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The Chemokine Receptor CXCR4 Is Required for the Retention of B Lineage and Granulocytic Precursors within the Bone Marrow Microenvironment

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Summary

We report that the chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within fetal liver and bone marrow microenvironment. In CXCR4-deficient embryos, pro-B cells are present in blood but hardly detectable in liver; myeloid cells are elevated in blood and reduced in liver compared to wild-type embryos. Mice reconstituted with CXCR4-deficient fetal liver cells have reduced donor-derived mature B lymphocytes in blood and lymphoid organs. The numbers of pro-B and pre-B cells are reduced in bone marrow and abnormally high in blood. Granulocytic cells are reduced in bone marrow but elevated and less mature in the blood. B lineage and granulocytic precursors are released into the periphery in absence of CXCR4.

Introduction

In late embryonic life, mouse hematopoiesis occurs predominantly in liver and then shifts to bone marrow, which becomes the major definitive hematopoietic organ throughout life (Ikuta et al., 1992). Hematopoiesis is a highly dynamic and finely regulated process in which the hematopoietic stem cells lose their self-renewing capability, commit to specific lineages, and then mature along specific pathways. For instance, B lymphopoiesis is accompanied by a series of ordered immunoglobulin gene rearrangements and characteristic changes in cell surface marker expression (Rolink and Melchers, 1993; Kee and Paige, 1995). Myelopoiesis is regulated by sequential expression of transcription factors and accompanied by morphological changes including increasing nuclear segmentation and acquisition of specific cytoplasmic granules (Tenen et al., 1997; Berliner, 1998; Marmont et al., 1988). After appropriate maturation, leukocyte subsets emigrate from the bone marrow and are released into the bloodstream.

These complex events of hematopoiesis are affected by the bone marrow microenvironment and specifically by stromal cells located in the bone marrow medullary cavity (Dorshkind, 1990). These cells include a diverse nonhematopoietic, fixed tissue population of fibroblasts, adventitial reticular cells, preadipocytes, and macrophages. Stromal cells regulate hematopoiesis by binding directly to hematopoietic cells and providing numerous secreted factors. In long-term bone marrow culture, both B lineage and myeloid precursors grow in close physical association with stromal cells (Kincade et al., 1989; Kee and Paige, 1995). Stromal cells also secrete various soluble mediators essential for the growth and differentiation of these precursors (Dorshkind, 1990), such as interleukin-7 (IL-7), stem cell factor, and insulin-like growth factor for B lymphopoiesis, and GM-CSF, G-CSF, and IL-3 for myelopoiesis. Thus, it has been hypothesized that hematopoietic precursors need to stay in the bone marrow to receive necessary differentiation and proliferation signals delivered by this microenvironment.

CXCR4 is the chemokine receptor for stromal cell–derived factor 1 (SDF-1), a CXC chemokine that is highly expressed by bone marrow stromal cells (Tashiro et al., 1993; Nagasawa et al., 1994; Bleul et al., 1996a, 1996b; Oberlin et al., 1996). CXCR4 is expressed by, and SDF-1 is chemoattractive for, $CD34^+$ hematopoietic progenitors, B cell precursors, mature B and T lymphocytes, and monocytes (Aiuti et al., 1997; Bleul et al., 1997, 1998; D'Apuzzo et al., 1997; Deichmann et al., 1997; Hori et al., 1998). CXCR4 is a G protein–coupled seven-transmembrane receptor that also functions as a coreceptor for the entry of T-tropic strains of HIV-1 into $CD4^+$ cells (Bleul et al., 1996a; Feng et al., 1996; Oberlin et al., 1996). Both CXCR4- and SDF-1-deficient mice die perinatally and display identical defects in neuron migration, organ vascularization, and hematopoiesis (Nagasawa et al., 1996; Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998). Although T lymphopoiesis is unaffected, B lymphopoiesis and myelopoiesis are impaired in these mice. B cell precursors are severely reduced in fetal liver and bone marrow. Myelopoiesis is quantitatively decreased in fetal liver (Ma et al., 1998) and virtually absent in bone marrow (Nagasawa et al., 1996; Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998).

While CXCR4 and SDF-1 have been demonstrated to regulate B lymphopoiesis and myelopoiesis, the mechanism remains unclear. SDF-1 was first described as a pre-B cell growth-stimulating factor in vitro (Nagasawa et al., 1994), and mice deficient in CXCR4 or SDF-1 display defects in B cell development (Nagasawa et al., 1996; Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998). Because SDF-1 has a dual function as a growth factor and a chemoattractant, it is unclear whether it is more important for the direct delivery of essential maturation signals to hematopoietic cells or for the appropriate localization of these cells in microenvironments where they can receive other maturation-promoting signals. To clarify the role of CXCR4 in B lymphopoiesis and myelopoiesis in vivo, we have examined fetal blood in CXCR4-deficient mice and have reconstituted lethally irradiated mice with CXCR4-deficient fetal liver cells. Our results suggest that CXCR4 regulates B lymphopoiesis and myelopoiesis by confining precursors within the supportive fetal liver and bone marrow microenvironment for further maturation.

