CXCR4 is required for the quiescence of primitive hematopoietic cells

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The quiescence of hematopoietic stem cells (HSCs) is critical for preserving a lifelong steady pool of HSCs to sustain the highly regenerative hematopoietic system. It is thought that specialized niches in which HSCs reside control the balance between HSC quiescence and self-renewal, yet little is known about the extrinsic signals provided by the niche and how these niche signals regulate such a balance. We report that CXCL12 produced by bone marrow (BM) stromal cells is not only the major chemoattractant for HSCs but also a regulatory factor that controls the quiescence of primitive hematopoietic cells. Addition of CXCL12 into the culture inhibits entry of primitive hematopoietic cells into the cell cycle, and inactivation of its receptor CXCR4 in HSCs causes excessive HSC proliferation. Notably, the hyperproliferative *Cxcr4*$^{-/-}$ HSCs are able to maintain a stable stem cell compartment and sustain hematopoiesis. Thus, we propose that CXCR4/CXCL12 signaling is essential to confine HSCs in the proper niche and controls their proliferation.

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Hematopoietic stem cells (HSCs) have robust proliferative potential, as they can undergo extensive expansion to quickly restore hematopoiesis after transplantation or histological injury. However, under steady state, HSCs proliferate at a very low rate and most HSCs are kept in the G_0 phase of the cell cycle (1). Disruption of HSC quiescence leads to premature exhaustion of the stem cell pool and causes hematological failure under stress conditions (2, 3). Thus, HSC self-renewal and quiescence have to be finely balanced to maintain a stable HSC pool that is capable of producing blood cells for the lifetime of the organism. Although numerous transcription factors and cell cycle molecules have been identified to regulate HSC self-renewal, it is not understood how nuclear regulatory factors adjust the HSC self-renewal rate to accommodate hematopoiesis under homeostatic and cytopenic conditions. It has been reported that HSCs are relocated from the osteoblastic niche to vascular zones in the BM after myeloablation (4). The translocation of HSCs is accompanied with an increase in HSC proliferation, suggesting that signals emanating from the BM niche where HSCs reside determine the balance between quiescence and self-renewal of HSCs.

The chemokine CXCL12 is the major chemoattractant for HSCs (5). It is expressed at a high level by osteoblasts, endothelial cells, and by a subset of reticular cells scattered throughout the BM (6, 7). Inactivation of CXCL12 or its receptor CXCR4 impairs the translocation of HSCs from the fetal liver to the BM during embryogenesis (8–11), and direct ablation of CXCR4 signaling or indirect modulation of CXCL12 level by proteases results in mobilization of primitive hematopoietic cells and compromises their engrafting activity (4, 12–14). This suggests an important role for CXCR4/CXCL12 in BM retention of primitive hematopoietic cells. Additional effects of CXCR4 on HSCs are still not fully understood, and studies evaluating its regulatory role in the cell cycle yielded contradictory results (7, 15). To better understand the function of CXCR4 in HSCs, we deleted the *Cxcr4* gene during adult hematopoiesis. We found that the compartment of primitive hematopoietic cells (Flt3$^-$ Lin$^-$ Sca-1$^+$ c-Kit$^+$ cells) was stably maintained in the BM in the absence of CXCR4 and sustained long-term hematopoiesis. These CXCR4-deficient primitive hematopoietic cells proliferated vigorously and outcompeted the coexisting WT counterpart in the same host. CXCL12 directly inhibited the cell cycle of WT, but not *Cxcr4*$^{-/-}$, primitive hematopoietic cells. Thus, our results demonstrate a critical role of CXCR4 in restraining HSCs in the quiescent state.

The online version of this article contains supplemental material.

RESULTS AND DISCUSSION

The population of *Cxcr4*$^{-/-}$ primitive hematopoietic cells is stably maintained

To investigate the function of CXCR4 at early hematopoietic developmental stages, we conditionally ablated CXCR4 function in adult primitive hematopoietic cells. We crossed *Cxcr4*-floxed mice (*Cxcr4*$^{f/f}$) to tamoxifen-inducible Cre transgenic mice (*ROSA*$^{CRE\text{-}ERT2}$) (16, 17), and we activated Cre by injecting tamoxifen. 6 tamoxifen injections over 9 d led to >99% deletion of *Cxcr4* in HSCs (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072513/DC1). Hereafter, we refer to *ROSA*$^{CRE\text{-}ERT2}$ *Cxcr4*$^{f/f}$ mice before tamoxifen induction as *Cxcr4*$^{C/C}$ mice, and after tamoxifen treatment as *Cxcr4*$^{-/-}$ mice. Control animals used in the following studies are Cre^{-} *Cxcr4*$^{f/f}$ mice because *ROSA*$^{CRE\text{-}ERT2}$ *Cxcr4*$^{+/f}$ and Cre^{-} *Cxcr4*$^{f/f}$ are phenotypically identical (Fig. S2).

