-type control cells (**Figure 5.2.2 A**), suggesting the differential impacts of CEBPβ, RelB, Irf5 and JunB deficiency on neutrophil gene expression in response to stimulation

Gene ontology (GO) annotation analysis for those downregulated genes demonstrated that CEBPβ is more likely to involve the regulation of biological cellular processes, as evidenced that genes that are significantly downregulated in CEBPβ-deficient HoxB8 neutrophils are more enriched in the GO terms for mitotic cell cycle process (GO: 1903047) and regulation of cellular process (GO: 0022402) (**Figure 5.2.2 B**), while RelB, Irf5 and JunB are more important in mediating neutrophil inflammatory responses. For instance, GO terms for immune system process (GO: 0002376), immune process (GO: 0006955), defence response (GO: 0006952), and response to external stimulus (GO: 0009605) are commonly highly enriched from the list of genes that were significantly downregulated in RelB-deficient, Irf5-deficient and JunB-deficient HoxB8 neutrophils in comparison to wild-type cells (**Figure 5.2.2C-E**). Notably, JunB also participates in regulating some key inflammatory responses, as evidenced by the enrichment of downregulated genes in the GO terms for positive regulation of immune system process (GO: 0002684), positive regulation of cytokine production (GO: 0001819), and positive regulation of response to stimulation (GO: 0048584) (**Figure 5.2.2E**). Overall, these GO annotation analysis of genes downregulated in TF-deficient HoxB8 neutrophils suggested that RelB, Irf5 and JunB might involve neutrophil effector functions and their deficiency in neutrophils affect neutrophils inflammatory responses to stimulation.

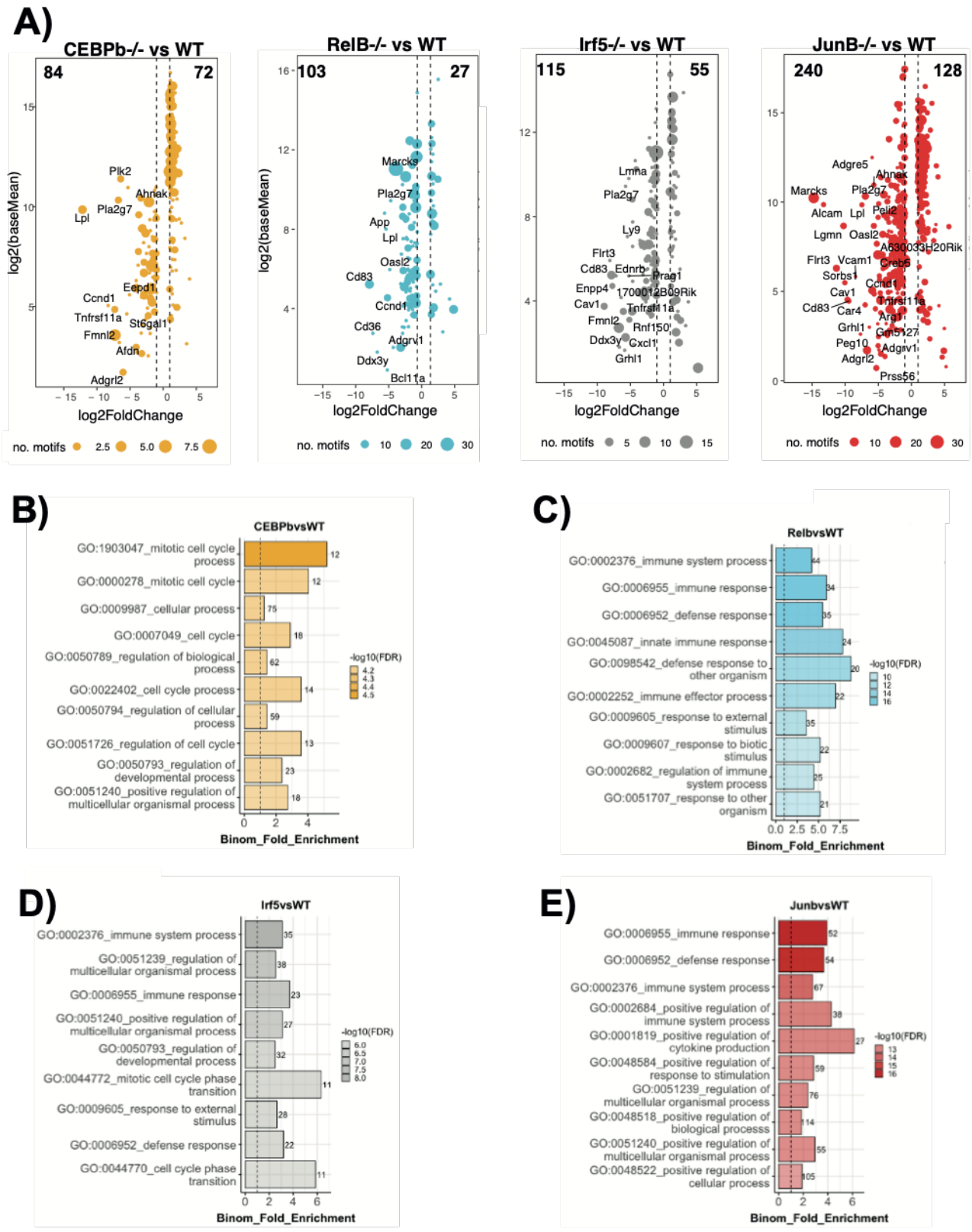


Figure 5.2.2 RelB, Irf5 and JunB mediate neutrophil inflammatory responses.
(A) Volcano plot of putative target genes from wild-type and TF-deficient HoxB8 neutrophils (n=2) differentiated for 5 days and then stimulated with 25 µg/ml Zymosan (triangle) or left un-stimulated (circle) for 2 hours. Putative target genes for each TF were identified by stratifying the differentially expressed genes (adjusted $p < 0.05$, fold change > 2) between wild-type and TF-deficient HoxB8 neutrophils with the identified binding sites for corresponding TFs (FIMO p-value < 0.0001) within differentially accessible ATAC-seq peaks (padj < 0.05, fold change > 1.5) in gene vicinity from neutrophils from air pouch model of acute inflammation. Figure A was made by Dr Tariq Khoyratty. (**B-E**) The top GO biological-process terms enriched for pu-

tative target genes downregulated in CEBPβ-deficient (**B**), RelB-deficient (**C**), Irf5-deficient (**D**) and JunB-deficient (**E**) HoxB8 neutrophils with the highest statistical significance. The vertical axis was the GO biological process terms, and the horizontal axis was the Binom fold enrichment between corresponding TF knockout and wild-type cells. The number of affected genes involved in the GO terms was labelled in corresponding bars. WT (day 5 differentiation of wild -type Hoxb8 myeloid progenitor cells). (**B-E**) Feature counts were made with the assistance from Dr Tariq Khoyratty.

5.2.3 RelB, Irf5 and JunB regulate neutrophil phagocytosis

Neutrophils are major phagocytic cells that execute rapid inflammatory responses, including phagocytosis of invading pathogens. To functionally validate the importance of specific TFs in neutrophil activation, we determined the functional consequence of selected TF knockout in neutrophil effector functions, such as phagocytosis, ROS generation, NETosis and bacterial killing effects. In previous sections, HoxB8 neutrophils highly recapitulate neutrophil inflammatory responses, therefore, are useful for *in vitro* studies of neutrophil inflammatory responses. In current section, we firstly investigated the phagocytic capacity of wild-type and TF-deficient HoxB8 neutrophils to see if TF knockout affects neutrophil phagocytosis (**Figure5.2.3 A**). Wild-type and TF-deficient HoxB8 myeloid progenitor cells were differentiated for 5 day, followed by incubation with fluorescent *E. coli* at a multiplicity of infection (MOI) of 10 in complete RPMI 1640 medium for 15 minutes. Flow cytometry assessment and quantification of green fluorescence demonstrated that both wild-type and CEBPβ-deficient neutrophils are similar in their capacity of phagocytosis, as both were capable of phagocytizing *E.coli* efficiently and produced comparable levels of fluorescent *E. coli*, while RelB, Irf5 and JunB deficiency negatively affected the phagocytic ability of neutrophils, as neutrophils with RelB, Irf5 or JunB deficiency were less capable of phagocytizing *E.coli*. Having generated transcriptional profiling of wild-type and TF-deficient HoxB8 neutrophils, we looked into the differentially expressed genes enriched for the GO term for phagocytosis (GO:0006909) and found that HoxB8 neutrophils with CEBPβ, RelB , Irf5 and JunB deficiency have a number of genes that are downregulated in comparison to wild-type cells. For instance, decreased expression of phagocytic genes *Pear1*, *Lyar*, *P2rx7*, *Mesd* were commonly observed in RelB-deficient, Irf5-deficient and JunB-deficient neutrophils. Additionally, JunB deficiency had the additionally effect on a number of genes involved in phagocytosis, such as *Cd302, Icam5, Itgb, Tgm2* (**Figure5.2.3 B**). Overall, these results suggest an important role of Relb, Irf5 and JunB in neutrophil phagocytosis.

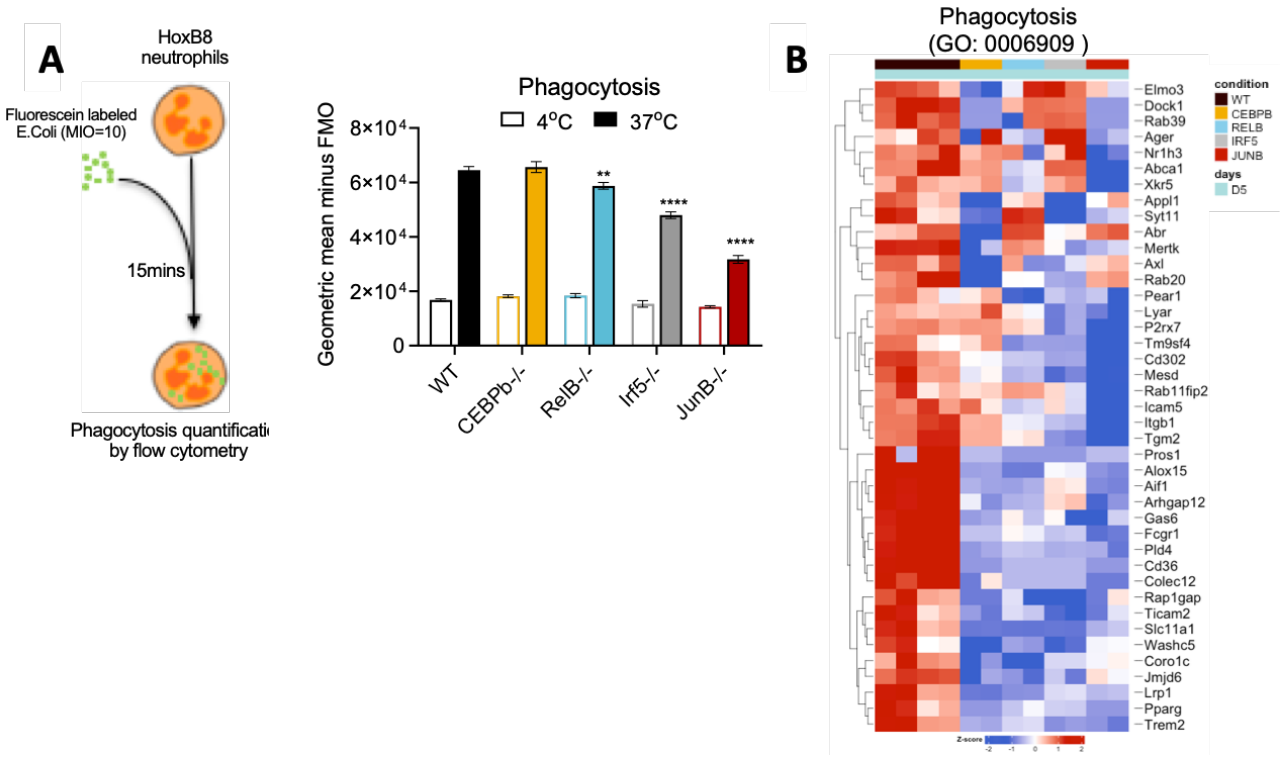


Figure 5.2.3 RelB, Irf5 and JunB regulate neutrophil phagocytosis.
(A) Phagocytosis of fluorescent E. coli by WT and CEBPβ-deficient, RelB-deficient, Irf5-deficient or JunB-deficient HoxB8 neutrophils, measured by flow cytometry. Wild-type and TF-deficient HoxB8 myeloid progenitor cells were incubated in medium supplemented with G-CSF to induce the differentiation for 5 days and it is followed by the incubation with fluorescent E. coli at a multiplicity of infection (MOI) of 10 in complete RPMI 1640 medium for 15 minutes at 37°C and 4°C. 4°C incubation is the negative control. Phagocytosis was quantified by the fluorescence of fluorescein conjugated E. coli across the neutrophil populations and analysed by normalising fluorescence signals of stained cells to unstained cells. (**B**) Z-scores of differentially expressed genes enriched for phagocytosis (GO: 0006909). Wild-type and TF-deficient HoxB8 myeloid progenitors cells were incubated int the medium supplemented with G-CSF to induce the differentiation for 5 days.Total RNA samples were collected from wild-type and TF-deficient HoxB8 neutrophils for mRNA sequencing. Differentially expressed genes were defined by the threshold of adjusted $p < 0.05$ and fold change >2.

5.2.4 RelB and JunB mediate neutrophil ROS generation and NETosis

ROS generation by neutrophils play an important role in antimicrobial host defence and inflammation, and deficient ROS generation results in recurrent and severe bacterial infections. . We next looked at ROS generation by wild-type and TF-deficient HoxB8 neutrophils. Wild-type and TF-deficient HoxB8 myeloid progenitor cells were differentiated for 5 days followed by incubation with PMA and intracellular ROS indicator DHR123, to induce intracellular ROS generation in complete RPMI 1640 medium for 15 minutes. Flow cytometry quantification of intracellular ROS generation demonstrated that wild-type neutrophils were efficient in producing intracellular ROS in response to PMA stimulation, and that CEBPβ-deficient and Irf5-deficient neutrophils produced intracellular ROS at levels comparable to wild-type cells. Notably, HoxB8 neutrophils with RelB and JunB deficiency are impaired in generating intracellular ROS, as evidenced the lower levels of intracellular ROS generation in response to PMA

stimulation (**Figure5.2.4 A**). To determine how RelB and JunB deficiency affect neutrophil intracellular ROS generation, we analysed the mRNA sequencing results generated from wild-type and TF-deficient neutrophils with a focus on the differentially expressed genes that are enriched for the GO term for ROS synthesis (GO: 1903409). It was found that decreased expression levels of key genes involved in phagocytosis were found in RelB-deficient and JunB-deficient neutrophils. For instance, RelB deficiency specifically reduced the expression of *Cyba* and *Ncf1*, two components of the NADPH oxidase complex. Additionally, both RelB-deficient and JunB-neutrophils expressed higher levels of genes that relate to the anti-oxidant system, such as *Ctns* and *Foxo3* (**Figure5.2.4 B**), suggesting the impaired capacity of intracellular ROS production in RelB-deficient and JunB-deficient neutrophils.

Induction of the classical microbicidal mechanism NETosis has been shown to be dependent on ROS generation, in which NADPH oxidase is the main source, to extrude de-condensed chromatin and granule components, a mechanism to eliminate extracellular pathogens. It was demonstrated in previous section that HoxB8 neutrophils replicate neutrophil inflammatory responses, including NETosis. Having demonstrated the involvement of RelB and JunB in neutrophil ROS generation, we subsequently determined the capacity of wild-type and TF-deficient neutrophils. Wild-type and TF-deficient HoxB8 myeloid progenitor cells were differentiated with G-CSF for 5 days and NETosis of neutrophils were induced by the overnight incubation with PMA and ionomycin, followed by fluorescent staining for citrullinated histone and neutrophil elastase. Fluorescent imaging and quantification suggested that in comparison to wild-type neutrophils that produce NETosis efficiently, neutrophils with RelB and JunB knockout are deficient in producing NETosis, as evidenced by the significantly lower percentages of neutrophils that undergo NETosis in response to PMA and ionomycin stimulation, whereas CEBPβ and IRF5 deficiency do not affect neutrophil NETosis (**Figure5.2.4 C**). This is consistent with lower intracellular ROS generation observed in RelB-deficient and JunB-deficient neutrophils. Overall, these results support the notion that RelB and JunB are crucial for neutrophil ROS generation and NETosis in response to stimulation.

