

**Accelerated apoptosis of peripheral blood monocytes
in *Cebpb*-deficient mice**

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Running title: C/EBP β protects peripheral blood monocytes from apoptosis

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Abstract

The CCAAT/enhancer-binding protein β (C/EBP β) transcription factor is required for granulopoiesis under stress conditions. However, little is known about its roles in steady state hematopoiesis. Here, we analyzed the peripheral blood and bone marrow of *Cebpb*^{-/-} mice at steady state by flow cytometry and unexpectedly found that the number of peripheral blood monocytes was severely reduced, while the number of bone marrow monocytes was maintained. The ability of *Cebpb*^{-/-} bone marrow cells to give rise to macrophages/monocytes *in vitro* was comparable to that of wild-type bone marrow cells. Apoptosis of monocytes was enhanced in the peripheral blood, but not in the bone marrow of *Cebpb*^{-/-} mice. These results indicate that C/EBP β is required for the survival of monocytes in peripheral blood.

Keywords

CCAAT/enhancer-binding protein β (C/EBP β), monocytes, apoptosis

Abbreviations

7-AAD, 7-aminoactinomycin D; C/EBP β , CCAAT/enhancer-binding protein β ; DC, dendritic cell; LC, Langerhans cell; M-CSF, macrophage-colony stimulating factor; WT, wild type

1. Introduction

The leucine zipper basic region-containing transcription factor

CCAAT/enhancer-binding protein β (C/EBP β) is a member of the C/EBP family

[1,2,3,4]. C/EBP β regulates the proliferation, differentiation, survival, and metabolism

of many cell types through the activation or repression of target genes [5,6,7,8,9,10].

Within the hematopoietic system, C/EBP β is involved in regulation of the development

and function of macrophages [11,12,13,14]. In addition, we and others previously

reported that C/EBP β is required for granulopoiesis during emergencies such as

infection and cytokine stimulation, but is dispensable at steady state [15,16]. Under

stress conditions, C/EBP β is induced or activated and elicits its activities to facilitate

proliferation and differentiation of granulocyte precursors [15,16,17,18,19,20,21,22].

By contrast, the role of C/EBP β in steady state hematopoiesis is largely unknown.

Therefore, we carefully analyzed the peripheral blood and bone marrow of *Cebpb*^{-/-}

mice by flow cytometry and unexpectedly found that the number of peripheral blood

monocytes was much lower in *Cebpb*^{-/-} mice than in wild-type (WT) littermate controls.

We discuss the mechanisms by which C/EBP β is involved in the regulation of monocyte

homeostasis.

2. Materials and Methods

2.1. Mice

C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). CD45.1⁺ mice (a kind gift from Dr. Shigekazu Nagata, Osaka University, Japan) and *Cebpb*^{-/-} mice were bred and maintained under specific pathogen-free conditions in Kyoto University. Littermates were used as controls in all experiments involving *Cebpb*^{-/-} mice. The animal protocols were approved by the Committee on Animal Research of Kyoto University Faculty of Medicine.

2.2. Manual differential counts of peripheral blood

Smears of mouse peripheral blood were stained using a Diff-Quik Kit (Sysmex, Kobe, Japan), which is a modified Wright Giemsa staining system. Images were obtained using an Olympus BX43 microscope (Olympus, Tokyo, Japan) at 100× magnification. For differential analysis of peripheral blood smears, 100 cells were counted per sample.

2.3. *In vitro* differentiation of bone marrow cells into monocytes/macrophages

In vitro differentiation of mouse bone marrow cells was carried out as previously described [23]. In brief, freshly isolated bone marrow cells were suspended in

RPMI1640 medium supplemented with 10% fetal calf serum and 20 ng/ml recombinant mouse macrophage-colony stimulating factor (M-CSF) (R&D Systems, Minneapolis, MN, USA). Cells were cultured in a well of 6-well ultra-low attachment surface plates (Corning Costar; Schiphol-Rijk, Netherlands) at a density of 1×10^6 cells/ml for 3 days. Non-adherent cells were collected by gentle pipetting, and adherent cells were collected by cell scraping for subsequent flow cytometric analysis.