The involvement of the chemokine CXCL12 in HSC function was first documented by a study showing that colonization of HSCs in the fetal BM was abolished in CXCL12$^{-/-}$ animals (8). Extensive studies have been conducted ever since to define the role of CXCL12 and CXCR4 in homing and retention of HSCs. Early transplantation experiments demonstrated that primitive hematopoietic cells required CXCR4 and CXCL12 interaction for efficient engraftment (12, 18). Later studies involved transplantation of CXCR4-inactivated primitive hematopoietic cells into irradiated hosts, and showed that the recovery of these cells in the BM within 24 h was quantitatively normal compared with that of WT cells (13, 19). These results imply that early BM homing of primitive hematopoietic cells might be CXCR4 independent, but BM retention of these cells requires CXCR4. However, it is not clear from these experiments whether HSCs behave in the same way as hematopoietic progenitors. Specifically, these studies did not directly examine whether extravasation of CXCR4-inactivated primitive hematopoietic cells from the circulation into the BM stroma, indeed, occurred. Moreover, we cannot conclude from these transfer experiments as to whether BM homing and retention of HSCs under the steady state requires CXCR4 because the BM microenvironment of the recipients used in these studies had been altered by irradiation (20).

To fully evaluate this issue, we inactivated CXCR4 in 8-wk-old mice in which HSCs had colonized in the BM and had reached a steady state, and then determined the content of phenotypically defined HSCs in the BM and periphery at different times after the final tamoxifen injection. Flow cytometry analysis revealed that a population of cells bearing characteristic markers of hematopoietic primitive cells, including long-term HSCs (Flt3^{-} Lin^{-} Sca-1^{+} c-Kit^{+} [Flt3^{-}LSK]), was markedly increased in the peripheral blood and spleen in mutant but not in WT animals 12 d after tamoxifen treatment (Fig. 1 B). This population continued to be high in the periphery, even 32 wk later (Fig. 1 C). During the same period, the cell counts of *Cxcr4*$^{-/-}$ Flt3^{-}LSK cells in the BM remained stable and were even slightly higher than that of WT (Fig. 1 C). These data showed that the compartment of phenotypic HSCs was stably retained in the BM in the absence of CXCR4, even though a substantial amount of *Cxcr4*$^{-/-}$ Flt3^{-}LSK cells emerged into the periphery.

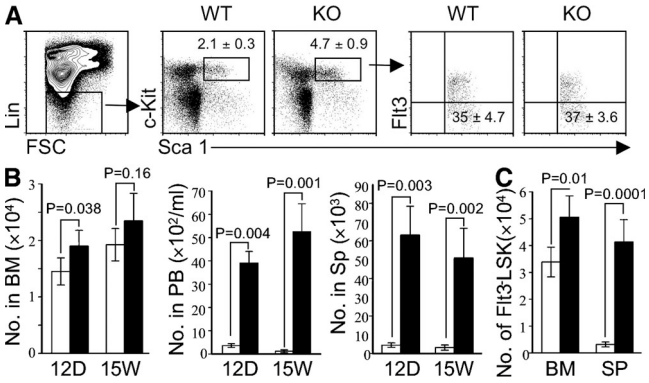

Figure 1. *Cxcr4*$^{-/-}$ HSCs are retained in the BM. (A) Dot plots represent the Flt3^{-}LSK population in the BM of *Cxcr4*$^{-/-}$ (KO) and control mice (WT) at day 12 after tamoxifen treatment. The percentages of gated populations of Flt3^{-}LSK cells ± the SD are shown. (B) Absolute numbers of Flt3^{-}LSK cells in the femur (BM), peripheral blood (PB), and spleen (Sp) of *Cxcr4*$^{-/-}$ and control mice at day 12 and week 15 after tamoxifen treatment. Values are the mean ± the SD ($n = 6$). (C) Absolute numbers of Flt3^{-}LSK cells in the femur and tibia (BM) and spleen (SP) 32 wk after tamoxifen treatment. Values are the mean ± the SD ($n = 4$). Open bars represent data of WT mice; filled bars show data of mutant animals in B and C.

