Vif mutants were generated by a PCR-based method with properly mutated primers. HA-tagged expression plasmids for A3F and A3G were previously described (Shirakawa et al., 2006). N-terminally myc-tagged expression plasmids for A3F, pcDNA3-myc-A3F, was generated by amplifying coding sequences of human A3F with primers CTA GCT AGC ATG GAG CAG AAA CTC ATC TCT GAA GAG GAT CTG ATG AAG CCT CAC TTC AGA AAC ACA GTG G and GGG GTA CCT CAC TCG AGA ATC TCC TGC AGC TTG CTG, and inserting it into pcDNA3.1 (Invitrogen) at Nhe I/Kpn I sites. C-terminally myc-tagged expression plasmids for A3G, pcDNA3-A3G-myc, was generated by amplifying coding sequences of human A3G with primers ATA CTC GAG AAT GAA GCC TAC TTC AGA AAC ACA GTG and GGG GTA CCC TAC AGA TCC TCT TCA GAG ATG AGT TTC TGC TCG CAG TTT TCC TGA TTC TGG AGA ATG GC, and inserting it into pcDNA3.1 (Invitrogen) at Xho I/Kpn I sites. The luciferase-reporter HIV-1 plasmids for single-cycle infection, pNL43/ΔEnv-Luc and pNL43/ΔEnvΔvif-Luc were previously described (Shindo et al., 2003). Mutations in vif region of pNL43/ΔEnv-Luc and pNL4-3 were introduced by a PCR-based method using internal restriction sites, MSC I at position 4553, and EcoR I at position 5743.

Cell culture and transfection

293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and penicillin, streptomycin and glutamine (PSG). CEM and CEM-SS cells were maintained in RPMI1640 medium supplemented with 10% FBS and PSG. 293T cells on 6-well plates were transfected with about 1 μg of plasmid DNA in total using X-tremegene HP DNA transfection reagent (Roche) according to manufacturer's instruction.

Immunoblotting

Primary antibodies for immunoblotting against Vif, A3G and $p24^{Gag}$ were obtained from the NIH AIDS Research and Reference Reagent Program. Rabbit anti-CBFβ serum and mouse anti-Cul5 and anti-HA antibodies were purchased from Santa Cruz. Rabbit anti-myc serum was purchased from Sigma. Mouse anti-tubulin antibody was purchased from Covance. HRP-conjugated secondary antibodies against mouse and rabbit were purchased from GE Healthcare. We used a standard chemiluminescence protocol for immunoblotting with PVDF membrane (Millipore).

Immunoprecipitation

For co-immunoprecipitation to test interaction of Vif mutant to CBFβ, 293T cells were transfected with pDON-Vif-myc or its derivative mutant, treated with MG132 at concentration of 2.5 μM for 16 h, and lysed with co-IP buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1mM $MgCl_2$) supplemented with protease inhibitor cocktail (Nacalai) and MG132. After centrifugation at 20,000 × *g* for 10 min, supernatant was mixed with 2 μg anti-myc rabbit serum (Sigma) for 1 h, and then mixed with 20 μl protein A sepharose (Pharmacia) for 1 h. Beads were washed with co-IP buffer 3 times, and bound protein was eluted with 1 x SDS sample buffer. Samples were analyzed by immunoblotting as described above.

Single-cycle infection

Luciferase encoding HIV-1 particles were produced by transiently transfecting 293T cells at 50% confluency using 0.8 μg pNL43/ΔEnv-Luc or derivative mutant, 0.2 μg pVSV-G and 0.02 μg pcDNA3/HA-A3F, pcDNA3/HA-A3G, or empty vector. After 48 h, virus-containing supernatants were harvested through PVDF filter with 0.45 μm pores (Millipore), and challenged to fresh 293T cells. After 48 h, cells were lysed with Passive lysis buffer (Promega) and luciferase activity was determined by luminometer (2030 Arvo X, Perkin Elmer) using Luciferase Assay System (Promega). Sample preparation of producer cells and virus for immunoblotting was performed as described (Haché et al., 2008).

Spreading infection

293T cells were transfected with NL4-3 molecular clone or derivative mutant, and virus-containing supernatant was harvested through PVDF filters with 0.45 μm pores (Millipore) after 2-day incubation. CEM-SS and CEM cells were inoculated with the supernatant at MOI of 0.005. The culture supernatants were harvested periodically, and analyzed for p24 by an HIV-1 p24 antigen ELISA kit (Zeptometrix).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.11.004.

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C/EBPβ Expressed by Bone Marrow Mesenchymal Stromal Cells Regulates Early B-cell Lymphopoiesis

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Key Words. B lymphocytes • bone marrow • CCAAT/enhancer-binding protein β • mesenchymal stromal cells.

ABSTRACT

The transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) regulates the differentiation of a variety of cell types. Here, the role of C/EBPβ expressed by bone marrow mesenchymal stromal cells (BMMSCs) in B-cell lymphopoiesis was examined. The size of the precursor B-cell population in bone marrow was reduced in C/EBPβ-knockout (KO) mice. When bone marrow cells from C/EBPβ-KO mice were transplanted into lethally irradiated wild-type (WT) mice, which provide a

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normal bone marrow microenvironment, the size of the precursor B-cell population was restored to a level equivalent to that generated by WT bone marrow cells. In co-culture experiments, BMMSCs from C/EBPβ-KO mice did not support the differentiation of WT c-Kit$^+$ Sca-1$^+$ Lineage$^-$ hematopoietic stem cells (KSL cells) into precursor B-cells, whereas BMMSCs from WT mice did. The impaired differentiation of KSL cells correlated with the reduced production of CXCL12/stromal cell-derived factor-1 by the co-cultured C/EBPβ-deficient BMMSCs. The ability of C/EBPβ-deficient BMMSCs to undergo osteogenic and adipogenic differentiation was also defective. The survival of leukemic precursor B-cells was poorer when they were co-cultured with C/EBPβ-deficient BMMSCs than when they were co-cultured with WT BMMSCs. These results indicate that C/EBPβ expressed by BMMSCs plays a crucial role in early B-cell lymphopoiesis.

