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IRF8 inhibits C/EBP α activity to restrain mononuclear phagocyte progenitors from differentiating into neutrophils

Daisuke Kurotaki^{1,*}, Michio Yamamoto^{1,*}, Akira Nishiyama¹, Kazuhiro Uno¹, Tatsuma Ban¹, Motohide Ichino¹, Haruka Sasaki¹, Satoko Matsunaga², Masahiro Yoshinari¹, Akihide Ryo², Masatoshi Nakazawa³, Keiko Ozato⁴ & Tomohiko Tamura¹

Myeloid progenitors lose their potential to generate neutrophils when they adopt the mononuclear phagocyte lineage. The mechanism underlying this lineage restriction remains unknown. We here report that the protein expression of IRF8, an essential transcription factor for the development of dendritic cells (DCs) and monocytes, sharply increases at the monocyte-DC progenitor (MDP) stage and remains high in common monocyte progenitors (cMoPs). *Irf8*^{-/-} MDPs and cMoPs accumulate but fail to efficiently generate their downstream populations, instead giving rise to neutrophils *in vivo*. IRF8 physically interacts with the transcription factor C/EBP α and prevents its binding to chromatin in MDPs and cMoPs, blocking the ability of C/EBP α to stimulate transcription and neutrophil differentiation. A partial inhibition of C/EBP activity in *Irf8*^{-/-} haematopoietic progenitors alleviates the neutrophil overproduction *in vivo*. Thus, IRF8 not only bestows monocyte and DC differentiation potential upon mononuclear phagocyte progenitors but also restrains these progenitors from differentiating into neutrophils.

¹Department of Immunology, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan. ²Department of Microbiology, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan. ³Department of Experimental Animal Science, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan. ⁴Program in Genomics of Differentiation, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA. * These authors are co-first authors. Correspondence and requests for materials should be addressed to T.T. (email: tamurat@yokohama-cu.ac.jp).

Hematopoietic stem cells (HSCs) in the bone marrow self-renew and ultimately differentiate into all types of mature blood and immune cells via several intermediate progenitor stages^{1,2}. In these processes, intermediate progenitors progress to subsequent stages at the expense of a gradual loss of multi- or oligo-potency. During myelopoiesis, common myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs) lose their ability to generate neutrophils as they progress to monocyte-dendritic cell (DC) progenitors (MDPs)³ that give rise to mononuclear phagocytes, that is, monocytes, macrophages and DCs. However, the mechanism by which this lineage restriction occurs is unknown.

Cell differentiation requires dynamic changes in gene expression patterns, and these are tightly regulated by cell type-specific transcription factors. The interplay between such transcription factors is important not only to stimulate the differentiation of certain cell lineages but also to inhibit that of other lineages⁴. Defects in these processes in haematopoiesis can result in immunological or haematological disorders such as immunodeficiency and leukaemias⁵. Interferon regulatory factor-8 (IRF8) is a haematopoietic cell-specific member of the IRF transcription factor family. *Irf8*^{-/-} mice develop immunodeficiency and a chronic myeloid leukaemia-like syndrome^{6,7}. Subsequent studies revealed that IRF8 regulates the development of multiple immune cell types⁸. IRF8 is required for the generation of mouse monocytes (particularly Ly6C⁺ monocytes)^{9,10}, classical DCs (cDCs; particularly CD8α⁺ DCs) and plasmacytoid DCs (pDCs)^{11–13} that express high levels of IRF8 (refs 14,15). At the progenitor level, *Irf8*^{-/-} mice harbour increased numbers of GMPs and MDPs but lack common DC progenitors (CDPs)^{16,17}. Recent studies have suggested that *Irf8* transcript expression is relatively high in MDPs, CDPs and common monocyte progenitors (cMoPs)^{18–20}. Importantly, mutations in the human *IRF8* gene are associated with DC and monocyte deficiency²¹.

On the other hand, IRF8 deficiencies cause severe neutrophilia in mice and humans^{6,21}. As a possible explanation for this phenotype, we have previously demonstrated that IRF8 strongly inhibits growth and neutrophil differentiation of granulocyte colony-stimulating factor (G-CSF)-treated myeloid progenitors^{10,22}. Of note, neutrophils do not express *Irf8* (refs 15,18), suggesting that IRF8 may act at progenitor stages to inhibit this lineage. However, the exact differentiation stage at which IRF8 inhibits neutrophil differentiation remains elusive.

IRF8 binds to specific DNA sequences only when it forms heterodimers with partner transcription factors. The IRF8–PU.1 heterodimer transcriptionally activates genes containing the ETS-IRF composite element (EICE) or the IRF-ETS composite sequence (IECS)^{8,9}. Since PU.1 is essential for the development of myeloid cells, particularly monocytes and DCs, PU.1 is undoubtedly a critical partner of IRF8 in promoting their differentiation. In addition, the IRF8–BATF heterodimer binds to the activating protein-1-IRF composite element to promote gene activation during DC differentiation²³. It has been reported in overexpression experiments that IRF8 also acts as a transcriptional repressor when it binds to the interferon-stimulated response element in association with IRF1 or IRF2 (ref. 24). However, our recent analysis of genome-wide IRF8 behaviour in myeloid progenitors has suggested that IRF8, as a DNA binding protein, mainly functions as an activator in association with PU.1, inducing a chromatin signature of enhancers (that is, histone H3 lysine 4 monomethylation) when promoting monocyte differentiation⁹. In contrast to the well-studied mode of action of IRF8 in stimulating differentiation, nothing is yet known regarding the molecular mechanism by which IRF8 inhibits neutrophil differentiation.

In our current study, we found that IRF8 expression is sharply increased at the MDP stage and is essential *in vivo* for restraining MDPs and cMoPs from developing into neutrophils. We further demonstrate that IRF8 physically interacts with and inhibits the transcriptional activity of CCAAT/enhancer-binding protein α (C/EBPα), a transcription factor that strongly induces neutrophil differentiation. A partial inhibition of C/EBP activity in *Irf8*^{-/-} haematopoietic progenitors alleviates the overproduction of neutrophils *in vivo*. These results clarified how and the stage at which IRF8 inhibits neutrophil differentiation, thus shedding light on the mechanism underlying the commitment of the mononuclear phagocyte lineage.

Results

IRF8 protein expression in myeloid progenitor populations.

We examined IRF8 expression in bone marrow myeloid progenitor cell populations on a per cell basis using intracellular immunostaining followed by flow cytometry (see Supplementary Fig. 1 for antibody validation). IRF8 protein was found to be highly expressed in MDPs (Fig. 1a). MDPs were originally identified as myeloid progenitor cells expressing CD117 and a *Cx3cr1* promoter-driven GFP (*Cx3cr1*-GFP)²⁵ reporter, and subsequently described as a CD115⁺ myeloid progenitor population²⁶. More than 80% of '*Cx3cr1*-GFP⁺' MDPs and essentially all '*CD115*⁺' MDPs expressed high levels of IRF8. Subpopulations of CMPs and GMPs also expressed graded levels of IRF8 that closely correlated with that of CD115 (Fig. 1b). This suggests that these subpopulations are becoming or are already MDPs, which is consistent with the fact that significant portions of CMPs and GMPs overlap with MDPs^{25,26}. The HSC-enriched population (lineage markers-negative (Lin⁻) Sca-1⁺ c-Kit/CD117⁺; LSK) barely expressed IRF8 (Fig. 1c). Hence, IRF8 expression in LSK cells and CD115⁻ early myeloid progenitors (MPs) is low or absent, but sharply increases when they differentiate to MDPs. IRF8 expression continued to be high in CDPs (Fig. 1d). CD115⁻ CDPs and cMoPs, recently identified as additional populations with prominent pDC and monocyte differentiation potentials, respectively^{20,27}, also highly expressed IRF8 protein (Fig. 1d,e).

Irf8^{-/-} MDPs and cMoPs aberrantly give rise to neutrophils.