2.4. Flow cytometry

Peripheral blood and bone marrow samples were treated with Pharm Lyse reagent (BD Biosciences, San Jose, CA, USA) to lyse red blood cells. Cells were stained with fluorescent marker-conjugated antibodies and analyzed using a FACSCantoII instrument (BD Biosciences). The antibodies used were PE-Cy7-conjugated anti-CD11b (M1/70), APC-conjugated anti-CD115 (AFS98), APC-conjugated anti-CD3 (145-2C11), FITC-conjugated anti-B220 (RA3-6B2), PE-conjugated anti-Ly6G (1A8), PE-conjugated anti-NK1.1 (PK 136), APC-conjugated anti-CD11b (M1/70), PE-conjugated anti-CD11c (HL3), V450-conjugated anti-CD45.1 (A20), and APC-Cy7-conjugated anti-CD45.2 (104). To exclude dead cells, samples were stained with propidium iodide. All antibodies were purchased from BD Biosciences,

eBiosciences (San Diego, CA, USA), or Biolegend (San Diego, CA, USA). Data were obtained using BD FACSCanto II or FACS Aria II and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

2.5. Mouse bone marrow transplantation model

To evaluate the involvement of cell-extrinsic factors in the observed phenotypes, 5×10^5 bone marrow cells from WT or *Cebpb*^{-/-} mice (CD45.2⁺) were transplanted into lethally irradiated CD45.1⁺ WT mice. Mice were analyzed 6–10 weeks after reconstitution.

2.7. Annexin V staining

Bone marrow and peripheral blood cells were stained with V450-Annexin V (BD Biosciences) and 7-aminoactinomycin D (7-AAD) according to the manufacturer's protocols after staining with an APC-conjugated anti-CD115 antibody. Cells were then analyzed by flow cytometry.

2.8. Statistical analysis

Statistical significance was determined using the Student's t test. A *p* value less than 0.05 was considered statistically significant.

3. Results

3.1. Monocytes in peripheral blood are reduced in *Cebpb*^{-/-} mice

We first analyzed the complete blood cell count and differential counts of peripheral blood from WT and *Cebpb*^{-/-} mice. The total white blood cell count was significantly higher in *Cebpb*^{-/-} mice than in WT mice, while the numbers of red blood cells and platelets were comparable between WT and *Cebpb*^{-/-} mice. In analyses of manual differential counts, the frequencies of neutrophils, eosinophils, and lymphocytes were almost the same in WT and *Cebpb*^{-/-} mice (Table 1). Notably, both the frequency and absolute number of monocytes were significantly reduced in *Cebpb*^{-/-} mice (Table 1). Flow cytometric analysis of peripheral blood of these mice further corroborated these data. There was no difference in the frequencies of CD3⁺ T cells, B220⁺ B cells, NK1.1⁺ NK cells, or CD11b⁺ Ly6G⁺ neutrophils between WT and *Cebpb*^{-/-} mice (Fig. 1A). In addition, the frequencies of dendritic cells (DCs) in the spleen and Langerhans cells (LCs) in the epidermis did not differ between WT and *Cebpb*^{-/-} mice (Supplementary Fig. 1). By contrast, the frequency of CD115⁺ CD11b⁺ monocytes in peripheral blood was significantly lower in *Cebpb*^{-/-} mice than in WT mice ($4.24 \pm 2.71\%$ in WT mice vs. $0.72 \pm 0.50\%$ in *Cebpb*^{-/-} mice, $p < 0.001$; Fig. 1A). Peripheral blood monocytes

supposedly originate from bone marrow monocytes. Surprisingly, the frequency of CD115⁺ CD11b⁺ monocytes in bone marrow was comparable between *Cebpb*^{-/-} and WT mice (Fig. 1B), suggesting that production of monocytes in bone marrow was not impaired in *Cebpb*^{-/-} mice. Collectively, these results suggest that C/EBPβ is required for the homeostasis of monocytes.

3.2. C/EBPβ is cell-intrinsically required by peripheral blood monocytes

To determine whether the monocytopenia observed in *Cebpb*^{-/-} mice is due to defects in hematopoietic cells or the hematopoietic microenvironment, we transplanted CD45.2⁺ WT or *Cebpb*^{-/-} bone marrow cells into lethally irradiated CD45.1⁺ WT mice. Six to ten weeks after bone marrow transplantation, peripheral blood and bone marrow of recipient mice were analyzed using flow cytometry. Within CD45.2⁺ donor-derived cells, the frequency of peripheral blood monocytes was significantly reduced when *Cebpb*^{-/-} mice were used as the donor (Fig. 2A). By contrast, the frequency of monocytes within CD45.2⁺ cells in the bone marrow of recipient mice did not vary, irrespective of the donor genotype (Fig. 2B). Unfortunately, *Cebpb*^{-/-} mice are highly sensitive to irradiation and cannot be used as recipients in bone marrow transplantation. Therefore, we were unable to evaluate the ability of the *Cebpb*^{-/-} microenvironment to support

monopoiesis. These results indicate that C/EBP β is required for homeostasis of monocytes in a cell-autonomous manner.

