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Cellular Niches Controlling B Lymphocyte Behavior within Bone Marrow during Development

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Summary

In bone marrow, hematopoiesis is thought to depend on special microenvironments known as niches that maintain blood cells. However, the identity of niches and interaction of blood cells with niches remain poorly understood. Here we identify stage-specific cellular niches for B lymphopoiesis. The earliest precursors, pre-pro-B cells and end-stage B cells, plasma cells require CXC chemokine ligand (CXCL)12. CXCL12-expressing cells are a small population of stromal cells, scattered throughout bone marrow and located some distance from the cells expressing interleukin (IL)-7. Multipotent hematopoietic progenitors are attached to the processes of CXCL12-expressing cells and pre-pro-B cells adjoin their cell bodies. Maturer pro-B cells that require IL-7 have moved away and adjoin the IL-7-expressing cells. Plasma cells again seed CXCL12-expressing cells. We demonstrate the B lymphocyte characteristic location and movement between specific niches within bone marrow during development and suggest that CXCL12 maintains the cells in the niche.

Introduction

Hematopoiesis occurs within a complex bone marrow microenvironment. It has been assumed that the bone marrow contains stromal cells that create the special microenvironments known as niches that maintain blood cells and supply the requisite factors for their development. However, the lack of distinctive characteristics of stromal cells in bone marrow makes it difficult to identify the niches for hematopoiesis. Spatial microenvironmental organization of blood cells and their interaction with stromal cell niches within bone marrow are important for understanding the regulatory mechanisms of hematopoiesis. It has been previously reported that hematopoietic precursors migrate from the subendosteal region (the inner bone surface) toward the central region of the bone marrow cavity during development (Lord et al., 1975; Hermans et al., 1989; Jacobsen and Osmond, 1990). Consistent with this, it has been reported that primitive hematopoietic progenitors are localized in close contact to the endosteum of the bone marrow (Lord et al., 1975; Nilsson et al., 2001; Calvi et al., 2003; Zhang et al., 2003). In contrast with these results, it has been shown that RAG-1$^+$ primitive lymphoid precursors are uniformly scattered throughout the bone marrow (Hirose et al., 2002). It will remain difficult to unravel blood cell behavior within bone marrow until specific niches can be identified.

Mobilization and stromal cell interactions of hematopoietic precursors are thought to be controlled by cytokines, particularly, chemokines, structurally related small chemoattractive proteins (Baggiolini et al., 1997). Among chemokines, CXC chemokine ligand (CXCL)12 (stromal cell-derived factor (SDF)-1/pre-B-cell-growth-stimulating factor (PBSF)) is particularly intriguing because it has been shown definitively to be involved in various developmental processes including hematopoiesis (Nagasawa et al., 1996, 1999; Nagasawa, 2000). CXCL12 was first characterized as a pre-B-cell growth-stimulating factor (Nagasawa et al., 1994, 1999) and the primary physiologic receptor for CXCL12 is CXCR4, which also functions as an entry receptor for strains of HIV-1 (Bleul et al., 1996; Feng et al., 1996; Nagasawa et al., 1996; Oberlin et al., 1996; Tachibana et al., 1998; Zou et al., 1998). The studies using mutant mice with targeted gene disruption have revealed that CXCL12 and CXCR4 are essential for B cell development and colonization of bone marrow by hematopoietic stem cells (HSCs) and myeloid lineage cells during ontogeny as well as blood vessel formation in gastrointestinal tract, cardiac ventricular septum formation, and cerebellar development (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998; Egawa et al., 2001; Ara et al., 2003b). Recently, it has been shown that CXCL12 and CXCR4 are involved in mobilization and homing of primordial germ cells (PGCs) into gonads during their development in mammals and fish (Doitsidou et al., 2002; Ara et al., 2003a; Knaut et al., 2003). Consistent with these, the studies using transfilter migration assay have revealed that various kinds of cells including hematopoietic progenitors migrate in response to CXCL12 in vitro (Bleul et al., 1996; Aiuti et al., 1997; Nagasawa et al., 1999). In hematopoiesis, it has been reported that the numbers of B lymphoid and myeloid lineage precursors are decreased in bone marrow but increased in the peripheral blood in chimeric mice reconstituted by CXCR4$^{-/-}$ fetal liver cells compared with control animals, suggesting that CXCR4 is involved in retaining

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hematopoietic precursors in bone marrow (Ma et al., 1999). However, the role of CXCL12 and CXCR4 in the regulation of blood cell behavior within bone marrow remains unclear.

B cell precursors are among the best characterized and have contributed an important model system for hematopoiesis. B lymphocytes are generated from HSCs and develop in bone marrow before they egress into peripheral blood to reach peripheral lymphoid organs. Of note, end-stage B cells, plasma cells again colonize bone marrow. In this study, we examined the B lymphocyte behavior within bone marrow during development at the cellular and molecular level. We focused our analysis on the stromal cells expressing CXCL12 and/or interleukin (IL)-7, cytokines that are essential for B lymphopoiesis (von Freeden-Jeffry et al., 1995; Nagasawa et al., 1996; Egawa et al., 2001) and their association with precursors in successive stages of the B lymphoid lineage using mice in which GFP gene was knocked into the CXCL12 locus (CXCL12/GFP knockin mice; Ara et al., 2003a, 2003b). We identified stage specific cellular niches for B cell development including CXCL12-expressing cells and IL-7-expressing cells and demonstrate that B cell precursors and plasma cells reside in the specific niches and move between the niches as development proceeds. Furthermore, our results strongly suggest that CXCL12 plays a role in maintaining the cells in the specific niche.

Results

CXCL12-Expressing Cells Are a Small Population of Stromal Cells, Scattered throughout Bone Marrow and Located Some Distance from IL-7-Expressing Cells

B cell precursors can be divided into several populations by the use of the cell surface markers differentially expressed during development in bone marrow (Hardy and Hayakawa, 1991). $B220^+CD19^-NK1.1^-CD43^+HSA^{lo/-}$ cells have been shown to progress to $B220^+CD19^+CD43^+HSA^+$ phenotypes during in vitro stromal cell culture and suggested to be the earliest identifiable B cell precursors, termed pre-pro-B cells (Hardy and Hayakawa, 1991; Li et al., 1996; Rolink et al., 1996; Allman et al., 1999). $B220^+CD19^+CD43^+HSA^+$ cells consist mainly of large mitotically active cells, termed pro-B cells (Hardy and Hayakawa, 1991; Li et al., 1996). Maturer $B220^+CD19^+CD43^-$ cells consist mainly of small resting cells, termed pre-B cells (Hardy and Hayakawa, 1991).