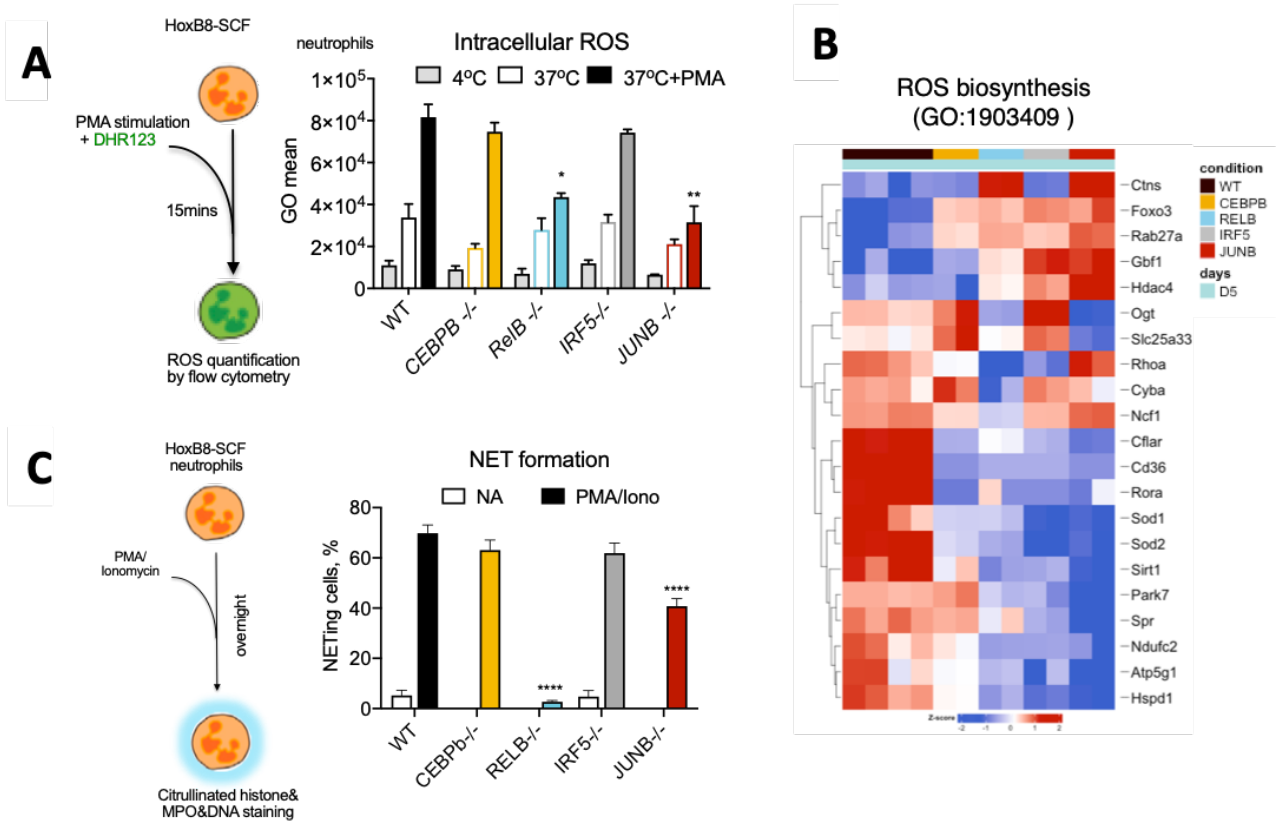


Figure 5.2.4 RelB and JunB mediate neutrophil ROS generation and NETosis.
(A) PMA induced intracellular ROS production by HoxB8 neutrophils were assessed by flow cytometry with dihydrorhodamine 123 (DHR123), and then analysed by normalising fluorescence signals of stained cells to unstained cells. Wild-type and TF-knockout HoxB8 myeloid progenitors were differentiated in medium supplemented with G-CSF to induce differentiation for 5 days, and it is followed by the overnight incubation with PMA to stimulate intracellular ROS generation. Data are shown as means and SD from three independent experiments with replicates. (**B**) Z-scores of differentially expressed genes enriched for phagocytosis (GO:1903409). Wild-type and TF-deficient HoxB8 myeloid progenitors cells were incubated int the medium supplemented with G-CSF to induce the differentiation for 5 days.Total RNA samples were collected from wild-type and TF-deficient HoxB8 neutrophils for mRNA sequencing. Differentially expressed genes were defined by the threshold of adjusted p < 0.05 and fold change >2. (**C**) NETOsis quantification in differentiating HoxB8 neutrophils. Wild-type and TF-knockout HoxB8 myeloid progenitors were differentiated in medium supplemented with G-CSF to induce differentiation for 5 days, and it is followed by the overnight incubation with PMA and ionomycin to induce NETosis. NET formation in response to PMA and ionomycin stimulation were assessed by staining with fluorescently labelled anti-citrullinated-His3 and anti-Mpo antibodies and SYTOX DNA staining. The data are expressed as percentages of neutrophils undergoing NETosis out of at least 200 cells counted from different fields and independent replicates. Data are shown as means and SD derived from three independent experiments. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.

5.2.5 RelB and JunB mediate neutrophil bacterial killing activities.

Neutrophils are professional effector cells that control bacterial and fungal infection by phagocytosis, ROS generation, and NETosis. Following phagocytosis by neutrophils, microbes are exposed to reactive oxygen species and antimicrobial peptides, which effectively kill and digest most microorganisms (9). Having demonstrated the importance of RelB and JunB in neutrophil inflammatory responses, we subsequently measure the bactericidal capacity of wild-type and TF-deficient HoxB8 neutrophils by measuring *S. aureus* survival after phagocytic interaction with neutrophils. Opsonised *S. aureus* were combined with PMN at a ratio of 10:1 (CFU:PMN ratio) and incubated at 37°C for up to 90 minutes. After incubation, *S. aureus* phagocytosed by neutrophils were released and plated on LB agar for overnight incubation. Quantification of surviving *S. aureus* demonstrated that RelB and JunB deficiency impair the capacity of neutrophils to kill *S. aureus*, as evidenced by higher numbers of *S. aureus* colonies after incubation with RelB-deficient and JunB-deficient neutrophils in comparison to wild-type cells (**Figure5.2.5**). This is consistent with the interior capacity of RelB-deficient and JunB-deficient neutrophils in phagocytosis, ROS generation and NETosis.

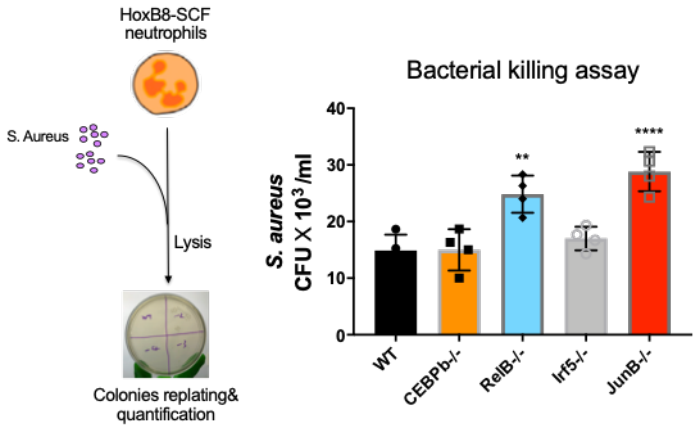


Figure 5.2.5 RelB and JunB mediate neutrophil bacterial killing activities.
Bactericidal activities of HoxB8 neutrophils toward *S. aureus*. Wild-type and TF-deficient HoxB8 myeloid progenitor cells were differentiated in medium supplemented with G-CSF to induce the differentiation for 5 days. Then, neutrophils in suspension were incubated with *S. aureus* at a CFU:PMN ratio of 1:10 at 37°C for up to 90 minutes. After incubation, HoxB8 neutrophils were washed to remove excessive *S. aureus* and then lysed to release surviving bacteria, which were plated into the LB agar plate for overnight incubation before quantification of surviving *S. aureus* colonies. Values represent absolute CFU counts generated by surviving bacteria. Data are shown as means and SD derived from at least three independent experiments. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.

5.2.6 RelB, Irf5 and JunB mediate pro-inflammatory cytokine and chemokine production.

Besides their involvement in phagocytosis, ROS generation and NETosis, neutrophils participate in regulating the inflammation processes through the *de novo* production of inflammatory cytokines and chemokines. To investigate the functional role of specific TFs in the ability of neutrophils to produce inflammatory mediators, we next compared the transcriptome of wild-type and TF-deficient HoxB8 neutrophils by mRNA sequencing to check differentially expressed genes that are enriched for the GO terms for cytokine production (GO: 0001816) and chemokine production (GO: 0032602), two crucial neutrophil inflammatory processes. Such a GO annotation analysis demonstrated that wild-type HoxB8 neutrophils respond to zymosan stimulation with strong regulation of a wide variety of inflammatory molecules, including pro-inflammatory cytokines, interleukins (ILs), colony-stimulating factors as well as CC chemokines (**Figure 5.2.6 A&B**). Notably, RelB, Irf5 and JunB deficient neutrophils demonstrated reduced expression of cytokines and chemokines, including but not limited to C-X-C motif) ligand 1 (Cxcl1), Cxcl2, C–C motif chemokine ligand 2 (Ccl2), TNF-α and Il-6, whereas CEBPβ does not affect the production of cytokines and chemokines by neutrophils, as evidenced by the similar expressional levels of cytokines and chemokines between CEBPβ-deficient and wild-type cells (**Figure 5.2.6 A&B**).

To complement the transcriptional profiling carried out in wild-type and TF-deficient HoxB8 neutrophils, we next determined the mRNA levels of pro-inflammatory cytokines, such as Il1a, Il1β, TNF-α, Il-6, and chemokines, such as Ccl2, Cxcl2, in HoxB8 neutrophils under zymosan stimulation. RNA samples were collected from wild-type and TF-deficient neutrophils at 1h and 2h post stimulation with zymosan for qPCR analysis. Consistent with mRNA sequencing results, the expression al levels of pro-inflammatory cytokines and chemokines, such as *Il1a*, *Il1β*, *Il6*, *Ccl2*, *TNF-α* and *Cxcl2* were rapidly and significantly increased with Zymosan stimulation in wild-type HoxB8 neutrophils, while no significant difference was observed between wild-type and CEBPβ-deficient cells (**Figure 5.2.6 C**). In comparison to wild-type cells, RelB-deficient, Irf5-deficient and JunB-deficient neutrophils exhibited decreased mRNA expression of *Il1b*, *TNF-α*, *Ccl2*, *Il1a*, *Il6*, *Cxcl2* in response to zymosan stimulation (**Figure 5.2.6 C**).

To further consolidate the involvement of RelB, Irf5 and JunB in cytokine and chemokine production, we next made use of proteome arrays, which is an antibody-pair-based method to simultaneously detect the relative levels of 36 different extracellular cytokines and chemokines

on nitrocellulose membranes (**Figure 5.2.6D**). Wild-type and TF-deficient HoxB8 myeloid progenitor cells were differentiated for 5 days into mature neutrophils, followed by zymosan stimulation for a further 2 hours. Then, supernatants were collected for the analysis by cytokine arrays. In response to zymosan stimulation, wild-type HoxB8 neutrophils secreted large amounts of inflammatory cytokines, including TNF-α and Il1-ra, and chemokines, such as Ccl2, Ccl3, Ccl4, and Cxcl10 into the supernatants, and comparable levels of those cytokines and chemokine were observed in the supernatant from CEBPβ-deficient HoxB8 neutrophils (**Figure 5.2.6 D**). JunB and RelB have strong impacts on neutrophil cytokine and chemokine production, as evidenced by the lower levels of Ccl2, Ccl3, Ccl4, Cxcl2, Il1-ra production by JunB-deficient cells and lower levels of Ccl2, Il1-ra, TNF-α and Cxcl10 secreted from RelB-deficient cells, while Irf5 deficiency produced lower levels of Ccl2 and Il1-ra production in comparison to wild-type cells (**Figure 5.2.6 D**). These results are consistent with previous mRNA sequencing and qPCR results and collectively support the importance of RelB, Irf5 and JunB in producing inflammatory cytokines and chemokines by neutrophils.

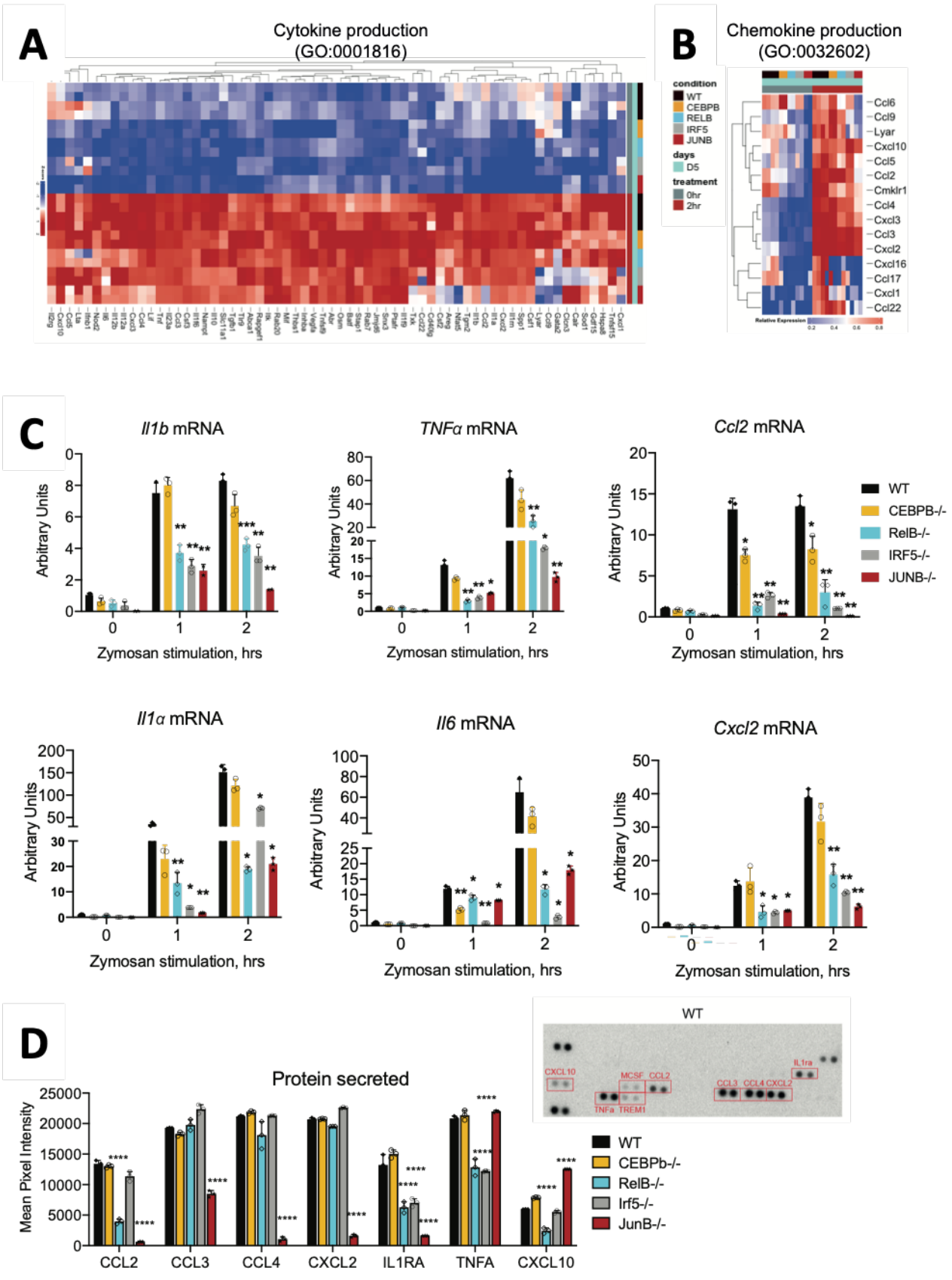


Figure 5.2.6 RelB, Irf5 and JunB mediate neutrophil pro-inflammatory production. (A&B) Z-scores of the differentially expressed genes enriched for cytokine production (GO:0001816) and chemokine production (GO:0032602). Wild-type and TF-deficient HoxB8 myeloid progenitors cells were incubated int the medium supplemented with G-CSF to induce the differentiation for 5 days.Total RNA samples were collected from stimulated or unstimu-

lated wild-type and TF-deficient HoxB8 neutrophils for mRNA sequencing. Differentially expressed genes were defined by the threshold of adjusted $p < 0.05$ and fold change >2. (**C**) Cytokine and chemokine levels mRNA profiles of wild-type HoxB8 neutrophils stimulated with Zymosan (25µg/ml) for one and two hours. Total RNA was extracted and 200ng total RNA (as determined by Nanodrop quantification) were reversely transcribed to cDNA. Transcript levels of Il1a, Il1β, Il6, Ccl2, TNF-α, Cxcl2 were measured with real-time PCR and gene expression was analysed in normalisation to the housekeeping gene Hrpt and using the change in threshold ΔΔCt-method. Data are shown as means and SD from three independent experiments. Statistical comparison was analyzed by two-way ANOVA, **P<0.01, ***<0.001, ****P<0.0001. (**D**) Cytokine and chemokine protein profiles of wild-type and TF-deficient HoxB8 neutrophils. Two million of wild-type and TF-deficient HoxB8 neutrophils were seeded into 2ml of complete RPMI1640 medium before they are subject to Zymosan stimulation for two hours. Supernatants from each type of HoxB8 neutrophils were then collected for the analysis by proteome arrays. Experiments were performed in duplicates and results are shown as means and SD. Statistical comparison was made by two-way ANOVA. **P<0.01, ***<0.001, ****P<0.0001. Top-right corner: Cytokines and chemokines secreted from wild-type HoxB8 neutrophils challenged with Zymosan for two hours, measured by the proteome array.

