

IMMUNOBIOLOGY AND IMMUNOTHERAPY

C/EBP β is required for survival of Ly6C⁻ monocytes

Akihiro Tamura,¹ Hideyo Hirai,¹ Asumi Yokota,¹ Naoka Kamio,¹ Atsushi Sato,¹ Tsukimi Shoji,¹ Takahiro Kashiwagi,¹ Yusuke Torikoshi,¹ Yasuo Miura,¹ Daniel G. Tenen,^{2,3} and Taira Maekawa¹

¹Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, Kyoto, Japan; ²Cancer Science Institute, National University of Singapore, Singapore; and ³Harvard Stem Cell Institute, Boston, MA

Key Points

- In *Cebpb*^{-/-} mice, the number of Ly6C⁻ monocytes was specifically decreased in a cell-intrinsic manner due to their accelerated death.
- C/EBP β supports the survival of Ly6C⁻ monocytes, at least in part through direct upregulation of *Csf1r*.

The transcription factor CCAAT/enhancer-binding protein β (C/EBP β) is highly expressed in monocytes/macrophages. However, its roles in monopoiesis are largely unknown. Here, we investigated the roles of C/EBP β in monopoiesis. Further subdivision of monocytes revealed that *Cebpb* messenger RNA was highly upregulated in Ly6C⁻ monocytes in bone marrow. Accordingly, the number of Ly6C⁻ monocytes was significantly reduced in *Cebpb*^{-/-} mice. Bone marrow chimera experiments and *Mx1*-Cre-mediated deletion of *Cebpb* revealed a cell-intrinsic and monocyte-specific requirement for C/EBP β in monopoiesis. In *Cebpb*^{-/-} mice, turnover of Ly6C⁻ monocytes was highly accelerated and apoptosis of Ly6C⁻ monocytes was increased. Expression of *Csf1r*, which encodes a receptor for macrophage colony-stimulating factor, was significantly reduced in Ly6C⁻ monocytes of *Cebpb*^{-/-} mice. C/EBP β bound to positive regulatory elements of *Csf1r* and promoted its transcription. Collectively, these results indicate that C/EBP β is a critical factor for Ly6C⁻ monocyte survival, at least in part through upregulation of *Csf1r*. (*Blood*. 2017;130(16):1809-1818)

Introduction

Monocytes are mononuclear phagocytic cells that are involved in host defense and maintenance of tissue homeostasis.¹ The half-life of monocytes is short; therefore, they must be continuously replenished by proliferation and differentiation of monocyte precursors, a process called monopoiesis. Common myeloid progenitors, granulocyte-macrophage progenitors, macrophage dendritic cell precursors (MDPs), and common monocyte progenitors (cMoPs) are intermediates during the differentiation of hematopoietic stem cells (HSCs) into mature monocytes, although it is disputed whether MDPs are clonal common precursors of monocytes and dendritic cells.²⁻⁵ Recently, functionally and phenotypically distinct subsets of mature monocytes have been identified in mice and humans.⁶⁻⁹ In mice, CD11b⁺ CD115⁺ monocytes are subdivided into 2 subsets based on the expression of Ly6C. Ly6C⁺ monocytes (also called inflammatory or classical monocytes) are rapidly recruited to tissues during inflammation and exhibit antimicrobial activity,¹⁰ whereas Ly6C⁻ monocytes (also called nonclassical, resident, or patrolling monocytes) are often found attached to or crawling on the luminal wall of the endothelium.^{11,12} Recent data show the involvement of nonclassical monocytes in a variety of pathophysiological conditions,^{9,13-16} although their precise significance is not fully understood. Developmentally, Ly6C⁻ monocytes are considered to be downstream of Ly6C⁺ monocytes or direct progeny of MDPs or cMoPs under some circumstances.¹⁷⁻²⁰ The molecular mechanisms that regulate the development of these monocyte subsets remain largely unknown.

CCAAT/enhancer-binding protein β (C/EBP β) is a member of the C/EBP leucine zipper transcription factor family.²¹ In hematopoiesis,

we previously reported that C/EBP β is required for granulopoiesis under stressed conditions.²²⁻²⁴ At steady state, C/EBP β is an important regulator of the differentiation and function of macrophages.²⁵⁻²⁸ Despite the well-characterized importance of C/EBP β in macrophages, its precise roles in monopoiesis have remained largely elusive. Recently, we found that the level of monocytes in peripheral blood (PB) is severely reduced in *Cebpb*^{-/-} mice.²⁹ In this study, we further investigated monopoiesis in *Cebpb*^{-/-} mice and found that C/EBP β plays critical roles in the survival of Ly6C⁻ nonclassical monocytes.

Methods

Mice

C57BL/6 mice (7-12 weeks old) were purchased from CLEA Japan (Tokyo, Japan). CD45.1⁺ mice were a kind gift from Shigekazu Nagata (Kyoto University). *Cebpb*^{-/-} mice were bred and maintained under specific pathogen-free conditions at Kyoto University. *Cebpb*^{fl/fl} mice (*Cebpb* is flanked with loxP) crossed with *Mx1*-Cre transgenic mice were kindly provided by Esta Sterneck (US National Cancer Institute). Littermates were used as controls in all experiments, except for the 5-bromo-2'-deoxyuridine (BrdU) pulsing experiments shown in Figure 3 and supplemental Figure 5 (available on the *Blood* Web site). The animal protocols were approved by the Committee on Animal Research of the Kyoto University Faculty of Medicine.

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Cell lines

EML cells, a kind gift from Schickwahn Tsai (University of Utah), were maintained in Iscove modified Dulbecco medium supplemented with 20% heat-inactivated horse serum and 10% BHK/MKL cell-conditioned medium as a source of stem cell factor.³⁰ Cos-7 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum.

Vectors

The Csf1r-EGFP-long Fms-intronic regulatory element (FIRE) vector was a kind gift from Clare Pridans and David A. Hume (The Roslin Institute, University of Edinburgh).³¹ To avoid long terminal repeats in the vector interfering with reporter expression, the Csf1r promoter-green fluorescent protein (GFP)-FIRE expression cassette was excised and cloned into the BamHI/ApaI sites of pBluescript SKII. Next, the GFP sequence in the vector was replaced with a sequence encoding luciferase obtained from the pGL4.20 vector (Promega, Fitchburg, WI). A KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan) was used to mutate the consensus binding sites for C/EBP β in the promoter and FIRE regions. The mutations in the promoter changed the sequence from ATATTTCCAAA to GGTACCATAAAA, and those in the FIRE changed the sequence from TTCCCTAAGA to TTCCCTTTGA. A mouse Cebpb expression vector was generated by cloning polymerase chain reaction (PCR)-amplified Cebpb complementary DNA (cDNA) obtained from mouse bone marrow (BM) cells into the pCDNA3.1 vector. The entire PCR-amplified region in the vector was verified by sequencing. pBABEPuro and pBABEPuroC/EBP β ER, which express estrogen receptor (ER) or C/EBP β fused to ER, respectively, were described previously.³²