Early B cell development is known to require cytokines CXCL12 and IL-7 (von Freeden-Jeffry et al., 1995; Nagasawa et al., 1996; Egawa et al., 2001). Previous detailed analysis using radiation chimeras reconstituted with $CXCR4^{+/-}$ or $CXCR4^{-/-}$ fetal liver cells have revealed that CXCL12 is required for pre-pro-B cells (Egawa et al., 2001). On the other hand, IL-7 has been shown to be essential for pro-B cells but not pre-pro-B cells (von Freeden-Jeffry et al., 1995). The different cytokine requirement between two early B cell precursor fractions prompted us to examine the localization of cells expressing CXCL12 or IL-7 (or both) in bone marrow. First, we located and analyzed the CXCL12-expressing cells using CXCL12/GFP knockin mice (Ara et al., 2003a, 2003b). Strong CXCL12/GFP expression was seen in a small population of stromal cells and the CXCL12-expressing cells had several processes, occurred singly and were uniformly scattered throughout the bone marrow (Figure 1A). Further characterization of the CXCL12-expressing cells was done on CXCL12/GFP knockin mice using immunohistochemical staining with antibodies to the pan-endothelial marker PECAM-1, the smooth muscle cell marker, smooth muscle α-actin (SMαA), vascular cell adhesion molecule (VCAM)-1, and the markers for osteoblasts including osteopontin and osteocalcin. It has been reported previously that expression of CXCL12 was observed in osteoblasts as well as stromal cells and endothelial cells in human bone marrow (Ponomaryov et al., 2000). However, almost all of CXCL12-expressing cells did not express PECAM-1 (data not shown) or the markers for osteoblasts (Figure 1C), indicating that CXCL12-expressing cells are different from endothelial cells and osteoblasts. Consistent with this, CXCL12-expressing cells were located some distance from bone surface and osteoblasts (Figure 1C). Furthermore, almost all of CXCL12-expressing cells did not express SMαA (data not shown). In contrast, almost all of CXCL12-expressing cells expressed VCAM-1 but constitute only 17% of VCAM-1^+ stromal cells ($n = 600$) (Figure 1D). Considering that VCAM-1 has been shown to be expressed in the fibroblast-like cells morphologically termed reticular cells as well as sinusoid endothelial cells within bone marrow (Jacobsen et al., 1996), CXCL12-expressing cells might be a subset of bone marrow reticular cells.

Next, we analyzed the expression of IL-7 within bone marrow by immunohistochemical staining with an antibody to IL-7. Since both CXCL12 and IL-7 are required for B lymphopoiesis, combined expression of CXCL12 and IL-7 in stromal cells for their synergistic or additive functions on B cell precursors would be expected (von Freeden-Jeffry et al., 1995; Nagasawa et al., 1996; Ma et al., 1999; Egawa et al., 2001). However, surprisingly, analysis of bone marrow from CXCL12/GFP knockin mice revealed that IL-7 was not expressed in CXCL12-expressing cells (Figure 1A). The IL-7-expressing cells were scattered throughout bone marrow as has been previously reported (Funk et al., 1995) and located some distance from the CXCL12-expressing cells (Figure 1A). Immunohistochemical staining revealed that IL-7-expressing cells also expressed VCAM-1 (data not shown) and about 46% of VCAM-1^+ stromal cells ($n = 121$) were IL-7-expressing cells.

B Cell Precursors Migrate from CXCL12-Expressing Cells to IL-7-Expressing Cells as Development Proceeds

These results prompted us to examine the association between early B cell precursors and CXCL12- or IL-7-expressing cells. We found, using flow cytometric analysis, that cell surface expression of flk2 was restricted, within $B220^+$ B lymphocytes, to pre-pro-B cells, so that virtually all $B220^+flk2^+$ cells were pre-pro-B cells and vice versa (Figure 2A; data not shown). On the other hand, it has been shown previously that expression of c-kit is restricted, within B lymphocytes, to pro-B cells and $B220^+c\text{-}kit^+$ cells are the principal pro-B cells (Löf-

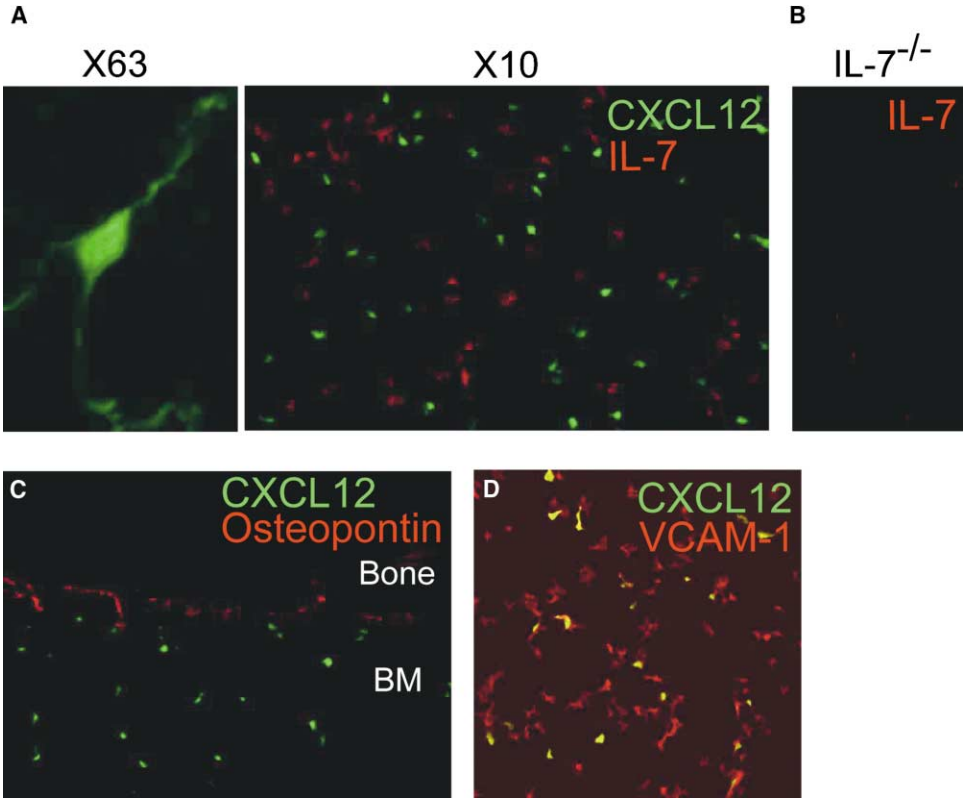

Figure 1. Localization and Characteristics of CXCL12-Expressing Cells within Bone Marrow

(A and B) The distribution of CXCL12-expressing cells and IL-7-expressing cells. The bone marrow sections (10× and 63× magnification) from CXCL12/GFP knockin mice (A) or sections (10×) from control IL-7$^{-/-}$ mice (B) were stained with an antibody against IL-7. (A) The CXCL12-expressing cells (green) are uniformly scattered throughout the marrow and located some distance from IL-7-expressing cells (red). The cells expressing both CXCL12 and IL-7 (yellow because of the overlap of green and red) are not observed.