5.2.7 RelB, Irf5 and JunB mediate neutrophil activation *in vivo*.

Neutrophils from the blood can be rapidly infiltrated into sites of infection or inflammation, where they modify their functional responses after being exposed to multiple inflammatory signals, through the process termed neutrophil activation (9). In previous section, we demonstrated the involvement of RelB, Irf5 and JunB in cytokine and chemokine production. To determine whether the selected TFs affect the ability of neutrophils to produce inflammatory mediators *in vivo*, we again utilised adoptive neutrophil transfer into an acute inflammation model, as described previously (chapter 4). Briefly, wild-type and TF-deficient HoxB8 myeloid progenitor cells were differentiated for 5 days and labelled with CellTracker™ Red and CellTracker™ CFSE. Cells were mixed at an equal ratio before adoptive transfer into mice subject to air pouch model of acute inflammation, in which wild-type and TF-deficient HoxB8 neutrophils competitively migrate toward the air pouch membrane and exudate. 4 hours post zymosan challenge, pro-IL1b levels in HoxB8 neutrophils recruited into the blood, air pouch membrane and exudate were measured by flow cytometry (**Figure 5.2.7 A**). Consistent with *in vitro* stimulation experiments, wild-type HoxB8 neutrophils rapidly increased the expression of pro-IL1b when infiltrated into inflammatory sites, suggesting the process of neutrophil activation. In comparison, RelB-deficient, Irf5-deficient and JunB-deficient HoxB8 neutrophils produced lower levels of pro-IL1b during the transition from the blood into air pouch membrane and air pouch exudate whereas no significant difference was observed between wild-type and TF-deficient neutrophils in circulation (**Figure 5.2.7 B**). In comparison, CEBPβ-deficient neutrophils were similar to wild-type cells in the levels of neutrophil recruitment and activation at inflammatory sites (**Figure 5.2.7 B**). Overall, these results suggest that RelB, Irf5 and JunB significantly contribute the ability of neutrophils to produce inflammatory mediators at the sites of inflammation *in vivo.*

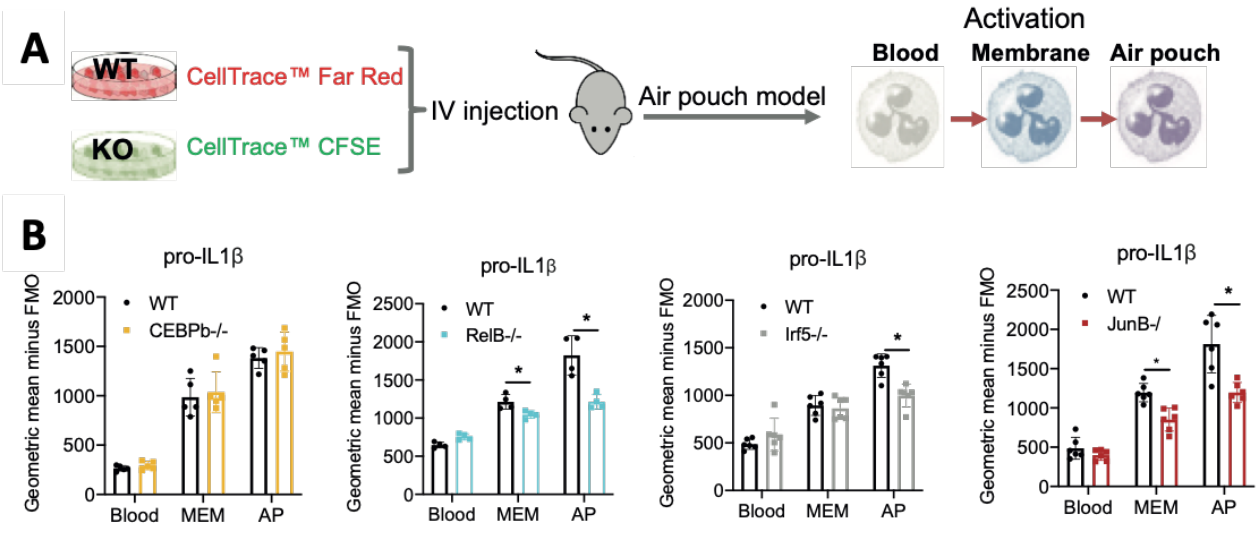


Figure 5.2.7 RelB, Irf5 and JunB mediate neutrophil activation *in vivo*.
(**A**) Experimental set-up of differentially labelled wild-type and TF-deficient HoxB8 neutrophils adoptively transferred into mice subject to air pouch model of acute inflammation. 4 hours post zymosan challenge, the activation of neutrophils was assessed by the levels of pro-IL1b in differentially labeled HoxB8 neutrophils recovered from the blood, air-pouch membrane and air pouch exudate. (**B**) Expression of pro-IL1β, as measured by flow cytometry, in HoxB8 neutrophils recovered from the bone marrow, blood, air pouch membrane and air pouch exudate of mice subjected to the air pouch model and zymosan challenge. All results are shown as means and SD derived from at least three mice within one experiment. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.

5.3 Conclusion

Neutrophils migrate from the blood into inflammatory sites in response to inflammation, during which they become activated for augmented effector functionality and protect the host by phagocytizing, killing and digesting bacterial and fungal pathogens. However, the transcriptional regulatory networks underlining neutrophil activation remain largely unexplored. Prior to the current study, we made use of integrated transcriptional and chromatin analyses of neutrophils during acute inflammation and identified distinct sets of putative TFs associated with control of neutrophil inflammatory responses. In the current section, we have demonstrated the important role of RelB, Irf5 and JunB in mediating neutrophil inflammatory responses. Analysis of the differentially expressed genes in wild-type and TF-deficient neutrophils revealed decreased expressions of key genes that relate to phagocytosis, ROS generation and cytokine production in RelB-, Irf5-, and JunB-deficient neutrophils (**Figure 5.2.8**). Additionally, using neutrophil function assays, such as phagocytosis, ROS generation, NETosis and bacterial killing assays, we have validated the functional relevance of RelB, Irf5 and JunB in promoting neutrophil inflammatory responses. Furthermore, using *ex vivo* and *in vivo* approaches, we have demonstrated the lower capacity of neutrophils with RelB, Irf5 or JunB knockout to produce pro-inflammatory mediators *in vivo*, whereas CEBPβ do not significantly affect neutrophil inflammatory responses.

Neutrophil activation is accompanied by enhanced effector functions, which result from increased expression of genes that associate with pathogen clearance. For instance, in bacteria-challenged host, bacterial infection triggered significant unregulations of genes that relate to synthesis of granular proteins, NADPH oxidase complex, and phagocytosis, suggesting the immune adaption of neutrophils in response to bacterial challenge In current study, using HoxB8 neutrophils, we obtained gene expression profiles of unstimulated neutrophils and zymosan-stimulated neutrophils, to determine changes in gene expression characteristic of neutrophil activation, and consequently found the overall up-regulation of neutrophil functions related to bactericidal activities, including phagocytosis, ROS generation and cytokine production in activated HoxB8 neutrophils. This is consistent with augmented neutrophil functionality observed in other studies using human and mouse primary cells (9, 61) and corroborate the notion that HoxB8 neutrophils represent a physiologically relevant platform for investigating neutrophil inflammatory responses *in vitro*. Additionally, enhanced expression of genes involved in immune effector processes observed in wild-type neutrophils has also provided the

molecular basis for investigating how specific TF knockout affects neutrophil inflammatory responses.

To elucidate the transcriptional regulatory networks that shape neutrophil functions during inflammation, we used the air pouch model of acute inflammation and conducted integrated chromatin accessibility and transcriptomic profiling of neutrophils from the bone marrow, which led us to the discovery of putative key transcriptional regulators of neutrophil function *en route* to inflamed tissue. In differentially accessible chromatin regions between blood and air pouch membrane and exudate, we identified binding motifs for TFs, such as CEBPβ, RELB, IRF5, JUNB which promote neutrophil maturation. As previously mentioned, we used a loss-of-function approach to investigate how specific TFs affect neutrophil phenotype and function, using CRISPR/Cas9 targeted deletion in the *ex vivo* system of HoxB8 neutrophils Deficient expression of these TFs do not affect neutrophil maturation, nor do they impair the capacity of neutrophils to migrate into tissues. These TFs instead contributed to cytokine and chemokine expression and production and various effector functions, such as phagocytosis, generation of ROS, bacterial killing or NETosis. In current section, transcriptional profiling by mRNA sequencing and *in vitro* functional assays were used for evaluating the functional role of CEBPβ, RelB, Irf5 and JunB in mediating neutrophil inflammatory responses. Our transcriptomic analysis of wild-type and TF-deficient HoxB8 neutrophils demonstrated RelB, Irf5 and JunB preferentially participate in regulating immune effector processes, whereas CEBPβ regulates cell cycle and cellular processes. CEBPβ is known to regulate the cell cycle to increase neutrophil generation during inflammation (93). However, the functional relevances of RelB, Irf5 and JunB are yet to be established in the context of neutrophil inflammatory responses.

Transcriptomic analysis of wild-type and TF-deficient HoxB8 neutrophils demonstrated RelB, Irf5 and JunB preferentially participate in regulating immune effector processes, whereas CEBPβ regulate cell cycle and cellular processes. CEBPβ is known to regulate the cell cycle to increase neutrophil generation during inflammation (93). JunB demonstrated the strongest overall effect on neutrophil effector functions, consistent with previously reported role of JunB in setting up neutrophil inflammatory response (87). The functional role of Irf5 in neutrophil inflammatory responses has been reported by analysing the expression of neutrophils upon activation (61). Consistently, we confirmed that IRF5 is important for regulating neutrophil inflammatory production in response to zymosan stimulation. Additionally, Irf5 contributes to

regulation of neutrophil phagocytosis, although its involvement in ROS production and NETosis is limited (**Figure 5.2.8**).

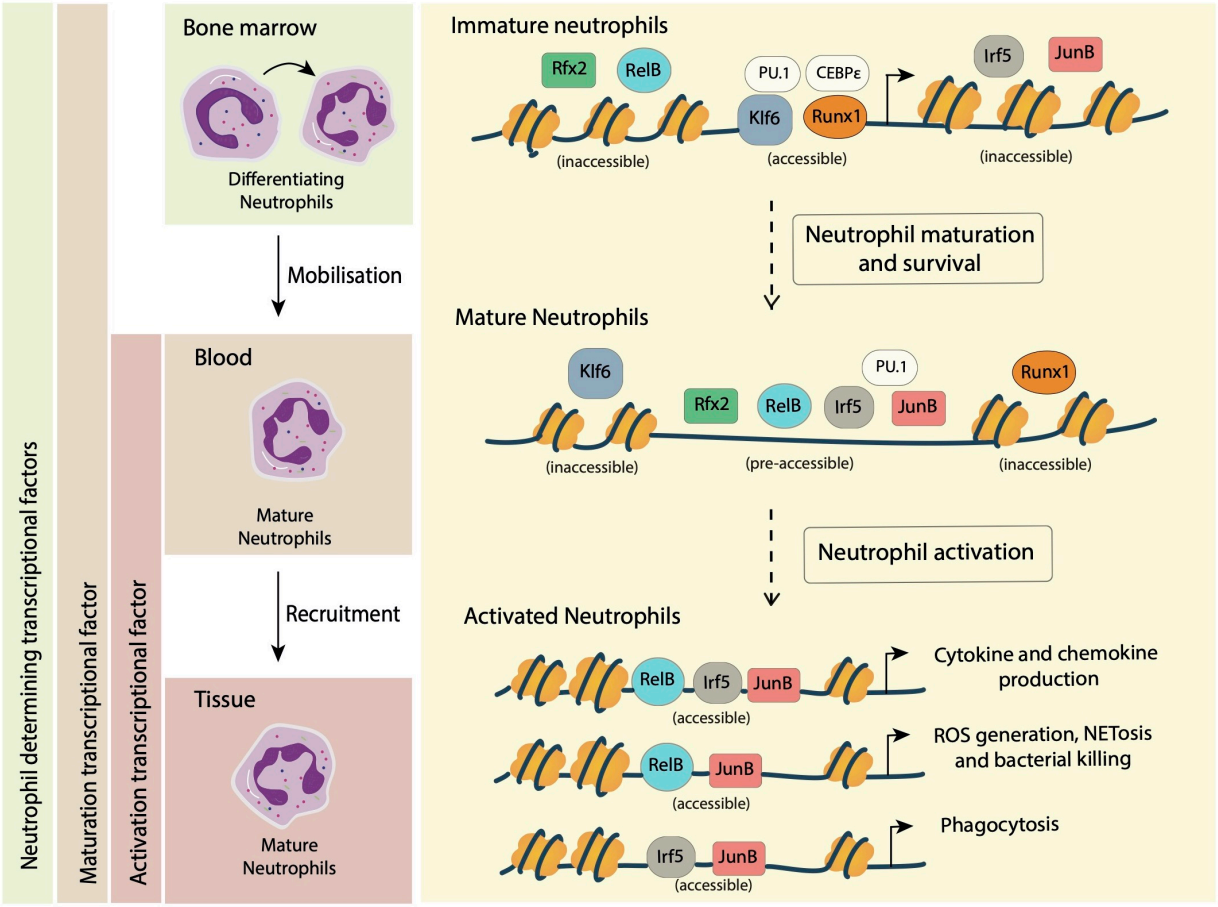


Figure 5.2.8 Model of transcriptional regulation of neutrophils during inflammation (1)**.**
In the process of differentiation in bone marrow, lineage-determining transcriptional factors, including RUNX1, KLF6, C/EBPe, and PU.1, are highly expressed and ensure gene expression programmes that promote proper neutrophil maturation. During the mobilization from the bone marrow into the blood, RFX2, RELB, IRF5 and JUNB become upregulated and transcriptionally accessible to support neutrophil cell survival and establish their effector function repertoire, whereas RUNX1 and KLF6 expression are silenced. Upon inflammation, circulating neutrophils migrate into the inflammatory sites, where they are exposed to inflammation-derived signals and become activated. Neutrophil activation leads to the activation of TFs, including RELB, IRF5 and JUNB, and subsequent TF binding to already accessible binding sites, thereby resulting in diverse TFs genomic occupancy and distinct transcriptional outputs.

ROS released by the NADPH oxidase complex can activate granular proteases and induce the formation of neutrophil extracellular traps (NETs) (237). Although NET formation is not a process controlled by TF transcriptional regulation (238), we hypothesised that some of the mechanisms that underline formation of NETs, such as activation of NADPH oxidase or citrullination (237), might be regulated by RelB. RelB-deficient neutrophils have lower levels of ROS production and low expression levels of *Cyba*, encoding one of the main subunits (p22-phox) of the catalytic NOX2 complex of NADPH, as well as its cofactor *Ncf1* (p47-phox), supporting the notion that RelB regulate neutrophil NET formation through ROS production.

A functional consequence of neutrophil activation is to produce various inflammatory mediators in the inflammatory sites to initiate and amplify the inflammatory process (9). In current section, our gene expression profiling of wild-type and TF-deficient HoxB8 neutrophils demonstrated that pro-inflammatory cytokines and chemokines were upregulated in wild-type neutrophils stimulated with zymosan, whereas RelB-deficient, Irf5-deficient and JunB-deficient neutrophils are more deficient in cytokine and chemokine expression. Here, we have utilised quantitative transcriptional analysis supplemented with proteomic arrays to determine the cytokine and chemokine expression in response to zymosan stimulation, and demonstrated that RelB, Irf5 and JunB promote the production of pro-inflammatory cytokines and chemokines in neutrophils. In alignment with these finding, adoptive neutrophils transfer into mice subject to air pouch model of in acute inflammation has provided further evidence supporting that RelB, Irf5 and JunB mediate pro-inflammatory cytokine and chemokine production.

To summarise, we have demonstrated that Irf5, RelB and JunB have limited roles in neutrophil maturation, but they significantly participate in promoting neutrophil effector functions and producing pro inflammatory cytokine and chemokines both *in vitro* and *in vivo* at inflammatory sites (**Figure 5.2.8**). Work conducted by our collaborators from the Hidalgo's group has demonstrated that neutrophil-specific JunB ablation significantly reduced the extent of infarct in the model of acute myocardial infarction. We further analysed neutrophil-specific deletion of JUNB (*S100a8^{cre} x Junb$^{fl/fl}$*) at steady state and confirmed that the mice had no defect in neutrophil maturation. We also generated mixed chimeric mice harboring both control and JUNB-deficient neutrophils by BM transplantation into WT recipient mice, induced cardiac ischemic-reperfusion injury and analyzed neutrophils in the myocardia. We found consistent reductions both in intracellular levels of pro-IL1b and ROS in JUNB-deficient neutrophils compared to

control neutrophils, corroborating our *in vitro* and *in vivo* data on the absence of alterations in neutrophil development and migration in the JUNB mutants and impairment of inflammatory-related properties (1). Future work should test how Irf5, RelB deficiency in neutrophils affect pathological inflammation *in vivo*. Overall, our study provides a significant advance in understanding the molecular mechanisms underlining neutrophil development and function during inflammation by depicting key TF modules that modulate development vs inflammatory responses or survival.

6.0 Zfp263 function in neutrophils

6.1 Introduction

Meeting the daily demand for functionally mature neutrophils requires the extensive proliferation of hematopoietic myeloid progenitor cells and subsequent differentiation into neutrophils. In the bone marrow, differentiating neutrophils undergo prominent changes in the surface marker phenotype, nuclear shape, granular enzymes, and storage vesicles in the cytoplasm, simultaneously acquiring the full range of neutrophil effector functions and loss of their proliferative capacity (9). The differentiation from hematopoietic progenitor cells into neutrophils is a well-balanced process between proliferation, self-renewal and differentiation. However, the transcriptional regulatory networks that regulate the renewal of the progenitor pool and the production of neutrophils remain still largely unexplored.