Flow cytometric analysis and cell sorting

PB samples were obtained from the tail vein or caudal vena cava, and blood cell counts were analyzed using an SE-9000 instrument (Sysmex, Kobe, Japan). BM cells and PB cells were stained with fluorescently conjugated antibodies and analyzed using a FACSCantoII, FACSARIA, or FACSARIA II instrument (BD Biosciences, San Jose, CA). Antibodies used to detect monocytes were fluorescein isothiocyanate (FITC)-conjugated anti-Ly6C (AL-21), allophycocyanin (APC)-conjugated anti-CD115 (AFS98), phycoerythrin (PE)-Cy7-conjugated anti-CD11b (M1/70), PE-conjugated anti-Ly6G (1A8), PE-conjugated anti-NK1.1 (PK136), PE-conjugated anti-CD4 (GK1.5), PE-conjugated anti-CD8 (53-6.7), and PE-conjugated anti-B220 (RA3-6B2). The following reagents were used to detect MDPs: APC-conjugated anti-CD115, PE-conjugated anti-CD135 (A2F10.1), and PE-Cy7-conjugated anti-c-kit (ACK2) antibodies; PerCP-Cy5.5-conjugated antibodies specific for lineage markers (CD11b [M1/70], CD3 [17A2], Ter119 [TER-119], and CD19 [eBio1D3]); biotin-conjugated antibodies specific for lineage markers (interleukin-7-R α [B12-1], CD11c [HL3], NK1.1 [PK136], and Gr-1 [RB6-8C5]); and streptavidin-PerCP-Cy5.5. MDPs were regarded as CD135⁺ CD115⁺ c-kit⁺ lineage marker-negative BM cells. For experiments utilizing Mxl-Cre Cebpb^{fl/fl} mice, APC-conjugated anti-CD115, PE-conjugated anti-c-kit (2B8), V450-conjugated anti-Ly6C (AL-21), and biotin-conjugated anti-CD135 (A2F10) antibodies; PerCP-Cy5.5-conjugated antibodies specific for lineage markers (CD3, CD19, NK1.1 [PK136], and Ly6G [1A8]); and streptavidin-PE-Cy7 were used to identify MDPs and monocytes. In this case, monocytes were regarded as CD115⁺ c-kit⁻ CD135⁻ lineage marker-negative BM cells. For HSCs and myeloid progenitors, APC-conjugated anti-c-kit (2B8), PE-Cy7-conjugated anti-Sca-1 (D7), PE-conjugated anti-CD16/32 (93), and FITC-conjugated anti-CD34 (RAM34) antibodies and PerCP-Cy5.5-conjugated antibodies specific for lineage markers, including CD11b, CD4 (RM4-5), CD8 (53-6.7), CD19, B220 (RA3-6B2), and Ter119, were used. V450-conjugated anti-CD45.1 (A20) and APC-Cy7-conjugated anti-CD45.2 (104) antibodies were used to distinguish donor type cells from recipient type cells for the experiments involving BM transplantation. To exclude dead cells, samples were stained with propidium iodide unless otherwise specified. All antibodies were purchased from BD Biosciences, eBioscience (San Diego, CA), or BioLegend (San Diego, CA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Quantitative reverse transcription PCR

Total RNA was extracted using an RNeasy Micro Kit (Qiagen, Valencia, CA) and converted to cDNA using random primers. The cDNA was amplified using a Step One Plus thermal cycler (Applied Biosystems, Waltham, MA). The following parameters were used: 95°C for 20 s, followed by 45 to 50 cycles at 95°C for 1 s and 60°C for 20 s. Primers and probes from the Universal Probe Library (Roche Applied Science, Mannheim, Germany) were used. The sequences of the primers and probes are listed in supplemental Table 1. Results were normalized against the level of Gapdh messenger RNA (mRNA).

Giemsa staining

Smears of mouse PB and cytospin slides of sorted cells were stained using a Diff-Quik Kit (Sysmex), which is a modified Wright Giemsa staining system. Images were obtained using an Olympus BX43 microscope (Olympus, Tokyo, Japan) at original magnification $\times 400$.

BM transplantation

To evaluate the cell-intrinsic requirement for C/EBP β , 5×10^5 BM cells from wild-type (WT; Cebpb^{+/+}), Cebpb^{-/-} (CD45.2⁺), or Mxl-Cre Cebpb^{fl/fl} mice (CD45.2⁺) were transplanted into lethally irradiated (8 Gy) CD45.1⁺ recipient mice. Mice were analyzed 6 to 8 weeks after BM transplantation.

BrdU pulsing

For BrdU pulsing, mice were intraperitoneally injected with 2 mg BrdU (BD Biosciences) 3 times 3 hours apart, as previously described.¹⁹ BM or PB cells were harvested and stained with fluorescently conjugated antibodies. The antibodies used to label monocytes were FITC-conjugated anti-Ly6C, APC-conjugated anti-CD115, PerCP-Cy5.5-conjugated anti-CD11b, PE-conjugated anti-Ly6G, PE-conjugated anti-NK1.1, PE-conjugated anti-CD4, PE-conjugated anti-CD8, and PE-conjugated anti-B220. APC-conjugated anti-CD115, PE-conjugated anti-CD135, and FITC-conjugated anti-c-kit (2B8) antibodies, PerCP-Cy5.5-conjugated antibodies specific for lineage markers (CD11b, CD3, Ter119, and CD19), and biotin-conjugated antibodies specific for lineage markers (IL7-R α , CD11c, NK1.1, and Gr-1) were used to label MDPs. Biotinylated antibodies were labeled by subsequent staining with streptavidin-PerCP-Cy5.5. Labeled BM cells and PB cells were fixed, permeabilized, and treated with DNase to expose incorporated BrdU using a BrdU Flow Kit (BD Biosciences). Cells were then stained with a V450-labeled anti-BrdU (3D4) antibody and analyzed using a FACSCantoII instrument.

Annexin V staining

Annexin V and 7-amino-actinomycin D (7-AAD; BD Biosciences) were used to identify apoptotic and dead cell populations by flow cytometry. Following staining of surface markers, BM and PB cells were stained with V450-Annexin V (BD Biosciences) and 7-AAD according to the manufacturers' protocols. The antibodies used to stain surface markers were FITC-conjugated anti-Ly6C and APC-conjugated anti-CD115. Forward scatter^{low-int} side scatter^{low} CD115⁺ cells were defined as monocytes in this analysis.

Reporter assay

Cos-7 cells (1×10^4) were seeded in 24-well plates 24 hours before transfection. Cells were transfected with 950 ng of Csf1r reporter vectors and 0, 1, 5, or 10 ng mouse Cebpb expression vector together with 2 ng pRL-null vector (Promega) using FuGENE6 reagent (Promega). Luciferase assays were performed 24 hours after transfection using the dual luciferase reporter assay system (Promega). Firefly luciferase activity was normalized against renilla activity to control for variation in the transfection efficiency.