(C and D) Characterization of CXCL12-expressing cells. The bone marrow sections from CXCL12/GFP knockin mice were stained with an antibody against the markers for osteoblasts, osteopontin (red) (C) and VCAM-1 (red) (D). (C) Almost all of CXCL12-expressing cells (green) are different and located some distance from osteopontin^{+} osteoblasts (red). (D) Almost all of CXCL12-expressing cells express VCAM-1 (yellow because of the overlap of green and red) but constitute only a small population of VCAM-1^{+} cells.

fert et al., 1994; Allman et al., 1999; ten Boekel et al., 1997). These results allowed detailed observation of a characteristic localization of pre-pro-B cells and pro-B cells. Immunohistochemical analysis with antibodies against B220, flk2, and c-kit revealed that B220^{+}flk2^{+} pre-pro-B cells and B220^{+}c-kit^{+} pro-B cells occurred singly and were uniformly scattered throughout bone marrow (Figures 2B–2D; data not shown). In CXCL12/GFP knockin mice, most of pre-pro-B cells were in contact with the bodies of CXCL12-expressing cells (Figure 2B; Table 1). CXCL12-expressing cells had only one pre-pro-B cells in contact. However, all B220^{+}c-kit^{+} pro-B cells were located at a great distance from CXCL12-expressing cells (Figure 2C; Table 1). In contrast, immunohistochemical analysis revealed that most of pre-pro-B cells were not in contact with IL-7-expressing cells but almost all B220^{+}c-kit^{+} pro-B cells adjoined the IL-7-expressing cells (Figure 2D; Table 1). Subsequently, most of B220^{+}IL-7receptor (R)$^{+}$ cells, consisting mainly of pre-B cells (Löffert et al., 1994), were not in contact with CXCL12-expressing cells or IL-7-expressing cells (Figure 2E; Table 1), suggesting that the cells that differentiate into pre-B cells leave IL-7-expressing cells. Furthermore, most B220^{+}IgM^{+} cells comprising newly generated B cells that develop from pre-B cells and mature B cells (Hardy and Hayakawa, 1991; Löffert et al., 1994) were not in contact with CXCL12-expressing cells or IL-7-expressing cells (Table 1).

Multipotent Hematopoietic Progenitors Are in Contact with the Processes of CXCL12-Expressing Cells

Pre-pro-B cells are generated directly or indirectly from multipotent hematopoietic progenitors. We therefore sought to analyze the association between multipotent hematopoietic progenitors and CXCL12-expressing cells. It remains difficult to visualize HSCs or common lymphoid progenitors (CLPs) that can give rise to T lymphoid, B lymphoid, and natural killer (NK) cells but not myeloid cells (Kondo et al., 1997; Gounari et al., 2002; Martin et al., 2003). Previous studies have shown that Lin^{-}c-kit^{+}Sca-1^{+} fraction is highly enriched in variety of multipotent hematopoietic progenitors including HSCs (Ikuta and Weissman, 1992). Because flow cytometric analysis revealed that the c-kit^{+}Sca-1^{+} cells were Lin^{-} (data not shown), immunohistochemical staining analysis using antibodies against c-kit and Sca-1 was performed to visualize multipotent hematopoietic pro-

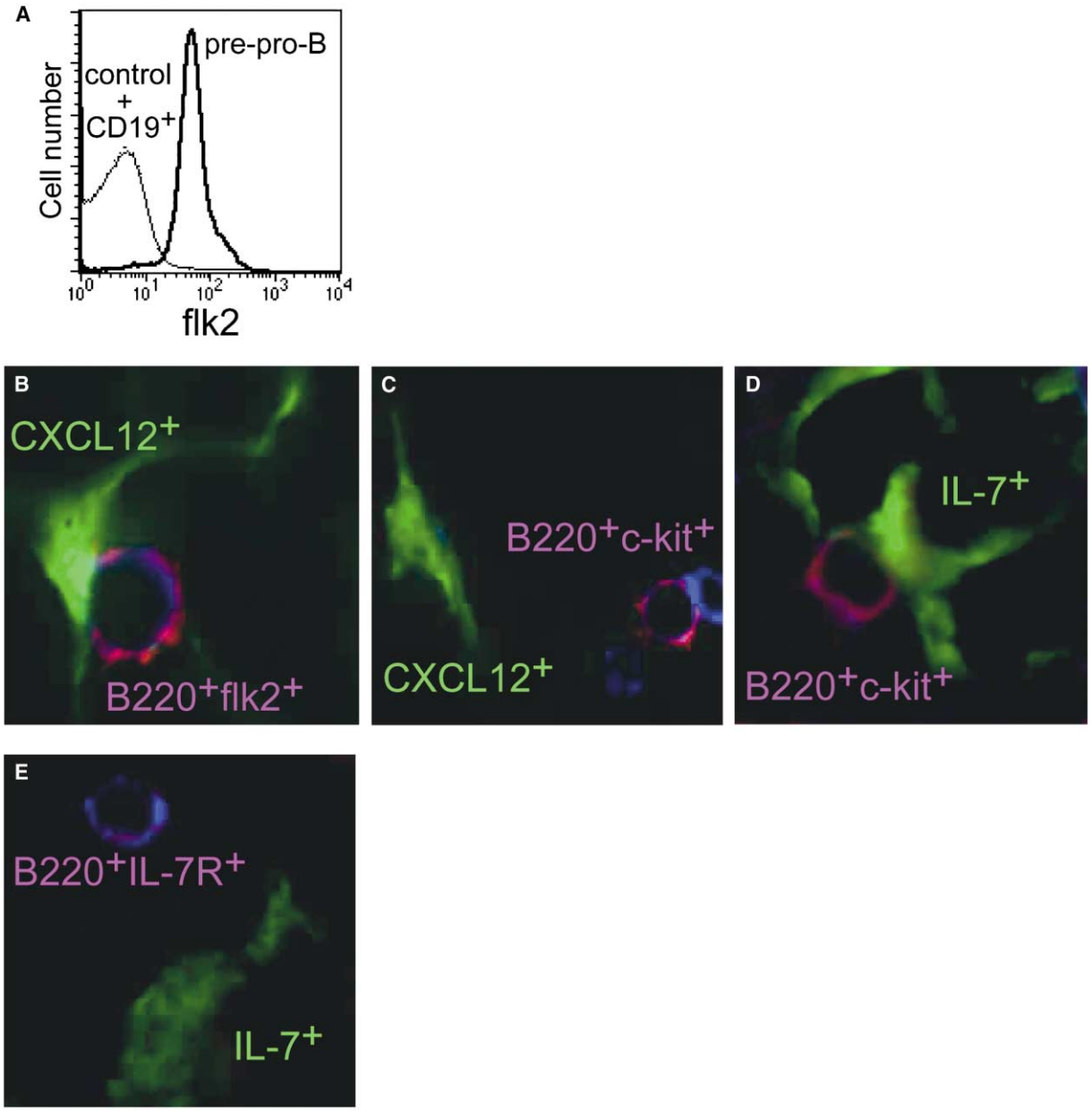

Figure 2. The Interaction of CXCL12-Expressing Cells and IL-7-Expressing Cells with B Cell Precursors during Development

(A) Cell surface expression of flk2 is restricted, within B lymphocytes, to pre-pro-B cells. Gated bone marrow $B220^+CD19^-NK1.1^-HSA^{lo/-}$ (pre-pro-B) or $CD19^+$ cells were analyzed for flk2 expression by flow cytometry. $CD19^+$ cells contain all $B220^+$ B lymphocytes other than pre-pro-B cells. The results shown are representative of three experiments.