Comprehensive analysis comparing wild-type (WT) and *Irf8*^{-/-} myeloid progenitor populations is shown in Fig. 2. We confirmed previously reported findings¹⁸ that *Irf8*^{-/-} myeloid progenitors accumulate in MDPs and lack CD117^{low} MDPs/CDPs²⁶ (Fig. 2a,b). We refer to the CD117^{low} MDP population as CD117^{low} MDPs/CDPs because most CDPs reside in the CD117^{low} MDP population, yet CD117^{low} MDPs still produce some macrophages²⁶. *Irf8*^{-/-} MDPs were found to express another MDP marker, *Cx3cr1*-GFP, suggesting that they are *bona fide* MDPs (Supplementary Fig. 2). We observed that the numbers of CD115⁻ CDPs and pre-cDCs (committed cDC precursors) were also severely diminished in *Irf8*^{-/-} mice (Fig. 2b,c). Furthermore, we found that *Irf8*^{-/-} mice accumulate cMoPs (6.9-fold compared with WT cMoPs) to an even greater extent than MDPs (3.6-fold compared with WT MDPs), and, as we have reported previously⁹, lack Ly6C⁺ monocytes (Fig. 2d). These data suggest that the differentiation arrest in *Irf8*^{-/-} mice occurs at the transition from MDPs to CD117^{low} MDPs/CDPs and from cMoPs to Ly6C⁺ monocytes.

The fate of *Irf8*^{-/-} MDPs and cMoPs then became a key question. To answer this question, we isolated CD117⁺ MDPs and cMoPs from WT and *Irf8*^{-/-} mice and then transplanted these cells into lethally irradiated mice (1.0 × 10⁴ cells per mouse). As expected, WT MDPs generated monocytes (mostly

Figure 1 | Mononuclear phagocyte progenitors express high levels of IRF8 protein. (a–e) IRF8 protein expression in bone marrow progenitor populations was examined by immunostaining followed by flow cytometry. Bone marrow progenitors were defined as follows: *Cx3cr1*-GFP⁺ MDPs, Lin[−] IL-7R[−] Sca-1[−] CD117⁺ *Cx3cr1*-GFP⁺ (a); MDPs, Lin[−] IL-7R[−] Sca-1[−] CD117⁺ CD115⁺ (a); CD115[−] MDPs, Lin[−] IL-7R[−] Sca-1[−] CD117⁺ CD115[−] (a); CD117^{low} MDPs/CDPs, Lin[−] IL-7R[−] Sca-1[−] CD117^{low} CD115⁺ (a); GMPs, Lin[−] IL-7R[−] Sca-1[−] CD117⁺ CD34⁺ CD16/32⁺ (b); CMPs, Lin[−] IL-7R[−] Sca-1[−] CD117⁺ CD34⁺ CD16/32^{low} (b); LSKs, Lin[−] IL-7R[−] Sca-1⁺ CD117⁺ (c); CDPs, Lin[−] IL-7R[−] CD117^{low} CD115⁺ Flt3⁺ (d); CD115[−] CDPs, Lin[−] IL-7R[−] CD117^{low} CD115[−] Flt3⁺ (d); cMoPs, CD3[−] CD19[−] Ly6G[−] NK1.1[−] CD117⁺ CD115⁺ Flt3[−] cells (e). Histograms indicate anti-IRF8 antibody (Ab; red) and control (Ctrl) Ab (grey shaded) staining. Similar results were obtained from four independent experiments.

Ly6C⁺) in the bone marrow and cDCs in the spleen (Fig. 3a). MDP-derived pDCs were not detectable under our experimental conditions, consistent with a previous report²⁵. On the other hand, *Irf8*^{−/−} MDPs failed to efficiently yield bone marrow monocytes and splenic cDCs, especially the CD8α⁺ subset, but, intriguingly, instead gave rise to a large number of neutrophils. In mice transplanted with WT MDPs, almost no neutrophils could be detected (only 0.064×10^4 and 0.058×10^4 neutrophils in the lower limb bones and spleen on average, respectively). However, in mice transplanted with *Irf8*^{−/−} MDPs, we could detect an average of 3.2×10^4 and 7.1×10^4 neutrophils in the lower limb bones (a 50-fold increase) and spleen (a 121-fold increase), respectively. Moreover, *Irf8*^{−/−} cMoPs failed to efficiently produce monocytes and, again, aberrantly gave rise to a large number of neutrophils (Fig. 3b). Thus, many *Irf8*^{−/−} MDPs may pass through cMoPs to produce neutrophils, although it is also possible that some *Irf8*^{−/−} MDPs directly differentiate into neutrophils. We also cultured MDPs *in vitro* in the presence of stem cell factor (SCF), macrophage colony-stimulating factor (M-CSF), FMS-like tyrosine kinase 3 ligand (Flt3L) and G-CSF. We found that ~50% of the cells generated from *Irf8*^{−/−} MDPs, but only 3% of those derived from WT MDPs, were neutrophils (Fig. 3c). These results suggest that IRF8 is essential not only to bestow monocyte and DC differentiation potential upon MDPs and cMoPs, respectively, but also to restrain these populations from differentiating into neutrophils.

Transcriptome analysis of MDPs and cMoPs in *Irf8*^{−/−} mice.

To investigate the mechanism by which IRF8 inhibits neutrophil differentiation, we performed transcriptome analysis by microarray of MDPs and cMoPs in WT and *Irf8*^{−/−} mice (the quality control for the microarray data is shown in Supplementary Fig. 3). Although it is reasonable to speculate that the loss of IRF8 results in the upregulation of genes critical for neutrophil differentiation, given the results of our previous genome-wide analysis on the behaviour of IRF8 in myeloid progenitors⁹, it seemed unlikely that IRF8 directly acts as a transcriptional repressor. To predict the transcription factor(s) responsible for the aberrant production of neutrophils from *Irf8*^{−/−} MDPs and cMoPs, we used Ingenuity Pathway Analysis (IPA) software. In both MDPs and cMoPs, C/EBPs were identified by IPA as candidate transcription factors activated in the absence of IRF8 (Supplementary Fig. 3c,d). Examples of known C/EBP target genes with significantly higher expression in *Irf8*^{−/−} than WT progenitors included *Mmp8*, *S100a8* and *Hsd11b1* (Supplementary Fig. 4a). Among the C/EBPs, C/EBPα and C/EBPε have been extensively shown to be capable of driving myeloid progenitor cell differentiation into neutrophils^{4,5}. Despite the upregulation of C/EBP target genes, neither the expression of *Cebpa* nor *Cebpe* *per se* was found to be drastically upregulated in *Irf8*^{−/−} MDPs (Supplementary Fig. 4a), suggesting that IRF8 may regulate the activity, rather than the expression levels, of the C/EBP(s). In *Irf8*^{−/−} cMoPs, *Cebpe*

Figure 2 | *Irf8*^{-/-} mice have increased MDP and cMoP counts but lack CDPs and Ly6C⁺ monocytes. (a–d) Bone marrow cells from WT and *Irf8*^{-/-} mice were analysed for myeloid progenitors and Ly6C⁺ monocytes by flow cytometry. The cell numbers of each population per lower mouse limb were calculated. Values are the mean ± s.d. from three or four mice. ****P* < 0.001 (Student's *t*-test). Similar results were obtained from four independent experiments in total.

expression was increased compared with that in WT cMoPs by 3.45-fold, presumably because *Cepbe* is a target gene of *C/EBPα*. Consistent with these transcript levels, the expression of *C/EBPα* protein was comparable in *Irf8*^{-/-} MDPs and only modestly elevated in cMoPs compared with WT counterparts (Supplementary Fig. 4b). Hence, we focused on *C/EBPα* in our subsequent experiments. We note here that NF-κB was also identified as a transcription factor complex activated in *Irf8*^{-/-} MDPs and cMoPs, and this may warrant future investigation, especially in terms of the regulation of apoptosis by IRF8.

