Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches

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Summary

In the bone marrow, the special microenvironment niches nurture a pool of hematopoietic stem cells (HSCs). Many HSCs reside near the vasculature, but the molecular regulatory mechanism of niches for HSC maintenance remains unclear. Here we showed that the induced deletion of CXCR4, a receptor for CXC chemokine ligand (CXCL) 12 in adult mice, resulted in severe reduction of HSC numbers and increased sensitivity to myelotoxic injury, although it did not impair expansion of the more mature progenitors. Most HSCs were found in contact with the cells expressing high amounts of CXCL12, which we have called CXCL12-abundant reticular (CAR) cells. CAR cells surrounded sinusoidal endothelial cells or were located near the endosteum. CXCL12-CXCR4 signaling plays an essential role in maintaining the quiescent HSC pool, and CAR cells appear to be a key component of HSC niches, including both vascular and endosteal niches in adult bone marrow.

Introduction

Hematopoietic stem cells (HSCs) give rise to all types of blood cells including lymphocytes and myeloid cells. In the bone marrow, the niches where HSCs reside are thought to supply the requisite factors and play an essential role in maintaining a pool of HSCs to provide the appropriate numbers of mature blood cells throughout life (Moore and Lemischka, 2006; Wilson and Trumpp, 2006). Most primitive hematopoietic cells, including HSCs, are thought to be in a quiescent G_0 state in these niches, probably to prevent depletion of the regenerative cell pool or sensitivity to myelotoxic injury (Cheshier et al., 1999). Therefore, it is important to understand the molecular regulatory mechanism of niches for maintenance of the stem cell pool, not only for stem cell and developmental biology of lymphohematopoietic cells but also for bone marrow transplantation and gene therapy requiring HSCs. It was reported previously that osteoblasts lining the bone surface act as HSC niches (Calvi et al., 2003; Zhang et al., 2003) and that Angiopoietin-1 (Ang-1), observed exclusively in osteoblasts, maintains HSC quiescence (Arai et al., 2004). In contrast, imaging of HSCs reveal that many HSCs are associated

with sinusoidal endothelium although only some HSCs adjoin the endosteum (Kiel et al., 2005). Nevertheless, the precise cellular components and niche-derived factors regulating HSCs in the vascular niches remain unclear.

CXC chemokine ligand (CXCL)12 (also known as stromal cell-derived factor [SDF]-1 or pre-B-cell-growth-stimulating factor [PBSF]) is a member of a large family of structurally related chemoattractive cytokines and was first characterized as a growth-stimulating factor for the B cell precursor clone (Nagasawa et al., 1994). The primary physiologic receptor for CXCL12 is CXCR4, a hepta helical receptor coupled to heterotrimeric guanosine triphosphate (GTP) binding proteins, which also functions as an entry receptor for the HIV-1 virus (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). Studies of mutant mice with targeted gene disruption have revealed that CXCL12-CXCR4 signaling is essential for hematopoiesis, including B cell development and colonization of bone marrow by hematopoietic progenitors, including HSCs, during ontogeny as well as cardiovascular formation and neurogenesis (Ara et al., 2003; Nagasawa et al., 1996; Nagasawa, 2006; Tachibana et al., 1998; Zou et al., 1998). Lethality caused by deficiencies of CXCL12 and CXCR4 prevents immediate analysis of their role in adult hematopoiesis. Treatment with CXCR4-selective antagonist induces increase in HSCs in the peripheral blood, suggesting a role for CXCL12 in retaining HSCs in hematopoietic organs (Broxmeyer et al., 2005). In addition, treatment of immature human bone marrow $CD34^+$ cells with CXCR4 antibodies prevents the engraftment of primitive human nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mouse repopulating cells (SRCs) (Peled et al., 1999). However, experiments with radiation chimeras have revealed only a modest reduction in long-term myeloid reconstitution by $Cxcr4^{-/-}$ fetal liver cells (Kawabata et al., 1999; Ma et al., 1999). Therefore, the roles of CXCL12-CXCR4 signaling in HSCs within adult bone marrow remain unclear.

Here we show that CXCL12-CXCR4 signaling is essential in adult bone marrow to maintain the HSC pool and suggest that many HSCs are in contact with a small population of reticular cells expressing high amounts of CXCL12 (Tokoyoda et al., 2004). In addition, almost all HSCs near the sinusoidal endothelium appear to be in contact with these reticular cells surrounding endothelial cells in the extravascular spaces, suggesting that these cells are the key cellular components of HSC vascular niches.

Results

Induced Deletion of CXCR4 in Adult Bone Marrow
To determine the roles of CXCL12-CXCR4 signaling in hematopoiesis in adult animals, we generated CXCR4 conditionally deficient mice. We crossed mice with a loxP-CXCR4 conditional targeting allele (CXCR4[flox(f)/null] mice) (Tokoyoda et al., 2004) to MxCre mice (Kuhn et al., 1995) in which Cre was expressed after the
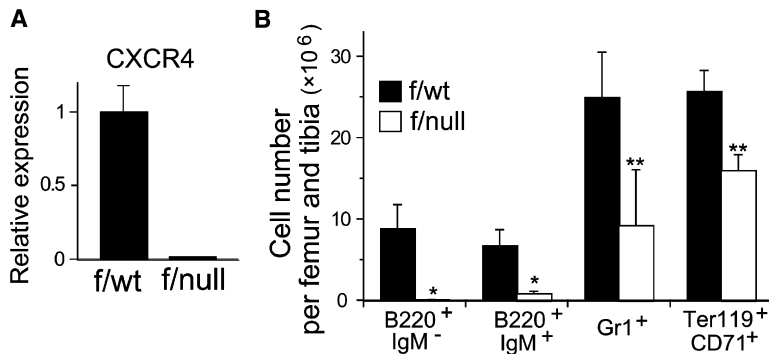
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A

B

Figure 1. Hematopoiesis in Adult Bone Marrow from CXCR4 Conditionally Deficient Mice

(A) QRT-PCR analysis of mRNA expression of CXCR4 in c-kit$^+$Sca-1$^+$Lin$^-$ cells in the bone marrow from pIpC-treated MxCre-CXCR4$^{f/null}$ (f/null) and MxCre-CXCR4$^{f/wt}$ (f/wt) mice at 16 weeks after the final pIpC treatment (n = 3, p = 0.009).

(B) Flow cytometric analysis of the numbers of B220$^+$IgM$^-$ B cell precursors, B220$^+$IgM$^+$ B cells, Gr-1$^+$ myeloid lineage cells and Ter119$^+$CD71$^+$ erythroblasts in bone marrow from pIpC-treated MxCre-CXCR4$^{f/null}$ and MxCre-CXCR4$^{f/wt}$ mice at 16 weeks after final pIpC treatment (n = 3; *p < 0.02; **p < 0.05). Error bars represent SD of the mean.

induction of interferon by the administration of poly(I)-poly(C) (pIpC) to inactivate the CXCR4 gene in the adult animals. The floxed allele was excised almost completely in bone marrow c-kit$^+$Sca-1$^+$Lineage (Lin)$^-$ primitive hematopoietic cells and myeloid lineage cells of some pIpC-treated MxCre-CXCR4$^{f/null}$ mice as analyzed by quantitative, real-time polymerase chain reaction with reverse transcription (qRT-PCR) and flow cytometry (Figure 1A and data not shown). Flow cytometric analysis revealed that the bone marrow of pIpC-treated MxCre-CXCR4$^{f/null}$ mice contained severely reduced numbers of B cells but modestly reduced numbers of myeloid or erythroid lineage cells compared with control MxCre-CXCR4$^{wt/wt}$ or MxCre-CXCR4$^{f/wt}$ mice (Figure 1B). In peripheral blood, most pIpC-treated MxCre-CXCR4$^{f/null}$ mice showed increased myeloid lineage cells (data not shown). These results are consistent with the known phenotype of mice reconstituted with CXCR4-deficient fetal liver cells (Kawabata et al., 1999; Ma et al., 1999).