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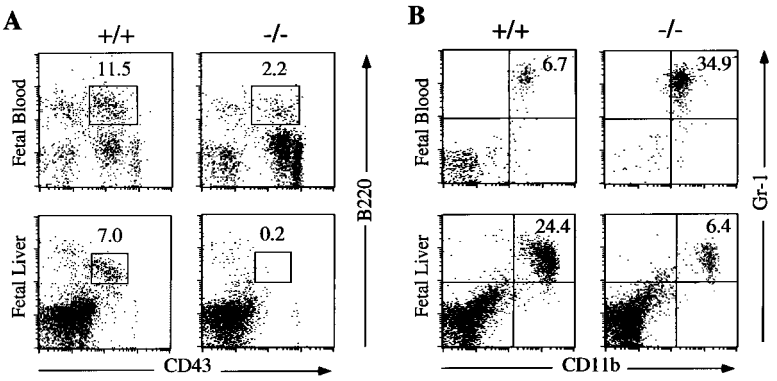


Figure 1. Immunofluorescent Flow Cytometry of B Lymphoid and Myeloid Cells in E18.5 Fetal Blood and Liver

The percentages of total cells within the box are shown. The data are representative of six independent experiments. Genotypes are +/+, wild-type control, and −/−, CXCR4-deficient embryos. (A) B lineage cells. Pro-B cells ($B220^+CD43^+$) within the lymphocyte gate are boxed. (B) Myeloid lineage cells. Myeloid lineage cells were identified by Gr-1 and CD11b mAbs.

Results

B and Myeloid Lineage Cells in the Fetal Blood of CXCR4-Deficient Embryos

We examined whether B and myeloid lineage cells were present in the bloodstream of E18.5 CXCR4-deficient embryos and their wild-type littermates, in comparison with liver tissue from the same mice. Pro-B cells ($B220^+CD43^+$) constituted 11.5% ± 3% of fetal nucleated blood cells and 7% ± 2% of fetal liver cells in wild-type embryos, while in CXCR4-deficient embryos, this population was almost absent in liver (0.2% ± 0.2%) (Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998) but was definitely present in blood (2.2% ± 1.1%) (Figure 1A). The findings with myeloid cells were even more dramatic (Figure 1B). Myeloid lineage cells ($Gr\text{-}1^+CD11b^+$) were elevated in CXCR4-deficient blood (34.9% ± 4.0% versus 6.7% ± 1.8%, respectively) but reduced in CXCR4-deficient liver (6.4% ± 1.6% versus 24.4% ± 3.1%, respectively) compared to the wild-type control. Similar results were seen in six CXCR4-deficient embryos from four different litters. The data suggest that in the absence of CXCR4, B cell and myeloid precursors are generated but are inefficiently retained in fetal liver, the primary site of B lymphopoiesis and myelopoiesis in late embryonic life (Ikuta et al., 1992).

Bone Marrow Reconstitution with CXCR4-Deficient Fetal Liver Cells

To further characterize the role of CXCR4 in hematopoiesis, especially in bone marrow, we generated hematopoietic chimeras by transferring E15.5 fetal liver cells into lethally irradiated C57BL/6-CD45.1 recipient mice. The fetal liver-derived donor cells could be distinguished by their surface expression of the leukocyte common antigen CD45.2 allele. The recipient mice survived the radiation challenge, and hematopoiesis was successfully reconstituted with either wild-type or CXCR4-deficient fetal liver cells. The peripheral blood of mice 4 weeks post transplant was evaluated by morphologic analysis and flow cytometry. Mice reconstituted with wild-type or CXCR4-deficient cells had normal ranges of total white blood cells (Figure 2). B lymphocytes ($CD45.2^+B220^+$) and granulocytes ($CD45.2^+Gr\text{-}1^+$) were predominantly of donor origin, 99.5% and 97.2%, respectively. Compared to mice reconstituted with wild-type cells, those reconstituted with CXCR4-deficient cells showed a consistent decrease in the absolute number of donor-derived B lymphocytes and an increase in the absolute number of donor-derived granulocytes in the blood (Figure 2). However, the absolute numbers of monocytes were approximately the same, and the red blood cell and platelet counts were similar (data not shown). The thymus was well reconstituted and contained mostly donor-derived immature T cells 4 weeks post transplant with CXCR4-deficient cells (data not shown).

B Lymphopoiesis in CXCR4-Deficient Chimeras

Although B lineage cells in the bone marrow of mice reconstituted with CXCR4-deficient cells were mainly donor derived ($CD45.2^+B220^+$), the percentage of B lineage cells was markedly reduced compared to the wild-type control (2.2% ± 1.3% versus 16.8% ± 2.3%, respectively) (Figure 3A). However, CXCR4-deficient pro-B ($B220^+CD43^+$), pre-B ($B220^+CD43^-$), and IgM^+ B ($B220^+IgM^+$) cell populations could be readily detected in bone marrow (Figure 3A), indicating that B cell development could occur in the absence of CXCR4.