INTRODUCTION

Early B-cell lymphopoiesis occurs in bone marrow, during which hematopoietic cells in the bone marrow interact with non-hematopoietic cells in the bone marrow microenvironment [1]. The molecular mechanisms in hematopoietic cells that underlie early B-cell lymphopoiesis have been well investigated. Several transcription factors in hematopoietic cells are essential for early B-cell lymphopoiesis such as PU.1, Ikaros, E2A, early B-cell factor, and paired box protein 5 [2]. By contrast, the transcription factors in non-hematopoietic cells that are important for early B-cell lymphopoiesis remain unclear.

The transcription factor CCAAT/enhancer-binding protein (C/EBPβ) is crucial for the differentiation of a variety of cell types, including adipocytes [3], hepatocytes, keratinocytes and mammary epithelial cells [4, 5]. C/EBPβ regulates myelopoiesis and granulopoiesis in hematopoiesis [6-8]. However, the role(s) of C/EBPβ expressed by bone marrow mesenchymal stromal cells (BMMSCs), one of the major hematopoiesis-supporting cellular constituents in the bone marrow microenvironment [9-11], in B-cell lymphopoiesis is unknown. In this study, detailed analysis of B-cell subpopulations in C/EBPβ-knockout (KO) mice revealed that the level of precursor B-cells was decreased in the bone marrow of C/EBPβ-KO mice. The bone marrow microenvironment, rather than hematopoietic cells, contributed to the defective generation of precursor B-cells in C/EBPβ-KO mice. C/EBPβ-deficient BMMSCs did not support the differentiation of precursor B-cells from hematopoietic stem cells (HSCs), whereas BMMSCs from wild-type (WT) mice did. In addition, the proliferation and survival of leukemic precursor B-cells co-cultured with C/EBPβ-deficient BMMSCs was examined.

MATERIALS AND METHODS

Mice. C57BL/6 (Ly5.2) and SCID (C.B-17/Icr-*scid*/*scid*Jcl) mice were purchased from CLEA Japan. C/EBPβ-KO (Ly5.2) mice [12] were back-crossed to C57BL/6 (Ly5.2) mice at least eight times. C/EBPβ-KO (Ly5.2)

mice and WT (Ly5.2) mice were obtained by intercrossing heterozygous (C/EBPβ$^{+/-}$) mice. Transgenic GFP-expressing (GFP^{+}) mice were kindly provided by Dr. Masaru Okabe (Osaka University) [13]. To obtain GFP^{+} C/EBPβ-KO and GFP^{+} WT mice, GFP+ mice were crossed with C/EBPβ$^{+/-}$ mice. C57BL/6 (Ly5.1) mice were kindly provided by Dr. Shigekazu Nagata (Kyoto University). All mice used in the experiments were 7–12 weeks old. All mice were maintained under specific pathogen-free conditions at the Institute of Laboratory Animals, Kyoto University. All animal experiments were approved by the Committee on Animal Research of the Kyoto University Faculty of Medicine.

Bone marrow transplantation. Bone marrow cells (1×10^6 cells/mouse) were administrated intravenously through the tail vein into WT mice that received 10 Gy of total body irradiation prior to transplantation. For competitive transplantation experiments, lethally irradiated (10 Gy) WT mice received bone marrow cells from both GFP^{-} WT mice (1×10^6 cells/mouse) and either GFP^{+} C/EBPβ-KO or GFP^{+} WT mice (1×10^6 cells/mouse). B-cell lymphopoiesis was evaluated by flow cytometric analysis at 14–20 weeks after transplantation.

BMMSC culture and in vivo bone formation assays. Murine BMMSCs were isolated as previously described [14-16]. Briefly, murine bone marrow cells (1.5×10^7) from long bones was seeded into 10 cm culture dishes, incubated for 3 hours at 37°C to allow adherent cells to attach, and then washed twice with PBS to remove non-adherent cells. BMMSCs formed adherent colonies and adherent cells were collected after 12–15 days of culture. Primary cultures were passaged to disperse the colony-forming cells (passage 1). Cells at passage 1 were used in experiments. The culture medium consisted of α-MEM supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 55 μM 2-mercaptoethanol (all from Gibco-BRL). For osteogenic induction *in vitro*, 2 mM β-glycerophosphate (Sigma), 100 μM L-ascorbic acid 2-phosphate (Wako Pure Chemical Industries Ltd.), and 10 nM dexamethasone (Sigma) were added to the culture media. Calcium deposition was evaluated by staining with 1% Alizarin Red S after 4 weeks of osteogenesis-inducing culture. For adipogenic induction *in vitro*, 0.5 mM isobutylmethylxanthine, 60 μM indomethacin, 0.5 μM hydrocortisone and 10 μg/mL insulin were added to the culture media. Oil Red O staining was used to assess lipid-laden fat cells after 1–2 week of adipogenesis-inducing culture. The area of the mineralized areas and the number of Oil Red O^{+} cells were quantitated using Image J software. In some experiments, the differentiated BMMSCs were used in further experiments, including immunoblot analysis or quantitative real-time PCR to analyze the expression of osteogenesis- or adipogenesis-associated molecules. For *in vivo* bone formation assays, cultured BMMSCs ($1–4\times10^6$ cells) were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer). The mixture was implanted subcutaneously into the dorsal surface of 7–9-weeks-old SCID mice, and the implants were harvested 8 weeks later. Histological analysis and quantification of bone

formation in the harvested implants were performed as previously described [17, 18]. Human bone marrow samples were obtained with informed consent and the approval of the ethical committee of Kyoto University Hospital. Human BMMSCs were isolated from bone marrow samples as previously described [19, 20] and cultured in α-MEM containing 15% FBS, 100 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. To exclude the possibility that hematopoietic cells in BMMSC cultures affect the results, $CD45^+$ cells were isolated from primary cultures of BMMSCs using anti-CD45 immunomagnetic microbeads (Miltenyi Biotech). These cells did not have multi-differentiation capabilities *in vitro* and did not express C-X-C motif chemokine 12 (CXCL12) / stromal cell-derived factor-1 (SDF-1), stem cell factor (SCF), and interleukin-7 (IL-7), whereas CD45- cells did (Fig. S1). In some experiments, human bone marrow cells and BMMSCs were purchased from AllCells or Lonza. The clinical features of the precursor B-cell acute lymphoblastic leukemia (B-ALL) samples are shown in Table S2.

