Transcriptional regulation of neutrophil differentiation and function during inflammation

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Abbreviations

BM
bone marrow

C/EBP
CAAT/enhancer binding protein

CMPs
common myeloid progenitors

CXCR
CXC-chemokine receptor

fMLP
N-formylmethionyl-leucyl-phenylalanine

HSC
hematopoietic stem cells

JNK
c-Jun N-terminal kinase

LPS
lipopolysaccharide

MPO
myeloperoxidase

NE
elastase

NETs
neutrophil extracellular traps

ROS
reactive oxygen species

PRR
pattern recognition receptor

PAMPs
pathogen‐associated molecular patterns

TFs
transcription factors

TNF-α
tumor necrosis factor alpha

Abstract

Neutrophils are the most abundant leukocytes in innate immunity where they elicit powerful effector functions to eliminate invading pathogens and modulate the adaptive as well as the innate immune response. Neutrophil function must be tightly regulated during inflammation and infection to avoid additional tissue damage. Increasing evidence suggests that transcription factors (TFs) function as key regulators to modulate transcriptional output, thereby controlling cell fate decision and the inflammatory responses. However, the molecular mechanisms underlying neutrophil differentiation and function during inflammation remain largely uncharacterized. Here, we provide a comprehensive overview of TFs known to be crucial for neutrophil maturation and in the signalling pathways that control neutrophil differentiation and activation. We also outline how emerging genomic and single-cell technologies may facilitate further discovery of neutrophil transcriptional regulators.

Introduction

Neutrophils are the most abundant leukocytes in the innate immune system and eliminate invading pathogens via various inflammatory responses, such as the release of cytotoxic products, reactive oxygen species (ROS), and neutrophil extracellular traps (NETs) (1). On one hand, quantitative and qualitative neutrophil defects contribute to various types of agranulocytosis, consequently resulting in a high risk of invasive infection (2). On the other hand, excessive infiltration and activation of neutrophils at the site of inflammation can cause tissue damage, leading to intense local and systematic inflammation (3). Neutrophils can initiate and amplify immune responses through the release of cytokines, chemokines and other pro-inflammatory mediators and by promoting the recruitment and activation of additional neutrophils as well as 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) (4). Neutrophils are traditionally considered to be a homogenous population of cells. However, recent findings have expanded our knowledge on the roles of neutrophils as these cells exhibit phenotypic and functional heterogeneity in various immune disorders and cancers (3, 5) and on their ability to reorganise their genome and transcriptional programme *en route* to the tissue(6-8). Nevertheless, the molecular mechanisms controlling the outcome of neutrophil differentiation and inflammatory responses have not been fully explored.

Transcription factors (TFs) have been recognised as major regulators in the commitment and functional regulation of several myeloid subtypes(9). This can be appreciated by recognising that the leukemogenesis frequently associated with acute myeloid leukaemia mostly involves aberrant expression of TFs crucial for maintaining cell development and differentiation(10). Further evidence comes from studies using disrupted expression of TF in myeloid progenitor cells(11) and targeted deletion of genes encoding TFs in mice(12, 13). Many studies have also supported many master TFs involved in regulating neutrophil maturation(6, 8, 14) and in the signalling pathways

mediating neutrophil activation(6). For example, some TFs, such as PU.1, CAAT/enhancer binding protein α (C/EBPα) are essential during the early granulocytopoiesis(12, 15) while others, such as C/EBPε, function in promoting neutrophil terminal differentiation of neutrophils (16). Moreover, several studies using human neutrophils have demonstrated the involvement of NF-κB subunits and STAT3 proteins in neutrophil activation(17, 18). 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. In this review, we discuss the current knowledge on which the specific TFs that mediate neutrophil differentiation and activation.

Granulocytopoiesis

Neutrophils are generated by granulopoiesis in the bone marrow (BM). Like other leukocytes, they are produced via the haematopoietic stem cells (HSCs), which possess self-renewal capacity and further develop into multi-potent progenitors (MPPs). In turn, MPPs develop into common myeloid progenitors (CMPs) and then granulocytes/monocyte progenitors (GMPs) with limited potential of proliferation and increased differentiation. GMPs possess 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. With respest to granulocytopoiesis (Figure 1), ongoing differentiation of the CMPs and GMPs produces $CXCR4^{+}$ $CXCR2^{-}$ proliferative preneutrophils (promyelocytes), which progresses through CXCR4- $CXCR2^{low}$ non-proliferating immature neutrophils (myelocytes, metamyelocytes and band neutrophils) and finally develops into $CXCR2^{high}$ segmented mature neutrophils(8).