HSCs Are Severely Reduced in Adult Bone Marrow of CXCR4 Conditionally Deficient Mice

HSCs can be highly purified as CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ cells (Osawa et al., 1996). In addition, stem cells have the differential ability to efflux Hoechst dye, which defines a small subset of side population (SP) cells (Goodell et al., 1996, 1997). SP cells with higher amounts of Hoechst efflux (lower SP) are a nearly homogeneous subset of bone marrow HSCs (Camargo et al., 2006), and they can reconstitute myeloablated mice with absolute efficiency (Matsuzaki et al., 2004). Consistent with these, the numbers of CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ cells were similar to those of lower SP (SP^{low}) cells and increased with age in wild-type bone marrow (data not shown; Sudo et al., 2000).

pIpC reversibly inhibited expansion of primitive hematopoietic cells, probably reflecting a transient antiproliferative and/or apoptotic effect of interferon induced by pIpC treatment (Gidali et al., 1981). In addition, the numbers of CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ and SP^{low} cells were reduced in pIpC-treated wild-type and control MxCre-CXCR4$^{f/wt}$ mice as well as MxCre-CXCR4$^{f/null}$ mice at early time points after injection of pIpC (see Figure S1 in the Supplemental Data available online and data not shown). Therefore, we analyzed the role of CXCR4 in HSCs via pIpC-treated MxCre-CXCR4$^{f/null}$ and control mice at the late stages after pIpC injection

when the mice recovered from the effects of pIpC in terms of the HSC numbers. Multiparameter flow cytometric analysis revealed that the numbers of CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ and SP^{low} cells in bone marrow were comparable in pIpC-treated MxCre-CXCR4$^{f/wt}$ mice but severely reduced in pIpC-treated MxCre-CXCR4$^{f/null}$ mice compared with pIpC-treated MxCre-CXCR4$^{wt/wt}$ mice and untreated animals at 16 to 36 weeks after the final pIpC injection (Figures 2A and 2B, and data not shown), indicating that CXCL12-CXCR4 signaling is required to maintain the HSC pool within bone marrow.

Next we compared the expression of genes that were highly expressed in HSCs in the bone marrow c-kit$^+$Sca-1$^+$Lin$^-$ cell population in pIpC-treated MxCre-CXCR4$^{f/null}$ and control mice. Among the genes involved in HSC regulation, the expressions of *Tek* (encoding Tie2), which is a physiologic receptor for Ang-1 (Arai et al., 2004), *Vegfa* (Gerber et al., 2002), and *Junb* (Passegue et al., 2004) increased in CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ HSCs compared with CD34$^+$c-kit$^+$Sca-1$^+$Lin$^-$ cells, which were enriched for non-self-renewing primitive hematopoietic progenitors including multipotent progenitors (MPPs) (Nakauchi et al., 1999; Osawa et al., 1996) in wild-type mice via qRT-PCR (Figure 2C, left). Gene expression analysis revealed that the expressions of *Tek*, *Vegfa*, and *Junb* were markedly reduced in pIpC-treated MxCre-CXCR4$^{f/null}$ mice compared with control MxCre-CXCR4$^{wt/wt}$ or MxCre-CXCR4$^{f/wt}$ mice (Figure 2C, right; and data not shown). These results support the idea that the reduction of CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ cell numbers in pIpC-treated MxCre-CXCR4$^{f/null}$ mice reflected the decreased numbers of HSCs, but not merely as a consequence of upregulated CD34 expression in HSCs based on the amount of cell activation (Sato et al., 1999) in these mutants.

The Numbers of LTC-ICs and CRUs in Adult Bone Marrow of CXCR4 Conditionally Deficient Mice

We performed long-term in vitro cultures (LTCs) on primary bone marrow stromal cells via limiting dilution LTC-initiating cell (IC) assays (Ploemacher et al., 1989; Stier et al., 2005). The frequency of LTC-ICs has been shown to correlate with in vivo repopulating potential (Ploemacher et al., 1989). A marked reduction of LTC-ICs was observed in bone marrow from pIpC-treated MxCre-CXCR4$^{f/null}$ mice compared with control animals (Figure 2D). This suggests that the numbers of HSCs were reduced in CXCR4 conditionally deficient mice
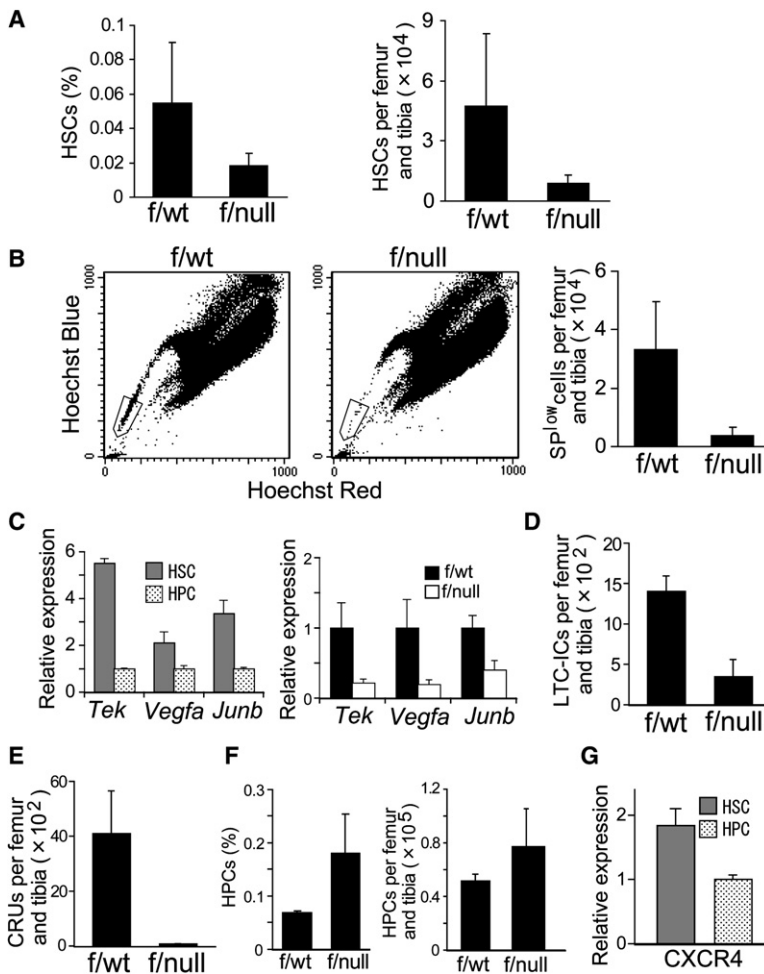
Figure 2. Reduced HSC Pool in Adult Bone Marrow in the Absence of CXCR4

(A and B) Flow cytometric analysis of HSCs in bone marrow from pIpC-treated MxCre-CXCR4$^{f/null}$ and MxCre-CXCR4$^{f/wt}$ mice.

(A) The frequencies (left) and the absolute numbers (right) of CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ cells in the bone marrow (left, n = 6, p = 0.026; right, n = 6, p = 0.031).

(B) Fluorescence staining profile of Lin$^-$ SP cells, where the boxed region is the lower SP (SP^{low}) region (left and middle). The numbers of SP^{low} cells in bone marrow (right; n = 6, p = 0.0061).

(C) QRT-PCR analysis of mRNA expression of Tek, Vegfa, and Junb in CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ HSCs and CD34$^+$c-kit$^+$Sca-1$^+$Lin$^-$ HPCs in bone marrow from wild-type mice (left, n = 3). QRT-PCR analysis of mRNA expression of Tek, Vegfa, and Junb in c-kit$^+$Sca-1$^+$Lin$^-$ cells in bone marrow from pIpC-treated MxCre-CXCR4$^{f/null}$ and MxCre-CXCR4$^{f/wt}$ mice (right, n = 4).

(D) The numbers of LTC-ICs in bone marrow from pIpC-treated MxCre-CXCR4$^{f/null}$ and MxCre-CXCR4$^{f/wt}$ mice (n = 4, p = 0.019). LTC-IC numbers per femur and tibia were assayed by limiting dilution analysis.

(E) The numbers of CRU in the bone marrow from pIpC-treated MxCre-CXCR4$^{f/null}$ and MxCre-CXCR4$^{f/wt}$ mice (n = 2). Long-term in vivo competitive repopulating unit (CRU) numbers per femurs and tibias were assayed by limiting dilution analysis.