Sorting of KSL cells and co-culture of KSL cells with BMMSCs. Single-cell suspensions of bone marrow cells from WT (Ly5.1) mice were labeled with allophycocyanin (APC)-conjugated anti-mouse c-Kit (2B8), fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ly6A/E [Sca-1] (D7), and PerCP-Cy5.5-conjugated anti-mouse lineage markers, including CD3e (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD19 (1D3), B220 (RA3-6B2), CD11b (M1/70), Gr-1 (RB6-8C5) and Ter119 (TER119). All antibodies were purchased from eBioscience. Sorting of $c\text{-}Kit^+$ $Sca\text{-}1^+$ Lineage- (KSL) HSCs was performed with a FACSAria cell sorter (Becton Dickinson). KSL cells (1×10^4) were co-cultured with BMMSCs (2×10^6) in 3 mL of RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 ng/mL SCF, 10 ng/mL fms-like tyrosine kinase 3-ligand (Flt3-L), and 10 ng/mL IL-7 in a 6 cm dish. The medium was replenished after 6 days, and B-cell lymphopoiesis was analyzed after 10 days of co-culture. For CXCL12/SDF-1 rescue experiments, recombinant CXCL12/SDF-1 (R&D Systems) was added to the medium to be a final concentration of 10 ng/mL.

Co-culture of murine precursor B-ALL cells with BMMSCs. Murine precursor B-ALL cells, BaF3 that were transfected with Bcr/Abl^{p185} (BaF3/Bcr-Abl), were generated as previously described [21]. BaF3/Bcr-Abl cells were co-cultured with BMMSCs derived from either C/EBPβ-KO or WT mice using a co-culture system as previously reported [22]. BMMSCs (1×10^6) were attached to the reverse side of the membrane of a 6-well cell culture insert (BD Falcon) and cultured for 3 days. Then, BaF3/Bcr-Abl cells (5×10^4) were seeded onto the upper side of the insert membrane. In co-culture experiments, cells were cultured in RPMI medium containing 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The number of BaF3/Bcr-Abl cells on the upper side of the insert was counted after 3 days of co-culture. BaF3/Bcr-Abl cells were stained with propidium iodide (PI) after 2 days of co-culture

to label DNA for cell cycle analysis. After co-culture with BMMSCs for 3 days, the percentage of apoptotic BaF3/Bcr-Abl cells was determined by performing APC-conjugated Annexin V and PI (BD Bioscience) co-staining according to the manufacturer's instructions.

BrdU incorporation assay. BaF3/Bcr-Abl cells (2×10^4 cells/well in a 96-well plate) were cultured in the medium supplemented with 0, 0.01, 0.1, 1, or 10 ng/mL CXCL12/SDF-1. After 24 hours of culture, BrdU incorporation was analyzed by using the colorimetric immunoassay Cell Proliferation ELISA, BrdU (Roche Applied Science) according to the manufacturer's instructions.

Flow cytometric analysis. Single-cell suspensions of bone marrow cells were stained with fluorescence-conjugated antibodies and analyzed with a FACSCanto II (Becton Dickinson). B-cell populations were identified based on the Hardy Fraction [23]. The antibodies used were FITC-conjugated anti-mouse CD43 (R2/60), FITC-conjugated anti-mouse CD45.1/Ly5.1 (A20), phycoerythrin (PE)-conjugated anti-mouse BP-1 (6C3), PE-conjugated anti-mouse IgD (11-26c), APC-conjugated anti-mouse B220 (RA3-6B2), APC-conjugated anti-mouse CD11b (M1/70), PE-Cy7-conjugated anti-mouse CD24 (M1/69), and PE-Cy7-conjugated anti-mouse IgM (II/41). All antibodies were purchased from eBioscience. Dead cells were excluded by staining with PI. Data were analyzed using FlowJo software (Tree Star).

Quantitative real-time PCR. Total RNA was extracted using the QIAamp RNA Blood Mini Kit (Qiagen). cDNA was prepared using the PrimeScript RT reagent kit (Perfect Real-Time) (Takara). Real-time PCR was performed using the StepOnePlus real-time PCR system (Applied Biosystems) and a Universal ProbeLibrary (Roche). The primer sets and universal probes used are listed in Table S1. Gene expression levels were normalized to the mRNA level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All samples were analyzed in duplicate.

Immunoblot analysis. Cells lysates were boiled in SDS sample buffer, separated by SDS-PAGE, and transferred to PVDF membranes. Primary antibodies against runt-related transcription factor 2 (Runx2, Abcam), alkaline phosphatase (ALP, Abcam), lipoprotein lipase (Lpl, Abcam), fatty acid-binding protein 4 (FABP4, Abcam), peroxisome proliferator-activated receptor γ (PPARγ, Cell Signaling Technology), and β-actin (Sigma-Aldrich) were used. Immunoreactive proteins were detected using horseradish peroxidase-conjugated anti-mouse (for anti-Runx2, -Lpl, and -β-actin) or anti-rabbit (for anti-ALP, -PPARγ, and -FABP4) immunoglobulin G (GE Healthcare) and visualized using enhanced chemiluminescence or enhanced chemiluminescence prime kits (GE Healthcare). Relative protein expression levels were measured using Quantity One software (Bio-Rad).

Histological analysis. Histological analysis and quantification of bone formation were

performed as previously described [17]. Briefly, bone specimens from the lower vertebrae of mice were stained with hematoxylin and eosin (HE), and trabecular bone areas were measured at 100× magnification using Image J software. Images were acquired using a DP71 digital camera and DP Controller software in combination with a CX41 microscope and a PlanCN objective lens (10×, 0.25 numerical aperture) (Olympus).

