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C/EBPβ promotes BCR-ABL-mediated myeloid expansion and leukemic stem cell exhaustion

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Running title: C/EBPβ drives BCR-ABL-mediated myeloid expansion

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The BCR-ABL fusion oncoprotein accelerates differentiation and proliferation of myeloid cells during the chronic phase of chronic myeloid leukemia (CP-CML). Here, the role of C/EBPβ, a regulator for 'emergency granulopoiesis', in the pathogenesis of CP-CML was examined. C/EBPβ expression was upregulated in Lineage⁻ CD34⁺ CD38⁻ hematopoietic stem cells and myeloid progenitors isolated from bone marrow of patients with CP-CML. In EML cells, a mouse hematopoietic stem cell line, BCR-ABL upregulated C/EBPβ, at least in part, through the activation of STAT5. Myeloid differentiation and proliferation induced by BCR-ABL was significantly impaired in C/EBPβ-deficient bone marrow cells in vitro. Mice that were transplanted with BCR-ABL-transduced C/EBPβ knockout bone marrow cells survived longer than mice that received BCR-ABL-transduced wild-type bone marrow cells. Significantly higher levels of leukemic stem cells were maintained in BCR-ABL-transduced C/EBPβ-deficient cells than in BCR-ABL-transduced wild-type cells. These results suggest that C/EBPβ is involved in BCR-ABL-mediated myeloid expansion. Further elucidation of the molecular mechanisms underlying the C/EBPβ-mediated stem cell loss might reveal a novel therapeutic strategy for eradication of CML stem cells.
Keywords: C/EBPβ; BCR-ABL; chronic myeloid leukemia
Introduction

Chronic phase chronic myeloid leukemia (CP-CML) is characterized by massive proliferation and differentiation of myeloid cells.\textsuperscript{1, 2} In sharp contrast to acute myeloid leukemia with leukemic hiatus, both myeloid progenitors and mature granulocytes accumulate in the bone marrow, peripheral blood and spleen in CP-CML.\textsuperscript{1} The myeloid expansion in CP-CML has been attributed to the BCR-ABL fusion protein resulting from a translocation between chromosomes 9 and 22.\textsuperscript{2-4} Experiments using transgenic mouse models have shown that the BCR-ABL-mediated leukemic status is reversed by suppression of BCR-ABL,\textsuperscript{5-7} suggesting that BCR-ABL is the sole cause of the myeloid expansion in CP-CML. In practice, inhibition of the BCR-ABL tyrosine kinase activity effectively controls the disease during the chronic phase in most cases.\textsuperscript{8-12} Recent findings suggested a hierarchical organization of CML hematopoiesis, with CML stem cells giving rise to heterogeneous progeny.\textsuperscript{13-15} Accumulating clinical experiences, together with experimental data, have shown that leukemic stem cells in CP-CML are resistant to tyrosine kinase inhibitors (TKIs) and sometimes causes a relapse of the disease after discontinuation of TKI treatment.\textsuperscript{16-19} Progression of CML toward the accelerated phase and blast crisis is considered to be a consequence of further acquisition of genetic mutations, which makes
the disease more resistant to TKIs and results in an extremely poor prognosis.\textsuperscript{20, 21}

Therefore, a better understanding of the characteristics of leukemic stem cells, as well as the pathogenesis of CP-CML, are essential for establishing a novel therapeutic strategy for CML.

Granulopoiesis is a process in which hematopoietic stem cells give rise to mature granulocytes throughout life. Our previous findings revealed that a member of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors, C/EBP\(\beta\), is required for 'emergency granulopoiesis', which is characterized by the accelerated differentiation and proliferation of granulocytic precursors in response to infections or cytokine stimulation.\textsuperscript{22} Myeloid expansion is a common feature of both emergency granulopoiesis and CP-CML. However, little is known regarding the role of C/EBP\(\beta\) in the pathogenesis of CP-CML. In this study, the effects of BCR-ABL on the expression and function of C/EBP\(\beta\) in BCR-ABL-induced myeloid expansion was determined and the therapeutic implications of these data are discussed.
Materials and methods

Primary human bone marrow cells and cell lines

Frozen samples of human bone marrow cells from healthy donors or untreated CP-CML patients were purchased from AllCells LLC (Emeryville, CA, USA). In all cases, written informed consent was obtained according to the Institutional Review Board or Human Subject Committee approved donor program. The characteristics of the patients are shown in Supplementary Table 1. EML cells (a kind gift from Dr. Schickwann Tsai at the University of Utah, UT, USA) were maintained in Iscove modified Dulbecco’s medium (IMDM) supplemented with 20% heat-inactivated horse serum and 15% BHK/MKL cell-conditioned medium.

Mice

C57BL/6 mice were purchased from Clea Japan (Tokyo, Japan). C/EBPβ knockout (KO) mice were back crossed to C57BL/6 strain mice at least 8 times. Whenever C/EBPβ KO mice were analyzed, wild-type (WT) littermates were used as control. All mice were maintained under specific pathogen-free conditions in the Institute of Laboratory Animals, Kyoto University. All experiments were performed according to the institutional guidelines.
of Kyoto University.