TFs involved myeloid lineage commitment

Lineage commitment and subsequent differentiation of CMPs and GMPs involve 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. One example of such a transcriptional network is the lineage choice between myeloid versus lymphoid cell commitment downstream of HSCs and MPPs, which is regulated by the haematopoietic TFs *PU.1*, early B cell factor (Ebf) and Notch(19). The protein PU.1 consists of an N-terminal transactivation domain, a PEST domain and a conserved ETS domain at the C-terminus(20). PU.1 is expressed by myeloid and B lymphoid lineages and its ETS domain mediates binding to DNA and interactions with other proteins, including C/EBPα and C/EBPβ, in a stage- and lineage-specific manner (20). PU.1 is highly detectable in the nucleus of erythroid and neutrophil precursors but is absent during the late maturation stages(21). In contrast, mature macrophages but not monocytes, express *PU.1* at low levels (21). For lymphoid lineages, a low level

of PU.1 expression is needed for early B lymphoid lineages but not T lymphoid lineages(21). Based on the phenotypic analysis of *PU.1*-knockout mice, 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(21). Enforced expression of *PU.1* induce the commitment of multiple myeloid lineages(22), suggesting that *PU.1* is a lymphoid and myeloid lineage determinant. Unlike PU.1, Ebf is restricted in promoting B lymphoid lineage commitment, antagonises the expression of genes encoding *C/EBPa* and *PU.1*, and initiates the B lymphoid lineage-specific program of gene expression (e.g. expression of *B29*, *VpreB* and *Pax5*). Similarly, the T lymphoid-specific determinant factor Notch promotes T cell differentiation by antagonising B lymphoid development via promoting the repression of *Ebf*, *B29*, *Pax5* and other B-cell-lineage genes(23).

TFs promoting granulocytic lineage commitment

The differentiation of GMPs leads to commitment to monocytic and granulocytic lineages. Several studies have demonstrated that the inducible knockout of *C/EBPα* in the BM results in a complete loss of mature neutrophils and a decreased frequency of monocytes, leading to a differentiation block in the transition from CMP to GMP(24). Furthermore, systematic knockout of *C/EBPα* selectively blocks myeloid differentiation in the newborn liver and BM. In agreement with this finding, a mouse study showed that a lack of C/EBPα expression resulted in significant reductions in the population of GMP, neutrophils, monocyte and macrophage populations (12). C/EBPα consists of three N-terminal transactivation domains and a C-terminal DNA-binding domain that mediates the binding to the CCAAT motif(25, 26). C/EBPα also has been shown to regulate several myeloid-specific genes and it specifically interacts with functional regions proximal to the promoters of the G-CSF receptor (G-CSFR) and GM-CSF receptors to regulate the expression of the receptors crucial for early myeloid differentiation(27).

Further commitment of the GMPs into the granulocytic lineage primarily depends on the expression levels of the 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, even in the presence of cycloheximide, suggesting that *C/EBPα* directly activates the expression of *PU.1* expression (28). Indeed, C/EBPα has been demonstrated to directly activate *PU.1* transcription by interacting with its proximal promoter(29). Induction of *PU.1* expression by C/EBPα during haematopoiesis contributes to the development of myeloid lineages and further commitment in monopoiesis. Consistent with this finding, forced *C/EBPα* expression in myeloid progenitors favours monopoiesis but not granulopoiesis, whereas enhanced expression of *C/EBPα* in myeloid progenitors lacking *PU.1* promotes granulocytopoiesis at the expense of monopoiesis(29).

The indispensable role of *C/EBPα* in granulocytopoiesis has been additionally supported by the C/EBPα-induced transcriptional upregulation of several granulocyte-specific TFs, such as the transcriptional repressor growth factor independent-1 (Gfi-1). C/EBPα recognises the C/EBP binding site flanked in the *Gfi-1* region to enhance *Gfi-1* transcriptional levels(6). Upregulation of *Gfi-1* expression represses the expression of genes encoding monocyte lineage-promoting factors such as *Egr2* and *Csf1*, leading to the progression of granulocyte differentiation(30). Another C/EBPα-driven lineage mechanism involves the downregulation of lineage-repressing microRNAs (miRNAs), such as miRNA-21, miRNA-196b(31), and the transcriptional activation of miRNA-223, which in turn suppresses the nuclear translocation of nuclear factor I-A to favour granulocytic maturation(32).

Taken together, the current studies implicate the transcriptional network in deciding monocyte versus granulocyte lineage choice (Figure 1). First, both C/EBPα and PU.1 are essential for granulocyte development and a number of lineage specific genes depend on both TFs for transactivation(33). Second, the ratio of C/EBPα to PU.1 expression is crucial for the granulocytic cell commitment. C/EBPα, which is induced by G-CSF signalling, has been shown to antagonise the ability of PU.1 to

activate a minimal promoter containing multiple PU.1 binding sites and as a result increase the threshold of PU.1 concentration needed for guiding macrophage differentiation(34). By upregulating C/EBPα expression, G-CSF pretreatment of myeloid cell lines blocks the usual macrophage differentiation and instead results in granulocyte differentiation(35). However, high *PU.1* expression favours monopoiesis due to the interaction with other monocyte-specific TFs, such as members of activating protein-1 (AP-1) family and others(35) whereas insufficient transcription of *PU.1* allows C/EBPα to promote the granulopoiesis-specific transcriptional programme, which is accompanied by suppression of monocyte development(36).