CXCL12/SDF-1 ELISA. Culture supernatant was collected after culturing murine BMMSCs (2×10^6 cells in 3mL per dish) for 3 days, or after co-culturing WT KSL cells with the same number of BMMSCs for 10 days in 6 cm dishes. The level of CXCL12/SDF-1 protein in the culture supernatant was measured using the Quantikine mouse CXCL12/SDF-1α ELISA kit (R&D Systems) according to the manufacturer's instructions.

Statistical analyses. The unpaired Student's *t*-test was used for analysis. Data in bar graphs indicate the mean ± SD, and statistical significance is expressed as follows: *, $P < 0.05$; **, $P < 0.01$; n.s., not significant.

RESULTS

The level of precursor B-cells is decreased in the bone marrow of C/EBPβ-KO mice.

The levels of B-cell subsets in C/EBPβ-KO mice were analyzed by flow cytometric analysis based on the expression patterns of B220, CD43, BP-1, CD24, surface IgM (sIgM), and surface IgD (sIgD) [23]. The percentage of $B220^+$ B-cells in the bone marrow was significantly lower in C/EBPβ-KO mice than in WT mice (Fig. 1A: WT, 26.5 ± 7.3%; C/EBPβ-KO, 19.1 ± 7.1%; *$P < 0.05$). The percentage of $B220^+$ B-cells in the spleen was similar in WT and C/EBPβ-KO mice (Fig. S2A–C), which is consistent with a previous study [24]. The percentage of B220+CD43+ precursor B-cells in the bone marrow was significantly lower in C/EBPβ-KO mice than in WT mice (Fig. 1B). In detail, the percentages of pre-pro-B-cells ($B220^+CD43^+BP\text{-}1^-CD24^-$; Fraction A), pro-B-cells ($B220^+CD43^+BP\text{-}1^-CD24^+$ and $B220^+CD43^+BP\text{-}1^+CD24^{low}$; Fraction B/C), and pre-BI cells ($B220^+CD43^+BP\text{-}1^+CD24^{high}$; Fraction C') were all significantly lower in C/EBPβ-KO mice than in WT mice (Fig. 1C, D). The percentages of pre-BII cells ($B220^+CD43^-sIgM^-sIgD^-$; Fraction D), immature B-cells ($B220^+CD43^-sIgM^+sIgD^{low}$; Fraction E), and mature B-cells ($B220^+CD43^+sIgM^+sIgD^{high}$; Fraction F) in bone marrow were not significantly different between C/EBPβ-KO mice and WT mice (Fig. S3A, B).

The bone marrow microenvironment contributes to the impairment of B-cell lymphopoiesis in C/EBPβ-KO mice.

The bone marrow microenvironment is crucial for early B-cell development [1]; therefore, we hypothesized that the bone marrow microenvironment contributes to the decreased level of precursor B-cells in C/EBPβ-KO mice. We conducted bone marrow transplantation experiments to test this hypothesis (Fig. 2A). Bone marrow cells from C/EBPβ-KO mice were transplanted into lethally irradiated (10 Gy) WT mice (KO→WT). As a control, bone marrow cells from WT mice were transplanted

into WT mice (WT→WT). The recipient WT mice should provide a normal bone marrow microenvironment. Fourteen weeks after transplantation, the percentage of B220+ B-cells in bone marrow was similar in recipients that received C/EBPβ-deficient bone marrow cells and those who received WT bone marrow cells (Fig. 2B). Detailed analysis of B-cell subsets showed that the levels of pre-pro B-cells (Fraction A), pro-B-cells (Fraction B/C), and pre-BI cells (Fraction C') were similar in the two groups (Fig. 2C). The size of the B-cell population in the spleen was similar in both groups (Fig. S4*A*, B).

Next, we performed competitive bone marrow transplantation experiments. The same number of GFP^- WT bone marrow cells and either GFP^+ WT bone marrow cells (Transplantation-A) or GFP^+ C/EBPβ-deficient bone marrow cells (Transplantation-B) were co-transplanted into lethally irradiated WT mice (Fig. 2D). GFP^+ C/EBPβ-deficient hematopoietic cells engrafted into recipient bone marrow with approximately 50% cellularity (Fig. 2E, BM, Transplantation-B), which was equivalent to the engraftment rate of GFP^+ WT hematopoietic cells (Fig. 2E, BM, Transplantation-A). The percentage of GFP^+ cells in the spleens of recipients was also similar in both transplantations (Fig. 2E, SP). GFP^+ C/EBPβ-deficient bone marrow cells and GFP^- WT bone marrow cells gave rise to a similar percentage of $B220^+$ B-cells in the recipient bone marrow when they were co-transplanted (Fig. 2F, Transplantation-B, open bar versus closed bar). GFP^+ WT bone marrow cells and GFP^- WT bone marrow cells also generated a similar percentage of $B220^+$ B-cells (Fig. 2F, Transplantation-A, dotted bar versus closed bar). These findings demonstrate that C/EBPβ-deficient bone marrow hematopoietic cells are able to generate B-cells as efficiently as normal WT bone marrow hematopoietic cells when transplanted into a normal bone marrow microenvironment. Taken together, these results indicate that the impairment of B-cell lymphopoiesis in C/EBPβ-KO mice is due, at least in part, to the bone marrow microenvironment.

Reverse transplantation from WT mice to C/EBPβ-KO mice was also performed (Fig. S5A). However, donor-derived (Ly5.1) B-cell reconstitution after transplantation could not be evaluated in most C/EBPβ-KO recipient mice because of early death, although an irradiation dose was reduced. When an irradiation dose of 5 Gy was used, donor cells did not engraft (Fig. S5B, C). When an irradiation dose of 7 Gy was used, the level of donor-derived (Ly5.1) $B220^+$ cells in peripheral blood and the bone marrow was lower in surviving C/EBPβ-KO recipients than in WT recipients (Fig. S5D, E). Perhaps the C/EBPβ-deficient bone marrow microenvironment cannot support hematopoietic and/or immune recover after transplantation.

C/EBPβ-deficient BMMSCs have an impaired ability to support the differentiation of HSCs into precursor B-cells.