**Reagents**

A STAT5 inhibitor (N’-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide) and Ly294002, a PI3K inhibitor, were purchased from Merck (Darmstadt, Germany). U0126, a MEK inhibitor, was purchased from Cell Signaling Technology (Danvers, MA, USA). Stock solutions of the STAT5 inhibitor, Ly294002, and U0126 were made in dimethyl sulfoxide (DMSO).

**Plasmids**

The pMSCVneo vector and pMSCV-internal ribosome entry site-green fluorescent protein (GFP) vector (MIG), and their derivatives for the expression of BCR-ABL (p210), were kind gifts from Dr. Keiko Okuda (Kyoto Prefectural University of Medicine, Kyoto, Japan). Retroviruses expressing the constitutively-active STAT5 mutant (STAT5<sup>166</sup>) and the dominant negative STAT5 mutant (STAT5<sup>Δ749</sup>) were kind gifts from Dr. Toshio Kitamura (University of Tokyo, Tokyo, Japan).
Retrovirus infection

Plat-E packaging cells were transfected with retrovirus vectors using FuGENE6 (Roche Diagnostics, Mannheim, Germany) as previously described. Bone marrow cells were harvested from mice (4 to 8 weeks of age) treated with 5-fluorouracil (5-FU) (150 mg/kg, intraperitoneally). The bone marrow cells were cultured at 37 °C for 48 h in IMDM containing 15% FBS, 50 μM 2-mercaptoethanol, 50 ng/ml mouse stem cell factor (SCF), 50 ng/ml human thrombopoietin (TPO) (a kind gift from Kyowa Hakko Kirin Co., Ltd), 50 ng/ml mouse fms-like tyrosine kinase 3 ligand (FL), and 10 ng/ml mouse interleukin-6 (IL-6). First round retroviral infection was carried out using RetroNectin (Takara Bio, Otsu, Japan) in the same medium. For the second round infection, polybrene was added with the retroviral supernatant.

Bone marrow transplantation

Recipient C57BL/6 mice (8 to 10 weeks of age) were lethally irradiated (10 Gy). MIG or MIG-BCR-ABL-transduced bone marrow cells (0.5 to 1 × 10^5 GFP-positive cells per mouse) were injected into the tail vein of primary recipient mice. For radioprotection, 2 × 10^5 cells of freshly harvested whole bone marrow were co-transplanted. For secondary
transplantation, bone marrow cells were harvested from mice that received the primary transplants and 0.2 to 2 × 10^6 GFP-positive cells were intravenously injected into sublethally irradiated secondary recipient mice. The frequencies of leukemic stem cells were calculated using the L-Calc software (StemCell Technologies, Vancouver, Canada).

**Methylcellulose colony-forming assay**

MIG-BCR-ABL-transduced mouse bone marrow cells from C/EBPβ KO or WT mice were subjected to a methylcellulose colony-forming assay using the cytokine-free medium Methocult 3231 (StemCell Technologies). The fluorescence images of the colonies were obtained using AxioCam MRm digital camera and Axio Vision software in combination with SteREO Lumar V12 microscope and Neolumar S objective lens (0.8×) (Carl Zeiss, Oberkochen, Germany).

**Wright Giemsa staining**

Smears of mice peripheral blood or bone marrow cells and cytospin slides of EML cells or colony-forming cells were stained using a Diff-Quik kit (Sysmex, Kobe, Japan), a modified Wright Giemsa staining system. Images were obtained using DP71 digital camera and DP
Controller software in combination with CX41 microscope and PlanCN objective lens (40×/0.65 numerical aperture) (Olympus, Tokyo, Japan).

Flow cytometric analysis

Flow cytometric analysis and cell sorting were performed with a FACSCalibur, FACSCanto II, or FACSAria instrument (BD Biosciences, San Jose, CA, USA). As lineage markers for human bone marrow mononuclear cells, phycoerythrin (PE)-Cy5-conjugated anti-CD235, biotin-conjugated anti-CD3, CD4, CD8, CD11b, CD14, CD19, CD20 (eBioscience, San Diego, CA, USA), and anti-CD56 antibodies (Bio Legend, San Diego, CA, USA) were used and followed by staining with streptavidin-PE-Cy5. Cells were further stained with allophycocyanin (APC)-conjugated anti-CD34 (8G12), phycoerythrin (PE)-conjugated anti-CD38 (HIT2), fluorescein isothiocyanate (FITC)-conjugated anti-CD45RA (HI100) (all from BD Pharmingen, San Diego, CA, USA) and PE-Cy7-conjugated anti-CD123 antibodies (eBioscience) for definition of hematopoietic stem cells and myeloid progenitors.

For the staining of mouse cells, PerCP-Cy5.5-conjugated anti-CD3, CD4, CD11b, B220, and Ter119 antibodies, and PE-Cy5.5-conjugated anti-CD8 and Gr-1 antibodies (all from eBioscience) were used as lineage markers. PE-Cy7-conjugated anti-Sca-1, PE-conjugated
anti-CD11b and APC-conjugated anti-c-kit antibodies (all from eBioscience) were used for
definition of hematopoietic stem cells and myeloid cells. Data were analyzed with the
FlowJo software (Tree Star, Ashland, OR, USA).