IRF8 inhibits transcriptional activation by *C/EBPα*. To examine the effects of IRF8 on *C/EBPα*-dependent transcriptional activation, we first performed a reporter assay. We transduced 293T cells with a reporter lentivirus harbouring four copies of the *C/EBP* response element, followed by a minimal cytomegalovirus (CMV) promoter and cDNA insert encoding GFP (GreenFire1-*C/EBPα*). These reporter cells were then transfected with

expression plasmids for *C/EBPα* and IRF8. *C/EBPα* strongly activated the reporter and IRF8 inhibited this activation in a dose-dependent manner (Supplementary Fig. 5). We next tested IRF8^{K79E} and IRF8^{R289E}, which have a mutation in the DNA binding domain (DBD) and IRF association domain, respectively. IRF8^{K79E} is defective in DNA binding, while IRF8^{R289E} cannot interact with PU.1 and other IRFs²⁸. Neither mutant is capable of inducing macrophage and DC differentiation^{10,28,29}. Interestingly, IRF8^{R289E} still blocked the transactivation by *C/EBPα*, whereas IRF8^{K79E} lost this inhibitory effect, suggesting the involvement of DBD in the inhibition of *C/EBPα* activity. IRF8 did not downregulate the expression of exogenous *C/EBPα* (see immunoblotting analysis in the section following the next).

IRF8 inhibits *C/EBPα*-induced neutrophil differentiation. To investigate whether IRF8 has indeed any antagonistic effects on *C/EBPα* in neutrophil differentiation, we used the 32Dcl.3 myeloid progenitor cell line that has been shown to differentiate

Figure 3 | *Irf8*^{-/-} MDPs and cMoPs aberrantly produce a large number of neutrophils. (a,b) Flow cytometric analysis of bone marrow monocytes, splenic cDCs and splenic neutrophils 5 and 2 days after intravenous transplantation of MDPs (a) and cMoPs (b), respectively. Ten thousand progenitor cells from WT or *Irf8*^{-/-} mice (CD45.2⁺) were transplanted into irradiated Ly5.1 mice (CD45.2⁻), and donor-derived (CD45.2⁺) cells were analysed. Bone marrow CD117⁺ cells were gated out to exclude progenitor cells. Absolute cell numbers (per spleen or lower limbs of a mouse) of the indicated progeny cells derived from transplanted MDPs and cMoPs are shown in the bar graphs. Values are the mean $\pm$ s.d. from three or four mice. Similar results were obtained from three independent experiments. (c) Five thousand MDPs were cultured for 7 days in the presence of SCF, M-CSF, Flt3L and G-CSF. Neutrophils derived from these MDPs were analysed by flow cytometry. Values in the bar graph are the mean $\pm$ s.d. of triplicate determinations. Similar results were obtained from three independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001 (Student's *t*-test).

into neutrophils upon the exogenous expression of C/EBP α ^{30,31}. 32Dcl.3 cells were retrovirally transduced with IRF8 and/or C/EBP α , and their morphology was monitored. When transduced with C/EBP α alone, 40% of these cells exhibited typical

neutrophil features such as segmented nuclei by day 4 (Fig. 4a). IRF8 alone did not affect cell morphology as previously reported¹⁰. However, when IRF8 was coexpressed, only 12% of the cells displayed a neutrophil-like morphology and some

displayed cytoplasmic vacuoles that are a feature of monocytes. The expression level of C/EBP α protein was found not to be downregulated by IRF8 (Fig. 4b). These results indicate that IRF8 is capable of inhibiting C/EBP α -induced neutrophil differentiation.

We next performed quantitative PCR with reverse transcription (RT-qPCR) to analyse the expression of target genes of C/EBP α and IRF8. The C/EBP α target genes such as *Csf3r*, *Elane*, *Gfi1* and *Ltf* were markedly suppressed by IRF8 transduction. On the other hand, the induction of IRF8 target genes such as *Aif1*, *Irf5*, *Ctss* and *Entpd1* was not suppressed by C/EBP α (Fig. 4c). Transcriptome analysis by microarray (Fig. 4d) revealed that among the 1,018 genes upregulated by C/EBP α (fold change (FC) > 3 and false discovery rate (FDR) < 0.05), the expression of 408 genes (40.1%) was suppressed by coexpression of IRF8 (FC < 0.5 and FDR < 0.05). On the other hand, among the 343 IRF8-upregulated genes (FC > 3 and FDR < 0.05), the expression of only 42 genes (12.2%) was suppressed by coexpression of C/EBP α (FC < 0.5 and FDR < 0.05). We also found that IRF8^{R289E} but not IRF8^{K79E} suppressed target gene induction and neutrophil differentiation by C/EBP α (Fig. 4e–g), which is consistent with the results in 293T cells. Of note, neither IRF8^{K79E} nor IRF8^{R289E} induces monocyte differentiation-related IRF8 target genes^{22,29}, suggesting that the modes of IRF8 action in promoting monocyte differentiation and inhibiting neutrophil differentiation are indeed distinct.

Cell growth was found to be markedly inhibited by C/EBP α as expected from previous findings^{32,33} (Fig. 4h). IRF8 alone had no impact on cell growth in 32Dcl.3 cells as also previously reported¹⁰. Interestingly, despite the fact that IRF8 and IRF8^{R289E} inhibited neutrophil differentiation in our current analysis, neither of them affected the growth arrest induced by C/EBP α (Fig. 4h; see Discussion section).

IRF8 physically interacts with C/EBP α . To clarify the mechanism by which IRF8 inhibits C/EBP α activity, we examined whether these factors physically interact. By using 293T cells transfected with IRF8 and C/EBP α , we found that IRF8 co-immunoprecipitated C/EBP α (Fig. 5a). Interestingly, IRF8^{R289E} but not IRF8^{K79E} also co-immunoprecipitated C/EBP α , which is congruent with our reporter assay data. These results suggest that IRF8, via its DBD, physically interacts with C/EBP α to inhibit its activity. The interaction between IRF8 and C/EBP α was also detected by an amplified luminescence proximity homogeneous assay screen (AlphaScreen) using recombinant IRF8 and C/EBP α generated by the wheat-germ cell-free protein production system (Fig. 5b), suggesting that this interaction is direct.

To identify the domain of C/EBP α that is required for the interaction with IRF8, we constructed mutant forms of C/EBP α . These included deletion mutants of the transactivation element-I

(Δ TE-I), Δ TE-II, Δ TE-III and of the DBD (Δ DBD) (Fig. 5c). Because the DBD contains a region required for nuclear entry, we also constructed a mutant in which a nuclear localization signal (NLS) was combined with the DBD deletion (Δ DBD + NLS). The subcellular localization of C/EBP α mutants was validated by imaging of yellow fluorescent protein (YFP) signals in 293T cells transfected with YFP-C/EBP α chimeras (Supplementary Fig. 6). Using 293T cells transfected with IRF8 and the C/EBP α mutants, we found that IRF8 co-immunoprecipitated the Δ TE-I, Δ TE-II and Δ TE-III mutants, but not the Δ DBD and Δ DBD + NLS products (Fig. 5c). These results indicate that IRF8 and C/EBP α interact with each other through their DBDs. The DBD of C/EBP α is subdivided into a basic region (BR) and leucine zipper (LZ) that are necessary for DNA binding and dimerization, respectively³⁴. We also constructed Δ BR and Δ LZ mutants combined with an NLS (Supplementary Fig. 7a,b), which we cotransfected with IRF8. Both of the Δ BR and Δ LZ mutants could be co-immunoprecipitated by IRF8, suggesting that each of these regions can independently interact with IRF8 (Supplementary Fig. 7c).

We further examined whether such interactions would occur between endogenous proteins in MDPs and cMoPs. To this end, we performed an *in situ* proximity ligation assay (PLA) in purified MDPs and cMoPs. Multiple strong signals were observed, indicating the association between endogenous IRF8 and C/EBP α in WT MDPs and cMoPs (Fig. 5d). As a further endorsement of the specificity of these signals, essentially no PLA signals between IRF8 and C/EBP α were observed in *Irf8*^{-/-} MDPs and cMoPs.