Chromatin immunoprecipitation (ChIP) PCR assay

EML cells were retrovirally transduced with the pBABEPuro or pBABEPuroC/EBP β ER vector, and transduced cells were selected using puromycin.³³ Once established, nuclear translocation of ER or C/EBP β -ER in the transduced cells was induced by addition of 1 μ M 4-hydroxytamoxifen to the medium. These

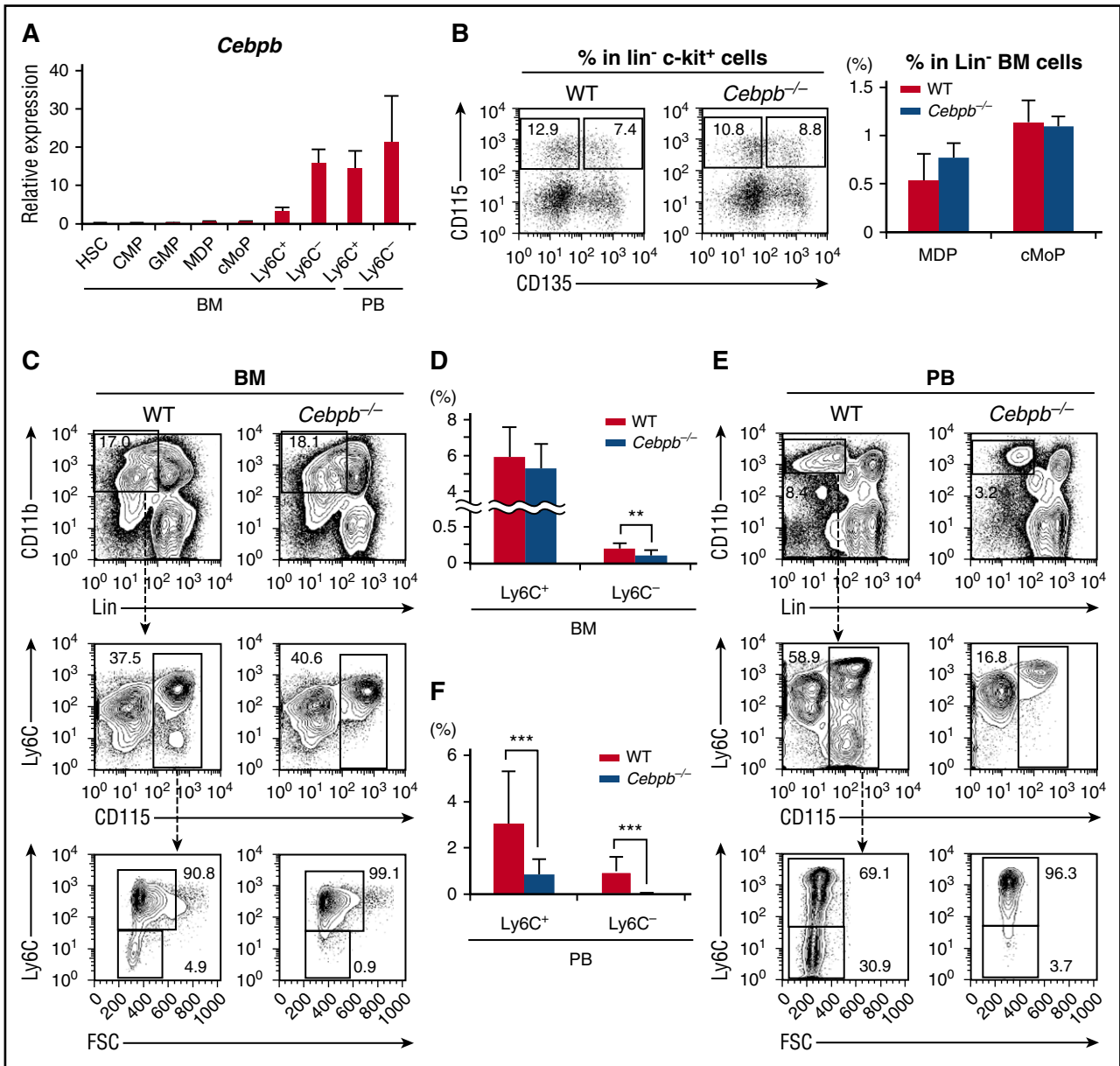


Figure 1. *Cebpb*^{-/-} mice lack Ly6C⁻ monocytes. (A) *Cebpb* mRNA expression in purified hematopoietic populations (n = 3). (B) Expression of CD115 and CD135 in BM cells among c-kit⁺ lineage marker (CD11b, Gr-1, CD11c, Ter119, IL-7R, CD3, CD19, and NK1.1)⁻ cells. The frequencies of MDPs (CD115⁺ CD135⁻) and cMoPs (CD115⁺ CD135⁺) among lineage⁻ BM cells are shown in the right panel (n = 5). Data were pooled from 4 independent experiments. (C,E) Gating strategy to define Ly6C⁺ and Ly6C⁻ monocytes in the BM (C) and PB (E). Lineage marker (Ly6G, B220, CD4, CD8, and NK1.1)⁻ CD11b⁺ CD115⁺ monocytes were subdivided on the basis of Ly6C expression. (D,F) Frequencies of Ly6C⁺ and Ly6C⁻ monocytes in the BM (D) and PB (F) of WT or *Cebpb*^{-/-} mice (n = 10 for BM and n = 19 for PB). Data were pooled from 7 independent experiments and are presented as the mean ± standard deviation (SD). **P < .01, ***P < .001. Numbers indicate the percentage of cells in the adjacent boxed area. FSC, forward scatter; Lin, lineage.

cells were subjected to ChIP-PCR as previously described.³⁴ In brief, cells were fixed with 1% formaldehyde for 5 min at room temperature. Soluble chromatin-containing DNA 200 to 500 bp in length was immunoprecipitated using 2 μg anti-C/EBPβ antibody (sc-150X; Santa Cruz Biotechnology, Dallas, TX) or 2 μg normal rabbit immunoglobulin G (sc2027X; Santa Cruz) overnight at 4°C. Purified ChIP and input DNA was measured by real-time quantitative PCR. The levels of ChIP DNA were normalized to those of input DNA. In each experiment, samples were analyzed in duplicate. The following oligonucleotide primers were used for amplification: *Csf1r* promoter, 5'-TTGAGCCTGGCCCCAGAT-3' (forward), 5'-GGTTGCCTGAAAGGGAACACTAC-3' (reverse); FIRE, 5'-GCCCTCAGAGGCTGTGAATC-3' (forward), 5'-CTCAAACCCCTGTCCAGGTC-3' (reverse); negative region-1, 5'-GCTGGGAAGTAGGACTCGTG-3' (forward), 5'-GCCCTGATCCCTCCATGTTC-3' (reverse); negative region-2, 5'-GCTAGCGGACTCTCTAGTG-3' (forward), 5'-GTCCCATCATTCCTCCACCC-3' (reverse).

Statistical analysis

Statistical differences were determined using the Student *t* test. Values of *P* < .05 were considered statistically significant.