Cxcr4$^{-/-}$ primitive hematopoietic cells are multipotent and sustain hematopoiesis

To examine stem cell function of *Cxcr4*$^{-/-}$ Flt3^{-}LSK cells, we evaluated their reconstitution efficiency using a competitive repopulating assay. Different numbers of *Cxcr4*$^{-/-}$ BM cells (CD45.2) were transplanted, along with a constant dose (2×10^5) of competitive BM cells (CD45.1), into lethally irradiated mice. Regeneration of HSCs and blood cells in the recipients was measured by flow cytometry 8 wk after transplantation. In mice that had received equal numbers (2×10^5) of *Cxcr4*$^{-/-}$ and competitor BM cells, no more than 10% of the Flt3^{-}LSK cells were of *Cxcr4*$^{-/-}$ donor origin. Even a large dose (10^6) of *Cxcr4*$^{-/-}$ BM cells produced only 50% chimerism in the Flt3^{-}LSK compartment and provided little contribution to B and myeloid cells (Fig. 2 A). These results are in line with previous reports showing impaired engrafting capacity of *Cxcr4*$^{-/-}$ primitive hematopoietic progenitors (12, 18).

Compromised reconstitution activity of *Cxcr4*$^{-/-}$ HSCs could be attributed to defects in homing, self-renewal, or differentiation. To circumvent the requirement for CXCR4 in HSC homing and to directly assess the differentiation potential of *Cxcr4*$^{-/-}$ HSCs in vivo, we first transplanted equal numbers (2.5×10^6) of *Cxcr4*$^{C/C}$ (CD45.2, H-2$^{b/b}$) and WT (CD45.1, H-2$^{b/b}$) marrow cells into lethally irradiated hosts (H-2$^{b/d}$), and then deleted *Cxcr4* and examined frequencies of donor-derived hematopoietic cells 14 wk after *Cxcr4* ablation. In contrast to the reduced engraftment observed in mice that received *Cxcr4*$^{-/-}$ donor cells, *Cxcr4*$^{C/C}$ donor cells yielded a much higher proportion (71%) of Flt3^{-}LSK cells over WT donor cells (29%), and engrafted the myeloid compartment efficiently

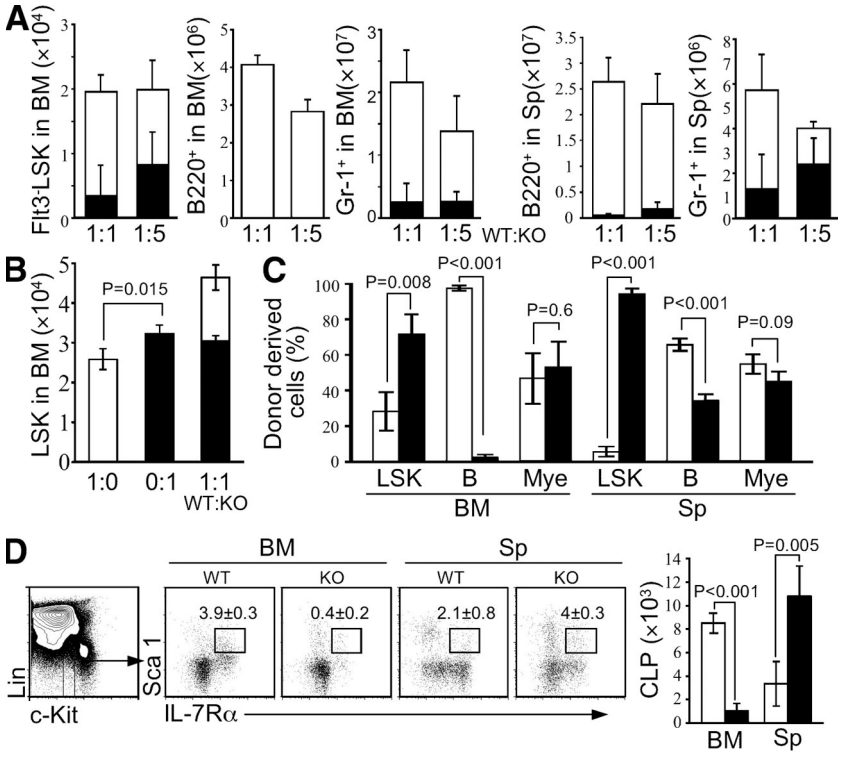

Figure 2. ***Cxcr4*$^{-/-}$ HSCs sustain hematopoiesis.** Open bars represent data of WT mice; filled bars show data of mutant animals. (A) BM cells were isolated from *Cxcr4*$^{C/C}$ and control mice 6 wk after tamoxifen treatment and cotransplanted into recipients at the indicated ratios (2 × 10^5 of WT cells). Hematopoiesis was analyzed 8 wk after transplantation. Bars with SD show cell counts of HSCs, B cells, and myeloid cells in the BM and spleen (Sp). (B) *Cxcr4*$^{C/C}$, WT, or an equal number (2.5 × 10^6) of *Cxcr4*$^{C/C}$ and WT BM cells were transferred into irradiated recipients. 2 mo after transplantation, mice were treated with tamoxifen to delete *Cxcr4*. LSK cells in BM chimeras were enumerated 14 wk after tamoxifen treatment (n = 3). (C) BM chimeras were generated as described in B. Bars (mean ± the SD; n = 3) show the frequencies of donor-derived LSK, B cells, and myeloid cells of either mutant or WT origin. (D) Total cell numbers of CLPs in the BM and spleen 15 wk after tamoxifen treatment.

