Distinct transcription factor networks control neutrophil-driven inflammation

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Neutrophils display distinct gene expression patterns depending on their developmental stage, activation state and tissue microenvironment. To determine the transcription factor networks that shape these responses in a mouse model, we integrated transcriptional and chromatin analyses of neutrophils during acute inflammation. We showed active chromatin remodeling at two transition stages: bone marrow-to-blood and blood-to-tissue. Analysis of differentially accessible regions revealed distinct sets of putative transcription factors associated with control of neutrophil inflammatory responses. Using ex vivo and in vivo approaches, we confirmed that RUNX1 and KLF6 modulate neutrophil maturation, whereas RELB, IRF5 and JUNB drive neutrophil effector responses and RFX2 and RELB promote survival. Interfering with neutrophil activation by targeting one of these factors, JUNB, reduced pathological inflammation in a mouse model of myocardial infarction. Therefore, our study represents a blueprint for transcriptional control of neutrophil responses in acute inflammation and opens possibilities for stage-specific therapeutic modulation of neutrophil function in disease.
In this model, a cavity is created on the dorsal surface of mice and granulation tissue (membrane) is formed over a period of 6 d (Extended Data Fig. 1a), composed primarily of fibroblasts and macrophages. Injection of 1 mg of zymosan, a ligand for dectin-1 and Toll-like receptor 2/6, into the pouch cavity results in efficient infiltration of immune cells from the blood into the
membrane and pouch (Supplementary Data), a large proportion being neutrophils. Neutrophil recruitment initiated at 4 h (Supplementary Data), and peaked at 12 h after stimulation in the pouch (Extended Data Fig. 1c), while remaining up to 24 h in the membrane (Extended Data Fig. 1d). Phenotypic assessment of neutrophils from the blood, membrane, and pouch at 4 h post-zymosan challenge revealed progressively elevated levels of intracellular pro-interleukin 1-β (IL-1β) in the membrane and pouch, indicating that neutrophils undergo activation as they traverse to the tissue (Extended Data Fig. 1e).

We isolated Ly6Ghi CD11b+ cells from the bone marrow, blood, membrane, and air pouch exudate 4 h after challenge with zymosan (Extended Data Fig. 1f) and conducted small bulk messenger RNA-seq and assay for transposable-accessible chromatin using sequencing (ATAC-seq) analyses (Fig. 1a). In zymosan-challenged animals, a substantial proportion of neutrophils in the blood, air pouch membrane and exudate expressed CD101 (Extended Data Fig. 1g), a marker of mature neutrophils, whereas bone marrow neutrophils were largely CD101- and represented immature neutrophils (Extended Data Fig. 1g,h). Zymosan challenge induced egress of bone marrow immature neutrophils into the circulation, which were largely absent in naive mice (Extended Data Fig. 1h).

First, we mapped the transcriptional changes associated with neutrophil progression to the site of inflammation in vivo (Fig. 1a). Principal component analysis (PCA) of 1,865 differentially expressed genes (DEGs) (P_adj < 0.1) clearly separated neutrophils from the compartments and indicated that changes were incremental, initiated with the transition from the bone marrow to the blood and continuing gradually as neutrophils migrate into sites of inflammation (Fig. 1b). Comparison between the bone marrow and air pouch neutrophils revealed 445 upregulated and 1,303 downregulated genes (Extended Data Fig. 2a). Gene set enrichment analysis (GSEA) of DEGs between the air pouch and bone marrow (P_adj < 0.05 and fold change >1.5) revealed that chemotaxis, cytokine activity and signaling pathway genes are gradually upregulated along the maturation trajectory and the inflammatory cascade, while respiratory chain complex, mitochondrial matrix and fatty acid beta-oxidation genes are gradually downregulated (Fig. 1c and Extended Data Fig. 2b,c).

Hierarchical clustering of the DEGs across all the compartments identified five clusters (Fig. 1c), which encompass genes progressively upregulated in the blood, membrane and air pouch (cluster 1), transiently upregulated in the blood (cluster 3), rapidly downregulated in the membrane and air pouch (cluster 2), slowly downregulated in the air pouch (cluster 4) and transiently downregulated in the blood (cluster 5) (Fig. 1d). Gene ontology (GO) analysis revealed that downregulated genes in clusters 2 and 4 primarily correlated to metabolic processes (cluster 2: cellular lipid catabolism, fatty acid beta oxidation) and protein transport (cluster 4: cytosolic transport, Golgi vesicle transport) (Fig. 1c). The upregulated genes in cluster 3 represent stress response (JNK/SAP kinases; in cluster 1, they are pro-inflammatory and interferon response genes (cytokine secretion, response to interferon gamma) (Fig. 1c).

Overall, our global analyses of the transcriptional landscape during inflammation reveals loss of anabolic capacity as neutrophils leave the bone marrow, transient reduction in their toxic potential coupled with gain of signal transduction in the circulation and final acquisition of an inflammatory and effector profile at the site of challenge.

Chromatin changes underpin transcriptional activation. Second, we mapped chromatin changes associated with neutrophil activation in vivo (Fig. 1a). The PCA analysis of 11,881 differentially accessible peaks (DAPs) derived from ATAC-seq analysis (P_adj < 0.05) revealed marked diversity in chromatin landscapes between neutrophils in the bone marrow, blood and membrane (Fig. 2a); therefore, chromatin remodeling is likely to underpin the changes in transcriptional profiles seen along the inflammatory cascade. Remarkably, we observed no further chromatin remodeling events at the transition from inflamed tissue to exudate (Fig. 2a), suggesting that the detected change in gene expression at this final step of neutrophil migration does not require global alterations in transcriptional control.

The ATAC-seq analyses identified two distinct remodeling events, the transition of neutrophils from bone marrow to blood (481 opening and 356 closing peaks) and from blood to the inflamed tissue (2,150 opening and 878 closing peaks) (Extended Data Fig. 3a). For instance, accessibility of the myeloid differentiation marker Myadm increased as neutrophils were released into the blood,
ATAC-seq

Replicate

Condition

Myadm

Air pouch

Membrane

Blood

Bone marrow

Replicate

Condition

Myadm

Air pouch

Membrane

Blood

Bone marrow

ATAC-seq signal

(FPKM)

ATAC-seq log fold change

RNA Pol regulatory region

sequence-specific DNA binding

Integral component of plasma membrane

Intracellular membrane-bounded organelle

Neutrophil degranulation

Inflammatory response

Focal adhesion

Mitochondrial inner membrane

Golgi membrane

Peroxisome

Nuclear membrane

Chromatin DNA binding

Motif

Transcription factor family

Comparison

PU.1

1.4 \times 10^{-38}

Blood versus bone marrow

AP-1

3.4 \times 10^{-87}

Blood versus bone marrow

RFX

1.8 \times 10^{-11}

Blood versus bone marrow

AP-1

1.6 \times 10^{-283}

Membrane versus blood

PU.1

2.3 \times 10^{-69}

Membrane versus blood

NF-κB

9.5 \times 10^{-33}

Membrane versus blood

IRF

3.5 \times 10^{-3}

Membrane versus blood

Motif

Transcription factor family

Comparison

PU.1

2.3 \times 10^{-28}

Blood versus bone marrow

SP/KLF

2.2 \times 10^{-12}

Blood versus bone marrow

PU.1

7.1 \times 10^{-318}

Membrane versus blood

SP/KLF

1.4 \times 10^{-41}

Membrane versus blood

SP/KLF

1.5 \times 10^{-14}

Membrane versus blood

RUNX

1.1 \times 10^{-5}

Membrane versus blood
whereas the Thbs1 locus closed. In turn, the basally inaccessible promoter of Ccl3 opened drastically once neutrophils reached the site of inflammation (Fig. 2b). The analysis of DAPs over 5 kilobase (kb) regions centered on the peaks revealed comparable number of peaks with increased and decreased accessibility at the two transition points: bone marrow to blood and blood to tissue (Fig. 2c and Extended Data Fig. 3b). It suggested that neutrophil reprogramming is parsimonious and under strict control.
Hierarchical clustering of the DAPs identified six clusters of distinct patterns of chromatin behavior, including the ones that gained or lost accessibility at the bone marrow-to-blood transition (clusters 1 and 2), at the blood-to-tissue transition (clusters 5 and 3) or transiently in blood (clusters 4 and 6) (Extended Data Fig. 3c). When DAPs were mapped to the neighboring genes, the blood-to-tissue transition was linked to increased accessibility of inflammatory response regulators and signaling pathways, while the bone marrow-to-blood transition was mainly associated with antigen presentation and metabolic activity (Extended Data Fig. 3d). Next, we compared the change in accessibility in promoter proximal peaks to that of the associated gene expression levels (Fig. 2d). The categories of genes that showed both upregulation of gene expression and increase in chromatin accessibility included the inflammatory response, neutrophil degranulation and focal adhesion (Fig. 2e).

By conducting de novo motif discovery at DAPs from bone marrow to blood and blood to tissue (membrane) we identified putative transcription factors controlling neutrophil inflammatory responses (Fig. 2f). Consistent with the known role of PU.1 in myeloid cell development, PU.1 motifs were detected at DAPs at both transitions (Fig. 2f). Other transcription factors displayed greater selectivity: regulatory factor X (RFX) motifs were detected exclusively at peaks with increasing accessibility from bone marrow to blood, while interferon regulatory factor (IRF) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) motifs were detected at peaks with increased accessibility from blood to membrane (Fig. 2f). Activator protein 1 (AP-1) motifs were detected at both transitions but were more strongly enriched in the blood-to-membrane opening peaks. We also noted SP/KLF, and RUNX motifs at peaks with reduced accessibility from bone marrow to blood and blood to membrane. In search of transcription factors highly expressed in neutrophils, we checked the expression of each family member across immune cells (Extended Data Fig. 4a) or neutrophil populations (Extended Data Fig. 4b) at the Immunological Genome Project (ImmmGen). Rfx2 was the only gene of the RFX family and Runx1 of the RUNX family expressed in neutrophils, while Klf6 expression was strongest among KLF family members (Extended Data Fig. 4a). Furthermore, we queried an scRNA-seq dataset that mapped neutrophil subpopulations G0–G5 to progressively maturing neutrophils. Runx1 expression was highest at the earliest stages of neutrophil differentiation (G0–G3), whereas Relb, Irf5 and Junb expression increased with neutrophil maturation (G4 and G5) and remained unaffected by Escherichia coli challenge. Klf6 expression was detected throughout the G0–G5 populations (Extended Data Fig. 4c).

Taken together, these data suggest an incremental program of neutrophil activation, with Rfx2, Klf6 and Runx1 linked to the initial transition of neutrophils to blood and ongoing neutrophil maturation, whereas the signal-dependent transcription factors Junb, Relb and Irf5 are predicted to play a role in the inflamed tissue.

