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

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ABSTRACT

The CCAAT/enhancer-binding protein β (C/EBPβ) 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/EBPβ is required for the survival of monocytes in peripheral blood.

1. Introduction

The leucine zipper basic region-containing transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) is a member of the C/EBP family [1–4]. C/EBPβ regulates the proliferation, differentiation, survival, and metabolism of many cell types through the activation or repression of target genes [5–10]. Within the hematopoietic system, C/EBPβ is involved in regulation of the development and function of macrophages [11–14]. In addition, we and others previously reported that C/EBPβ is required for granulopoiesis during emergencies such as infection and cytokine stimulation, but is dispensable at steady state [15,16]. Under stress conditions, C/EBPβ is induced or activated and elicits its activities to facilitate proliferation and differentiation of granulocyte precursors [15–22]. By contrast, the role of C/EBPβ 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/EBPβ 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.

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

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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 FACSAria 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.

2.6. 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.

2.7. 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 ± 2.71% in WT mice vs. 0.72 ± 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/EBPβ 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

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

Cell type	Genotype		p value
	WT (n = 9)	*Cebpb*$^{-/-}$ (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.