IRF8 prevents C/EBP α from binding to chromatin. The above findings that DBDs are involved in the interaction between IRF8 and C/EBP α prompted us to examine the chromatin binding of these proteins. We performed a chromatin immunoprecipitation (ChIP)-qPCR assay using anti-C/EBP α and anti-IRF8 antibodies in 32Dcl.3 cells transduced with IRF8 and/or C/EBP α . ChIP signals for C/EBP α on the promoters of C/EBP α target genes *Csf3r*, *Elane* and *Ltf* were significantly suppressed by IRF8 (Fig. 6a). On the other hand, ChIP signals of IRF8 on the promoters of IRF8 target genes *Ctss* and *Entpd1* were not suppressed by C/EBP α . Both IRF8 and C/EBP α bound to the promoter of *Aif1*, which was induced either by IRF8 or C/EBP α . Again, the binding of C/EBP α to this promoter was repressed by IRF8, while that of IRF8 was instead augmented by C/EBP α . These results indicate that IRF8 inhibits chromatin binding by C/EBP α to thereby block neutrophil differentiation, but not the other way around.

We further examined whether an IRF8 deficiency causes enhanced binding of C/EBP α to chromatin *in vivo*. We isolated CD115⁺ progenitor cells (CD3⁻ CD11b⁻ CD19⁻ Ly6G⁻ NK1.1⁻ CD117⁺ CD115⁺), which comprised MDPs and cMoPs,

Figure 4 | IRF8 inhibits C/EBP α -induced neutrophil differentiation. (a) Wright-Giemsa staining (left; original magnification, $\times 600$) and polymorphonuclear cell frequencies (right bar graph). 32Dcl.3 cells were transduced with empty MSCV-CD8t or MSCV-IRF8-CD8t and then with empty MSCV-puro or MSCV-C/EBP α -puro retroviruses. Cells were analysed 4 days post-C/EBP α transduction. Arrowheads indicate typical polymorphonuclear cells. Bars, 10 μ m. Values in the bar graph are the mean $\pm$ s.d. from five independent experiments. *** P < 0.001 (Student's t -test). (b) Immunoblotting of C/EBP α and IRF8 in transduced 32Dcl.3 cells on day 3. (c) Expression of C/EBP α - or IRF8-inducible genes in 32Dcl.3 cells transduced with C/EBP α and/or IRF8. Transcript levels were analysed 3 days post-C/EBP α transduction by RT-qPCR using the $\Delta\Delta$ CT method. Data were normalized to the *Gapdh* levels, and the values shown are relative to those in empty vector-transduced cells (mean $\pm$ s.d. from three independent experiments). * P < 0.05, ** P < 0.01, *** P < 0.001 (Student's t -test). (d) Pie charts for the numbers of genes that displayed a more than threefold increase in expression induced by C/EBP α (upper panel) and IRF8 (lower panel), and those reversed by IRF8 (red) and C/EBP α (blue), respectively. (e) Expression of C/EBP α - or IRF8-inducible genes in 32Dcl.3 cells transduced with C/EBP α and/or IRF8 mutants. Transcript levels were analysed 3 days post-C/EBP α transduction by RT-qPCR. * P < 0.05, ** P < 0.01, *** P < 0.001 (Student's t -test). (f) Polymorphonuclear cell frequencies in 32Dcl.3 cells transduced with C/EBP α and/or IRF8 (WT or mutants) examined by Wright-Giemsa staining. Values in the bar graph are the mean $\pm$ s.d. from three independent experiments. * P < 0.05 (Student's t -test). (g) Immunoblotting analysis of IRF8 mutants. (h) Growth rate analysis. Data are expressed as the mean $\pm$ s.d. of triplicate determinations. Ctrl, control; NS, not significant.

Figure 5 | Direct interaction between IRF8 and C/EBP α . (a) Co-immunoprecipitation of C/EBP α and IRF8, IRF8^{K79E} or IRF8^{R289E} in 293T cells. IP, immunoprecipitation; WCL, whole-cell lysate; IB, immunoblot. (b) *In vitro* binding analysis of IRF8 with C/EBP α using AlphaScreen. *Escherichia coli* dihydrofolate reductase (DHFR) was used as a negative control (Ctrl). The emission from the acceptor beads was measured and is shown as AlphaScreen signals. All values are the mean $\pm$ s.d. from three independent reactions. *** P < 0.001 (Student's t -test). (c) Co-immunoprecipitation of IRF8 and C/EBP α or its deletion mutants. Diagrams of C/EBP α and its mutants are shown on the top. The results are representative of three independent experiments. (d) *In situ* PLA in purified MDPs and cMoPs. Interaction between IRF8 and C/EBP α were visualized as punctate fluorescent spots (PLA signals) detected by confocal microscopy. In the box plot, the numbers of PLA spots per cell were counted in 100 cells each. The results are representative of two independent experiments. Scale bars, 10 μ m. *** P < 0.001 (Student's t -test).

Figure 6 | IRF8 prevents C/EBP α from binding to chromatin. (a,b) ChIP-qPCR assay using anti-C/EBP α and anti-IRF8 antibodies. (a) 32Dcl.3 cells were transduced with C/EBP α and/or IRF8 as described in Fig. 4. Cells were analysed 3 days post-C/EBP α transduction. Values are the mean $\pm$ s.d. from four independent experiments. (b) CD115 $^{+}$ progenitor cells (CD3 $^{-}$ CD11b $^{-}$ CD19 $^{-}$ Ly6G $^{-}$ NK1.1 $^{-}$ CD117 $^{+}$ CD115 $^{+}$ cells) were freshly isolated and analysed. Values are the mean $\pm$ s.d. from three independent experiments. * P <0.05, ** P <0.01, *** P <0.001 (Student's t -test). Ctrl, control; NS, not significant.

from WT and *Irf8* $^{-/-}$ bone marrow and performed ChIP-qPCR. The binding of C/EBP α to its target genes *Hsd11b1*, *S100a8* and *Cebpe* was significantly augmented in *Irf8* $^{-/-}$ CD115 $^{+}$ progenitor cells compared with WT cells (Fig. 6b), which is consistent with the increased expression of these genes in *Irf8* $^{-/-}$ MDPs or cMoPs (Supplementary Fig. 4a). The binding of IRF8 to these C/EBP α target genes in WT CD115 $^{+}$ progenitor cells was at near background levels, while IRF8 strongly bound to its authentic target, the *Klf4* enhancer⁹. The ChIP signals for C/EBP α on the IRF8-bound *Klf4* enhancer region were relatively low. These *in vivo* data further support our notion that IRF8 inhibits C/EBP α binding to chromatin to suppress its transcriptional activity.

To gain insight into how IRF8 suppresses C/EBP α binding to chromatin, we examined whether IRF8 prevents C/EBP α homodimer formation. FLAG-tagged C/EBP α very efficiently co-immunoprecipitated haemagglutinin (HA)-tagged C/EBP α , which was not inhibited by IRF8 (Supplementary Fig. 7d). These results suggest that IRF8 does not inhibit the dimerization of C/EBP α , but probably interacts with the C/EBP α dimer to prevent its binding to chromatin.