The importance of TFs in instructing neutrophil differentiation are continuously unraveled. Under healthy condition, hematopoietic TFs, PU.1 and C/EBPα, are essential for myeloid lineage commitment and early stage of neutrophil differentiation (49, 239), whereas C/EBPe and Gfi-1 function in promoting multi-potent precursor commitment to the granulocytic lineage (11, 89). Under inflammatory condition, C/EBPβ is sufficient to promote emergency granulopoiesis to meet the increased demand for functionally mature neutrophils (78). These TFs, together with others, including but not limited to C/EBPγ and C/EBPδ (81), form a TF regulatory network that tightly regulates the developmental transition from neutrophil precursors into mature neutrophils. Recent single-cell RNA sequencing of murine neutrophils revealed a heterogeneous and complex neutrophil population under steady-state and bacterial infection conditions, and proposed neutrophil-specific transcriptional networks, including previously reported TFs and uncharacterised regulons, involving the transitioning between consecutive neutrophil differentiation stages (15). Further mapping of transcriptional control of neutrophil differentiation trajectory and functional validation of TFs involved would provide new insights into the current model of neutrophil development.

Using integrated transcriptional and chromatin accessibility analysis of neutrophils en route to sites of inflammation, we discovered the involvement of distinct sets of TFs across the process of neutrophil differentiation and activation. For instance, PU.1 and Runx1, highly expressed and accessible in neutrophils transiting from the bone marrow into the blood, were

associated with hematopoietic renewal and early lineage commitment. On the other hand, RelB, JunB and Irf5, upregulated in neutrophils that infiltrated into the inflammatory sites, mediated neutrophil inflammatory response and effector functions (**Chapter 5**). Importantly, the genomic analysis also identified a number of novel putative regulators of neutrophil biology, including Zinc Finger Protein 263 (Zfp263). Subsequently, we sought to examine the functional role of Zfp263 in neutrophil development and inflammatory responses.

Zfp263 is a TF that belongs to the family of kruppel-associated box-containing zinc-finger protein (KRAB-ZNF) that contains the largest subset of the C2H2 zinc-finger proteins with 423 members (240). In human chronic myelogenous leukaemia K562 cells with knock-down of ZNF263, ChIP analysis showed human ZNF263 targets a variety of genes, and can either positively or negatively regulate gene transcription (241). GO enrichment analysis for the differentially expressed genes suggested that the downregulated genes upon loss of ZNF263 expression was more enriched in the GO term for “Cellular component organisation and biogenesis”, whereas genes upregulated by ZNF263 knock-down were enriched for “negative regulation of biological processes” and “negative regulation of cellular processes”, suggesting that ZNF263 might be crucial for maintaining cell development and proliferation (241).

Several studies in humans have highlighted the importance of ZNF263 functioning as a transcriptional regulator. Previous studies have shown the differential expression of ZNF263 in hepatocellular carcinoma (242), cholangiocarcinoma (243), and gastric cancer (244). It has been recently demonstrated that ZNF263 functions as a transcriptional repressor to inhibit expression of tumour suppressor genes, such as SIX3, through the MAPK pathway, consequently enhancing tumorigenic activities. ZNF263 also negatively regulates transcription of genes involved in heparin and heparin sulphate (HS) biosynthesis (245). These studies support a possible role for ZNF263 as a key transcriptional regulator of gene expression. However, to date, no studies have shown a direct impact of Zfp263 on cell development or neutrophil biology. The aim of this chapter is to functionally characterise Zfp263 and elucidate its function in neutrophil phenotype and differentiation.

6.2 Results

6.2.1 CRISPR-Cas9-mediated knockout of Zfp263 in HoxB8 myeloid progenitor cells

In previous sections, CRISPR-Cas9 genetic modification of HoxB8 has proven successful for analysis of TF function in neutrophil differentiation and functions. Therefore, we used the same system to generate Zfp263 knockouts in HoxB8 myeloid progenitor cells. HoxB8 myeloid progenitor cells were transduced with lenti-CRISPR-Cas9-TLCV2, which allows for constitutive expression of gRNA specifically targeting the exon of Zfp263 gene and doxycycline-inducible Cas9 nuclease for introduction of double stranded DNA breaks. Lenti-CRISPR-Cas9-TLCV2 also confers the puromycin resistance and green fluorescent protein (GFP), two selection markers to allow enrichment for successfully transduced progenitor cells (**Figure 6.2.1 A**). Following puromycin selection and enrichment via cell sorting, cells were expanded for knockout validation. Wild-type and Zfp263-deficient Hoxb8 myeloid progenitor cells were incubated in medium supplemented with G-CSF for 5 days to induce cell differentiation. The cells were lysed and total protein fractions were collected to assess the knockout efficiency of Zfp263 by Western Blot analysis (**Figure 6.2.1 B**). The continuous upregulation of Zfp263 levels was observed with the maturation of wild-type HoxB8 neutrophils. Whereas, Zfp263-deficient neutrophils showed no Zfp263 expression at any point of cell differentiation.

To further confirm that the cells are genetically deficient in Zfp263, HoxB8 myeloid progenitor cells with and without Zfp263 knockout were subjected to Sanger sequencing. Primer pairs were designed to sequence roughly 200 base pairs (bps) around the protospacer adjacent motif (PAM) sequence of the gRNA. Sequencing results were aligned between wild type HoxB8 myeloid progenitor cells and cells with Zfp263 knockout (**Figure 6.2.1 C**). In the alignment, all the knockout lines displayed 64 base-pair insertion and 11 base-pair deletions preceding the PAM sequence, which could lead to amino acid deletions, insertions, or frameshift mutations leading to premature stop codons within the open reading frame of *Zfp263* genes, eventually causing a loss-of-function mutation within the targeted gene.

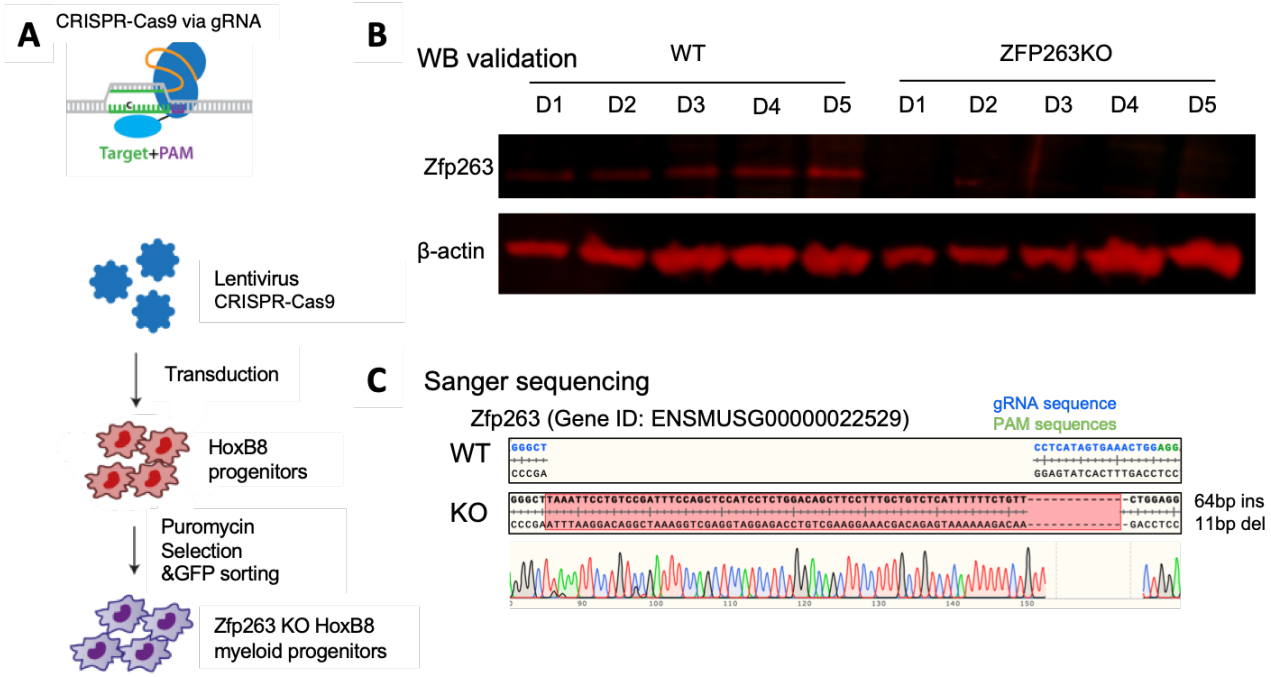


Figure 6.2.1 CRISPR-Cas9-mediated knockout of Zfp263.
(A) Overview of lentiviral CRISPR/Cas9 knockout system. HoxB8 myeloid progenitor cells were transduced with lentiviral particles containing the CRISPR/Cas9 and gRNA target Zfp263. 24 hours post lentiviral infection, transduced cells were selected for deletion by puromycin and GFP-mediated cell sorting. (**B**) Total protein samples were generated from wild-type and Zfp263-deficient HoxB8 neutrophils differentiated for 1-5 days for knockout validation. Western Blot were conducted for for validating the targeted knockout of Zfp263. For the experiments, 40 µg of total protein was collected from both wild-type and Zfp263-deficient cells by diluting cell lysates in Laemmli sample buffer and boiling at 100°C for 10 minutes. Samples were then loaded onto a 4-20% precast gel followed by SDS-PAGE. Verification of gene knockout was performed by immunoblotting for target proteins and β-actin or Gapdh as the loading control. Cells infected with a non-targeting construct were used as wild type (WT) control. (**C**) Sanger sequencing validation of Zfp263 gene editing. HoxB8 myeloid progenitors cells with/without genetic modification were harvested for genomic DNA isolation and then isolated genomic DNA were used for PCR amplification for amplify the targeted exon 1 of *Zfp263*. Then, the forward PCR primers were also used for Sanger sequencing. Gen6.e ID was annotated next to the gene name. D1-5 (day 1-5 of Hoxb8 neutrophil differentiation).

6.2.2 Surface marker expression and morphology of Zfp263-deficient neutrophils

In the previous chapters, HoxB8 myeloid progenitors have been shown to recapitulate normal neutrophil differentiation *in vitro* and become segmented mature neutrophils after 5 days of culture in the presence of G-CSF. To examine whether Zfp263 affects neutrophil differentiation, we investigated surface marker expression and morphology of Zfp263-deficient HoxB8 neutrophils. Cells deficient in expression of C/EBPe, a key TF in neutrophil terminal differentiation, were used as a positive control. The dynamic change in expression of neutrophil-specific markers during differentiation was assessed by flow cytometry. As stated in Chapter 2&3, neutrophil differentiation is characterised by the downregulation of immature surface makers c-Kit, CD49d and CXCR4, and the upregulation of mature surface markers Ly6C, Ly6G, CXCR2 and CD101. Consistently, wild-type neutrophils increased the expression of Ly6C and Ly6G with the progression through to mature neutrophils, with the highest expressions upon day 5 (**Figure 6.2.2 A**). Deficiency in Zfp263 led to a neutrophil differentiation block between day 2 and day 3 of differentiation, as evidenced by the reduction in upregulation of Ly6C and Ly6G, similar to the differentiation block observed in C/EBPe-deficient neutrophils (**Figure 6.2.2 A&B**).

Morphology assessment based on nuclear size and shape, density of chromatin, and presence of granular enzymes, was conducted to quantify the proportions of metamyelocyte, banded neutrophils and segmented neutrophils in wild-type, Zfp263-deficient or C/EBPe-deficient HoxB8 cells. After 5 days in culture, wild-type HoxB8 myeloid progenitors differentiate into mature neutrophils with high nuclear segmentation. Whereas Zfp263-deficient myeloid progenitors produced a mixed population of metamyelocytes and band neutrophils, morphologically similar to immature neutrophils generated from C/EBPe-deficient myeloid progenitors (**Figure 6.2.2 A**). The formal quantification shows a significantly lower proportion of segmented mature neutrophils in Zfp263-deficient and C/EBPe-deficient neutrophil cultures, 7.67 ± 10.79% and 2.77 ± 2.66 % (mean ± SD), respectively, in comparison to wild-type cells, 79.67 ± 2.08, (mean ± SD). Consistently, the same Zfp263-deficient and C/EBPe-deficient cultures contained higher levels of metamyelocytes (69.33 ± 10.50% and 43.67 ± 10.07%, mean ± SD, respectively) and banded neutrophils (25.33 ± 4.72% and 33.5 ± 4.92%, mean ± SD, respectively), whereas wild-type HoxB8 neutrophils had lower levels of metamyelocytes (20.33 ± 2.082%, mean ± SD) (**Figure 6.2.2 B**). Together, the data indicates that similar to C/EBPe, Zfp263 may be involved in to guiding neutrophil maturation.

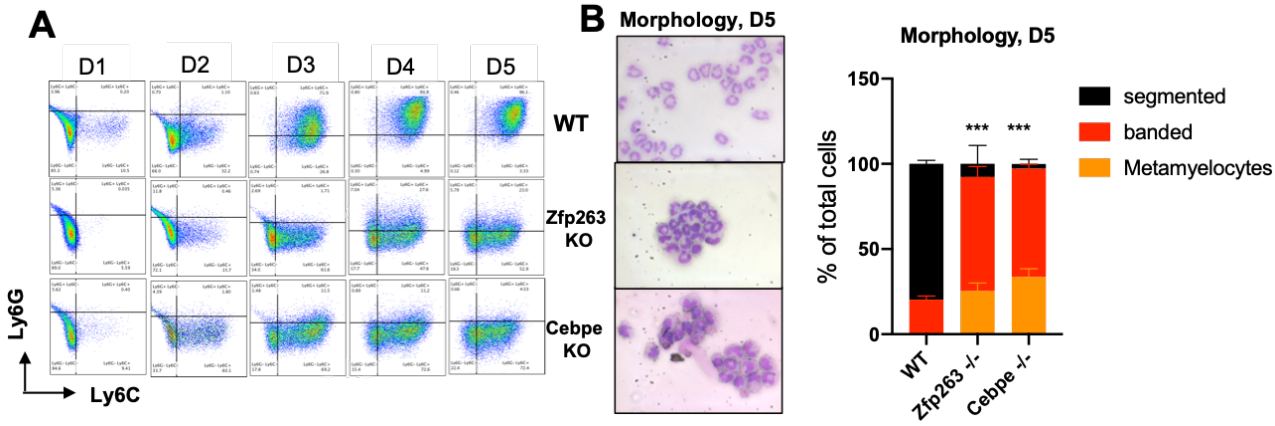


Figure 6.2.2 Phenotypes of HoxB8 neutrophils with Zfp263 deficiency.
Wild-type, Zfp263-deficient and C/EBPe-deficient HoxB8 myeloid progenitors were seeded into the medium containing G-CSF to induce differentiation. Following the differentiation, neutrophil-specific surface markers, such as Ly6C, Ly6G, were measured in differentiating neutrophils by flow cytometry on day 1-5 of differentiation. (**A**) Representative flow cytometry plots of wild-type Zfp263-deficient and C/EBPe-deficient HoxB8 neutrophils under different differentiation days after G-CSF treatment. One example in each group from three independent experiments is shown. (**B**) Morphology assessment of neutrophils generated from wild-type, Zfp263-deficient and C/EBPe-deficient neutrophils. Left: one representative image of neutrophils using Wright-Giemsa staining. Right: quantification of neutrophils under different maturation stage. The results are expressed as percentages of segmented, banded neutrophils and metamyelocyte out of at least 200 cells counted from different fields and independent replicates. Data are shown as means and SD derived from three mice from each group within one experiment. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.

6.2.3 Zfp263-deficient neutrophils are impaired in generation of ROS and NETs.

Normal neutrophil differentiation results in the production of mature neutrophils with full range of neutrophil effector function, such as ROS generation and NETosis. To determine whether Zfp263 deficiency affects neutrophil functionality, we next looked at the capacity of Zfp263-deficient neutrophils to produce ROS. Wild-type and Zfp263-deficient HoxB8 myeloid progenitor cells were incubated in medium supplemented with G-CSF to induce differentiation for 5 days. For measuring ROS generation, differentiated neutrophils were incubated with PMA to induce intracellular ROS generation and intracellular ROS indicator DHR123, in complete RPMI 1640 medium for 15 minutes. Flow cytometry quantification of intracellular ROS demonstrated that Zfp263 deficiency impaired the ability of neutrophils to produce ROS (**Figure 6.2.3 A**).

Induction of NETosis depends on ROS generation. It was demonstrated in previous section that HoxB8 neutrophils replicate inflammatory responses of primary neutrophils, including their ability to NETose. Having demonstrated the lower level of ROS generation in Zfp263-deficient neutrophils, we subsequently determined their capacity to produce NETosis. After 5 days of G-CSF-induced differentiation, wild-type and Zfp263-deficient neutrophils were incubated overnight with PMA and ionomycin to induce NETosis, followed by fluorescent staining for extracellular DNA content, citrullinated histone and neutrophil elastase. Fluorescent imaging and quantification of neutrophils under NETosis demonstrated that wild-type neutrophils produce NETosis efficiently in response to PMA and ionomycin stimulation. In comparison, neutrophils lacking Zfp263 were largely incapable to generate NETs in response to PMA and ionomycin stimulation (**Figure 6.2.3 B**). Overall, these results supported that Zfp263-deficient neutrophil are impaired in producing ROS and NETosis in response to stimulation.