Analysis of peripheral blood lymphocytes by flow cytometry not only confirmed reduced numbers of CXCR4-deficient B cells as compared to the wild-type control

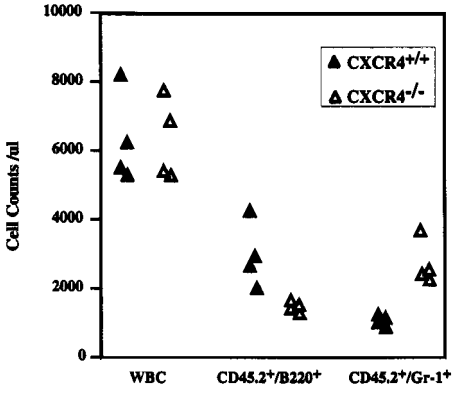


Figure 2. Cell Populations in Peripheral Blood of Mice 4 Weeks Post Transplant

Blood samples were collected from four different mice. Total white blood cells (WBC) were determined by Coulter counter. Donor-derived B cells ($CD45.2^+B220^+$) and granulocytes ($CD45.2^+Gr\text{-}1^+$) were determined by flow cytometry. Genotypes refer to the source of the donor fetal livers.

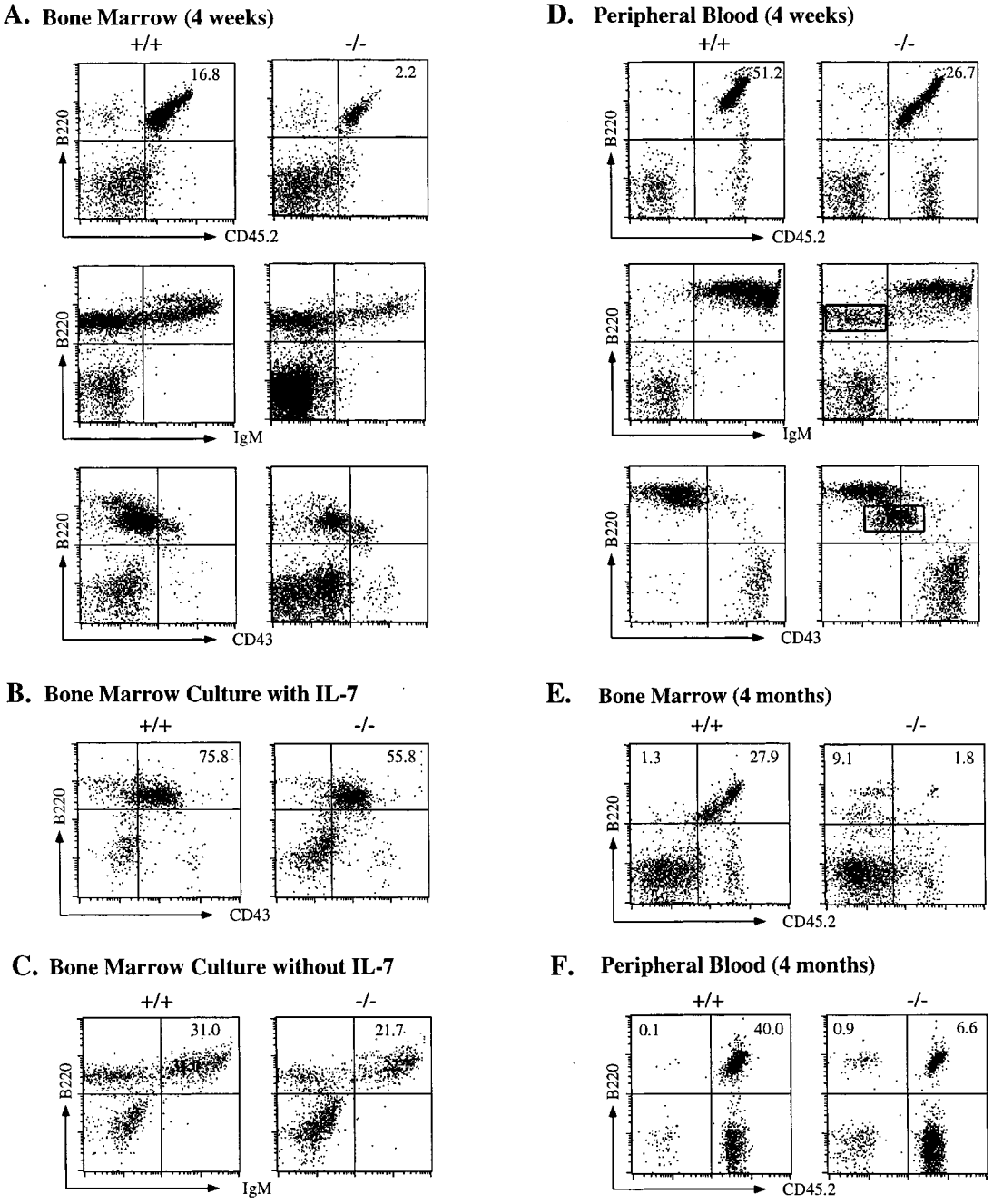


Figure 3. B Lymphopoiesis in Reconstituted Mice

In all experiments, cells were stained either with CD45.2 and B220 mAbs for donor-derived B cells ($CD45.2^+B220^+$) or with B220, CD43, and IgM mAbs for pro-B ($B220^+CD43^+IgM^-$), pre-B ($B220^+CD43^-IgM^-$), and IgM^+ B ($B220^+CD43^-IgM^+$) cell subpopulations; concordant results were obtained. The lower two panels in (A) and (D) show cells triple-stained with mAb to B220, CD43, and IgM. In all experiments with bone marrow cells from animals 4 weeks after transplant and after culture of these cells in vitro, $B220^+$ cells were more than 90% $CD45.2^+$. Genotypes above each column refer to the source of donor fetal livers. The data are representative of four independent experiments. The averages and SD values reported in the text are for all experiments. (A) Immunofluorescent flow cytometry of bone marrow. The percentages of total nucleated cells are shown in the upper panel. The percentages of cells within lymphoid side and forward angle scatter gates are shown in the middle and lower panels. (B and C) In vitro bone marrow culture under Whitlock-Witte conditions for 5 days in the presence (B) or absence (C) of IL-7. (D) B cell subpopulations in peripheral blood. The boxed areas in the two lower