C/EBP inhibition alleviates neutrophilia in *Irf8* $^{-/-}$ mice. If the failure to inhibit C/EBP α is a cause of neutrophilia in *Irf8* $^{-/-}$ mice, the symptom would be ameliorated by reducing C/EBP

Figure 7 | A dominant-negative C/EBP remedies the aberrant overproduction of neutrophils from *Irf8*^{-/-} haematopoietic progenitors. (a) WT or *Irf8*^{-/-} Lin⁻ cells were transduced with empty pGCSam-EGFP (Ctrl) or A-C/EBP-EGFP, and transplanted into irradiated WT mice. Retrovirus-transduced (GFP⁺) cells in the spleen of recipient mice were analysed by flow cytometry at 3 weeks post transplantation. Data are representative of four independent experiments with similar results. **(b)** The frequencies of the indicated progeny cells in GFP⁺ splenic cell populations. Values are the mean $\pm$ s.d. from eight mice of each group in four independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t*-test). NS, not significant.

activity *in vivo*. We transduced WT or *Irf8*^{-/-} bone marrow progenitor cells with a retrovirus encoding a dominant-negative form of C/EBP (A-C/EBP) along with enhanced GFP (EGFP), and transplanted these cells into irradiated mice. A-C/EBP contains the LZ domain conserved in the C/EBPs but lacks a functional DBD and TEs, forms stable inactive heterodimers with C/EBPs and thus prevents C/EBPs from binding to DNA³⁵. GFP-positive (that is, retrovirus-transduced) cells in the spleen were analysed by flow cytometry 4 weeks after transplantation. As expected, engraftment of empty retrovirus-transduced *Irf8*^{-/-} cells resulted in a significant increase in neutrophil counts, whereas the generation of Ly6C⁺ monocytes, pDCs and CD8 α ⁺ DCs was severely impaired compared with empty retrovirus-transduced WT cells (Fig. 7a,b). On the other hand, mice transplanted with A-C/EBP-transduced *Irf8*^{-/-} progenitor cells clearly showed inhibition of neutrophil overproduction, that is, the neutrophil counts returned to almost normal levels. The generation of Ly6C⁺ monocytes, pDCs and CD8 α ⁺ DCs was not restored by A-C/EBP. In mice transplanted with A-C/EBP-transduced WT progenitor cells, a

mild reduction of neutrophil and monocyte counts and a modest increase in DC counts were observed, consistent with the previously reported functions of C/EBP α ³⁶. This also suggests that the inhibition of C/EBP activity by A-C/EBP is partial. These results support the notion that the enhancement of C/EBP activity causes an overproduction of neutrophils in the absence of IRF8.

Discussion

Our current findings have unveiled a molecular mechanism that regulates the differentiation potential of MDPs and cMoPs. We pinpointed MDPs as the stage at which IRF8 expression becomes high. IRF8 is essential not only for MDP and cMoP progression to CD117^{low} MDPs/CDPs and Ly6C⁺ monocytes, respectively, but also for preventing these progenitors from developing into neutrophils. We show from our present data that IRF8 interacts with C/EBP α in MDPs and cMoPs, interferes with the ability of C/EBP α to bind to chromatin and thereby inhibits C/EBP α -induced transcription and neutrophil differentiation (Fig. 8).

Figure 8 | Schematic representation of haematopoiesis in WT and *Irf8*^{-/-} mice. WT MDPs and cMoPs express high levels of IRF8 protein. IRF8 binds to and inhibits the transcriptional activity of C/EBPα in these mononuclear phagocyte progenitors, thereby preventing C/EBPα-mediated neutrophil differentiation. In *Irf8*^{-/-} mice, MDPs and cMoPs accumulate, and due to the derepression of C/EBPα activity, *Irf8*^{-/-} MDPs and cMoPs give rise to massive numbers of neutrophils. IRF8 is also required for the development of Ly6C⁺ monocytes, CDPs, pre-cDCs, CD8α⁺ cDCs and pDCs.

Our present data indicate that essentially all 'CD115⁺' MDPs and >80% of 'Cx3cr1-GFP⁺' MDPs highly express IRF8, a finding which is somewhat distinct from the results of a recent study of transgenic mice harbouring a phage artificial chromosome (PAC) sequence containing three copies of the *Irf8* gene fused to internal ribosome entry site (IRES)-YFP. In that study, ~50% of 'Cx3cr1-GFP⁺' MDPs were found to express *Irf8* via a PU.1-dependent mechanism¹⁸. We also note that while our present data indicate that LSK cells barely express IRF8, the above-mentioned study reported that all LSK cells already express *Irf8* at even higher levels than MDPs. The reason for these discrepancies is unknown, but may be because the earlier report used the exogenous PAC sequence and monitored the transcription level of *Irf8*. Whatever the reason for the discrepancies, our current results indicate that IRF8 protein expression sharply increases only when myeloid progenitor cells progress to MDPs.

By comparing WT and *Irf8*^{-/-} mice, we have demonstrated herein that *Irf8*^{-/-} myeloid progenitors accumulate at the MDP and cMoP stages. *Irf8*^{-/-} mice lack CD117^{low} MDPs/CDPs, CD115⁻ CDPs, pre-cDC and Ly6C⁺ monocytes that pinpoints the steps at which differentiation arrest occurs in these mice. Indeed, when transplanted, *Irf8*^{-/-} MDPs and cMoPs failed to efficiently give rise to DCs and monocytes. Scheller *et al.*⁷ reported in 1999 through an *in vitro* colony-forming assay of

bone marrow cells that *Irf8*^{-/-} colonies are mostly of a neutrophil morphology, even in the presence of M-CSF. We have also previously demonstrated that a significant fraction of *Irf8*^{-/-} Lin⁻ cells respond to M-CSF to generate neutrophils²². These seemingly anomalous observations can now be explained by our present finding that MDPs and cMoPs, which are CD115 (M-CSF receptor)-expressing progenitor populations within Lin⁻ cells, give rise to neutrophils *in vivo* if IRF8 is lost. We infer from this that the production of neutrophils derived from MDPs and cMoPs is a major cause of neutrophilia in *Irf8*^{-/-} mice because mononuclear phagocyte progenitors are the first populations at which IRF8 is highly expressed in the myeloid lineage, and MDPs and cMoPs accumulate in *Irf8*^{-/-} mice. Whether all *Irf8*^{-/-} MDPs pass through cMoPs to produce neutrophils is unknown; some *Irf8*^{-/-} MDPs may directly differentiate into neutrophils. Interestingly, Bhattacharya and colleagues¹⁷ have recently reported that all lymphoid progenitors (ALPs) from *Irf8*^{-/-} mice can produce neutrophils. Therefore *Irf8*^{-/-} ALPs may also contribute to neutrophilia.

Our transcriptome analysis of MDPs and cMoPs from WT and *Irf8*^{-/-} mice predicted that C/EBP(s) is aberrantly activated in the absence of IRF8. Indeed, C/EBPα binding to its target genes was significantly increased in these *Irf8*^{-/-} CD115⁺ progenitor cells. From the results of co-immunoprecipitations of exogenously expressed proteins, an AlphaScreen using recombinant

proteins and PLA for endogenous proteins, we concluded that IRF8 directly interacts with C/EBP α , which is known to promote neutrophil differentiation, in MDPs and cMoPs. Domain analysis indicated that this interaction involves DBDs of IRF8 and C/EBP α . Consistent with these findings, IRF8 inhibits chromatin binding and transactivation activities of C/EBP α and thereby blocks C/EBP α -induced neutrophil differentiation. The ability of IRF8 to stimulate monocyte and DC differentiation is not a consequence of inhibiting C/EBP α activity and neutrophil differentiation, because the IRF8^{R289E} mutant can still do the latter but not the former, and the inhibition of C/EBP activity in *Ir8*^{-/-} haematopoietic progenitors selectively restored neutrophil overproduction. Interestingly, IRF8 did not reverse C/EBP α -induced growth arrest. This suggests the possibility that C/EBP α does not require its DNA binding activity to halt cell growth. Indeed, it has been reported previously that C/EBP α arrests cell proliferation by directly binding to E2F via the TE-I domain during adipogenesis and granulopoiesis³³, and to Cdk2 and Cdk4 via the TE-III domain in the liver³⁷. In support of such a notion, the introduction of C/EBP α significantly inhibited the expression of various known E2F target genes in 32Dcl.3 cells, and this inhibition was not affected by the coexpression of IRF8 (Supplementary Fig. 8a). Moreover, reporter assays in 32Dcl.3 cells transduced with a lentivirus carrying an E2F-responsive luciferase construct showed that IRF8 does not reverse the inhibitory effects of C/EBP α upon E2F transcriptional activity (Supplementary Fig. 8b). We note that in addition to the direct inhibition of C/EBP α activity by IRF8 via physical interaction, it is also possible that IRF8 induces the expression of genes encoding proteins that then inhibit chromatin binding of C/EBP α and/or neutrophil differentiation. Indeed, although the IRF8^{R289E} mutant, which can interact with C/EBP α but not with PU.1 or other IRFs, suppressed C/EBP α -mediated neutrophil differentiation, this inhibition was less potent than WT IRF8.