**Real-time RT-PCR**

Total RNA was extracted with an RNeasy Micro Kit (Qiagen, Valencia, CA, USA) and
converted to cDNA using random primers. The cDNA was amplified using an Applied
Biosystems Step One Plus thermal cycler. The following parameters were used: 95 °C for
20 sec, followed by 45 cycles at 95 °C for 1 sec and 60 °C for 20 sec. The following
primers, and probes from the Universal Probe Library (Roche Applied Science, Mannheim,
Germany) were used: for mouse C/EBPβ, probe #55, and primers
5′-ATCGACTTCAGCCCCTACCT-3′ and 5′-TAGTCGTCGGCGAAGAGG-3′; for mouse
GAPDH, probe #80, and primers 5′-TGTCGGTGATCTGAC-3′ and
5′-CCTGCTTCACCACCTTCTTG-3′; for human C/EBPβ, probe #74, and primers
5′-CGCTTACCTCGGCTACCA-3′ and 5′-ACGAGGAGGACGTGGAGAG-3′; and for
human GAPDH, probe #60, and primers 5′-AGCCACATCGCTCAGACAC-3′ and
5′-GCCCAATACGACCAATCC-3′. Results were normalized by the level of GAPDH
mRNA.

**Western blot analysis**

Cells were diluted with equal amounts of Laemmli sample buffer and boiled at 100 °C for 10 min. Samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. A “Can Get” Signal immunoreactions enhancer kit (Toyobo, Osaka, Japan) was used to dilute the primary and secondary antibodies. Antibodies specific for C/EBPβ (sc-150, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (sc-25778, Santa Cruz Biotechnology) were used as primary antibodies. Immunoreactive proteins were detected using horseradish peroxidase-conjugated anti-rabbit IgG (NA934V, GE Healthcare, Little Chalfont, UK) and visualized using enhanced chemiluminescence (ECL, GE Healthcare).

**Statistics**

Statistical analyses were performed using Student’s t-test. Survival of mice was analyzed using the log-rank test. *P* values < 0.05 were considered statistically significant.
Results

C/EBPβ is upregulated in bone marrow hematopoietic stem cells and myeloid progenitors from patients with CP-CML

C/EBPβ expression is upregulated or maintained in normal myeloid progenitors during emergency granulopoiesis, while the expression of all other C/EBP family members was downregulated.22 Therefore, the expression of C/EBPβ in bone marrow cells of patients with CP-CML was examined. Among the lineage marker negative (Lin⁻) bone marrow cells, the frequency of CD34⁺ CD38⁻ hematopoietic stem cells (HSCs) was lower and the frequency of CD34⁺ CD38⁺ myeloid progenitors was higher in CP-CML bone marrow than in bone marrow from healthy donors (Figure 1a and b, Supplementary Figure S1). The CD34⁺ CD38⁺ population was further subdivided into common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), and megakaryocyte-erythocyte progenitors (MEPs) based on the expression levels of CD123 and CD45RA 29 (Figure 1a). The frequency of GMPs was significantly lower and that of MEPs was significantly higher (approximately two-fold) in CP-CML bone marrow than in bone marrow from healthy donors (Figure 1c). The differences between normal and CP-CML bone marrow in the frequency of CD34⁺ CD38⁻ HSCs and of myeloid progenitors...
were consistent with previous findings.\textsuperscript{13,15,30} The expression of C/EBP\(\beta\) in the purified progenitors was measured. The levels of C/EBP\(\beta\) mRNA in HSCs and all the myeloid progenitors from CP-CML bone marrow were significantly higher than levels in bone marrow from healthy donors (Figure 1d, healthy donors, \(n = 6\); CP-CML, \(n = 5\); GMP, \(P < 0.01\); HSC, CMP, and MEPs, \(P < 0.05\)). These results suggest that C/EBP\(\beta\) is upregulated in HSCs and myeloid progenitors in patients with CP-CML.

221 \textbf{BCR-ABL upregulates C/EBP\(\beta\) in EML cells, a mouse hematopoietic stem cell line}

The presence of the BCR-ABL fusion protein is thought to be the only difference between normal hematopoiesis and BCR-ABL-mediated myeloid expansion in CP-CML.\textsuperscript{2,4} To assess whether BCR-ABL could upregulate C/EBP\(\beta\), the \textit{BCR-ABL} gene was retrovirally introduced into a factor-dependent mouse hematopoietic stem cell line, EML cells, and the expression of C/EBP\(\beta\) was compared with EML cells transduced with a control vector (EML-control). After transduction with BCR-ABL, EML cells (EML-BCR-ABL) became factor-independent and could be cultured long term in the absence of stem cell factor-containing conditioned medium. The morphologies of the EML-control and EML-BCR-ABL cells were indistinguishable by Giemsa staining (Figure
The parent EML cells expressed c-kit but not CD11b. Flow cytometric analysis of the transduced cells revealed that a small subset of EML-BCR-ABL expressed c-kit at a slightly lower intensity and expressed CD11b weakly (Figure 2b, Day 0). Myeloid differentiation of EML cells can be induced by the addition of interleukin-3, retinoic acid, and granulocyte-macrophage colony stimulating factor (GM-CSF). As shown in Figure 2a (Day 5), myeloid differentiation of both EML-control and EML-BCR-ABL cells was effectively induced. Lower c-kit and higher CD11b expression by EML-control cells was observed 5 days after the induction of differentiation and the extent of the changes in the expression of c-kit and CD11b was more evident in EML-BCR-ABL cells (Figure 2b). These results suggest that BCR-ABL enhanced myeloid differentiation of immature cells such as EML cells.