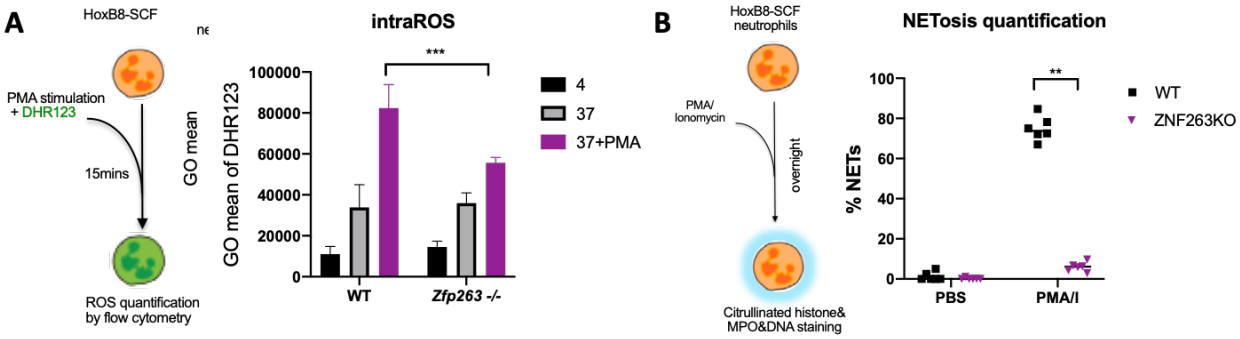


Figure 6.2.3 Zfp263-deficient neutrophils are impaired in ROS generation and NETosis. (A) PMA induced intracellular ROS production by HoxB8 neutrophils were assessed by flow cytometry with dihydrorhodamine 123 (DHR123), and then analysed by normalising fluorescence signals of stained cells to unstained cells. Wild-type and Zfp263-deficient HoxB8 myeloid progenitors were differentiated in medium supplemented with G-CSF to induce differentiation for 5 days, and it is followed by the overnight incubation with PMA to stimulate intracellular ROS generation. Comparison was made between wild-type and Zfp263-deficient neutrophils. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01. Data are shown as means and SD from three independent experiments with replicates. (**B**) NETOsis quantification in differentiating HoxB8 neutrophils. Wild-type and Zfp263-deficient HoxB8 myeloid progenitors were differentiated in medium supplemented with G-CSF to induce differentiation for 5 days, and it is followed by the overnight incubation with PMA and ionomycin to induce NETosis. NET formation in response to PMA and ionomycin stimulation were assessed by staining with fluorescently labelled anti-citrullinated-His3 and anti-Mpo antibodies and SYTOX DNA staining. The data are expressed as percentages of neutrophils undergoing NETosis out of at least 200 cells counted from different fields and independent replicates. Data are shown as means and SD derived from three independent experiments. Comparison was made between wild-type and Zfp263-deficient neutrophils. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.

6.2.4 Zfp263-deficiency caused deficient neutrophil infiltration and activation in vivo.

Mature neutrophils possess the superior capacity of neutrophil migration and infiltration into the inflammatory sites (9). Having demonstrated that Zfp263-deficient neutrophils are phenotypically and functionally immature, we next determined how Zfp263 knockout affects neutrophil recruitment and activation *in vivo* by making adoptive transfer of Hoxb8 neutrophils into mice subjected to the air pouch model and zymosan challenge. Wild-type and Zfp263-deficient HoxB8 myeloid progenitor cells were differentiated in medium supplemented with G-CSF to induce differentiation for 5 days. Before adoptive transfer, wild-type neutrophils were labelled with the red fluorescent dye CellTracker™ Red, to monitor cell movement or migration, while Zfp263-deficient cells were labeled with a green fluorescent dye CellTracker™ CFSE, to distinguish knockout cells from wild-type cells during acquisition by flow cytometry. Upon adoptive transfer, an equal mix of CellTracker™ Red-labelled wild-type and CellTracker™ CFSE-labelled Zfp263-deficient HoxB8 neutrophils was intravenously injected 10 minutes before injection of zymosan into the pouch cavity. Wild-type and Zfp263-deficient HoxB8 neutrophils competitively migrate toward sites of inflammation and tissues. 4 hours post zymosan challenge, the percentages of adoptively transferred HoxB8 neutrophils circulating in the blood and infiltrated into air pouch membrane and exudate, as well as tissue sites including lung and spleen, were measured by flow cytometry. Upon adoptive transfer into air pouch model of acute inflammation, wild-type neutrophils were efficiently infiltrated into air pouch membrane and exudate, occupying 0.156 ± 0.024 % and 0.147 ± 0.054% (mean ± SD), respectively, whereas Zfp263-deficient neutrophils were significantly less capable of infiltrating into the air pouch membrane and exudate, 0.034 ± 0.022 % and 0.035 ± 0.023% (mean ± SD), respectively, in comparison to wild-type neutrophils, 2.334 ± 0.72 % and 0.256 ± 0.132 %, (mean ± SD), respectively. Zfp263-deficient neutrophils are more frequently circulating in the circulation, 0.551 ± 0.105%, (mean ± SD), in comparison to wild-type cells, 0.151 ± 0.084 (mean ± SD) (**Figure 6.2.4 A**), suggesting deficient infiltration of Zfp263-deficient neutrophils into inflammatory sites and tissue sites.

Upon infiltration into inflammatory sites, Zfp263-deficient neutrophils produced lower levels of pro-IL1b during the transition from the blood into air pouch membrane and air pouch exudate, supporting the immature phenotype of Zfp263-deficient neutrophils. Overall, these results highlight that Zfp263 has a role in neutrophil infiltration and activation during acute inflammation *in vivo* (**Figure 6.2.4 B**).

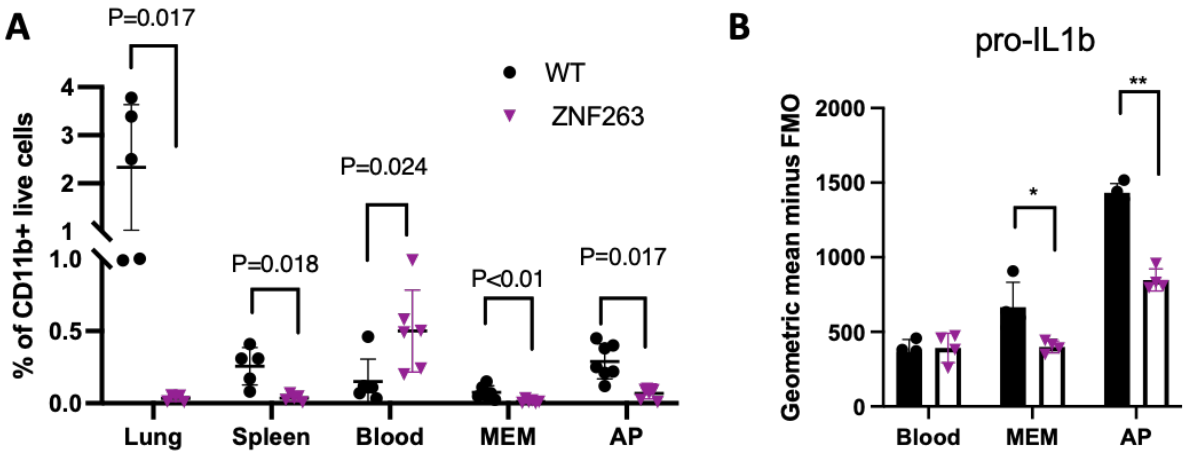


Figure 6.2.4 Impaired migration and activation of Zfp263-deficient neutrophils.
(A) the percentages of wild-type and Zfp263-deficient neutrophils recovered in the blood, the tissue sites, air pouch membrane and air pouch exudate recovered from mice subjected to the air pouch model and zymosan challenge. All results are shown as means and SD derived from at least three mice within one experiment. Statistical comparison was made between wild-type and Zfp263-deficient HoxB8 neutrophils. Statistical analysis was made by one-way ANOVA, n.s., not statistically significant; *P<0.05; **<0.01; ***<0.001; ****P<0.0001. (**B**) Expression of pro-IL1β, as measured by flow cytometry, in wild-type and Zfp263-deficient neutrophils recovered from the bone marrow, blood, air pouch membrane and air pouch exudate of mice subjected to the air pouch model and zymosan challenge. All results are shown as means and SD derived from at least three mice within one experiment. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.

6.2.5 Zfp263-deficiency caused deficient neutrophil infiltration and activation in vivo.

To this end, we have demonstrated that Zfp263 is required for neutrophil differentiation, as neutrophils with Zfp263 deficiency are phenotypically and functionally immature. Therefore, to better understand the molecular mechanisms of how Zfp263 regulates neutrophil maturation, we next sought to make use of transcriptional profiling by mRNA sequencing to analyze the global gene expression of wild-type and Zfp263-deficient neutrophils. Total RNA samples were collected from wild-type HoxB8 neutrophils differentiated for 0, 1, 3, 5 days and Zfp263-deficient neutrophils differentiated for 1,3, 5 days for mRNA sequencing. Unsupervised hierarchical clustering of the differentially expressed genes across different differentiation days between WT and Zfp263-deficient cells identified five clusters. These clusters encompassed genes progressively up-regulated with maturation (cluster 1), transiently up-regulated in the intermediate stage (cluster 3), slowly down-regulated with maturation (cluster 2), consistently down-regulated by Zfp263-deficient neutrophils (cluster 4), and upregulated by Zfp263-deficeint cells (cluster 5) (**Figure 6.2.5 A&B**). Genes associated with RNA synthesis and metabolic processes were among the genes upregulated in early differentiation stage, and genes related to antimicrobial activities and immune effector responses were highly expressed by neutrophils approaching mature stage, revealing an induction of strong inflammatory responses only when neutrophils become mature. Gene ontology analysis (GO) revealed that down-regulated genes in clusters 4 (downregulated by Zfp263-deficient cells) primarily correlated to stem cell maintenance and early lineage commitment, whereas the up-regulated genes in cluster 5 (upregulated by Zfp263-deficient cells) represented the cell surface receptor signalling pathways (MAPK kinases), and in immune response genes (**Figure 6.2.5 C**). These included genes encoding surface receptor and transmembrane precursor protein *APP*, MAP kinase negative regulator *Dusp6*, AMPK downstreameffector *Mapt* (**Figure 6.2.5 D**).

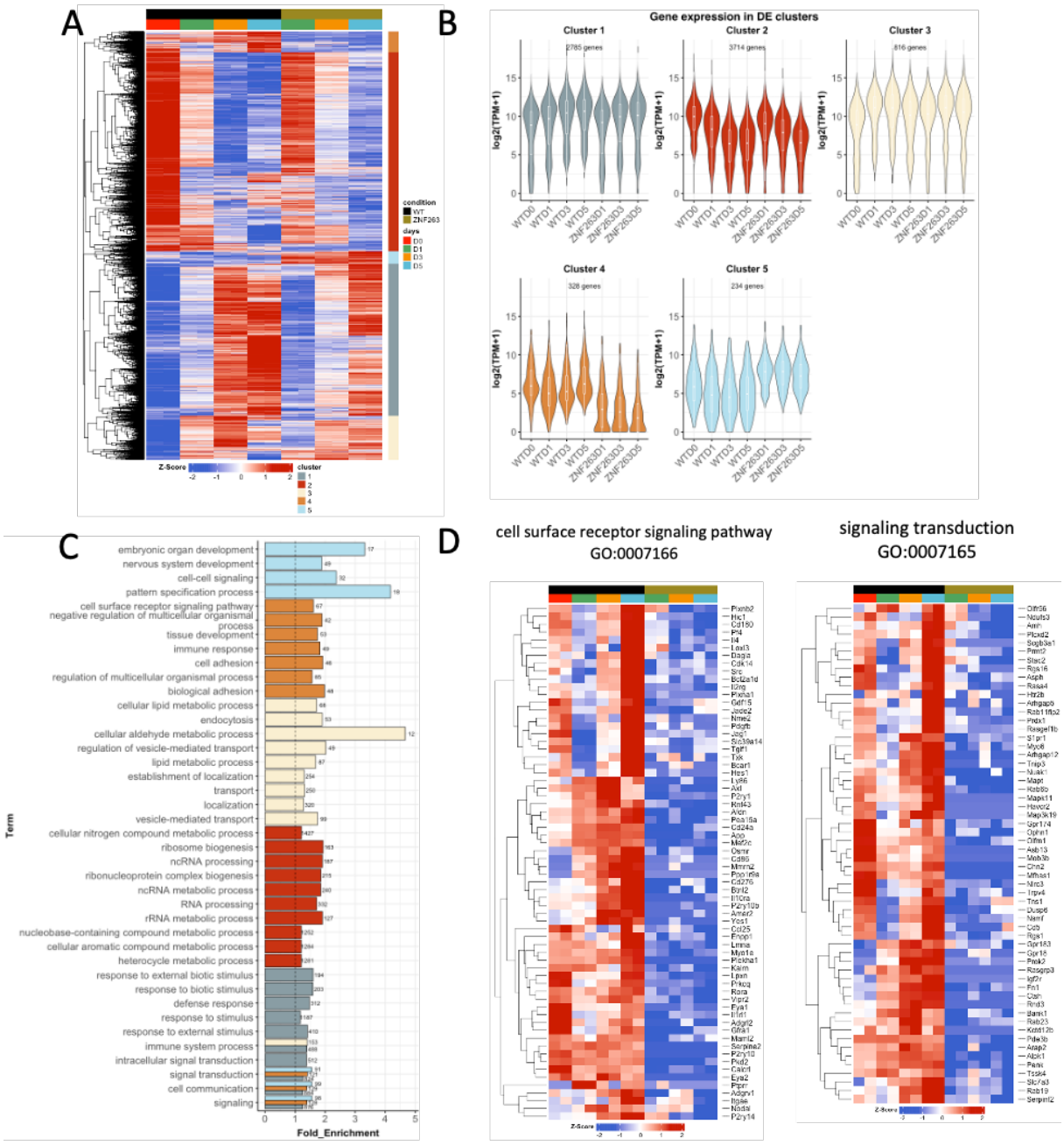


Figure 6.2.5 Zfp263-deficiency caused deficient neutrophil infiltration and activation.
(A) Hierarchical clustering of all differentially expressed genes (LRT test padj < 0.05, |FC|>2), based on Manhattan distances using the Ward method. Data are presented as heatmap normalised to the minimum and maximum of each row. B) Violin plots showing the expression of all genes within each cluster identified in (**A**) across different differentiation stages. (C) Gene ontology (GO) analysis, showing the top 5 enriched GO categories for each cluster from B). (D) Expression of genes that associate with cell surface signalling pathways and signalling transaction. Wild-type and Zfp263-deficient HoxB8 myeloid progenitors cells were incubated int the medium supplemented with G-CSF to induce the differentiation for different days. Total RNA samples were collected from wild-type and Zfp263-deficient HoxB8 neutrophils differentiation for 0, 1, 3, 5 days and with or without zymosan stimulation for mRNA sequencing. Line arrows indicate the the process of neutrophil maturation for different days and activation under zymosan stimulation.

I next compared the gene expression and chromatin region accessibility in proximal promoter peaks to that of the associated gene expression levels between wild-type and Zfp263-deficient neutrophils (**Figure 6.2.6A**). When regions of differential chromatin accessibility were mapped to the neighboring genes, we found a small overlap of the downregulated genes in Zfp263-deficient cells across the differentiation stages, and decreased chromatin accessibility (**Figure 6.2.6 B**). The categories of genes, which showed reduced expression and chromatin accessibility, included genes related to MAPK cascades and regulation of signal transduction (**Figure 6.2.6 C**).

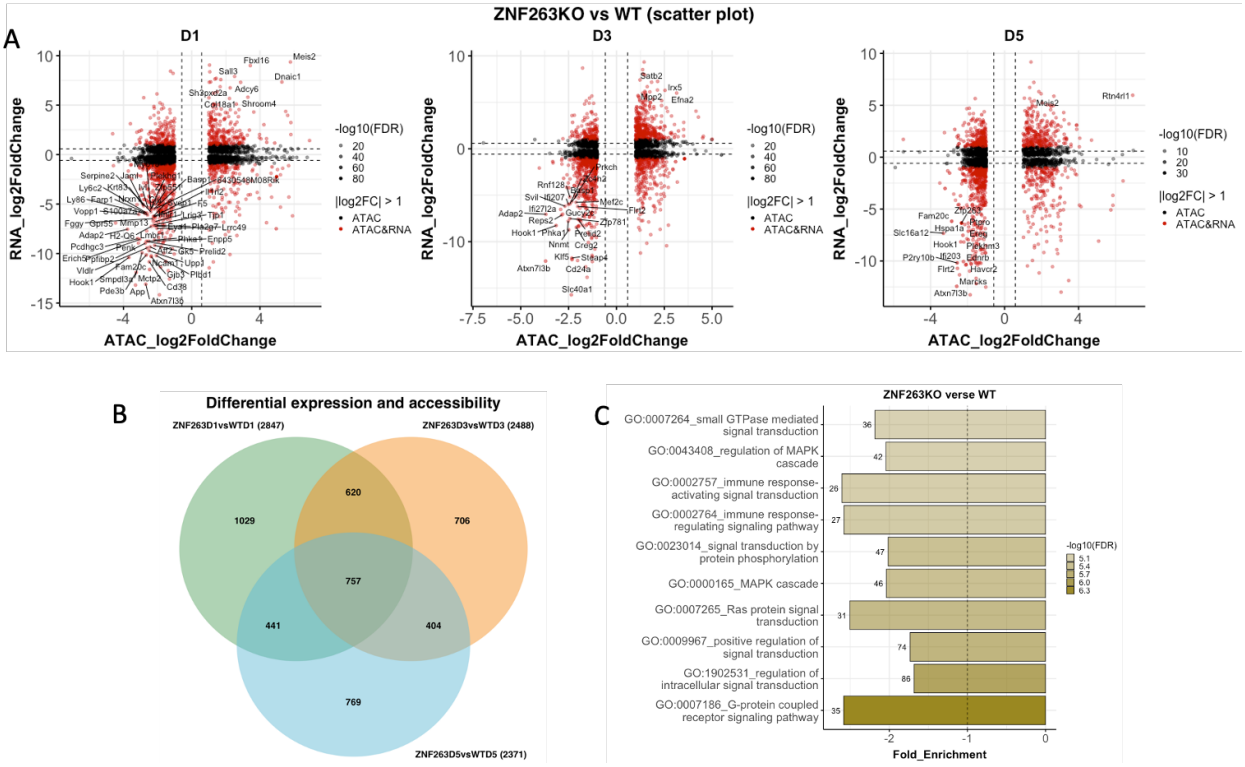


Figure 6.2.6 Integrated transcriptome and chromatin accessibility of WT and Zfp263-deficient neutrophils.
(A**)** MA plots depicting intersected RNA-seq and ATAC-seq data: genes which are both upregulated and located in the regions of opening ATAC-Seq peaks (increased chromatin accessibility), and downregulated and located in the regions of closing ATAC-Seq peaks (decreased chromatin accessibility) are highlighted in red (padj < 0.05, fold change > 1.5). (B) Venn diagrams demonstrate overlap of the down-regulated genes in Zfp263-deficient neutrophils across different differentiation stages. **(C)** Gene ontology analysis of genes that are differentially expressed and located in differentially accessible regions in Zfp263 deficient cells across different differentiation stages (as in C).