(B and C) The bone marrow sections from CXCL12/GFP knockin mice were stained with antibodies against B220 (blue) and flk2 (red, B) or c-kit (red, C). (B) The $B220^+flk2^+$ pre-pro-B cell (purple because of the overlap of blue and red) is in contact with the body of the CXCL12-expressing cell (green). (C) The $B220^+c\text{-}kit^+$ pro-B cell (purple) is located at a great distance from the CXCL12-expressing cell (green).

(D and E) Wild-type bone marrow sections were stained with antibodies against IL-7 (green), B220 (red) and c-kit (blue, D) or IL-7R (blue, E). (D) The pro-B cell (purple) adjoins the IL-7-expressing cell (green). (E) The $B220^+IL\text{-}7R^+$ cell (purple) is not in contact with the IL-7-expressing cell (green).

genitors (de Bruijn et al., 2002). $C\text{-}kit^+Sca\text{-}1^+$ cells were observed throughout bone marrow. Interestingly, in the bone marrow from CXCL12/GFP knockin mice, most of $c\text{-}kit^+Sca\text{-}1^+$ cells (88 cells out of 105 cells) were in contact with the course of the processes of CXCL12-expressing cells (Figure 3) but not with their cell bodies, raising the possibility that differentiating multipotent hematopoietic progenitors move along the processes of CXCL12-expressing cells.

CXCL12 Induces a Significant Increase in Adhesion of Pre-Pro-B Cells but Not Pro-B or Pre-B Cells to Stromal Cell-Derived Adhesion Molecules

To address the role of CXCL12 in the selective association between pre-pro-B cells and CXCL12-expressing cells, we compared the effect of CXCL12 on mobilization of pre-pro-B cells and pro-B cells. It has been shown previously that CXCL12 is a potent attractant for both pre-pro-B and pro-B cells (D'Apuzzo et al., 1997; Bow-

Table 1. Interactions of B Cell Precursors with Bodies of CXCL12-Expressing or IL-7-Expressing Cells

	Contacts with CXCL12$^+$ Cells	Contacts with IL-7$^+$ Cells
B220$^+$flk2$^+$ cells	68/105 (65%)	12/107 (11%)
B220$^+$c-kit$^+$ cells	0/117 (0.0%)	93/105 (89%)
B220$^+$IL-7R$^+$ cells	4/138 (2.9%)	62/201 (31%)
B220$^+$IgM$^+$ cells	4/100 (4.0%)	33/102 (32%)

We examined individual 105 or 107 B220$^+$flk2$^+$, 117 or 105 B220$^+$ c-kit$^+$, 138 or 201 B220$^+$IL-7R$^+$, and 100 or 102 B220$^+$IgM$^+$ cells in bone marrow sections (Figures 2B–2E). Most of B220$^+$IL-7R$^+$ cells belong to pre-B cells.

man et al., 2000). Transfilter migration assay revealed that pre-pro-B cells and pre-B cells were more responsive to CXCL12 compared to pro-B cells (Figure 4A; see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/20/6/707/DC1). On the other hand, it has been reported that in human bone marrow CXCL12 triggers a sustained adhesion response in B cell precursors (Glodek et al., 2003). We found that CXCL12 induced a significant increase in adhesion of pre-pro-B cells but not pro-B cells or pre-B cells to VCAM-1, which was expressed in both CXCL12-expressing and IL-7-expressing cells (Figure 4B). This suggests that CXCL12 acts selectively on pre-pro-B cells to increase adhesiveness of VCAM-1 ligands including $\alpha4\beta1$ integrin. Flow cytometric analysis revealed that pre-pro-B, pro-B, and pre-B cells displayed high surface expression of $\alpha4$ integrins (Figure 4C). It therefore appears that the responsiveness of B cell precursors to CXCL12 may not correlate with expression levels of $\alpha4$ integrins. On the other hand, we observed higher expression of CXCR4 mRNA in pre-pro-B cells and pre-B cells compared to pro-B cells by real-time quantitative RT-PCR analysis (Figure 4D). Flow cytometric analysis revealed that pro-B cells and pre-B cells displayed higher surface expression of CXCR4 (Figure 4E). Thus despite high-level expression of CXCR4 in pre-pro-B, pro-B, and pre-B cells, pre-pro-B cells were much more responsive to CXCL12 than the other B cell precursors with respect to their capacity to adhere to VCAM-1 in vitro.

Notch-1 Ligands Are Expressed in Some Bone Marrow Stromal Cells but Not in CXCL12-Expressing Cells

Notch-1 receptors are transmembrane proteins that are known to deliver key signals for T lymphoid lineage decision in hematopoietic progenitors, possibly by influencing lineage commitment from CLPs (Pui et al., 1999; Radtke et al., 1999). Enforced expression of constitutively active form of Notch-1 severely decreases the numbers of cells in all B lineage precursor subsets including pre-pro-B and pro-B cells in vivo (Pui et al., 1999). Real-time quantitative RT-PCR analysis revealed that Notch-1 was highly expressed in pre-pro-B cells but not in pro-B cells or pre-B cells (data not shown). These, together with the previous studies that have shown that Notch-1 ligands including Delta-like-1 and Jagged-1 are expressed in bone marrow (Li et al., 1998; Han et al., 2000), prompted us to analyze the expression of Notch-1 ligands in bone marrow stromal cells including CXCL12-expressing cells. Delta-like-1 and Jagged-1 are also transmembrane proteins and Delta-like-1 has been shown to induce the differentiation of multipotent hematopoietic progenitors into T lymphoid lineage cells while inhibiting the generation of B lymphoid lineage cells in vitro (Jaleco et al., 2001; Schmitt and Zuniga-Pflucker, 2002). Immunohistochemical analysis of bone marrow from CXCL12/GFP knockin mice revealed that Delta-like-1 and Jagged-1 were detectable in some stromal cells including IL-7-expressing cells but undetectable in CXCL12-expressing cells, suggesting that Notch-1 in pre-pro-B cells does not deliver a signal since pre-pro-B cells are in contact with CXCL12-expressing cells (data not shown).