Results

Requirement of C/EBPβ for development of monocyte subsets

To determine the precise developmental stages at which monopoiesis is impaired in *Cebpb*^{-/-} mice, we first measured *Cebpb* mRNA in purified cellular intermediates involved in the differentiation of HSCs into mature monocytes isolated from WT mice (Figure 1A). *Cebpb*

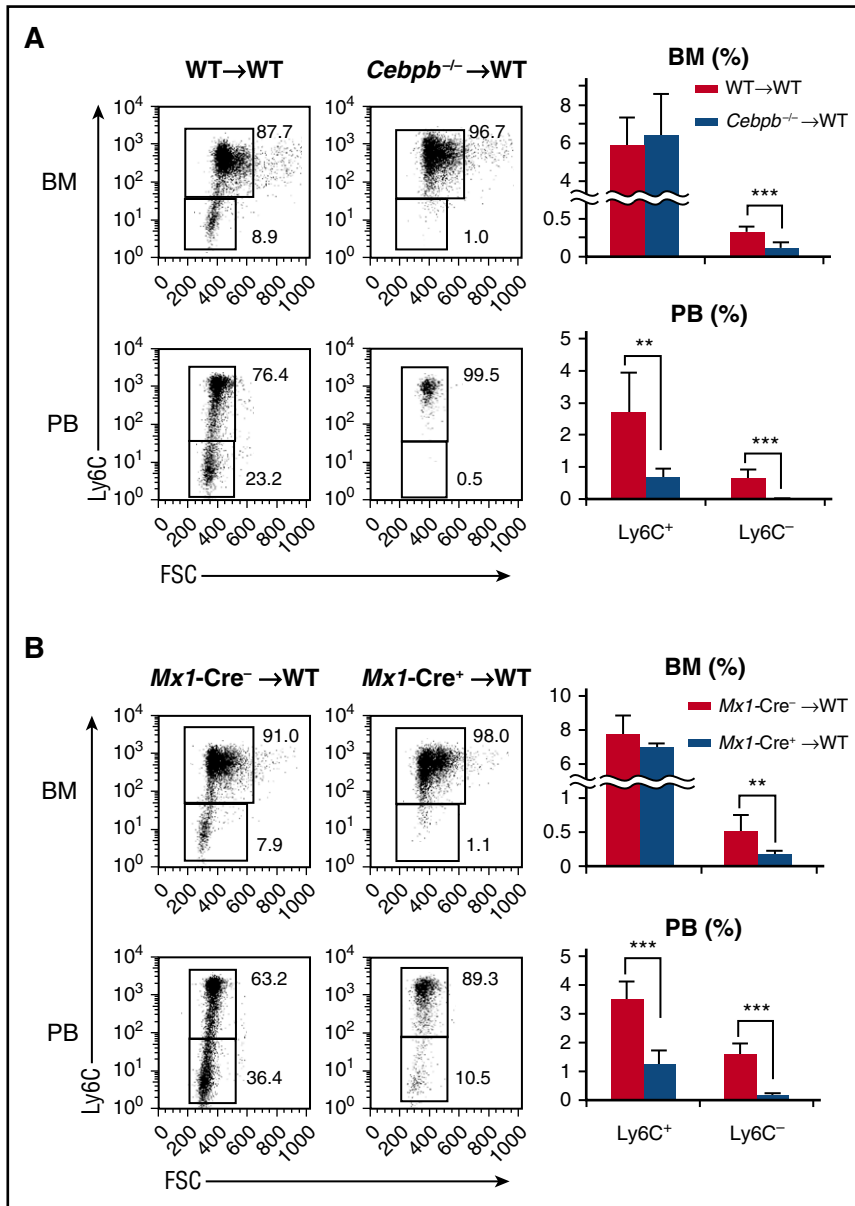


Figure 2. Cell-intrinsic requirement for C/EBP β in monoipoiesis. (A) Lethally irradiated CD45.1⁺ mice were reconstituted with BM cells obtained from CD45.2⁺ WT or *Cebpb*^{-/-} mice. Flow cytometric analyses of monocyte subsets among CD45.2⁺ lineage⁻ CD11b⁺ CD115⁺ cells in the recipients are shown in the left panel, and the frequencies of the indicated population are shown in the right panel (n = 7 for WT donors and n = 6 for *Cebpb*^{-/-} donors). (B) BM cells of *Mx1-Cre*⁺ *Cebpb*^{fl/fl} or *Mx1-Cre*⁻ *Cebpb*^{fl/fl} mice (CD45.2) were transplanted into lethally irradiated mice (CD45.1). Flow cytometric analysis of monocyte subsets among CD45.2⁺ lineage⁻ CD11b⁺ CD115⁺ cells in recipient mice is shown in the left panel. The frequencies of monocyte subsets in the BM and PB are shown in the right panel (n = 8). Data are presented as the mean \pm SD. ***P* < .01, ****P* < .001. Numbers indicate the percentage of cells in the adjacent boxed area. FSC, forward scatter.

mRNA expression remained relatively low during differentiation from HSCs to cMoPs, was induced in Ly6C⁺ monocytes, and was further upregulated in Ly6C⁻ monocytes in the BM. In PB, *Cebpb* mRNA levels remained high in both Ly6C⁺ and Ly6C⁻ monocytes.

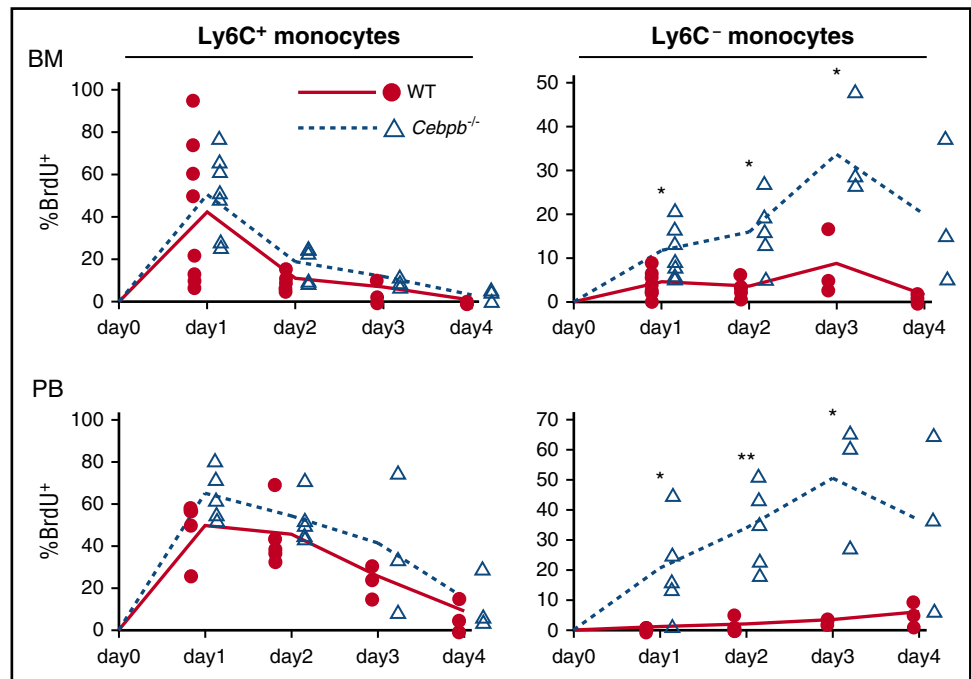
Next, BM and PB were closely compared between *Cebpb*^{-/-} and WT mice (Figure 1B-F; supplemental Figure 1). The frequencies of MDPs and cMoPs (Figure 1B), as well as HSCs, common myeloid progenitors, and granulocyte-macrophage progenitors, in *Cebpb*^{-/-} mice were comparable to those in WT mice (supplemental Figure 1A). Consistent with our previous report,²⁹ *Cebpb*^{-/-} and WT mice had a comparable number of total monocytes in the BM, whereas the frequency of monocytes in the PB was decreased in *Cebpb*^{-/-} mice (Figure 1C,E, middle panels). We analyzed Ly6C⁺ and Ly6C⁻ subsets of monocytes (Figure 1C-F). While there was no difference in the frequency of BM Ly6C⁺ monocytes, the frequency of Ly6C⁻ monocytes in the BM was significantly lower in *Cebpb*^{-/-} mice than in WT mice (0.22% \pm 0.071% in WT mice vs 0.12% \pm 0.073% in *Cebpb*^{-/-} mice, *P* < .01; Figure 1C-D). The difference was more evident in