(F) Flow cytometric analysis of HPCs in bone marrow from pIpC-treated MxCre-CXCR4$^{f/null}$ and MxCre-CXCR4$^{f/wt}$ mice. The frequencies (left) and the absolute numbers (right) of CD34$^+$c-kit$^+$Sca-1$^+$Lin$^-$ hematopoietic progenitor cells (HPCs) in the bone marrow (n = 4).

(G) QRT-PCR analysis of mRNA amounts of CXCR4 in CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ HSCs and CD34$^+$c-kit$^+$Sca-1$^+$Lin$^-$ HPCs in bone marrow from wild-type mice (n = 4, p = 0.020). Error bars represent SD of the mean.

because in vitro studies showed that colony formation per se in LTCs was unaffected by the administration of CXCR4 selective antagonist (data not shown).

To further define the roles of CXCR4 in HSCs, the limiting-dilution analysis was performed with long-term in vivo competitive repopulating unit (CRU) assay to detect and measure the frequency of the cells capable of long-term lympho-myeloid repopulation in bone marrow cells (Szilvassy et al., 1990). Repopulation of the hematopoietic system was analyzed for the presence of myeloid cells in peripheral blood because they were not obviously reduced in the absence of CXCR4 as mentioned above (Figure S2). The numbers of CRU in the bone marrow of pIpC-treated MxCre-CXCR4$^{f/null}$ mice was severely reduced compared with control animals (Figure 2E).

Development of More Mature Hematopoietic Progenitors in CXCR4 Conditionally Deficient Mice

In contrast to HSC reduction, CD34$^+$c-kit$^+$Sca-1$^+$Lin$^-$ progenitors were slightly increased in bone marrow from most pIpC-treated MxCre-CXCR4$^{f/null}$ mice compared with control pIpC-treated MxCre-CXCR4$^{wt/wt}$ or MxCre-CXCR4$^{f/wt}$ mice by flow cytometric analysis

(Figure 2F), suggesting that CXCL12 plays a specific role in HSCs within primitive hematopoietic cells. Consistent with this, qRT-PCR analysis revealed that CXCR4 mRNA expression in CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ HSCs was higher than in CD34$^+$c-kit$^+$Sca-1$^+$Lin$^-$ progenitors (Figure 2G).

Flow cytometric analysis of peripheral blood and spleen revealed that CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ cells were not observed in pIpC-treated MxCre-CXCR4$^{f/null}$ and control mice, although the numbers of c-kit$^+$Sca-1$^{lo/-}$Lin$^-$ cells were increased in the mutants (Figure S3). These results suggest that CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ cell reduction in bone marrow is not due to defective retention in the marrow cavity. In addition, it may be that CXCR4 is involved in the retention of more mature c-kit$^+$Sca-1$^{lo/-}$Lin$^-$ cells in bone marrow and/or peripheral blood. Alternatively, extramedullary hematopoiesis possibly occurs when HSCs in bone marrow are severely reduced in the absence of CXCR4.

Susceptibility of CXCR4 Conditionally Deficient Mice to Myelosuppressive Stress

The finding that CXCL12-CXCR4 signaling is essential for maintaining the HSC pool led us to examine
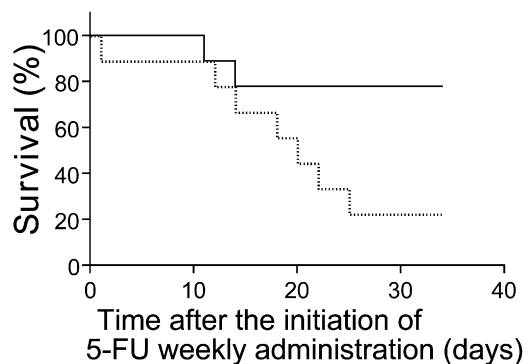
Figure 3. Reduced Survival Frequency after 5-FU Treatment in the Absence of CXCR4

5-FU was administered weekly, and survival data were analyzed by a log-rank nonparametric test and expressed as Kaplan-Meier survival curves (n = 9, p = 0.037). Unbroken black lines, pIpC-treated MxCre-CXCR4[f/wt] mice; broken black lines, pIpC-treated MxCre-CXCR4[f/null] mice.

susceptibility of pIpC-treated MxCre-CXCR4[f/null] mice to myelosuppressive stress. Treatment of mice with antimetabolite 5-fluorouracil (5-FU) is selectively cytotoxic for cycling hematopoietic cells, induces bone marrow hypoplasia, and can cause lethality. However, quiescent HSCs are thought to be resistant to the effect of 5-FU and play a role in restoring hematopoiesis. When animals were treated weekly with 5-FU at a dose of 120 mg/kg, the survival frequency of pIpC-treated MxCre-CXCR4[f/null] mice was much lower than that in control MxCre-CXCR4[f/wt] mice (Figure 3). Thus, CXCR4-deficient mice are vulnerable to myelosuppressive stress, consistent with dramatic reduction of the HSC pool.

Enhanced Exit from Quiescent G_0 Phase in the Primitive Hematopoietic Progenitors of CXCR4 Conditionally Deficient Mice

Because CD34+c-kit+Sca-1+Lin− cells in the bone marrow were slightly increased in most pIpC-treated MxCre-CXCR4[f/null] mice, the cell-cycling status of primitive hematopoietic cells was determined with the RNA-specific dye pyronin Y (PY) in conjunction with DNA-specific dye Hoechst 33342 or proliferation marker Ki67 in conjunction with the DNA-specific dye 7AAD as a measure of quiescence (Gerdes et al., 1984; Ladd et al., 1997). Quiescent cells in G_0 have a low RNA content (low PY uptake) (Ladd et al., 1997), and Ki67 is expressed in all actively dividing cells but not in quiescent (G_0) cells (Gerdes et al., 1984). Although most c-kit+Sca-1+Lin− cells in control mice were quiescent in G_0, the remaining c-kit+Sca-1+Lin− cells in pIpC-treated MxCre-CXCR4[f/null] mice demonstrated a smaller fraction of low PY uptake or Ki67-negative G_0 cells and a larger fraction of high PY uptake or Ki67-positive actively dividing (G_1+S+G_2/M) cells, respectively (Figures 4A–4C). Because HSCs were severely decreased in the c-kit+Sca-1+Lin− cell population in the absence of CXCR4, these results raised the possibility that exit from the quiescent G_0 phase was promoted in the remaining CD34+ c-kit+Sca-1+Lin− progenitors in CXCR4 conditionally deficient mice. To address this possibility, we analyzed the frequencies of cycling cells within the CD34+
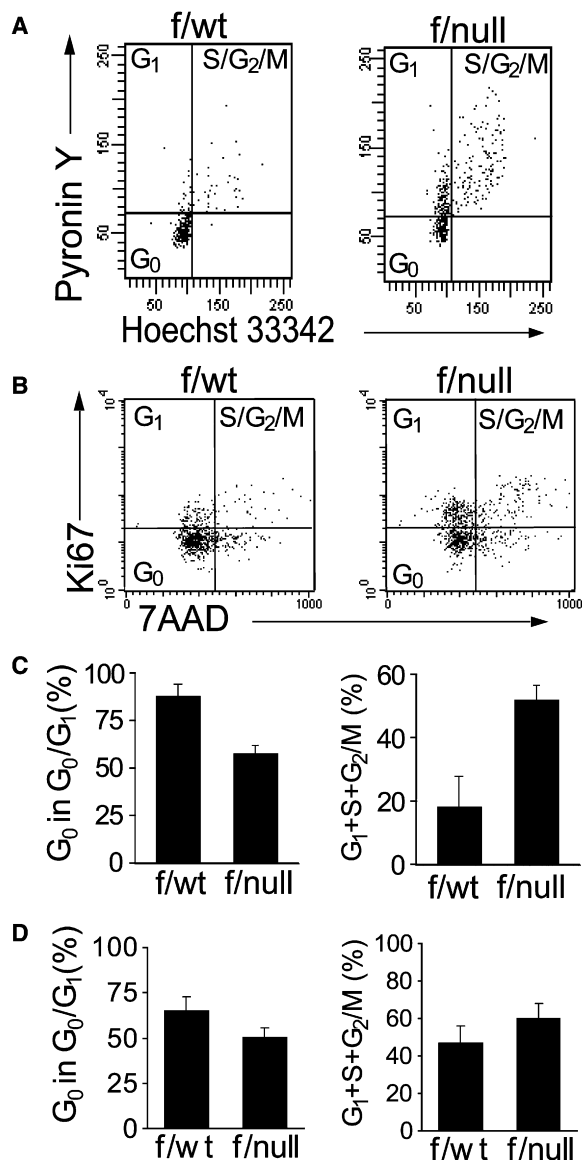

Figure 4. Increased Fraction of the Primitive Hematopoietic Progenitors in Active Cell Cycle in the Bone Marrow from CXCR4 Conditionally Deficient Mice

Flow cytometric analysis of the bone marrow from pIpC-treated MxCre-CXCR4[f/null] and MxCre-CXCR4[f/wt] mice.