**Bone marrow-to-blood transition transcription factors control neutrophil maturation.** For further functional validation of Rfx2, Klf6, Runx1, Junb, Relb and Irf5, as well as the previously noted neutrophil transcription factors C/EBPβ and CEBPε, we utilized a model system of immortalized myeloid HoxB8 progenitor cells, which differentiate into nonproliferating neutrophils after granulocyte colony-stimulating factor-induced ex vivo differentiation for 5 days. At day 1/day 2, they resemble myelocytes/meta-myelocytes and express c-Kit, CXCR4 and CD49d; at day 3/day 4 they resemble band neutrophils; at day 5, they resemble mature neutrophils expressing CXCR2 and CD101 (ref. 30). We confirmed the highest protein levels of Junb in day 5 neutrophils (Extended Data Fig. 4d).
Data Fig. 4d), matching the mRNA expression data (Extended Data Fig. 4c) Stimulation with zymosan led to JUNB phosphorylation without affecting its protein levels (Extended Data Fig. 4d), indicating the posttranslational role of inflammatory signals in the activation of signal-dependent transcription factors.

Using CRISPR–Cas9, we generated stable knockout lines for selected transcription factors in Hoxb8 progenitors and validated their deletion by western blot analysis (Extended Data Fig. 5a). After 5 days of ex vivo differentiation, HoxB8 progenitors with genetic deletion of the Irf5, JunB or Cebpb genes gave rise to...
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mitochondrial membrane potential (MMP), a key indicator of mitochondrial activity. Mature neutrophils possess a limited number of mitochondria and are defective in ATP production. Indeed, mitochondrial membrane potential (MMP), a key indicator of mitochondrial activity and the driving force behind ATP production, was significantly lower in day 5, compared to day 3 HoxB8 neutrophils (Extended Data Fig. 5d). Consistent with their arrested maturation, Klf6−/− and Runx1−/− neutrophils displayed higher MMP (Extended Data Fig. 5d) compared to WT cells. Interestingly, deletion of Junb also led to higher MMP, without affecting neutrophil maturation (Fig. 3a,b and Extended Data Fig. 5d). To further investigate a possible impact of selected transcription factors on the metabolic aspects of neutrophil development, we utilized the Seahorse assay to measure the oxygen consumption rate (OCR) as a key indicator of mitochondrial respiration (Supplementary Fig. 1). Klf6−/− or Runx1−/− neutrophils displayed higher levels of mitochondrial basal respiration, ATP turnover and mitochondrial maximal and spare capacities compared to WT cells (Fig. 3e). Other mutants had little impact on neutrophil mitochondrial activity, with the exception of Rfx2−/−, which also averaged a lower level in all metabolic measurements (Fig. 3e). We examined if Rfx2 deficiency may lead to spontaneous apoptosis, using annexin V staining, and observed a dramatic increase in apoptotic rate compared to WT (Extended Data Fig. 5e). Deficiency in Relb also led to an increase in neutrophil apoptosis, which is consistent with the known function of NF-kB in supporting neutrophil survival and blocking spontaneous apoptosis.

Akin to findings in the HoxB8 system in vitro, conditional deletion of Runx1 in myeloid populations produced a lower percentage (Fig. 3f) and absolute counts (Extended Data Fig. 6c) of Ly6G+CD101+ mature neutrophils in the bone marrow of naive Lys2Cre × Runx1loxP/loxP mice, whereas no significant difference was observed in either Irf5−/− or S100a8Cre × JunbloxP/loxP mice, with conditional deletion of Junb in neutrophils (Extended Data Fig. 6a,b). Corresponding percentages of immature Ly6G+CD101+ and c-kit+CXCR4+ preneutrophils were increased in the bone marrow of naive Lys2Cre × Runx1loxP/loxP mice (Fig. 3f). Morphological characterization revealed a significantly lower percentage of segmented neutrophils and increased number of metamyelocyte-like cells in the bone marrow of Lys2Cre × Runx1loxP/loxP mice (Extended Data Fig. 6d) but not S100a8Cre × JunbloxP/loxP mice (Extended Data Fig. 6e). We investigated dependence of known neutrophil transcription factor expression on Runx1 and found that expression of Cebpe was significantly reduced in Runx1−/− neutrophils (Fig. 3g), which is reminiscent of human data. Furthermore, Cebp protein levels were significantly reduced in bone marrow neutrophils of Lys2Cre × Runx1loxP/loxP mice (Extended Data Fig. 6f). Expression of Klf6 was independent of Runx1 (Fig. 3g).

Thus, Klf6 and Runx1, associated with neutrophil chomatins closing en route to the tissue, are independently involved in neutrophil maturation, Rfx2 and RELB, linked to chromatin opening, support neutrophil survival.

RUNX1 and KLF6 deficiency impairs neutrophil recruitment. Neutrophil precursors display reduced migratory capacity in tissue. We hypothesized that KLF6- and RUNX1-deficient neutrophils may display impaired (trans)migration properties. Using the Boyden chamber migration assay, we observed a significant reduction in the migration of day 5 Runx1−/− and Klf6−/− neutrophils, which was comparable to day 3 WT HoxB8 neutrophils (Fig. 4a).

To assess the impact of RUNX1 and KLF6 on the transcriptional programs involved in neutrophil maturation and migration, we performed mRNA-seq analysis of WT HoxB8 neutrophils at days 0, 1, 3 and 5 of Runx1−/− and Klf6−/− neutrophils at day 5. A total of 3,306 and 2,611 genes were differentially expressed in Runx1−/− and Klf6−/− neutrophils compared to day 5 WT cells, respectively. Hierarchical clustering of the DEGs identified five clusters, which encompassed genes downregulated (clusters 1 and 4) or upregulated with maturation (clusters 2, 3 and 5). Genes in clusters 2 and 5 were downregulated in Runx1−/− or Klf6−/− neutrophils, respectively (Extended Data Fig. 7a). GO annotation analysis revealed that these two clusters encompassed transcriptional programs of immune responses, cytokine production and leukocyte migration (Extended Data Fig. 7b). Genes in clusters 4 and 3 were upregulated in Runx1−/− or Klf6−/− neutrophils, respectively, with programs capturing metabolic and biosynthetic processes (Extended Data Fig. 7b). 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.

Fig. 5 | RELB, IRF5 and JUNB control the immune responses of neutrophils. a, Putative target genes for each transcription factor: DEGs (HoxB8 WT versus transcription factor knockout in zymosan-induced HoxB8 neutrophils, Padj < 0.0001) within differentially accessible ATAC-seq peaks (Padj < 0.05, fold change > 2) stratified by the identified binding sites for the corresponding transcription factors (FIMO P < 0.0001) within differentially accessible ATAC-seq peaks (Padj < 0.05, fold change > 1.5) in the gene vicinity. b, GO biological process terms enriched for putative target genes as in a. c, CAV1 expression in HoxB8 neutrophils. Top: One representative western blot probed with antibodies specific for CAV1 and β-actin is shown. Bottom: Statistical analysis of CAV1 expression normalized against the amount of β-actin in the lysates (and expressed as the arbitrary unit of CAV1). Data are shown as the mean and s.d. from three independent experiments. Significant differences compared with the unsensitized WT neutrophils are denoted as *P < 0.05, **P < 0.01 (ordinary one-way ANOVA with Dunnett’s multiple comparisons test). d, Phagocytosis of fluorescein-conjugated E. coli by WT and indicated knockout HoxB8 neutrophils, measured by flow cytometry. e, Intracellular ROS production by WT and indicated knockout HoxB8 neutrophils. f, Bacterial killing by HoxB8 neutrophils incubated with Staphylococcus aureus for 90 min before cell lysis. Values represent absolute CFU counts generated by surviving bacteria. Data are shown as the mean and s.d. derived from four independent experiments. g, NET formation in response to stimulation by PMA/ionomycin in WT and indicated knockout HoxB8 neutrophils. Data are expressed as the percentages of neutrophils undergoing NETosis out of at least 200 cells counted from different fields and independent replicates. Data are shown as the mean and s.d. derived from three independent experiments. d.e.g. Significant differences between knockout and WT neutrophils are denoted as *P < 0.05, **P < 0.01, ****P < 0.0001 (repeated measures one-way ANOVA with Dunnett’s multiple comparisons test).
for RUNX1 and Vcam1, CdB, C3ar1, Cx3cr1 and Icam1 for KLF6 (Extended Data Fig. 7c).

To examine the role of selected transcription factors in neutrophil recruitment in vivo, we injected intravenously an equal mix of CellTracker Far Red–labeled WT and CellTracker carboxyfluorescein succinimidyl ester (CFSE)–labeled knockout HoxB8 neutrophils into the air pouch cavity (Fig. 4b). The recruitment of WT and knockout cells was monitored at 4 h postzymosan injection.

![Graphs and diagrams showing gene expression and protein levels](NATURE IMMUNOLOGY | VOL 22 | SEPTEMBER 2021 | 1093–1106 | www.nature.com/natureimmunology)
In summary, our data place KLF6 and RUNX1 at the apex of the neutrophil differentiation cascade, where they may independently control multiple associated processes, including neutrophil migration, migration, granular content and metabolism.

RELB, IRF5 and JUNB control neutrophil activation and effector functions. Next, we investigated whether transcription factors associated with chromatin opening, expressed more highly in circulating, activated and tissue neutrophils, and not implicated in neutrophil development (C/EBPβ, RELB, IRF5, JUNB) regulated neutrophil activation. We conducted mRNA-seq analysis of WT and transcription factor–deficient HoxB8 neutrophils challenged with zymosan for 2 h. Expression of 4,887 genes was affected in the blood of Lyz2Ct/Ct × Runx1loxPloxP mice (Extended Data Fig. 7e). The total number of neutrophils in circulation was comparable between the genotypes but the number of neutrophils mobilized into the site of inflammation was significantly lower in the Lyz2Ct/Ct × Runx1loxPloxP mice (Extended Data Fig. 4d), most likely attributed to fewer circulating mature neutrophils (Extended Data Fig. 7e).

To validate the importance of selected transcription factors in neutrophil activation, we examined the consequence of their knock-out on neutrophil effector functions. IRF5 and JUNB deficiency affected the ability of neutrophils to phagocytose bacteria (Fig. 5d), while RELB and JUNB were important for ROS production (Fig. 5e), bacterial killing (Fig. 5f) and neutrophil extracellular trap (NET) formation (Fig. 5g and Extended Data Fig. 8c). This was supported by decreased expression of key genes involved in ROS generation and phagocytosis (Extended Data Fig. 8d). JUNB had the strongest effect on genes involved in phagocytosis, such as Csl302, lcam5, Ilgb and Tgm2 (Extended Data Fig. 8e).

Thus, among the transcription factors active in the blood-to-tissue transition, JUNB demonstrated the most consistent overall effect on neutrophil effector functions, while the contributions of IRF5 and RELB were more nuanced and that of C/EBPβ was minimal.

RELB, IRF5 and JUNB control the expression of inflammatory mediators. Expression of cytokines and chemokines by HoxB8 neutrophils was strongly and globally upregulated in response to zymosan treatment and affected by RELB, IRF5 and JUNB deficiency (Extended Data Fig. 9a,b). Zooming in, we observed a significant reduction in mRNA expression of the pro-inflammatory cytokines Il1a, Il1b, Tnf and Il6 and the chemokines Ccl2 and Cxcl2 in zymosan-stimulated Relb−/−, Irf5−/− or Junb−/− mice displayed reduced levels of CCL2, IL-1α, TNF and CXCL10; Irf5−/− cells displayed reduced levels of IL-1α and TNF (Fig. 6b). Once again, deficiency in JUNB affected the widest range of mediators (Fig. 6b). After a 30-min (long) exposure, we detected CCL2, CCL3, CCL4, CXCL2, CXCL10, TNF and IL-1α (Fig. 6b). Relb−/− cells displayed reduced levels of CCL2, IL-1α, TNF and CXCL10; Irf5−/− cells displayed reduced levels of IL-1α and TNF (Fig. 6b).