BMMSCs are important for the regulation of B-cell lymphopoiesis in the bone marrow microenvironment [1, 11, 25]; therefore, we explored whether C/EBPβ-deficient BMMSCs have an impaired ability to support B-cell

lymphopoiesis. KSL HSCs from the bone marrow of WT (Ly5.1) mice (WT-KSL cells) were co-cultured with BMMSCs from C/EBPβ-KO (Ly5.2) mice in the presence of SCF, Flt3-L, and IL-7 (Fig. 3A). BMMSCs from WT (Ly5.2) mice were used as a control in the co-culture experiments (Fig. 3A). The generation of hematopoietic cells from WT-KSL cells was significantly lower when cells were co-cultured with C/EBPβ-deficient BMMSCs than when they were co-cultured with WT BMMSCs (Fig. 3B). The generation of $B220^+$ B-cells from WT-KSL cells was also significantly lower when cells were co-cultured with C/EBPβ-deficient BMMSCs than when they were co-cultured with WT BMMSCs (Fig. 3C, D and S6A). Detailed analysis of B-cell subsets showed that differentiation of WT-KSL cells into precursor B-cells was reduced (Fig. 3E, left panels) and differentiation from pre-pro-B-cells (Fraction A) to pro-B-cells/pre-BI cells (Fraction B/C/C') was suppressed when cells were co-cultured with C/EBPβ-deficient BMMSCs compared to when they were co-cultured with WT BMMSCs (Fig. 3E, right panels and 3F). Therefore, C/EBPβ-deficient BMMSCs have an impaired ability to support the differentiation of normal HSCs into precursor B-cells.

Reduced production of CXCL12/SDF-1 by C/EBPβ-deficient BMMSCs partially contributes to impaired differentiation of HSCs into B-cells.

Next, the expression of B-cell lymphopoiesis-associated humoral factors in BMMSCs was examined. Levels of CXCL12/SDF-1 (Fig. 3G) and Flt3-L (Fig. S6C) mRNA were significantly lower in C/EBPβ-deficient BMMSCs than in WT BMMSCs. Levels of IL-7 (Fig. S6D) and SCF (Fig. S6E) mRNA tended to be lower in C/EBPβ-deficient BMMSCs than in WT BMMSCs, although the difference was not statistically significant. CXCL12/SDF-1 is essential for hematopoiesis, particularly B-cell lymphopoiesis [26, 27]. In addition to mRNA expression, the protein concentration of CXCL12/SDF-1 was significantly lower in the culture supernatant of C/EBPβ-deficient BMMSCs than that of WT BMMSCs (WT, $n = 5$, 9.90 ± 1.93 ng/mL; C/EBPβ-deficient, $n = 5$, 4.47 ± 1.16 ng/mL; $^{**}P < 0.01$) (Fig. 3H). The concentration of CXCL12/SDF-1 in the supernatant of BMMSC co-cultures correlated with the number of $B220^+$ B-cells that differentiated from WT-KSL cells (Fig. 3I). The addition of exogenous CXCL12/SDF-1 to the co-culture of C/EBPβ-deficient BMMSCs and WT-KSL cells slightly increased the total number of cells and the number of B-cells (Fig. 3B, D). The frequencies of pre-pro-B cells, pro-B and pre-BI cells (Fractions A, B and C+C') that differentiated from KSL cells were not apparently affected by the addition of CXCL12/SDF-1 to the culture medium (Fig. S6A, B). Thus, reduced production of CXCL12/SDF-1 by C/EBPβ-deficient BMMSCs is partially associated, and other functional abnormalities of C/EBPβ-deficient BMMSCs may be associated with the impaired differentiation of HSCs into B-cells in the co-culture.

C/EBPβ-deficient BMMSCs have an impaired multi-differentiation capability.

We sought to identify other differentiation characteristics in which C/EBPβ-deficient

BMMSCs were defective. The ability to differentiate into multiple cell types is a fundamental property of bone marrow mesenchymal stem cells [28, 29]; therefore, C/EBPβ-deficient BMMSCs were evaluated in osteogenic and adipogenic differentiation assays. When BMMSCs were cultured under osteogenesis-inducing conditions *in vitro*, calcium accumulation was significantly lower in C/EBPβ-deficient BMMSCs than in WT BMMSCs, as assessed by Alizarin Red S staining (Fig. 4A, B). The expression of the osteogenic master molecule Runx2 and of another crucial osteogenic marker ALP was down-regulated in C/EBPβ-deficient BMMSCs in this assay (Fig. 4C, D). Moreover, when BMMSCs were subcutaneously implanted with HA/TCP into SCID mice, C/EBPβ-deficient BMMSCs induced less bone formation than WT BMMSCs (Fig. 4E, F). Therefore, the osteogenic differentiation capability of C/EBPβ-deficient BMMSCs was defective compared to that of WT BMMSCs. Findings of skeletal examinations of C/EBPβ-KO mice were in agreement with this. Male and female C/EBPβ-KO mice had a shorter crown-rump length and total length than sex- and age-matched WT mice (Fig. S7A, B). C/EBPβ-KO mice had less trabecular bone (TB) and fewer bone-lining cells on the surface of TB that were positive for osteocalcin, a marker of osteoblasts, than age-matched WT mice (Fig. S7C-F). Next, *in vitro* adipogenic differentiation assays were performed. When BMMSCs were cultured under adipogenesis-inducing conditions *in vitro*, lipid deposition was significantly lower in C/EBPβ-deficient BMMSCs than in WT BMMSCs (Fig. 5A-C). Furthermore, expression of the adipogenic markers PPARγ, Lpl, and Fabp4 was down-regulated in C/EBPβ-deficient BMMSCs in this assay (Fig. 5D, E). Therefore, the adipogenic differentiation capability of C/EBPβ-deficient BMMSCs was defective compared to that of WT BMMSCs.

Taken together, these results demonstrate that C/EBPβ-deficient BMMSCs have an impaired multi-differentiation capability.

Survival of leukemic precursor B-cells is suppressed when co-cultured with C/EBPβ-deficient BMMSCs.