panels in (D) contain cells with pro-B and pre-B cell phenotypes, which are $B220^+IgM^-$ and are differentiated as $CD43^+$ or $CD43^-$, respectively. (E and F) Immunofluorescent flow cytometry of bone marrow cells (E) and peripheral blood leukocytes (F) in mice 4 months post transplant.

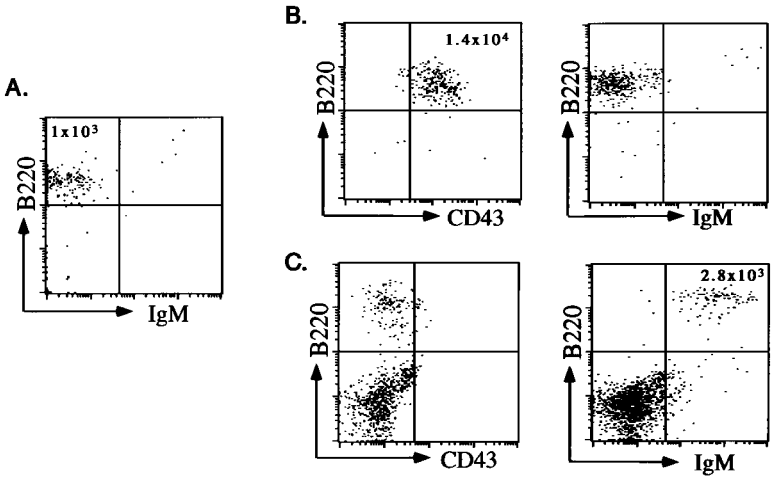


Figure 4. In Vitro Culture of IgM⁻ B Cells Isolated from the Peripheral Blood of Mice 4 Weeks Post Transplant with CXCR4-Deficient Fetal Liver Cells

The B220⁺IgM⁻ cells in peripheral blood purified by cell sorting were analyzed by flow cytometry (A). The purified cells were cultured on ST2 stromal cells under Whitlock-Witte conditions (Whitlock and Witte, 1982) with (B) and without (C) IL-7. Cells were collected 10 days after culture and analyzed by flow cytometry. The data represent two independent culture experiments. In cultures without IL-7, stromal cells were washed off the well and contributed to the B220⁻ population. The B220⁺ cells were more than 98% CD45.2⁺.

(26.7% ± 3.2% versus 51.2% ± 6.5%, respectively) but also revealed a strikingly different staining pattern of CXCR4-deficient B cells (Figure 3D). In addition to the circulating mature B lymphocytes (B220^{hi}IgM⁺), there were B220⁺IgM⁻ cells in the peripheral blood of mice reconstituted with CXCR4-deficient cells (Figure 3D, box in the middle panel). Further analysis showed that these cells expressed surface markers characteristic of pro-B (B220⁺CD43⁺IgM⁻) and pre-B (B220⁺CD43⁻IgM⁻) cells (Figure 3D, box in the lower panel), which normally reside in the adult bone marrow but are not released into the bloodstream. The spleen of mice reconstituted with CXCR4-deficient cells contained mainly IgM⁺ B cells, with only a few IgM⁻ B cells that were likely derived from the circulating blood; furthermore, no significant lymphopoiesis was noted in the liver (data not shown).