The amount of C/EBPβ mRNA in undifferentiated EML-BCR-ABL cells was 1.87-fold higher than in undifferentiated EML-control cells (Figure 2c). When the c-kit+ CD11b- fraction of the EML-control cells and EML-BCR-ABL cells was analyzed, a significant difference was still observed 2.26-fold higher in EML-BCR-ABL cells (Figure 2d), suggesting that the upregulation of C/EBPβ was not the result of contamination of differentiated cells. The level of C/EBPβ protein was 3.76-fold higher in EML-BCR-ABL
cells relative to EML-control cells (Figure 2e). When EML-BCR-ABL cells were treated
with imatinib mesylate, the upregulation of C/EBPβ by BCR-ABL was reduced (Figure 2f),
while the level of C/EBPβ in EML-control cells was not affected. These results suggest that
C/EBPβ is upregulated directly in response to signaling downstream of BCR-ABL.

STAT5 is involved in the BCR-ABL-mediated upregulation of C/EBPβ

Various signaling pathways are activated by BCR-ABL, including the JAK/STAT,
Raf/MEK/ERK, and PI3K/AKT pathways. To elucidate the signaling pathways
responsible for the upregulation of C/EBPβ, each of the known downstream signaling
pathways was inhibited. When EML-BCR-ABL cells were treated with the MEK inhibitor
U0126 or the PI3K inhibitor Ly294002, C/EBPβ expression was not affected (Figure 3a). In
contrast, treatment with the STAT5 inhibitor
(N’-((4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide) significantly reduced
C/EBPβ expression in EML-BCR-ABL cells (Figure 3b). A dominant negative STAT5
mutant, STAT5Δ749, was introduced into the EML-derived cell lines to inhibit STAT5.
STAT5Δ749 significantly repressed the expression of C/EBPβ in EML-BCR-ABL cells but
had no effect in EML-control cells (Figure 3c). Conversely, when a constitutively-active
STAT5 mutant, STAT5\textsuperscript{1*6}, was retrovirally transduced into the parental EML cells (EML-CA-STAT5), C/EBP\(\beta\) mRNA levels were significantly greater compared to the level in EML cells transduced with a control vector (Figure 3d). These results suggest that STAT5 is involved in the BCR-ABL-mediated upregulation of C/EBP\(\beta\).

**C/EBP\(\beta\) regulates BCR-ABL-mediated proliferation and differentiation of myeloid cells in vitro**

The upregulation of C/EBP\(\beta\) in the presence of BCR-ABL was confirmed using primary bone marrow cells. BCR-ABL was retrovirally introduced into bone marrow cells obtained from 5-FU-treated WT mice and RNA was extracted from purified c-kit\(^+\) Sca-1\(^+\) Lin\(^-\) (KSL) cells after the transduced cells were cultured for 2 days. The RNA was analyzed by quantitative RT-PCR. The expression of C/EBP\(\beta\) in BCR-ABL-transduced KSL cells was significantly greater than in control vector-transduced KSL cells (\(n = 3\) each, \(P < 0.01\)) (Figure 4a).

To understand the role of C/EBP\(\beta\) in BCR-ABL-mediated myeloid expansion, BCR-ABL and GFP were retrovirally introduced into bone marrow cells obtained from 5-FU-treated C/EBP\(\beta\) KO mice and their properties were compared to
BCR-ABL-transduced bone marrow cells from WT mice. The transduced cells were first subjected to cytokine-free semisolid methylcellulose culture. All the colonies formed on day 7 were GFP-positive and the numbers of colonies were equivalent between transduced bone marrow cells from WT and KO mice (Figure 4b and c), suggesting that the frequencies of clonogenic progenitors were similar. The majority of C/EBPβ KO cell-derived colonies were smaller in size (Figure 4b) and the cell number per C/EBPβ KO cell colony was significantly lower than WT cell colonies ($P < 0.01$) (Figure 4d). These findings suggest that the loss of C/EBPβ impaired BCR-ABL-mediated cell proliferation.

Having observed a difference between WT and C/EBPβ KO cells in proliferative capacity in the presence of the BCR-ABL fusion protein, the morphologies of the colony-forming cells were assessed by Giemsa staining (Figure 4e). The BCR-ABL-transduced C/EBPβ KO cell colonies contained more immature myeloid cells with larger nuclei and basophilic cytoplasm, whereas the BCR-ABL-transduced WT cell colonies contained more mature neutrophilic granulocytes and macrophages (Figure 4e). Flow cytometric analysis revealed that the BCR-ABL-transduced C/EBPβ KO cells gave rise to a significantly higher proportion of c-kit$^+$ cells and a lower proportion of CD11b$^+$ cells, relative to BCR-ABL-transduced WT cells (%c-kit$^+$ cells = 6.7 ± 1.0% vs. 2.0 ± 0.2%, $P =$
0.01; %CD11b$^+$ cells = 17.9 ± 4.4% vs. 41.3 ± 8.8%, $P = 0.039$) (Figure 4f and g). In addition, BCR-ABL-transduced C/EBPβ KO cells-derived colonies could be replated more than three times in cytokine-free medium, whereas BCR-ABL-transduced WT cells stopped growing after being replated only twice (Figure 4h and Supplementary Figure S2). These results suggest that the C/EBPβ deficiency abolished the BCR-ABL-mediated proliferation and differentiation of myeloid cells.