Terminal differentiation

The terminal differentiation of neutrophils begins with a transition between GMP and preneutrophil stage, where the cellular state switches from proliferation into differentiation and involves the loss of ability to undergo cell division beyond the preneutrophil stage and the formation of neutrophil-specific primary granules. Starting from the preneutrophil stage, myeloperoxidase (MPO) and neutrophil elastase (NE) are expressed and stored in the form of primary granules. As neutrophils progress into the immature stage , MPO and NE expression ceases and the formation of neutrophil secondary granules such as lactoferrin, cathelicidin, and collagenase begins (37). The most important TFs that govern the formation of primary granules are C/EBPα and Gfi-1, whose expression in GMPs favours granulocyte lineage commitment via transcriptional activation of granulocyte-specific genes including *MPO*, *NE* and *G-CSFR*(36, 38). The intrinsic requirement for Gfi-1 and NE in neutrophil terminal differentiation has been demonstrated by studies that analysed several congenital and cyclic neutropenias, in which mutations in the genes encoding *Gfi-1* or *NE* were linked to the defective production of mature neutrophils and the peripheral release of immunodeficient neutrophils that appear phenotypically immature (39-41). Furthermore, *Gfi-1*-knockout mice exhibit abnormal granulocytopoiesis with the absence of phenotypically mature neutrophils and lost of primary but not secondary granule expression in the neutrophilic lineages in the circulation(42). Gfi-1 has also been

shown to regulate the G-CSF/GCSF-R signalling by activating the Ras/MEK/Erk signalling pathway, ultimately contributing to the G-CSF–induced neutrophil differentiation(43). Another important TF in neutrophil terminal differentiation is the lymphoid enhancer factor-1 (Lef-1), which is highly expressed in neutrophil progenitors. A deficiency in Lef-1 expression is the most common cause of impaired production of mature neutrophils in patients with severe congenital neutropenia (SCN). *Lef-1* directly transactivates *C/EBPα* expression leading to granulopoiesis and differentiation of myeloid progenitors into mature granulocytes(44).

Ongoing differentiation beyond the preneutrophil stage leads to a developmental transition into immature and mature neutrophils. Such transition is characterised by the initiation of nuclear segmentation and formation of neutrophil secondary granules and secretory vesicles that correlate with neutrophil specific functions(45). The molecular mechanism regulating neutrophil terminal differentiation depends on the myeloid-specific TF C/EBPε, whose expression is upregulated during granulocytic, but not during monocytic differentiation (46). The importance of *C/EBPε* in myeloid differentiation has been supported by various *in vivo* murine models of C/EBPε deficiency, in which *C/EBPε* knockout commonly leads to abnormal granulopoiesis, the generation of hypo-segmented morphologically atypical neutrophils and a neutrophil-specific deficiency in phagocytosis, chemotaxis and cytokine production(36, 47, 48). Additionally, C/EBPε performs an essential function in terminal differentiation by promoting the formation of granular and secretory proteins and directly interacting with the broad-spectrum proteins Rb and E2f1 to repress neutrophilic proliferation and promote neutrophil terminal differentiation(16, 47). Neutrophils from patients with neutrophil-specific granule deficiency (SGD), who have a truncation in the C/EBPε 32-kD isoform, lack neutrophil secondary granule proteins and display defective neutrophil function in chemotaxis and bactericidal response(49).

The importance of TFs in the neutrophil differentiation process has prompted research on the transcriptional landscape during neutrophil differentiation and maturation. In human neutrophils, transcriptional and epigenetic analyses have revealed several TFs that are differentially expressed in the comparisons between different differentiation stages(50). Preneutrophils and immature neutrophils express high levels of C/EBPα, C/EBPε, Gfi1 and Klf5, which have been shown to function to promote the granulocytic lineage choice(12, 16, 31, 51), and several other TFs like Erg, Myb, which have implicated to associate with neutrophil terminal maturation(52, 53). Moreoever, TFs like Fosl1, Fosl2, Junb, Bcl6, Klf6 and Irf1 are constitutively expressed across differentiation stages and tend to reach the maximal expression in terminally differentiated neutrophils(50). Many of these TFs are zinc-finger proteins or leucine zipper motif-containing factors that play roles during inflammatory and oxidative stress responses. In murine neutrophils, multi-parametric analysis has classified BM neutrophils into GMPs, preneutrophils, immature and mature neutrophils and has demonstrated that GMPs highly express TFs involving granulopoiesis initiation, such as C/EBPα(54) and Runx1(55). Also, preneutrophils express high levels of C/EBPε and Gfi1, which is in the line with their role in neutrophil terminal differentiation(42, 47). Finally, mature neutrophils exhibit high expression of Cebpd and PU.1, two TFs crucial for normal neutrophil differentiation(8). 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 stage and identified several novel TFs , including but not limited to *Nfil3, Max, Mlx,* and *Xbp1*(56), although further functional analysis are needed to elucidate the molecular mechanism underlying the regulation of neutrophil differentiation by specific TFs.