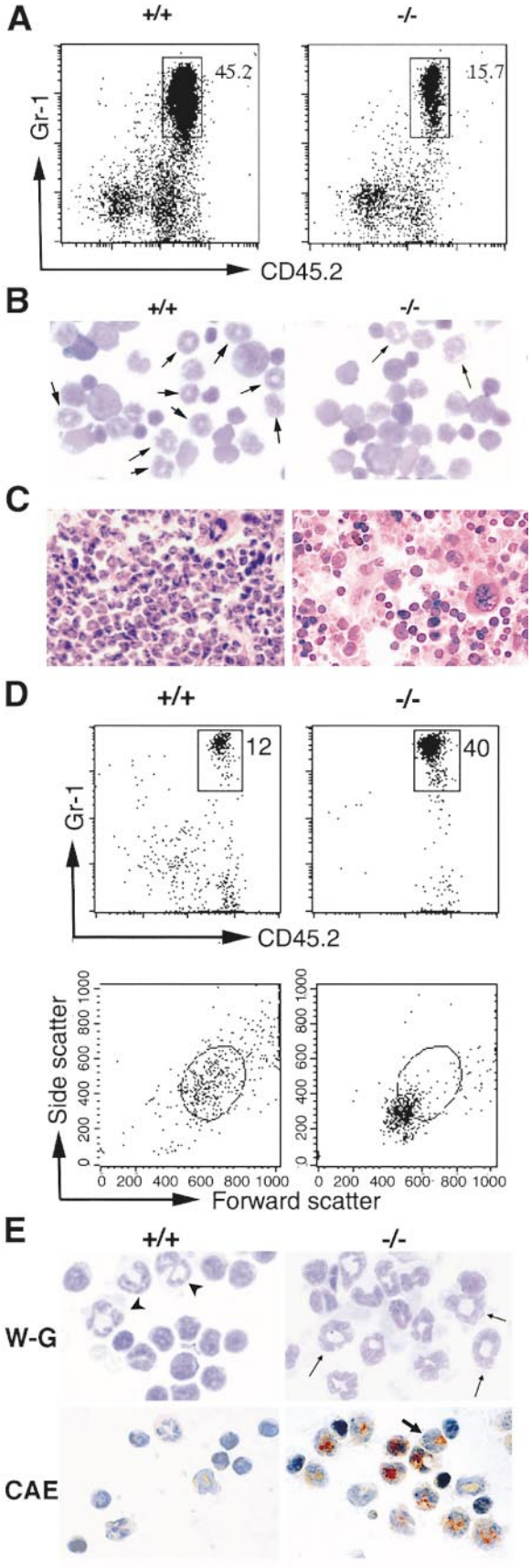
The above results suggested that CXCR4 is required to retain B cell precursors within the bone marrow microenvironment for further maturation, rather than to directly signal B cell development. To confirm this issue, bone marrow from mice 4 weeks post transplant was cultured in vitro for 5 days (Whitlock and Witte, 1982) to confine the B cell precursors in the supportive stromal environment. Although CXCR4-deficient pro-B cells (B220⁺CD43⁺IgM⁻) were present in a lower proportion in bone marrow, upon IL-7 stimulation they yielded a proportion of pro-B cells similar to wild-type (55.8% ± 8.1% and 75.8% ± 6.4%, respectively) (Figure 3B). Furthermore, based on the numbers of B220⁺CD43⁺IgM⁻ cells at the beginning and end of cultures, pro-B cells expanded 8.6-fold with CXCR4-deficient cells and 9.9-fold with wild-type cells (average of two experiments). Moreover, in the absence of IL-7, donor-derived CXCR4-deficient B cell precursors were fully able to differentiate into IgM⁺ B cells (Figure 3C). Compared to the total number of starting B220⁺ cells, the yield of B220⁺CD43⁺IgM⁺ cells was 2.2-fold for CXCR4-deficient cells and 2.3-fold for wild-type cells (average of two experiments).

Four months after transplantation, donor-derived B cells greatly outnumbered recipient-derived B cells in the bone marrow of wild-type recipients, but more recipient than donor-derived B cells were present in the bone marrow of mice reconstituted with CXCR4-deficient cells (Figure 3E). Although some recipient-derived B lymphocytes were present in the circulation, the CXCR4-deficient donor-derived B lymphocytes still predominated in the peripheral blood at this time (Figure 3F).

To characterize the B220⁺IgM⁻ cells in the peripheral blood of mice reconstituted with CXCR4-deficient cells, we purified the B220⁺IgM⁻CD45.2⁺ population from blood by cell sorting (Figure 4A). One thousand purified cells were cultured on ST2 stromal cells in vitro for 10 days, in the presence or absence of IL-7. With IL-7 stimulation, the B220⁺IgM⁻ cells proliferated 14-fold and retained their B220⁺CD43⁺IgM⁻ phenotype (Figure 4B). In the absence of IL-7, the B220⁺IgM⁻ cells differentiated into B220⁺CD43⁻IgM⁺ cells (Figure 4C) and proliferated 2.8-fold based on their yield. The B220⁻ cells evident in Figure 4C are ST2 stromal cells, which were more readily resuspended when cultured in the absence of IL-7. The data show that the CXCR4-deficient B220⁺IgM⁻ cells in the bloodstream can proliferate as well as differentiate into IgM⁺ cells. Therefore, they are B cell precursors.