the PB (Figure 1E-F). Subdivision of monocytes revealed a significant decrease in Ly6C⁺ monocytes and the almost complete absence of Ly6C⁻ monocytes in the PB of *Cebpb*^{-/-} mice (0.86% \pm 0.67% in WT mice vs 0.029% \pm 0.030% in *Cebpb*^{-/-} mice, *P* < .001; Figure 1E-F). The difference was observed not only in frequencies but also in the absolute number of cells (supplemental Figure 1B). Similar differences were observed in the levels of monocytes in the spleen of *Cebpb*^{-/-} mice (supplemental Figure 1C-E). These results clearly suggest that C/EBP β is essential for the development of monocytes, especially Ly6C⁻ monocytes.

Cell-autonomous and monocyte-specific requirement for C/EBP β in monoipoiesis

Next, we investigated whether the requirement for C/EBP β in monoipoiesis is cell autonomous. Lethally irradiated CD45.1⁺ mice were reconstituted with BM cells from either CD45.2⁺ WT or *Cebpb*^{-/-} mice (Figure 2A). Six weeks after reconstitution, the

Figure 3. Turnover of monocytes is accelerated in *Cebpb* $^{-/-}$ mice. Time-course analysis of the incorporation of BrdU into Ly6C $^+$ and Ly6C $^-$ monocytes in the BM and PB after BrdU pulsing (n = 3-8 mice per group). The percentages of BrdU $^+$ cells among the indicated monocyte subsets are shown. * $P < .05$, ** $P < .01$.



frequencies of monocyte subsets among CD45.2 $^+$ donor-derived BM cells were basically identical to those in the original donor mice, regardless of the donor cell genotype. In brief, when *Cebpb* $^{-/-}$ cells were transplanted, the frequencies of PB Ly6C $^+$ monocytes and BM Ly6C $^-$ monocytes were decreased, and Ly6C $^-$ monocytes in the PB almost disappeared among CD45.2 $^+$ cells (Figure 2A). Even when competitive BM transplantation was performed (supplemental Figure 2), the contribution of CD45.2 $^+$ *Cebpb* $^{-/-}$ cells in PB Ly6C $^+$ cells, BM and PB Ly6C $^-$ cells was significantly lower than the contribution of CD45.2 $^+$ WT cells, confirming the cell-intrinsic requirement of C/EBP β for proper monopoiesis.

A recent publication demonstrated that a conditional knockout system utilizing the *Mx1* promoter targets Ly6C $^+$ monocytes without inoculation of polyI:C, because *Mx1* is constitutively expressed in Ly6C $^+$ and Ly6C $^-$ monocytes³⁵ (supplemental Figure 3A). *Mx1*-Cre-mediated recombination is observed in ~40% of Ly6C $^+$ monocytes and 90% of Ly6C $^-$ monocytes.³⁵ Even if the developmental hierarchy between Ly6C $^+$ monocytes and Ly6C $^-$ monocytes is unclear, conditional targeting of *Cebpb* using the *Mx1* promoter is ideal to evaluate the monocyte-specific requirement for C/EBP β . Indeed, upregulation of *Cebpb* during monopoiesis was severely impaired in *Mx1*-Cre $^+$ *Cebpb* $^{fl/fl}$ mice (supplemental Figure 3B). We first analyzed non-manipulated *Mx1*-Cre $^+$ *Cebpb* $^{fl/fl}$ mice and found that their phenotypes were identical to those of *Cebpb* $^{-/-}$ mice (supplemental Figure 3C). To exclude the possibility of unexpected *Mx1* expression in nonhematopoietic tissue, BM cells of *Mx1*-Cre $^+$ *Cebpb* $^{fl/fl}$ mice (CD45.2 $^+$) were transplanted into lethally irradiated CD45.1 $^+$ mice. The frequency of Ly6C $^-$ monocytes was significantly lower in the recipients than in mice that received *Mx1*-Cre $^+$ *Cebpb* $^{fl/fl}$ BM cells (Figure 2B). These results strongly suggest that C/EBP β is specifically required after commitment to monocytes.

Extremely rapid turnover of Ly6C $^-$ monocytes in *Cebpb* $^{-/-}$ mice

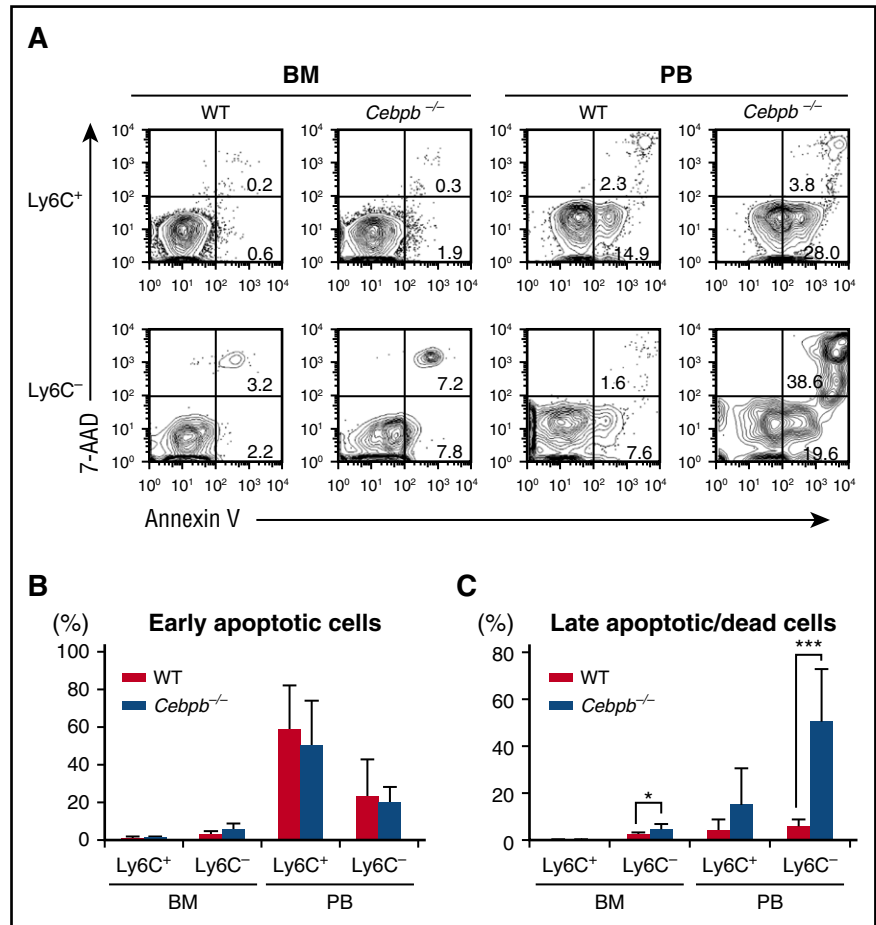
The absence of Ly6C $^-$ monocytes in *Cebpb* $^{-/-}$ mice was not accompanied by an increase in any of the upstream progenitors examined (Figure 1B; supplemental Figure 1A). Therefore, we hypothesized that the defects were due to impaired proliferation or