(A) Fluorescence staining profile of c-kit+Sca-1+Lin− cells after staining with PY and Hoechst 33342.

(B) Fluorescence staining profile of c-kit+Sca-1+ cells after staining with Ki67 and 7AAD.

(C) The percentage of G_0 cells (Ki67-negative) in G_0/G_1 fraction (left) and the percentage of G_1+S+G_2/M cells (Ki67-positive, right) in c-kit+Sca-1+ cell population (left, n = 3, p = 0.008; right, n = 3, p = 0.007). Error bars represent SD of the mean.

(D) The percentage of G_0 cells (Ki67-negative) in G_0/G_1 fraction (left) and the percentage of G_1+S+G_2/M cells (Ki67-positive, right) in CD34+c-kit+Sca-1+ cell population (left, n = 3, p = 0.045; right, n = 3, p = 0.043). Error bars represent SD of the mean.

c-kit+Sca-1+ compartment. CD34+c-kit+Sca-1+ cells in pIpC-treated MxCre-CXCR4[f/null] mice comprised a smaller fraction of Ki67-negative G_0 cells and a larger fraction of Ki67-positive actively dividing (G_1+S+G_2/M) cells, compared with CD34+c-kit+Sca-1+ cells in control

chimeras (Figure 4D). This indicated that increased percentages of G_1+S+G_2/M cells in c-kit$^+$Sca-1$^+$ cells were partly due to the promotion of mitotic cycling in the remaining CD34$^+$c-kit$^+$Sca-1$^+$ progenitors but not merely the reduction of G_0 HSC numbers in the mutants.

Cell-Intrinsic Alterations in HSCs and an Altered Microenvironment in the Absence of CXCR4

To examine the consequence of CXCR4 deficiency on hematopoietic cells and the contribution from a mutant microenvironment, we generated mice with mixed chimeric bone marrow cells in which conditionally targeted cells could be distinguished from wild-type competitors by surface marker expression. Bone marrow cells derived from MxCre-CXCR4$^{f/null}$ or MxCre-CXCR4$^{f/wt}$ mice carrying the Ly5.2$^+$Ly5.1$^-$ alleles were mixed with wild-type Ly5.2$^-$Ly5.1$^+$ competitor marrow cells and transferred into lethally irradiated Ly5.2$^-$Ly5.1$^+$ mice. Analysis of the surface expression of Ly5.1 by flow cytometry revealed that donor-derived MxCre-CXCR4$^{f/null}$ or MxCre-CXCR4$^{f/wt}$ cells contributed comparably to peripheral blood cells in these recipient mice 12 weeks after transfer. They maintained approximately 80% Ly5.2$^+$ Ly5.1$^-$ peripheral blood cells. Mice were then treated with pIpC to induce excision of the floxed allele 13 weeks after transfer. At 10 weeks after the final pIpC treatment, bone marrow was analyzed by flow cytometry, revealing that donor-derived Ly5.1$^-$CD34$^-$c-kit$^+$ Sca-1$^+$ HSCs were severely reduced in mutant chimeras compared with control chimeras (Figure 5A). In contrast, competitor-derived Ly5.1$^+$CD34$^-$c-kit$^+$Sca-1$^+$ HSCs in mutant chimeras were not impaired (Figure 5B). These results suggest that the deficits in maintenance of the HSC pool in the absence of CXCR4 are HSC autonomous.

Analysis of the cell-cycling status of primitive hematopoietic cells revealed that competitor-derived wild-type Ly5.1$^+$c-kit$^+$Sca-1$^+$ cells in the bone marrow from mutant chimeras demonstrated a smaller fraction in quiescent G_0 cells and a larger fraction in actively dividing (G_1+S+G_2/M) cells compared with c-kit$^+$Sca-1$^+$ cells in control chimeras (Figure 5C). This suggests that the altered cell-cycle status in mutant chimeras is due to an altered microenvironment.

CAR Cells Scattered throughout Bone Marrow Are the Major Producers of CXCL12

The result that CXCL12-CXCR4 signaling is required to maintain the HSC pool prompted us to examine further the expression of CXCL12 in bone marrow. Our study of mice with the GFP reporter gene knocked into the CXCL12 locus (CXCL12-GFP knockin mice) showed a population of reticular cells, which express CXCL12 at high amounts, with several long processes and scattered throughout adult bone marrow (Tokoyoda et al., 2004). Further, it has been shown previously that CXCL12 is expressed in osteoblasts lining the bone surface (Jung et al., 2006; Ponomaryov et al., 2000), in bone (Katayama et al., 2006), and in endothelial cells (Dar et al., 2005; Ponomaryov et al., 2000; Sipkins et al., 2005). Histological analysis of CXCL12-GFP knockin mice revealed that most cells expressing high amounts of CXCL12 were not in close proximity to the bone surface but were scattered throughout the intertra-
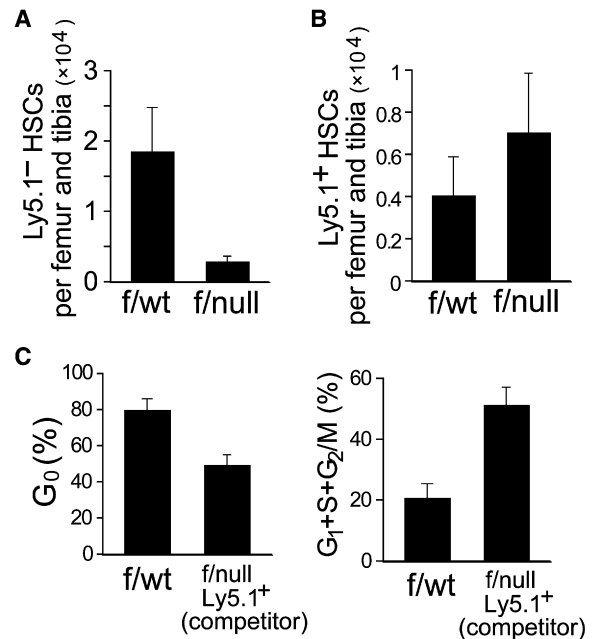
Figure 5. Reduced HSC Pool and Increased Fraction of the Primitive Hematopoietic Cells in Active Cell Cycle in Mice Reconstituted with CXCR4$^{f/null}$ Bone Marrow Cells

Bone marrow cells (Ly5.2$^+$Ly5.1$^-$) from MxCre-CXCR4$^{f/null}$ and MxCre-CXCR4$^{f/wt}$ mice were transferred along with wild-type bone marrow cells (Ly5.2$^-$Ly5.1$^+$) into lethally irradiated Ly5.2$^-$Ly5.1$^+$ recipients. Chimeric mice were treated with pIpC at 13 weeks after transfer and their bone marrow was analyzed by flow cytometry at 10 weeks after the treatment (n = 3).

(A) Numbers of MxCre-CXCR4$^{f/null}$ and MxCre-CXCR4$^{f/wt}$ Ly5.1$^-$ CD34$^-$c-kit$^+$Sca-1$^+$ HSCs in bone marrow (p = 0.028).

(B) Numbers of competitor-derived Ly5.1$^+$CD34$^-$c-kit$^+$Sca-1$^+$ HSCs in bone marrow (p = 0.082).

