MIP-1α/CCL3-mediated maintenance of leukemia-initiating cells in the initiation process of chronic myeloid leukemia

Tomohisa Baba,[1] Kazuhito Naka,[2] Soji Morishita,[4] Norio Komatsu,[5] Atsushi Hirao,[3] and Naofumi Mukaida[1]

[1]Division of Molecular Bioregulation, [2]Exploratory Project on Cancer Stem Cells, and [3]Division of Molecular Genetics, Cancer Research Institute, Kanazawa University, Kanazawa 920-1192, Ishikawa, Japan
[4]Department of Transfusion Medicine and Stem Cell Regulation and [5]Department of Hematology, Juntendo University School of Medicine, Tokyo 113-8421, Japan

In the initiation process of chronic myeloid leukemia (CML), a small number of transformed leukemia-initiating cells (LICs) coexist with a large number of normal hematopoietic cells, gradually increasing thereafter and eventually predominating in the hematopoietic space. However, the interaction between LICs and normal hematopoietic cells at the early phase has not been clearly delineated because of the lack of a suitable experimental model. In this study, we succeeded in causing a marked leukocytosis resembling CML from restricted foci of LICs in the normal hematopoietic system by direct transplantation of *BCR-ABL* gene–transduced LICs into the bone marrow (BM) cavity of nonirradiated mice. Herein, we observed that BCR-ABL$^+$lineage$^-$c-kit$^-$ immature leukemia cells produced high levels of an inflammatory chemokine, MIP-1α/CCL3, which promoted the development of CML. Conversely, ablation of the *CCL3* gene in LICs dramatically inhibited the development of CML and concomitantly reduced recurrence after the cessation of a short-term tyrosine kinase inhibitor treatment. Finally, normal hematopoietic stem/progenitor cells can directly impede the maintenance of LICs in BM in the absence of CCL3 signal.

CORRESPONDENCE
Tomohisa Baba:
sergenti@staff.kanazawa-u.ac.jp

Abbreviations used: Ab, antibody; CML, chronic myeloid leukemia; HSC, hematopoietic stem cell; HSPC, hematopoietic stem/progenitor cell; LIC, leukemia-initiating cell; MPN, myeloproliferative neoplasm; PB, peripheral blood; SP, spleen; TKI, tyrosine kinase inhibitor; WBC, white blood cell.

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm (MPN) resulting from the neoplastic transformation of hematopoietic stem cells (HSCs). CML undergoes a triphasic process, a chronic phase, an accelerated phase, and a terminal blast crisis (Lahaye et al., 2005). More than 90% of CML cases are associated with the presence of the Philadelphia chromosome. This chromosome arises from a reciprocal translocation between chromosomes 9 and 22 and forms the breakpoint cluster region with a constitutively activated tyrosine kinase, BCR-ABL fusion protein (Ren, 2005; Melo and Barnes, 2007). This protein is a pathogenic protein in CML (Sawyers, 1999), and maintenance of BCR-ABL–expressing leukemia-initiating cells (LICs) in the BM is crucial for initiating the chronic phase of CML (Koschmieder et al., 2005).

Zhang et al. (2012) observed several characteristic changes in the BM microenvironment of mice developing CML-like myeloproliferative disease, such as BM hypercellularity and myeloid cell infiltration into spleen (SP). Moreover, they detected an altered chemokine/cytokine expression pattern in the BM, including down-regulation of SDF-1/CXCL12 and up-regulation of MIP-1α/CCL3, MIP-1β/CCL4, IL-1α, IL-1β, and TNF. They further obtained similar observations on human CML patients. Based on these observations, they proposed that altered chemokine/cytokine expression in BM may contribute to the preferential proliferation of LICs in the BM microenvironment, to displace the normal hematopoietic cells, although they did not clarify the molecular and cellular mechanisms in more detail.

Chemokines are produced by a wide variety of hematological and stromal cells and exhibit diverse activities on various types of BM-derived cells. Evidence is accumulating to indicate that a CC chemokine, MIP-1α/CCL3,

The Rockefeller University Press $30.00
J. Exp. Med. 2013 Vol. 210 No. 12 2661-2673
www.jem.org/cgi/doi/10.1084/jem.20130112

The Journal of Experimental Medicine

has direct inhibitory activities on normal hematopoietic stem/progenitor cell (HSPC) growth (Graham et al., 1990; Dunlop et al., 1992; Maze et al., 1992; Broxmeyer et al., 1993). Induction of BCR-ABL expression in vivo can cause the aberrant expression of CCL3 in the BM (Zhang et al., 2012). Moreover, CCL3-mediated signal can regulate the in vitro proliferation of normal HSPCs and LICs in distinct ways (Eaves et al., 1993; Chasty et al., 1995), depending on the kinase activity of Abl protein (Wark et al., 1998). Furthermore, IFN-α–induced CCL3 production by BM-derived stromal cells enhanced β1 integrin–dependent adhesion of LICs to the stromal cells to restore normal hematopoiesis in CML (Bhatia et al., 1995). These observations suggest that CCL3 can contribute to the interaction between LICs and normal hematopoietic system in the initiation process of CML development (Zhang et al., 2012), but its precise roles remain unclear because of the lack of a suitable experimental model.

Murine CML-like myeloproliferative disease can be induced by transferring human-derived *BCR-ABL* oncogene–transduced primitive BM cells to a lethally irradiated host (Pear et al., 1998; Li et al., 1999). This experimental model has been widely used to examine the in vivo leukemogenic role of the *BCR-ABL* oncogene in CML development. However, in this model, lethal irradiation completely breaks down the normal hematopoietic system to enable intravenously injected BCR-ABL$^+$ leukemic cells to home to the BM to grow and develop CML. Thus, this model is not helpful in elucidating the role of the BM microenvironment in CML development. Furthermore, lethal irradiation induced a temporal leukopenia, a condition that can have a profound impact on CML pathology by compensatory overproduction of various growth factors (Singh et al., 2012). Hence, to observe the course of CML development under the steady-state, an inducible *BCR-ABL* transgenic mouse, which can express the *BCR-ABL* gene under the control of a Tet-regulated 3′ enhancer of the murine stem cell leukemia gene, was established (Koschmieder et al., 2005). This well-designed transgenic model enables the study of the function of LICs in the condition closely resembling that in CML patients. However, in this experimental model, it is not easy to selectively tag leukemia cells with mutated *BCR-ABL* gene for the examination of leukemia cell trafficking. Moreover, it is laborious to introduce a gene mutation into either leukemia cells or normal hematopoietic cells.

To circumvent these problems, we initially attempted to establish an experimental CML model under nonirradiated conditions. We transduced c-kit$^+$lineage$^-$Sca-1$^+$ (KLS$^+$) HSPCs with *BCR-ABL* oncogene using retroviral vector and injected the resultant cells directly into the BM cavity in nonirradiated immune-deficient nude mice. In the early phase of this model, only <500 BCR-ABL$^+$KLS$^+$ LICs are presumed to coexist with a large number of normal residual hematopoietic cells. However, this procedure succeeded in the development of a CML-like disease with a marked leukocytosis and splenomegaly in nonirradiated and BM-preserved host. Moreover, LICs moved to the contralateral site of BM while

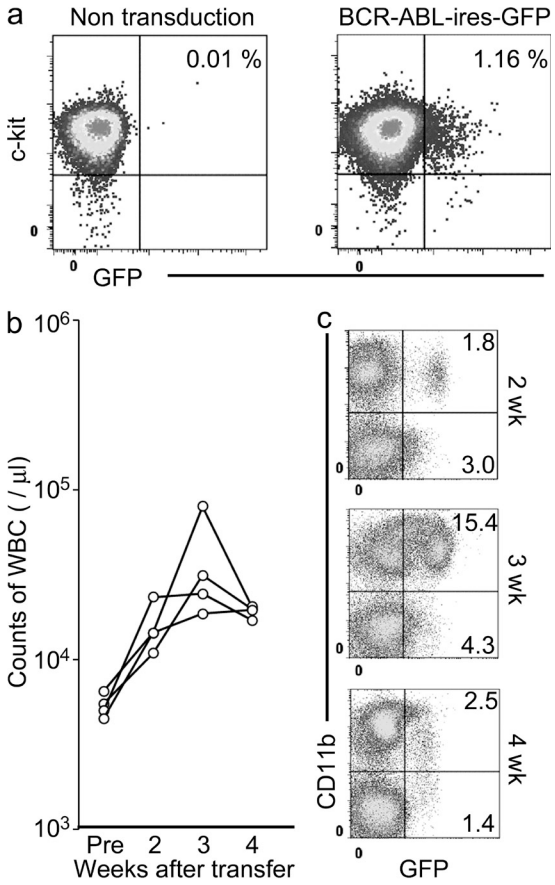

Figure 1. Transient CML-like leukocytosis in WT recipients. (a) At 4 d after the retroviral infection, BCR-ABL transduction efficiency was determined with GFP signal. Percentage of c-kit$^+$GFP$^+$ cells is shown in each panel. Representative results from three independent experiments are shown. (b) WT donor–derived LICs were transplanted directly into the BM cavity of nonirradiated WT mice. WBC numbers were determined at the indicated time points. Each symbol connected with solid lines represents an individual mouse (n = 4). (c) Expression of CD11b and GFP (BCR-ABL-ires-GFP) in WBCs was determined at the indicated time points. Percentages of CD11b$^+$GFP$^+$ and CD11b$^-$GFP$^+$ cells are shown in each panel. Representative results from four independent experiments are shown.

expanding in the injected site of BM. Thus, this novel model is quite helpful to clarify the interaction between normal hematopoietic system and leukemic cells, particularly in the early phase of CML development, and the trafficking of LICs to other hematopoietic tissues. By using this model, we have obtained definitive evidence to indicate an indispensable role of leukemia cell–derived CCL3 in the maintenance of LICs in BM for subsequent CML development.