Loss of C/EBPβ delays BCR-ABL-mediated myeloid expansion in vivo

BCR-ABL-transduced bone marrow cells from C/EBPβ KO mice or WT mice were transplanted into lethally irradiated recipient mice to clarify the role of C/EBPβ in the in vivo myeloproliferation induced by BCR-ABL. After transplantation of transduced cells, increases in neutrophilic granulocytes were observed in the peripheral blood of mice having received either WT cells or KO cells (Figure 5a). Flow cytometric analysis of peripheral blood also revealed that over 70% of nucleated cells were CD11b$^+$ in both WT and KO transplant recipients (Figure 5b). In mice transplanted with BCR-ABL-transduced C/EBPβ KO cells, the increase in white blood cell count (Figure 5c) and the development of splenomegaly (Figure 5d) were delayed compared to mice receiving transplants of WT
BCR-ABL-transduced cells. The median survival of mice transplanted with BCR-ABL-transduced WT cells was 19 days. In contrast, the median survival of mice transplanted with BCR-ABL-transduced C/EBPβ KO cells was 31 days, significantly longer than mice receiving transplants of BCR-ABL-transduced WT cells ($P = 0.0005$) (Figure 5e). These results suggest that C/EBPβ is involved in BCR-ABL-induced enhanced myelopoiesis *in vivo*.

**BCR-ABL-induced loss of self-renewing hematopoietic/leukemic stem cells was attenuated in the absence of C/EBPβ**

*In vitro* experiments revealed that C/EBPβ KO bone marrow cells retained more c-kit+ immature cells and could be replated more times after transduction with BCR-ABL (Figure 4f-h, Supplementary Figure S2), suggesting that C/EBPβ is involved in the BCR-ABL-mediated loss of self-renewing hematopoietic/leukemic stem cells. Consistent with these observations, BCR-ABL-transduced C/EBPβ KO cells had given rise to a higher proportion of c-kit+ cells than BCR-ABL-transduced WT cells on day 19 after transplantation (16.0 ± 2.6% vs. 5.5 ± 4.6%, $P = 0.01$) (Figure 6a and b).

Serial transplantation experiments were carried out to determine the role of
C/EBPβ in the loss of self-renewal capacity. When $2 \times 10^6$ GFP$^+$ bone marrow cells from the primary recipients were transplanted into sublethally irradiated secondary recipients, all the mice having received either BCR-ABL-transduced WT cells or BCR-ABL-transduced KO cells developed a myeloproliferative status, reminiscent of the first transplantation (Figure 6c). After transplantation of $1 \times 10^6$ GFP$^+$ bone marrow cells from the primary transplant recipients, one of the four recipient mice that received a secondary transplant of BCR-ABL-transduced WT cells developed a myeloproliferative status and four out of five recipient mice that received a secondary transplant of BCR-ABL-transduced C/EBPβ KO cells developed a myeloproliferative disorder (Figure 6d). On day 38 after the secondary transplantation of $1 \times 10^6$ GFP$^+$ bone marrow cells, BCR-ABL-transduced C/EBPβ KO cells achieved a significantly higher degree of chimerism in the peripheral blood of the secondary recipients than BCR-ABL-transduced WT cells ($n = 8$ each, $P = 0.013$) (Figure 6e). None of the mice that received less than $0.5 \times 10^6$ GFP$^+$ bone marrow cells from the primary recipients developed a myeloproliferative status. Based on these transplantation experiments, the estimated frequencies of leukemia-initiating cells in bone marrow of primary recipient mice transplanted with BCR-ABL-transduced WT cells and BCR-ABL-transduced C/EBPβ KO cells were 1 in 1 404 129 and 1 in 683 773,
respectively (Figure 6f). These results suggest that enhanced C/EBPβ expression induced
by BCR-ABL was involved in the loss of the self-renewal potential of leukemic stem cells.
Discussion

Proliferation and differentiation of myeloid cells are unique to CP-CML. This is the first report demonstrating that the BCR-ABL-mediated myeloid expansion in CP-CML is promoted by C/EBPβ, a regulator of 'emergency granulopoiesis'.

One of the major findings of this study is the upregulation of C/EBPβ by BCR-ABL in CP-CML. In EML cells or in immature mouse hematopoietic cells, BCR-ABL upregulated C/EBPβ and accelerated the differentiation of these cells (Figure 2b-e and Figure 4a). In contrast, previous reports showed that, in 32Dcl3 cells transduced with BCR-ABL, C/EBPβ was downregulated and granulocytic differentiation was blocked.37, 38

One explanation for the discrepancy between the observations in EML cells and 32Dcl3 cells may be due to the differentiation status. 32Dcl3 cells give rise only to neutrophilic granulocytes in the presence of G-CSF,39 while EML cells are multipotent and can give rise to monocytic, erythroid and lymphoid lineages, in addition to the granulocytic lineage, suggesting that EML cells represent more immature hematopoietic cells than 32Dcl3 cells.