(C) Analysis of cell-cycle status of c-kit$^+$Sca-1$^+$ cells after staining with Ki67. The percentage of G_0 cells (left) and G_1+S+G_2/M cells (right) in competitor-derived wild-type c-kit$^+$Sca-1$^+$ cell population in control and mutant chimeras (left, p = 0.005; right, p = 0.005). Error bars represent SD of the mean.

becular space of the bone marrow cavity and GFP expression was barely detectable in bone (Figure 6A). Immunohistochemical analysis with antibodies against the osteoblast marker osteocalcin revealed that the majority of osteoblastic cells that expressed osteocalcin and were in close proximity to the bone surface exhibited an undetectable expression of GFP (Figures 6B–6D; Figures S4A and S4B), but some of these cells exhibited a much lower expression of GFP relative to the reticular cells expressing high amounts of CXCL12 (Figures 6E–6G; Figures S4C and S4D). Thus, reticular cells that expressed high amounts of CXCL12 were termed CXCL12-abundant reticular (CAR) cells, and our results indicate that CAR cells are the major producer of CXCL12 in adult bone marrow. It has been reported previously that the cells lining the bone surface and expressing Jagged1 (Calvi et al., 2003) or N-cadherin (Zhang et al., 2003) act as HSC niches. Immunohistochemical analysis with antibodies against Jagged1 or N-cadherin revealed that CAR cells exhibited similar expression of Jagged1 and lower expression of N-cadherin relative to the cells lining the bone surface (Figures 6H–6K; Figures S4E–S4H). Additionally,
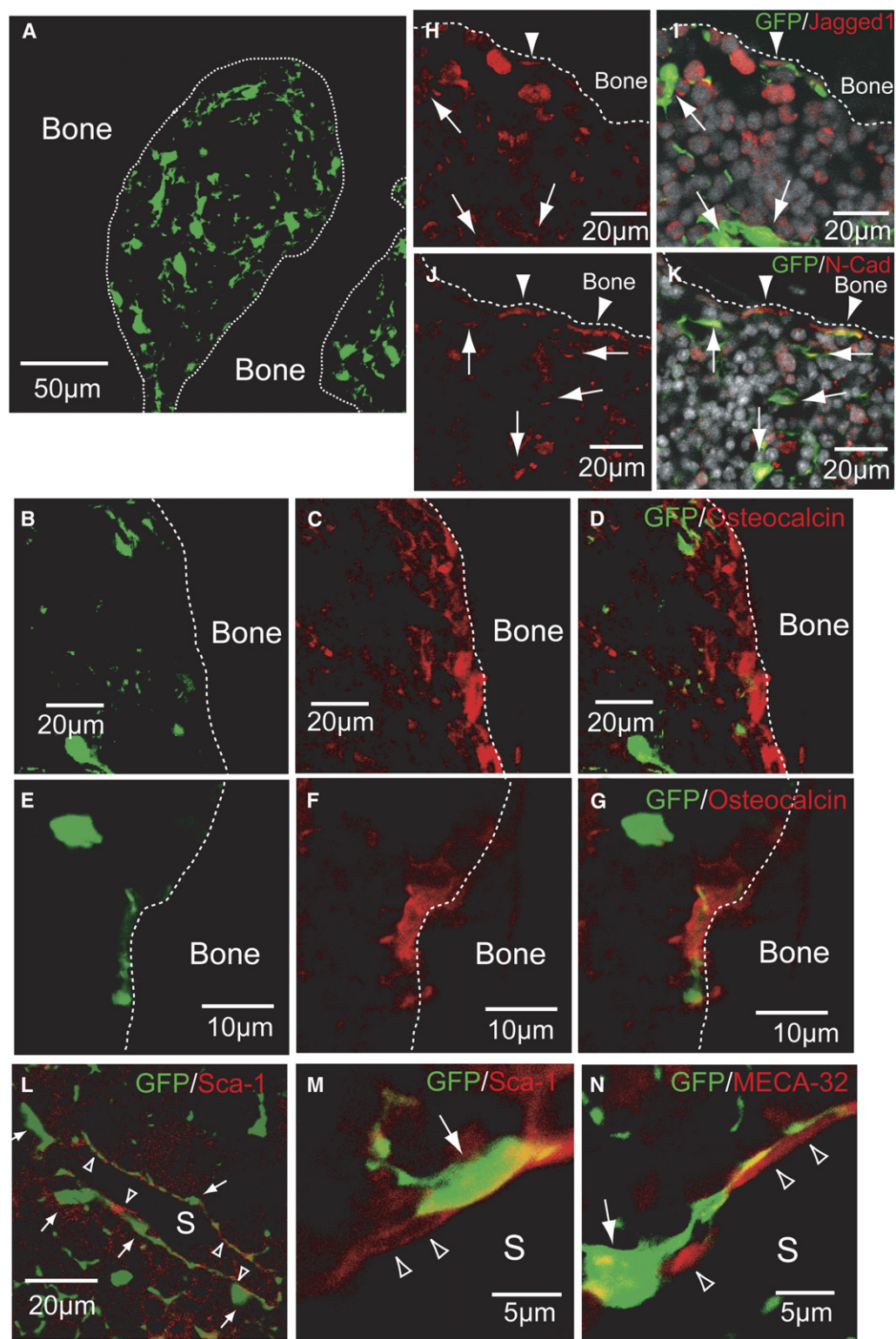
Figure 6. Expression of CXCL12 in Adult Bone Marrow

(A) A trabecular bone section from CXCL12-GFP knockin mice. Most cells expressing a high amount of GFP (green) are scattered throughout intertrabecular space of the bone marrow cavity, and GFP expression is barely detectable in bone.

(B–G) Bone marrow sections from CXCL12-GFP knockin mice stained with antibodies against osteocalcin (red). Osteoblastic cells lining the bone surface that exhibit undetectable expression of GFP (B–D) or a much lower expression of GFP relative to CAR cells (E–G) are shown.

(H–K) Bone marrow sections from CXCL12-GFP knockin mice stained with antibodies against Jagged1 (red; [H] and [I]) or N-cadherin (red; [J] and [K]). The nuclei of cells were labeled with TO-PRO3 dye (white; [I] and [K]). CAR cells (arrows; green), which exhibit similar expression of Jagged1 and lower expression of N-cadherin relative to the cells lining the bone surface (arrowheads; red), are shown.

immunohistochemical analysis of CXCL12-GFP knockin mice with antibodies against Sca-1 and the panendothelial markers PECAM-1 and MECA-32 antigen revealed that morphologicaly identifiable PECAM-1$^+$ endothelial cells in bone marrow expressed Sca-1 (data not shown; Suzuki et al., 2006). In addition, Sca-1$^+$ or MECA-32$^+$ endothelial cells exhibited a much lower to undetectable expression of GFP relative to CAR cells (Figures 6L–6N; Figures S4I–S4L). Interestingly, detailed observations revealed that Sca-1$^+$ or MECA-32$^+$ sinusoidal endothelial cells were often surrounded by cell bodies or the processes of some CAR cells (Figures 6L–6N; Figures S4I–S4L), as observed in fetal bone marrow (Ara et al., 2003).

HSCs near Endosteum or the Sinusoidal Endothelium Are in Contact with CAR Cells

Next we sought to elucidate interaction of HSCs with the cells expressing CXCL12 in aged mice because the proportion of HSCs in the c-kit$^+$Sca-1$^+$Lin$^-$ cell population increases with age (Morrison et al., 1996; Sudo et al., 2000). Flow cytometric analysis revealed that approximately 40% of c-kit$^+$Sca-1$^+$Lin$^-$ cells were CD34$^-$ in bone marrow from 13-month-old mice (data not shown; Sudo et al., 2000). Immunohistochemical analysis of bone marrow sections from 13-month-old CXCL12-GFP knockin mice with antibodies against c-kit and Sca-1 revealed that almost all c-kit$^+$Sca-1$^+$ cells (45 of 48; 94%), which were Lin$^-$ (Tokoyoda et al., 2004), adjoined CAR cells, consistent with our previous study (Figure S5 and data not shown; Tokoyoda et al., 2004). In addition, CD150$^+$CD48$^-$CD41$^-$ cells are enriched in HSC activity and can be imaged (Kiel et al., 2005). Immunohistochemical analysis of bone marrow sections from 13-month-old CXCL12-GFP knockin mice with antibodies against CD150, CD48, and CD41 revealed that 0.0264% of cells in the sections were CD150$^+$CD48$^-$CD41$^-$ and almost all of CD150$^+$CD48$^-$CD41$^-$ cells (34 of 35; 97%) were in contact with CAR cells (Figure S6 and data not shown). These results strongly suggest that many HSCs are associated with CAR cells. Consistent with previous studies (Kiel et al., 2005; Tokoyoda et al., 2004), many c-kit$^+$Sca-1$^+$ and CD150$^+$CD48$^-$CD41$^-$ cells were found scattered throughout the marrow, and the remaining cells resided near the bone surface (data not shown). Interestingly, almost all c-kit$^+$Sca-1$^+$ and CD150$^+$CD48$^-$CD41$^-$ cells near the endosteum (9 of 9; 100%) were also found in close association with CAR cells (Figures 7A and 7B, and data not shown).