Expression of pro-IL-1β, 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. Data are shown as the mean and s.d. from five mice. c, Significant differences between Irf5loxPloxP and S100a8loxPloxP mice subjected to the air pouch model and zymosan challenge. Data are shown as the mean and s.d. from five mice. d, Significant differences between Irf5loxPloxP and S100a8loxPloxP mice subjected to the air pouch model and zymosan challenge.
We used an adoptive HoxB8 neutrophil transfer into the air pouch model (Fig. 4c) to examine the effect of transcription factor knockout on neutrophil ability to produce inflammatory mediators in vivo (Fig. 6d). RelB−/−, Irf5−/− or JunB−/− HoxB8 neutrophils in the membrane and/or exudate produced less pro-IL-1β than WT neutrophils (Fig. 6e). When mice with conditional deletion of Irf5 in neutrophils, S100a8Cre×Irf5loxP/loxP, were subjected to the air pouch model, IRF5-deficient neutrophils displayed a significant reduction in pro-IL-1β at the site of inflammation (Fig. 6f), matching the adoptive transfer experiments (Fig. 6e).
Finally, we sought to demonstrate that interference with neutrophil activation would curb pathological inflammation in vivo by applying a neutrophil-dependent model of acute myocardial infarction (AMI) induced by ischemia-reperfusion of the left anterior descending coronary artery to \( S100a8^{loxP} \times JunB^{loxP/loxP} \) mice (Fig. 7a). The analysis revealed a significant reduction in infarct size in mice with neutrophil-specific ablation of JunB compared to littermate controls (Fig. 7b–d). To examine whether JUNB intrinsically controls neutrophil activation at inflamed sites, we generated mixed chimeric mice harboring both control and JUNB-deficient \( (S100a8^{loxP} \times JunB^{loxP/loxP}) \) neutrophils by bone marrow transplantation into WT recipient mice; 3h after induction of cardiac ischemia-reperfusion injury, we analyzed neutrophils in the myocardium (Fig. 7c) and confirmed no substantial change in the chemistry ratio in bone marrow:blood or blood:heart transitions (Extended Data Fig. 10a), thereby corroborating our finding that neutrophil recruitment to the site of inflammation is not affected by depletion of JUNB in neutrophils (Fig. 4c). In agreement with our in vitro and in vivo analyses (Figs. 5e and 6c,e), we found consistent reductions in the levels of pro-IL-1b (Fig. 7f) and intracellular ROS (Fig. 7g) in JUNB-deficient neutrophils compared to their WT counterparts.

Together, these data suggest that IRF5, RELB and JUNB have limited roles in neutrophil maturation but significantly regulate inflammatory cytokines production in vitro and at the sites of inflammation in vivo. Moreover, neutrophil-specific inhibition of JUNB limits pathological destruction of tissue during neutrophil-driven inflammation (Extended Data Fig. 10b).

**Discussion**

It is becoming increasingly clear that neutrophils, which have previously been considered as transcriptionally inactive cells, display distinct gene expression profiles at different developmental stages, activation states and microenvironments. Nevertheless, the transcriptional networks that shape neutrophil function have not yet been defined. In this study, we investigated the transcriptional and chromatin landscape of neutrophils during sterile inflammation and identified previously unappreciated transition points in neutrophil transcriptional regulation, from bone marrow to the blood and from the blood to the tissue, each associated with the involvement of a distinct set of transcription factors. Further functional validation of candidate transcription factors in vitro and in vivo, dissociated cell state-specific transcription factors that modulate neutrophil maturation (RUNX1, KLF6) from transcription factors driving neutrophil effector responses (REL, IRF5, JUNB) and/or neutrophil survival (RFX2, RELB), thus opening new possibilities for specific stage-specific modulation of neutrophil function in disease.

The capture of transcriptional changes related to neutrophil maturation at the transition from bone marrow to the blood likely reflects differences in proportion of immature Ly6G-CD101+ and mature Ly6G+CD101+ neutrophils in the bone marrow and blood samples, which is consistent with recently published studies.

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*Fig. 7 | Neutrophil-specific knockout of JunB limits pathological destruction of tissue during neutrophil-driven inflammation.**

(a) Mouse model of AMI. The left anterior descending coronary artery was occluded for 45 min; this was followed by reperfusion for 1 h. b, Representative images of heart slices stained with TTC and normalized to area at risk (AAR; negative Evans blue staining) from \( JunB^{loxP/loxP} \) and \( S100a8^{loxP} \times JunB^{loxP/loxP} \) mice subjected to ischemia-reperfusion injury. The dotted yellow lines highlight areas of dead myocardium. c, Histological evaluation of the left ventricle AAR (c) and infarct size (d) in mice subjected to ischemia-reperfusion injury. All results are shown as the mean and s.d. derived from four mice from each group in one experiment. Significant differences between \( JunB^{loxP/loxP} \) and \( S100a8^{loxP} \times JunB^{loxP/loxP} \) mice were determined by unpaired Student’s t-test analysis.

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Genomic and transcriptomic differences separating bone marrow and blood neutrophils may in part be the reason for a long held view on immaturity of mouse neutrophils and their differences with human neutrophils\textsuperscript{3,6,28,31} since most of mouse studies focused on bone marrow neutrophils and most human studies used blood neutrophils. This highlights the need for careful consideration of the experimental source of cells in neutrophil studies.

In differentially accessible chromatin regions between the bone marrow and blood, we identified binding motifs for transcription factors, such as RUNX1 and KLF6, which facilitate neutrophil maturation. RUNX1 is a transcription factor with a major role in myelopoiesis; it promotes neutrophil terminal differentiation in a Cebpε-dependent manner\textsuperscript{6}, which we confirmed in this study. KLF6 is a transcription factor linked to the pathogenesis of acute myeloid leukemia\textsuperscript{28}; its role in neutrophil maturation has not been previously addressed. Differentiating neutrophils undergo a metabolic reprogramming toward glycolysis on maturation\textsuperscript{5}, which ensures that neutrophils can function in an inflammatory environment where the oxygen tension may be low or even absent\textsuperscript{6,28}. At the same time, we and others observed the decrease in their mitochondrial activity during the final stages of neutrophil maturation. In line with their immature phenotype, KLF6- and RUNX1-deficient neutrophils display higher mitochondrial membrane potential, which is associated with respiratory chain activity and oxidative phosphorylation. They are also characterized by a higher level of mitochondrial basal respiration, ATP turnover and mitochondrial maximal and spare capacities compared to WT. Another consequence of neutrophil maturation is their ability to rapidly migrate toward sites of inflammation\textsuperscript{28}. Indeed, neutrophils deficient in RUNX1 or KLF6 could not efficiently reach the site of inflammation. RNA-seq analysis confirmed that these transcription factors positively control transcriptional programs upregulated with maturation, such as immune responses or leukocyte migration, but also inhibit programs related to metabolic and biosynthetic processes. Further mapping of transcriptional control of neutrophil differentiation trajectory and functional validation of the transcription factors involved would provide new insights into the current model of neutrophil development.

sRNA-seq profiling of neutrophils\textsuperscript{28} is the essential basis for such analysis, while emerging spatial genomic technologies would help to avoid the impact of cell isolation.

Importantly, factors regulating the transition from blood into the tissue (IRF5, JUNB and RELB) do not affect respiration and mitochondrial function of neutrophils, nor do they impair their migration into the tissue. Instead, they contribute to cytokine and chemokine expression and production and various effector functions, such as phagocytosis, generation of ROS, bacterial killing or NETosis. JUNB demonstrated the strongest overall effect on neutrophil effector functions, which is consistent with the previously reported role of JUNB in setting up the neutrophil inflammatory response\textsuperscript{29}. Neutrophil-specific ablation of JUNB has a profound effect on the extent of infarct in the model of AMI, supporting the notion that neutrophil transcriptional reprogramming could offer alternative therapeutic strategies to manage cardiovascular disease. As highlighted previously\textsuperscript{22}, IRF5 plays a role in regulating inflammatory gene expression but also contributes to regulation of neutrophil phagocytosis, while its involvement in ROS production and NETosis is limited. On the contrary, RELB is critical for the formation of NETs and drives ROS production via expression of specific subunits of the catalytic NOX2 complex of NADPH. RELA, another member of the NF-kB family, has also been implicated in ROS induction in myeloid cells via different NADPH complex subunits\textsuperscript{30}, raising the possibility for their coordinated action.

RFX2 and RELB are critical for neutrophil survival at the steady state and during inflammation. While the role of the NF-kB family in supporting neutrophil survival and blocking spontaneous apoptosis has been documented previously\textsuperscript{28}, the pro-survival function of RFX2 in immune cells has not been described previously. RFX2-deficient mice do not have an obvious embryonic phenotype but absence of RFX2 results in germ cell apoptosis\textsuperscript{31}, suggesting a general role for RFX2 in controlling cell survival across cell lineages.

In summary, our study provides a significant advance in understanding the molecular mechanisms underlying neutrophil development and function during inflammation by depicting key transcription factor modules that modulate development versus inflammatory responses or survival. It extends our understanding of neutrophil transcriptional reprogramming from adaptation to local tissue environment at homeostasis\textsuperscript{32}, to acquisition of specific effector functions under inflammation. We generated 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 transcription factors 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 postchemotherapy cancer patients. Inhibition of neutrophil activation, in contrast, may help to reduce the inflammatory burden suffered during inflammation-associated diseases, such as cardiovascular diseases. Induction of neutrophil effector functions may be needed in infectious diseases and early stages of cancer.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41590-021-00968-4.