Plasma Cells in Bone Marrow Require CXCL12 and Are in Contact with CXCL12-Expressing Cells

B lymphocytes positive for surface immunoglobulin that develop from pre-B cells in bone marrow egress into

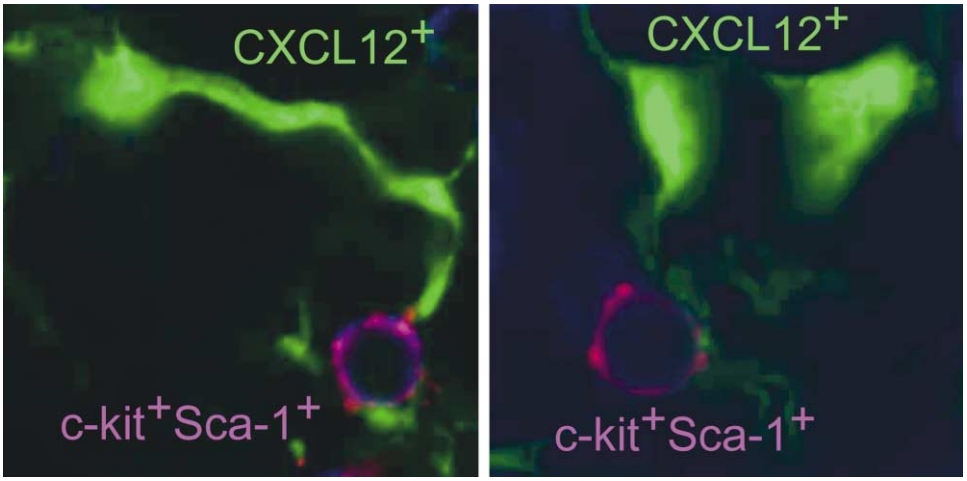

Figure 3. C-kit$^+$Sca-1$^+$ Multipotent Hematopoietic Progenitors Are in Contact with the Processes of CXCL12-Expressing Cells

The bone marrow sections from CXCL12/GFP knockin mice were stained with antibodies against c-kit (red) and Sca-1 (blue). Two c-kit$^+$Sca-1$^+$ cells (purple because of the overlap of blue and red) contacting the course of the processes of CXCL12-expressing cells (green) are shown.

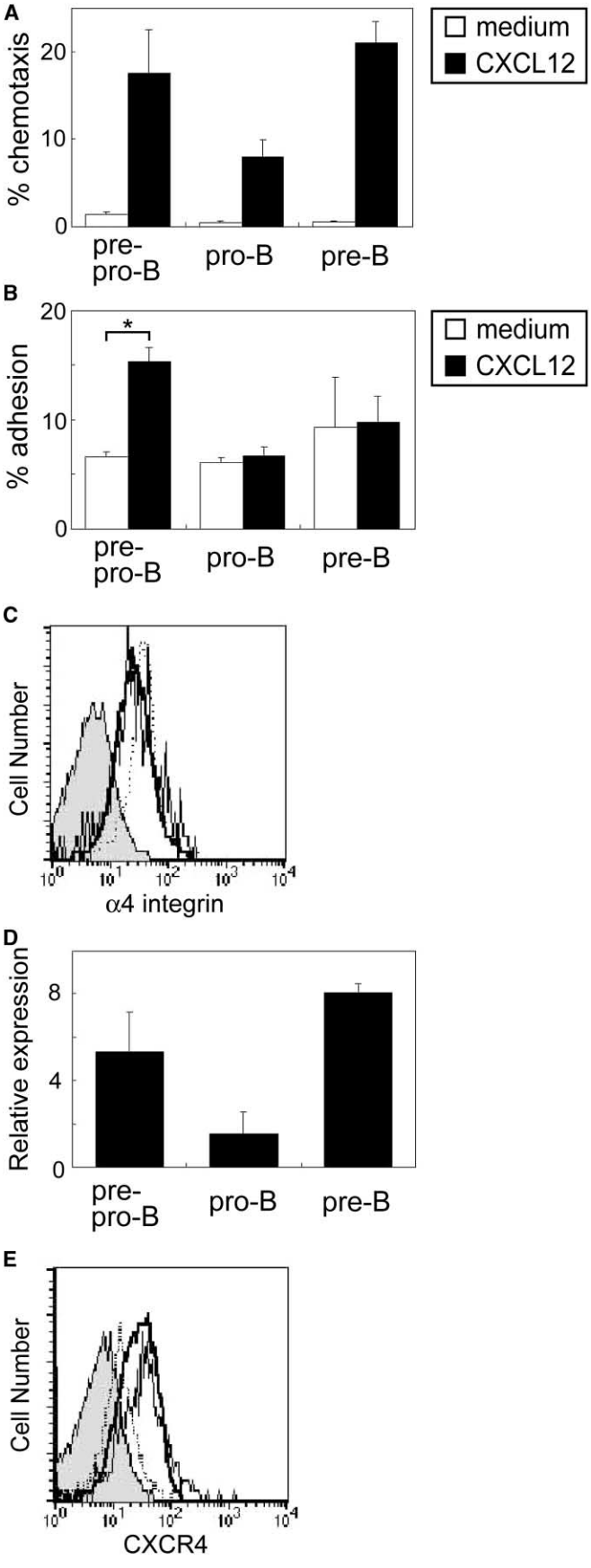

Figure 4. Pre-Pro-B Cells but not Pro-B or Pre-B Cells Exhibited Adhesive Activities to VCAM-1 in Response to CXCL12

(A) Pre-pro-B and pre-B cells were more responsive to CXCL12 compared to pro-B cells with respect to their capacity for chemotaxis. Shown are the frequencies of migrating cells (the mean and SD of data derived from three experiments).

(B) CXCL12 induced a significant increase in adhesion of pre-pro-B cells but not pro-B cells or pre-B cells to VCAM-1. The frequencies of adherent cells are shown. Shown are the frequencies of adherent cells (the mean and SD of data derived from six experiments). *, $p < 0.01$ by Student's t test.

(C) Flow cytometric analysis of $\alpha 4$ integrins surface expression in pre-pro-B (dotted), pro-B (plain) and pre-B cells (bold) along with the isotype control (tinted). The results shown are representative of three experiments.

(D) Higher expression of CXCR4 in pre-pro-B cells and pre-B cells compared to pro-B cells. The CXCR4 mRNA levels were quantitated by real-time quantitative RT-PCR. The mean and SD of data derived from three experiments is shown.

(E) Flow cytometric analysis of CXCR4 surface expression in pre-pro-B (dotted), pro-B (plain) and pre-B cells (bold) along with the isotype control (tinted). The results shown are representative of six experiments.