The results described so far demonstrate that C/EBPβ expressed by BMMSCs plays a crucial role in supporting physiological early B-cell lymphopoiesis; therefore, we explored whether C/EBPβ expressed by BMMSCs is involved in the proliferation and survival of leukemic precursor B-cells. The murine precursor B-ALL cell line BaF3/Bcr-Abl was co-cultured with BMMSCs from C/EBPβ-KO or WT mice (Fig. 6A). The number of BaF3/Bcr-Abl cells was higher when they were co-cultured with WT BMMSCs than when they were cultured alone (Fig. 6B). However, the number of BaF3/Bcr-Abl cells was similar when they were co-cultured with C/EBPβ-deficient BMMSCs and when they were cultured alone (Fig. 6B). This difference was not associated with cell cycle (Fig. 6C, D). The proportion of apoptotic BaF3/Bcr-Abl cells was significantly higher when they were co-cultured with C/EBPβ-deficient BMMSCs than when they were co-cultured with WT BMMSCs (Fig. 6E, F). Production of CXCL12/SDF-1 was reduced in C/EBPβ-deficient BMMSCs (Fig. 3G, H);

therefore, the response of BaF3/Bcr-Abl cells to CXCL12/SDF-1 was examined. Stimulation with CXCL12/SDF-1 increased the BrdU incorporation of BaF3/Bcr-Abl cells in a dose-dependent manner (Fig. 6G). These results suggest that reduced production of CXCL12/SDF-1 and impaired anti-apoptotic activity in C/EBPβ-deficient BMMSCs contribute to the reduced survival of BaF3/Bcr-Abl cells. In human BMMSCs derived from precursor B-ALL bone marrow samples, the mRNA levels of C/EBPβ and CXCL12/SDF-1 were increased in some cases (Fig. S8A, B). Thus, the expression level of C/EBPβ in BMMSCs might modulate the survival of leukemic precursor B-cells.

DISCUSSION

In this study, we showed that the bone marrow microenvironment contributed to the reduced level of precursor B-cells in the bone marrow of C/EBPβ-KO mice, and that C/EBPβ-deficient BMMSCs had an impaired ability to support differentiation of HSCs into precursor B-cells and an impaired multi-differentiation capability. We and others have previously shown that myelopoiesis and the number of hematopoietic stem and progenitor cells are comparable between C/EBPβ-KO and WT mice [6, 8]. Therefore, impaired B-cell lymphopoiesis in C/EBPβ-KO mice is not due to a reduced level of HSCs and is not associated with increased myelopoiesis.

It was previously reported that reduced production of IL-7 by bone marrow stromal cells and weak response of $B220^{+}IgM^{-}$ B-cells to IL-7 contribute to the impairment of B-cell lymphopoiesis in C/EBPβ-KO mice [24]. In this study, the expression of IL-7 tended to be lower in C/EBPβ-deficient BMMSCs than in WT BMMSCs; however, this difference was not statistically significant. Rather, C/EBPβ-deficient bone marrow hematopoietic cells were able to generate B-cells as efficiently as normal WT bone marrow hematopoietic cells when they were transplanted into the normal bone marrow microenvironment of WT mice. This finding demonstrates that the bone marrow microenvironment, rather than hematopoietic cells, is responsible for the impairment of B-cell lymphopoiesis in C/EBPβ-KO mice, at least at steady-state. The contributions of hematopoietic cells and the bone marrow microenvironment to the impairment of B-cell lymphopoiesis in C/EBPβ-KO mice reported here differ from those previously reported [24]; however, the reason(s) for this discrepancy is unclear. The two reports used different methods to evaluate B-cell generation; *in vitro* liquid culture was performed in the previous report [24] and *in vivo* reconstitution following hematopoietic cell transplantation was performed in our analysis. IL-7 is an essential cytokine for early B-cell lymphopoiesis that is expressed in the bone marrow microenvironment [30], and C/EBPβ-deficient hematopoietic cells exhibit impaired responses to various cytokines in addition to IL-7 [7]. Thus, both the bone marrow microenvironment and hematopoietic cells might contribute to the impairment of B-cell lymphopoiesis in C/EBPβ-KO mice, and the relative contribution of each might vary in different states.

Transcription factors expressed by hematopoietic cells that are essential for early B-cell lymphopoiesis have been well studied [2]. Whereas WT BMMSCs supported the differentiation of normal purified HSCs (WT-KSL cells) into precursor B-cells in co-culture experiments, C/EBPβ-deficient BMMSCs did not. Therefore, the ability of BMMSCs to support early B-cell lymphopoiesis is dependent upon C/EBPβ. Production of CXCL12/SDF-1 was reduced in C/EBPβ-deficient BMMSCs, and the level of differentiation of normal HSCs into precursor B-cells correlated with the concentration of CXCL12/SDF-1 in the supernatant of BMMSC and HSC co-cultures. Several lines of evidence indicate that CXCL12/SDF-1 has essential roles in early B-cell lymphopoiesis [26, 27, 31]. The current study did not investigate transcriptional regulation of CXCL12/SDF-1 by C/EBPβ directly; however, previous reports demonstrated that C/EBPβ is one of the major regulatory elements driving transcription of CXCL12/SDF-1 [32, 33]. The addition of exogenous CXCL12/SDF-1 to the co-culture of C/EBPβ-deficient BMMSCs and WT-KSL cells slightly increased the total number of B-cells. Hematopoiesis is regulated by the complicated interaction between hematopoietic cells and the bone marrow microenvironment via adhesion molecules and humoral factors including Flt3-L, IL-7, SCF [1, 10, 11]. Reduced production of CXCL12/SDF-1 by C/EBPβ-deficient BMMSCs is partially associated, but other functional abnormalities of C/EBPβ-deficient BMMSCs may be associated with the impaired differentiation of HSCs into B-cells in the co-culture. Further studies are needed to elucidate the contribution of C/EBPβ in BMMSCs to B-cell lymphopoiesis.