Many CD150$^+$CD48$^-$CD41$^-$ cells are associated with the sinusoidal endothelium in the bone marrow (Kiel et al., 2005). Thus, we examined the interaction among c-kit$^+$Sca-1$^+$ or CD150$^+$CD48$^-$CD41$^-$ cells, CAR cells, and bone marrow sinusoidal endothelial cells. Immunohistochemical analysis of CXCL12-GFP knockin mice with antibodies against c-kit and Sca-1 revealed that almost all c-kit$^+$Sca-1$^+$ (17 of 20; 85%) and CD150$^+$CD48$^-$CD41$^-$ cells (20 of 21; 95%) near the sinusoidal endothelium were in contact with CAR cells surrounding

endothelial cells in extravascular spaces (Figures 7C–7H). Additionally, some c-kit$^+$Sca-1$^+$ (15 of 48; 31%) and CD150$^+$CD48$^-$CD41$^-$ (14 of 35; 40%) cells were in contact with CAR cells that were not associated with sinusoidal endothelial cells (data not shown). Together, these results suggest that CAR cells might be a critical component of HSC niches, including vascular niches.

Contact of the Primitive Hematopoietic Progenitors with CAR Cells Is Maintained in the Absence of CXCR4

We next examined the interaction of c-kit$^+$Sca-1$^+$ cells with CAR cells in bone marrow from CXCR4 conditionally deficient mice. We crossed MxCre-CXCR4$^{f/null}$ mice with CXCL12-GFP knockin mice. Mice were then treated with pIpC to induce excision of the floxed allele and were analyzed 10 weeks after the final pIpC treatment. Flow cytometric analysis revealed that CD34$^-$ c-kit$^+$Sca-1$^+$Lin$^-$ HSCs were severely decreased in CXCL12-GFP-MxCre-CXCR4$^{f/null}$ mice compared with control animals, suggesting that the majority of c-kit$^+$Sca-1$^+$Lin$^-$ cells are CD34$^+$c-kit$^+$Sca-1$^+$Lin$^-$ progenitors in the mutants. Immunohistochemical analysis with antibodies against c-kit and Sca-1 revealed that even in the absence of CXCR4, almost all c-kit$^+$Sca-1$^+$ cells (76 of 84; 90%) adjoined CAR cells and the proportion of associated c-kit$^+$Sca-1$^+$ cells was not reduced, suggesting that CXCR4 is dispensable for the contact of CD34$^+$ c-kit$^+$Sca-1$^+$Lin$^-$ progenitors with CAR cells (Figures 7I and 7J; Figure S7).

Discussion

We have shown previously that CXCL12-CXCR4 signaling is involved in the colonization of bone marrow by HSCs during ontogeny (Ara et al., 2003). Here we have shown that, within adult bone marrow, CXCL12-CXCR4 signaling plays a critical role in maintaining a pool of HSCs in a cell-autonomous manner. It is possible that CXCL12 supports the survival and/or self-renewing divisions of HSCs, because CXCL12 has previously been shown to enhance the engrafting capacity of HSCs cultured in vitro with SCF and interleukin-6 (Broxmeyer et al., 2003). In addition, CXCL12 might act as an inhibitor of the cycling status of HSCs to maintain their pool. This possibility is consistent with the previous results that in vivo administration of CXCL12 arrested the cycling of primitive human hematopoietic progenitors and enhanced the engrafting ability of SRCs in NOD-SCID mice that were short-term reconstituted by human fetal liver cells (Cashman et al., 2002). Alternatively, CXCL12 might play a role in tethering HSCs in their niches in which essential factors act on HSCs for their maintenance. Although CXCL12 induced the migration of c-kit$^+$Sca-1$^+$Lin$^-$ cells and increased their adhesion to vascular cell adhesion molecule (VCAM)-1 and fibronectin, expressed in CAR cells (data not shown), the remaining c-kit$^+$Sca-1$^+$ cells stayed in contact with CAR cells in CXCR4 conditionally deficient

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(L–N) Bone marrow sections from CXCL12-GFP knockin mice stained with antibodies against Sca-1 (red; [L] and [M]) and MECA-32 antigen (red, [N]), expressed in sinusoidal endothelial cells. Endothelial cells (arrowheads; red), which exhibit a much lower to undetectable expression of GFP relative to CAR cells and are often surrounded by CAR cells (arrows; green), are shown. S, vascular sinus.
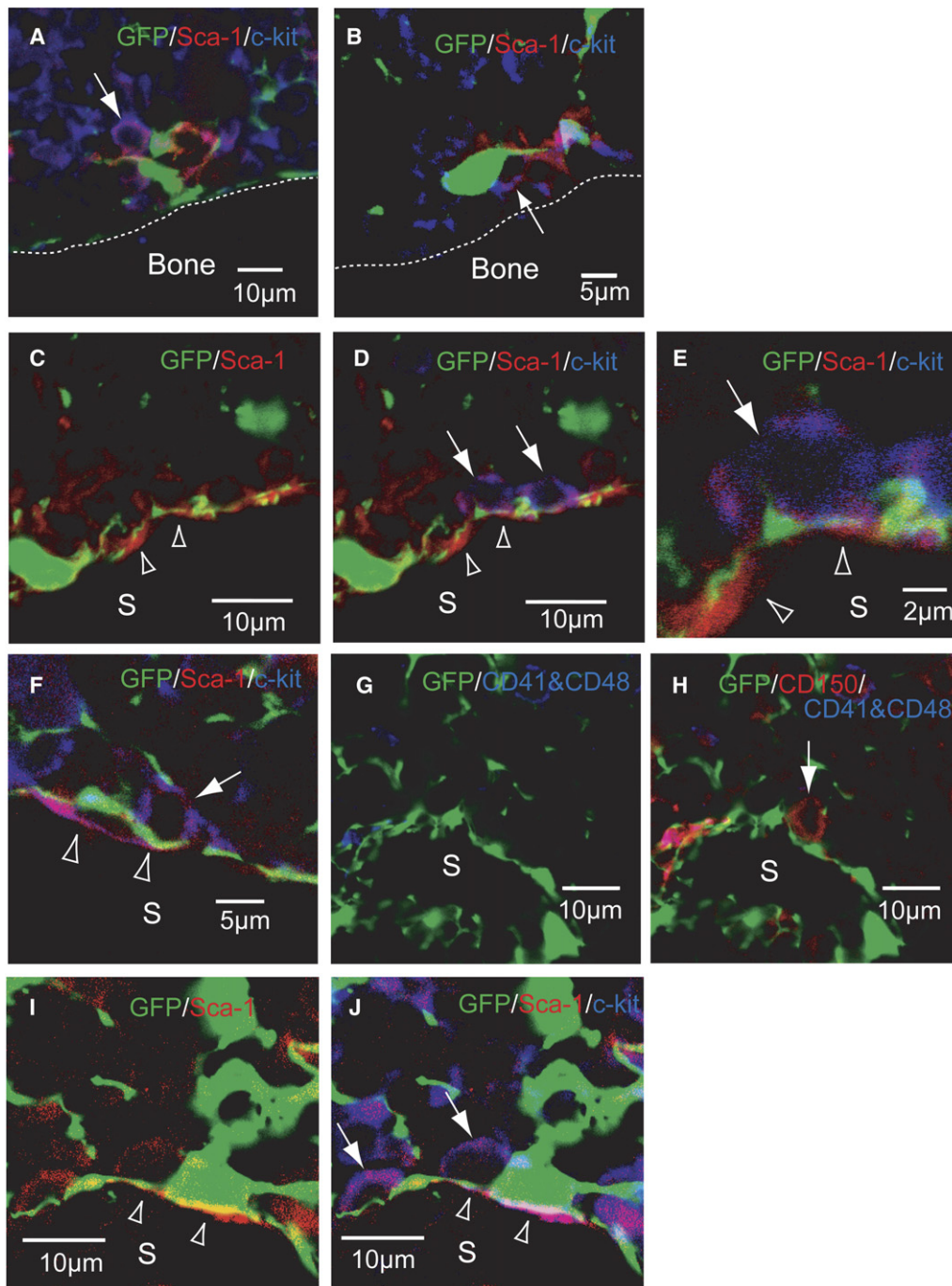
Figure 7. Interaction among HSCs, CAR Cells, and Bone Marrow Sinusoidal Endothelial Cells

(A and B) Bone marrow sections from CXCL12-GFP knockin mice stained with antibodies against c-kit (blue) and Sca-1 (red). c-kit⁺Sca-1⁺ cells (arrows; purple), which reside near the bone surface and are in close association with CAR cells, are shown.