peripheral blood to reach the spleen and mature to peripheral mature B cells. Plasma cells are end-stage B cells that develop following antigen activation of mature B cells and are critical for mediating the humoral immune response because they alone synthesize and secrete large amounts of antigen-specific antibodies. Long-term antibody production by long-term plasma cells is one of the hallmarks of effective vaccination and is an important characteristic of immunological memory. Of note, the majority of long-lived plasma cells reside in the bone marrow. The previous study using chimeric mice reconstituted with $CXCR4^{-/-}$ fetal liver cells has shown that $CXCR4^{-/-}$ plasma cells fail to home to the bone marrow (Hargreaves et al., 2001). Since the chimeric mice reconstituted with $CXCR4^{-/-}$ fetal liver cells display deficits in early B lymphopoiesis, we analyzed the role of CXCR4 in plasma cells using conditional CXCR4-deficient mice in which CXCR4 was specifically deleted from mature B cells. We generated the mice with a loxP-CXCR4 conditional targeting allele ($CXCR4^{flox/+}$ mice) (Figure 5A) and crossed them to mice that express Cre-recombinase under the control of the endogenous B cell-specific CD19 locus (Rickert et al., 1997). Deletion by CD19-Cre ongoing throughout B lymphopoiesis resulted in a lower deletion efficiency in pre-B cells compared to mature B cells in the spleen as previously described (Rickert et al., 1997; Figure 5C; data not shown). In $CXCR4^{flox/-}$/CD19-Cre mice, the numbers of mature B cells and plasma cells in spleen were normal (Figure 5D; data not shown) but the numbers of plasma cells in bone marrow were severely reduced compared with $CXCR4^{flox/+}$/CD19-Cre mice supporting the idea that bone marrow plasma cells require CXCR4 (Figure 5E). These results prompted us to determine the association of plasma cells with CXCL12-expressing cells within bone marrow. Immunohistological analysis using antibodies against IgG and the plasma cell marker, Syndecan-1 (Sanderson et al., 1989; Lalor et al., 1992), was performed to visualize plasma cells. The IgG^{+}Syndecan-1^{+} cells sorted from wild-type bone marrow and stained with May-Grünwald-Giemsa had the typical morphology of plasma cells, with enlarged cytoplasm and an eccentric nucleus (Figure 5F). The numbers of IgG^{+}Syndecan-1^{+} plasma cells in the bone marrow sections of $CXCR4^{flox/-}$/CD19-Cre mice were severely reduced compared to $CXCR4^{flox/+}$/CD19-Cre mice (Figure 5G). Analysis of bone marrow from CXCL12/GFP knockin mice revealed that almost all of IgG^{+}Syndecan-1^{+} plasma cells were in contact with the processes or bodies of CXCL12-expressing cells (93 cells out of 100 cells) (Figure 5H).

Discussion

In the present report, we assessed the cellular niches for B lymphopoiesis and the systemic movement of B lymphocytes within bone marrow during development. Since most of pre-pro-B cells were in contact with CXCL12-expressing cells, a small population of stromal cells, CXCL12-expressing cells would constitute a specific niche for precursors at the earliest stages of B lymphopoiesis. This raises the possibility that the niche supports B lymphoid lineage commitment and/or survival of the precursors. The precursors then would leave CXCL12-expressing cells and migrate through the mesenchyme to reach IL-7-expressing cells since most of pro-B cells adjoin IL-7-expressing cells (Figure 6). Considering that pro-B cells are mitotically active (Hardy and Hayakawa, 1991) and require IL-7 in their proliferation (von Freeden-Jeffry et al., 1995), the IL-7-expressing cells are likely to provide the niches that induce an expansion division. In this case, the precursors would move from the niche for cell fate decision to the niche for cell proliferation during B lymphopoiesis. Since one CXCL12-expressing cell or IL-7-expressing cell maintained only one pre-pro-B cell or pro-B cell (Figures 2B and 2D), there might be a strict regulation in space of the functions of the cellular niches in B lymphopoiesis. Our results have revealed that most of the cells that differentiate into pre-B cells were not in contact with CXCL12-expressing or IL-7-expressing cells. This is consistent with the idea that pre-B cells become independent on stromal microenvironment (Hardy and Hayakawa, 1991; Rolink et al., 2000). Why do pre-pro-B cells reside in the specific niches that are different from the niches maintaining pro-B cells? It is likely that appropriate combinations of microenvironmental factors that support the development of pre-pro-B and pro-B cells are different. It has been shown that stimulation with thymic environment or several cytokines induces the B cell precursors in the mice lacking a transcription factor Pax-5 to differentiate into T lymphoid, NK, or myeloid lineages, indicating that Pax-5 induces irreversible B lymphoid lineage commitment (Nutt et al., 1999; Rolink et al., 1999). Considering that Pax-5 is barely detectable in pre-pro-B cells and expressed from pro-B cells by real-time quantitative RT-PCR (data not shown), the pre-pro-B cells would be still responsive to lineage-inappropriate signals. These raise the possibility that pre-pro-B cells are protected against lineage-inappropriate signals in the specific niches. Consistent with the hypothesis, the ligands for Notch-1 that have been shown to induce T lymphoid lineage commitment (Pui et al., 1999; Radtke et al., 1999; Jaleco et al., 2001; Schmitt and Zuniga-Pflucker, 2002) are expressed in some stromal cells but not in CXCL12-expressing cells within bone marrow. Whether CXCL12-expressing cell niches are specific for B lymphoid lineage and whether in other hematopoietic lineages a similar scenario occurs, in which the earliest precursors reside in the specific niche distinct from the niche for their extensive proliferation are important questions for the future. Identification and visualization of early precursors for other hematopoietic lineages will be needed to address these issues.

Pre-pro-B cells are thought to generate from multipotent hematopoietic progenitors including CLPs and HSCs. The localization of CLPs or HSCs remains elusive due to the difficulty of visualizing these progenitors. However, our finding that most cells in c-kit^{+}Sca-1^{+} multipotent hematopoietic progenitor fraction adhere to the processes of CXCL12-expressing cells raises the possibility that cells during or immediately after commitment to B lymphoid lineage move centrally to the bodies of CXCL12-expressing cells along the processes of CXCL12-expressing cells (Figure 6). On the other hand, considering our previous findings that CXCL12-expressing cells might act as a hematopoietic stem cell niche in fetal bone marrow (Ara et al., 2003b), there is also