Received: 27 November 2020; Accepted: 4 June 2021; Published online: 19 July 2021

References

1. Cowland, J. B. & Borregaard, N. Granulopoiesis and granules of human neutrophils. Immunological Rev. 273, 11–28 (2016).
2. Evrard, M. et al. Developmental analysis of bone marrow neutrophils reveals populations specialized in expansion, trafficking, and effector functions. Immunity 48, 364–379.e8 (2018).
3. Lawrence, S. M., Corriden, R. & Nizet, V. The ontogeny of a neutrophil: mechanisms of granulopoiesis and homeostasis. Microbiol. Mol. Biol. Rev. 82, e00557-17 (2018).
4. Nauseef, W. M. & Borregaard, N. Neutrophils at work. Nat. Immunol. 15, 602–611 (2014).
5. Scapini, P. & Cassatella, M. A. Social networking of human neutrophils within the immune system. Blood 124, 710–719 (2014).
6. Adrover, J. M. et al. A neutrophil timer coordinates immune defense and vascular protection. Immunity 50, 390–402.e10 (2019).
7. Beyrav, M., Bodkin, J. V. & Nourshargh, S. Neutrophil heterogeneity in health and disease: a revitalized avenue in inflammation and immunity. Open Biol. 2, 120134 (2012).
8. Silvestre-Roig, C., Hidalgo, A. & Soehnlein, O. Neutrophil heterogeneity: implications for homeostasis and pathogenesis. Blood 127, 2173–2181 (2016).
9. Ericson, J. A. et al. Gene expression during the generation and activation of mouse neutrophils: implication of novel functional and regulatory pathways. PLoS ONE 9, e108553 (2014).
10. Ostuni, R., Natali, G., Cassatella, M. A. & Tamassia, N. Epigenetic regulation of neutrophil development and function. Semin. Immunol. 28, 83–93 (2016).
11. Xie, X. et al. Single-cell transcriptome profiling reveals neutrophil heterogeneity in homeostasis and infection. Nat. Immunol. 21, 1119–1133 (2020).
12. Zhu, Y. et al. Comprehensive characterization of neutrophil genome topology. Genes Dev. 31, 141–153 (2017).
13. Ballesteros, I. et al. Co-option of neutrophil fates by tissue environments. Cell 183, 1282–1297.e18 (2020).

NATURE IMMUNOLOGY | VOL 22 | SEPTEMBER 2021 | 1093-1106 | www.nature.com/natureimmunology

1105
14. Kolaczkowska, E. & Kubes, P. Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* **13**, 159–175 (2013).

15. Sagy, J. Y., Voels, S. & Granot, Z. Isolation and characterization of low- vs. high-density neutrophils in cancer. *Methods Mol. Biol.* **1458**, 179–193 (2016).

16. Yvan-Charvet, L. & Ng, L. G. Granulopoiesis and neutrophil homeostasis: a metabolic, daily balancing act. *Trends Immunol.* **40**, 598–612 (2019).

17. Hohaus, S. et al. PU.1 (Spi-1) and C/EBP alpha regulate expression of the granulocyte-macrophage colony-stimulating factor receptor alpha gene. *Mol. Cell. Biol.* **15**, 5813–5845 (1995).

18. Wilkinson, L. S., Moore, A. R., Pitsillides, A. A., Willoughby, D. A. & Edwards, J. C. Comparison of surface fibroblastic cells in subcutaneous air pouch model of acute gouty arthritis. *Arthritis Rheum.* **48**, 2310–2320 (2003).

19. Wilkinson, L. S., Moore, A. R., Pitsillides, A. A., Willoughby, D. A. & Edwards, J. C. Comparison of surface fibroblastic cells in subcutaneous air pouch and synovial lining: differences in uridine diphosphoglucose dehydrogenase activity. *Int. J. Exp. Pathol.* **74**, 113–115 (1993).

20. Blazek, K. et al. IFN-λ resolves inflammation via suppression of neutrophil infiltration and IL-1β production. *J. Exp. Med.* **212**, 845–853 (2015).

21. Monticelli, S. & Natoli, G. Transcriptional determination and functional specificity of myeloid cells: making sense of diversity. *Nat. Rev. Immunol.* **17**, 595–607 (2017).

22. Jojic, V. et al. Identification of transcriptional regulators in the mouse immune system. *Nat. Immunol.* **14**, 633–643 (2013).

23. Hirai, H. et al. C/EBPβ is required for ‘emergency’ granulopoiesis. *Nat. Immunol.* **7**, 732–739 (2006).

24. Paul, F. et al. Transcriptional heterogeneity and lineage commitment in myeloid progenitors. *Cell* **163**, 1663–1677 (2015).

25. Tessier, P. A. et al. Chemokine networks in vivo: involvement of C-X-C and C-C chemokines in neutrophil extravasation in vivo in response to TNF-alpha. *J. Immunol.* **1488**, 1495–1499 (2002).

26. Grassi, L. et al. Dynamics of transcription regulation in human bone marrow myeloid differentiation to mature blood neutrophils. *Cell Rep.* **24**, 2784–2794 (2018).

27. Harris, J. G., Flower, R. J. & Perretti, M. Endogenous corticosteroids mediate the neutrophilia caused by platelet-activating factor in the mouse. *Arthritis Rheum.* **48**, 2310–2320 (2003).

28. Wang, G. G. et al. Quantitative production of macrophages or neutrophils ex vivo using conditional HOXB8. *Nat. Methods* **3**, 287–293 (2006).

29. Wang, L. et al. ROS producing immature neutrophils in giant cell arteritis are linked to vascular pathologies. *JCI Insight* **5**, e139163 (2020).

30. Manley, H. R., Keightley, M. C. & Lieschke, G. J. The neutrophil nucleus: an important influence on neutrophil migration and function. *Front. Immunol.* **9**, 2867 (2018).

31. van Raam, B. J. et al. Mitochondrial membrane potential in human neutrophils is maintained by complex III activity in the absence of supercomplex organisation. *PLoS ONE* **3**, e2013 (2008).

32. Ward, C. et al. NF-kB activation is a critical regulator of human granulocyte apoptosis in vitro. *J. Biol. Chem.* **274**, 4309–4318 (1999).

33. Ng, K. P. et al. Runx1 deficiency permits granulocyte lineage commitment but impairs subsequent maturation. *Oncogenesis* **2**, e78 (2013).

34. Hu, G., Ye, R. D., Dinauer, M. C., Malik, A. B. & Minshall, R. D. Neutrophil caveolin-1 expression contributes to mechanism of lung inflammation and injury. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **294**, L178–L186 (2008).

35. Casanova-Acubes, M. et al. Rhythmic modulation of the hematopoietic niche through neutrophil clearance. *Cell* **153**, 1025–1035 (2013).

36. Sionov, R. V., Fridlender, Z. G. & Granot, Z. The multifaceted roles of neutrophils play in the tumor microenvironment. *Cancer Microenviron.* **8**, 125–158 (2015).

37. Borregaard, N. Neutrophils, from marrow to microbes. *Immunity* **33**, 657–670 (2010).

38. Kwok, I. et al. Combinatorial single-cell analyses of granulocyte-macrophage progenitor heterogeneity reveals an early uni-potent neutrophil progenitor. *Immunity* **53**, 303–318.e5 (2020).

39. Humbert, M. et al. Deregulated expression of Kruppel-like factors in acute myeloid leukemia. *Leuk. Res.** **35**, 909–913 (2011).

40. Riffelmacher, T. et al. Autophagy-dependent generation of free fatty acids is critical for normal neutrophil differentiation. *Immunity* **47**, 466–480.e5 (2017).

41. Borregaard, N. & Herlin, T. Energy metabolism of human neutrophils during phagocytosis. *J. Clin. Invest.* **70**, 550–557 (1982).

42. Manvelyan, L. et al. Clathrin-mediated mechanism of human neutrophil apoptosis. *J. Exp. Med.* **205**, 105–115 (2005).

43. Fischer, I. et al. Safeguard function of PU.1 shapes the inflammatory epigenome of neutrophils. *Nat. Commun.* **20**, 546–558 (2019).

44. Anrather, J., Racchumi, G. & Iadecola, C. NF-κB regulates phagocytic supercomplex organisation. *PLoS ONE* **3**, e2013 (2008).

45. Fischer, I. et al. NF-κB regulates phagocytic supercomplex organisation. *PLoS ONE* **3**, e2013 (2008).

46. Wu, Y. et al. Transcription factor RFX2 is a key regulator of mouse neutrophil chemokine production. *Nat. Immunol.* **15**, 274–283 (2014).

47. Wang, G. G. et al. Quantitative production of macrophages or neutrophils ex vivo using conditional HOXB8. *Nat. Methods* **3**, 287–293 (2006).

48. Wang, L. et al. ROS producing immature neutrophils in giant cell arteritis are linked to vascular pathologies. *JCI Insight* **5**, e139163 (2020).

49. Manley, H. R., Keightley, M. C. & Lieschke, G. J. The neutrophil nucleus: an important influence on neutrophil migration and function. *Front. Immunol.* **9**, 2867 (2018).

50. van Raam, B. J. et al. Mitochondrial membrane potential in human neutrophils is maintained by complex III activity in the absence of supercomplex organisation. *PLoS ONE* **3**, e2013 (2008).

51. Borregaard, N. Neutrophils, from marrow to microbes. *Immunity* **33**, 657–670 (2010).

52. Kwok, I. et al. Combinatorial single-cell analyses of granulocyte-macrophage progenitor heterogeneity reveals an early uni-potent neutrophil progenitor. *Immunity* **53**, 303–318.e5 (2020).

53. Humbert, M. et al. Deregulated expression of Kruppel-like factors in acute myeloid leukemia. *Leuk. Res.** **35**, 909–913 (2011).

54. Riffelmacher, T. et al. Autophagy-dependent generation of free fatty acids is critical for normal neutrophil differentiation. *Immunity* **47**, 466–480.e5 (2017).

55. Borregaard, N. & Herlin, T. Energy metabolism of human neutrophils during phagocytosis. *J. Clin. Invest.* **70**, 550–557 (1982).

56. Walmsey, S. R. et al. Lipoxin-induced neutrophil survival is mediated by HIF-1α-dependent NF-κB activity. *J. Exp. Med.* **201**, 105–115 (2005).

57. Fischer, I. et al. Safeguard function of PU.1 shapes the inflammatory epigenome of neutrophils. *Nat. Commun.* **20**, 546–558 (2019).

58. Anrather, J., Racchumi, G. & Iadecola, C. NF-κB regulates phagocytic NADPH oxidase by inducing the expression of gp91phox. *J. Biol. Chem.* **281**, 5657–5667 (2006).

59. Wu, Y. et al. Transcription factor RFX2 is a key regulator of mouse neutrophil chemokine production. *Nat. Immunol.* **15**, 274–283 (2014).

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Methods

Materials and reagents. Details of commercially available reagents and other materials used in this study are summarized in Supplementary Table 1.

Air pouch model of acute inflammation. Mice were bred and maintained under specific pathogen-free conditions in accredited animal facilities at the University of Oxford and were housed in individually ventilated cages at a constant 20–23 °C with a 12 h dark–light cycle and supplied with food and water ad libitum. All experimental procedures were approved by the local animal care and ethics committees (Oxford and Madrid). Animal housing, handling and experimental procedures in Oxford were conducted under a UK Home Office Project licence to I.A.U. The air pouch model of acute inflammation was established as described previously48. Briefly, C57BL/6J male mice (Charles River Laboratories) were maintained at nine weeks of age and allowed to acclimatize for one week. Mice were anesthetized with isoflurane and 3 ml of air was injected subcutaneously to create a dorsal air pouch with a top-up of air 3 d later. At 5 d after the creation of the air pouch, mice were challenged with 1 mg of zymosan injected directly into the pouch. Challenged mice were culled after 1, 2, 4, 12 and 24 h after zymosan injection and bone marrow, blood, membrane and exudate were collected.