RESULTS

Intra-BM injection of LICs temporarily causes leukocytosis in immune-competent recipient mice

Purified KLS$^+$ cells were infected with the retrovirus carrying MSCV-BCR-ABL-ires-GFP as previously described (Naka et al., 2010). At 4 d after the infection, ∼1% of c-kit$^+$ cells expressed GFP reporter protein (Fig. 1 a), and the positive cell

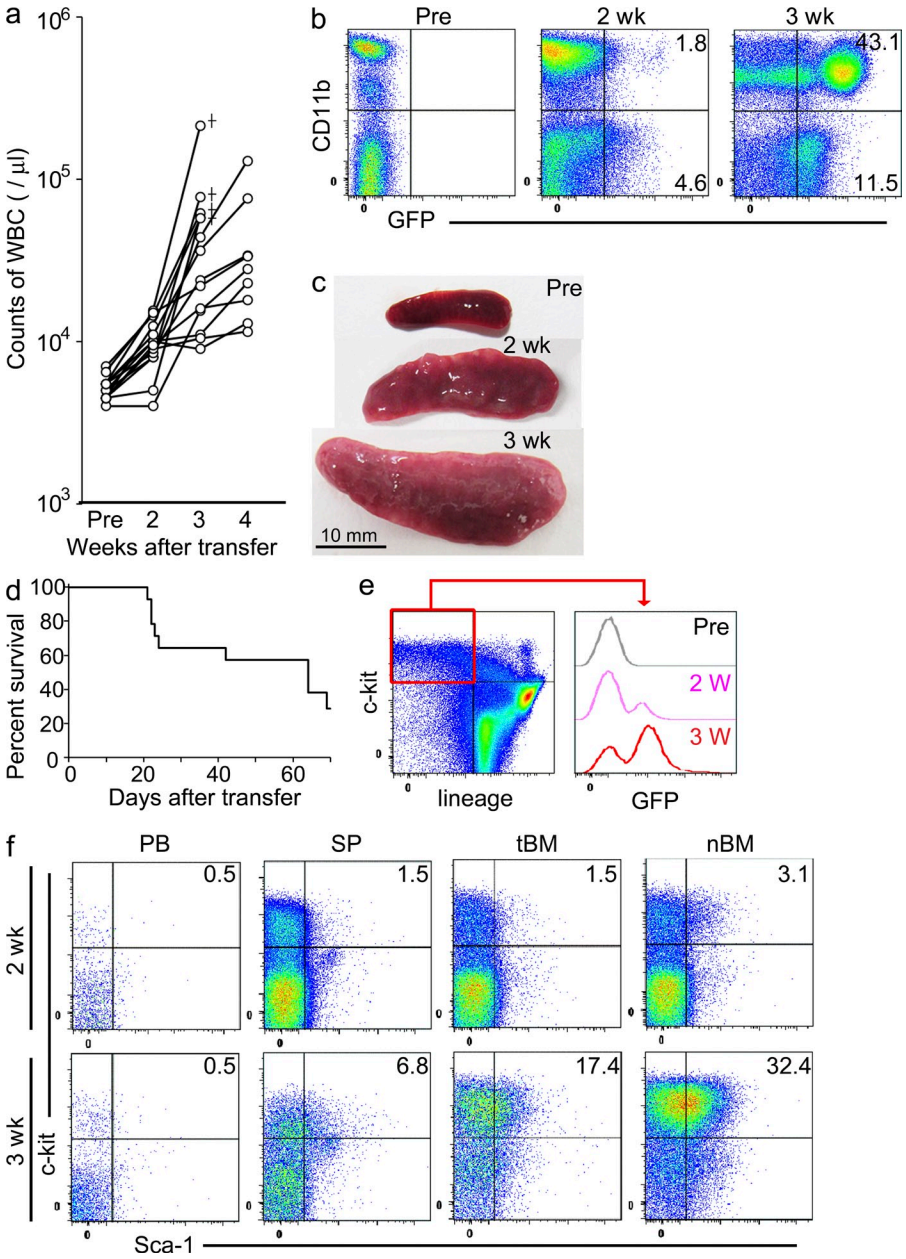

Figure 2. CML-like MPN development in immune-deficient recipients. WT donor-derived LICs were transplanted directly into the BM cavity of nonirradiated nude mice. (a) WBC numbers were determined at the indicated time points. Each symbol connected with solid lines represents an individual mouse (n = 14). A cross symbol indicates a mouse that died within 4 wk. (b) Expression of CD11b and GFP in WBCs was determined at the indicated time points. Percentages of $CD11b^+GFP^+$ and $CD11b^-GFP^+$ cells are shown in each panel. Representative results from 12 independent experiments are shown. (c) Macroscopic appearance of the recipient SP at the indicated time points. Representative results from three (Pre) or five (2 and 3 wk) independent experiments are shown here. (d) Survival rates within 70 d after BM transfer (n = 12). (e) $Lineage^-c\text{-}kit^+$ cells among total BM cells were gated to analyze the expression of GFP at the indicated time points. Representative results from five independent experiments are shown. (f) The expression of c-kit and Sca-1 among $lineage^-GFP^+$ cells was analyzed on total PB, SP, transferred (t), and nontransferred (n) BM cells at 2 or 3 wk after BM transfer. Percentage of $c\text{-}kit^+Sca\text{-}1^+$ LICs is shown in each panel. Representative results from five independent experiments are shown.

proportion did not increase by 7 d after the infection (not depicted). Because we injected ~4×10^4 total KLS^+ cells into the each BM cavity, we estimated that 400–500 resultant BCR-ABL-expressing LICs could be injected into each mouse. In the beginning, we injected LICs directly into the BM cavity of nonirradiated WT mice. The white blood cell (WBC) number in peripheral blood (PB) increased until 2–3 wk after the transplantation, decreasing thereafter (Fig. 1 b). $CD11b^+GFP^+$ leukemia cells consistently but transiently appeared in the PB from 2–3 wk, diminishing at 4 wk (Fig. 1 c).

Intra-BM injection of LICs can cause CML-like MPN in immune-deficient recipient mice

Given the capacity of exogenous proteins such as human BCR-ABL and GFP to induce an immune response in nonirradiated immune competent mice (Pear et al., 1998; Stripecke et al., 1999; Mumprecht et al., 2009), we presumed that $BCR\text{-}ABL^+$ leukemia cells could not survive T cell–dependent immune response in the WT recipient mice. To overcome this obstacle, we injected LICs directly into the BM cavity of a nonirradiated athymic nude mouse lacking T cell–dependent immune response. When LICs were given into the BM cavity, all nude mice developed a CML-like leukocytosis (Figs. 2 a and 3 a) and GFP^+ LIC-derived cells, particularly $Gr\text{-}1^+CD11b^+GFP^+$ cells, predominated in the PB (Figs. 2 b and 3 a). Splenomegaly, a pathognomonic sign, was evident in all nude mice receiving LICs (Fig. 2 c), and death ensued within 2 mo in most of the nude mice (Fig. 2 d). Moreover, histopathological examination demonstrated extramedullary hematopoiesis in the

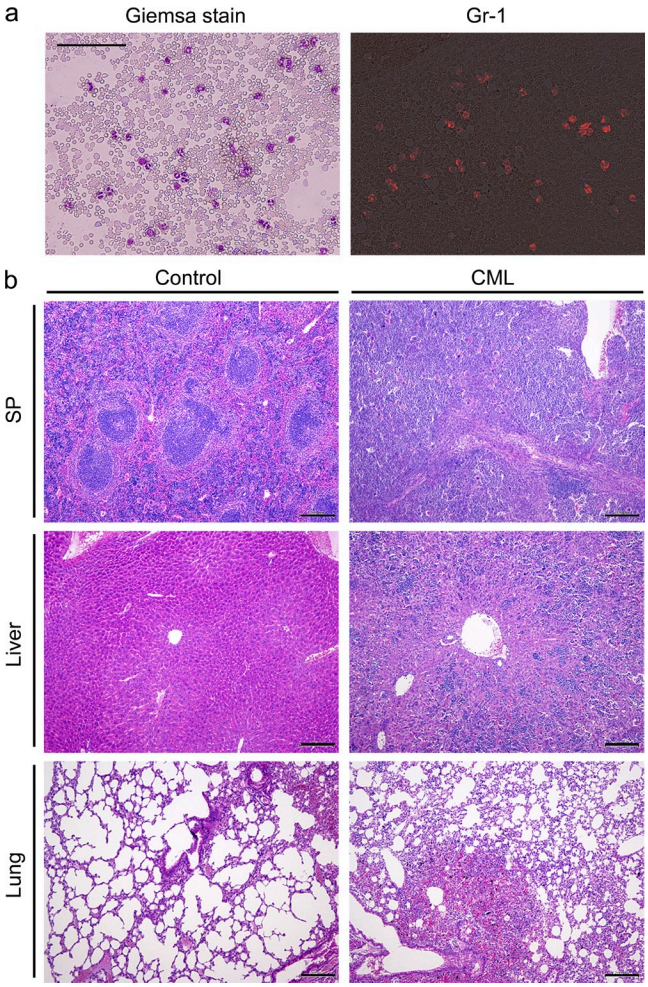

Figure 3. Histopathological analysis after LIC transfer to nonirradiated nude mice. (a) Whole blood was obtained at 3 wk after LIC transfer to nonirradiated nude mouse BM and was subjected to Giemsa stain (left) and fluorescent immunostaining for Gr-1 (right). (b) SP, liver, and lung tissues were harvested from normal nude mice and nude mice developing CML-like MPN and were subjected to H&E staining. Bars: (a) 100 µm; (b) 200 µm. Representative results from three independent experiments are shown.

SP and liver and abnormal leukocyte infiltration in the lung (Fig. 3 b). LIC injection into the BM cavity caused a progressive replacement of lineage$^-$c-kit$^+$ BM progenitors with BCR-ABL$^+$ cells, as indicated by an increase in GFP-positive cells in the lineage$^-$c-kit$^+$ population (Fig. 2 e). Furthermore, BCR-ABL$^+$KLS$^+$ LICs were distributed in the contralateral nontransferred BM as well as the transferred BM (Fig. 2 f). Thus, the present model can recapitulate a CML-like MPN in nonirradiated recipients.

Expression of an inflammatory chemokine, CCL3, is enhanced during the process of CML development

In the early phase of the present model, normal hematopoietic cells were preserved in the BM and PB. Given the accumulating evidence to indicate the effects of chemokines on BM HSPC mobilization (Pelus and Fukuda, 2008), we examined inflammatory chemokine expression in the serum of mice developing CML. Consistent with the previous report that MIP-1α/CCL3 expression is positively regulated by BCR-ABL expression (Zhang et al., 2012), we observed that serum CCL3 transiently increased at 2 wk after BM transplantation of LICs (Fig. 4 a). On the contrary, other chemokines such as CXCL2, CCL2, CCL4, and CCL5 did not change significantly (Fig. 4 a). Flow cytometric analysis demonstrated that CCL3 was mainly expressed by BCR-ABL$^+$lineage$^-$c-kit$^-$ immature leukemia cells and, to a lesser degree, by BCR-ABL$^-$ lineage$^-$c-kit$^-$ cells in the BM (Fig. 4 b). Moreover, these populations expressed constitutively high levels of CCL3 during CML development in the BM (not depicted) despite a transient increase in the serum concentration (Fig. 4 a).