(53%) in a competitive situation (Fig. 2, B and C). Although competent in myelopoiesis, mutant donor cells were severely impaired in generating B cells (Fig. 2 C). We then determined whether *Cxcr4*$^{-/-}$ HSCs were able to generate common lymphoid progenitors (CLPs; Lin$^-$ Sca^{lo} c-Kit^{lo} IL-7Rα$^+$). We found that although *Cxcr4*$^{-/-}$ CLPs were barely detectable in the BM, a large number of these cells emerged in the periphery and persisted for >3 mo (Fig. 2 D). Together, these data reveal that *Cxcr4*$^{-/-}$ Flt3$^-$LSK cells retained in the BM are multipotential and able to sustain myelopoiesis and lymphopoiesis up to the CLP stage for >3 mo.

In the absence of CXCR4, HSCs cannot home to the BM niche to reconstitute hematopoiesis (Fig. 2 A). To carry out a repopulating assay to confirm the existence of *Cxcr4*$^{-/-}$ long-term HSCs, we isolated BM cells 11 wk after *Cxcr4* deletion, infected them with retroviral vector expressing WT CXCR4, and then transplanted these infected cells into irradiated recipients. Our data presented in Fig. S3 (available at http://www.jem.org/cgi/content/full/jem.20072513/DC1) clearly show that a robust hematopoiesis was restored by *Cxcr4*$^{-/-}$ BM cells in which CXCR4 was reexpressed. This result unequivocally demonstrates that HSCs are maintained for at least 11 wk in the absence of CXCR4.

Cxcr4$^{-/-}$ primitive hematopoietic cells are hyperproliferative

In the mixed BM chimeras, the cellularity of *Cxcr4*$^{-/-}$ Flt3$^-$LSK cells was twofold higher than that of the WT (Fig. 2 B), suggesting that the expansion of these cells was cell intrinsic and could result from enhanced survival or self-renewal of HSCs. Because we did not observe changes in apoptosis detected by annexin V staining of freshly isolated *Cxcr4*$^{-/-}$ Flt3$^-$LSK cells (Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20072513/DC1), we decided to examine whether CXCR4 deficiency promoted HSC proliferation using a BrdU-uptake assay. In WT mice, we found that 4-d BrdU exposure yielded 28% of BrdU$^+$ LSK cells, and that a longer labeling period (15 d) raised this population to 63%. In contrast, the frequency of BrdU$^+$ LSK cells had increased from 64 to 91% during the same interval in mice that had *Cxcr4* deleted (Fig. 3 B). The proliferation rate of mutant primitive hematopoietic cells was ∼3-fold higher than that of WT 14 wk after *Cxcr4* deletion (Fig. 3 C). Analysis of the cell cycling status by measuring RNA and DNA content revealed that the number of cycling *Cxcr4*$^{-/-}$ LSK cells remained high even 32 wk after *Cxcr4* deletion (Fig. 3 D). In accordance with enhanced proliferation of *Cxcr4*$^{-/-}$ HSCs, *Cxcr4*$^{-/-}$