6.2.6 MAPK signalling genes exhibit ZNF263 binding motif

The lineage commitment of hematopoietic progenitor cells depend on the activation and transcriptional activities of intracellular signalling downstream of the cell surface cytokine receptors (246). To identify the potential signalling pathways underlying Zfp263 regulation, I analysed the genomic regions encompassing −400 to +100 bp around the transcription start sites of the genes involved in MAPK signalling pathways for the presence of Zfp263 binding sites. We used Hypergeometric Optimisation of Motif Enrichment (HOMER), which consists of an algorithm that identifies 8- to 20-bp motifs in large-scale genomics data (247). In the default settings, HOMER uses a hypergeometric model to score the enrichment of motifs in genomic sequences derived from chromatin immunoprecipitation sequencing (ChIP-seq) datasets compared with randomly selected sequences.

Assuming that MAPK signalling kinases could also be controlled by master regulators, we analysed genes involved in MAPK signalling cascades, including ERK1 and ERK2 cascade (GO:0070371), ERK5 cascade (GO:0070375), stress-activated MAPK cascade (GO:0051403), and regulation of MAPK cascade (GO:0043408). We found a motif associated with ZNF263 gained the greatest statistical significance, with predicted binding sites present on 45% of the input genes related to ERK1 and ERK2 cascade (GO:0070371) (**Figure 6.2.6 A&B**). ZNF263-regulated genes encoded enzymes involved in the negative regulation of MAPK signalling activation (*Dusp3*, *Dynlt1a*, *Dynlt1c*, and *Dynlt1f*), MAPK signalling kinases, such as Map3k12, representing the ZNF263 putative regulatory region (**Figure 6.2.6 C**).

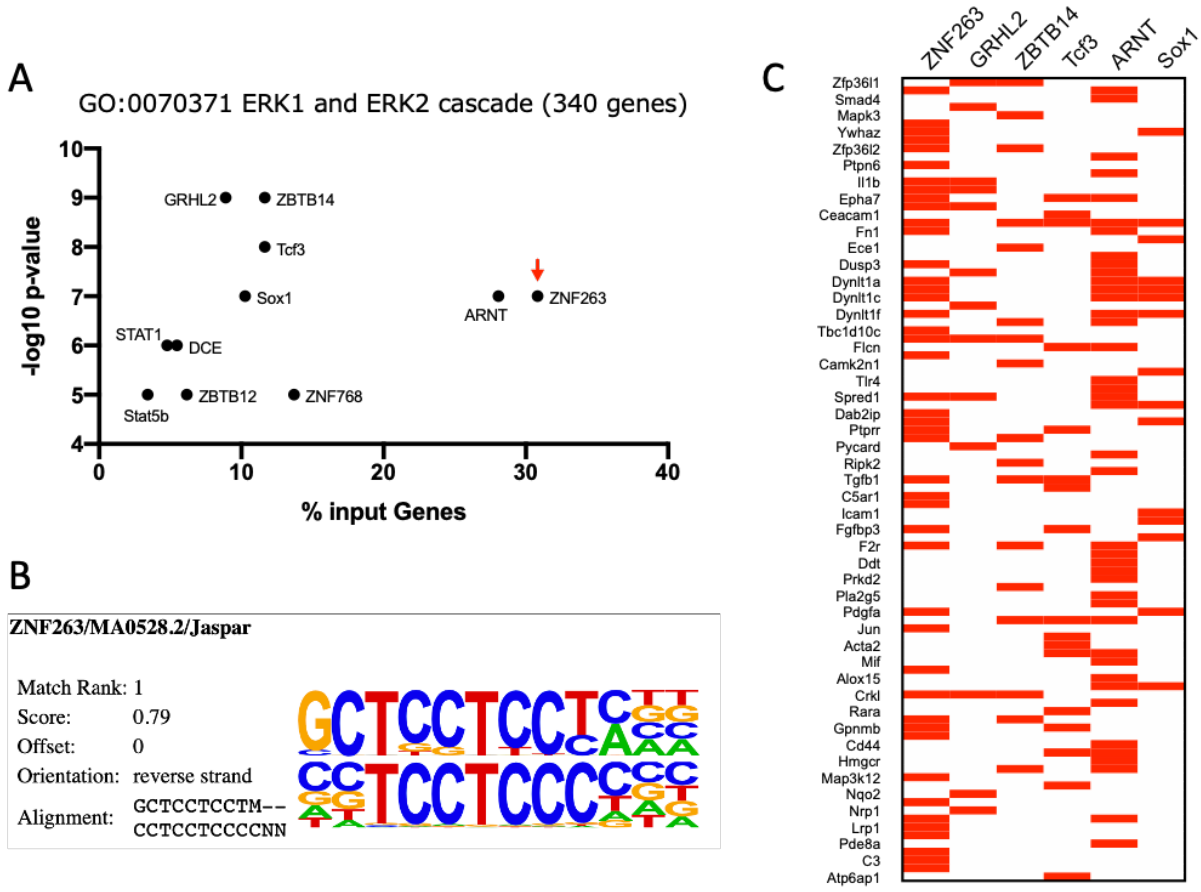


Figure 6.2.6 ZNF263-binding motifs enriched in the set of ERK1/2 cascade genes.

(A) HOMER motif enrichment analysis revealing TFs with predicted binding motifs in the set of ERK1/2 cascade genes. (B) ZNF263 motif enriched in the analysis of ERK1/2 cascade genes. (C) Heatmap showing the presence (red) or absence (gray) of ZNF263-binding motifs (y-axis) in regulatory regions of ERK1/2 cascade genes (x-axis), as predicted by HOMER.

6.2.7 ERK1/2 signalling in Zfp263-deficient neutrophils under differentiation.

After revealing the presence of ZNF263 binding sites in ERK1/2 cascade genes, we then asked whether Zfp263 may be involved in the regulation of ERK1/2 signalling pathways. To address this, I analysed the phosphorylation profile of proteins in the MAPK cascades in wild-type and Zfp263- deficient HoxB8 neutrophils under different differentiation stages (days 0-5), and found that the lack of ZNF263 did not cause notable changes in the levels of Jak/Stat kinase STAT3, MAPK kinase P38, but significantly increased the expression levels as well as the phosphorylation of ERK1/2 (**Figure 6.2.7**). Additionally, increased ERK1/2 phosphorylation was accompanied by prolonged expression of myeloid transcriptional regulator PU.1 (**Figure 6.2.7**), which has been shown to positively regulate monocyte development (246).

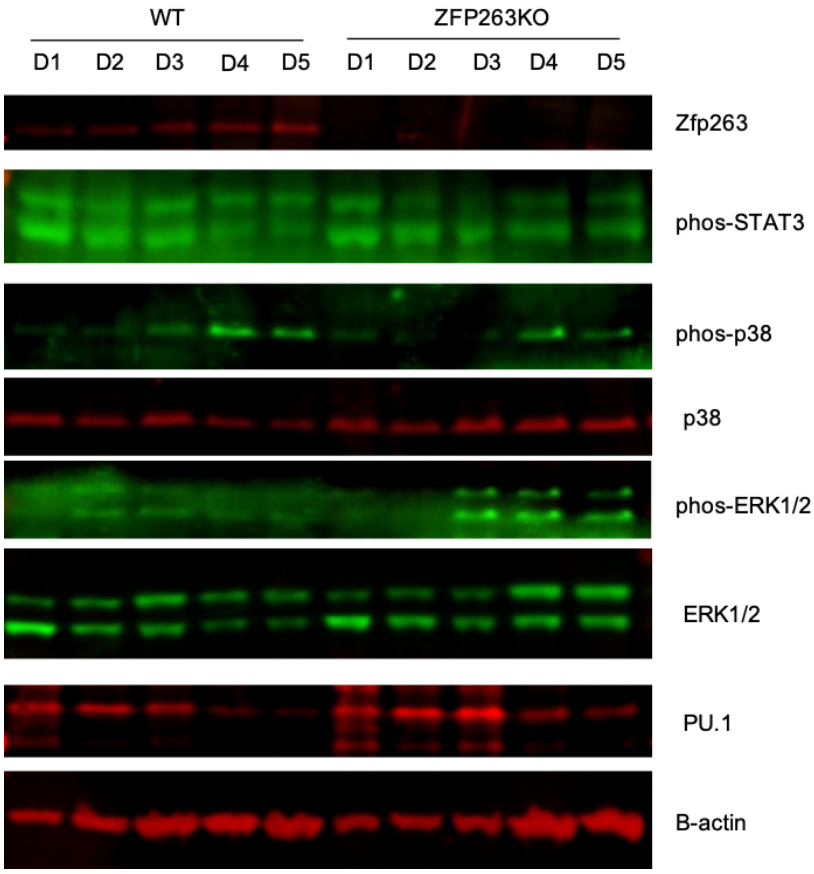


Figure 6.2.7 ERK1/2 signalling activation in Zfp263-deficient neutrophils.
ZFP263, STAT3, P38, ERK1/2, and PU.1 were immunoprecipitated from cell lysates from wild-type and Zfp263-deficient neutrophils differentiated for 1-5 days. STAT3, P38, ERK1/2 phosphorylation were also assessed by immunoblotting.

6.2.8 Endogenous Zfp263 activation enhances neutrophil maturation.

We next addressed whether Zfp263 favoured neutrophil development. As shown in **Figure 6.2.8 A**, the CRISPR-Cas9 activation system was used to generate HoxB8 myeloid progenitors with endogenous Zfp263 activation, and validated the transcriptional activation of Zfp263 before differentiation (**Figure 6.2.8 B**). Wild-type HoxB8 myeloid progenitor cells and cells with Zfp263 activation were incubated in medium supplemented with G-CSF to induce the differentiation for 5 days. During the process of differentiation, the surface marker phenotype of differentiating HoxB8 neutrophils was assessed by dynamic change in expression of neutrophil-specific markers by flow cytometry. As mentioned previously, neutrophil differentiation is featured with the downregulation of immature surface makers c-Kit, CD49d and CXCR4, and the upregulation of mature surface markers Ly6C, Ly6G, CXCR2 and CD101. Throughout this chapter, wild-type HoxB8 neutrophils increase the expression of Ly6C and Ly6G with maturation and become mature neutrophils with high expressions of Ly6C and Ly6G upon day 5 of differentiation, whereas Zfp263 activation enhanced expression of neutrophil mature surface markers, such as Ly6C and Ly6G, and reduced expression of neutrophil immature surface marker like CD49d (**Figure 6.2.8 C&D**), indicating accelerated neutrophil differentiation. Morphology assessment, based on nuclear size and shape, staining density of chromatin, presence of granular enzymes, was conducted to quantify the proportion of metamyelocyte, banded neutrophils and segmented neutrophils generated from wild-type and Zfp263-activated HoxB8 myeloid progenitor cells. After 5 days of differentiation, wild-type HoxB8 myeloid progenitors differentiate into a mixture of banded and segmented neutrophils, whereas Zfp263-activated myeloid progenitors produced a population of highly segmented neutrophils (**Figure 6.2.8 D&E**), confirming the notion that Zfp263 activation leads to accelerated neutrophil maturation.

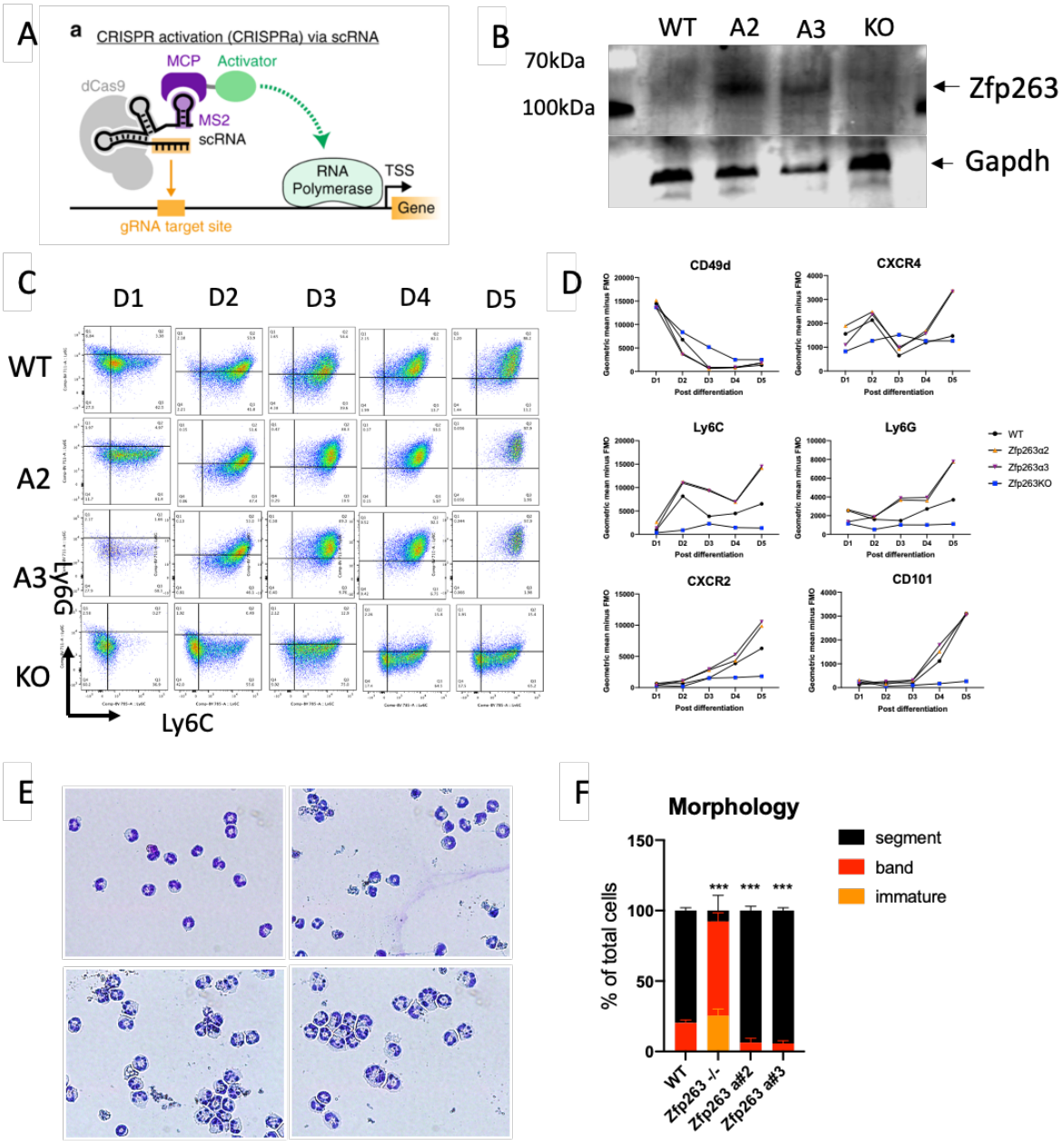


Figure 6.2.8 Endogenous Zfp263 activation promoted neutrophil maturation.
(A) Schematic of generating cells with endogenous activation. (B) Zfp263 immunoblotting using cell lysates from wild-type and Zfp263-activated HoxB8 myeloid progenitor cells. (C) Representative flow cytometry plots of wild-type and Zfp263-activated HoxB8 neutrophils under different differentiation days after G-CSF treatment. One example in each group from three independent experiments is shown. (D) Expressional levels of neutrophil surface markers, as assessed by flow cytometry. (**E**) Morphology assessment of neutrophils generated from wild-type, Zfp263-activated neutrophils. One representative image of neutrophils using Wright-Giemsa staining. (**F**) Morphological quantification of neutrophils under different maturity. The results are expressed as percentages of segmented, banded neutrophils and metamyelocyte out of at least 200 cells counted from different fields and independent replicates. Data are shown as means and SD derived from three mice from each group within one experiment. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001.