Our finding that IRF8 inhibits chromatin binding of C/EBP α but not the other way around has implications for the interplay between three key myeloid transcription factors, IRF8, C/EBP α and PU.1, that have now all been found to interact with each other. Both C/EBP α -PU.1 (ref. 38) and C/EBP α -IRF8 interactions involve the DBDs of these three transcription factors. On the other hand, the IRF8-PU.1 interaction occurs between the IRF association domain and the PEST domain, respectively³⁹. We infer from this that the binding affinity of the IRF8-PU.1 heterodimer for its target DNA element may be higher than that for its interaction with C/EBP α homodimer. This notion is supported by our two findings, that is, the chromatin binding of IRF8 is not inhibited by C/EBP α , and these two transcription factors do not colocalize on chromatin. This model also suggests that the expression level of IRF8 may need to be high enough to inhibit C/EBP α activity, as in MDPs and cMoPs.

Because the DBDs of the C/EBP family are conserved and A-C/EBP can inhibit any C/EBP, the activity of C/EBPs other than C/EBP α might be also inhibited by IRF8. When overexpressed in 293T cells, IRF8 did bind to and inhibit transcriptional activation by C/EBP ϵ , and IRF8 was found to inhibit the C/EBP ϵ -mediated induction of target genes and neutrophil differentiation in 32Dcl.3 cells (Supplementary Fig. 9). However, since *Cebpe* expression levels are low in cells highly expressing IRF8, the interaction between IRF8 and C/EBP ϵ may not occur *in vivo*. Another possibility would be an involvement of C/EBP β , which has been shown to be required for emergency granulopoiesis⁴⁰.

The inhibition of C/EBP by IRF8 may also operate in other cell lineages where both IRF8 and C/EBP are expressed. For example, IRF8 is expressed in pre-osteoclasts and inhibits their differentiation, whereas C/EBP α is expressed in both pre-osteoclasts and

osteoclasts to promote osteoclastogenesis^{41,42}. Moreover, because the DBDs are well conserved in both the IRF and C/EBP families, other IRF-C/EBP interactions may also occur. Adipocytes are a good candidate because they express multiple IRFs and C/EBPs, and IRF3 and IRF4 repress, while some C/EBPs stimulate, adipogenesis^{43,44}.

In conclusion, IRF8 was found to have a critical role in determining the differentiation characteristics of mononuclear phagocyte progenitor populations. IRF8 thus appears to have a dual role in stimulating and inhibiting cell differentiation within myeloid cell lineages by cooperating with other key transcription factors.

Methods

Mice. Male and female Ly5.1, Ly5.2, *Ir8*^{-/-} and *Cx3cr1*^{gfp/+} mice⁴⁵ in a C57BL/6 background were used at 7–9 weeks of age. All animal experiments were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan), and all protocols were approved by institutional review boards of Yokohama City University (protocol no. F11-85).

Flow cytometry and separation of haematopoietic progenitors.

Splenocytes were isolated by liberase and DNase I (Roche) treatment. Bone marrow cells were harvested by flushing femurs and tibias. Flow cytometry was performed using a FACSCanto II (BD Biosciences), and data were analysed using FlowJo software (Tree Star). For staining lineage marker-positive cells, the Lineage Cell Detection Cocktail (biotinylated anti-CD5, CD45R (B220), CD11b, Gr-1 (Ly6G/C), Ter-119 and 7-4 antibodies; Miltenyi Biotec) was used. Other antibodies and their clone names were anti-CD117 (2B8), Sca-1 (D7), CD16/32 (93), CD115 (ASF98), Flt3 (A2F10), CD3 (145-2C11), CD11b (M1/70), CD19 (6D5), Ly6C (HK1.4), MHC-class II (AF6-120.1), CD11c (N418), CD8 α (53-6.7), CD4 (GK1.5) and Ly6G (1A8) antibodies from Biolegend and anti-CD34 (RAM34) and NK1.1 (PK136) antibodies from eBioscience. Anti-IRF8 (C-19) and C/EBP α (AA14) antibodies for intracellular immunostaining were purchased from Santa Cruz Biotechnology. The antibodies used including dilutions are listed in Supplementary Table 1. For staining surface makers, cells were incubated for 30 min at 4 °C with appropriately diluted fluorochrome-conjugated antibodies or biotinylated antibodies, and then with fluorochrome-conjugated streptavidin. 7-amino-actinomycin D (Sigma) was used to exclude dead cells. Intracellular staining of transcription factors was performed as described previously¹⁴. Briefly, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% saponin (Sigma-Aldrich), blocked with 10% normal donkey serum and stained with goat anti-IRF8, rabbit anti-C/EBP α antibody, control normal goat IgG or normal rabbit IgG (Jackson ImmunoResearch). This was followed by incubation with fluorochrome-conjugated donkey anti-goat or anti-rabbit antibody. For isolation of bone marrow MDPs (defined as Lin⁻ IL-7R⁻ Sca-1⁻ CD117⁺ CD115⁺ cells)^{26,46}, Lin⁻ cells were first enriched using the Lineage Cell Depletion Kit and magnetic-activated cell sorting (MACS) system (Miltenyi Biotec). For the isolation of cMoPs (defined as CD3⁻ CD11b⁻ CD19⁻ Ly6G⁻ NK1.1⁻ Flt3⁻ CD117⁺ CD115⁺ Ly6C⁺ cells)²⁰ and CD115⁺ progenitor cells (defined as CD3⁻ CD11b⁻ CD19⁻ Ly6G⁻ NK1.1⁻ CD117⁺ CD115⁺ cells), bone marrow cells were first incubated with biotinylated anti-CD3, CD19, Ly6G and NK1.1 antibodies and then with anti-Biotin MicroBeads (Miltenyi Biotec), followed by negative selection. Enriched Lin⁻ cells or CD3⁻ CD19⁻ Ly6G⁻ NK1.1⁻ cells were then stained with appropriate antibodies and sorted using FACSARIA II (BD Biosciences). The purity of the sorted cells was always >99%.

Adoptive transfer and culture of bone marrow progenitors. MDPs or cMoPs purified from Ly5.2 mice (CD45.2⁺) were injected intravenously into lethally irradiated (950 rad) Ly5.1 mice (CD45.2⁻; 1.0×10^4 cells per mouse). These mice were killed 5 and 2 days after the MDP and cMoP transfer, respectively, and the progeny cells in the bone marrow and spleen were analysed. For *in vitro* culture, sorted MDPs (5.0×10^3 cells ml⁻¹) were treated with SCF (100 ng ml⁻¹), M-CSF (20 ng ml⁻¹), Flt3L (100 ng ml⁻¹) and G-CSF (20 ng ml⁻¹) in RPMI1640 medium (Nacalai Tesque) supplemented with 20% heat-inactivated fetal bovine serum. Harvested cells were counted and stained with anti-CD11b and Ly6G antibodies, followed by flow cytometric analyses.

Microarray and IPA. MDPs, cMoPs and 32Dcl.3 cells¹⁰ were subjected to microarray analysis using the RNeasy Micro Kit (Qiagen), the Low Input Quick Amp Labeling Kit (Agilent), a Whole Mouse Genome 8 × 60K Microarray (Agilent) and an Agilent DNA Microarray Scanner, in accordance with the manufacturer's protocols. All data were normalized using quantile methods and analysed by NIA Array Analysis software (<http://lgsun.grc.nia.nih.gov/ANOVA/>). Visualization of hierarchical clustering and primary component analysis were also performed using the NIA Array Analysis software. *P* values were determined by one-way analysis of variance test. Among the microarray probes, only those

associated with RefSeq or Ensembl protein-coding transcripts were selected for analysis. To predict transcription factors that could explain the upregulation of certain genes ($FC > 3$ and $FDR < 0.05$) in *Irf8*^{-/-} progenitors compared with WT progenitors, Ingenuity Upstream Regulator Analysis was performed using IPA software version 8.8 (Ingenuity Systems; www.ingenuity.com). This analysis was based on compiled knowledge from the literature describing relationships between regulators and their target genes (Ingenuity Knowledge Base), and calculates a Z-score using a prediction algorithm (http://pages.ingenuity.com/rs/ingenuity/images/0812%20upstream_regulator_analysis_whitepaper.pdf). In accordance with the manufacturer's instructions, Z-scores > 2.0 were considered significant. All microarray data are available at the GEO/NCBI database (accession code GSE50052). Microarray data for HSCs have been previously published⁴⁷.