enhanced cell loss rather than a block in differentiation. The cell cycle status of MDPs, Ly6C $^+$ monocytes, and Ly6C $^-$ monocytes in the BM was assessed by the incorporation of BrdU or propidium iodide staining (supplemental Figure 4). The proportion of cycling cells among Ly6C $^-$ monocytes was slightly higher in *Cebpb* $^{-/-}$ mice but much lower than the proportion among Ly6C $^+$ monocytes. These changes suggest that a feedback mechanism compensates for the loss of Ly6C $^-$ monocytes in *Cebpb* $^{-/-}$ mice but could not compensate for the loss. Next, turnover of monocyte subsets was evaluated by performing *in vivo* BrdU pulsing experiments (Figure 3; supplemental Figure 5).¹⁹ In WT mice (Figure 3, filled lines), BrdU $^+$ Ly6C $^+$ monocytes appeared in the BM and PB on day 1 and then gradually lost this labeling (Figure 3, left). In accordance with a previous report,¹⁹ WT Ly6C $^-$ monocytes slowly became BrdU $^+$ in the BM and PB, probably reflecting the developmental hierarchy between Ly6C $^+$ and Ly6C $^-$ monocytes (Figure 3, right, filled lines). In sharp contrast, in *Cebpb* $^{-/-}$ mice, much higher frequencies of Ly6C $^-$ monocytes in the BM and PB became BrdU $^+$ (dotted lines in Figure 3, right), while the frequency of BrdU $^+$ Ly6C $^+$ monocytes did not significantly differ between WT and *Cebpb* $^{-/-}$ mice. These results indicated that the turnover of Ly6C $^-$ monocytes was rapid in *Cebpb* $^{-/-}$ mice.

Apoptosis of Ly6C $^-$ monocytes is accelerated in *Cebpb* $^{-/-}$ mice

We assessed the death of mature monocytes in the BM and PB by staining with Annexin V and 7-AAD (Figure 4). Among Ly6C $^+$ monocytes, the frequencies of early apoptotic cells (Annexin V $^+$ 7-AAD $^-$) and late apoptotic or dead cells (7-AAD $^+$ Annexin V $^+$) were equivalent in WT and *Cebpb* $^{-/-}$ mice (Figure 4A-C). By contrast, the frequency of late apoptotic or dead Ly6C $^-$ monocytes was significantly higher in *Cebpb* $^{-/-}$ mice than in WT mice in the BM, and this difference was even more pronounced in the PB (5.84% \pm 2.90% in WT mice vs 50.4% \pm 22.4% in *Cebpb* $^{-/-}$ mice, $P < .001$; Figure 4A,C). In addition, the frequency of Ly6C $^-$ monocytes with a depolarized mitochondrial membrane in the PB was higher in *Cebpb* $^{-/-}$ mice than in WT mice (supplemental Figure 6A-B), suggesting that apoptosis of Ly6C $^-$ monocytes was accelerated in *Cebpb* $^{-/-}$ mice.

Figure 4. Death of Ly6C⁻ monocytes is accelerated in *Cebpb*^{-/-} mice. (A) Flow cytometric analysis of Ly6C⁺ and Ly6C⁻ monocytes in the BM and PB stained with Annexin V and 7-AAD. (B-C) Frequencies of early apoptotic cells (Annexin V⁺ and 7-AAD⁻) and late apoptotic/dead cells (Annexin V⁺ and 7-AAD⁺) among the Ly6C⁺ and Ly6C⁻ monocyte populations (n = 7). Data are presented as mean ± SD. **P* < .05, ****P* < .001.



Upregulation of *Csf1r* in monocytes is impaired in *Cebpb*^{-/-} mice

Macrophage colony-stimulating factor (M-CSF) is a critical regulator of the proliferation, differentiation, and survival of monocytes and macrophages.³⁶⁻³⁸ We investigated the involvement of *Csf1r* in altered monopoiesis in *Cebpb*^{-/-} mice. During the process of monopoiesis in WT mice, *Csf1r* mRNA expression was highly induced after differentiation into Ly6C⁻ monocytes in the BM (Figure 5A). Interestingly, this expression pattern resembled that of *Cebpb* mRNA shown in Figure 1. When *Cebpb*^{-/-} BM cells were analyzed, upregulation of *Csf1r* mRNA in Ly6C⁻ monocytes was significantly impaired (Figure 5B). In the PB of WT mice, *Csf1r* was highly expressed in both Ly6C⁺ and Ly6C⁻ monocytes (Figure 5A), and this expression was significantly decreased in *Cebpb*^{-/-} mice (Figure 5C). Accordingly, surface expression of CD115 (*Csf1r*) on Ly6C⁻ monocytes in the PB was reduced in *Cebpb*^{-/-} mice (Figure 5D). These data suggest that *Csf1r* is a target of C/EBP β during the terminal stage of monopoiesis.

Csf1r is a downstream target of C/EBP β

Previous reports revealed a minimum *cis*-regulatory element of *Csf1r*, a combination of a 0.5-kb promoter region and a 1.8-kb FIRE located in the second intron^{31,39} (Figure 6A). There are at least 2 consensus binding sites for C/EBPs within the regulatory elements (one in the promoter and the other in the FIRE region). We hypothesized that C/EBP β binds to these sites, activates transcription and promotes survival of Ly6C⁻ monocytes. When a construct harboring the luciferase gene flanked by the *Csf1r* promoter and FIRE was subjected

to the reporter assay using Cos-7 cells, C/EBP β enhanced luciferase activity in a dose-dependent manner (Figure 6B). This enhancement was substantially weakened by mutation of either of the binding sites and completely abrogated by mutation of both binding sites, suggesting that these binding sites are functional in terms of activating transcription of *Csf1r* by C/EBP β .

To investigate whether C/EBP β can upregulate *Csf1r*, EML cells (a mouse HSC line) were engineered to express the C/EBP β -ER fusion protein or ER alone. Nuclear translocation of C/EBP β -ER induced by addition of tamoxifen resulted in significantly higher levels of *Csf1r* mRNA and protein, while ER alone had no effect on *Csf1r* expression (Figure 6C-D). Using these cell lines, we performed ChIP-PCR to confirm the binding of C/EBP β to the consensus binding sites in the regulatory elements mentioned above. C/EBP β was enriched at the promoter and FIRE regions by almost twofold (Figure 6E). Binding of C/EBP β to these regions was further confirmed by ChIP-sequencing analysis using monocyte-derived cells⁴⁰ (Figure 6F). Collectively, these data suggest that C/EBP β positively regulates *Csf1r* expression, at least in part through direct binding to these regulatory elements.