Cell and tissue preparation. Blood was obtained via cardiac puncture; then, red blood cells were lysed in ACK lysis buffer (Thermo Fisher Scientific). For bone marrow cells, mice femurs were flushed using a 23-G needle in PBS and passed through a 70-μm nylon mesh sieve. Membrane tissue was collected from mice dissected and digested with Cell Dissection Kit (RPMI) to membrane 10% FCS + 1% PS (penicillin streptomycin) + 2.5 μl/ml of collagenase type VIII (Sigma-Aldrich) + 2 μl/ml of DNase I (Roche) and passed through a 70-μm nylon mesh sieve. Exudate was passed through a 70-μm nylon mesh sieve. Cells were pelleted by centrifugation at 400 res for 5 min before counting.

Flow cytometry and cell sorting. To identify preneutrophils, immature and mature neutrophils, cells were washed and preincubated with Fc Block (BD Biosciences) before surface staining with 1:200-diluted fluorochrome-conjugated anti-mouse antibodies against CD11b (clone M1/70), cKl2 (clone 2B8), Ly6C (clone HK1.4), Ly6G (clone 1A8), CXCGr2 (clone SA404G4), CXCGr4 (clone 2B11) and CD101 (clone Moushi101), together with exclusion lineage markers that include CD3e (clone 145-2C11), B220 (clone RA3-6B2), NK.1.1 (clone PK136), Sca-1 (clone D7), CD11c (clone N418), CD19 (clone 6D5) and Siglec-F (clone E50-2440) (Supplementary Table 1). For surface staining, neutrophils were segregated into preneutrophils and immature and mature neutrophils (Supplementary Fig. 3).

For intracellular cytokine staining, surface staining was followed by fixation and then permeabilization to allow for intracellular staining with pro-IL-1β (clone NITEN3). Flow cytometry acquisition was performed using the FACSDiva v6.1.3 software (on an LSR and LSRFortessa X-20; BD Biosciences). Data analysis was performed using FlowJo v10 (FlowJo LLC).

RNA-seq analysis. Neutrophils from bone marrow, blood, membrane and exudate were sorted from CX3CR1GFP, Ly6G–cd–tdTomato mice subjected to air pouch of inflammation, based on the gating strategy depicted in Extended Data Fig. 1f. Three hundred cell samples were sorted through a 100-μm diameter nozzle into 2 μl of lysis buffer and amplified complementary DNA was prepared for small RNA-seq using the Smart-seq2 protocol52. Libraries were prepared using Nextera XT kits (Illumina) and sequenced to a mean depth of 17.4 read pairs (illumina HiSeq 4000).

HoxB8 neutrophils differentiated for 5 d were incubated with zymosan (50 μg/ml) or dimethylsulfoxide (DMSO) vehicle for 2 h. Total RNA was extracted using the RNeasy Mini Kit (QiAGEN) according to the manufacturer’s instructions. Subsequently, poly-A selected mRNA libraries were sequenced on an Illumina HiSeq 4000 yielding 20–30 × 10^6 base pair (bp) paired-end reads per sample. The libraries were mapped to the mm10 genome using STAR v2.7.5b with the options: --local--X 2000. PCR duplicates were removed with Picard Tools v2.24.2; additionally, reads mapping to chrM with a mapping quality < 20 were filtered. Reads were then performed using MEME-Chip v5.3.1 (ref. 49) with the options: -meme-minw 5 -meme-maxw 30 -meme-mmotifs 10. The motif discovery mode (-meme-mod) was set to zero or one occurrence per sequence (000). To find the occurrences of known motifs within ATAC-seq peaks, FIMO (find individual motifs) occurrences was used with 1,000-bp sequences centered on the ATAC-seq peak summits; peak flanking regions of equal lengths were used for the background, as above.

Generation of HoxB8 neutrophils with targeted knockouts. HoxB8 C57BL/6N myeloid progenitors and stem cell factor (SCF)-producing CHO cells were kindly provided by B. Walzog (LMU Biomedizinsches Zentrum). HoxB8 myeloid progenitors were tested for Mycophasia and routinely cultured in RPMI 1640 medium supplemented with 10% FCS, 30 μM of β-mercaptoethanol (Thermo Fisher Scientific) or SCF-supersaturant from CHO cells and 100 ng/ml β-estradiol (Sigma-Aldrich). Differentiation was induced by estrogen removal and culture in medium containing 1% SCF supernatant. Progenitor cells were differentiated into neutrophils by culture with complete RPMI 1640 medium supplemented with 30 μM of β-mercaptoethanol, 4% SCF containing supernatant and 20 ng/ml granulocyte colony-stimulating factor in a 5% CO2 tissue culture incubator at 37 °C. To generate CRISPR-Cas9-mediated knockout, progenitors were transduced with lentCas9-v2 lentiviruses targeting ex1 of Cbp (ENSMUSG00000056501; guide RNA (gRNA) AGCCCTCAGTCTAGATCGATGTCA, exon1 of KL6 (ENSMUSG00000000708, gRNA TGCTGTGGAGAAACACG), exon5 of Runx1 (ENSMUSG00000022952, gRNA TAGCCGAGATTCAACGACCTC), exon5 of RFX2 (ENSMUSG00000023206, gRNA CTGTCTGGGGCTAAGTGG), exon4 of RELB (ENSMUSG00000002983, gRNA CTGCGACGACGGCTCTGAC), exon2 of IRF5 (ENSMUSG00000029771, gRNA ACCCTGGGCGCATGAGACGACGAGG) and exon1 of JUNB (ENSMUSG000000052837, gRNA GGAACCGGAGACGTCGAGCA).

Cytoxin. For morphological analysis, differentiated HoxB8 neutrophils were spun onto glass slides by centrifugal forces using a Thermo Shandon CytoSpin 3 Cytocentrifuge (Thermo Fisher Scientific) at 400 g for 5 min. The slides were stained with ready-to-use modified Wright–Giemsa stain from Sigma-Aldrich (catalog no. WG16) according to the manufacturer’s protocol. Images were obtained from stained slides under bright field using an Olympus BX51 fluorescence microscope (Olympus).

Western blots. Cells were lysed in 1% Triton X-100 lysis buffer (1% v/v Triton X-100, 10% v/v glycerol, 1 ml/m of EDTA, 150 mM of NaCl, 50 mM of Tris, pH 7.8) supplemented with protease inhibitor cocktails (Roche). Lysates were incubated on ice for 30 min and cleared by centrifugation at 13,000 × g for 10 min at 4 °C. Protein quantification was performed with the Qubit assay (Thermo Fisher Scientific) according to the manufacturer’s protocol; 20 μg of lysates were boiled in Laemml sample buffer (Bio-Rad Laboratories), resolved on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and transferred onto a polyvinylidene difluoride membrane (GE Healthcare) by western blot. Membranes were blocked with 5% non-fat milk in TBS-T and incubated with primary antibodies (Supplementary Table 1), followed by IRDye-conjugated secondary antibodies. Complexes were detected with the Odyssey Infrared Imaging System (LI-COR) and analyzed using Image Studio Lite v5.2.5 (LI-COR).

Estimation of mitochondrial transmembrane potential. Mitochondrial transmembrane potential was monitored with the voltage-sensitive fluorescence indicator tetramethylrhodamine, methyl ester (TMRM). For that, HoxB8 neutrophils (2 × 10^6 cells/ml) were loaded with 200 nM of TMRM for 20 min. TMRM fluorescence was estimated using a BD LSR II flow cytometer.

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Intracellular ROS measurement. Intracellular ROS were measured using a FACSc-based method. HoxB8 neutrophils were incubated with 2.5 μg/ml (7 μM) dihydrodihoromidine 123 (Thermo Fisher Scientific) in complete RPMI 1640 medium and stimulated with 50 nM of phorbol 12-myristate 13-actetate (PMA) (Sigma-Aldrich) for 20 min at 37°C. Cells were subsequently washed with PBS and the fluorescence intensity of each subset/cells was measured by flow cytometry.

Phagocytosis. The phagocytosis capacity of HoxB8 neutrophils was measured by FACSc-based method using fluorescent E. coli (product no. 25922GFP; ATCC). Briefly, HoxB8 neutrophils were incubated with fluorescent E. coli at a multiplicity of infection of 10 in complete RPMI 1640 medium for 15 min at 37°C. Neutrophils were subsequently washed with PBS and the fluorescence intensity of each subset/cells was measured by flow cytometry.

Bacterial killing assay. The bacterial killing assay was performed with S. aureus (NCTC no. 6571), which was used at a multiplicity of infection of 10. For the bacterial killing assay, neutrophils were seeded in 24-well plates and then lysed in 1% Triton X-100 buffer; 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 colony-forming unit (CFU) count.

NEToxis. To induce NEToxis, HoxB8 neutrophils were seeded into an 8-well Nunc Lab-Tek II Chamber Slide (VWR international) coated with 2% poly-lysine (Sigma-Aldrich) at a volume of 100 μl at the density of 1 x 10⁵/ml. Neutrophils were stimulated with 5 μM of oxonornycin and PMA (Sigma-Aldrich) overnight at 37°C in a 5% CO₂ tissue culture incubator and were subsequently fixed with 4% paraformaldehyde (Sigma-Aldrich) in Dubbceco’s PBS for 30 min at room temperature. After blocking, the primary antibodies rabbit anti-citrullinated histone 4 (catalog no. ab5103; Abcam) and mouse anti-mouse MPO (catalog no. HM1051BT; Hyclut) were added at a 1:100 dilution overnight at 4°C. Cells were washed with Dubbceco’s PBS before adding secondary antibodies: mouse anti-rabbit DyLight 647-labeled secondary antibody (Thermo Fisher Scientific) and rabbit anti-mouse IgG secondary antibody conjugated with Alexa Fluor 488 (Thermo Fisher Scientific). Images were obtained using an Olympus BX51 fluorescence microscope. Neutrophils with a clear formation of fibers stained by citrullinated histone 3, colocalized with a diffuse nucleus stained by SYTOX and colocalization with MPO, were counted as neutrophils under NEToxis. Images were analyzed using Fiji v2.1.0-1.53h.

Cell viability. A total of 10,000 cells per well HoxB8 myeloid progenitors were seeded and differentiated for 5 d. Cell viability was assessed using annexin V staining and Fixable Viability Dye eFlour 780 LIVE/DEAD staining (Thermo Fisher Scientific) and analyzed by flow cytometry to determine the percentage undergoing apoptosis. Samples were treated in triplicate and normalized to fluorescent minus one (FMO) wells.

Neutrophil migration assay with Boyden chamber. For the neutrophil migration assay, HoxB8 neutrophils were washed with the migration medium RPMI 1640 containing 2.5 mM of HEPES and 0.1% BSA and tested for migration in a 96-well microchamber using 2 μm pore size polycarbonate filter (Neuro Probe). The lower wells of the Boyden chamber were filled with the migration medium supplemented with either PBS or 10% of murine recombinant chemokine (C-C motif) ligand 3 (CCL3) (R&D). In the upper wells, 5 x 10⁴ HoxB8 neutrophils were added and the chamber was incubated for 90 min at 37°C and 5% CO₂. After incubation, nonmigrating cells in the upper chamber were removed using a cotton-tipped applicator. Cells that had migrated to the bottom of the membrane were fixed in 70% ethanol and stained with 4,6-diamidino-2-phenylindole before mounting onto poly-L-lysine-coated slides. Each chamber was counted in duplicate. The number of migrated neutrophils counted in five random fields was analyzed using Fiji.