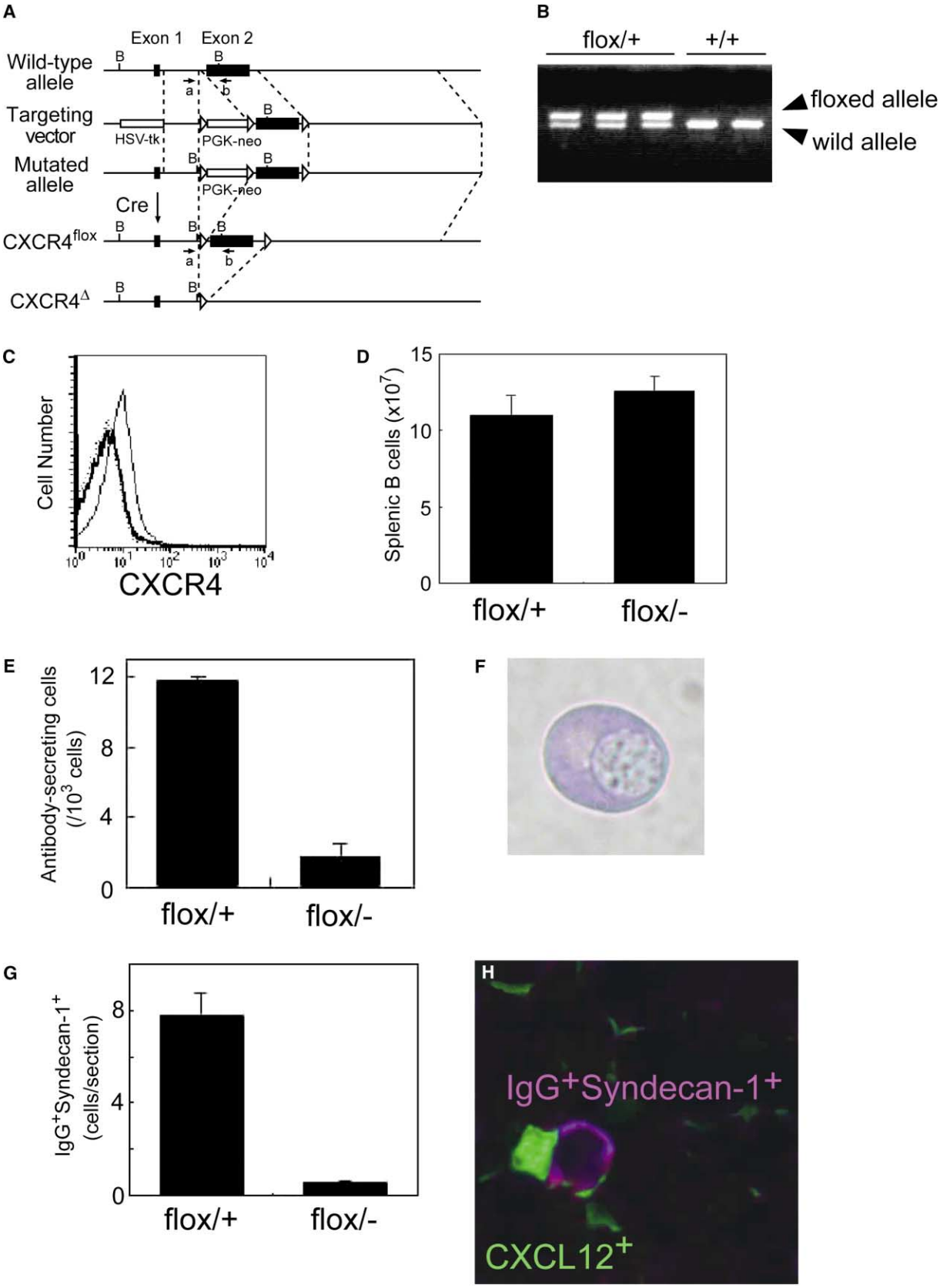

Figure 5. Plasma Cells in Bone Marrow Require CXCL12 and Are in Contact with CXCL12-Expressing Cells

(A) Generation of the floxed CXCR4 locus. Restriction endonuclease map of the wild-type CXCR4 locus, the targeting vector, the initial targeted locus, floxed locus after neo deletion and targeted locus after complete deletion.

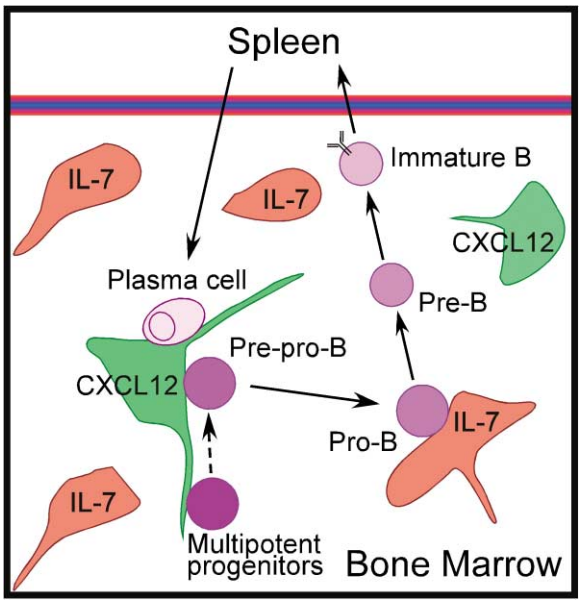

Figure 6. Model of the Movement of Multipotent Hematopoietic Progenitors and B Lymphocytes, and Their Association with Specific Cellular Niches within Bone Marrow during Development

the possibility that HSCs remain adjacent to CXCL12-expressing cells in adult bone marrow. Recent studies suggest that HSCs are located near the osteoblastic cells lining the bone surface (Calvi et al., 2003; Zhang et al., 2003). In this case, intermediates between HSCs and pre-pro-B cells (Tudor et al., 2000) would move from the bone surface toward the CXCL12-expressing cells that are located some distance from osteoblasts and dispersed throughout bone marrow. Recently, it has been shown that a small population of pre-pro-B cells have the capacity to colonize the thymus and differentiate into all lymphoid lineages (Martin et al., 2003). Further studies will be needed to see if the cells in this population are in contact with CXCL12-expressing cells.

Since donor-derived pre-pro-B cells were almost absent in the bone marrow from chimeric mice long-term reconstituted with CXCR4$^{-/-}$ fetal liver cells as compared with control chimeras (Egawa et al., 2001), CXCL12 is likely to play a role in maintaining the precursors in their specific niche. The results that CXCL12 induced the migration of pre-pro-B, pro-B, and pre-B cells but increased adhesiveness of VCAM-1 ligands only on pre-pro-B cells in vitro and only pre-pro-B cells were in contact with CXCL12-expressing cells in vivo raise the possibility that the precursors require CXCL12-induced increase in adhesiveness of VCAM-1 ligands but not migration to CXCL12 for residing in CXCL12-expressing cell niche. In this case, CXCL12 would control the anchorage of pre-pro-B cells in the niche. α4 integrin, a subunit of VCAM-1 ligands, is a candidate for targets for the CXCL12 function because α4 integrin has been shown to be essential for early B lymphopoiesis in bone marrow (Arroyo et al., 1996). There is also the possibility that CXCL12 acts directly to promote survival or differentiation of the precursors. Furthermore, CXCL12 might attract the progenitors along the processes of the CXCL12-expressing cells. On the other hand, as development proceeds, the cells lose the contact with CXCL12-expressing cells (Figure 6). Of note, pro-B cells and pre-B cells that are located at a great distance from CXCL12-expressing cells express high level of CXCR4 but do not display CXCL12-mediated upregulation of adhesion to VCAM-1 (Figure 4). The underlying mechanisms are not well understood, but cells would become less responsive to CXCL12 with respect to their capacity to adhere to CXCL12-expressing cells due to alterations in intracellular signaling pathways stimulated by CXCR4. Additionally, IL-7-expressing cells are likely to secrete another attractant(s) for pro-B cells.

We have shown that plasma cells in bone marrow require CXCL12 and are in contact with CXCL12-expressing cells. Together with the previous studies (Hargreaves et al., 2001; Cassese et al., 2003), these suggest that CXCL12 supports homing and survival of plasma cells in the niches in bone marrow. It is intriguing that the earliest B cell precursors and end-stage B cells share the common cellular niche. This may be a reason for the result that patients with multiple myeloma, a B cell malignancy characterized by excess plasma cells in the bone marrow show severely reduced numbers of B lymphocytes (Pilarski et al., 1984).