3.3 *Cebpb*^{-/-} and WT bone marrow cells give rise to a similar level of

monocytes/macrophages *in vitro*

To assess the developmental potential of *Cebpb*^{-/-} bone marrow cells toward monocytes/macrophages, we performed an *in vitro* differentiation assay. Bone marrow cells isolated from WT or *Cebpb*^{-/-} mice were cultured in the presence of recombinant mouse M-CSF. On day 3, non-adherent and adherent cells were recovered separately and analyzed. Monocytes and macrophages were enriched among non-adherent and adherent cells, respectively. In total, $2.5 \pm 1.0 \times 10^5$ non-adherent cells and $2.5 \pm 0.7 \times 10^6$ adherent cells were recovered from a culture of 5.0×10^6 WT bone marrow cells, while $2.3 \pm 1.4 \times 10^5$ non-adherent cells and $2.2 \pm 0.4 \times 10^6$ adherent cells were recovered from a culture of 5.0×10^6 *Cebpb*^{-/-} bone marrow cells. There was no statistically significant difference between WT and *Cebpb*^{-/-} bone marrow cells. The frequencies of CD11b⁺ CD115⁺ monocytes/macrophages among the recovered cells were analyzed by flow cytometry. Both WT and *Cebpb*^{-/-} bone marrow cells gave rise to non-adherent and adherent monocytes/macrophages at equivalent frequencies (Fig. 3),

indicating that *Cebpb*^{-/-} bone marrow cells have the potential to give rise to monocytes/macrophages in the presence of M-CSF.

3.3. Apoptosis is enhanced in peripheral blood *Cebpb*^{-/-} monocytes

We observed that the level of bone marrow monocytes was not decreased in *Cebpb*^{-/-} mice and that *Cebpb*^{-/-} bone marrow cells could give rise to monocytes *in vitro*; therefore, we speculated that apoptosis of monocytes in *Cebpb*^{-/-} mice might be enhanced *in vivo*. In the bone marrow, the frequencies of early (Annexin V⁺ and 7-AAD⁻) and late (Annexin V⁺ and 7-AAD⁺) apoptotic cells among monocytes did not differ between WT and *Cebpb*^{-/-} mice (Fig. 4). By contrast, the frequency of late apoptotic cells among peripheral blood monocytes was significantly higher in *Cebpb*^{-/-} mice than in WT mice. These results indicate that enhanced apoptosis of monocytes in the peripheral blood of *Cebpb*^{-/-} mice underlies the monocytopenia.

4. Discussion

Monocytes are bone marrow-derived phagocytes that circulate in peripheral blood and give rise to macrophages [24]. The process of monocyte production is controlled by interplay among multiple factors including transcription factors [25,26,27,28,29,30,31].

In the present study, we found that C/EBP β is required for the survival of peripheral blood monocytes, while it is dispensable for the development and/or maintenance of mature cells of other lineages at steady state. Our previous report showed that C/EBP β plays important roles in the bone marrow microenvironment [32]. Therefore, we transplanted *Cebpb*^{-/-} bone marrow cells into WT recipients to evaluate the cell-extrinsic roles of C/EBP β in monopoiesis. *Cebpb*^{-/-} bone marrow cells failed to give rise to monocytes in the peripheral blood of recipients at a comparable level to WT bone marrow donor cells, suggesting that defects in monopoiesis are largely due to cell-intrinsic defects in *Cebpb*^{-/-} monocytes or their precursors.

One interesting feature of monocytopenia in *Cebpb*^{-/-} mice was its tissue distribution. The number of monocytes was reduced only in the peripheral blood, not in the bone marrow of *Cebpb*^{-/-} mice. Our *in vitro* differentiation studies demonstrated that monocytes/macrophages were efficiently induced from *Cebpb*^{-/-} bone marrow cells. These results suggest that bone marrow production of monocytes is not majorly

defective in *Cebpb*^{-/-} mice. Instead, apoptosis of monocytes was accelerated in the peripheral blood, but not in the bone marrow of *Cebpb*^{-/-} mice, suggesting that C/EBPβ is specifically required for the survival of monocytes in peripheral blood. Indeed, C/EBPβ has an anti-apoptotic effect on many cell types including neutrophils and keratinocytes [8,9,17]. Further investigations are needed to elucidate the molecular mechanisms underlying apoptosis of *Cebpb*^{-/-} monocytes.