RNA extraction and quantitative PCR with reverse transcription. Total RNAs were isolated from cells using the RNeasy Mini Kit and reverse-transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. RNA from sorted cells was isolated utilizing the RNeasy Micro Kit (QIAGEN). Real-time PCR reactions were performed on a ViiA7 system (Thermo Fisher Scientific) with TaqMan primer sets (Supplementary Table 1). Gene expression was analyzed using the comparative Ct (ΔΔCt) method and normalized against the levels of the housekeeping gene Hprt as the arbitrary unit.

Measurement of cytokine production. For the cytokine array, 1 x 10⁶ HoxB8 neutrophils seeded in 2 ml of RPMI 1640 were stimulated with 50 μg/ml zymosan or DMSO vehicle for 2 h. The Mouse Cytokine Array, Panel A (catalog no. ARY006; R&D Systems) is used to simultaneously detect 20 different cytokines and chemokines in 400 μl of supernatant of stimulated HoxB8 neutrophils, according to the manufacturer’s instructions. Signals were detected by chemical luminescence and subsequently quantitated with Image Studio.

Adaptive transfer of HoxB8 neutrophils. HoxB8 neutrophils were transferred intravenously into mice subjected to the air pouch model of in vivo inflammation, as shown in Supplementary Fig. 2. WT and knockout HoxB8 neutrophils were labeled with CellTrace Far Red (Thermo Fisher Scientific) and CellTrace CFSE (Thermo Fisher Scientific), respectively, at a final concentration of 5 μM according to the manufacturer’s instructions. Next, 10 x 10⁶ differentially labeled WT and knock out neutrophils were mixed at an equal ratio and adoptively transferred into the air pouch model of in vivo inflammation at a volume of 300 μl followed by subcutaneous zymosan stimulation. Four hours postzymosan challenge, blood, membrane and exudate were collected for flow cytometry.

Model of AMI. Female 8–12-week-old mice were subjected to 45 min occlusion of the left anterior descending (LAD) coronary artery followed by 1 h reperfusion (for infant size), as described previously. Briefly, fully anesthetized animals were intubated and temperature-controlled throughout the experiment at 36.5°C to prevent hypothermic cardioprotection. Thoracotomy was then performed and the LAD was ligated with a 7-0 nylon filament suture for 45 min. To define infant size, mice were re-anesthetized and reperfused and the LAD coronary artery was reoccluded by ligating the suture in the same position as the original infarction. Animals were then humanely killed and 1 ml of 1% Evans Blue dye (Sigma-Aldrich) was infused intravenously to delineate the AAR (myocardium lacking blood flow, that is, negative for blue dye staining). The left ventricle was isolated, cut into transverse slices (5–7 mm thick slices used for left ventricle) and both sides were imaged. To delineate the infarcted (necrotic) myocardium, slices were incubated in TTC (Sigma-Aldrich) at 37°C for 15 min. The slices were then rephotographed, weighed and regions negative for Evans Blue staining (AARs) and for TTC (infarcted myocardium) were quantified using ImageJ v2.1.0.33h (NIH). The percentage values for AARs and infarcted myocardium were corrected against the mg of AAR:left ventricle and infarcted myocardium:AAR, respectively.

Mixed bone marrow chimera and AMI. Recipient WT C57BL/6 mice were lethally irradiated (two 6-Gy doses, 3h apart) before receiving 1 million bone marrow nucleated cells by intravenous injection. For mixed chimeras, equal numbers of donor CD45.1 or ST10*Ala* Rb* bone marrow cells, collected by flushing the femur with PBS, were mixed before intravenous injection. Engraftment of recipient animals was assessed six weeks after transplantation by analysis of the percentage of CD45.1 or CD45.2 leukocytes in the blood by flow cytometry. Eight weeks after transplantation, mice were subjected to 45 min occlusion of the LAD coronary artery followed by 3 h reperfusion. Heart, blood and bone marrow were collected and processed for flow cytometry analysis. Briefly, blood was taken through cardiac puncture, the bone marrow cells of mice were flushed from femurs using a 23-G needle and hearts were collected and cut down into small pieces before being digested in liberase TM (Roche) and DNase1 (Sigma-Aldrich) for 45 min at 37°C and homogenized into single-cell suspensions using 70-mm nylon mesh sieves and syringe plungers. Single-cell suspensions were lysed in red blood cell lysis buffer (eBioscience) for 4 min, stained with fluorochrome-conjugated antibodies and then fixed and permeabilized using the Fix & Perm and Perm buffers (eBioscience) according to the manufacturer’s instructions to determine pro-IL-1β production or neutrophils in the Gene Expression Omnibus (GEO) under accession no. GSE161765. The previously published RNA-seq data with accession nos. GSE147910 and GSE109125 were subjected to air pouch and zymosan stimulation and the RNA-seq data using the Gene Expression Omnibus (GEO) accession no. GSE147910 and GSE109125 were retrieved from the GEO. To align the neutrophil subpopulations from the...
bone marrow, blood, air pouch membrane and exudate in the current study (Extended Data Fig. 1i), we also utilized published scRNA-seq data (accession no. GSE137540). Source data are provided with this paper.

**Code availability**
All code used in current study has been stored at https://github.com/Tariq-K?
tab=repositories.

**References**
53. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).
54. Buenrostro, J. D., Giresi, P. G., Zaba, L. C., Chang, H. Y. & Greenleaf, W. J. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat. Methods* **10**, 1213–1218 (2013).
55. McLean, C. Y. et al. GREAT improves functional interpretation of cis-regulatory regions. *Nat. Biotechnol.* **28**, 495–501 (2010).
56. Machanick, P. & Bailey, T. L. MEME-ChIP: motif analysis of large DNA datasets. *Bioinformatics* **27**, 1696–1697 (2011).

**Acknowledgements**
We are grateful to Jonathan Webber (Kennedy Institute of Rheumatology (KIR), Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences (NDORMS)) for providing the cell sorting service, T. Nicol and M. Crabtree (Nuffield Department of Medicine) for help with the Seahorse assay the and Genomic core facility (Wellcome Centre for Human Genetics) for sample sequencing and technical support. We thank F. Prowse for scientific discussions and E. Thompson and D. Berthold (KIR, NDORMS) for helpful suggestions on the manuscript and assistance with figures. This work was supported by the Oxford-Cellgene fellowship (T.E.K.); the Chinese Science Council (Z.A.); Wellcome Trust Investigator Award no. 209422/Z/17/Z to I.A.U., E.v.G. and H.E.; Novo Nordisk Foundation Tripartite Immunometabolism Consortium (no. NNF15CC0018486 to S.M. and L.W.); and the Erasmus Foundation (N.W. and V.v.W.).

**Author contributions**
I.A.U. conceptualized the study. I.A.U., S.M., H.E., T.E.K. and Z.A. devised the methodology. T.E.K., Z.A., S.M., H.E., I.B., S.M-S., L.W., N.W., V.v.W. and A. Hemmings carried out the experiments. T.E.K., Z.A., I.B., E.v.G. and I.A.U. analyzed and/or interpreted the experimental data. T.E.K. and Z.A. carried out the computational genomic analysis. A.Z. and B.W. provided the materials. I.A.U., Z.A., A. Hidalgo, T.E.K., E.v.G. and H.E. wrote, reviewed and edited the manuscript.

**Competing interests**
The authors declare no competing interests.

**Additional information**
Extended data is available for this paper at https://doi.org/10.1038/s41590-021-00968-4.

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41590-021-00968-4.

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**Peer review information** *Nature Immunology* thanks the anonymous reviewers for their contribution to the peer review of this work. Z. Fehervari was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team. Peer reviewer reports are available.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Administration of zymosan into the air pouch cavity induces neutrophil transcriptional remodeling. a, Representative H&E staining showing the formation of the granulation tissue (membrane) and infiltration of leukocytes. Representative (left) and zoomed-in (right) images of air pouch cavity from mice subjected to air pouch and zymosan challenge are shown with indicated scale bars. b, Representative image of immunofluorescence imaging on the pouch cavity of Ly6gcre-tdTomato x Cx3cr1GFP illustrates typical neutrophil infiltration. Neutrophils and macrophages appear reddish and green, respectively, and cell nuclei stained with DAPI appear blue. a, b) Representative images from four mice are shown. c, Total number of neutrophils per milliliter of air-pouch exudate (AP) post challenge with zymosan. d, Total number of neutrophils per milligram of air-pouch membrane (MEM) post challenge with zymosan. e, Expression of pro-IL1β, as measured by flow cytometry, in neutrophils from indicated tissues recovered from mice subjected to the air pouch model and zymosan challenge. c, d, e) Results are the means and SD of 15 mice. Significant differences are denoted as: "P < 0.05, ****P < 0.0001; (DM one-way ANOVA with Dunnett’s multiple comparisons test). f, Gating strategy of neutrophil sorting from Ly6gcre-tdTomato x Cx3cr1GFP mice for subsequent RNA sequencing analysis and ATAC sequencing analysis. g, Gating strategy used to quantify neutrophils under different maturation stages. h, Percentage distribution of neutrophil subsets in BM and blood between steady condition and zymosan-induced inflammation. Data are shown as means and SD from three naïve mice and four mice with zymosan challenge. Significant differences are denoted as: "**P < 0.01, ****P < 0.0001 (RM two-way ANOVA with Šidák’s multiple comparisons test). i, Correlation between indicated neutrophil samples with scRNA-seq-defined neutrophil populations reported by Xie et al.11. The mean fraction of indicated scRNA-seq defined clusters (G0-G5) in each group of neutrophil samples. G0-G4: BM neutrophils (G0-CMP, G1-GMP, G2-pre-neutrophils; G3-immature neutrophils; G4-mature neutrophils). G5a, G5b, G5c: peripheral mature neutrophils.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Analysis of DEG expression reveals gene set enriched pathways. a, Global changes in gene expression between the bone marrow and air pouch (padj < 0.05, fold change > 1.5). b, Top gene ontology terms associated with the bone marrow and air pouch identified by gene set enrichment analysis (GSEA) padj < 0.01. c, Gene set enrichment analysis (GSEA) of genes differentially expressed between the bone marrow and air pouch. Line plot shows the distribution of genes ranked by their normalised enrichment score (ES). Heatmaps show variance stabilised counts of the leading-edge genes most associated with each tissue.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Discrete changes occur in neutrophil chromatin landscape en route to the site of inflammation. a, Venn diagrams showing the overlap of differentially accessible peaks (padj < 0.05, fold change > 1.5) between the indicated transitions. b, Heat maps of normalised read counts (FPKM) over 2.5 k.b regions centred on ATAC-seq peaks from each tissue. Only differentially accessible peaks between the blood and bone marrow, and between blood and membrane are shown (padj < 0.05, fold change > 1.5). c, Hierarchical clustering of all differentially accessible peaks (LRT test padj < 0.01) based on Manhattan distances using the Ward method. Data are presented as a heatmap normalised to the minimum and maximum of each row. d, Gene ontology analysis for differentially accessible peaks between the blood and bone marrow, and between blood and membrane (padj < 0.05, fold change > 1.5). The top 10 most significant results are shown and the number of differentially accessible peaks within each GO term are annotated.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Expression of predicted transcription factor family members in neutrophils. 