Crucial contribution of leukemia cell–derived CCL3 to the early phase of CML development

When KLS$^+$ cells purified from CCL3$^{-/-}$ mice were transduced with the *BCR-ABL* gene, the transduction efficiency was ∼1.5% (not depicted), similarly as observed when using WT mouse–derived KLS$^+$ cells. However, CCL3$^{-/-}$-derived LICs failed to induce CML-like leukocytosis (Fig. 5 a) and splenomegaly (Fig. 5 b) when administered to the nonirradiated nude mouse BM cavity. A small number of CD11b$^+$GFP$^+$ leukemia cells appeared in the PB at 3 wk after BM transplantation but disappeared by 4 wk (Fig. 5 c). GFP$^+$lineage^{high} mature leukemia cells were generated from CCL3$^{-/-}$-derived as well as WT-derived LICs at 2 wk after the transplantation, but this cell population disappeared in BM transplanted with CCL3$^{-/-}$- but not WT-derived LICs at 3 wk after the transplantation (Fig. 5 d). Moreover, CCL3$^{-/-}$-derived GFP$^+$lineage$^-$ immature leukemia cells in BM and KLS$^+$ LICs in SP and BM markedly decreased compared with WT-derived cells (Fig. 5, d and e). To exclude the possibility of the defect in the leukemogenesis in CCL3$^{-/-}$-derived LICs by the gene modification, we extrinsically injected CCL3 into nude mice that were transplanted with CCL3$^{-/-}$-derived LICs (illustrated in Fig. 6 a). The administration of CCL3 restored most of the CML phenotypes such as CML-like leukocytosis (Fig. 6 b), splenomegaly (Fig. 6 c), and the maintenance of GFP$^+$ LICs in BM (Fig. 6 d). After the termination of CCL3 administration, three out of five mice succumbed to leukemia within 8 wk, whereas all five PBS-treated mice survived (Fig. 6 e). Moreover, CCL3$^{-/-}$-derived LICs induced a CML-like MPN in irradiated recipients, accompanied with leukocytosis containing CD11b$^+$GFP$^+$ cells and splenomegaly, but to a lesser degree than WT LICs did (Fig. 7, a–c). However, the LIC numbers in BM were much less with the use of CCL3$^{-/-}$-derived LICs than that of WT LICs (Fig. 7 d). Thus, CCL3 can be crucial for the early phase of CML development, at least in part by maintaining LICs in the BM.

Crucial contribution of CCL3 to recurrence of CML after the cessation of tyrosine kinase inhibitor (TKI) treatment

Because CCL3$^{-/-}$-derived LICs can only reproduce the CML-like MPN in irradiated WT mice (Fig. 7) but not in nonirradiated nude mice (Fig. 5), we next examined the synergistic

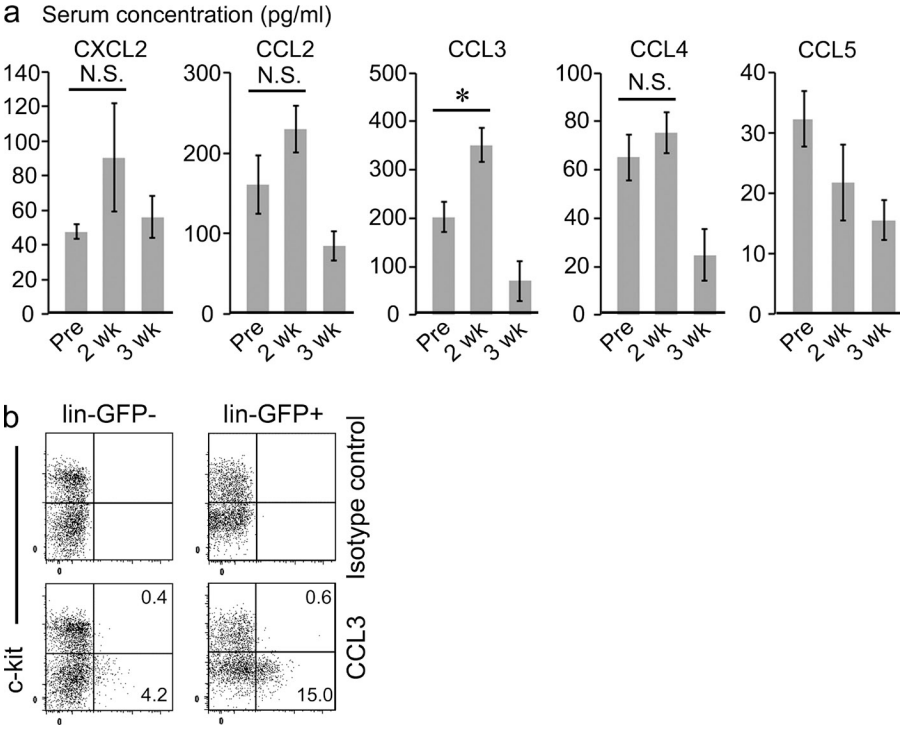

Figure 4. Enhanced CCL3 expression during CML development. WT donor–derived LICs were transplanted directly into the BM cavity of nonirradiated nude mice. (a) Sera were collected at the indicated time points after transplantation. The concentrations of KC/CXCL2, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5 were determined using the Bioplex system. Data represent mean ± SD from three independent experiments. P-values were calculated with the unpaired Student's *t* test: *, $P < 0.01$; N.S., no significant difference. (b) Total BM cells were isolated at 2–3 wk after transplantation of WT LICs and were subjected to the intracellular CCL3 staining. Lineage$^-$GFP$^-$ and lineage$^-$GFP$^+$ cells among total BM cells were gated to analyze the expression of c-kit and intracellular CCL3. PE-conjugated rat IgG2a was used as an isotype control. Percentages of c-kit$^+$CCL3$^+$ and c-kit$^-$CCL3$^+$ cells are shown in each panel. Representative results from three independent experiments are shown here.

effects of *CCL3* gene loss on TKI treatment using irradiated WT mice. Daily treatment with a TKI, imatinib, from 8 d after LIC transplantation, reduced CML-like leukocytosis and splenomegaly (Fig. 7, a and c), but LICs survived in the BM until 3 wk, regardless of the loss of *CCL3* gene (Fig. 7 d). However, survival after the cessation of imatinib was significantly prolonged in the mice injected with CCL3$^{-/-}$ mouse–derived LICs compared with those injected with WT mouse–derived LICs (Fig. 7 e). Moreover, immediately after the cessation of imatinib treatment, CCL3$^{-/-}$ mouse–derived LICs were present in the BM at similar levels as WT mouse–derived LICs, but the numbers were remarkably decreased in the mice surviving until 60 d after the injection (Fig. 7 d). Thus, CCL3 can also crucially contribute to the recurrence of CML after TKI discontinuation by supporting the maintenance of LICs in BM.

CCR1 and CCR5 expressed on both donor and recipient cells are indispensable for the maintenance of LICs in BM

Leukocytosis persisted with the appearance of LIC-derived cells in PB until 3 wk after intra-BM injection of LICs into immune competent WT mice (Fig. 1). Moreover, when WT mouse–derived BCR-ABL–transduced KLS$^+$ cells were transferred into nonirradiated WT mice, splenomegaly transiently appeared, reaching a maximum at 2 wk after the transplantation (not depicted). Concomitantly, lineage$^-$GFP$^+$ cells were abundant in the BM (Fig. 8 a) at 2 wk after the transplantation. On the contrary, the transplantation of CCL3$^{-/-}$–derived and BCR-ABL–transduced KLS$^+$ cells yielded reduced lineage$^-$GFP$^+$ cell numbers in BM (Fig. 8 a). Because these observations paralleled the results with nude mice, we assume that it is reasonable to compare the graft efficiency of LICs at 2 wk after BM transplantation to nonirradiated BALB/c mice and those deficient in specific receptor genes for *CCL3*, *CCR1*, or *CCR5*. After transplantation, WT-derived GFP$^+$lineage$^-$ immature cells and GFP$^+$KLS$^+$ LICs were efficiently maintained in the CCL3$^{-/-}$ recipient BM as well as in the WT one (Fig. 8, c and d), suggesting that donor cell–derived CCL3 is sufficient for their maintenance. In contrast, when WT-derived LICs were transferred into the BM of nonirradiated CCR1$^{-/-}$ or CCR5$^{-/-}$ mice, both strains failed to maintain GFP$^+$lineage$^-$ immature cells and GFP$^+$KLS$^+$ LICs (Fig. 8, c and d). Thus, the maintenance of LICs may require CCL3-mediated signals through both CCR1 and CCR5 expressed on BCR-ABL$^-$ normal recipient cells. However, the transfer of CCR1$^{-/-}$ or CCR5$^{-/-}$ mouse–derived LICs failed to maintain GFP$^+$lineage$^-$ immature cells and GFP$^+$KLS$^+$ LICs even in the WT recipients (Fig. 8, c and d), suggesting the requirement of CCR1 and CCR5 expression on both donor and recipient cells for the maintenance of LICs. However, BCR-ABL infection generated GFP$^+$lineage$^+$ mature cells at a similar proportion among all strains (Fig. 8 b). These observations would indicate that LICs were successfully transplanted but could not be maintained to generate mature leukemia cells in the absence of CCL3-mediated signals.

Normal HSPCs can potentially inhibit the occupation of BM by LICs in the absence of CCL3 signal

We observed that CCR1 and CCR5 expressed on both donor and recipient cells were required for the maintenance of LICs (Fig. 8 d). Considering a low efficiency of BCR-ABL transduction (Fig. 1 a), a small number of BCR-ABL$^+$KLS$^+$