Emergency granulopoiesis

Given their short lifespan and post-mitotic nature, mature neutrophils are constantly generated from upstream progenitors in the BM and then released into circulation(4). Such a program can be enhanced under pathological conditions, such as systematic infection, in which the compensatory mechanisms of granulocytopoiesis are triggered mainly through the IL-23/IL-17/G-CSF axis to enhance *de novo* generation of neutrophils and fulfil the increased demand for mature neutrophils(57). Given the temporal activation and function of TFs frequently observed in granulopoiesis under steady-state condition, the intracellular signalling cascades and the transcriptional networks underlining emergency granulopoiesis are less well understood.

C/EBPα serves as a key TF in granulocytic development as evidenced by a differentiation block in the transition from the common myeloid progenitors to the GMPs in *C/EBPα*-deficient mice(12). Such a neutrophil differentiation block partially results from a selective loss of the C/EBPα target gene, the G-CSF receptor, which supported the importance of C/EBPα in steady condition. However, *in vitro* stimulation with granulopoietic cytokines, such as G-CSF, GM-CSF and IL-3(58), markedly enhances the expression of the *G-CSFR* and restores neutrophil differentiation in *C/EBPα*-deficient myeloid progenitors, indicating the existence of a *C/EBPα*-independent pathway of granulopoiesis(58). Subsequent analysis of C/EBP TFs during cytokine- or infection-induced granulopoiesis demonstrated that C/EBPα is dispensable whereas C/EBPβ is essential, for the emergency granulopoietic response(11).

Both C/EBPα and C/EBPβ favour granulopoiesis but have different effects on cell cycle progression in neutrophil progenitors. Upon G-CSFR-induced signalling, *C/EBPα* functions as a master regulator of granulopoiesis under the steady-state condition by promoting neutrophil differentiation and simultaneously arresting cell proliferation by inhibiting expression of the cyclin D proteins *Cdk2* and *Cdk4*, and the cell cycle accelerator *c-Myc*, consequently leading to neutrophil production homeostasis (59). During emergency conditions, granulopoietic cytokines, mostly G-CSF and, to less

extent, GM-CSF and others, signal through the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathways, which directly promote cell cycle progression and activate *C/EBPβ*, the master TF of emergency granulopoiesis(11). Notably, upon G-CSF-induced signalling activation, STAT3 links the granulopoietic signals to C/EBPβ and function together with *C/EBPβ* to directly promote the expression of *Cdk2*, *Cdk4* and *c-Myc* expression and inhibit *C/EBPα* binding to the promoters of target genes, thereby accelerating the generation and mobilisation of neutrophils under emergency conditions(60).

Neutrophil ageing

After being released from the BM, neutrophils shuttle between the blood and the tissues and display a phenotypic shift that follows a strict diurnal pattern(61). In humans, neutrophils display diurnal changes in the expressions of CD11b, intercellular adhesion molecule 1 (ICAM-1), CD62L, and CXCR4(62). In mice, diurnal fluctuations have also been observed in P-selectin glycoprotein ligand-1 (PSGL1), CD62L and the chemokine receptors CXCR2 and CXCR4 (63). Specifically, neutrophils released from the BM express high levels of CD62L, a surface marker whose expression is reduced upon ageing (Figure 2). During the approximate 6-hour course of peripheral circulation, neutrophils gradually downregulate CD62L expression and increase CD11b and CXCR4 surface marker expressions, which mediate neutrophil migration into or within the BM, a process termed neutrophil ageing (61).

Ageing-associated TFs

The process of neutrophil ageing has been proposed to be intrinsically driven by the clock-related TFs, which also regulate neutrophil clearance and its physiological consequences. One major example of such TFs is Brain and muscle aryl hydrocarbon receptor nuclear translocator-like (Arnt) 1 (Bmal1), which is an intrinsic circadian-clock regulator and the only TF whose deficiency disrupts circadian rhythmicity in response to light (13). The molecular mechanisms of the Bmal1-controlled circadian

clock involve auto-regulatory transcriptional feedback loops, in which the TFs Bmal1 and Clock form transcriptional activator complexes to activate the transcription of the cryptochrome and period genes in the core feedback loop to inhibit Bmal1-Clock complexes. Additional feedback loops driven by Bmal1-Clock complexes involve the transcription of the retinoic acid receptor - related orphan nuclear receptors REVERBs and RORs to repress *Bmal1* expression (64). In human peripheral neutrophils, the relative expressional ratio of *Bmal1* is significantly higher in comparison to that of other clock components and was found to regulate the rhythmic expression of adhesion molecules and time-dependent production of cytokines(65). In mice, a conditional knockout of *Bmal1* abolishes the rhythmic emigration of leukocyte subsets from the blood under both physiological and systemic inflammatory conditions, suggesting a central role for the Bmal1-induced circadian clock in neutrophil rhythmic ageing (63). Mechanistically, Bmal1 regulates the temporal production of Cxcl2, a CXCR2 ligand that activates CXCR2 signalling in an autocrine manner, thereby regulating cell-intrinsic rhythms of diurnal neutrophil ageing (Figure 2). Targeted deletion of *Bmal1* or CXCR2 dramatically prevents diurnal neutrophil ageing, which is characterised by the loss of neutrophil transcriptional and migratory oscillations in the response to pathogenic infections(66).