The higher C/EBPβ expression in 32Dcl3 than in EML cells (Supplementary Figure S3) also supports this idea, as C/EBPβ is upregulated during myeloid differentiation.40, 41

Taking into account the previous findings that HSCs are the target cell population for
BCR-ABL during chronic phase, the data from EML cells are most likely to reflect conditions of CP-CML. Consistent with our observations, Minami et al. found that C/EBPβ is one of the markedly upregulated genes in a pluripotent hematopoietic cell line transduced with BCR-ABL. Most importantly, C/EBPβ is upregulated in purified HSCs and myeloid progenitors obtained from patients with CP-CML, as shown in Figure 1d. The differences in BCR-ABL-mediated regulation of C/EBPβ in EML cells and in 32Dcl3 cells also suggested the involvement of cell context-dependent machinery. Guerzoni et al. described a downregulation of C/EBPβ associated with the progression of CML toward a blast crisis, whereas our study focused on the upregulation of C/EBPβ in the chronic phase. The changes in the BCR-ABL-mediated regulation of C/EBPβ during the progression of CML may be a consequence of genetic or epigenetic changes, which result in a cell context similar to 32Dcl3 cells, in terms of the regulation of C/EBPβ.

BCR-ABL activates a number of signaling cascades through its tyrosine kinase activity. STAT5 is a well known target of BCR-ABL. STAT5 is phosphorylated by JAK2 and is thought to activate target genes to induce or maintain CP-CML. Our current data strongly suggest that C/EBPβ resides downstream of STAT5. Transplantation of BCR-ABL-transduced STAT5 KO bone marrow cells resulted in delayed progression of a
myeloproliferative disorder in mice, a phenotype highly similar to our CP-CML model using C/EBPβ KO cells. The similarities between the behavior of BCR-ABL-transduced STAT5 KO cells and BCR-ABL-transduced C/EBPβ KO cells strongly suggested that these two molecules act sequentially in the same pathway. STAT5 and C/EBPβ are both essential for cytokine-induced granulopoiesis, suggesting that STAT5 and C/EBPβ may also interact when emergency granulopoiesis is stimulated. There has been no evidence, thus far, to demonstrate that STAT5 directly regulates C/EBPβ. The consensus binding site for STAT5 (TTCN3GAA) is not found in the proximal promoter region of C/EBPβ. Recently, Zhang et al. showed that STAT3 regulates C/EBPβ transcription through binding to the IL-6 response element II (CTGGGA) located at -1180 base pairs in the C/EBPβ promoter. However, our preliminary analysis of the 2000 base pairs proximal promoter region of C/EBPβ by reporter assay failed to identify the positive regulatory elements which respond to STAT5 (data not shown). In addition, specific binding of STAT5 to the particular regulatory element was not observed in published chromatin immunoprecipitation sequencing data analyzing T cells stimulated with interleukin-2 (GSE12346 (ref. 49) and GSE26553 (ref. 50)). These data suggest that STAT5 might upregulate C/EBPβ through binding to regulatory elements outside this IL-6 response element II or through other
indirect mechanisms. Elucidation of the direct or indirect interactions between STAT5 and C/EBPβ is necessary for further understanding of BCR-ABL-mediated myeloid expansion and emergency granulopoiesis.

Our present data showed that myeloid expansion was delayed when BCR-ABL was transduced into C/EBPβ KO bone marrow cells, clearly suggesting the involvement of C/EBPβ in the enhanced myelopoiesis observed in patients with CP-CML. The effects of BCR-ABL on the self-renewing potential of HSCs are still controversial. Schemionek et al recently reported that BCR-ABL partially impaired long term HSCs. In the present study, a phenotypically and functionally immature status was maintained in a greater fraction of BCR-ABL-transduced C/EBPβ KO cells than in BCR-ABL-transduced WT cells both in vitro and in vivo. These data suggested that C/EBPβ is involved in the BCR-ABL-mediated loss of self-renewing potential of the HSCs. From the therapeutic point of view, repression of C/EBPβ in CP-CML patients would delay the progression of the disease, although leaving leukemic stem cells relatively intact. This strategy might be effective for patients with BCR-ABL mutations that are resistant to tyrosine kinase inhibitors, as the role of C/EBPβ in the pathogenesis of CP-CML should be common to all BCR-ABL mutants. Alternatively, upregulation of C/EBPβ in leukemic stem cells might induce exhaustion of
the leukemic stem cells, leading to a complete cure for the disease. Actually, Guerzoni et al. showed that transduction of C/EBPβ promotes differentiation of BCR-ABL expressing cells. The effects of upregulation of endogenous C/EBPβ on leukemic stem cells should be determined in the future. Further understanding of the regulation of the self-renewal and differentiation of leukemic stem cells in CP-CML may lead to identification of novel therapies for CML based on the regulation of C/EBPβ. 

The leukocytosis observed during infections (=emergency granulopoiesis) is sometimes called a 'leukemoid' reaction because of the great increase in the number of myeloid cells with a 'left shift' in the shape of the nucleus. Here, a molecular link between leukemoid reactions and CP-CML was identified. C/EBPβ fine tunes the proliferation and differentiation of HSCs and CML leukemic stem cells in response to their respective upstream signals. Further elucidation of the molecular mechanisms that regulate the self-renewal and differentiation of stem cells in CP-CML and emergency granulopoiesis will facilitate the development of novel strategies for the treatment of CML.
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Conflict of Interest

The authors declare no conflict of interest.