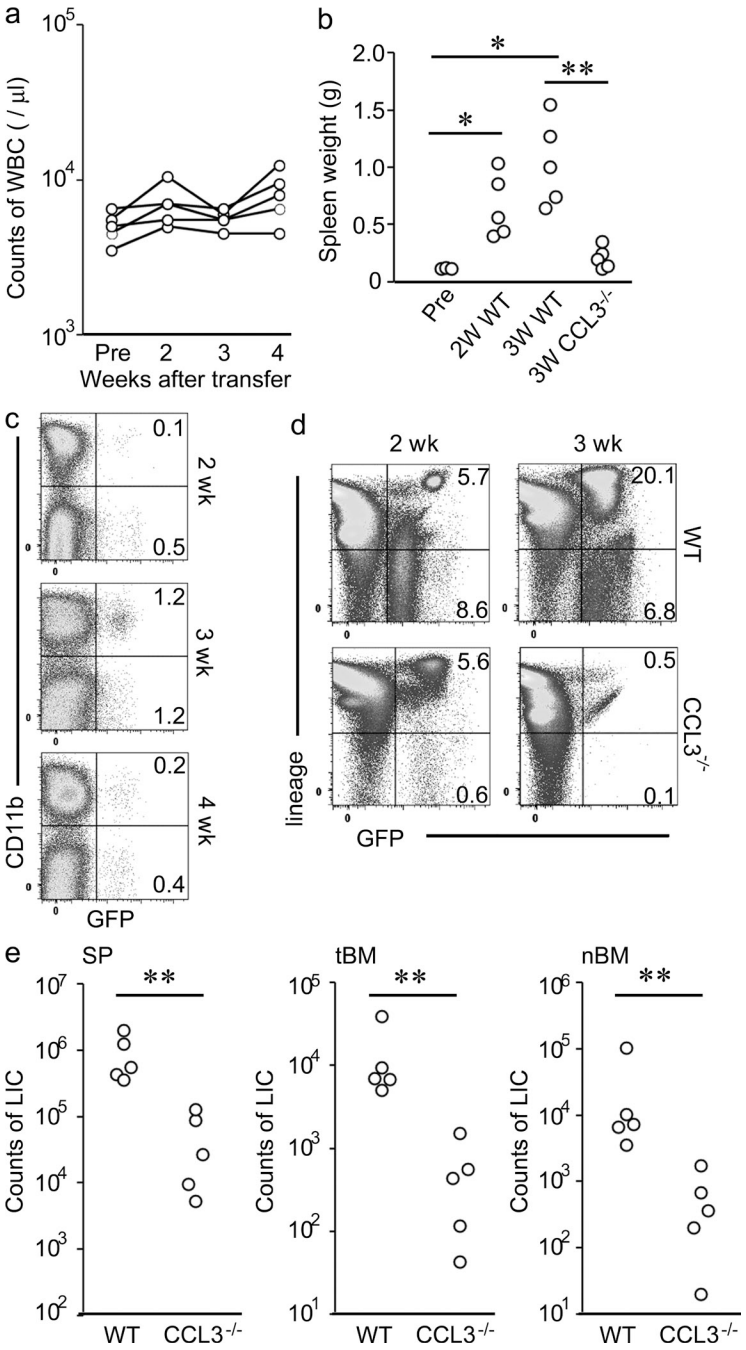

Figure 5. CCL3-deficient LICs fail to develop CML-like MPN. WT or CCL3$^{-/-}$ donor-derived LICs were transplanted directly into the BM cavity of nonirradiated nude mice. (a and b) The number of WBCs (a) and SP weights (b) were determined at the indicated time points after transplantation of CCL3$^{-/-}$ LICs. Each symbol represents an individual mouse (n = 5). (c) CD11b and GFP expression in WBCs was determined at the indicated time points after transplantation of CCL3$^{-/-}$ LICs. Percentages of CD11b^{+}GFP^{+} and CD11b^{-}GFP^{+} cells are shown in each panel. Representative results from five independent experiments are shown. (d) Lineage marker and GFP expression in total BM cells was determined at the indicated time points after the transplantation of WT or CCL3$^{-/-}$ LICs. Percentages of lineage^{high}GFP^{+} and lineage^{low}GFP^{+} cells are shown in each panel. Representative results from five independent experiments are shown. (e) The number of GFP^{+}KLS^{+} LICs in SP, transferred (t), and nontransferred (n) BM were determined at 3 wk after transplantation of WT- or CCL3$^{-/-}$-derived LICs. Each symbol represents an individual mouse (n = 5). P-values were calculated with the Mann–Whitney's U test (b and e): *, $P < 0.05$; **, $P < 0.01$.

LICs coexist with a relatively larger numbers of donor KLS^{+} cells (illustrated in Fig. 9 a). Thus, we assumed that LICs should survive the competition with both recipient- and donor-derived normal hematopoietic cells by using the CCL3/CCR1 and CCL3/CCR5 axes. To test this hypothesis, WT LICs were mixed with an equal number of nontransduced WT, CCR1$^{-/-}$, or CCR5$^{-/-}$ KLS^{+} cells, and the resultant cell population was injected into the BM cavity of nonirradiated nude mice (illustrated in Fig. 9 b). WT LICs efficiently induced CML-like leukocytosis and splenomegaly in the absence of competitive cells (Fig. 9, c and d). The admixture of nontransduced CCR1$^{-/-}$- and CCR5$^{-/-}$-derived KLS^{+} cells, but not WT-derived KLS^{+} cells, attenuated leukocytosis (Fig. 9 c) and splenomegaly (Fig. 9 d) and markedly reduced the numbers of LICs in BM (Fig. 9 e). Thus, the interaction of leukemia cell–derived CCL3 with CCR1 and CCR5 expressed on normal hematopoietic cells can confer LICs the advantage to survive in BM in a paracrine manner.

CCL3 can mobilize normal HSPCs expressing CCR1 and CCR5 from the BM to PB

At 3 wk after the transplantation with WT-derived LICs, the numbers of GFP-nonexpressing total cells and lineage^{-}c-kit^{+} progenitor cells in BM were markedly reduced, which were

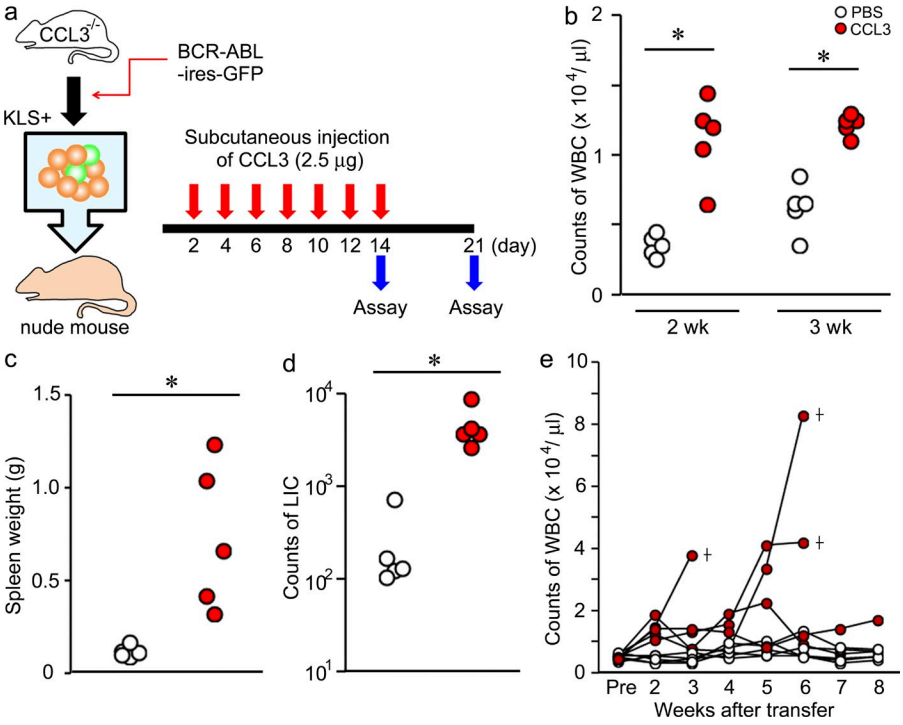

Figure 6. Administration of CCL3 recovers the leukemogenic capability of CCL3$^{-/-}$-derived LICs. (a) Nonirradiated nude mice were transplanted with CCL3$^{-/-}$ donor–derived LICs and were subcutaneously injected with human recombinant CCL3 (Lu et al., 2003; provided by Ohtsuka Pharmaceutical Co., Ltd.) at the indicated time points. PBS was injected as a control. (b–d) The numbers of WBCs (b), SP weights (c), and numbers of GFP$^+$KLS$^+$ LICs in the transferred BM (d) were determined (b, 2 and 3 wk; c and d, 3 wk). P-values were calculated with the Mann–Whitney's *U* test: *, $P < 0.01$. (e) Alteration of the numbers of WBCs within 8 wk after BM transfer. A cross symbol indicates a mouse that died within 8 wk. Each symbol represents an individual mouse ($n = 5$).

attenuated when CCL3$^{-/-}$-derived LICs were transplanted (Fig. 10 a). Likewise, the reduction of GFP-nonexpressing BM progenitor cells was attenuated when WT-derived LICs were transplanted into CCR1$^{-/-}$ and CCR5$^{-/-}$ mice (not depicted). CCR1 and CCR5 were constitutively expressed on lineage$^-$c-kit$^+$ progenitor cells as well as lineage$^+$ mature leukocytes in nontreated WT mice (Fig. 10 b). Moreover, BCR-ABL$^-$ normal BM progenitor cells expressed both CCR1 and CCR5. However, CCR5 but not CCR1 expression was abrogated in BCR-ABL$^+$ progenitor cells in the BM of CML mice (Fig. 10, c and d). Thus, CCL3 may interact with CCR5 and CCR1 expressed on normal BM progenitor cells. The capacity of CCL3 to mobilize HSPCs from the BM (Lord et al., 1995; Broxmeyer et al., 1999) prompted us to examine the effects of intra-BM injection of CCL3. This treatment decreased the number of lineage$^-$c-kit$^+$ progenitors in BM by half in WT mice, but not in CCR1$^{-/-}$ or CCR5$^{-/-}$ mice (Fig. 10 e). Moreover, the circulating lineage$^-$c-kit$^+$ progenitor cells significantly increased in the PB after intra-BM injection of CCL3 (Fig. 10 f). However, intra-BM injection of CCL3 did not change the composition of long-term HSCs, short-term HSCs, and multipotent progenitors among lineage$^-$c-kit$^+$ BM progenitors (not depicted). Thus, CCL3 can interact with CCR1 and CCR5 expressed on normal HSPCs to accelerate their mobilization from BM to PB.

DISCUSSION

In the present study, we established a novel mouse model developing CML-like MPN in a nonirradiated host with preserved BM architecture. Previous CML mouse models have difficulties in elucidating the interaction between normal hematopoietic cells and LICs because of irradiation-induced destruction of BM architecture, whereas our model can facilitate the analysis on this point, particularly at the early phase of CML development. By using this model, we demonstrated that an inflammatory chemokine, CCL3, was expressed abundantly at the early phase of CML development and that BCR-ABL-expressing lineage$^-$ immature leukemia cells were a major source of CCL3. Moreover, we showed that CCL3-deficient BCR-ABL–expressing LICs failed to populate the BM and could not cause CML-like leukocytosis and splenomegaly when given directly into the BM of nude mice. Furthermore, WT mouse–derived LICs exhibited reduced capacity to survive in the BM of mice deficient in CCR1 or CCR5, specific receptors for CCL3, even when BCR-ABL–expressing LICs were directly injected into the BM of these mice. These observations implicate the crucial involvement of the interaction between CCL3 and its receptors, CCR1 and CCR5, in the initiation of CML development.