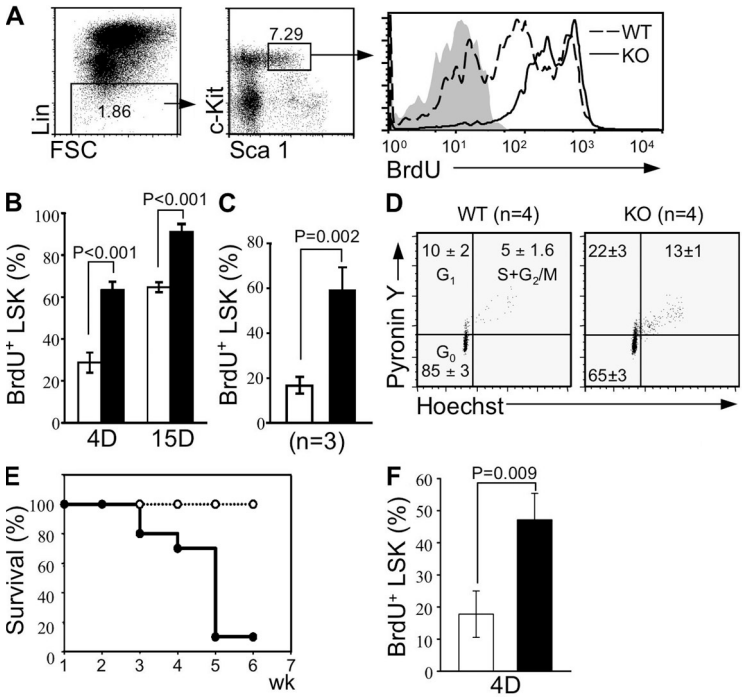

Figure 3. CXCR4 deficiency causes hyperproliferation of primitive hematopoietic cells. Open bars represent data of WT mice; filled bars show data of mutant mice. (A) The histogram shows representative profiles of BrdU+ LSK cells. The shaded histogram represents background staining using an Ig isotype-matched control antibody. (B) 2 wk after *Cxcr4* deletion, *Cxcr4*$^{-/-}$ and WT mice were labeled with BrdU for 4 or 15 d. Bars ± the SD (n = 4) represent percentages of BrdU+ cells within the LSK compartment. (C) Bars ± the SD show percentages of BrdU+ LSK cells 15 wk after *Cxcr4* deletion. Mice were given BrdU for 20 h. (D) Cell cycle profiles revealed by pyronin Y/Hoechst staining of Flt3$^-$LSK cells in mice 32 wk after *Cxcr4* deletion (n = 4). The percentage of cells in the given quadrants represents the means ± the SD. (E) 2 wk after tamoxifen treatment, mice were injected weekly with 5-fluorouracil (100 mg/kg bodyweight). The survival rate of WT (n = 5, open circle) and *Cxcr4*$^{-/-}$ (n = 10, filled circle) mice was monitored. (F) Equal numbers (2.5×10^6) of *Cxcr4*$^{C/C}$ and WT BM cells were cotransplanted into BDF1 recipients. 4 wk after transplantation, mice were treated with tamoxifen. 14 wk later, mice were labeled with BrdU for 4 d. LSK frequencies of BrdU+ cells are shown (n = 3).

mice died more readily from hematological failure after depletion of cycling HSCs by weekly challenge with the cell-cycle cytotoxic agent 5-fluorouracil (Fig. 3 E).

To address whether hyperproliferation was a cell autonomous property of *Cxcr4*$^{-/-}$ HSCs, we transplanted an equal number (2.5×10^6) of *Cxcr4*$^{C/C}$ (CD45.2, H2$^{b/b}$) and WT (CD45.1, H2$^{b/b}$) marrow cells into recipients (H2$^{b/d}$), and then deleted *Cxcr4* 4 wk after transplantation. The proliferation rate of HSCs was examined by a BrdU-uptake assay 14 wk after *Cxcr4* deletion. We found that 47% of *Cxcr4*$^{-/-}$ LSK cells had incorporated BrdU over a 4-d period, whereas only 18% of WT LSK cells were BrdU+ (Fig. 3 F). Because *Cxcr4*$^{-/-}$ LSK cells proliferated at a higher rate than the accompanied WT LSK cells in the same BM, we conclude that CXCR4 acts intrinsically in primitive hematopoietic cells to enforce quiescence.

CXCR4 signaling inhibits cell-cycle progression of primitive hematopoietic cells

Next, we investigated whether CXCR4 signaling directly inhibited cell-cycle progression of HSCs. The cell cycling profile of primitive hematopoietic cells were analyzed 24 h after BM cells cultured with different doses of CXCL12. We found that the proportion of WT primitive hematopoietic cells arrested in the G_0 phase was progressively increased in the presence of increasing doses of CXCL12. In contrast, the percentage of cycling *Cxcr4*$^{-/-}$ primitive hematopoietic cells was not affected at all by even the highest dose of CXCL12 (Fig. 4 A). These results thus demonstrate that CXCL12 prevents the entry of HSCs into the active cell cycle. Furthermore, because the proliferation of *Cxcr4*$^{-/-}$ primitive hematopoietic cells cannot be suppressed by CXCL12, we conclude that this action is mediated solely by CXCR4 and not by CXCR7, which is a newly identified receptor of CXCL12 (21).