Neutrophils also make use of ROS, which are produced in a cyclic pattern, to regulate neutrophil circadian oscillations. Both murine and human neutrophils are both stimulated by various physiological stresses during the light phase that induce p38/MK2 phosphorylation. Subsequently, p38/MK2 signalling activation stimulates differential intracellular ROS production between murine and human neutrophils, possibly resulting from the opposite function of MK2 in human and mice (67). Downstream production of ROS positively regulates the transcriptional activities of hypoxia-induced factor-1α (Hif-1α) and its interaction with Bmal1, thereby promoting the circadian expression of CXCR4 and hence neutrophil trafficking (67). Additionally, Hif-1α TF activation significantly increases the abundance and functional activities of NF-kB p65 (RelA), which in turn regulates the expression of several genes associated with neutrophil function and migration(68).

These findings collectively support a novel transcriptional network involving mitogen-activated protein kinase (MAPK) and NF-kB kinases, cellular oxidative stress, clock-related TFs and the chemokine system CXCR2/CXCR4 system, which collectively coordinate to control neutrophil circadian oscillations. However, the molecular mechanisms of how the clock-related TFs regulate the chemokine system remain yet to be fully elucidated.

Environmental factors

In contrast to clock-related TFs that intrinsically control neutrophil ageing, oscillation in the expression of adhesion molecules in humans(65) and microbiota-derived metabolites in mice(69) has been proposed to regulate diurnal ageing of neutrophils. In humans, time-of-day variations in the plasmas levels of Cxcl12/SDF-1 controls the mobilisation or immobilisation of the immature fraction of neutrophils from the BM, thereby diurnally regulating the redistribution and function of circulating neutrophils in the body. In mice, microbiota-derived molecules that cross the intestinal barrier have been shown to directly activate neutrophils via Toll-like receptors (TLRs) and MyD88-dependent signalling, thereby inducing circulating neutrophils to become more functionally active (69). Indeed, depletion of the gut microbiota abolishes this microbiota-driven neutrophil ageing and significantly lowers the number of aged neutrophils in the circulation, improving neutrophil-related inflammation and pathogenesis(69).

Neutrophil activation

Neutrophil activation is considered as a multiple-step process initiated by specific priming that is induced by exposure to pro-inflammatory chemoattractants or direct interaction with activated endothelial cells during the transmigration process, allowing for rapid and maximal neutrophil activation. Upon infiltration into inflammation sites, neutrophils respond to pro-inflammatory stimuli and become fully activated, a state that features the secretion of a variety of cytokines and

chemokines(70, 71), and the expression of co-stimulatory molecules and major histocompatibility complex (MHC) molecules(72), thereby influencing the activation of other leukocyte types, including T/B cells and dendritic cells. Neutrophil-mediated regulations of T/B cells and dendritic cells has been reviewed elsewhere (73, 74). Furthermore, recognition of microbial products by specific surface receptors, such as pattern recognition receptors (PPRs) induces an intensively interacting network of downstream signalling and subsequent nuclear translocation of signal-dependent TFs, such as NF-κB, IRFs and AP-1, to induce the expression of genes that associate with neutrophil specific functions (Figure 3).

NF‐κB

NF‐κB is a ubiquitous TF that is involved in multiple inflammatory and immune responses and regulates the expression of many other genes that control cell survival and proliferation. Neutrophils constitutively express NF‐κB subunits, such as RelA/p65, c-Rel, and p52 in both the cytoplasm and the nucleus where these molecules are sequestered via their interaction with inhibitory IkB kinases(such as IKK-α and IKK-β) (75). When exposed to pro-inflammatory stimuli, such asTNF-α, lipopolysaccharide (LPS) or N-formylmethionyl-leucyl-phenylalanine (fMLP), neutrophil activation induces the phosphorylation of the IKK kinases and in parallel with the degradation of inhibitory IKK-α in the cytoplasm and the nucleus. These events lead to the activation and subsequent nuclear translocation of specific NF-κB dimers as well as NF-κB downstream of transcription(76).

NF‐κB activation in neutrophils regulates the expression of genes that closely associate with the functions of producing pro-inflammatory mediators, enhancing cell adhesion and migration and inhibiting cell apoptosis. Among the NF-κB-regulated targets are the cytokines TNF-α, IL-1, IL-6(77, 78), the CXC chemokine family Cxcl-2, Cxcl-8 and Cxcl-10(79) and other immune effectors, such as neutrophil-derived microparticles(80), which predominately act in an autocrine manner to induce

further NF-κB activation, forming a positive feedback loop of neutrophil activation that initiates and amplifies pro-inflammatory responses (75).

Another set of genes upregulated by NF‐κB activation include adhesion molecules, such as integrin αLβ2 (CD11b/CD18), which require p50/p65 to arrest the rolling of neutrophils and facilitate subsequent transmigration(81, 82), as well as CXC chemokine receptors, such as CXCR2, which involve p60/p65 and the NF-κB-mediated induction of *C/EBPα* to mediate neutrophil release from the BM (83). Importantly, upon activation, NF-κB shifts the balance between the pro-apoptotic and anti-apoptotic signalling pathways by inducing the expression of many anti-apoptotic proteins, such as Mcl-1 and Bcl2, promoting extended neutrophil survival and decreased cell apoptosis (84, 85).