a, Expression of identified transcription factor (TF) family members across immune cell populations (ImmGen), subpopulation level counts are averaged. Highlighted TFs have high levels of neutrophil expression. 
b, Expression of predicted transcription factor (TF) family members across neutrophil sub-populations (ImmGen). Highlighted TFs have high levels of neutrophil expression and/or neutrophil specific expression compared with other family members. 
c, Expression of predicted transcription factors in each of G0 to G5 clusters, mapped to progressively maturing neutrophils in scRNA-Seq analysis\(^1\), before and after intraperitoneal *E. coli* challenge, coloured by the average expression of each gene in each cluster scaled across all clusters. 
d, Western blot analysis of JUNB activation (P-JUNB) and levels of expression (JUNB) over the time course of HoxB8 neutrophil differentiation/maturation (D0 to DS) and under zymosan stimulation, and representative blot from three independent experiments is shown.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Validation and maturation of TF knockout neutrophils. 

a, Targeted TF knockout of HoxB8 neutrophils. Left: Schematic of in vitro generation, CRISPR-Cas9 lentivirus transduction and subsequent differentiation into neutrophils in the presence of G-CSF. Right: Immunoblots for validating TF knockout from HoxB8 neutrophils, and representative blots from three independent experiments are shown.

b, Representative flow cytometry plots (top) of WT or CEBPβ−/−, Klf6−/−, Runx1−/−, Rfx2−/−, RelB−/−, Irf5−/− or JunB−/− HoxB8 neutrophils co-labelled with Ly6C, Ly6G and CD101. Quantification of flow cytometry data as percentages of preneutrophils Ly6G CXCR4+, immature Ly6G−CD101− and mature Ly6G−CD101+ neutrophils (bottom). Data are shown as means and SD and are representative of three independent experiments. Significant differences compared with the WT control group are denoted as: ****P < 0.0001 (two-way ANOVA with Dunnett’s multiple comparisons test).

c, Myeloperoxidase (MPO) expression in HoxB8 neutrophils. A representative Western blot probed with antibodies specific for MPO and β-Actin is shown. MPO expression is normalized against β-Actin amount in the lysates (and expressed as arbitrary unit of MPO).

d, Mitochondrial transmembrane potential of WT and indicated KO HoxB8 neutrophils measured by flow cytometry using TMRM.

e, Early apoptosis rates of HoxB8 neutrophils differentiated for five days as assessed by the percentage of cells positive for the Annexin V staining and negative for the live/dead staining. Data are shown as means and SD from three independent experiments, each with duplicate. (c, d, e) Data are shown as means and SD and are representative of three independent experiments. Significant differences compared with WT neutrophils are denoted as: **P < 0.01, ***P < 0.001, ****P < 0.0001; (Ordinary one-way ANOVA with Dunnett’s multiple comparisons test).
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | RUNX1 deficiency impairs neutrophil maturation. **a**, Irf5 deficiency does not affect neutrophil maturation. Left: representative flow cytometry of neutrophil subsets (pre-neutrophils, immature and mature neutrophils) from WT and Irf5−/− mice. **b**, JunB deficiency does not affect neutrophil maturation. Left: representative flow cytometry of neutrophil subsets (pre-neutrophils, immature and mature neutrophils) from JunBfl/fl and S100a8tm敲除xJunBfl/fl mice. Right: Statistical analysis of percentages of indicated neutrophil subsets. Data are shown as means and SD derived from three mice from each group. Statistical comparison was made by ordinary one-way ANOVA with Dunnett’s multiple comparisons test: ns, no significant difference. **c**, Absolute quantification of neutrophil subsets (pre-neutrophils, immature and mature neutrophils) in the bone marrow (left) and the blood (right) from Runx1fl/fl and Lyz2tm敲除xRunx1fl/fl mice. **d**, Morphology assessment of CD11b+Ly6G+ neutrophils sorted from Runx1fl/fl and Lyz2tm敲除xRunx1fl/fl mice. **e**, Morphology assessment of CD11b+Ly6G+ neutrophils sorted from JunBfl/fl and S100a8tm敲除xJunBfl/fl mice. (d, e) Results are expressed as percentages of segmented, banded neutrophils and metamyelocyte out of at least 200 cells counted from different fields and independent replicates. Scale bars represent 10 μm. **a, c, d, e** Data are shown as means and SD from three mice from each group within one experiment. Significant differences compared between two individual groups are denoted as: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 (RM two-way ANOVA with Šidák’s multiple comparisons test). **f**, Differential expression of Cebp in CD11b+Ly6G+ neutrophils sorted from Runx1fl/fl and Lyz2tm敲除xRunx1fl/fl mice, measured by flow cytometry. Gating strategy (top) of neutrophils for identifying CD11b+Ly6G+ neutrophils. Fluorescence histogram (middle) and geometric mean (bottom) of CEBPE expression in CD11b+Ly6G+ neutrophils. Data are shown as means and SD derived from three mice from each group. Significant differences compared with Runx1fl/fl and Lyz2tm敲除xRunx1fl/fl mice are denoted as: **P < 0.01 (unpaired student t test).
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | RUNX1 and KLF6 in transcriptional control of neutrophil migration. a, Hierarchical clustering of all differentially expressed genes (LRT test padj < 0.01, |log2FC|>1), based on Manhattan distances using the Ward method. Data are presented as heatmap normalised to the minimum and maximum of each row. b, Gene ontology (GO) analysis, showing the top 10 enriched GO categories for each cluster from Extended Data Fig. 7a. c, Leukocyte-migration-related gene expression in WT, Klf6−/−, or Runx1−/− HoxB8 neutrophils. (b, c) Significant differences compared with Runx1fl/fl and Lyz2cre×Runx1fl/fl mice are denoted as: **P < 0.01, ****P < 0.0001 (RM two-way ANOVA with Šidák’s multiple comparisons test). d, Early apoptosis rates of HoxB8 neutrophils recovered from blood, air pouch membrane and exudate from mice subjected to into air pouch model of acute inflammation and zymosan stimulation, assessed by the percentage of cells positive for the Annexin V staining and negative for the live/dead staining. Data are shown as means and SD from four mice from each group. e, Percentage distribution of neutrophil subsets (pre-neutrophils, immature and mature neutrophils) in BM and blood under zymosan-induced inflammation. Data are shown as means and SD from six mice from each group.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | RELB, IRF5, and JUNB deficiency impair neutrophil inflammatory responses. a, 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. c, Representative immunofluorescence images of NETosis from WT, RelB−/− and JunB−/− HoxB8 neutrophils stimulated with 5 μM 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 10 μm. d, Phagocytosis-related gene expression in Cebpβ−/−, RelB−/−, Irf5−/− or JunB−/− HoxB neutrophils. e, ROS-biosynthetic-process-related gene expression in WT, Cebpβ−/−, RelB−/−, Irf5−/− or JunB−/− HoxB neutrophils.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | RELB, IRF5, and JUNB affect neutrophil inflammatory gene expression and production. a, Cytokine-production-related gene expression in WT, Cebpβ−/−, RelB−/−, Irf5−/− or JunB−/− HoxB8 neutrophils. b, Chemokine-production-related gene expression in Cebpβ−/−, RelB−/−, Irf5−/− or JunB−/− HoxB8 neutrophils. c, Il1α, Il6 and Cxcl2 mRNA induction in HoxB8 neutrophils stimulated with zymosan (50ug/ml) for 0, 1, 2 hours. Gene expression was measured by qPCR. Data are shown as means and SD from three independent experiments. Significant differences compared between KO and WT neutrophils are denoted as: *P < 0.05, **P < 0.01, ****P < 0.0001, and *****P < 0.00001; (DM one-way ANOVA with Dunnett’s multiple comparisons test). d, Cytokines and chemokines secreted from WT HoxB8 neutrophils challenged with Zymosan for 2h, measured by the proteome array shown in Fig. 6b. e, Densitometric quantification of IL1β secretion from HoxB8 neutrophils stimulated with zymosan (50ug/ml) for 2hours, measured with 30 minutes exposure time. Data are shown as mean and SD of three biologically independent samples. Significant differences compared between KO and WT neutrophils are denoted as: *P < 0.05, **P < 0.01; (Ordinary one-way ANOVA with Dunnett’s multiple comparisons test).
Extended Data Fig. 10 | See next page for caption.
**Extended Data Fig. 10 | Assessment of chimerism in MBMC and proposed TF blueprint.**

**a**, Representative flow cytometry (left) and the chimerism ratio (right) of neutrophils in the bone marrow, blood, and heart in mice subjected to permanent myocardial infarction 6 weeks after bone marrow transplant. Data are shown as means and SD derived from three mice from each group within one experiment. Statistical comparison was made by paired student-t test. ns, no significant difference. **b**, Model of transcriptional regulation of neutrophils during inflammation. In the process of differentiation in bone marrow, lineage-determining transcriptional factors, including RUNX1, KLF6, CEBPE, 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.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a, Confirmed

☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ The statistical test(s) used AND whether they are one- or two-sided

*Only common tests should be described solely by name; describe more complex techniques in the Methods section.*

☐ A description of all covariates tested

☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

*Give P values as exact values whenever suitable.*

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

*Our web collection on statistics for biologists contains articles on many of the points above.*

Software and code

Policy information about availability of computer code

Data collection

Flow cytometry and sorting were performed using FACs DIVA (v 6.1.3 on BD LSR and BD LSRFortessa X20). Sequencing data were collected on a illumina HiSeq 4000. Morphological images were obtained using Olympus BX51 microscope. Metabolic flux analysis were acquired on Agilent Seahorse XFe96 Analyser [Wave software 2.4]

Data analysis

All flow cytometry data were analysed on FlowJo v10. Statistical analysis were performed on GraphPad Prism 8.0 and R 3.6 (R studio as plateform). Raw RNA sequencing reads were processed with STAR Aligner (https://github.com/alexdobin/STAR), Cutadapt (https://cutadapt.readthedocs.io/en/stable/), PicardTools (https://broadinstitute.github.io/picard/), DEXSeq2 (v 1.24.0), Bioconductor. Motif analysis was performed using MEME-Chip (https://meme-suite.org/doc/meme-chip.html). Gene ontology analysis was performed using GENEONTOLOGY (http://geneontology.org/docs/go-enrichment-analysis/). The coding resource is available at https://github.com/Tariq-K?tab=repositories

Western Blotting Quantification was conducted using Image Studio™ Lite (Version 5.2.5). Confocal microscopy images were processed using Fiji (Fiji is just Image), version 2.1.0/1.53h.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data.

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

RNA-seq (Figure 1) and ATAC-seq (Figure 2) data using neutrophils from mice subject to air pouch and zymosan stimulation. RNASeq data using HoxB8 neutrophils (Figure 5, Extended Figure 7-9) are publicly available with the GEO accession number GSE161765.