To gain further insight into the mechanisms by which CXCR4 deficiency affected cell cycle regulation, we purified Flt3$^-$LSK cells from WT and *Cxcr4*$^{-/-}$ BM, and quantified expression of various cell cycle regulators by quantitative RT-PCR (qRT-PCR). Consistent with the hyperproliferative status of mutant cells, the expression of cyclin D1 was increased fourfold in *Cxcr4*$^{-/-}$ Flt3$^-$LSK cells over control cells. Previous reports have shown that p21$^{cip1/waf1}$ and Gfi-1 were required to impose G_0 arrest of HSCs. However, these molecules were expressed at similar levels in WT and *Cxcr4*$^{-/-}$ primitive hematopoietic cells. Interestingly, the cyclin-dependent kinase inhibitor p57^{kip2}, which was expressed at a particularly high

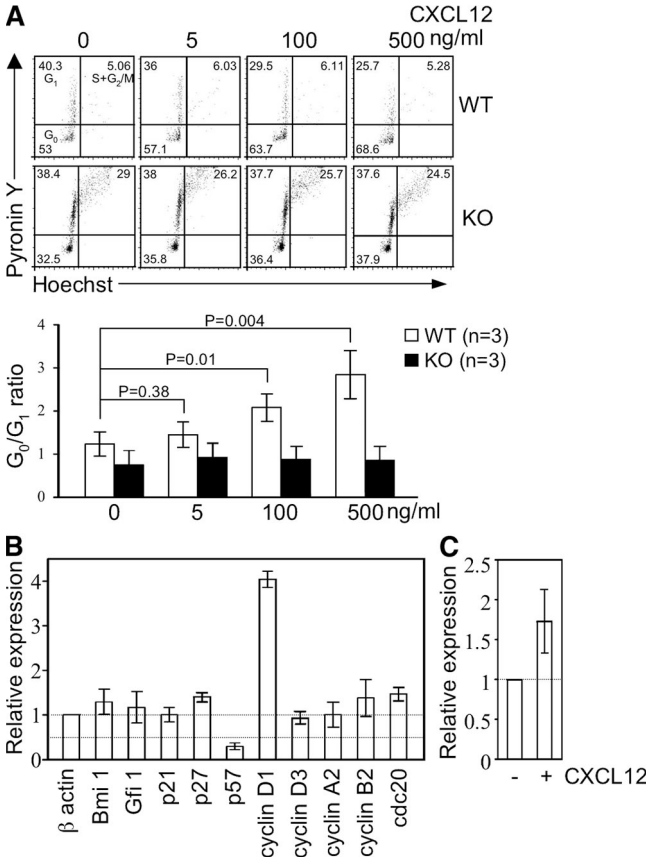

Figure 4. CXCL12 inhibits cell cycle progression of HSCs. (A) *Cxcr4*$^{-/-}$ and control BM cells were cultured for 24 h in the presence of cytokines and with CXCL12 at the indicated concentrations. Dot plots show cycling CD48^{-}Flt3^{-}LSK cells detected by pyronin Y/Hoechst staining. Bars (means ± the SD) show the ratio of LSK cells in the G_0 versus G_1 phase of cell cycle from three independent experiments. (B) Relative expression levels represent the ratio of each gene transcript in *Cxcr4*$^{-/-}$ versus WT Flt3^{-}LSK cells. cDNA input was normalized to the level of β-actin. Values are the means ± the SD of three experiments. (C) CD48^{-}Flt3^{-}LSK BM cells were sorted from WT mice and cultured in the presence of cytokines with or without 300 ng/ml of CXCL12 for 24 h. Each value was normalized to β-actin expression levels and is presented as fold induction compared with the p57^{kip2} expression level (set to 1) detected in CXCL12-untreated cells. Results (means ± the SD) are obtained from three experiments using independently sorted cells. P = 0.019.

level in long-term HSCs (22), was reduced to one third of the normal level in *Cxcr4*$^{-/-}$ primitive hematopoietic cells.

To assess whether CXCR4 signaling controls p57^{kip2} expression, sorted HSCs (Flt3^{-}CD48^{-}LSK cells) were incubated with CXCL12 for 24 h and p57^{kip2} expression levels were determined by qRT-PCR. As shown in Fig. 4 C, CXCL12 treatment significantly elevated the p57^{kip2} expression level in HSCs, thus establishing p57^{kip2} as one of the direct targets downstream of CXCR4 signaling pathway.