Activation of NF‐κB activation engages a negative feedback that promote the down-modulation of NF-κB activity through the re-expression of the NF-κB inhibitory kinase IκBα (17) and NF‐κB-counter regulators, such as RIPK1, leading to the degradation of NF-κB activating TFs(86). Failure to downregulate the NF-κB-driven transcriptional responses results in extended survival of neutrophils and subsequent tissue damage, which is closely associated with the pathogenesis of neutrophil-mediated inflammatory diseases, such as sepsis and RA (87).

Defects in NF‐κB signalling have been shown to influence neutrophil generation and functions. Homozygous deletion of NF–κB subunit *RelA/p65* is embryonically lethal (88) whereas a heterozygous deletion of *RelA/p65* does not result in a significant phenotype in mice. Mice lacking *RelB/p50* show a significant decrease in the level of neutrophil progenitor levels and a high susceptibility to infection (89). 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(83, 90). These examples underline the importance of specific NF‑κB subunits in neutrophil biology and explain the recurrent and persistent infections that patients who lack any one of NF-κB signalling mediators may suffer from (91). However, the molecular mechanisms by which NF-κB signalling loss disrupts neutrophil functions remain largely unexplored.

MAPK-downstream TFs

Another prominent set of signalling pathways mediating neutrophil activation is the MAPK subtype cascades, which are activated upon stimulation of G-coupled protein receptor (GPCR). Neutrophils express all three types of MAPK subtypes, including p38 MAPK, ERK and c-Jun terminal kinase (JNK) cascades(92). Activation of each MAPK subtype is mediated by dual phosphorylation of conserved threonine and tyrosine residues by upstream MAPK kinases, such as MAPK/ERK kinase (MEKK) and MAPK kinase (MKK) 1, 3, 6 or 4/7. Upon activation, neutrophils adopt selective activation of distinct MAPK subtype cascades depending on the type of stimulatory agents used. G-CSF exclusively activates the MEK-ERK cascade and GM-CSF preferentially induces activation of the MEK-ERK over the MKK3/6-p38 MAPK cascade to mediate signals modulating neutrophil activation(93). Activation of the p38 and MEK/ERK and JNK cascades regulate a wide range of TFs, which include and are not limited to c-Jun, JunB, ATF2, p53 and c-Myc that are regulated by JNKs(94); Sap1, CAMP responsive element binding protein 1 (CREB1*)*, p53 and C/EBPβ regulated by p38 MAPKs(95) and Elk1, c-Fos, c-Myc, c-Myb regulated by ERKs(96). These TFs act as transcriptional regulators of several genes that control morphology determination, inflammatory responses, survival and apoptosis upon neutrophil activation(97-99). 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 activator whereas the 20-kDa form of C/EBPβ with a truncated transactivation domain inhibits the transcription (100). As discussed above, in response to cytokine stimulation and/or infection, *C/EBPβ*-deficient mice dispaly deffects in emergency granulopoeisis (11). Moreover, neutrophils with C/EBPβ deficiency shows enhanced apoptosis(101), 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 (102).

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 homodimers or heterodimers to regulate the transcription(103). Upon cell stimulation, CREB proteins are rapidly phosphorylated on their signal domain by p38 MAPK kinases, which allows for increased transactivation potential(104). CREB1 is strongly expressed by resting human neutrophils and constitutively associated with the promoters of several chemoattractants, such as CXCL8 and CCL3 (103). Neutrophil activation leads to the phosphorylation of CREB-1/ATF-1 by p38 MAPKs and increased production of CXCL8, CCL3, CCL4 and TNF (103). CREB1 phosphorylation also enhances the expression of matrix

metallopeptidase 9 (MMP9), which is highly expressed by tumour infiltrating neutrophils (105), 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(106, 107). 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 (108). 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 (109, 110) 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 (111). Neutrophils constitutively express a number of the Jun family proteins. Among them, *JunB* is found to be mostly expressed upon activation(112, 113). 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 (113). *PU.1* inhibits *JunB* binding by restricting the accessibility of enhancers via the recruitment of histone deacetylase 1 (HDAC1), and consequently restraining neutrophil activity (113).

Others families

P53 is another TF critical for neutrophils' stress response. Maintained at low expressional levels in resting cells through targeted degradation, p53 is stabilized and activated in response to cellular stress and and/or inflammatory agents, such as TNF-α(114) and ROS (115). It promotes the expression of anti-apoptotic protein Mcl-1(116). In fact, *p53*-deficient neutrophils demonstrate extended lifespans and enhanced capacity of phagocytosis in response to inflammatory stimulation (117, 118), likely via induction of anti-apoptotic mechanisms. Recent studies demonstrated the mutual regulation of p53

and NF-κB in immune cells (119, 120). p53 inhibits NF-κB transcriptional activity and attenuates the NF-κB–dependent cytokine production by neutrophils (119). Loss of *p53* expression results in enhanced NF-κB DNA-binding activity that induces the expression of many pro-inflammatory cytokines by neutrophils(119). Additionally, p53 regulates cell migration through the activity of the phosphatidylinositol 3-kinase/Rac1 pathway (121) and neutrophils with *p53* deficiency demonstrated increased tissue infiltration and enhanced bacterial killing in response to infection(119), supporting the inhibitory role of p53 in neutrophil functions during inflammation.