Discussion

In this study, we revealed a novel role of C/EBP β in monopoiesis. *Cebpb* was highly upregulated in mature monocytes, and *Cebpb*^{-/-} mice had a severely reduced number of Ly6C⁻ monocytes (Figure 1). BM chimera experiments in which *Cebpb*^{-/-} cells

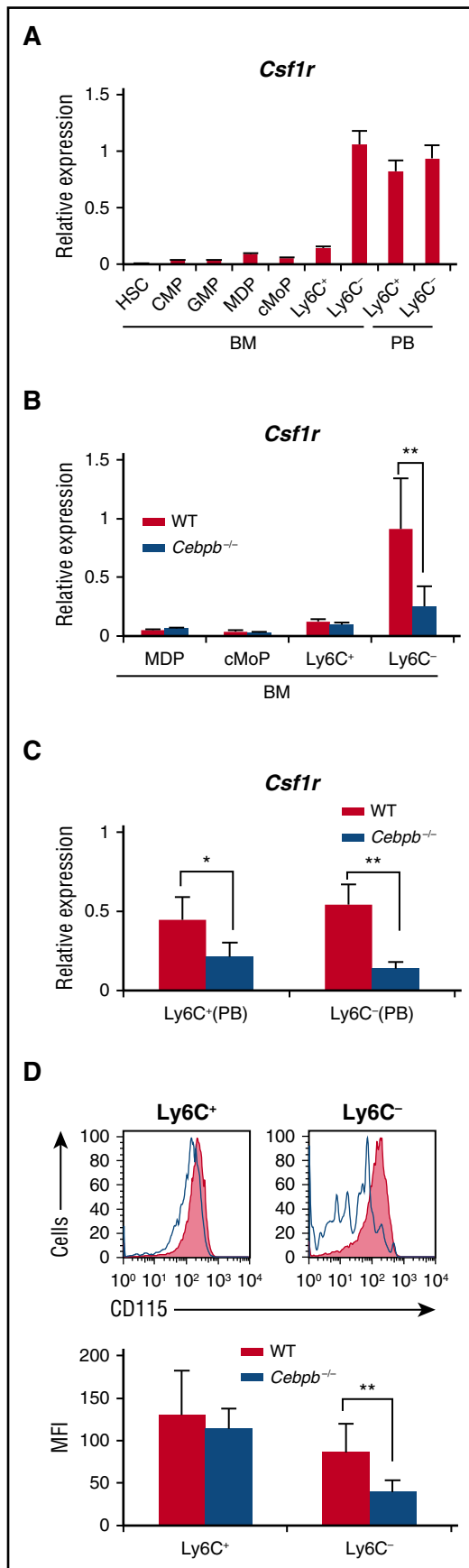


Figure 5.

were transplanted into WT mice showed similar defects in Ly6C⁻ monocytes (Figure 2A), demonstrating that C/EBPβ is a cell-intrinsic factor required for Ly6C⁻ monocyte development. In addition, *Mxl*-Cre-mediated targeting of *Cebpb* resulted in a decrease in the frequency of Ly6C⁻ monocytes (Figure 2B). These findings clearly demonstrated that C/EBPβ is required by mature monocytes, in which expression of this transcription factor is strongly induced.

Our results clearly showed that death of Ly6C⁻ monocytes was accelerated and that these cells were rapidly turned over in *Cebpb*^{-/-} mice, clearly suggesting that C/EBPβ is required for the survival of Ly6C⁻ monocytes. To identify a factor required for the survival of Ly6C⁻ monocytes, we focused on *Csf1r*, which encodes the receptor for M-CSF. We found that *Csf1r* is a critical downstream target of C/EBPβ, while the serum level of Csf1 was not reduced in *Cebpb*^{-/-} mice (supplemental Figure 7). Accordingly, recent reports show that administration of an antibody against Csf1r depletes PB Ly6C⁻ monocytes, but not Ly6C⁺ monocytes, in vivo, suggesting the higher dependency of Ly6C⁻ monocyte survival on M-CSF receptor signaling.⁴¹⁻⁴³

Cell surface expression of Csf1r (CD115) was first observed at the MDP stage, even in the absence of C/EBPβ (Figure 1B). Interestingly, surface expression of Csf1r was maintained at a similar level during differentiation into monocytes, while the mRNA level of *Csf1r* was highly upregulated in mature monocytes, in which *Cebpb* mRNA was also highly expressed. Discrepancies between mRNA and protein expression of *Csf1r* might reflect the instability of surface Csf1r expression in mature monocytes.⁴⁴ Collectively, these results suggest that the initial induction of *Csf1r* mRNA in MDPs is regulated by other factors including C/EBPα and that the subsequent sharp increase in *Csf1r* mRNA in monocytes, which might be required for survival of Ly6C⁻ monocytes, is highly dependent on C/EBPβ.⁴⁵⁻⁴⁸ Further elucidation of the molecular mechanism regulating Csf1r and C/EBPβ is the next key step to understand the regulation of the monocyte/macrophage system.

Recently, 3 molecules have been reported to be involved in the homeostasis of Ly6C⁻ monocytes, namely, Nr4a1, S1PR5, and CX3CR1. Mice in which one of these molecules is knocked out have a reduced number of Ly6C⁻ monocytes.⁴⁹⁻⁵¹ Interestingly, expression of all these molecules was highly induced during monocyte differentiation and severely decreased in *Cebpb*^{-/-} monocytes (supplemental Figure 8). Of these molecules, S1PR5 functions in the egress of Ly6C⁻ monocytes from BM.⁵⁰ However, the egress of Ly6C⁻ monocytes from BM was not impaired in *Cebpb*^{-/-} mice (supplemental Figure 9). By contrast, in Nr4a1- or CX3CR1-knockout mice, the decreased level of Ly6C⁻ monocytes has been suggested to be due to the accelerated death of these cells.^{49,51} This suggests that C/EBPβ can control the survival of Ly6C⁻ monocytes through the regulation of Nr4a1 and/or CX3CR1. Indeed, a recent study showed that C/EBPβ binds to the promoter region of *Nr4a1* and controls the expression of this gene.⁵²

In this study, we focused on Ly6C⁻ monocytes. However, we noticed that the number of PB Ly6C⁺ monocytes was also significantly

Figure 5. Upregulation of Csf1r in monocyte subsets is impaired in *Cebpb*^{-/-} mice. (A) *Csf1r* mRNA expression in purified hematopoietic populations (n = 3). (B) *Csf1r* mRNA expression in purified hematopoietic populations obtained from BM of WT or *Cebpb*^{-/-} mice (n = 3). (C) *Csf1r* mRNA expression in purified monocyte subsets obtained from the PB of WT or *Cebpb*^{-/-} mice (n = 3). (D) Flow cytometric analysis of CD115 (Csf1r) expression on Ly6C⁺ (left) and Ly6C⁻ (right) monocytes in the PB of WT (filled lines, n = 3) and *Cebpb*^{-/-} (unfilled lines, n = 3) mice. Data are presented as mean ± SD. **P < .01, ***P < .001. Numbers indicate the percentage of cells in the adjacent boxed area. MFI, mean fluorescence intensity.