Concluding remarks

In this study, we ablated CXCR4 after HSCs had seeded in the BM and directly assessed whether BM retention of HSCs was affected by CXCR4 deficiency under the steady state. Our results showed that hematopoiesis was sustained for at least 8 mo, indicating the persistence of functional HSCs in the BM after CXCR4 inactivation. Indeed, we observed that the phenotypic *Cxcr4*$^{-/-}$ HSCs (Flt3^{-}LSK cells) were stably retained. Remarkably, when both *Cxcr4*$^{-/-}$ and WT HSCs were present in the same BM, the cellularity of *Cxcr4*$^{-/-}$ HSCs exceeded that of the WT, and the expansion of mutant HSCs was at the expense of competitive WT HSCs. In marked contrast, when *Cxcr4* deletion preceded transplantation, even fivefold more *Cxcr4*$^{-/-}$ HSCs could not compete with the cotransplanted WT HSCs. Together, our findings suggest that CXCR4 plays a critical role in guiding HSCs into the proper BM niche. However, after seeding in the stem cell niche, HSCs can be retained through a CXCR4-independent mechanism. We also noted that a prominent fraction of HSCs appeared in the periphery after *Cxcr4* deletion, similar to a previous study showing that the CXCR4 antagonist AMD3100 rapidly mobilized HSCs (14). It remains to be determined whether these results reflect that there are two subsets of HSCs that have different requirements for BM retention or simply indicate that overly proliferated HSCs cannot be contained in the BM niche. Recently, a new CXCL12 receptor, CXCR7, has been identified, and its binding to CXCL12 is unaffected by AMD3100 (21). It will be interesting to elucidate whether different HSC subsets differentially express CXCR4 and CXCR7, and whether CXCR7 and CXCR4 have distinct roles in homing and BM retention of HSCs.

It has been proposed that the stem cell niche in BM regulates self-renewal and differentiation of HSCs. However, the niche signals that restrain HSCs in the quiescent state have not been identified. Our data demonstrate an inhibitory effect of CXCR4 signaling on proliferation of primitive hematopoietic cells, as increased doses of CXCL12 progressively inhibit $G_0 \rightarrow G_1$ cell cycle progression of LSK cells. Consistent with the in vitro data, we found many more cycling cells in the *Cxcr4*$^{-/-}$ Flt3^{-}LSK compartment than in the cotransplanted WT population. It is noteworthy that hematopoiesis remained robust even 8 mo after *Cxcr4* deletion, suggesting that extensive proliferation of *Cxcr4*$^{-/-}$ HSCs did not exhaust the mutant stem cell pool during this period. In this regard, *Cxcr4*$^{-/-}$ HSCs differ from those carrying mutations in cell cycle regulators like p21$^{cip1/waf1}$ and Gfi-1, in which hyperproliferation causes depletion of HSCs in a competitive environment (2, 3). Indeed, both p21$^{cip1/waf1}$ and Gfi-1 were expressed at similar levels in WT and *Cxcr4*$^{-/-}$ HSCs. Interestingly, our study identified p57^{kip2} as one of the putative targets of CXCR4 signaling pathways. These data suggest different functions of cell cycle regulators in HSCs. Further experiments are required to determine whether p57^{kip2} specifically inhibits HSC proliferation, whereas p21$^{cip1/waf1}$ plays additional roles in HSC self-renewal.

Recently, results of an independently derived line of CXCR4 conditional knockout mice were published, which showed that in the absence of CXCR4 primitive hematopoietic cells (LSK cells) were retained in the BM and became

hyperproliferative, a phenotype nearly identical to that described herein (7). However, this report noted a drastically reduced HSC compartment ($CD34^-$ LSK cells) in the BM accompanied by impaired hematopoiesis 4 mo after *Cxcr4* deletion. In this mouse model, the *Cxcr4* gene was deleted by poly I/poly C–activated Mx-Cre. It should be pointed out that the poly I/poly C treatment used in this study is deleterious to HSCs, because injections of poly I/poly C (300 μg/mouse, 4 times) wiped out WT HSCs in 2 d and HSCs in the BM did not recover even 3 wk after the treatment. On the contrary, tamoxifen administration used in our system did not cause noticeable toxic effect on the WT HSC compartment. Under these different conditions, although the $Cxcr4^{-/-}$ HSC compartment was preserved in mice treated with tamoxifen, it was "lost" in mice that received poly I/poly C. At present, the precise reason for this discrepancy cannot be ascertained, but might be related to a distinct role of CXCR4 in HSC survival in homeostatic state or under hematologic stress caused by poly I/poly C treatment. CXCL12 has been reported to enhance survival of primitive hematopoietic cells (23, 24), and of myeloid progenitor cells after cytokine withdrawal (25). Although we did not observe any changes in apoptosis of freshly isolated $Cxcr4^{-/-}$ HSCs as compared with the WT HSCs, it is possible that the survival of $Cxcr4^{-/-}$ HSCs is impaired under stress conditions, thereby compromising hematologic recovery from chemoablation such as poly I/poly C treatment. It is also conceivable that both WT and $Cxcr4^{-/-}$ HSCs could no longer reside in the stem cell niche under stress condition after poly I/poly C treatment. Although WT HSCs may repopulate the BM niche, $Cxcr4^{-/-}$ HCSs could not, thereby resulting in depletion of the mutant HSCs.