T cell precursors migrate from region of the perimedullary cortex to the subcapsular region through the cortex in thymus as development proceeds (Norment and Bevan, 2000; Lind et al., 2001). In contrast, we have shown that the early B cell precursors and plasma cells reside in the specific cellular niches which were uniformly scattered throughout bone marrow and move between the niches during development in vivo. This study provides a novel basis for probing the mechanisms by which physical microenvironments support hematopoiesis within bone marrow.

(B) PCR genotyping analysis of adult wild-type and CXCR4$^{flox/+}$/CD19-Cre mice.
(C) Flow cytometric analysis of CXCR4 surface expression in mature B cells in the spleen of CXCR4$^{flox/+}$/CD19-Cre (plain) and CXCR4$^{flox/-}$/CD19-Cre (bold) mice along with the isotype control (dotted). The results shown are representative of eight experiments.
(D) The numbers of mature B cells in the spleen of CXCR4$^{flox/+}$/CD19-Cre and CXCR4$^{flox/-}$/CD19-Cre mice were determined by flow cytometric analysis. n = 4.
(E) The numbers of IgG plasma cells in the bone marrow of CXCR4$^{flox/+}$/CD19-Cre and CXCR4$^{flox/-}$/CD19-Cre mice were determined by ELISPOT. n = 3.
(F) Cytospin preparations of FACS-sorted IgG^{+}Syndecan-1^{+} cells were stained with May-Grünwald-Giemsa.
(G) The numbers of IgG^{+}Syndecan-1^{+} cells in bone marrow sections of CXCR4$^{flox/+}$/CD19-Cre and CXCR4$^{flox/-}$/CD19-Cre mice were quantified. The data presented are the average of 12 sections.
(H) The bone marrow sections from CXCL12/GFP knockin mice were stained with antibodies against IgG (blue) and Syndecan-1 (red). The IgG^{+}Syndecan-1^{+} plasma cell (purple because of the overlap of blue and red) is in contact with the process and body of the CXCL12-expressing cell (green).

Experimental Procedures

Mice

The generation of CXCL12/GFP knockin mice has been described previously (Ara et al., 2003a, 2003b). CXCL12/GFP knockin and CXCR4$^{flox/+}$ mice were backcrossed more than seven times with C57BL/6-Ly5.2 mice. IL-7$^{-/-}$ mice were a gift from Dr. H. Kiyono. CXCR4$^{flox/+}$ mice were generated according to the standard two-step procedure. The CXCR4 locus was modified by conditional gene targeting in E14.1 embryonic stem (ES) cells derived from 129 mice (Nagasawa et al., 1996). The targeting construct introduced a floxed neomycin resistance cassette in the intron preceding the exon 2 and a loxP site downstream of the exon 2. The neomycin cassette was removed subsequently from targeted ES cell clones by transient expression of Cre, yielding the final floxed allele. For conditional inactivation, we have crossed them to mice that express Cre-recombinase under the control of the endogenous B cell-specific CD19 locus. Mice hemizygous for this Cre insertion are phenotypically normal, as they still retain one functional CD19 allele.

Flow Cytometry Analysis, Cell Sorting, and May-Grünwald-Giemsa Staining

Monoclonal antibodies against α4 integrin, B220, CD19, CD43, c-kit, CXCR4, flt3/flk2, HSA, IgM, IgD, NK1.1, and rat IgG isotype control were obtained from BD PharMingen. Biotinylated antibodies were visualized with streptavidin-PerCP (BD PharMingen). Stained cells were analyzed with FACSCalibur or sorted with FACSVantageSE (Becton Dickinson). Cytospin preparations were made by Cytospin 4 (ThermoShandon). The slides of each sort were stained with May-Grünwald-Giemsa stain after fixation with methanol. The slides were examined with a light microscope.

Immunohistochemical Staining and Confocal Microscopy

For section staining, samples were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose/PBS. Cryostat sections of adult femurs were stained and mounted with PERMAFLUOR (Beckman Coulter). All confocal microscopy was carried out on a LSM 510 META (Carl Zeiss). Monoclonal antibodies against c-kit, IL-7R were a gift from Dr. S-I. Nishikawa, and antibodies against B220, flk2, IgM, PECAM-1, Sca-1, Syndecan-1, VCAM-1 (BD PharMingen), IL-7 (Genzyme Techne), Delta-like-1, Jagged-1 (Santa Cruz), mouse IgG (Jackson Laboratories), SMαA (NeoMarkers) and osteopontin (LSL) were used. For secondary antibodies, Alexa 546 goat anti-rat or rabbit IgG, Alexa 546 donkey anti-goat IgG (Molecular Probes) and Cy5 donkey anti-rat IgG (Jackson Laboratories) were used. Biotinylated antibodies were visualized with streptavidin-Alexa 488 (Molecular Probes) or streptavidin-Cy5 (Jackson Laboratories).

Quantitative RT-PCR Analysis

Total RNA was isolated using Isogen (Nippon Gene) from sorted cells. Following DNase I (GibcoBRL) treatment, cDNA was synthesized with MMLV-reverse transcriptase (GibcoBRL) and oligo-dT primer following the manufacturer's protocol. The cDNA was quantified by real-time PCR with SYBR Green system (Applied Biosystems). The primers used for the PCR reaction were as follows. CXCR4: 5′-TAGGATCTTCCTGCCCACCAT-3′, 5′-TGACCAGGATCA CCAATCCA-3′, Values for each gene were normalized to the relative quantity of G3PDH mRNA in each sample.

Migration Assay

Fresh bone marrow cells were transferred to the upper layer of 5 μm pore polycarbonate membrane (Transwell, Corning) and the overlaid lower chamber containing 100 ng/ml CXCL12. After 2 hr, a fraction of the cells that migrated to the lower chamber was stained and analyzed by flow cytometry.

Adhesion Assay

Fresh bone marrow cells were isolated into RPMI/5% FCS, incubated for 30 min at 37°C on tissue culture plates to eliminate adherent macrophages, washed, analyzed by flow cytometry and then allowed to adhere to ligand-coated 96-well plates (Costar) in the presence or absence of 100 ng/ml CXCL12 for 30 min at 37°C at 5% CO2. After 30 min, nonadherent cells were removed by a standardized washing procedure (four cycles) in an automated plate washer (Bio-Tek) using HBSS (10 mM Hepes, 1 mM Mg^{2+}, and 1 mM Ca^{2+} [pH 7.4]). Adherent cells were collected, stained, and analyzed by flow cytometry. Adhesion was calculated as a percentage of input samples. Mouse VCAM-1/Fc and human IgG1 were purchased from R&D Systems and Sigma, respectively.

ELISPOT Assay

Plates were coated with anti-IgG (1 μg/ml in 0.05 M carbonate buffer [pH 9.6]; Southern Biotechnology) and blocked with 1% BSA in PBS. Fresh bone marrow cells were then added in RPMI/5% FCS and incubated 5 hr at 37°C. The plates were then incubated with alkaline phosphatase-conjugated anti-IgG (Southern Biotechnology) followed by the substrate 5-bromo-4-chloro-3-indolylphosphate (Sigma) until spots were clearly visible.

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