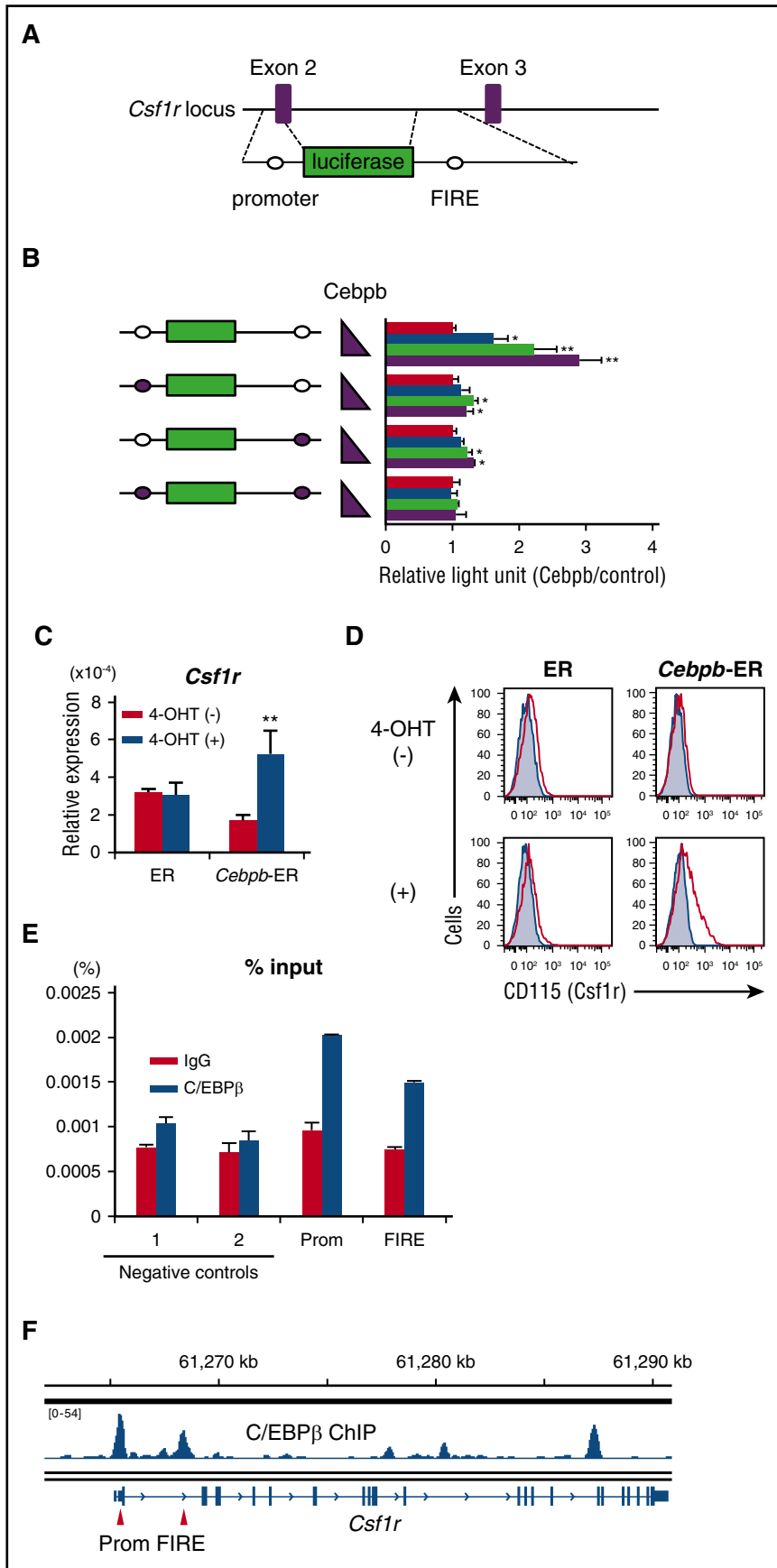


Figure 6. *Csf1r* is a downstream target of C/EBP β .

(A) Schematic illustration of the *Csf1r* reporter vector containing the promoter region and FIRE. Open circles indicate the consensus binding sites for C/EBP β . (B) Activity of the luciferase reporter gene under the control of a combination of the promoter and FIRE of the *Csf1r* gene in response to different amounts of the C/EBP β expression vector (0, 1, 5, and 10 ng) (n = 4). Open circles indicate the consensus binding sites for C/EBP β , and closed circles indicate sites that were mutated to disrupt the binding of C/EBP β . Data are representative of 2 independent experiments. (C-D) mRNA (C) and surface (D) expression of *Csf1r* (CD115) in EML cells that were engineered to express ER alone or ER fused to C/EBP β . 4-OHT, 4-hydroxytamoxifen. Shaded histograms indicate isotype controls. Data are representative of 3 independent experiments. (E) ChIP-PCR to analyze the direct binding of C/EBP β to the promoter and FIRE region in the *Csf1r* locus (n = 2). EML cells expressing C/EBP β -ER were used for this analysis. Data are representative of 2 independent experiments and are presented as mean \pm SD. (F) ChIP analysis showing binding of C/EBP β to the mouse *Csf1r* locus in monocyte-derived cells. Data were taken from a study by Bornstein et al.⁴⁰ Red arrows indicate promoter (Prom) and FIRE regions. IgG, immunoglobulin G.

reduced in *Cebpb*^{-/-} mice (Figures 1 and 2), while turnover and apoptosis of Ly6C⁺ monocytes were not altered. One plausible explanation for this is that C/EBP β also regulates the survival of Ly6C⁺ monocytes in the PB and that the extremely rapid turnover of Ly6C⁺ monocytes in the PB and their constant release from the BM allow for their detection in the PB of *Cebpb*^{-/-} mice. mRNA expression of *Csf1r* was decreased in *Cebpb*^{-/-} PB Ly6C⁺ monocytes (Figure 5C). This could be due to a decreased level of *Csf1r* in PB Ly6C⁺ monocytes or detection of BM Ly6C⁺ monocytes, which expressed *Csf1r* at low level (Figure 5A). In any case, regulation of *Csf1r* by C/EBP β may be critical for the survival of monocyte subsets, including PB Ly6C⁺ and BM and PB Ly6C⁻ cells, all of which expressed *Cebpb* at an extremely high level (Figure 1A). Further studies are required to elucidate the significance of C/EBP β in PB Ly6C⁺ monocytes. In addition, C/EBP β is required for differentiation of a newly identified Ly6C⁻ monocyte subset called segregated nucleus-containing atypical monocytes (SatMs).⁵³ Because there are many similarities between Ly6C⁻ non-classical monocytes and SatMs, it will be of interest to elucidate the molecular mechanisms by which C/EBP β induces differentiation of SatMs.

In summary, we showed that C/EBP β is involved in the survival of Ly6C⁻ monocytes, in part via *Csf1r* regulation. These results will facilitate understanding of the mechanisms that regulate monocyte/macrophage homeostasis and uncover the pathophysiologies of various diseases in which monocytes are involved.

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Authorship

Contribution: A.T. acquired and analyzed data and drafted the manuscript; H.H. conceived and designed the study, acquired, analyzed, and interpreted data, drafted the manuscript, and obtained funding; A.Y. acquired, analyzed, and interpreted data and drafted the manuscript; N.K., A.S., T.S., T.K., Y.T., and Y.M. acquired and analyzed data; and D.G.T. and T.M. supervised the study and interpreted data.

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Correspondence: Hideyo Hirai, Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, 54 Kawahara-cho, Shogo-in, Sakyo-ku, Kyoto 606-8507, Japan; e-mail: hhirai@kuhp.kyoto-u.ac.jp.

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