In summary, we demonstrate that the CXCL12–CXCR4 axis is essential for HSCs homing into the BM, but less critical for the BM retention. Our results also indicate that CXCR4 signaling restricts HSCs in quiescence, and it probably does so through up-regulating the cell cycle inhibitor $p57^{kip2}$. Interestingly, HIV/gp120, which is a viral ligand of CXCR4, has been shown to inhibit neural progenitor cell proliferation (26). This result thus suggests a universal role of CXCR4 signaling in the control of the quiescence of other somatic stem cells. Further investigation will be required to determine whether this inhibition also involves the same cell cycle regulators. It is also worthwhile to examine whether this mechanism may also contribute to HIV-1–associated dementia and immunodeficiency.

MATERIALS AND METHODS

Mice. $Cxcr4^{fl/fl}$ mice were crossed to $Cxcr4^{fl/fl}ROSA^{CRE\text{-}ERT2/+}$ mice (provided by T. Ludwig, Columbia University, New York, NY) to generate $Cxcr4^{C/C}$ mice. To delete *Cxcr4*, two sets of three consecutive administrations of tamoxifen (5 mg/mouse, i.p.) were delivered 3 d apart. Mice were maintained under specific pathogen–free conditions, and used according to the protocol approved by the Columbia University Institutional Animal Care and Use Committee.

Flow cytometry. BM cells were stained with PE-Cy5–conjugated antibodies against lineage markers (Lin), including B220, CD3, CD4, CD8, CD11b, Gr1, and Ter119; PE-Cy7–conjugated anti–c-Kit; and APC-conjugated anti-Sca1. Long-term HSCs defined as $Flt3^-$ LSK were visualized by staining BM cells with biotin-Flt3 followed by streptavidin-FITC with the combination of the aforementioned antibodies. Common lymphoid progenitors (Lin^- c-Kit^{lo} and Sca-1^{lo} IL-$7R\alpha^+$) were distinguished by biotinylated anti–IL-7Rα followed by streptavidin-FITC and antibodies against Lin, c-Kit, and Sca-1, as described. For lineage analyses, cells were stained with APC-B220, PE-TCR, and PE-Cy5-Gr1. All antibodies were obtained from eBioscience. BM cellularity was calculated from two femurs.

Proliferation analysis. $Cxcr4^{-/-}$ and WT BM cells were cultured in medium (OptiMEM; Invitrogen) with 7% FCS (HyClone), 50 ng/ml SCF, 10 ng/ml IL-3, and 10 ng/ml IL-6. All cytokines were purchased from R&D Systems. 24 h after incubation with different doses of CXCL12, cells were harvested for cell cycle analysis. For BrdU uptake assay, $Cxcr4^{-/-}$ mice were injected with BrdU (1 mg/mouse, i.p.) and fed with drinking water containing 1mg/ml BrdU for various periods, as indicated. BM cells were stained with antibodies against PE-Cy5-Lin, APC-Sca1, and PE-Cy7-c-Kit, and permeabilized, followed by staining with FITC-BrdU. For cell cycle analysis, cells were fixed with 4% paraformaldehyde and labeled with 0.5 μg/ml pyronin Y (Sigma-Aldrich) and 1 μg/ml Hoechst 33342 (Fluka), and analyzed on an LSR II flow cytometer (BD Biosciences).

Gene expression analysis. LSK cells were purified from $Cxcr4^{-/-}$ and WT mice by FACS sorting. After mRNA extraction with TRIzol (Life Technologies), cDNA was synthesized using Superscript II (Invitrogen). qRT-PCR was done with the ABI7700 Sequence Detection System (Applied Biosystems). Sequences of the primers used for qRT-PCR are listed in Table S1 (available at http://www.jem.org/cgi/content/full/jem.20072513/DC1).

Transplantation. 8-wk-old BDF1 recipient mice (B6D2F1/J; The Jackson Laboratory; $H2\text{-}D^d$) received 2 doses of 500 rads within a 6-h interval. Donor BM cells from WT (CD45.1), $Cxcr4^{-/-}$, and $Cxcr4^{C/C}$ mice (CD45.2) were then transferred into irradiated recipients. Recipient mice were injected with tamoxifen to delete *Cxcr4* at various times after transplantation, as indicated in the text.

Online supplemental material. Fig. S1 shows efficient deletion of *Cxcr4* in primitive hematopoietic cells. Fig. S2 shows a normal HSC compartment in $ROSA^{CRE\text{-}ERT2}$ mice. Fig. S3 shows that CXCR4-deficient primitive hematopoietic cells are indeed functional HSCs. Fig. S4 shows that CXCR4 deficiency does not affect HSC survival. Table S1 lists primers for qRT-PCR. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20072513/DC1.

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