To review GSE161765, please go to: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE161765

Figures associated with GSE161765 are listed below:
Bone marrow, Blood, air pouch membrane and exudate neutrophil RNAseq, GSE161765: Figure 1, Figure 2C, Extended Figure 11,
Bone marrow, Blood, air pouch membrane and exudate neutrophil ATACseq, GSE161765: Figure 2, Extended Figure 2, Extended Figure 3.
HoxB8 neutrophil RNAseq, GSE161765: Figure 5A&B, Extended Figure 7E&F&G, Extended Figure 9, Extended Figure 10A&B.

Previous published datasets:
GSE147910, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147910
GSE109125, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE109125

To align the neutrophil subpopulations from bone marrow, blood, air pouch membrane and exudate in current study (Extended Figure 11), we also utilized the published scRNA-seq data: GSE137540.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-list.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
- Sample size was chosen based on the prior experiment with the same experimental design to ensure the possibility of statistical analysis and to minimize the use of experimental animals based on the 3R principles.

Data exclusions
- No data were excluded from the analysis.

Replication
- All experiments were performed independently, multiple times, with different cohorts of animals. Results shown are always the result of two or more independent experiments. All the reported results are from experiments in which every repeat gave similar results.
- N number describes biological replicates, such as mice and cells. Technical replicates were present as a single experimental point for visualization and statistical analysis. Mean and standard deviation of technical replicate was used.

Randomization
- Animals from the same cage were randomly selected for different treatment. For in vitro experiments, cells from the same pool of HoxB8 neutrophils or sorted neutrophils were randomly split into separate wells and subjected to different treatment.

Blinding
- Assessment of pathology in wt and KO mice was performed blind to mouse type and condition during data collection. For all other analyses, blinding was not performed as the experimental endpoints and readouts are quantitative and not subjective.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.
## Antibodies

| Antibodies used                                      |
|-----------------------------------------------------|
| Anti-mouse B220 (Clone RA3-6B2) Thermo Fisher Cat# 102326; RRID: AB_893354 1:200 dilution |
| Anti-mouse CD3e (Clone 145-2C11) Bioreagent Cat# 45-0031-82; RRID: AB_1107000 1:200 dilution |
| Anti-mouse CD11b (Clone M1/70) Bioreagent Cat# 102145; RRID: AB_2561390 1:200 dilution |
| Anti-mouse CD11c (Clone N418) Thermo Fisher Cat# 45-0114-82; RRID: AB_925727 1:200 dilution |
| Anti-mouse CD19 (Clone 6D5) Bioreagent Cat# 115534; RRID: AB_2072929 1:200 dilution |
| Anti-mouse ckit (Clone 2B8) BD Biosciences Cat# 105828; RRID: AB_11204256 1:100 dilution |
| Anti-mouse CXCR2 (Clone SA4044) Bioreagent Cat# 149304; RRID: AB_2565692 1:200 dilution |
| Anti-mouse CXCR4 (Clone 2B11) Thermo Fisher Cat# 17-9991-82; RRID: AB_10670878 1:200 dilution |
| Anti-mouse CD10 (Clone Moushi-101) Thermo Fisher Cat# 25-1011-82' RRID: AB_2573378 1:200 dilution |
| Anti-mouse CD115 (Clone AF5598) Thermo Fisher Cat# 135526; RRID: AB_2566462 1:200 dilution |
| Anti-mouse Ly6C (Clone HK1.4) Bioreagent Cat# 128041; RRID: AB_2565852 1:200 dilution |
| Anti-mouse Ly6G (Clone IA8) Bioreagent Cat# 127643; RRID: AB_2565971 1:200 dilution |
| Anti-mouse NK1.1 (Clone PK136) Thermo Fisher Cat# 45-5941-82; RRID: AB_914361 1:200 dilution |
| Anti-mouse Siglec-F (Clone E50-2440) BD Biosciences Cat# 565526; RRID: AB_2739281 1:200 dilution |
| Anti-mouse TER119 (Clone TER-119) Bioreagent Cat# 116202; RRID: AB_313703 1:200 dilution |
| Anti-mouse Sca-1 (Clone D7) Bioreagent Cat# 108124; RRID: AB_893615 1:200 dilution |
| Anti-mouse pro-IL-1 beta (Clone NITEN3) Thermo Fisher Cat# 25-7114-82; RRID: AB_2573526 1:200 dilution |
| Anti-mouse CEBPa (Clone C-10) Santa Cruz Cat# sc-515192 AF647 1:500 dilution |
| Rabbit anti-C/EBPβ (Clone E299) Abcam Cat# ab32358; RRID: AB_726796 1:100 dilution |
| Mouse anti-KL6 (Clone E-10) Santa Cruz Cat# sc-365633; RRID: AB_10841903 1:1,000 dilution |
| Mouse anti-RUNX1 (Clone 1CS516) Bioreagent Cat# 659302; RRID: AB_2563194 1:1,000 dilution |
| Rabbit anti-RK2Abcam Cat# ab79241; RRID: AB_2042797 1:1,000 dilution |
| Mouse anti-RelB (Clone D-4) Santa Cruz Cat# sc-48366; RRID: AB_628212 1:1,000 dilution |
| Rabbit anti-IRF5 Abcam Cat# ab21689; RRID: AB_446483 1:1,000 dilution |
| Rabbit Phospho-JunB (Thr122/Thr123) (D3C6) Rabbit mAb Antibody Cell Signaling Cat# 8053; RRID: AB_10950322 1:500 dilution |
| Rabbit JunB-AP-1 Antibody (C37F9) Cell Signaling Cat# 7375; RRID: AB_2130002 1:500 dilution |
| Rabbit anti-Lamin B2 (Clone EP97001(b)) Abcam Cat# ab15773; RRID: AB_2827514 1:500 dilution |
| Mouse anti-MPO (Clone BF4) Cell Bioscience Cat# HM10518T; RRID: Ab_2146342 1:1,000 dilution |
| Rabbit anti-Histone H3 (citricline R2 + R8 + R17) Abcam Cat# ab5103 1:500 dilution |
| Mouse anti-CD44 (Clone 7G3) Pharmingen Cat# 550173 1:500 dilution |

## Validation

All the antibodies used for flow cytometry and cell sorting were well-established lineage and cell surface marker antibodies. Staining patterns were consistent with the manufacturer product information as well as published results.

Vendor-specific information:
All antibodies from Bioreagent were QC-tested by immunofluorescent staining with flow cytometry analysis. ([www.bioreagent.com/en-uk/reproducibility](http://www.bioreagent.com/en-uk/reproducibility))
All antibodies from Thermo Fisher were tested for target specificity and for the use of flow cytometry ([https://www.thermofisher.com/c/en/home/life-sciences/antibodies/inventron-antibody-validation.html](https://www.thermofisher.com/c/en/home/life-sciences/antibodies/inventron-antibody-validation.html))
All antibodies from abcam were tested for specificity and reproducibility with a range of experiment-based approaches. ([https://docs.abcam.com/pdf/primer-antibodies-raising-antibody-standards-whitepaper-abcam-2016.pdf](https://docs.abcam.com/pdf/primer-antibodies-raising-antibody-standards-whitepaper-abcam-2016.pdf))

All antibodies used for Western-Blotting and immunocytochemistry staining were purchased from commercial vendors. Validation of antibodies used in current study is described in technical data sheets provided by manufacturer’s websites. Validation of secondary antibodies was performed by staining of slides in the presence or absence of primary antibodies. Validation information below:

**Rabbit anti-C/EBPβ (Clone E299):** WB on manufacturer’s websites and WB used for current study. Antibody validated for WB used in immunohistochemistry knockout validation on mouse samples (fig 4b) and in western blot knockout validation on mouse samples (fig 2a). Neena et al. Skelet Muscle (2016).

**Mouse anti-KL6 (Clone E-10):** WB on manufacturer’s websites and WB used for current study. Antibody validated for immunohistochemistry using frozen section on mouse sample. (fig 5a). Hiroki et al. J Reprod Dev (2014).
Eukaryotic cell lines
Policy information about cell lines

Cell line source(s)  
HoxB8 SCF myeloid progenitors and SCF-producing CHO cells were kindly provided by Barbara Walzog (LMU München). HEK-293FT cells were obtained from ATCC (Cat# PTA-5077, RRID: CVCL_6911).

Authentication  
Cell lines were validated by morphology and surface marker phenotype.

Mycoplasma contamination  
Frozen batch used for the study was tested for mycoplasma as negative.

Commonly misidentified lines  
No misidentified cell lines were used in according to the version 9 of the register of misidentified cell lines.

Animals and other organisms
Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals  
All experimental animal used were C57BL/6 mice, both males and females were used at similar ratio, and the age of the mice is ranged from 2 to 4 months. Mice were bred and maintained under SPF conditions in accredited animal facilities at the Kennedy Institute of Rheumatology, University of Oxford. Animals were housed in individually ventilated cages at a constant 20-23.3 degree Celsius with 12h dark/light cycle, supplied with food and water and libitum.

Below is the specific genotypes used for current study:
Mouse: WT, Charles River, C57BL/6
Mouse: Ly2Cre/Cre Runx1f/f, This paper (JAX 0106/73, JAX 004781)
Mouse: Runx1f/f Dillon Lab - MRC LMS (JAX 010673)
Mouse: If5f/- Mak Lab – University of Toronto (MGI 3576384)
Mouse: SaldBcre Jun8If/I
Mouse: Jun8If/I
Mouse: SaldBcre If5f/I
Mouse: If5f/I

Wild animals  
This study does not use wild animals.

Field-collected samples  
This study does not use field-collected samples.

Ethics oversight  
All experimental procedures were approved by the local Animal Care and Ethics Committees (Oxford and Madrid). Animal housing, handling and experimental procedures in Oxford were conducted under the UK Home Office Project licence to I Udalova.

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

- The axis labels state the marker and fluorochrome used (e.g., CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Cells were prepared from the bone marrow are processed with mechanical disaggregation of the tissue through a 70 um strainer using a syringe plunger. Blood cells were incubated in red cell lysis buffer to remove erythrocytes from cell suspension. Air pouch membrane and exudate were digested using DNase I and Liberase TM for tissue digestion. See the Method section for detailed sample preparation and processing.

Instrument

BD LSR II and BD LSR Fortessa X20

Software

FACs DIVA (v 6.1.3) for acquisition and FlowJo V10 for analysis

Cell population abundance

All sorted samples were checked for after-sorting purity (>99%)

Gating strategy

All samples were gated on size by FSC-A/SSC-A. Doublets were removed with both FSC-H/FSC-A and SSC-H/SSC-A. On sorting for RNA-seq and ATAC-seq, neutrophils were identified as CD11b+ Ly6G+. For distinguishing different neutrophil subsets, after exclusion of cell doublets and dead cells, pre-neutrophils were identified as Lineage- Siglec-F- CD11b- Ly6Cint CXCR4+ cKit+ CXCR2-, and immature neutrophils were identified as Lineage- Siglec-F- CD11b- Ly6Cint CXCR4- cKit- CXCR2+ Ly6G+ CD101-, and mature neutrophils were identified as Lineage- Siglec-F- CD11b+ Ly6Cint CXCR4- cKit- CXCR2+ Ly6G+ CD101+, as exemplified in Supplementary Fig.3.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.