Another chemokine, CXCL12, is abundantly present in the BM and has diverse effects on BM HSPCs (Aiuti et al., 1997; Zou et al., 1998). Basu and Broxmeyer (2009) recently demonstrated that CCL3, CCL4, and CCL5 can enhance CXCL12-induced chemotaxis and concomitantly reduce CXCL12-induced adhesion to VCAM-1 of human HSPCs. This may account for the capacity of CCL3 and its genetically engineered variant, BB-10010, to potently and rapidly mobilize HSPCs from the BM to PB (Lord et al., 1995). We also observed that intra-BM injection of CCL3 efficiently induced the mobilization of HSPCs from BM to PB in WT but not CCR1$^{-/-}$ or CCR5$^{-/-}$ mice. These observations would indicate that CCL3 can act cooperatively on CCR1 and

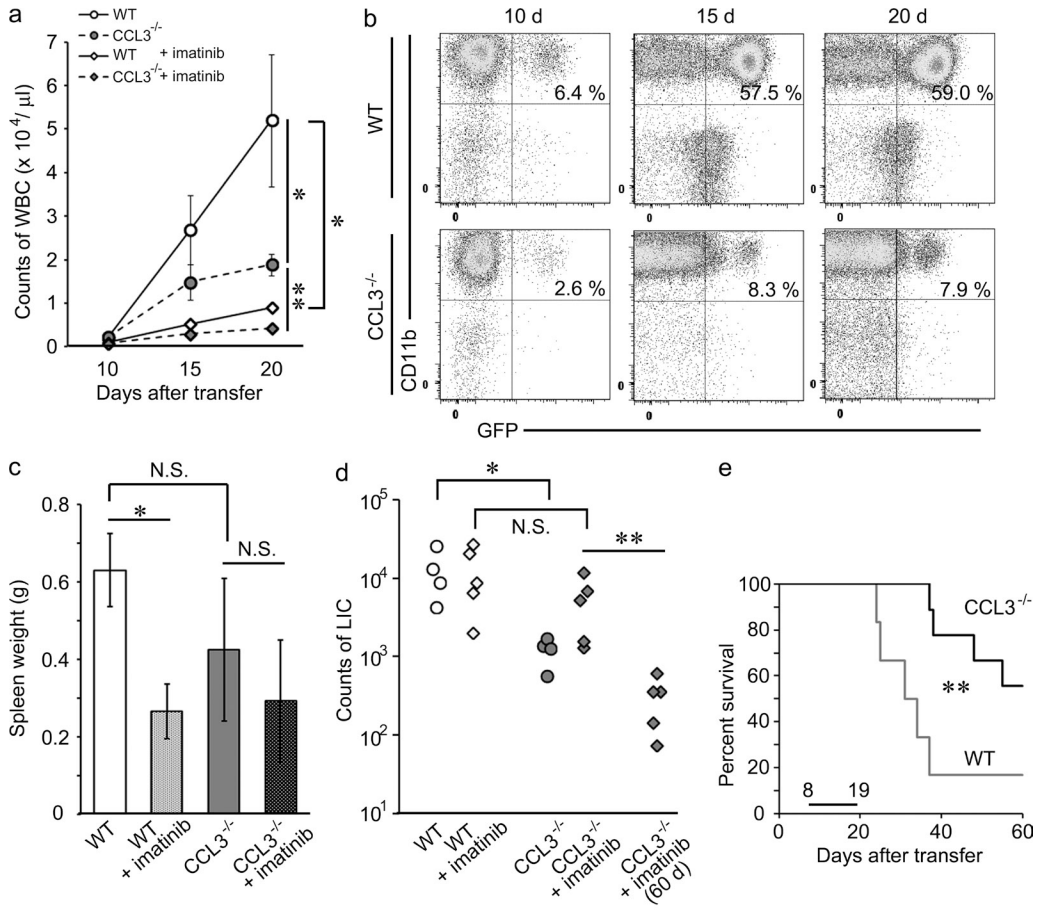

Figure 7. Combined effects of TKI and loss of *CCL3* gene on the irradiated CML model. WT or CCL3$^{-/-}$ donor–derived LICs were intravenously injected into irradiated WT mice, according to the previously described method (Naka et al., 2010). Imatinib or vehicle was daily administered by oral gavage from 8–19 d after LIC transplantation (150 mg/kg of body weight/day in water). (a–d) The numbers of WBCs (a), percentage of CD11b^{+}GFP^{+} cells in PB (vehicle control; b), SP weights (c), and numbers of GFP^{+}KLS^{+} LICs in BM (d) were determined (c and d, 3 wk). Data represent mean ± SEM (a) and mean ± SD (c) from four (vehicle control) or five (imatinib) individual animals. Representative results from four individual animals are shown in b. Each symbol in d represents an individual mouse (vehicle control, $n = 4$; imatinib, $n = 5$). P-values were calculated with the Mann–Whitney *U* test. (e) Survival rates until 60 d after BM transfer (WT, $n = 6$; CCL3$^{-/-}$, $n = 9$). The horizontal bar indicates a term of imatinib treatment. P-value was calculated with the log-rank test. Five mice transplanted with CCL3$^{-/-}$ donor–derived LICs survived until 60 d and were sacrificed for the determination of the numbers of GFP^{+}KLS^{+} LICs in BM. The data are additionally shown in d. *, $P < 0.05$; **, $P < 0.01$; N.S., no significant difference.

CCR5 on HSPCs as observed on dendritic precursor cells (Zhang et al., 2004). Moreover, WT-derived but not CCL3$^{-/-}$-derived BCR-ABL–expressing LICs markedly reduced the numbers of BCR-ABL^{-}lin^{-}c-kit^{+} normal HSPCs in BM when injected into the BM of nude mice. Given the failure of BCR-ABL–expressing LICs to populate the BM of CCR1$^{-/-}$ or CCR5$^{-/-}$ mice, we postulated that BCR-ABL–expressing leukemia cell–derived CCL3 can displace from the BM normal CCR1- and CCR5-expressing HSPCs, which are present in both donor cell population as well as recipient cell population, thereby boosting the growth of LICs. This notion is supported by our observation that the admixture of CCR1$^{-/-}$- and CCR5$^{-/-}$-derived HSPCs but not WT-derived HSPCs to BCR-ABL–expressing LICs attenuated the development of CML-like MPN when injected into the BM of nude mice.

In the developing wing imaginal disc of *Drosophila melanogaster*, normal epithelial cells can eliminate a small number of transformed cells with mutations in oncogenes and/or tumor-suppressor genes within a normal epithelial monolayer (Prober and Edgar, 2000; Hogan et al., 2009). Similar observations were obtained by using in vitro mammalian cell culture system (Hogan et al., 2009). The phenomenon is proposed to be termed as "cell competition," which can function as a potent intrinsic tumor suppression system. However, evidence is lacking to indicate the presence of cell competition in mammals in vivo. Our present observations would indicate that the CCL3 axis crucially contributes to the maintenance of BCR-ABL–expressing LICs in BM by mobilizing normal HSPCs from the BM and eventually reducing their numbers in BM. Accumulating evidence indicates that the niche construction is required for the maintenance and colonization of

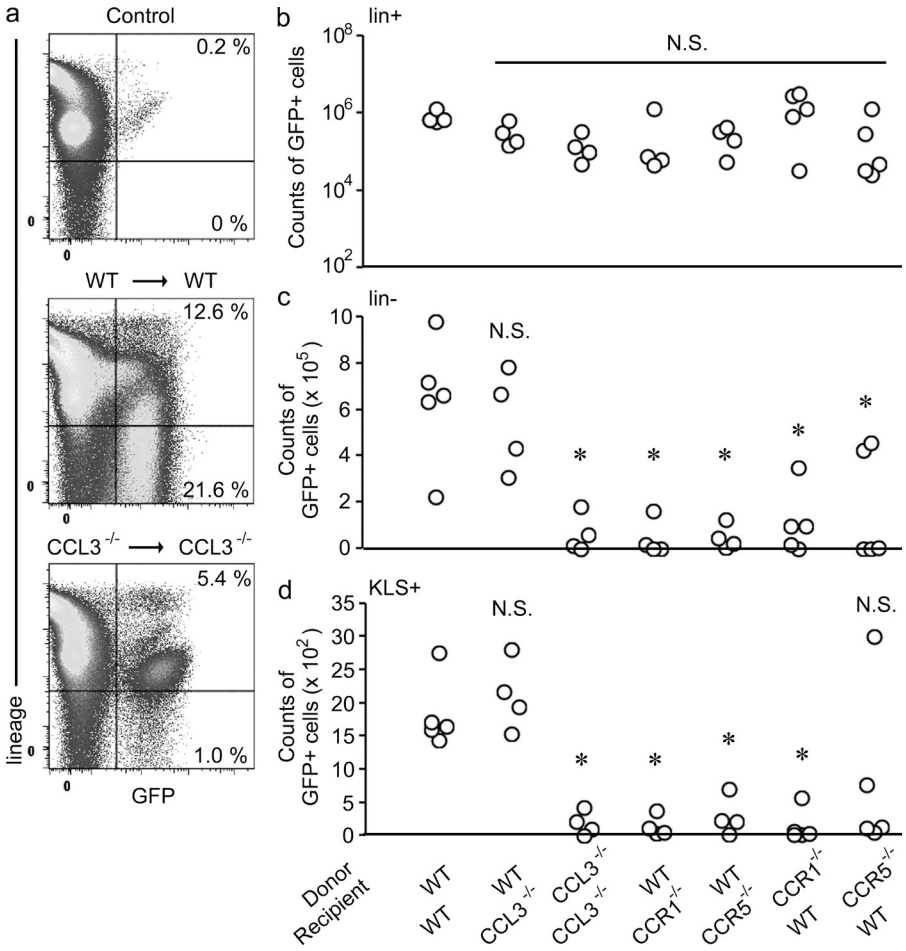

Figure 8. Requirement of CCR1 and CCR5 for CCL3-mediated maintenance of LICs in the BM. (a) WT and CCL3$^{-/-}$ donor-derived LICs were transplanted directly into the BM cavity of nonirradiated WT and CCL3$^{-/-}$ recipient mice, respectively. Total cells were isolated from the transferred BM to determine the expression of lineage marker and GFP at 2 wk after the transplantation. Percentages of lineage^{+}GFP^{+} and lineage^{-}GFP^{+} cells are shown in each panel. Representative results from four independent experiments are shown. Because of the presence of autofluorescent cells, nontreated WT mouse–derived BM cells are shown as a control. WT, CCL3$^{-/-}$, CCR1$^{-/-}$, or CCR5$^{-/-}$ donor–derived LICs were transplanted directly into the BM cavity of WT, CCL3$^{-/-}$, CCR1$^{-/-}$, or CCR5$^{-/-}$ recipient mice in the indicated combinations. (b–d) The number of lineage^{+}GFP^{+} differentiated leukemia cells (b), lineage^{-}GFP^{+} undifferentiated leukemia cells (c), and GFP^{+}KLS^{+} LICs (d) in the nontransferred BM were determined at 2 wk after transplantation. Each symbol represents an individual mouse (n = 4 or 5). P-values were calculated with the Dunnett's test: *, $P < 0.01$; N.S., no significant difference.

both normal BM stem/progenitor cells (Zhang et al., 2003) and LICs (Wertheim et al., 2002; Nair et al., 2010). Thus, our present observations would imply the presence of cell competition between normal HSPCs and LICs in the limited space of the BM cavity under the control of CCL3.