Reporter assay. The pcDNA3.1-HA-C/EBP α vector was constructed by inserting a restriction enzyme site-tagged *Cebpa* cDNA obtained by PCR from a pMSV-C/EBP α plasmid into pcDNA3.1(+) (Invitrogen) in which a HA epitope (HA tag) had been inserted. pcDNA3.1-IRF8, pcDNA3.1-IRF8^{K79E} and pcDNA3.1-IRF8^{R289E} were constructed by the subcloning of cDNAs from pMSCV plasmids into pcDNA3.1(+). The C/EBP reporter lentiviral vector, pGreenFire1-C/EBP α -EF1-puro (System Biosciences) and two packaging vectors (pCMV-VSV-G and pCMV Δ R8.2; RIKEN BioResource Center) were transiently transfected into HEK293T cells. After 48 h, the lentivirus-containing supernatants were harvested and used to transduce 293T cells, which were then selected by puromycin treatment at $2 \mu\text{g ml}^{-1}$. The reporter 293T cells were transfected with pcDNA3.1-C/EBP α and/or pcDNA3.1-IRF8, pcDNA3.1-IRF8^{K79E} or pcDNA3.1-IRF8^{R289E} using Lipofectamine LTX (Invitrogen). Twenty-four hours after transfection, GFP expression was analysed by flow cytometry. For the E2F reporter assay, 32Dcl.3 cells were transfected with Cignal E2F Reporter lentivirus (Qiagen), and selected under puromycin at $2 \mu\text{g ml}^{-1}$. The reporter 32Dcl.3 cells were transfected with empty MSCV-neo or MSCV-IRF8-neo vectors, and then with empty MSCV-CD8t or MSCV-C/EBP α -CD8t. Transduced cells were selected using $500 \mu\text{g ml}^{-1}$ G-418 (Roche) and MACS sorting. Two days after C/EBP α transduction, cells were lysed using the Dual-Glo Luciferase Assay System and analysed in a GloMax 96 Microplate Luminometer (Promega).

Quantitative PCR with reverse transcription. Total RNA was prepared using NucleoSpin RNA II (Macherey-Nagel) and reverse transcribed using the PrimeScript RT reagent kit (Takara) in accordance with the manufacturer's instructions. The following primers were used in the RT-qPCR analyses: *Csf3r* (sense, 5'-TTGCCCACCATCATGACAGA-3'; antisense, 5'-GAATCCCAGTGGGTCCGTTT-3'), *Elane* (sense, 5'-TGACTAATCATGTGCCCGCT-3'; antisense, 5'-GAGTCCCCGAAGCAGATGCC-3'), *Gfi1* (sense, 5'-ACAGGTGAGAAGCCCCACAA-3'; antisense, 5'-CCACACAGGTCACAGCCAAA-3'), *Ltf* (sense, 5'-TGCTGCTCCCAACAGCAAGA-3'; antisense, 5'-GCCTTCTCAGCCAGACACCTTA-3'), *Aif1* (sense, 5'-CAGAATGATGCTGGGCAAGA-3'; antisense, 5'-GGACCAGTTGGCTCTCTTG-3'), *Irf5* (sense, 5'-ATGTCCTGGACCGTGGGCTC-3'; antisense, 5'-GAACACCTTACACTGGCAGACAGC-3'), *Ctss* (sense, 5'-TGCCAAAGATTACTGGCTTG-3'; antisense, 5'-GCCATCCGAATGTATCCTTGA-3') and *Entpd1* (sense, 5'-GGGCAAGATCAAAGACAGCAA-3'; antisense, 5'-GTTTCAGCTGGGATCATGTTGG-3').

Retroviral transduction. The pMSCV-IRF8-CD8t construct that carries the *Irf8* cDNA in pMSCV-CD8t (MSCV-IRES-human-truncated CD8; a kind gift from Dr Ricardo A. Feldman) has been described previously^{15,48}. pMSCV-IRF8^{K79E}-CD8t and pMSCV-IRF8^{R289E}-CD8t were generated by subcloning of the cDNAs from pMSCV-IRF8^{K79E}-puro and pMSCV-IRF8^{R289E}-puro, respectively¹⁰. pMSCV-C/EBP α -puro was constructed by inserting restriction enzyme site-tagged *Cebpa* cDNA obtained by PCR from pMSV-C/EBP α , a kind gift from Dr Alan D. Friedman, into pMSCV-puro (MSCV-PGK-puromycin resistant gene; Clontech). To construct pMSCV-C/EBP α -puro, restriction enzyme site-tagged *Cebpe* cDNA, obtained by RT-PCR from C/EBP α -transduced 32Dcl.3 cells, was inserted into pMSCV-puro. pGCSam-IRES-EGFP and pGCSam-A-C/EBP-IRES-EGFP vectors³⁵ were kindly provided by Dr Atsushi Iwama. For retroviral transduction¹⁵, 293T cells were transiently transfected with retroviral vectors and the pCL-Eco packaging vector using Lipofectamine 2000 (Life Technologies) in accordance with the manufacturer's protocol. Retroviral supernatants were collected at 48 and 72 h. Cells were transduced by spinoculation ($1,300 \times g$, 30°C , 90 min) in a retroviral supernatant supplemented with cytokines and $4 \mu\text{g ml}^{-1}$ polybrene. Transduced cells were selected by puromycin treatment ($2 \mu\text{g ml}^{-1}$) or MACS sorting.

Co-immunoprecipitation. pcDNA3.1(+) plasmids carrying HA-tagged C/EBP α mutants, $\Delta 11$ -70 (Δ TE-I), $\Delta 70$ -97 (Δ TE-II) and $\Delta 97$ -257 (Δ TE-III) were constructed by inserting restriction enzyme site-tagged cDNAs obtained by PCR from the pMSV-C/EBP α $\Delta 1$ -2, pMSV-C/EBP α $\Delta 1$ -2 and pMSV-C/EBP α $\Delta 4$ -9 vectors, respectively, kindly provided by Dr Alan D. Friedman⁴⁹ into pcDNA3.1(+) with a HA tag. cDNAs encoding C/EBP α Δ DBD, C/EBP α Δ DBD + NLS from SV40 large T antigen, C/EBP α Δ BR + NLS and C/EBP α Δ LZ + NLS were generated by PCR using primers tagged with restriction enzyme sites (and the NLS sequence) and inserted into pcDNA3.1(+) with a HA tag. The pcDNA3.1-FLAG-C/EBP α vector was generated by subcloning the cDNA from pcDNA3.1-HA-C/EBP α into

pcDNA3.1(+) in which a FLAG tag had been inserted. 293T cells were transfected with empty pcDNA3.1, pcDNA3.1-HA-C/EBP α (WT or its mutants) and/or pcDNA3.1-IRF8 (WT or its point mutants) using Lipofectamine LTX. Forty-eight hours after transfection, cells were lysed with lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 2 mM EDTA and 1% Nonidet P-40). Protein G-coated Dynabeads (Invitrogen) were washed twice in lysis buffer, followed by incubation with a goat anti-IRF8 antibody (C-19; Santa Cruz Biotechnology). After washing in lysis buffer, the primary antibody-conjugated Protein G Dynabeads were incubated with cell lysates at 4°C for 16 h with gentle shaking, followed by washing twice in lysis buffer. For FLAG-tagged C/EBP α immunoprecipitation, anti-FLAG M2 magnetic beads (Sigma-Aldrich) were added to the lysates and incubated at 4°C for 16 h with gentle shaking, followed by five washes in lysis buffer. Immunoprecipitates were eluted with SDS sample buffer, boiled for 10 min, subjected to SDS-polyacrylamide gel electrophoresis and blotted with antibodies for IRF8 (C-19), C/EBP α antibody (14AA; Santa Cruz Biotechnology), HA (12CA5; Roche), β -actin (AC-15; Sigma-Aldrich) or FLAG (M2; Sigma-Aldrich). Uncropped scans of the western blots are shown in Supplementary Figs 10–12.