Myelopoiesis in CXCR4-Deficient Chimeras

In bone marrow, myeloid progenitors give rise to both monocytic and granulocytic precursors. Granulocytic precursors undergo morphological and functional changes during maturation (Berliner, 1998). The bone marrow maintains a large pool of metamyelocytes/ring forms and segmented granulocytes, which exceed the total number of granulocytes present in the peripheral circulation (Marmont et al., 1988). Four weeks post transplant, donor-derived myeloid precursors (CD45.2⁺Gr-1⁺) were markedly reduced in bone marrow from mice reconstituted with CXCR4-deficient cells (15.7% ± 3.1%) compared to the wild-type control (45.2% ± 4.2%) (Figure 5A), although the cellularity of bone marrow was similar. The results were confirmed by bone marrow cytospins (Figure 5B) and histologic sections (Figure 5C), which revealed a noticeable reduction of more mature myeloid elements and a marked erythroid predominance. Morphologic analysis showed fewer mature granulocytes including metamyelocytes/ring forms and segmented granulocytes in marrow reconstituted with CXCR4-deficient cells, whereas the percentages of more immature



myeloid cells, i.e., promyelocytes and myelocytes, were similar to the wild-type control.

In contrast to the marked reduction of granulocytic cells in bone marrow, the absolute number of CXCR4-deficient granulocytes in peripheral blood was 2- to 3-fold increased as compared to the wild-type control (Figures 2 and 5D). The CXCR4-deficient peripheral granulocytes displayed reduced forward and side scatter on the flow cytometry plot, indicative of morphological differences of these cells (Figure 5D) (Otten and Yokoyama, 1992). In contrast to the mature granulocytes with hypersegmented nuclei that predominated in the blood of wild-type reconstituted mice, CXCR4-deficient blood granulocytes contained a markedly increased fraction of metamyelocytes/ring forms and fewer mature segmented forms (Figure 5E). Scattered immature myeloid forms including myelocytes were also present in the blood of mice reconstituted with CXCR4-deficient cells (Figure 5E, thick arrow in lower right panel). In comparison to the diffuse pattern of chloroacetate esterase staining that reflects the finely dispersed cytoplasmic granules in typical mature granulocytes, the CXCR4-deficient peripheral granulocytes displayed a prominent granular staining pattern that resembled that seen in marrow granulocytic precursors (Figure 5E) (Marmont et al., 1988). These data suggest that a population of CXCR4-deficient granulocytic precursors are prematurely released into bloodstream, resulting in a reduction of myeloid elements in bone marrow and an overabundance of granulocytes in peripheral blood.

Localization of CXCR4-Deficient Lymphocytes in Secondary Lymphoid Organs

Because mature T and B lymphocytes express CXCR4 and migrate to SDF-1, a possible role of CXCR4 in lymphocyte trafficking has been postulated. To examine this hypothesis, the peripheral lymph organs of mice reconstituted with CXCR4-deficient cells were examined. Four months after transplantation, flow cytometry

Figure 5. Myelopoiesis in Mice 4 Weeks Post Transplant

Genotypes refer to the source of donor fetal livers. (A) Analysis of myeloid cells in bone marrow by flow cytometry. The yields of cells per femur from wild-type and CXCR4-deficient transplants were similar and ranged from 2.4×10^7 to 4.0×10^7 from a total of eight femurs from four sets of independent experiments. The percentages of Gr-1^+CD45.2^+ population (boxed) of total cells are shown. (B) Bone marrow cytospin samples stained with Wright-Giemsa. Arrows indicate myeloid elements (magnification 1000×). (C) Bone marrow sections stained with hematoxylin/eosin (magnification 400×). Arrows indicate myeloid elements. (D) Immunofluorescent flow cytometry of peripheral granulocytes. The upper panel dot plots display the cells within a forward and side scatter gate that was set broadly to include granulocytic cells. The Gr-1^+CD45.2^+ cells are boxed, and the percentages of these populations of the total nucleated cells are shown. The lower panel dot plots display the forward scatter and side scatter of the cells within the box in the corresponding upper panel. For reference, the circle including the scatter distribution of the wild-type granulocytes is shown in both plots. (E) Blood cytospin samples stained with Wright-Giemsa (W-G) and for chloroacetate esterase activity (CAE) (magnification 1000×). In the W-G stained smears, arrowheads indicate segmented granulocytes and thin arrows indicate metamyelocytes/ring forms. In the CAE stained smears, thick arrow indicates myelocyte.