6.2.9 Endogenous Zfp263 activation potentiates neutrophil inflammatory responses.

It was demonstrated in previous section that HoxB8 neutrophils replicate neutrophil inflammatory responses, including ROS generation, cytokine production, and NETosis. Having demonstrated the involvement of Zfp263 in neutrophil development, we subsequently determined how endogenous Zfp263 activation affects neutrophil inflammatory responses. Wild-type and Zfp263-activated HoxB8 myeloid progenitor cells were incubated in medium supplemented with G-CSF for 5 days to differentiate into mature neutrophils. Next, we determined the capacity of HoxB8 neutrophils to generate ROS in response to PMA stimulation, produce pro-IL1b in response to stimulation *in vitro* and *in vivo*, and produce NETs under PMA and ionomycin stimulation. Zfp263-activated neutrophils exhibited superior capacity to produce intracellular ROS in response to PMA stimulation (**Figure 6.2.9 A**), and extracellular ROS under fLMP stimulation (**Figure 6.2.9 B**). Upon *in vitro* stimulation with Zymosan and adoptive transfer into air pouch model with zymosan challenge, Zfp263-activated neutrophils produced higher levels of inflammatory mediators, such as pro-IL1B and TNF-α (**Figure 6.2.9 C&D**). Zfp263-activated neutrophils were more capable of producing NETs, as evidenced by the significantly higher percentages of neutrophils that undergo NETosis in response to PMA and ionomycin (**Figure 6.2.9 E&F**). Overall, these results suggest that endogenous Zfp263 activation enhances neutrophil inflammatory responses.

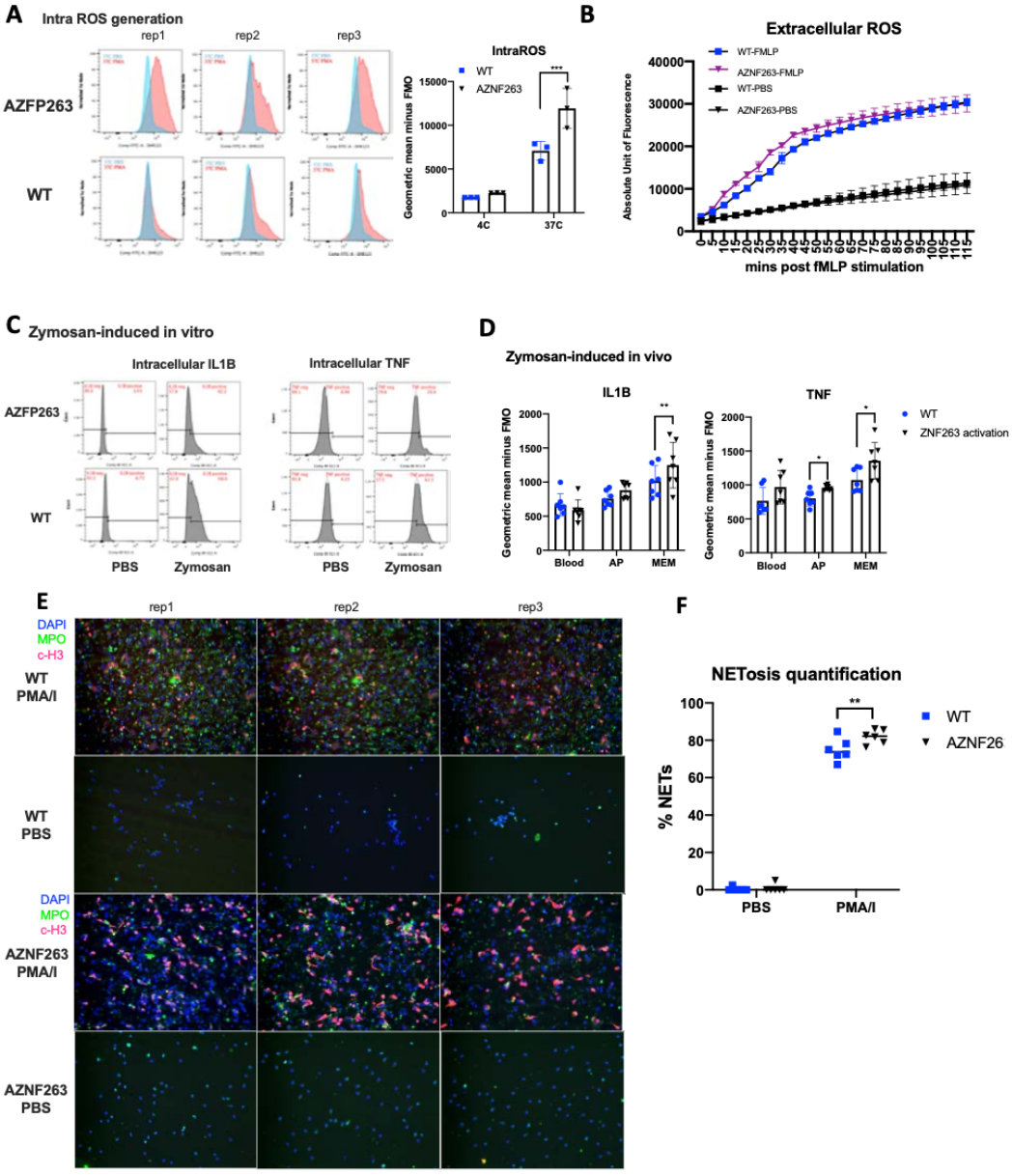


Figure 6.2.9 Functional analysis of wild-type and Zfp263-activated neutrophils
(A) PMA induced intracellular ROS production by HoxB8 neutrophils differentiated by flow cytometry with dihydrorhodamine 123 (DHR123)., and then analysed by normalising fluorescence signals of stained cells to unstained cells. Data are shown as means and SD from three independent experiments with replicates. (B) fMLP-induced extracellular ROS production by HoxB8 neutrophils NETOsis quantification in differentiating HoxB8 neutrophils. Data are shown as means and SD from three independent experiments with replicates. (C) Intracellular pro-IL1b and TNF-α in HoxB8 neutrophils were measured by flow cytometry, and then analysed by normalising fluorescence signals of stained cells to untreated cells. (D) Expression of pro-IL1b and TNF-α, as measured by flow cytometry, in wild-type and Zfp263-activated neutrophils recovered from the blood, air pouch membrane and air pouch exudate of mice subjected to the air pouch model and zymosan challenge. (E) HoxB8 myeloid progenitors differentiated in medium supplemented with G-CSF to induce differentiation for 1-5 days, and are NET formation in response to PMA and ionomycin stimulation were assessed by staining with fluorescently labelled anti-citrullinated-His3 and anti-Mpo antibodies and SYTOX DNA staining. (F) Quantification of neutrophils under NETOSIS. The data are expressed as percentages of neutrophils undergoing NETosis out of at least 200 cells counted from different fields and independent replicates. Data are shown as means and SD derived from three independent experiments. Statistical comparison was made by one-way ANOVA, *P<0.05, **<0.01, ***<0.001, ****P<0.0001.

6.3 Conclusion

In previous section, we have demonstrated that TFs function as key regulators of neutrophil differentiation and activation during inflammation. Based on integrated genomic analyses of neutrophils under inflammation, we have prioritised Zfp263 as a candidate TF, as Zfp263 is increasingly expressed and accessible in neutrophils transiting from the bone marrow into sites of inflammation. However, the functional role of Zfp263 in neutrophils is yet to be established. Using phenotypic and functional assessment of neutrophils with Zfp263 deficiency, we show that Zfp263 is required for the differentiation of mature neutrophils with full effector functions. Consistently, analysis of the differentially expressed genes between wild-type and Zfp263-deficient neutrophils has shown the differentiation block and impaired responsiveness to zymosan stimulation in Zfp263-deficient neutrophils.

Zfp263 is a KRAB- and SCAN-containing protein with nine zinc fingers and it has been shown to functions both as as an activator and a repressor, and might play a critical role in maintaining cell development and proliferation (241). In current study, we have discovered a functional role for Zfp263 in maintaining neutrophil differentiation. The CRISPR-Cas9 deletion of Zfp263 in myeloid progenitors produced the arrest of neutrophil differentiation in the early differentiation stage, which is very similar to the differentiation block observed in neutrophils with deficient expression of C/EBPe, a TF that mediate neutrophil terminal differentiation (11), suggesting the requirement of Zfp263 expression for normal neutrophil differentiation.

Neutrophil differentiation is accompanied with the progressive functional maturation of neutrophils (11). As we detected impaired maturation of Zfp263-deficient neutrophils, we hypothesised that Zfp263 could be involved in neutrophil functional maturation. To test this, we conducted functional assessment of neutrophils with Zfp263 deficiency. Consistent with the immature phenotype of morphology and surface markers, the interior capacity of Zfp263-deficient neutrophils in neutrophil effector functions, such as ROS generation and NETOsis were observed. Additionally, endogenous Zfp263 activation accelerates the process of neutrophil differentiation and enhances neutrophil inflammatory responses, suggesting that Zfp263 could be a key transcriptional regulator in neutrophil differentiation and inflammatory responses.

TFs regulate neutrophil maturation by upregulating and silencing of lineage-specific and irrelevant genes, respectively (57). However, how Zfp263 regulates neutrophil maturation remains unclear. Our preliminary analysis of the differentially expressed genes revealed the progressive maturation of wild-type neutrophils and the rapid activation in response to zymosan stimulation. The differentiation arrest and lower responsiveness to stimulation of Zfp263-deficient neutrophils suggest that these cells are transcriptionally distinct to wild-type mature neutrophils.

We hypothesise that Zfp263 might act as a crucial regulator in mediating neutrophil differentiation and function. Current section is a preliminary study and further studies will have to be conducted for validating the functionality of Zfp263 in vivo. To examine this, we have generated mice with conditional and global knockout of Zfp263, which could be used as a model to shed light on the function and mechanisms of Zfp263 in neutrophils. These are yet to be assessed. First, the investigation of Zfp263 function in vivo would add important insight into whether and how Zfp263 deficiency affect myeloid and neutrophil development. Flow cytometry-based assessment could be performed in the bone marrow and the blood, the tissue sites where neutrophils are generated and released into, respectively. Additionally, neutrophil infiltration and activation of neutrophils would be assessed in mice subjected to air pouch model of inflammation, with the aim to provide insight into how Zfp263 affect neutrophil phenotype and function during inflammation.

Furthermore, we hypothesise that the signalling pathway, such as the MAPKs, is likely to contribute to the function of ZFP263, based on the finding that human ZNF263 functions as a substrate of the MAPK kinases to modulate gene expression through DNA methylation (248). First, understanding the signalling pathway involving ZFP263 activation would provide important insights into the dynamic involvement of Zfp263 during neutrophil development and to determine whether the Zfp263 function repertoire is specific to the myeloid lineage. Integrated transcriptional and chromatin accessibility analysis of Zfp263-deficient neutrophils would provide insights into the Zfp263-dependent target genes or signalling pathways, which can be used for future experimental validation. Second, identification of Zfp263-dependent targets would add important insight into ZFP263 function to determine whether the binding repertoire is dynamic across development. ChIP-seq could be performed using wild-type and Zfp263-deficient HoxB8 neutrophils, a physiologically relevant platform that provides less heterogeneity than in vivo neutrophils. Human ZNF263 has been shown to interact with DNMT3A and

DNMT1, two main DNA methyltransferase for de novo DNA methylation (248). We hypothesise that Zfp263 might regulate gene transcription through the initiation and maintenance of DNA methylation. Future work should focus on functional validation of the involvement of Zfp263 in inflammatory signalling pathways and neutrophil biology in general.

7.0 General discussion

The results of my current thesis have provided insights into the transcriptional regulation in neutrophils during inflammation. In particular, I have focused on the functional role of recently identified TFs by our lab involved in neutrophil differentiation and activity. Increasing evidence have supported the notion that neutrophils are transcriptionally active and display distinct gene expression that shape their phenotype and function upon different differentiation stages (11), activation statuses (163), and infiltration into different tissue micro-environments (13). In line with this, neutrophils undergo transcriptional reprogramming powered by both known and uncharacterised TFs to acquire microbicidal capabilities, representing TF networks that regulate neutrophil inflammatory responses (15). Previous research using air pouch model of *in vivo* acute inflammation has revealed an active transcriptional and epigenetic remodelling of neutrophils during inflammation. Moreover, we have identified previously unappreciated transition points in neutrophil transcriptional regulation, from the bone marrow to the blood and from the blood to the tissue, each associated with the involvement of a distinct set of TFs (1). The purpose of current study was to validate the specific TFs as key regulators for neutrophil differentiation and function during the inflammatory conditions.

It is well accepted that neutrophils do not seem to be a homogeneous population and they are transcriptionally active cells with the potential to change the expression of several membrane molecules, and to produce cytokines, consequently performing different cell functions depending on the tissues where they are found. However, the molecular mechanisms underlining neutrophil transcriptional plasticity during inflammation remain yet to be fully elucidated. Additionally, the specific function of circulating neutrophils lies on a continuum due to their rapid ageing, the effect of the gastrointestinal microbiome, and accrued cellular damage as neutrophils traverse narrow capillaries. Recent single-cell RNA sequencing (scRNA-seq) of murine neutrophils revealed a heterogeneous and complex neutrophil population under steady-state and bacterial infection conditions and proposed neutrophil-specific networks, including previously reported transcription factors and new regulons (15). However, which of the proposed multitude of regulators actually control neutrophil differentiation and activation is unknown.

TFs function as nuclear effectors that direct signals of the signalling pathways to modulate transcriptional output, thereby controlling cell fate decision and inflammatory responses. The

importance of TFs in neutrophils has been appreciated by recognising the aberrant expression of TFs crucial for regulating myeloid development in leukemogenesis (16). Further evidence have also supported many master TFs involved in regulating neutrophil maturation(11, 61, 249) and in the signalling pathways mediating neutrophil activation(61). For example, hematopoietic TFs, such as PU.1, C/EBPα are essential during early granulocytopoiesis(31, 239) while others, such as C/EBPe, function in promoting neutrophil terminal differentiation of neutrophils (79). Moreover, several studies using human neutrophils have demonstrated the involvement of NF-κB subunits and STAT3 proteins in neutrophil activation(250, 251). Each of these TFs induces the activation of a selective set of lineage-specific genes as well as the silencing of lineage-foreign genes in a defined order. In the current study, we have demonstrated that hematopoietic TFs Runx1 and Klf6 are required for neutrophil differentiation, while signal-dependent TFs RelB, Irf5 and JunB are crucial to mediate neutrophil inflammatory responses. Additionally, we have provided preliminary evidence that support the requirement of Zfp263 in neutrophil differentiation.

Previous studies have shown that tightly-controlled transcriptional programmes regulate neutrophil differentiation (11, 20). This is likely to reflect on the transcriptome and transcriptional differences that occurs across the differentiation stages. For instance, the development of pre-neutrophils to mature neutrophils is accompanied with change of TF involved at different stages of myeloid cell development (11). Consistently, through integrated genomic and transcriptional analysis of neutrophils en route to sites of inflammation, our study has discovered the involvement of distinct cluster of TFs during the bone marrow-blood and blood-inflammatory-site transition. For example, I noted SP/KLF, and RUNX motifs at peaks with reduced accessibility from BM-to-blood and blood-to-membrane. Additionally, our analysis also revealed regulatory factor X (RFX) motifs exclusively at peaks with increasing accessibility from BM-to-blood, while Interferon regulatory factor (IRF) and Nuclear Factor kB (NFkB) motifs, and Activator protein 1 (AP-1) motifs were more detected at peaks with increased accessibility from blood-to-membrane transition. These data suggest an incremental program of neutrophil activation, with RFX2, KLF6, Runx1 linked to the initial transition of neutrophils to blood, and probably ongoing neutrophil maturation, whereas signal dependent transcription factors JunB, RelB, Irf5 are predicted to play a greater role as they transition to the inflamed tissue.

To investigate the role of specific TFs in mediating neutrophil differentiation and inflammatory responses, we made use of HoxB8 myeloid progenitors as a physiologically relevant platform

for generating murine neutrophils *in vitro*. With given growth factors and estrogen deprivation, HoxB8 myeloid progenitor cells undergo distinct morphological change and gradually differentiate into mature neutrophils (204). We utilised flow-cytometry analysis to determine the differentially expressed surface markers on HoxB8 neutrophils under different differentiation stages. The surface marker phenotype was then incorporated into subsequent molecular and functional characterisation, which led us to discover the phenotypic and functional differences that occurs across the different stages of HoxB8 neutrophil differentiation. For instance, HoxB8 myeloid progenitor cells recapitulate the highly coordinated process of normal neutrophil differentiation, which is characterised by increasing nuclear segmentation, differential expression of neutrophil-specific surface markers, sequential formation of granular enzymes with the progress of differentiation. Additionally, we found a progressive functional maturation of HoxB8 neutrophils under differentiation, such that mature neutrophils possessed the full range of neutrophil effector functions, such as ROS generation, phagocytosis and NETosis, consistent with functional changes of neutrophil lineages observed in the bone marrow (11). Importantly, HoxB8 myeloid progenitor cells could be amenable to CRISPR-Cas9-mediated genetic modification, which allows for the loss-of-function approach for analysis of gene function in neutrophils. In line with these findings, we believe that phenotypic and functional characterisation of HoxB8 neutrophils with specific TF knockout would provide importance insights into how specific TFs affect neutrophil differentiation and function.