(C–F) Bone marrow sections from CXCL12-GFP knockin mice stained with antibodies against c-kit (blue) and Sca-1 (red). c-kit⁺Sca-1⁺ cells (arrows; purple), which contact CAR cells (green) surrounding morphological identifiable Sca-1⁺ endothelial cells (arrowheads; red), are shown (D–F). Higher magnification of (D) is shown in (E). S, vascular sinus.

(G and H) A bone marrow section from CXCL12-GFP knockin mice stained with antibodies against CD150 (red), CD48 (blue), and CD41 (blue). A CD150⁺CD48⁻CD41⁻ cell (arrow; red), which contacts the morphologically identifiable CAR cells (green) surrounding the vascular sinus is shown (H). S, vascular sinus.

(I and J) A bone marrow section from CXCL12-GFP-MxCre-CXCR4^{f/null} mice stained with antibodies against c-kit (blue) and Sca-1 (red). c-kit⁺Sca-1⁺ cells (arrows; purple), which contact CAR cells (green) surrounding morphological identifiable Sca-1⁺ endothelial cells (arrowheads; red) are shown (J). S, vascular sinus.

mice. This raises the possibility that CXCL12-CXCR4 signaling is dispensable for adhesive interaction of HSCs as well as c-kit+Sca-1+ hematopoietic progenitors with CAR cells; however, it has been difficult to assess directly the impact of CXCR4 deficiency on HSC contact with CAR cells because HSCs were severely reduced in CXCR4 mutants. On the other hand, considering that deficiencies in long-term repopulation by CXCR4-deficient bone marrow cells might be partly due to the homing defects of CXCR4-deficient HSCs and the fact that CXCL12 is involved in homing of HSCs into bone marrow through endothelial cells during ontogeny (Ara et al., 2003), there is also the possibility that CXCL12 plays a critical role in homing of HSCs in the previously reported constitutive physiological circulation of HSCs in adult life (Schwarz and Bhandoola, 2004; Wright et al., 2001). Further investigations will be needed to clarify the mechanisms through which CXCL12 maintains the HSC pool.

Analysis of CXCL12-GFP knockin mice has shown that CAR cells are the major producers of CXCL12 within bone marrow. Thus, the results that almost all c-kit+ Sca-1+ and CD150+CD48−CD41− cells, which contain substantial numbers of HSCs, adjoined CAR cells suggest that many HSCs are in contact with CAR cells and receive CXCL12 signals mainly from these cells. On the other hand, sinusoidal endothelial cells were often surrounded by cell bodies or the processes of some CAR cells and exhibited a much lower to undetectable expression of CXCL12 relative to CAR cells. This seems consistent with the recent findings of the studies of in vivo confocal immunoimaging and immunohistochemistry that CXCL12 is found in bone marrow vasculature (Dar et al., 2005; Ponomaryov et al., 2000; Sipkins et al., 2005) because CAR cells are closely associated with endothelial cells. Considering that many HSCs are associated with sinusoidal endothelium (Kiel et al., 2005), our findings that almost all c-kit+Sca-1+ and CD150+CD48− CD41− cells near the sinusoidal endothelium were in contact with CAR cells surrounding endothelial cells in the extravascular space suggest that HSCs in vascular niches are in contact with CAR cells, raising the possibility that CAR cells are a critical cellular component of vascular niches (Ara et al., 2003; Avecilla et al., 2004).

It has been reported previously that HSCs are in contact with osteoblasts in the endosteum of bone marrow (Arai et al., 2004; Calvi et al., 2003; Wilson and Trumpp, 2006; Zhang et al., 2003). CXCL12 is expressed in osteoblasts lining the bone surface (Jung et al., 2006; Ponomaryov et al., 2000) and is likely deposited in the bone matrix, although there is no evidence that osteocytes express CXCL12 (Katayama et al., 2006). Our study with CXCL12-GFP knockin mice revealed that a small population of osteoblastic cells lining the bone surface expressed CXCL12, albeit at a substantially lower amount than CAR cells. Thus, we cannot rule out the possibility that CXCL12 produced by osteoblasts lining the bone surface acts on HSCs. However, because most c-kit+Sca-1+ and CD150+CD48−CD41− cells residing near the bone surface were also in contact with CAR cells, we speculated that CAR cells might play a role in the regulation of HSCs in osteoblastic as well as vascular niches in bone marrow. Ang-1, a ligand for the receptor tyrosine kinase Tie2, is expressed exclusively in osteoblasts lining the bone surface based on immunohistochemical analysis and on the fact that overexpression of kinase dead (KD)-Tie2 as a dominant-negative form of Tie2 affects the quiescent state of HSCs, suggesting that Ang-1-Tie2 signaling is involved in maintaining HSCs in a quiescent state in the endosteum (Arai et al., 2004). Thus, HSCs are possibly supported by different combinations of environmental factors in osteoblastic and vascular niches. Ang-1 was also reported to be produced by HSCs (Takakura et al., 2000). It is important to know how Ang-1 and CXCL12 influence HSC fate in these niches.

What is the lineage nature of CAR cells? Depleting osteogenic lineage cells with a 2.3 kb fragment from the rat Col1a1 gene promoter (pOBCol2.3) driving thymidine kinase (Col2.3ΔTK) induced a marked defect on bone marrow hematopoiesis (Visnjic et al., 2004). Although bone marrow reticular cells have been thought to be fibroblastic in nature and the expression of the osteoblast marker osteocalcin was below detection in CAR cells in immunohistochemical analysis, CAR cells are possibly progenitors of the osteogenic lineage considering that osteogenic lineage cells with long processes were previously reported to be present in the intertrabecular space of bone marrow (Rouleau et al., 1990). Further studies on the phenotypic characterization of CAR cells in wild-type and Col2.3ΔTK animals would help to address this issue.

In CXCR4 conditionally deficient mice, G_0 exit and G_1 entry was substantially promoted in the primitive hematopoietic progenitors compared with wild-type mice in which most primitive hematopoietic progenitors are quiescent in the G_0 phase. It is likely that lack of CXCR4 results in HSC pool reduction, leading to an expansion of the more differentiated progenitors and compensating for HSC defects. Experiments with chimeric mice have suggested that these defects are due to an altered microenvironment. In this case, the expression of cytokines that enhance the entry of primitive hematopoietic progenitors into the active cell cycle might be upregulated in the microenvironment of the mutants.

This study revealed the environmental factor essential for maintaining the HSC pool and produced by bone marrow stromal cell niches, including vascular niches, where many HSCs might reside. It is known that CXCL12 also acts on other organ-specific stem cells including primordial germ cells (Raz, 2003). Our findings provide a basis for probing the molecular mechanisms that control maintenance of the stem cell pool within microenvironmental niches in mammals.