Supplementary information is available at the Leukemia’s website.
References


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Figure Legends

Figure 1. C/EBPβ expression in hematopoietic stem and progenitor cells from the bone marrow of CP-CML patients.

(a) Flow cytometric analysis of lineage marker negative (Lin−) bone marrow cells from healthy donors and CP-CML patients identified CD34+ CD38− hematopoietic stem cells (HSC) and CD34+ CD38+ myeloid progenitors (upper panels). Myeloid progenitors were further subdivided into common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythocyte progenitors (MEP) based on the expression of CD45RA and CD123 (lower panels). The data shown are representative of six independent experiments. (b) Frequency of CD34+ CD38− HSCs within the Lin− fraction of bone marrow cells. Error bars indicate SD (healthy donors, n = 6; CP-CML, n = 5; *P < 0.05). (c) Percentage of CMPs, GMPs, and MEPs in the Lin− CD34+ CD38+ fraction. Error bars indicate SD (healthy donors, n = 6; CP-CML, n = 5; *P < 0.001). (d) C/EBPβ mRNA levels in purified HSCs, CMPs, GMPs, and MEPs. Results were normalized to the expression level of HSCs from healthy donors (healthy donors, n = 6; CP-CML, n = 5; *P < 0.05; **P < 0.01).
**Figure 2.** Effects of BCR-ABL on the expression of C/EBPβ in EML cells.

(a) Wright Giemsa staining of pMSCVneo vector-transduced EML cells (EML-control) and BCR-ABL-containing pMSCVneo vector-transduced EML cells (EML-BCR-ABL) before (Day 0) and after (Day 5) the induction of myeloid differentiation (scale bar, 20 μm; original magnification, 400×). (b) Flow cytometric analysis of c-kit and CD11b expression in EML-control and EML-BCR-ABL cells (Day 0 and Day 5 after myeloid differentiation). Numbers in each quadrant indicate the percentage of live cells. (c) C/EBPβ mRNA levels in EML-control and EML-BCR-ABL cells. Results were normalized to the expression level of control. Error bars indicate SD from duplicate samples. Results are representative of three independent experiments. *P = 0.011. (d) C/EBPβ mRNA levels in c-kit^+ CD11b^- fraction of the EML-control cells and EML-BCR-ABL cells. Results were normalized to the expression level of control. Error bars indicate SD from duplicate samples. *P < 0.01. (e) C/EBPβ protein levels in EML-control and EML-BCR-ABL cells. Results were normalized to the expression level of control. Error bars indicate SD from duplicate samples. Results are representative of three independent experiments. *P < 0.01. (f) C/EBPβ mRNA levels in EML-control and EML-BCR-ABL cells with or without a 48 h treatment with imatinib mesylate (100 nM). Dimethyl sulfoxide (DMSO) was used as the control. Results were
normalized to the expression level of control. Error bars indicate SD from duplicate samples. Results are representative of three independent experiments. \( *P = 0.016. \)

**Figure 3.** Involvement of BCR-ABL downstream signaling pathways in the upregulation of C/EBP\(\beta\).

Changes in C/EBP\(\beta\) mRNA in EML-BCR-ABL cells 24 h after treatment with the PI3K inhibitor Ly294002 (2.5 \(\mu\)M), the MEK inhibitor U0126 (25 \(\mu\)M) (a), or a STAT5 inhibitor (40 \(\mu\)M) (b). DMSO was used as the control. Results were normalized to the expression level of control. Error bars indicate SD from duplicate samples. Results are representative of two independent experiments. \( *P < 0.01. \) (c) C/EBP\(\beta\) mRNA in EML-control and EML-BCR-ABL cells transduced with a dominant negative STAT5 mutant, STAT5\(\Delta749\). Results were normalized to the expression level of control. Error bars indicate SD from duplicate samples. Results are representative of two independent experiments. \( *P < 0.05. \) (d) C/EBP\(\beta\) mRNA levels in EML cells transduced with a constitutively-active STAT5 mutant (CA-STAT5: STAT5\(^{1*6}\)) or empty-vector. Results were normalized to the expression level of control. Error bars indicate the SD from duplicate samples. Results are representative of two independent experiments. \( *P < 0.01. \)
Figure 4. BCR-ABL-mediated in vitro colony formation in the absence of C/EBPβ.

(a) C/EBPβ mRNA levels in c-kit⁺ Sca-1⁺ Lin⁻ cells from WT bone marrow cells transduced with a control MIG vector or a MIG-BCR-ABL vector (n = 3 each, P < 0.01).