Neutrophil lineage commitment and maturation involves the selective activation of lineage-specific genes and silencing of lineage-external genes, which are ultimately controlled by a set of master TFs that exert collaborative or competitive effects for lineage-determining commitment. In differentially accessible chromatin regions between the bone marrow and blood we identified binding motifs for transcription factors, such as Runx1 and Klf6, which promote neutrophil maturation (1). Both Klf6 and Runx1 are hematopoietic TFs, whose deficiency closely associates with the development of acute myeloid leukaemia. Klf6 is a TF linked to the pathogenesis of acute myeloid leukaemia (30), while Runx1 participates in myelopoiesis and it promotes neutrophil terminal differentiation through promoting the expression of C/EBPe (33). In the current study, we confirmed the importance of Klf6 and Runx1 in neutrophil differentiation. For instance, phenotypic comparison between wild-type and TF-deficient HoxB8 neutrophils demonstrated that Klf6 and Runx1 are crucial for neutrophil maturation, because Klf6- and Runx1-deficient myeloid progenitors failed to develop into segmented mature neutrophils and demonstrated immature surface marker phenotype with impaired expression of granular en-

zymes and nuclear envelope protein. We also observed a decreased proportion of mature neutrophils in the bone marrow of mice with Runx1 conditional myeloid knockout and confirmed impaired expression of C/EBPe in them, which corroborates the notion that Runx1 regulates neutrophil differentiation in a C/EBPe-dependent manner. Thus, one importance of our results is the capture of cell-state-specific TFs that modulate neutrophil maturation (Runx1, Klf6), as underlined by the difference in chromatin landscape and expression profiles at the transition from the bone marrow to the blood.

The progressive differentiation of myeloid progenitors into mature neutrophils involves mitochondrial reprogramming to meet their energy requirements (212). For instance, differentiating neutrophils engage a metabolic reprogramming toward glycolysis upon terminal maturation, such that mature neutrophils could function with limited availability of glucose and oxygen, such as sites of inflammation (252). Consistently, our mitochondrial function assessments demonstrated the decrease of mitochondrial activities with the progress of maturation, as evidenced by the significantly lower level of mitochondrial membrane potential and mitochondrial oxidative phosphorylation in neutrophils during the terminal differentiation stage. It matches the steep decreases of proteins that mitochondrial oxidative phosphorylation and electron transport chain during neutrophil differentiation (218). In alignment with these findings, Klf6- and Runx1-deficient neutrophils were characterised to be more mitochondrially active, as evidenced by higher levels of mitochondrial respiration and mitochondrial membrane potential, further supporting that they are more phenotypically immature.

In contrast to neutrophil precursors, mature neutrophils are non-proliferative and possess superior capacity for rapidly migrating toward sites of inflammation (9). Consistently, our migration assay and adoptive transfer model demonstrated that Klf6- and Runx1-deficient neutrophils migrated into inflammatory sites less efficiently as mature neutrophils, corroborating the observations that dysregulated neutrophil production and trafficking associated with leukaemia is aligned with factors such as aberrant production of systemic or local inflammatory mediators or diminished local anti-inflammatory mechanisms (253, 254). These results suggest that Klf6 and Runx1 orchestrate many programmes during neutrophil maturation, including increasing nuclear segmentation, expression of granular protein, mitochondrial reprogramming and migration machinery, such that they promote the differentiation of mature neutrophils in the bone marrow. Therefore, current study highlights the important role of TFs in regulating neutrophil differentiation. and further mapping of transcriptional control of neutrophil

differentiation trajectory and functional validation of TFs involved would provide new insights into the current model of neutrophil development. Important, the newly generated single-cell sequencing profile of differentiating and mature neutrophils under steady and infection conditions have provided a basis for further studying transcriptional regulation of neutrophil differentiation.

Neutrophil activation, induced by inflammatory conditions like bacterial infection, promotes significant upregulation of genes related to pathogen clearance, including cytokine production, phagocytosis, ROS generation (15). However, the TF networks that mediate neutrophil inflammatory responses are poorly defined. Integrated analysis of neutrophils en route to inflammation revealed the upregulation and increased chromatin accessibility of signal-dependent TFs, such as RelB, Irf5 and JunB. In my current study, deficient expression of RelB, Irf5 and JunB in HoxB8 neutrophils did not inhibit phenotypic and functional maturation of neutrophils, nor do they affect neutrophil mitochondria respiration and migration capacity, suggesting that neutrophils with RelB, Irf5 or JunB deficiency are capable of migrating out of the blood and enter the inflammatory sites where they are activated. Our transcriptomic analysis of HoxB8 neutrophils revealed deficient expression of genes that relate to neutrophil effector functions, such as phagocytosis, ROS generation, and cytokine production in neutrophils with RelB, Irf5 or JunB deficiency. Further functional assays suggested that JunB has the strongest global impact on neutrophil effector functions, as evidenced by the deficient capacity of JunB-deficient neutrophils to produce phagocytosis, ROS generation, bacterial killing, and NETosis. It matches the function role of JunB in modulating neutrophil inflammatory responses (87). The contribution of Irf5 in neutrophil cytokine production have previously been highlighted by the gene expression of neutrophils under various activation conditions, in which Irf5 regulate the secretion of pro-inflammatory mediators in response to TLR9 stimulation (61). My study has provided further evidence supporting the importance of Irf5 in regulating inflammatory gene expression and in promoting phagocytosis, cytokine and chemokine production in neutrophils. In alignment with these finding, we believe *in vivo* functional validation in models of neutrophils-mediated inflammation could provide further insights into the regulatory role of JunB and Irf5 in neutrophil inflammatory responses.

Neutrophils undergo a program for formation of neutrophil extracellular traps during infection (255). Although several key elements of NETosis, such as NADPH oxidase-dependent and -independent signalling pathways, have been described, the mechanistic details of NETosis

have not been fully elucidated. We hypothesised that the activation of the NAPDH-oxidase-dependent pathways, which promote NETosis, are dependent on RelB. Transcriptional investigation of RelB-deficient HoxB8 neutrophils revealed the impaired expression of the NAPDH oxidase complex, such as *Cyba* and *Ncf1* and the enhanced upregulation of the anti-oxidant system, such as *Ctns* and *Foxo3*, suggesting the deficient activation of NADPH oxidase in RelB-deficient neutrophils. Functional validation also demonstrated the lower level of intracellular ROS generation and deficient NETosis in RelB-deficient cells, suggesting the requirement of RelB-dependent transcription for NETosis. In previous report, RelA, another TF of the NF-kB family, was shown to be required for Cybb induction in other myeloid lineages (228). Therefore, it is possible that RelA and RelB function through different molecular mechanisms to regulating the activation of the NADPH oxidase system.

Mature neutrophils are terminally differentiated cells that tend to undergo constitutive apoptosis, which is conferred by balanced intracellular pro-survival and pro-apoptotic signals (256). However, the TF networks that modulate neutrophil survival and apoptosis remains unclear. Here, we suggested that both RelB and Rfx2 are critical for maintaining neutrophil survival, as evidenced by the significantly higher level of apoptosis in neutrophils with RelB and Rfx2 deficiency. The role of NF-κB TF family in supporting neutrophil survival and suppressing spontaneous apoptosis has been previously documented (257), the pro-survival functions of Rfx2 in neutrophils or any other immune cells remain obscure. The RFX proteins were originally cloned and characterised due to their high affinity for a cis-acting promoter sequence, called the Xbox, found in all MHC class II genes (258). Knockdown of *Rfx2* in mice did not have an obvious embryonic phenotype but results in the loss of spermatogenesis accompanied with increased germ cell apoptosis (259), suggesting a general role for Rfx2 in controlling cell survival across cell lineages. Therefore, based on the high expression of Rfx2 in mouse neutrophils (230), and the potential role of Rfx2 in regulating numerous germ-line-specific genes (259). Future studies will be needed to examine how Rfx2-deficient mice control infection and inflammation.

An important result of integrative transcriptional regulation associated with neutrophil transitions during inflammation is the identification of the activation-associated TFs, such as RelB, Irf5, JunB and others, as demonstrated by the increased transcription and accessibility at the blood-tissue (membrane) transition (1). Earlier study utilising scRNA-seq analysis of neutrophils during bacterial infection found a transcriptional switch from cellular-and-metabolic

programmes into inflammatory-response programmes, perhaps driven by the upregulation of several inflammatory genes and TFs that potentially mediate neutrophil maturation and activation (15). It provides the basis for further analysis of the molecular mechanisms underlying neutrophil differentiation and activation, using similar approaches as in this thesis but scaled up to a larger number of TFs. Another way of defining key neutrophil TFs may involve screening of CRISPR-Cas9 libraries for neutrophil phenotype alteration, which we are currently developing.

I have validated the usefulness of HoxB8 neutrophils for *in vivo* functional validation of specific TFs in neutrophil inflammatory responses and demonstrated that neutrophils with RelB, Irf5 or JunB deficiency reached inflammatory sites efficiently but are less capable of producing inflammatory mediators. Thus, those results from *in vitro* functional assessments and *in vivo* validation highlight the importance of RelB, Irf5 and JunB in setting up neutrophil effector functions, including phagocytosis, ROS generation, NETosis and cytokine production, allowing neutrophils to eliminate invading pathogens. This is consistent with the coherent drift of the gene regulator network, driven by both known and previously uncharacterised TF, toward augmented neutrophil functionality induced by bacterial infection (15). Further functional validation of crucial TFs underlining neutrophil inflammatory responses along with the evolution of pharmaceutical technologies may lead to the development of novel therapeutic strategies to selectively target neutrophils engaged in the pathogenesis of inflammatory diseases, such as arthritis and vasculitis.

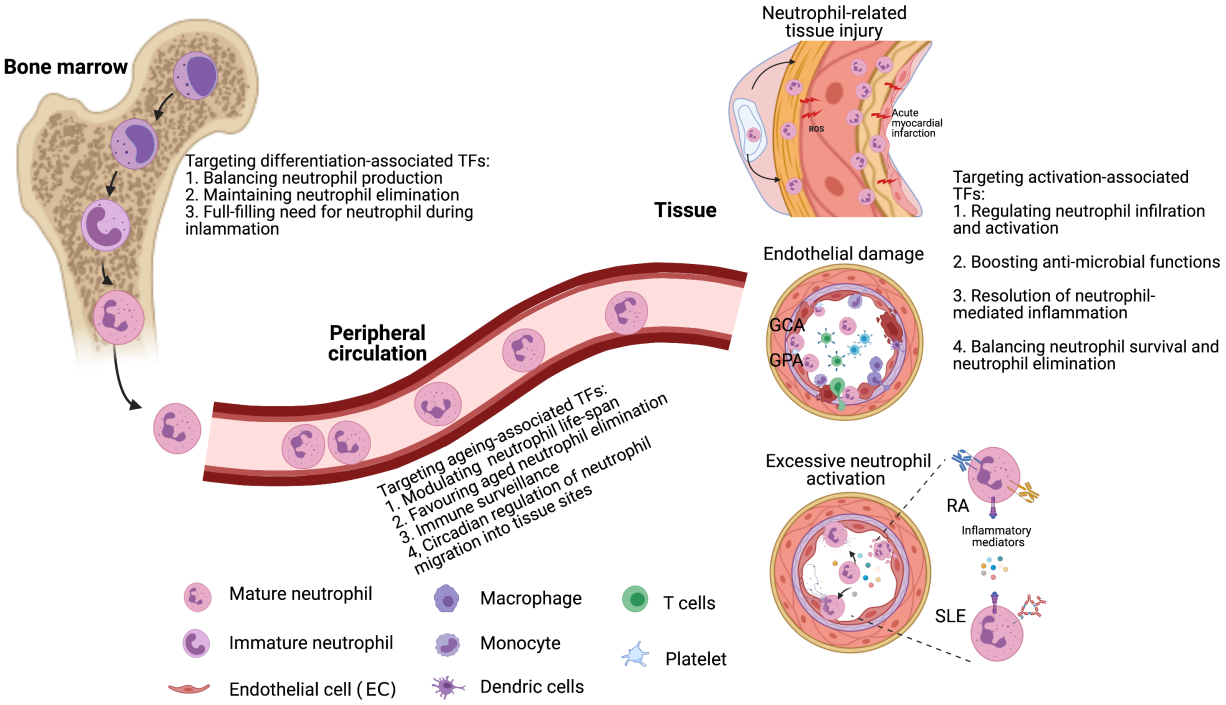


Figure 7.1 Therapeutic targeting TFs in neutrophils for neutrophil-related diseases.
Therapeutic targeting of neutrophils might occur via different mechanisms, including regulating neutrophil production in bone marrow, coordinating the processes of neutrophil ageing and survival, and modulating neutrophil infiltration and inflammation. When neutrophil generation and function are insufficient to the combat invading pathogens, the overall output of neutrophil subsets needs to be enhanced, whereas the overall neutrophil responses need to be reduced when there are excessive infiltration and activation of neutrophils.

In summary, our study provides a significant advance in understanding the molecular mechanisms underlining neutrophil development and function during inflammation by depicting key TF modules that modulate development vs inflammatory responses or survival (**Figure 7.1**). It extends our understanding of neutrophil transcriptional reprogramming from adaptation to local tissue environment at homeostasis, to acquisition of specific effector functions under inflammation (13). We generate the first draft of the neutrophil transcriptional blueprint in the context of in vivo inflammation, which will undoubtedly be edited and filled in with more details in the future. Moreover, the distinct repertoires of TFs controlling neutrophil maturation and activation may lead to multiple therapeutic strategies tailored to specific conditions. For example, stimulation of neutrophil maturation may be beneficial for boosting neutrophil re-generation in post-chemotherapy cancer patients. Another example to restore neutrophil function is in sepsis, in which IL-33 attenuated sepsis by preventing CXCR2 downregulation, restoring normal neutrophil recruitment and activation (181). Inhibition of neutrophil activation, in contrast, may help to reduce the inflammatory burden suffered during inflammation-associated diseases, such as cardiovascular diseases (260), and in severe COVID19 cases, which are

linked to abnormal neutrophil function (261). Induction of neutrophil effector functions may be needed in infectious diseases and early stages of cancer, as shown in lung adenocarcinoma studies (262).

In the final part of current study, Zfp263 was further validated as a TF that mediate neutrophil differentiation. Zfp263-deficient myeloid progenitors show deficient differentiation toward mature neutrophils, and phenocopies C/EBPe deficiency in terms of the block of neutrophil terminal differentiation. However, in contrast to C/EBPe deficiency, neutrophils lacking Zfp263 remained viable albeit halted at immature stage. They were characterized by reduced ROS and NET production, as well as impaired ability to trans-migrate and infiltrate sites of inflammation in vivo.

Our pilot gene expression analysis of Zfp263-deficient cells identified a number of signaling pathways that might be dependent on its presence. Combined with previously published reports, we propose the following model of Zfp263 regulation of neutrophil differentiation: G-CSFR signalling triggers the activation of Erk1/2-dependent signalling pathway. Upon activation, Erk1/2 interacts with Zfp263, leading to the activation and stabilisation of Zfp263. Then, Zfp263 might bind to the core promoter region of Erk1/2 target genes to induce their transcriptional silencing through chromatin and histone modification. Further studies should explore whether Zfp263 is capable of physical and functional interactions with MAPK Erk1/2 kinases and whether Zfp263 affects recruitment Erk1/2 or downstream TFs to target promoters. Although human ZNF263 has been described as a transcriptional regulator of some Erk1/2 target genes, such as tumour suppressor gene SIX3 (248), the list of target genes silenced by Zfp263 in neutrophils remains to be formally defined. This will help to highlight the major cellular functions of Zfp263 in neutrophils. We hypothesise that activation of Zfp263 in myeloid progenitors triggers or enhances the neutrophil-linage commitment, while inhibition of Zfp263 expression halts neutrophil differentiation.

To date, very little is known about the function of Zfp263 in the mouse genome, and no studies have shown a direct impact of Zfp263 on cell development. Having gained the preliminary view on the role of Zfp263 in neutrophil differentiation, we have generated a new strain of mice *Zfp263* $^{fl/fl}$ for conditional deletion of Zfp263. These have been crossed with PGK1^{-Cre} for a global deletion of Zfp263, and with S100A8^{-Cre} for neutrophil-specific deletion of Zfp263. Both strains are currently being examined for neutrophil development and phenotype, with a

focus on neutrophils in the bone marrow, blood and the tissue sites will be conducted to determine how Zfp263 affects neutrophil differentiation and phenotype. Postnatal growth and comparative immune analysis will be made to investigate whether Zfp263 deficiency has a global effect on growth, viability and the development of other immune cells. Further *in vivo* functional validation will be applied to characterise how Zfp263-deficient mice control infection and inflammation.

In summary, the results presented in current study have made the functional validation of candidate TFs, by applying CRISPR/Cas9 targeted deletion in the *ex vivo* system of HoxB8 neutrophils, followed by *in vivo* functional validation, and have confirmed the importance of Runx1 and Klf6 in modulating neutrophil maturation, Rfx2, RelB in neutrophil survival, and RelB, Irf5 and JunB in driving neutrophil inflammatory responses, thereby representing the first transcriptional blueprint of neutrophil differentiation and activation during inflammation. The identification of Zfp263 as a key TF will provide additional insights into the TF networks that underline neutrophil differentiation and function. My study has opened new avenues for investigation of transcriptional control of neutrophil development and function, in various tissues and under various pathological conditions.

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