C/EBP α -YFP chimeric proteins. The pEYFP-C1 vectors carrying C/EBP α and its mutants, $\Delta 11$ -70 (Δ TE-I), $\Delta 70$ -97 (Δ TE-II) and $\Delta 97$ -257 (Δ TE-III) were constructed by inserting restriction enzyme site-tagged cDNAs obtained by PCR from pMSV-C/EBP α , pMSV-C/EBP α $\Delta 1$ -2, pMSV-C/EBP α $\Delta 3$ and pMSV-C/EBP α $\Delta 4$ -9, respectively, into pEYFP-C1 (Clontech). cDNAs encoding C/EBP α Δ DBD and C/EBP α Δ DBD + NLS were generated by PCR using primers tagged with restriction enzyme sites (and the NLS sequence) and inserted into pEYFP-C1. The pEYFP-C1 vectors carrying C/EBP α Δ BR + NLS and C/EBP α Δ LZ + NLS sequences were generated by subcloning the cDNA insert from pcDNA3.1-HA-C/EBP α Δ BR + NLS and pcDNA3.1-HA-C/EBP α Δ LZ + NLS, respectively, into pEYFP-C1. HeLa cells were transfected with pEYFP-C1-C/EBP α (WT or its mutants) and pHcRed-C1 (Clontech) using Lipofectamine LTX. Forty-eight hours after transfection, cells were stained with Hoechst 33342, followed by confocal microscopy.

AlphaScreen. The pEU-E01-IRF8-FLAG construct was generated by subcloning the cDNA from pMSCV-IRF8-FLAG-puro¹⁵. The pEU-E01 vector (Cell Free Science) is a plasmid vector specifically developed for the wheat-germ cell-free protein synthesis system⁵⁰. pEU-E01-C/EBP α biotin-ligating sequence was constructed by inserting restriction enzyme site-tagged *Cebpa* cDNA obtained by PCR from pMSV-C/EBP α into pEU-E01 into which a biotin-ligating sequence had been inserted. These plasmids were then used as templates for *in vitro* protein synthesis using the wheat-germ expression kit (CellFree Science). FLAG-tagged IRF8 proteins were mixed with biotinylated C/EBP α in $15 \mu\text{l}$ of reaction buffer (20 mM Tris-HCl, pH 7.6, 5 mM MgCl₂ and 1 mM dithiothreitol in a well of 384-well optiplates (PerkinElmer) and incubated at 26°C for 1 h. The mixture was then added to AlphaScreen buffer containing anti-immunoglobulin G (protein A) acceptor beads, streptavidin-coated donor beads (PerkinElmer) and the anti-FLAG M2 antibody (Sigma), and further incubated at 26°C . One hour later, AlphaScreen signals from the mixture were detected using an EnVision device (PerkinElmer) with the AlphaScreen signal detection program.

In situ PLA. Purified MDPs and cMoPs were fixed by 4% paraformaldehyde and cytospin on Matsunami adhesive silane (MAS)-coated glass slides. Cells were then permeabilized in 100% methanol and PLA was performed using Duolink *in situ* PLA (OLINK Biosciences) in accordance with the manufacturer's instructions. As primary antibodies, goat anti-IRF8 (C-19) and rabbit anti-C/EBP α (2295; Cell Signaling Technology) antibodies were used. For negative control experiments, normal goat IgG and normal rabbit IgG (Santa Cruz Biotechnology) were used. The secondary antibodies used were anti-goat (for IRF8) and anti-rabbit (for C/EBP α) antibodies conjugated to complementary synthetic oligonucleotides. PLA signals were visualized by confocal microscopy (FV1000-D; Olympus).

ChIP-qPCR. ChIP-qPCR assays were performed as described previously⁹, except that cells were fixed with 1% formaldehyde for 10 min at room temperature and the reaction was quenched with 125 mM glycine. The lysates were sonicated 20 times for 1 min each with a Bioruptor sonicator (Cosmo Bio). Immunoprecipitation was then performed using Dynabeads Protein G (Veritas) coated with a goat anti-IRF8 antibody (C-19), a rabbit anti-C/EBP α antibody (14AA), control normal goat IgG or normal rabbit IgG. The following primers were used in the qPCR analysis: *Aif1* (sense, 5'-CTTCCACTCATTCCTTGCTAACT-3'; antisense, 5'-TCAGACAT TACTTCTCATCTCCTC-3'), *Ltf* (sense, 5'-TTAGCCAACGTGTGTGTTCTTC ACA-3'; antisense, 5'-AGGGTCCCTCATTTGGAAACAATA-3'), *Ctss* (sense, 5'-CAACATGGAGCAAGCCAGTG-3'; antisense, 5'-CTTCCGGAACATTCCA CACC-3'), *Entpd1* (sense, 5'-CCCCAGCTAGTACTCACC-3'; antisense, 5'-GTGGGCTGACTCCAGGATAA-3'), *Elane* (sense, 5'-TGCTGACATGGAG ACCCTGA-3'; antisense, 5'-ACCAGAGGCGCTGTATTGG-3'), *Csf3r* (sense, 5'-TTTCCCTCAACCTCCTTCTCTG-3'; antisense, 5'-CCAGAATTGCGACA CCTTCC-3'), *Hsd11b1* (sense, 5'-GTAAATGTTCCAACTCCCACTTCC-3'; antisense, 5'-GCTGCTTTTATAGAGAAAGCTTGG-3'), *S100a8* (sense, 5'-GTAAATGTTCCAACTCCCACTTCC-3'; antisense, 5'-GCTGCTTTTATA GAGGAAAGCTTGG-3'), *Cebpe* (sense, 5'-GGATCTTGCAACTGGGATG-3'; antisense, 5'-AGGTGAGTCTACCAAGGTCAGTCAG-3'), and *Klf4* (sense,

5'-AGACGAGATTAAGCTAAAGGGGACA-3'; antisense, 5'-AGAGTAGTTGA GCTCTTGCTGTGG-3'). C/EBP α binding sites on the *Cebpe* gene were predicted from previously published ChIP followed by deep sequencing (ChIP-seq) data for histone H3 lysine 27 acetylation and histone H3 lysine 4 monomethylation in bone marrow cells⁵¹.

Adoptive transfer of retrovirus-transduced progenitor cells. Lin⁻ cells isolated from WT or *Irf8*^{-/-} bone marrow were cultured with interleukin (IL)-6 (10 ng ml⁻¹), SCF (100 ng ml⁻¹) and IL-3 (10 ng ml⁻¹) in RPMI1640 supplemented with 20% heat-inactivated fetal bovine serum for 24 h, and then transduced with retroviral vectors (GCsam-IRES-EGFP or GCsam-A-C/EBP-IRES-EGFP) by spinoculation. Four hours after transduction, cells were washed and transplanted into irradiated (950 rad) WT mice. Three weeks after transplantation, splenocytes were isolated by liberase and DNase I treatment and analysed by flow cytometry.

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Author contributions

D.K., M.Ya. and T.T. designed the study; D.K., M.Ya., A.N., K.U., T.B., M.I., H.S., S.M. and A.R. performed the experiments; D.K., M.Ya., A.N. and T.T. analysed the data; D.K., M.Ya. and T.T. wrote the manuscript; M.Yo. and M.N. performed critical animal maintenance; K.O. provided key reagents; and T.T. supervised the project.

Additional information

Accession codes: Microarray data have been deposited in the NCBI Gene Expression Omnibus under accession code GSE50052.

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