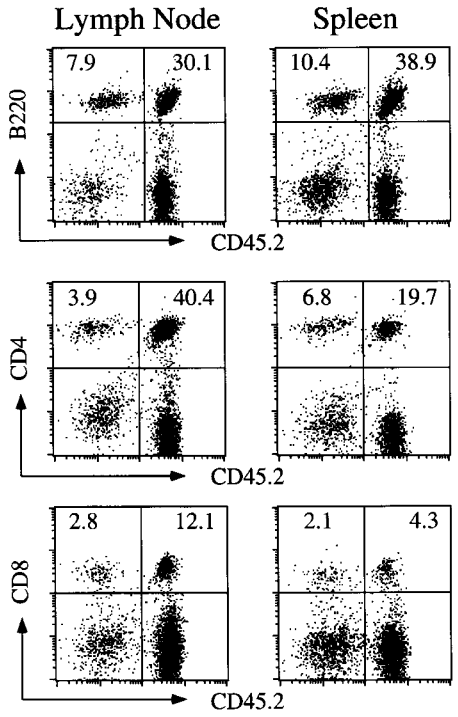


Figure 6. Immunofluorescent Flow Cytometry of B and T Lymphocytes in Lymph Node and Spleen from Mice 4 Months Post Transplant with CXCR4-Deficient Fetal Liver Cells

The dot plots display cells within the lymphocyte gate. The percentages of total gated cells within the box are shown.

showed that the mature B lymphocytes repopulating lymph nodes and spleen were predominantly of CXCR4-deficient donor origin (Figure 6). Furthermore, CXCR4-deficient T cells developed normally and populated secondary lymphoid organs efficiently, with only a minor portion of donor-derived residual T lymphocytes (Figure 6).

Histologic sections of lymph nodes revealed well formed architecture with numerous primary and secondary follicles (Figure 7A), as well as normal interfollicular areas and abundant plasma cells in the medullary areas. Immunohistochemical staining revealed a predominance of $B220^+$ B cells within follicles (Figure 7B) and $CD3^+$ T cells in the interfollicular regions (Figure 7C). Confirming the flow cytometric data (Figure 6), immunohistochemistry with CD45.2 mAb showed that these lymphocytes were predominately donor derived (data not shown). Splenic architecture was normal, with distinct B cell follicles in the white pulp and T cells located around central arterioles (Figures 7D and 7E). Histological sections also showed well organized Peyer's patches in the small bowel (Figure 7F). These findings suggest that CXCR4 is not required for the efficient localization of B and T lymphocytes in secondary lymphoid organs.

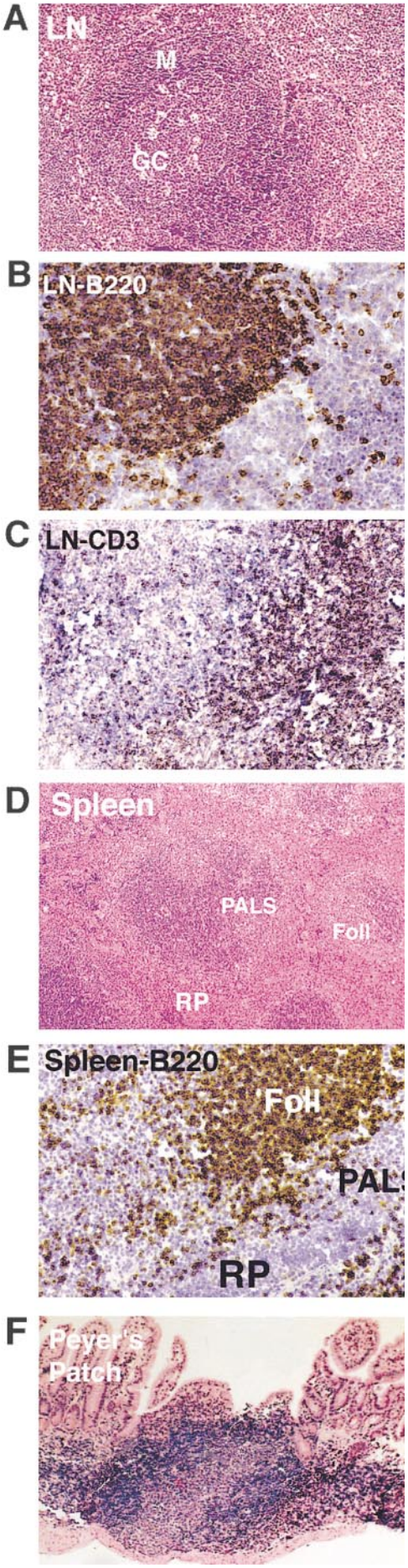
Discussion

We have studied the role of CXCR4 in hematopoiesis. Mice reconstituted with CXCR4-deficient cells have markedly reduced B cell precursors and myeloid elements in bone marrow. Remarkably, a significant proportion of donor B cells in the circulation are pro-B and pre-B cells. Furthermore, there is marked granulocytosis, with a predominance of immature granulocytic forms. Similar observations were made with blood from CXCR4-deficient embryos. Our results show that in the absence of CXCR4, growth and maturation of B lineage precursors and granulocytic cells can occur, and suggest that CXCR4 has an important function in retaining these cells within the bone marrow microenvironment. Our studies implicate CXCR4 in the retention of precursors within a hematopoietic compartment and thus demonstrate a novel function for chemokine receptors.