Here, we demonstrated that BCR-ABL–expressing leukemia cell–derived CCL3 can have a crucial role in CML development by mobilizing normal HSPCs from the BM and eventually making space for LICs in the BM. Several lines of evidence indicate that CCL3 has negative impacts on the proliferation of normal HSPCs. On the contrary, BCR-ABL–expressing LICs are rather resistant to CCL3-mediated growth inhibition arising from constitutive Abl tyrosine kinase activation–mediated desensitization (Wark et al., 1998). Consequently, CCL3 may provide BCR-ABL–expressing LICs with growth advantage over normal HSPCs. Thus, LICs may compete with normal HSPCs in the BM, particularly in the early phase of CML development, through these two distinct mechanisms, mobilization of normal HSPCs and inhibition of their growth.

The treatment with TKIs against BCR-ABL exhibits marked therapeutic effects, resulting in frequent remission at cytological and even at the molecular levels and has markedly improved the prognosis of CML patients (Druker et al., 2006; Kantarjian et al., 2010; Saglio et al., 2010). However, the cessation of the treatment often leads to the relapse of leukemia after a certain period of latency (Michor et al., 2005). Evidence is accumulating to indicate that LICs are relatively resistant to TKI therapy compared with mature leukemia cells (Corbin et al., 2011). As a result, LICs can survive in the BM even after extensive TKI treatment, and a residual number of TKI-resistant LICs can proliferate massively after the cessation of the treatment. Several signaling pathways have been proposed to account for LIC survival after TKI treatment (Seke Etet et al., 2012), including Hedgehog (Dierks et al., 2008; Zhao et al., 2009; Long et al., 2011) and TGF-β–FOXO3 pathways (Naka et al., 2010). Given a potent capacity of CCL3 to provide LICs with a growth advantage over normal HSPCs in the initiation process of CML, we also assessed the effects of CCL3 ablation on the TKI treatment. Under our experimental conditions, CCL3 ablation failed to decrease LICs on the TKI treatment. However, the cessation of TKI treatment induced CML to rapidly recur, whereas lack of CCL3 remarkably prolonged the survival. This prolongation can be ascribed to a sustained decrease in LICs in BM transplanted with CCL3$^{-/-}$-derived LICs, even after the cessation of imatinib treatment. Thus, CCL3 can also crucially contribute to the maintenance of LICs of CML after TKI discontinuation.

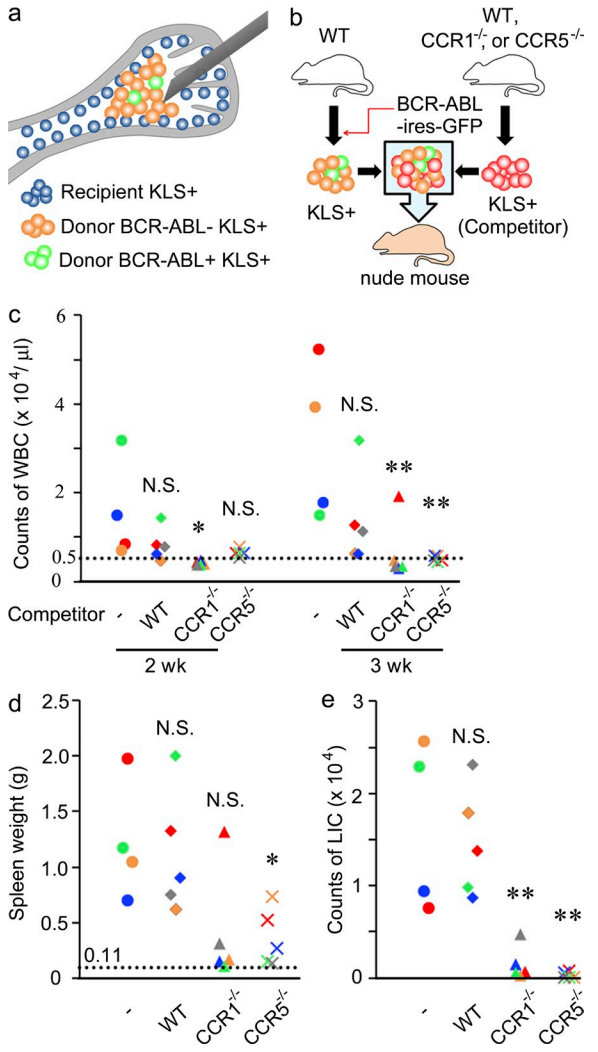

Figure 9. Admixture with CCR1- and CCR5-deficient normal HSPCs blunts the leukemogenic capability of LICs. (a) The image of cellular composition after BM transplantation in nonirradiated recipient. (b) The schema of experimental procedure of "normal KLS$^+$ cell/LIC competition assay." WT donor–derived LICs were mixed with nontransduced WT, CCR1$^{-/-}$, or CCR5$^{-/-}$ KLS$^+$ cells before BM transplantation. (c–e) The numbers of WBCs (c), SP weights (d), and numbers of GFP$^+$KLS$^+$ LICs in the transferred BM (e) were determined (c, 2 and 3 wk; d and e, 3 wk). Each mouse is indicated by using the same symbol with the same color (without competitors, $n = 4$; with competitors, $n = 5$). P-values were calculated with the Dunnett's test: *, $P < 0.05$; **, $P < 0.01$; N.S., no significant difference. Horizontal dashed lines in c and d indicate the mean of nontreated nude mice.

We identified BCR-ABL$^+$lineage$^-$c-kit$^-$ immature leukemia cells as the main CCL3-producing cells in CML mice. Moreover, we observed that after 3-mo treatment with TKI, human CML patients exhibited enhanced CCL3 mRNA expression in BM with a marked reduction in the copy number of *BCR-ABL* gene (unpublished data). Because immature leukemia cells are more resistant to TKI treatment than differentiated leukemia cells, at its initiation phase (Tang et al., 2011), CCL3-expressing immature leukemia cells might have an advantage in survival in the initiation phase of TKI treatment. Thus, more detailed analysis on CCL3-expressing cells in human CML BM can pave the way to develop a novel type of treatment against CML to supplement present TKI treatment.

MATERIALS AND METHODS

Mice. Specific pathogen–free 5–7-wk-old male BALB/c and athymic BALB/c-nu mice were purchased from Charles River and designated as WT and nude mice, respectively. CCL3$^{-/-}$ mice were obtained from the Jackson Laboratory. CCR1$^{-/-}$ and CCR5$^{-/-}$ mice were provided by P.M. Murphy (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) and K. Matsushima (University of Tokyo, Tokyo, Japan), respectively. These gene-deficient mice were backcrossed with BALB/c for more than eight generations. Animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University. All mice were kept under the specific pathogen–free conditions, and all the animal experiments in this study complied with the Guidelines for the Care and Use of Laboratory Animal of Kanazawa University.

Antibodies (Abs). The following rat anti–mouse mAbs were used: anti-CCR1 (643854; R&D Systems), anti-CD4 (RM4-5; eBioscience), anti-CD8 (53-6.7; eBioscience), anti-CD11b (M1/70; eBioscience), anti-CD34 (RAM34; eBioscience), anti-CD45R/B220 (RA3-6B2; eBioscience), anti-CD117/c-kit (ACK2; eBioscience), anti–Ly-6A/E/Sca-1 (D7; eBioscience), anti–Ly-6G/Gr-1 (RB6-8C5; eBioscience), anti–MIP-1α/CCL3 (39624; R&D Systems), and anti–TER-119 (TER-119; eBioscience). Mouse lineage Ab cocktail with isotype control set was purchased from BD. Rabbit anti–human CCR5 polyclonal Ab with a cross reactivity to mouse CCR5 was purchased from Novus Biologicals. Isotype-matched control IgGs for individual rat mAbs and control rabbit IgG were purchased from BD and Dako, respectively.

Cell preparation. Total BM cells were flushed out with cold magnetic-activated cell separation (MACS) buffer (PBS supplemented with 2 mM EDTA and 3% FBS) from the femoral and/or tibial bones. Total BM cells were separated by density gradient centrifugation using Histopaque-1083 reagent (Sigma-Aldrich), and then lineage marker (CD4, CD8, CD11b, Gr-1, B220, and TER-119)$^-$c-kit$^+$Sca-1$^+$ cells were sorted by using the FACSAria cell sorter (BD) and were used as KLS$^+$ BM cells. Total SP cells were isolated by mechanical digestion from SP. Flow cytometric analysis on BM, SP, and PB was conducted after erythrocytes were depleted by ammonium chloride lysing buffer containing 0.826% NH_4Cl, 0.1% KHCO3, and 0.004% EDTA-4Na.

Preparation of retrovirus. MSCV-BCR-ABL-ires-GFP plasmid was prepared as described previously (Naka et al., 2010). Retroviral packaging cells (Phoenix 293T) were transiently transfected with the MSCV-BCR-ABL-ires-GFP plasmid using jetPRIME transfection reagent (Polyplus transfection) to produce the retrovirus in the culture supernatant, which was subjected to the infection into KLS$^+$ BM cells.

Generation of CML model. KLS$^+$ cells purified from BM of nontreated WT or each gene-deficient mice were cultured in serum-free S-Clone SF-03 medium (Sanko Junyaku) supplemented with 1% BSA, 100 ng/ml stem cell factor (Wako Chemicals USA), 100 ng/ml thrombopoietin, 25 ng/ml *fms*-like tyrosine kinase-3 ligand (R&D Systems), 10 ng/ml IL-6 (PeproTech), and 10 ng/ml IL-3 (PeproTech) for 1 d. Cultured KLS$^+$ cells were infected with the retrovirus carrying MSCV-BCR-ABL-ires-GFP using ViroMag R/L kit (OZ Bioscience) to obtain LICs. The right knee joint was flexed, and a hole was made on the top of tibial bone above the patellar ligament, using a 29-G needle. LICs in a volume of 30 µl were subsequently injected with a 29-G needle–conjugated insulin syringe (Terumo). Hence, the right and left tibia are designated as the transferred and nontransferred BM, respectively. In the imatinib (Novartis) administration experiment, LICs were intravenously injected into the mice, which were irradiated in advance, in an essentially same way as described previously (Naka et al., 2010).