Experimental Procedures

Mice
CXCL12-GFP knockin mice and CXCR4[f/null] mice (Ara et al., 2003; Tokoyoda et al., 2004) were backcrossed more than seven times with C57BL/6-Ly5.2+Ly5.1− mice. CXCR4[f/null] mice were crossed with MxCre mice (Kuhn et al., 1995) to get MxCre-CXCR4[f/null] mice. Cre expression was induced by intraperitoneally injection of pIpC (300 μg per body for 4 times at 2 day interval; Sigma). Mice were then injected with the same amount of pIpC for four more times at 1 week intervals (eight injections in total). Deletion of the floxed CXCR4 gene in the target cells was detected by flow cytometer or qRT-PCR, and mice younger than 13 months of age were analyzed. All animal experimentation was conducted in accordance with institutional guidelines.

Flow Cytometric Analysis and Cell Sorting

The bone marrow cell suspensions were flushed from femurs and tibias. Monoclonal antibodies against c-kit (2B8), Sca-1 (E13-161.7), CD34 (RAM34), CD3e (145-2C11), CD4 (GK1.5), CD8a (53-6.7), CD11b (M1/70), Gr-1 (RB6-8C5), B220 (RA3-6B2), Ter119, CXCR4 (2B11), and Ly5.2 (104); and rat IgG isotype controls were obtained from BD Biosciences. Biotinylated antibodies were visualized with streptavidin-PerCP or streptavidin-PerCPCy5.5 (BD Biosciences). CD34$^-$c-kit$^+$Sca-1$^+$Lin$^-$ cells were stained as previously described (Osawa et al., 1996). Hoechst 33342 (Sigma) staining for SP cells was performed as described (Goodell et al., 1996). Stained cells were analyzed with FACSCalibur or sorted with FACSVantageSE (BD Biosciences).

Cell-Cycle Analysis

To analyze the cell-cycle status by PY staining, cells were first stained with APC-conjugated c-kit antibody, PE-conjugated Sca-1 antibody, and biotinylated lineage antibodies against CD3e, CD4, CD8a, B220, Gr-1, Mac-1, and Ter119. Biotinylated antibodies were visualized with streptavidin-PerCPCy5.5 and then fixed with 4% PFA. Fixed cells were stained with 10 μg/ml Hoechst33342 and 0.5 μg/ml PY (Sigma) as previously described (Ladd et al., 1997). Cells were analyzed with FACSAria (BD Biosciences). To analyze the cell-cycle status by Ki67, cells were stained with APC-conjugated c-kit antibody, PE-conjugated Sca-1 antibody, biotinylated CD34 antibody, FITC-conjugated Ki-67 antibody (B56; BD Biosciences), and 7AAD. Biotinylated antibodies were visualized with streptavidin-Pacific Blue (Invitrogen). Intranucleus staining was performed with BrdU flow kit (BD Biosciences) according to the manufacturer's instructions without DNase treatment. In the case of some competitive repopulation mice, cells were stained with PECy5-conjugated Ly5.1 antibody (A20; eBioscience) as well as antibodies against c-kit, Sca-1, and Ki67. Cells were analyzed with FACSCalibur or FACSAria.

Long-Term Culture-Initiating Cell Assay

The long-term culture-initiating cell (LTC-IC) assay was performed as described previously (Stier et al., 2005). To prepare stromal layers, murine bone marrow nucleated cells were cultured in 96-well flat-bottomed plates in long-term culture medium (M5300; StemCell Technologies). Irradiated stromal layers were seeded with serially diluted bone marrow single-cell suspensions in the same medium. Marrow was seeded at 2-fold dilutions (1,560–200,000 cells per well) for nucleated bone marrow cells. To measure LTC-ICs, the semisolid cytokine-containing methylcellulose medium for CFC (Methocult M3434; StemCell Technologies) was overlaid into the wells at 6 weeks and the colonies were counted at day 12. A limiting dilution analysis software program (L-Calc; StemCell Technologies) was used to calculate the frequency of LTC-ICs in the cell population.

Competitive Repopulation Units Assay

Limiting dilutions of bone marrow cells to be tested were mixed with 2×10^5 bone marrow cells from 8-week-old C57BL/6-Ly5.2$^-$Ly5.1$^+$ mice and transplanted into lethally irradiated (10Gy) C57BL/6-Ly5.2$^-$Ly5.1$^+$ mice. CRU amounts were determined in two separate experiments, by injecting 4 to 8 recipients at each cell dose in a dilution series. Cell doses ranged from 7.4×10^3 to 6.0×10^5 cells. Peripheral blood cells of the recipient mice were taken 4 months after transplantation, and lineage cells were analyzed by flow cytometer. CRU frequencies in the test bone marrow samples were calculated by applying Poisson statistics to the proportion of negative recipients at different dilutions via L-Calc.

Adoptive Transfer

Unfractionated 2×10^6 bone marrow cells to be tested were mixed with 1×10^6 bone marrow cells of C57BL/6-Ly5.2$^-$Ly5.1$^+$ mice as competitor cells and were transplanted into lethally irradiated (10Gy) C57BL/6-Ly5.2$^-$Ly5.1$^+$ mice. At 13 weeks after transplantation, recipients were treated with pIpC, resulting in excision of the conditionally targeted alleles. Mice were analyzed at 10 weeks after the final pIpC injection.

5-FU Exposure In Vivo

The mice were used at 16 to 32 weeks after the final pIpC injection. After we determined the sublethal dose of weekly administration of 5-FU (Kyowa Hakko), pIpC-treated MxCre-CXCR4$^{f/wt}$ or MxCre-CXCR4$^{f/null}$ mice were administered 5-FU intraperitoneally weekly at a dose of 120 mg/kg four times. The survival rates of the groups were defined.

Immunohistochemical Analysis

Immunostaining was performed as described in our previous publication (Tokoyoda et al., 2004). In brief, bone samples were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose/phosphate buffered saline (PBS). Fixed samples were embedded in OCT medium (Sakura) and frozen in cooled hexane. Sections of undecalcified femoral bone were generated via Kawamoto's film method (Cryofilm transfer kit; FINETEC). The 7 μm thick cryostat sections were first blocked with 5% fetal calf serum (FCS)/PBS and then stained with monoclonal antibodies in blocking buffer. The sections were mounted with PERMAFLUOR (Beckman Coulter), and confocal microscopy was performed with a LSM 510 META (Carl Zeiss). The following antibodies were used for immunostaining: purified monoclonal antibodies against CD150 (TC15-12F12.2) (Biolegend), Sca-1 (E13-161.8), CD41 (MWReg30), and CD48 (HM48-1); biotinylated monoclonal antibodies against c-kit (2B8), PECAM-1 (MEC13.3), panendothelial cell antigen (MECA-32) (BD Biosciences); and purified rabbit polyclonal antibody against Osteocalcin (M173) (Takara Bio), Jagged1 (H-114) (Santa Cruz Biotechnology), and N-Cadherin (YS) (IBL). Antibodies against Sca-1 and CD150 were conjugated with Alexa Fluor 555 by antibody-labeling kit (Invitrogen). For secondary antibodies, Cy5-conjugated goat anti-rat IgG antibody and Cy5-conjugated goat anti-Armenian Hamster IgG antibody (Jackson ImmunoResearch Laboratories) were used. Biotinylated antibodies were visualized with streptavidin-Alexa Fluor 546 or streptavidin-Alexa Fluor 647 (Invitrogen). Immunostaining with rabbit polyclonal antibody was performed with Zenon rabbit IgG labeling kit (Invitrogen) according to the manufacturer's instructions. The nuclei of cells were labeled with TO-PRO3 or SYTO62 dye (Invitrogen).

QRT-PCR Analysis

QRT-PCR was performed as described in our previous publication (Tokoyoda et al., 2004). The primers used for the PCR reaction were as follows: *Junb*: 5-CACCCGGCACAGCTGG-3, 5-TACGGTCT GCGGTTCCTCTTTAA-3, *Vegfa*: 5-CAGAGCGGGAGAAAGCATTTGT-3, 5-CGCGAGTCTGTGTTTTTGCA-3, *Tek*: 5-TTGCTCTGGGAGATTGT TAGCTT-3, 5-GCTCCGCGCACGTCA-3. Values for each gene were normalized to the relative quantity of *Gapdh* mRNA in each sample.

Statistical Analysis

The significance of the difference between groups in the experiments was evaluated by analysis of variance followed by a one-tailed Student's t test.

Supplemental Data

Eight Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/25/6/977/DC1/.

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