Collectively, these data suggest that C/EBPβ is required for homeostasis of monocytes through protecting monocytes from apoptosis in peripheral blood. Further studies are necessary to elucidate the molecular mechanisms underlying the survival of monocytes and the roles of C/EBPβ in pathophysiological conditions in which monocytes are involved.

Conflicts of interest

The authors have no conflicts of interest.

Acknowledgments

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Table 1**Peripheral blood cell counts and differential analysis of WT and *Cebpb*^{-/-} mice**

Cell type	Genotype		<i>p</i> value
	WT (n = 9)	<i>Cebpb</i> ^{-/-} (n = 9)	
RBC (×10 ¹² cells/L)	10.4 ± 0.6	10.3 ± 0.4	0.62
WBC (×10 ⁹ cells/L)	8.4 ± 1.5	11.5 ± 1.8	<0.01
Lymphocytes (%)	80.7 ± 7.3	76.9 ± 15.5	0.52
Neutrophils (%)	15.2 ± 7.4	21.3 ± 14.6	0.28
Monocytes (%)	2.6 ± 1.8	0.8 ± 0.7	0.01
Eosinophils (%)	1.6 ± 1.4	1.0 ± 1.1	0.37
Platelets (×10 ⁹ cells/L)	388 ± 293	574 ± 153	0.11

Data are presented as mean ± SD.

RBC, red blood cell; WBC, white blood cell; WT, wild type.

Figure legends

Figure 1

Specific decrease in the frequency of monocytes in peripheral blood of *Cebpb*^{-/-} mice

Flow cytometric analysis of peripheral blood (A) and bone marrow (B) cells obtained from WT or *Cebpb*^{-/-} mice. Numbers indicate the percentage of cells in the adjacent boxed area. The frequencies of CD3⁺ T cells, B220⁺ B cells, NK1.1⁺ NK cells, CD11b⁺ Ly6G⁺ neutrophils, and CD11b⁺ CD115⁺ monocytes in the peripheral blood or bone marrow from WT (n = 3–13) and *Cebpb*^{-/-} (n = 3–13) mice are shown in the right panels. The frequency of monocytes was decreased in the peripheral blood, but not in the bone marrow, of *Cebpb*^{-/-} mice.

Figure 2

Cell-intrinsic requirement of C/EBP β for the maintenance of peripheral blood monocytes

Bone marrow cells from WT or *Cebpb*^{-/-} mice (CD45.2⁺) were transplanted into lethally irradiated CD45.1⁺ WT mice. Six to ten weeks after transplantation, CD45.2⁺ cells among peripheral blood (A) or bone marrow (B) cells of recipient mice were analyzed

by flow cytometry. The frequencies of monocytes among CD45.2⁺ peripheral blood or bone marrow cells of recipient mice are shown in the right panels (n = 3–9 for WT mice and n = 5–11 for *Cebpb*^{-/-} mice). Data are presented as mean ± SD. ***, *p* < 0.001.

Figure 3

The *in vitro* ability of *Cebpb*^{-/-} bone marrow cells to give rise to monocytes/macrophages is not impaired

In vitro differentiation of mouse bone marrow cells was induced in the presence of recombinant mouse M-CSF. On day 3, both non-adherent and adherent cells derived from WT or *Cebpb*^{-/-} bone marrow cells were analyzed by flow cytometry. The numbers adjacent to the boxed area indicate the percentage of cells. The frequencies of CD11b⁺ CD115⁺ monocytes/macrophages are shown in the right panels (n = 2–3). Data are presented as mean ± SD.

Figure 4

Enhanced apoptosis of *Cebpb*^{-/-} peripheral blood monocytes

Peripheral blood or bone marrow cells obtained from WT or *Cebpb*^{-/-} mice were stained with Annexin V and 7-AAD and analyzed by flow cytometry. The frequencies of early

(Annexin V⁺ and 7-AAD⁻) and late (Annexin V⁺ and 7-AAD⁺) apoptotic cells among CD115⁺ monocytes obtained from peripheral blood and bone marrow of WT or *Cebpb*^{-/-} mice (n = 7) are shown in the right panels. Apoptosis of *Cebpb*^{-/-} monocytes was enhanced in peripheral blood, but not in bone marrow. Data are presented as mean ± SD.