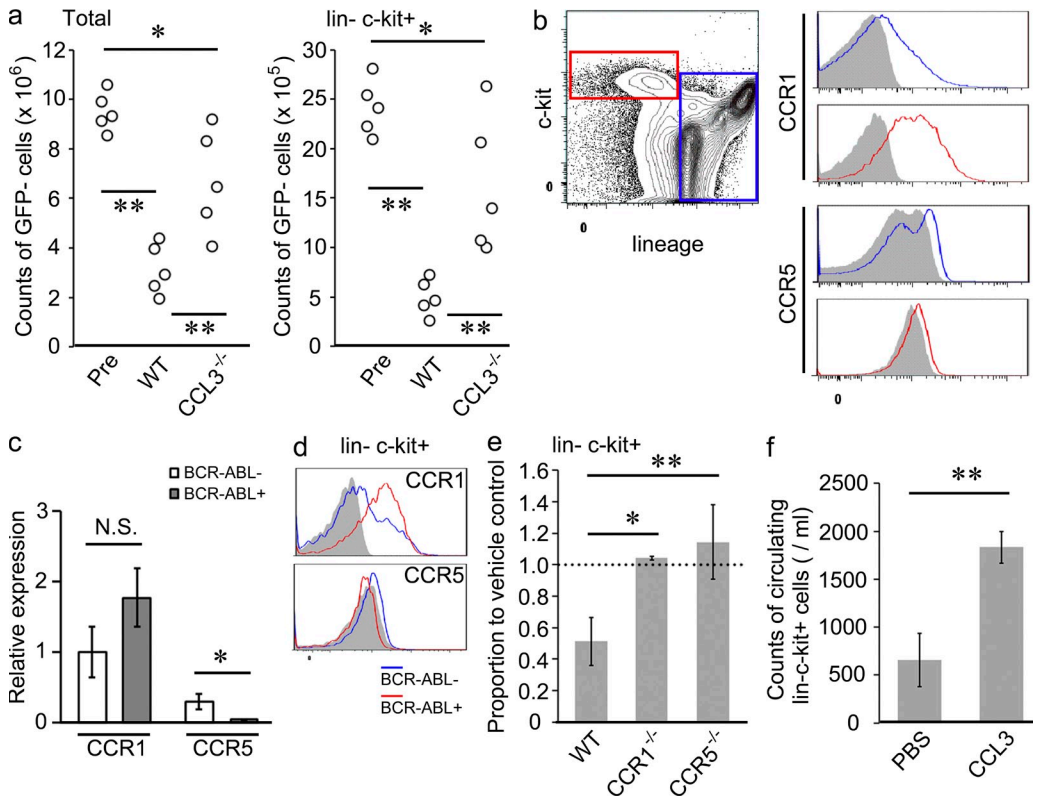

Figure 10. Intra-BM stimulation with CCL3 mobilizes normal HSPCs from the BM to PB. (a) WT or CCL3$^{-/-}$ donor–derived LICs were transplanted directly into the BM cavity of nude mice. The numbers of total GFP$^-$ cells and GFP$^-$c-kit$^+$lineage$^-$ HSPCs in the transferred BM were determined at 3 wk after the transplantation. Each symbol represents an individual mouse (n = 5). P-values were calculated with the Turkey-Kramer test. (b) Lineage$^+$ mature and lineage$^-$c-kit$^+$ progenitor cells in nontreated WT mouse–derived total BM cells are gated with blue and red squares, respectively. The expression of CCR1 and CCR5 on the gated cells are shown by the color-matched histograms. Isotype controls are shown by the gray-filled histograms. Representative results from three independent experiments are shown here. (c) GFP$^+$ or GFP$^-$c-kit$^+$lineage$^-$ cells were isolated from the BM at 2 wk after transplantation of WT LICs, using a FACSAria cell sorter, and were subsequently subjected to quantitative real-time PCR analysis for CCR1 and CCR5 expression. Data represent mean ± SD from three independent experiments. P-values were calculated with the unpaired Student's t test. (d) The expression of CCR1 and CCR5 on the gated GFP$^+$ (red) or GFP$^-$lineage$^-$c-kit$^+$ BM progenitor cells (blue) was examined at 2 or 3 wk after transplantation of WT LICs. Isotype controls are shown by the gray-filled histograms. Representative results from three independent experiments are shown here. (e) 5 ng of recombinant mouse CCL3 (PeproTech) in 30 µl PBS was directly injected into the BM cavity of WT, CCR1$^{-/-}$, or CCR5$^{-/-}$ mice. PBS was injected as a control. The numbers of lineage$^-$c-kit$^+$ HSPCs in the injected BM were determined at 6 h after injection. Proportion to vehicle control = the number of cells after CCL3 injection/that after control treatment. Data represent mean ± SD from three independent experiments. P-values were calculated with the Dunnett's test. (f) 5 ng CCL3 was injected into both the right and left tibial BM cavity of WT mice. The numbers of lineage$^-$c-kit$^+$ HSPCs in PB were determined at 6 h after injection. Data represent mean ± SD from three independent experiments. P-value was calculated with the unpaired Student's t test: *, $P < 0.05$; **, $P < 0.01$; N.S., no significant difference.

RNA isolation, cDNA synthesis, and quantitative real-time PCR. Total RNAs were extracted from cells using RNeasy Mini kit (QIAGEN) and then reverse transcribed using the SuperScript III First-Strand synthesis system (Invitrogen). Quantitative real-time PCR was performed on an Applied Biosystems StepOne real-time PCR system using SYBR Select Master Mix (Applied Biosystems) and the specific primer sets for *GAPDH* gene (sense, 5′-GCGGCACGTCAGATCCA-3′; and antisense, 5′-CATGGCCTTCCGTGTTTCCTA-3′), *CCR1* gene (sense, 5′-TTTGTGGGTGAACGGTTCTG-3′; and antisense, 5′-TGGTATAGCCACATGCCTTTGA-3′), and *CCR5* gene (sense, 5′-CATCCGTTCCCCCTACAAGA-3′; and antisense, 5′-GGAACTGACCCTTGAAAATCCA-3′). Thermal cycling was initiated with an initial activation step for 2 min at 50°C and 20 s at 95°C, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Immediately after the amplification, melt curve protocols were performed to ensure that primer-dimers and other nonspecific products had been minimized. Relative expression of target gene was analyzed by the ΔΔCt method, using the Ct value of *GAPDH* gene.

Flow cytometry. Isolated leukocytes were stained with various combinations of fluorescent dye–conjugated Abs in MACS buffer. For the intracellular MIP-1α/CCL3 staining, leukocytes were incubated in serum-free S-Clone SF-03 medium supplemented with 0.1% GolgiStop reagent (BD) for 4 h. Subsequently, intracellular CCL3 was stained with PE-conjugated anti-CCL3 Ab and the help of Intracellular Cytokine Staining Starter kit (BD). Expression of each molecule was determined using FACSCanto II (BD) and analyzed with FlowJo software (Tree Star).

Histopathology. Tissue samples were fixed in Ufix reagent (Sakura) and embedded in paraffin blocks. Each 4-µm section was stained with hematoxylin and eosin (H&E). Whole blood samples were smeared on to glass slides and fixed with 100% methanol, which were subjected to the Giemsa stain or fluorescent immunostaining. Histopathological images and fluorescent images were acquired using a fluorescence microscope (BX50; Olympus). DP Controller software (Olympus) was used for image processing.

Chemokine measurement. Chemokine expression in the serum was determined on a Bioplex reader (Bio-Rad Laboratories) using a Mouse Cytokine Standard 23-plex assay kit (Bio-Rad Laboratories). All procedures were conducted according to the manufacturer's instructions.

Statistical analysis. Data were analyzed statistically using methods indicated in each figure legend. $P < 0.05$ was considered statistically significant.

We would like to express our gratitude to Dr. Joost J. Oppenheim (National Cancer Institute–Frederick, Frederick, MD) for his critical review of the manuscript.

This work was supported in part by Japan Society for the Promotion of Science KAKENHI grant number 25460492.

The authors have no conflicting financial interests.

Submitted: 15 January 2013
Accepted: 26 September 2013

REFERENCES

Aiuti, A., I.J. Webb, C. Bleul, T. Springer, and J.C. Gutierrez-Ramos. 1997. The chemokine SDF-1 is a chemoattractant for human $CD34^+$ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of $CD34^+$ progenitors to peripheral blood. *J. Exp. Med.* 185:111–120. http://dx.doi.org/10.1084/jem.185.1.111

Basu, S., and H.E. Broxmeyer. 2009. CCR5 ligands modulate CXCL12-induced chemotaxis, adhesion, and Akt phosphorylation of human cord blood CD34+ cells. *J. Immunol.* 183:7478–7488. http://dx.doi.org/10.4049/jimmunol.0900542

Bhatia, R., P.B. McGlave, and C.M. Verfaillie. 1995. Treatment of marrow stroma with interferon-alpha restores normal beta 1 integrin-dependent adhesion of chronic myelogenous leukemia hematopoietic progenitors. Role of MIP-1 alpha. *J. Clin. Invest.* 96:931–939. http://dx.doi.org/10.1172/JCI118141

Broxmeyer, H.E., B. Sherry, S. Cooper, L. Lu, R. Maze, M.P. Beckmann, A. Cerami, and P. Ralph. 1993. Comparative analysis of the human macrophage inflammatory protein family of cytokines (chemokines) on proliferation of human myeloid progenitor cells. Interacting effects involving suppression, synergistic suppression, and blocking of suppression. *J. Immunol.* 150:3448–3458.

Broxmeyer, H.E., S. Cooper, G. Hangoc, J.L. Gao, and P.M. Murphy. 1999. Dominant myelopoietic effector functions mediated by chemokine receptor CCR1. *J. Exp. Med.* 189:1987–1992. http://dx.doi.org/10.1084/jem.189.12.1987

Chasty, R.C., G.S. Lucas, P.J. Owen-Lynch, A. Pierce, and A.D. Whetton. 1995. Macrophage inflammatory protein-1 alpha receptors are present on cells enriched for CD34 expression from patients with chronic myeloid leukemia. *Blood.* 86:4270–4277.

Corbin, A.S., A. Agarwal, M. Loriaux, J. Cortes, M.W. Deininger, and B.J. Druker. 2011. Human chronic myeloid leukemia stem cells are insensitive to imatinib despite inhibition of BCR-ABL activity. *J. Clin. Invest.* 121:396–409. http://dx.doi.org/10.1172/JCI35721

Dierks, C., R. Beigi, G.R. Guo, K. Zirlik, M.R. Stegert, P. Manley, C. Trussell, A. Schmitt-Graeff, K. Landwerlin, H. Veelken, and M. Warmuth. 2008. Expansion of Bcr-Abl-positive leukemic stem cells is dependent on Hedgehog pathway activation. *Cancer Cell.* 14:238–249. http://dx.doi.org/10.1016/j.ccr.2008.08.003

Druker, B.J., F. Guilhot, S.G. O'Brien, I. Gathmann, H. Kantarjian, N. Gattermann, M.W. Deininger, R.T. Silver, J.M. Goldman, R.M. Stone, et al; IRIS Investigators. 2006. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N. Engl. J. Med.* 355:2408–2417. http://dx.doi.org/10.1056/NEJMoa062867

Dunlop, D.J., E.G. Wright, S. Lorimore, G.J. Graham, T. Holyoake, D.J. Kerr, S.D. Wolpe, and I.B. Pragnell. 1992. Demonstration of stem cell inhibition and myeloprotective effects of SCI/rhMIP1 alpha in vivo. *Blood.* 79:2221–2225.