(b) Colonies formed by BCR-ABL-transduced C/EBPβ KO bone marrow cells and WT bone marrow cells after culture for 7 days in cytokine-free methylcellulose (scale bar, 1 mm). Colony numbers (c), cell numbers per colony (d), and Wright Giemsa staining (scale bar, 20 μm; original magnification, 400×) (e) of the colony-forming cells are shown. Error bars indicate SD from triplicate cultures. Results are representative of three independent experiments. *P < 0.01. (f) Flow cytometric analysis of the cells forming primary colonies by day 7. Numbers in each quadrant indicate the percentage of live cells. (g) Frequency of c-kit⁺ cells and CD11b⁺ cells in the cells forming primary colonies by day 7. Results are representative of two independent experiments. *P < 0.05. (h) Serial colony-replating of 1 × 10⁴ BCR-ABL-transduced C/EBPβ WT and KO bone marrow cells. The colonies were counted and collected on day 10 (2nd) or 14 (3rd), respectively. Error bars indicate SD from triplicate cultures. Results are representative of three independent experiments. *P < 0.01.
**Figure 5.** C/EBPβ deficiency alters the BCR-ABL-dependent myeloproliferative status *in vivo.*

Wright Giemsa staining (scale bar, 20 μm; original magnification, 400×) (a), 20 days post-transplantation, and flow cytometric analysis (b), 28 days post-transplantation, of peripheral blood from recipient mice transplanted with BCR-ABL-transduced WT cells or BCR-ABL-transduced C/EBPβ KO cells. Numbers in quadrants indicate the percentages within peripheral blood nucleated cells. (c) Peripheral white blood cell count of recipients after transplantation. Data are mean ± SD (n = 4 each; *P < 0.05). (d) Splenomegaly observed in recipients at day 19 after transplantation (scale bar, 10 mm). Spleen weights are presented as mean ± SD (WT, n = 9; KO, n = 6; *P < 0.01). (e) Survival of recipients transplanted with BCR-ABL-transduced WT cells or BCR-ABL-transduced C/EBPβ KO cells (WT, n = 12; KO, n = 11; *P = 0.0005).

**Figure 6.** Involvement of C/EBPβ in BCR-ABL-mediated loss of immature hematopoietic cells.

Flow cytometric analysis of GFP⁺ bone marrow cells from recipients 19 days after
transplantation of BCR-ABL-transduced WT cells or BCR-ABL-transduced C/EBPβ KO cells (a and b). Numbers in quadrants indicate the percentages within GFP+ bone marrow cells (WT, n = 2; KO, n = 3; *P = 0.01). (c and d) Survival of secondary transplantation recipients. 2 × 10^6 (e; WT, n = 7; KO, n = 7) or 1 × 10^6 BCR-ABL-transduced GFP+ cells (d; WT, n = 4; KO, n = 5) from the first recipients were transplanted. (e) GFP+ cell chimerism on day 38 after the secondary transplantation of BCR-ABL-transduced WT cells or BCR-ABL-transduced C/EBPβ KO cells (n = 8 each; *P = 0.013). (f) The frequencies of leukemia-initiating cells in bone marrow of primary recipient mice transplanted with BCR-ABL-transduced WT cells or BCR-ABL-transduced C/EBPβ KO cells.
Figure 1

(a) Healthy donor and CP-CML cells stained for CD34 and CD38, showing a distinct distribution of HSCs and Lin- cells.

(b) Graph comparing the percentage of CD34+CD38- / Lin- cells in Healthy donor and CP-CML, with significant difference indicated by *.

(c) Comparison of CMP, GMP, and MEP cells in Healthy donor and CP-CML, showing increased percentage of Lin- cells in CP-CML.

(d) Bar chart showing relative expression of C/EBPβ mRNA in HSC, CMP, GMP, and MEP cells, with Healthy donor and CP-CML groups, and significant differences indicated by * and **.
Figure 2

(a) Control vs. BCR/ABL at Day 0 and Day 5.

(b) Flow cytometry analysis of C-kit and CD11b expression.

(c) Relative expression of C/EBPβ mRNA in EML.

(d) Relative expression of C/EBPβ mRNA in EML.

(e) Western blot analysis of C/EBPβ and GAPDH.

(f) Relative expression of C/EBPβ mRNA in EML under DMSO and Imatinib treatment.
Figure 3
Figure 4
Figure 5
Supplementary Figure Legend

**Figure S1.** Frequency of myeloid progenitors in bone marrow of healthy donors and patients with CP-CML.

Frequency of CD34+ CD38+ myeloid progenitors within the Lin− fraction of bone marrow cells. Error bars indicate SD (healthy donors, n = 6; CP-CML, n = 5; *P < 0.05).

**Figure S2.** BCR-ABL-transduced C/EBPβ KO cells could be replated at least up to 5 times.

Serial colony-replating of 1 × 10⁴ BCR-ABL-transduced C/EBPβ WT and KO bone marrow cells. The colonies were counted and collected on day 10 (2nd) or 14 (3rd, 4th, and 5th), respectively. Error bars indicate SD from triplicate cultures.

**Figure S3.** C/EBPβ expression in EML cells and 32Dcl3 cells.

C/EBPβ mRNA levels in EML cells and 32Dcl3 cells. Results were mean values of two independent experiments and normalized to the expression level of EML cells. Error bar indicates SD. *P < 0.01.
Supplementary Figure S1

Healthy donor  CP-CML

%CD34+CD38+ / Lin cells

*
Supplementary Figure S3

C/EBPβ mRNA Relative expression

0 2.5 5 7.5 10

EML 32Dcl3

*
**Supplementary Table 1.** The characteristics of the patients

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CP, chronic phase; WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; PLT, platelet. NA indicates not available.