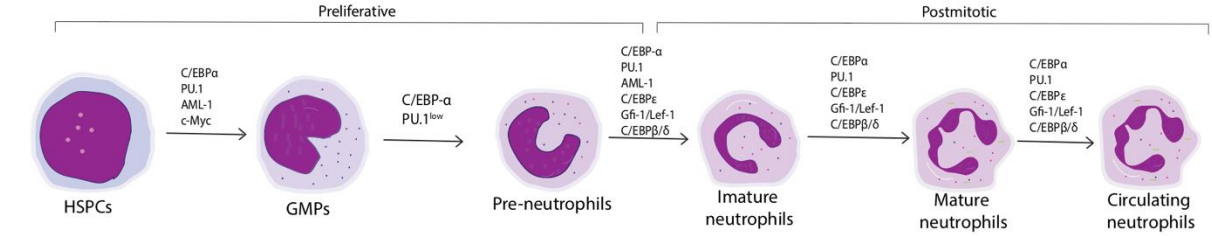
Interferon regulatory factor-5 (Irf5) functions downstream of the TLR-MyD88 signalling pathway to induce the target gene expression (122). Upregulation of *Irf5* has been detected in neutrophils activated via the TLR signalling and cytosolic DNA-sensing pathways (6), as well as the pathways induced by bacterial infection (56). Upon TLR9 stimulation, *Irf5*-deficient bone marrow isolated neutrophils produced significantly lower levels of inflammatory cytokines, such as TNF-α (6), supporting the potential role of *Irf5* as a novel regulator in neutrophil inflammatory responses, although more studies are needed for elucidating the underlying molecular mechanism.

Conclusive remark and future direction

Overall, neutrophil differentiation and activation are complex processes comprising several changes at the morphological to transcriptional levels. Different 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. Moreover, the recently described diverse neutrophil phenotypes, such as surface markers, immunoregulatory function, NETosis and phagocytic capacity, align with the increasing evidence supporting the roles of TFs in modulating the phenotypic and functional properties of neutrophils(4, 57). Thus, further studies about neutrophil transcriptional network should provide a more comprehensive picture of the pathways as well as the functional relevance of specific TFs underlying neutrophil differentiation and

activation process. Identification of crucial TFs 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 legends



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. Transcription factors(TFs) involved in neutrophil differentiation. 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 neutrophilic lineages. C/EBPε 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 preneutrophils 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 defense responses, thereby accelerating neutrophil maturation (56).