*, $p < 0.05$.

Figure 1

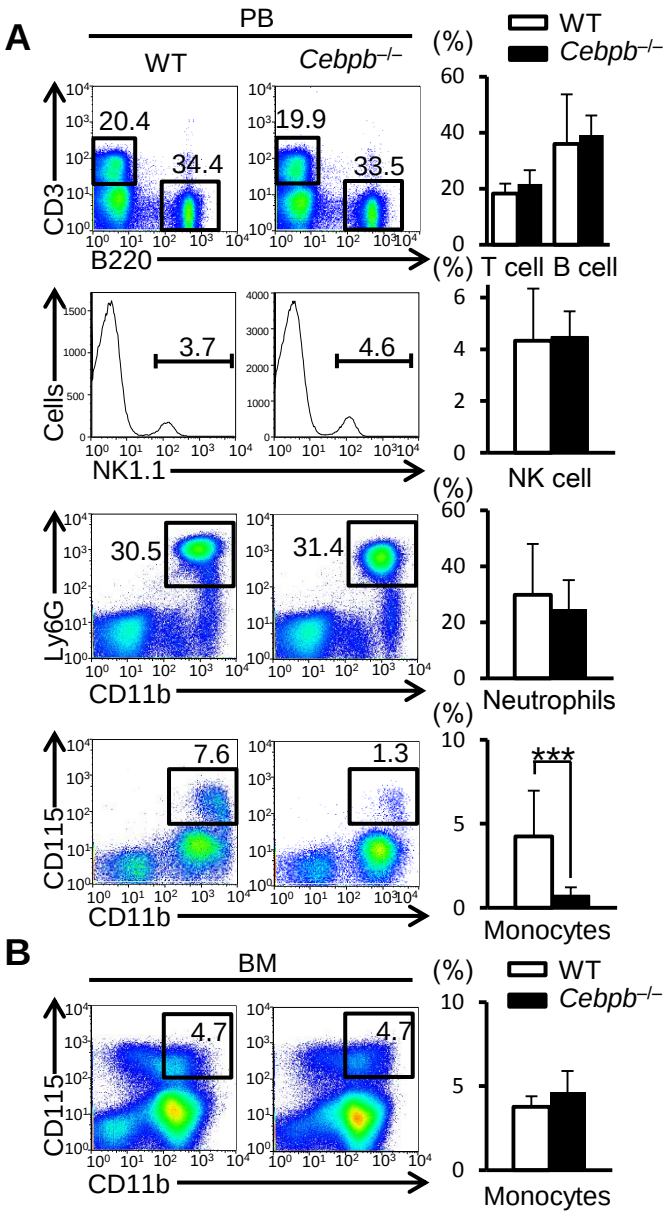


Figure 2

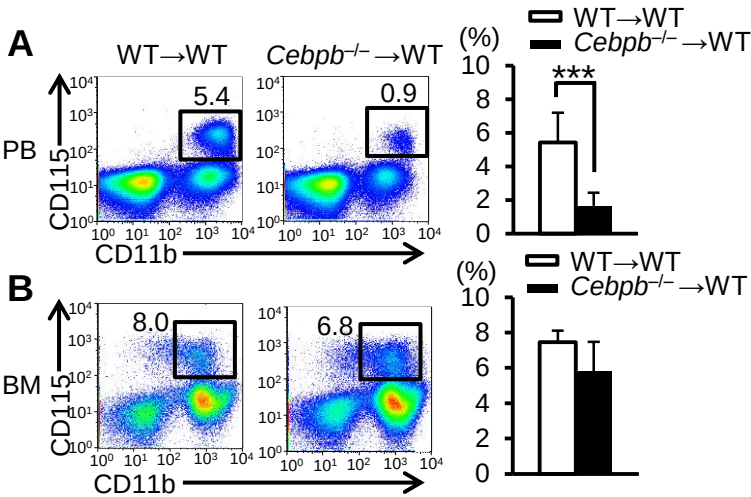


Figure 3

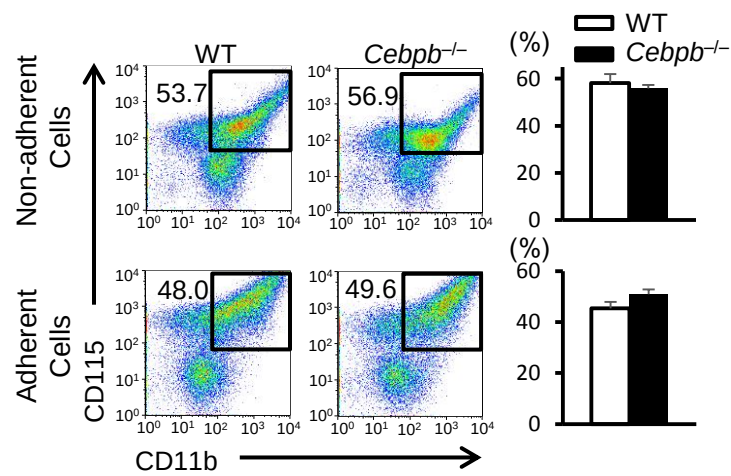
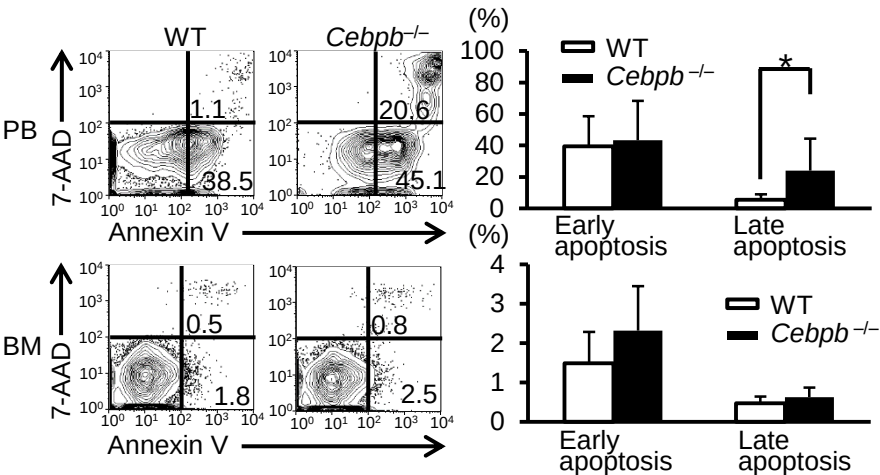
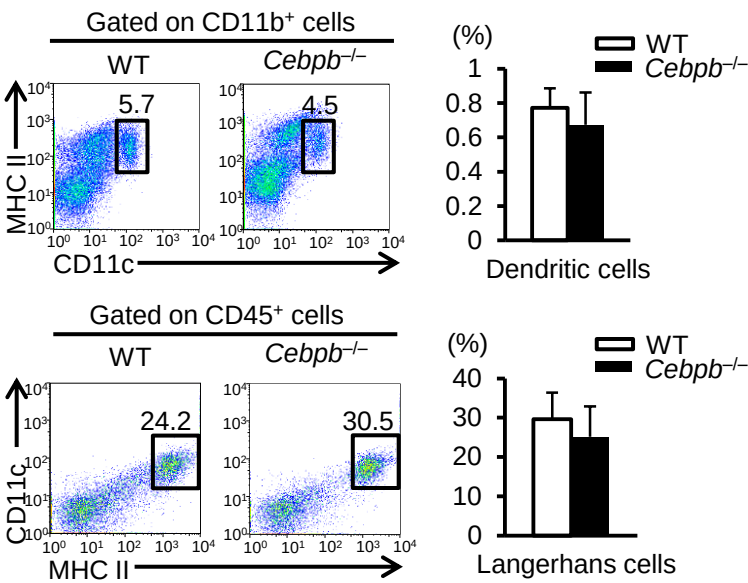


Figure 4



Supplementary Figure 1



Supplementary Figure 1

The numbers of Dendritic cells (DCs) and Langerhans cells (LCs) are not altered in *Cebpb*^{-/-} mice

Spleen cells or epidermal cells were prepared from WT or *Cebpb*^{-/-} mice and analyzed. The frequencies of CD11b⁺ CD11c⁺ MHC II⁺ DCs in the spleen (upper panels) and CD11c⁺ MHC II⁺ LCs in the CD45⁺ epidermal cell suspension (lower panels) were analyzed by flow cytometry (n = 3–4 for WT mice and n = 3–4 for *Cebpb*^{-/-} mice). Numbers indicate the percentage of cells in the adjacent boxed area. Data are presented as mean ± SD.