BMMSCs are multi-potent non-hematopoietic cells capable of differentiating into a variety of cell types, including osteoblasts, adipocytes and chondrocytes [34-37]. Given that C/EBPβ-deficient BMMSCs had an impaired ability to support B-cell lymphopoiesis, we hypothesized that their multi-differentiation capability was also impaired. C/EBPβ-deficient BMMSCs had an impaired osteogenic differentiation capability compared to WT BMMSCs, which was observed concomitant with reduced expression of the osteogenic master molecule Runx2 *in vitro*, and reduced formation of TB bone and a reduced number of osteoblasts *in vivo*. Moreover, C/EBPβ-deficient BMMSCs had an impaired adipogenic differentiation capability compared to WT BMMSCs. In summary, C/EBPβ-deficient BMMSCs have an impaired ability to support B-cell lymphopoiesis and reduced osteogenic/adipogenic differentiation capabilities; thus, C/EBPβ is presumably an important regulatory transcription factor needed for BMMSCs to exert their biological effects. Several links have been reported between B-cell lymphopoiesis and osteogenic/adipogenic cells. Osteoblasts support B-cell differentiation and commitment from HSCs [38]. Furthermore, CXCL12 abundant reticular cells, which have characteristics of adipo-osteogenic progenitors, are essential for the proliferation and survival of precursor B-cells [25, 39]. The impaired osteogenic and adipogenic differentiation capabilities of C/EBPβ-deficient BMMSCs seem to be associated with their impaired ability to support B-cell lymphopoiesis.

Recently, the contribution of the bone marrow microenvironment to leukemogenesis and chemo-resistance, called the "leukemic niche", has been clarified [40-43]. The association of the leukemic niche with pathogenesis and its potential as a therapeutic target in precursor B-ALL have also been described [44-46]. In the present study, the survival of precursor B-ALL cells was suppressed when they were co-cultured with C/EBPβ-deficient BMMSCs which produced CXCL12/SDF-1 less than WT BMMSCs. The involvement of CXCL12/SDF-1 in the suppressive effect of C/EBPβ-deficient BMMSCs on BaF3/Bcr-Abl cell survival is in agreement with several studies reporting that the CXCL12 (SDF-1)/CXCR4 axis contributes to the pathogenesis of precursor B-ALL [47-49]. In addition, our cell cycle analyses are consistent with a previous study reporting that the promotion of cell survival through CXCL12 (SDF-1)/CXCR4 axis is independent of cell cycle progression [49]. Several CXCL12/CXCR4 antagonists have been investigated as leukemic niche-targeting therapies [47, 49]. C/EBPβ is a regulator of CXCL12/SDF-1 [32, 33]; therefore, C/EBPβ might also be a therapeutic target in some cases in which C/EBPβ is highly expressed in BMMSCs. The anti-apoptotic activity of mesenchymal stem cells in some conditions has been reported [50], although the detail mechanism underlying this remains unknown. Further studies are needed to elucidate the anti-apoptotic effect of BMMSCs on precursor B-ALL cells. Among the various adult hematological malignancies, precursor B-ALL remains difficult to treat with conventional chemotherapy. Better understanding of the contribution of altered C/EBPβ expression in BMMSCs to the pathogenesis of precursor B-ALL may help identify a novel therapeutic target(s) for this disease.

Conclusion

C/EBPβ expressed by BMMSCs regulates B-cell lymphopoiesis, particularly precursor B-cell differentiation. In co-culture experiments, survival of leukemic precursor B-cells is associated with the expression level of C/EBPβ in BMMSCs. Further studies are needed to elucidate the contribution of C/EBPβ in BMMSCs to the regulation of physiological and pathological B-cell lymphopoiesis in the bone marrow microenvironment.

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Disclosure of potential conflicts of interest

The authors have no potential conflicts of interest.

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Figure 1. The level of precursor B-cells is reduced in the bone marrow of C/EBPβ-KO mice. The percentage of $B220^+$ B-cells (A) and $B220^+CD43^+$ precursor B-cells (B) in the bone marrow (BM) of WT mice (WT, n = 14) and C/EBPβ-KO mice (KO, n = 13), as determined by flow cytometric analysis. (C, D) Detailed flow cytometric analysis of subsets of B-cells in the BM of WT and C/EBPβ-KO mice. (C) Representative counter-plots. Numbers indicate the percentage of cells in boxes. (D) Percentages of pre-pro-B-cells (Fraction A), pro-B-cells (Fraction B/C), and pre-BI cells (Fraction C'). *, $P < 0.05$; **, $P < 0.01$.