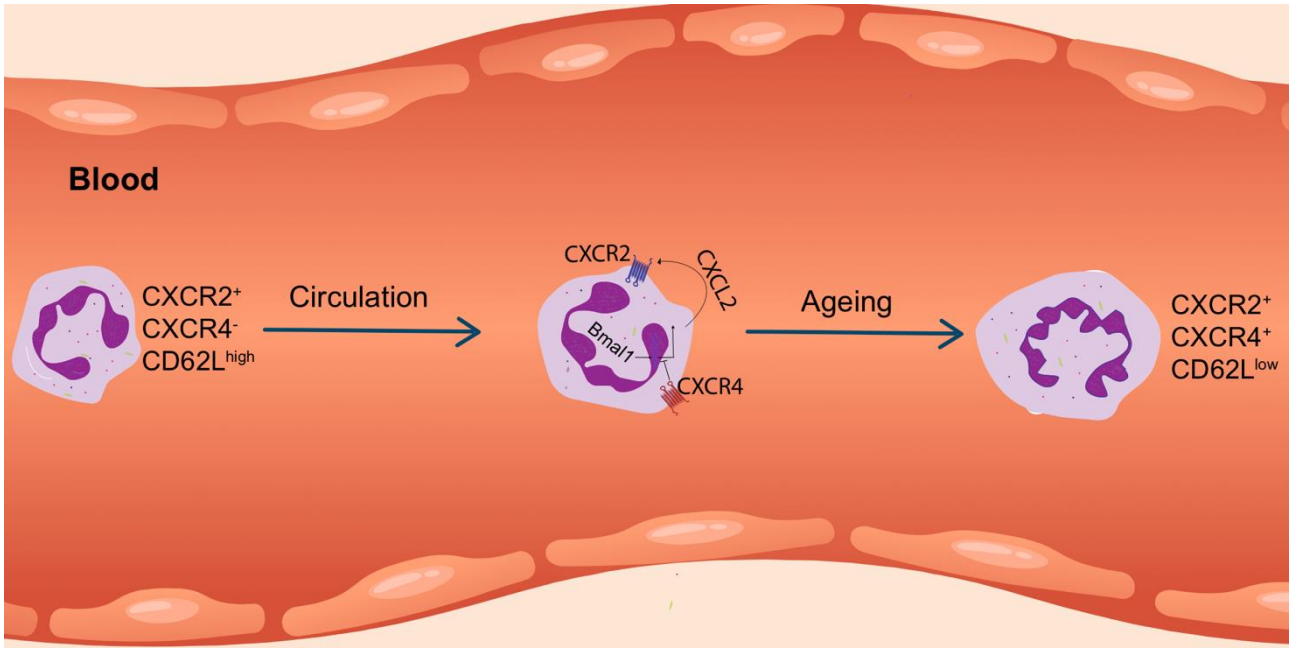


Figure 2. Transcriptional regulation of neutrophil ageing (66). Neutrophils released from the bone marrow display a fresh phenotype featuring with high CD62L expression, after which CD62L is gradually downregulated and CXCR4 expression is upregulated during ageing. Bmal1 intrinsically regulates neutrophil ageing by modulating the expression of CXCL2, a CXCR2 ligand that functions to promote neutrophil ageing in an autocrine manner.

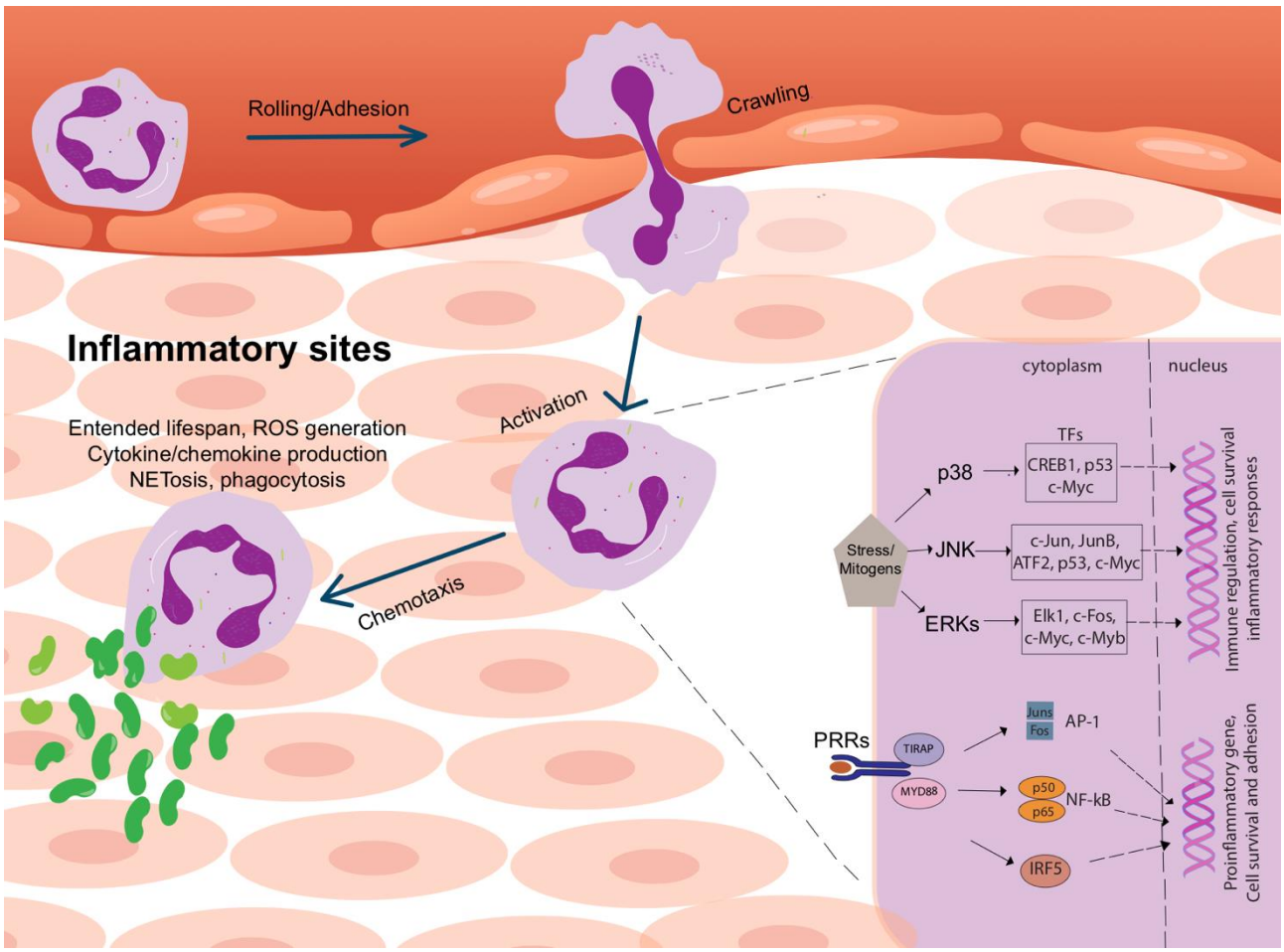


Figure 3. Transcriptional regulation of neutrophil activation in the inflammatory sites. Upon infiltration into sites of inflammation, direct interaction between neutrophils and activated endothelial cells and subsequent exposure to pro-inflammatory stimulation, fully activates neutrophils. Several TFs 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(6, 56, 71).

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