Eaves, C.J., J.D. Cashman, S.D. Wolpe, and A.C. Eaves. 1993. Unresponsiveness of primitive chronic myeloid leukemia cells to macrophage inflammatory protein 1 alpha, an inhibitor of primitive normal hematopoietic cells. *Proc. Natl. Acad. Sci. USA.* 90:12015–12019. http://dx.doi.org/10.1073/pnas.90.24.12015

Graham, G.J., E.G. Wright, R. Hewick, S.D. Wolpe, N.M. Wilkie, D. Donaldson, S. Lorimore, and I.B. Pragnell. 1990. Identification and characterization of an inhibitor of haemopoietic stem cell proliferation. *Nature.* 344:442–444. http://dx.doi.org/10.1038/344442a0

Hogan, C., S. Dupré-Crochet, M. Norman, M. Kajita, C. Zimmermann, A.E. Pelling, E. Piddini, L.A. Baena-López, J.P. Vincent, Y. Itoh, et al. 2009. Characterization of the interface between normal and transformed epithelial cells. *Nat. Cell Biol.* 11:460–467. http://dx.doi.org/10.1038/ncb1853

Kantarjian, H., N.P. Shah, A. Hochhaus, J. Cortes, S. Shah, M. Ayala, B. Moiraghi, Z. Shen, J. Mayer, R. Pasquini, et al. 2010. Dasatinib versus imatinib in newly diagnosed chronic-phase chronic myeloid leukemia. *N. Engl. J. Med.* 362:2260–2270. http://dx.doi.org/10.1056/NEJMoa1002315

Koschmieder, S., B. Göttgens, P. Zhang, J. Iwasaki-Arai, K. Akashi, J.L. Kutok, T. Dayaram, K. Geary, A.R. Green, D.G. Tenen, and C.S. Huettner. 2005. Inducible chronic phase of myeloid leukemia with expansion of hematopoietic stem cells in a transgenic model of BCR-ABL leukemogenesis. *Blood.* 105:324–334. http://dx.doi.org/10.1182/blood-2003-12-4369

Lahaye, T., B. Riehm, U. Berger, P. Paschka, M.C. Müller, S. Kreil, K. Merx, U. Schwindel, C. Schoch, R. Hehlmann, and A. Hochhaus. 2005. Response and resistance in 300 patients with BCR-ABL-positive leukemias treated with imatinib in a single center: a 4.5-year follow-up. *Cancer.* 103:1659–1669. http://dx.doi.org/10.1002/cncr.20922

Li, S., R.L. Ilaria Jr., R.P. Million, G.Q. Daley, and R.A. Van Etten. 1999. The P190, P210, and P230 forms of the *BCR/ABL* oncogene induce a similar chronic myeloid leukemia–like syndrome in mice but have different lymphoid leukemogenic activity. *J. Exp. Med.* 189:1399–1412. http://dx.doi.org/10.1084/jem.189.9.1399

Long, B., H. Zhu, C. Zhu, T. Liu, and W. Meng. 2011. Activation of the Hedgehog pathway in chronic myelogeneous leukemia patients. *J. Exp. Clin. Cancer Res.* 30:8. http://dx.doi.org/10.1186/1756-9966-30-8

Lord, B.I., L.B. Woolford, L.M. Wood, L.G. Czaplewski, M. McCourt, M.G. Hunter, and R.M. Edwards. 1995. Mobilization of early hematopoietic progenitor cells with BB-10010: a genetically engineered variant of human macrophage inflammatory protein-1 alpha. *Blood.* 85:3412–3415.

Lu, P., Y. Nakamoto, Y. Nemoto-Sasaki, C. Fujii, H. Wang, M. Hashii, Y. Ohmoto, S. Kaneko, K. Kobayashi, and N. Mukaida. 2003. Potential interaction between CCR1 and its ligand, CCL3, induced by endogenously produced interleukin-1 in human hepatomas. *Am. J. Pathol.* 162:1249–1258. http://dx.doi.org/10.1016/S0002-9440(10)63921-1

Maze, R., B. Sherry, B.S. Kwon, A. Cerami, and H.E. Broxmeyer. 1992. Myelosuppressive effects in vivo of purified recombinant murine macrophage inflammatory protein-1 alpha. *J. Immunol.* 149:1004–1009.

Melo, J.V., and D.J. Barnes. 2007. Chronic myeloid leukaemia as a model of disease evolution in human cancer. *Nat. Rev. Cancer.* 7:441–453. http://dx.doi.org/10.1038/nrc2147

Michor, F., T.P. Hughes, Y. Iwasa, S. Branford, N.P. Shah, C.L. Sawyers, and M.A. Nowak. 2005. Dynamics of chronic myeloid leukaemia. *Nature.* 435:1267–1270. http://dx.doi.org/10.1038/nature03669

Mumprecht, S., C. Schürch, J. Schwaller, M. Solenthaler, and A.F. Ochsenbein. 2009. Programmed death 1 signaling on chronic myeloid leukemia-specific T cells results in T-cell exhaustion and disease progression. *Blood.* 114:1528–1536. http://dx.doi.org/10.1182/blood-2008-09-179697

Nair, R.R., J. Tolentino, and L.A. Hazlehurst. 2010. The bone marrow microenvironment as a sanctuary for minimal residual disease in CML. *Biochem. Pharmacol.* 80:602–612. http://dx.doi.org/10.1016/j.bcp.2010.04.003

Naka, K., T. Hoshii, T. Muraguchi, Y. Tadokoro, T. Ooshio, Y. Kondo, S. Nakao, N. Motoyama, and A. Hirao. 2010. TGF-beta-FOXO signalling maintains leukaemia-initiating cells in chronic myeloid leukaemia. *Nature.* 463:676–680. http://dx.doi.org/10.1038/nature08734

Pear, W.S., J.P. Miller, L. Xu, J.C. Pui, B. Soffer, R.C. Quackenbush, A.M. Pendergast, R. Bronson, J.C. Aster, M.L. Scott, and D. Baltimore. 1998. Efficient and rapid induction of a chronic myelogenous leukemia-like myeloproliferative disease in mice receiving P210 bcr/abl-transduced bone marrow. *Blood.* 92:3780–3792.

Pelus, L.M., and S. Fukuda. 2008. Chemokine-mobilized adult stem cells; defining a better hematopoietic graft. *Leukemia*. 22:466–473. http://dx.doi.org/10.1038/sj.leu.2405021

Prober, D.A., and B.A. Edgar. 2000. Ras1 promotes cellular growth in the *Drosophila* wing. *Cell*. 100:435–446. http://dx.doi.org/10.1016/S0092-8674(00)80679-0

Ren, R. 2005. Mechanisms of BCR-ABL in the pathogenesis of chronic myelogenous leukaemia. *Nat. Rev. Cancer*. 5:172–183. http://dx.doi.org/10.1038/nrc1567

Saglio, G., D.W. Kim, S. Issaragrisil, P. le Coutre, G. Etienne, C. Lobo, R. Pasquini, R.E. Clark, A. Hochhaus, T.P. Hughes, et al; ENESTnd Investigators. 2010. Nilotinib versus imatinib for newly diagnosed chronic myeloid leukemia. *N. Engl. J. Med*. 362:2251–2259. http://dx.doi.org/10.1056/NEJMoa0912614

Sawyers, C.L. 1999. Chronic myeloid leukemia. *N. Engl. J. Med*. 340:1330–1340. http://dx.doi.org/10.1056/NEJM199904293401706

Seke Etet, P.F., L. Vecchio, and A.H. Nwabo Kamdje. 2012. Signaling pathways in chronic myeloid leukemia and leukemic stem cell maintenance: key role of stromal microenvironment. *Cell. Signal*. 24:1883–1888. http://dx.doi.org/10.1016/j.cellsig.2012.05.015

Singh, V.K., O.O. Fatanmi, P.K. Singh, and M.H. Whitnall. 2012. Role of radiation-induced granulocyte colony-stimulating factor in recovery from whole body gamma-irradiation. *Cytokine*. 58:406–414. http://dx.doi.org/10.1016/j.cyto.2012.03.011

Stripecke, R., M. Carmen Villacres, D. Skelton, N. Satake, S. Halene, and D. Kohn. 1999. Immune response to green fluorescent protein: implications for gene therapy. *Gene Ther*. 6:1305–1312. http://dx.doi.org/10.1038/sj.gt.3300951

Tang, M., M. Gonen, A. Quintas-Cardama, J. Cortes, H. Kantarjian, C. Field, T.P. Hughes, S. Branford, and F. Michor. 2011. Dynamics of chronic myeloid leukemia response to long-term targeted therapy reveal treatment effects on leukemic stem cells. *Blood*. 118:1622–1631. http://dx.doi.org/10.1182/blood-2011-02-339267

Wark, G., C.M. Heyworth, E. Spooncer, L. Czaplewski, J.M. Francis, T.M. Dexter, and A.D. Whetton. 1998. Abl protein kinase abrogates the response of multipotent haemopoietic cells to the growth inhibitor macrophage inflammatory protein-1 alpha. *Oncogene*. 16:1319–1324. http://dx.doi.org/10.1038/sj.onc.1201914

Wertheim, J.A., K. Forsythe, B.J. Druker, D. Hammer, D. Boettiger, and W.S. Pear. 2002. BCR-ABL-induced adhesion defects are tyrosine kinase-independent. *Blood*. 99:4122–4130. http://dx.doi.org/10.1182/blood.V99.11.4122

Zhang, B., Y.W. Ho, Q. Huang, T. Maeda, A. Lin, S.U. Lee, A. Hair, T.L. Holyoake, C. Huettner, and R. Bhatia. 2012. Altered microenvironmental regulation of leukemic and normal stem cells in chronic myelogenous leukemia. *Cancer Cell*. 21:577–592. http://dx.doi.org/10.1016/j.ccr.2012.02.018

Zhang, J., C. Niu, L. Ye, H. Huang, X. He, W.G. Tong, J. Ross, J. Haug, T. Johnson, J.Q. Feng, et al. 2003. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*. 425:836–841. http://dx.doi.org/10.1038/nature02041

Zhang, Y., H. Yoneyama, Y. Wang, S. Ishikawa, S. Hashimoto, J.L. Gao, P. Murphy, and K. Matsushima. 2004. Mobilization of dendritic cell precursors into the circulation by administration of MIP-1alpha in mice. *J. Natl. Cancer Inst*. 96:201–209. http://dx.doi.org/10.1093/jnci/djh024

Zhao, C., A. Chen, C.H. Jamieson, M. Fereshteh, A. Abrahamsson, J. Blum, H.Y. Kwon, J. Kim, J.P. Chute, D. Rizzieri, et al. 2009. Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature*. 458:776–779. http://dx.doi.org/10.1038/nature07737

Zou, Y.R., A.H. Kottmann, M. Kuroda, I. Taniuchi, and D.R. Littman. 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature*. 393:595–599. http://dx.doi.org/10.1038/31269