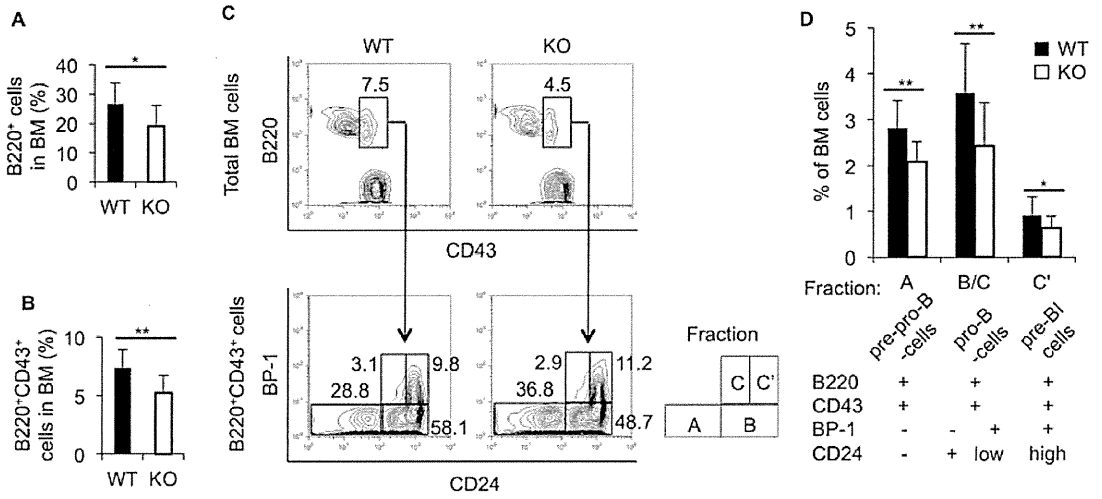

Figure 2. C/EBPβ-deficient bone marrow cells generate an equivalent level of B-cells as WT bone marrow cells when transplanted into WT mice. (A□C) BM transplantation experiments. (A) Schema of the BM transplantation experiments. WT mice, which provide a normal BM microenvironment, received BM hematopoietic cells (1×10^6 cells) from WT mice (WT→WT, n = 5) or C/EBPβ-KO mice (KO→WT, n = 3). The level of B-cells in the BM of recipient mice was analyzed 14 weeks after transplantation. (B) Percentage of $B220^+$ B-cells in the recipient BM of the WT→WT (closed bar) and the KO→WT (open bar) groups, as determined by flow cytometric analysis. (C) Detailed flow cytometric analysis of subsets of B-cells. Percentages of pre-pro-B-cells (Fraction A), pre-B-cells (Fraction B/C), and pre-BI cells (Fraction C') in the recipient BM of the WT→WT and KO→WT groups. (D□F) Competitive BM transplantation experiments. (D) Schema of the competitive BM transplantation experiments. GFP^- WT mice, which provide a normal bone marrow microenvironment, received BM cells from GFP^- WT mice (1×10^6 cells) and either GFP^+ WT mice (1×10^6 cells) (Transplantation-A, n = 5) or GFP^+ C/EBPβ-KO mice (1×10^6 cells) (Transplantation-B, n = 5). BM cells were analyzed 20 weeks after transplantation. (E) Percentage of GFP^+ cells engrafted in the recipient BM and spleen (SP), as determined by flow cytometric analysis. (F) Percentage of $B220^+$ cells engrafted in the recipient bone marrow (BM), as determined by flow cytometric analysis.

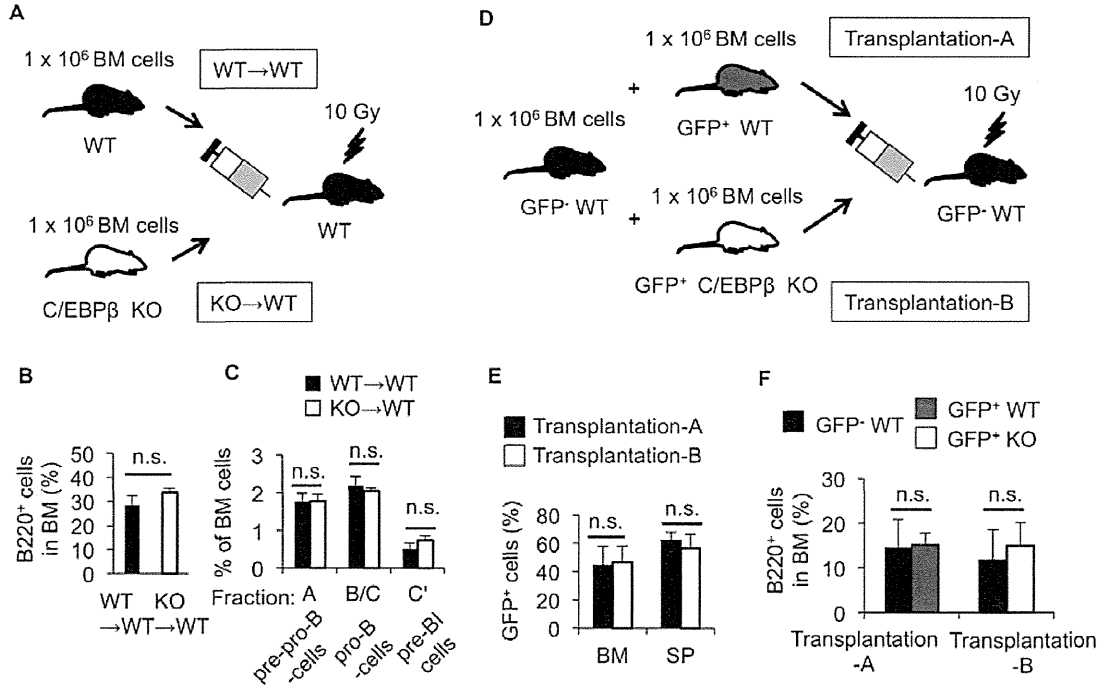

Figure 3. Differentiation of HSCs into precursor B-cells is impaired when co-cultured with C/EBPβ-deficient BMMSCs, in which CXCL12/SDF-1 production is reduced. KSL HSCs derived from WT (Ly5.1) mice (WT (Ly5.1)-KSL cells) were co-cultured with WT (Ly5.2) or C/EBPβ-deficient (KO) (Ly5.2) BMMSCs *in vitro* in the presence of SCF, Flt3-L and IL-7 with (SDF-1 +) or without (SDF-1 -) addition of CXCL12/SDF-1. Three experiments were performed in each condition. B-cell lymphopoiesis was analyzed after 10 days of co-culture. (A) Schema of co-culture experiments. (B) The number of hematopoietic cells generated from WT (Ly5.1)-KSL cells when co-cultured with WT (Ly5.2) or C/EBPβ-deficient (KO) (Ly5.2) BMMSCs, or cultured alone (BMMSCs (-)). (C, D) Flow cytometric analysis showing levels of $B220^+$ B-cells generated from WT (Ly5.1)-KSL cells when co-cultured with WT (Ly5.2) or C/EBPβ-deficient (KO) (Ly5.2) BMMSCs. (C) Representative counter plots. (E, F) Detailed flow cytometric analysis of the precursor B-cell population. Representative counter plots (E) and the percentages of B-cell subsets in $B220^+CD43^+$ precursor B-cells (F). (G) Quantitative real-time PCR analysis examining CXCL12/SDF-1 mRNA expression in BMMSCs. WT: WT BMMSCs (n = 6), KO: C/EBPβ-deficient BMMSCs (n = 6). **(H)** The protein concentration of CXCL12/SDF-1 was measured in the culture supernatant of WT BMMSCs (WT, n = 5) and C/EBPβ-deficient BMMSCs (KO, n = 5) by ELISA. **(I)** The number of $B220^+$ B-cells generated from WT-KSL cells strongly correlated with the concentration of CXCL12/SDF-1 in the culture supernatant. Numbers in each box indicate the percentage of cells (C, E). *, $P < 0.05$; **, $P < 0.01$.