The finding of CXCR4-deficient B cell precursors including pro-B and pre-B populations in the peripheral blood is striking, since these cells normally reside in adult bone marrow. Spleen was occupied mainly by mature B cells and no hepatic extramedullary hematopoiesis was noted; therefore, the B cell precursors in the peripheral blood appeared to be derived from bone marrow.

The bona fide marrow-derived B cell precursors in blood retained the ability to proliferate and mature in the presence of stromal cells in Whitlock-Witte culture. As recovery from transplant proceeded after 4 weeks, the CXCR4-deficient B cell precursors in peripheral blood continued to be detected but declined with time. The decline in circulating B cell precursors with time appears related to the decline in B lymphopoiesis in the bone marrow, which in turn may be related to the premature release of marrow B cell precursors. The fate of B cell precursors in the periphery is uncertain. It is likely that these cells gradually died once deprived of their proliferative and maturation signals in bone marrow. The premature release of B cell precursors prior to proper differentiation and expansion likely resulted in the reduction of B lymphopoiesis in bone marrow and the decrease of absolute B cell number in peripheral blood.

The data presented here demonstrate that CXCR4 is not directly required for the differentiation and proliferation of B cells. We detected CXCR4-deficient pro-B, pre-B, and IgM^+ B cell populations in reconstituted bone marrow and mature B lymphocytes in peripheral lymphoid organs. Furthermore, the CXCR4-deficient B cell precursors that resided in the marrow were fully capable of proliferation and maturation when cultured in vitro. The proliferation of pro-B cells, as assessed by the measurement of the number of $B220^+CD43^+IgM^-$ cells at the beginning and end of Whitlock-Witte cultures in the presence of IL-7, was 8.6-fold and 9.9-fold, respectively, for CXCR4-deficient and wild-type cells. Similarly, in the absence of IL-7, the number of $B220^+IgM^+$ cells at the end of culture, compared to the total number of $B220^+$ cells that were IgM^+ or IgM^- at the beginning of culture, demonstrated a 2.2-fold and 2.3-fold increase, respectively, for CXCR4-deficient and wild-type cells. During in vitro culture, the hematopoietic cells and stromal cells remain in the same compartment, and this may be the reason why we find no requirement for CXCR4 for growth or maturation of B cells in vitro. We cannot rule out an influence of SDF-1 on B cell proliferation under certain conditions. Previously, B cell proliferation was shown to be increased 3-fold when the combination of IL-7



plus 40% conditioned medium from COS cells transfected with SDF-1 cDNA was compared to IL-7 alone (Nagasawa et al., 1994). However, our results show that there clearly is no absolute requirement for CXCR4 for B cell proliferation in vivo. Rather, there is a quantitative decrease in vivo in B cell precursors in the bone marrow and in mature B cells in the periphery. We find abnormally high levels of CXCR4-deficient precursor B cells in the bloodstream and suggest that premature release of these cells from the bone marrow is the most important reason for the quantitative decrease in B lymphopoiesis. Thus, the primary, although not necessarily the exclusive, role of CXCR4 in B lymphopoiesis is to retain B lineage cells within the marrow microenvironment. This is consistent with the finding that CXCR4 selectively mediates chemotaxis of early B cell precursors to SDF-1 (D'Apuzzo et al., 1997).

Granulocytes are normally released into peripheral blood after maturation in bone marrow. There is also a large pool of less mature granulocytes including metamyelocytes/ring forms, which are reserved in bone marrow and can be released into the periphery in response to acute inflammation (Marmont et al., 1988). The granulocytosis we observed in peripheral blood of mice reconstituted with CXCR4-deficient cells appears distinct from that seen under stress-induced conditions, such as infection, in which myeloid hyperplasia of the bone marrow is invariably seen. By contrast, granulocytosis associated with CXCR4 deficiency was accompanied by reduced myeloid lineage cells in primary hematopoietic organs, both in the fetal liver of CXCR4-deficient embryos and in adult bone marrow of mice reconstituted with CXCR4-deficient cells. Furthermore, mice reconstituted with CXCR4-deficient cells were maintained under pathogen-free conditions and displayed persistence of peripheral granulocytosis over 6 months in the absence of any signs of infection. Morphological analysis and chloroacetate esterase staining demonstrated that CXCR4-deficient granulocytes in the peripheral blood were relatively immature. These findings suggest that the increased numbers of granulocytes seen in the periphery were due to the premature release of the granulocytic pool, which is normally reserved in marrow.

It is widely accepted that the proper differentiation and proliferation of hematopoietic precursors requires a supportive bone marrow microenvironment, provided in part by stromal elements and blood vessels (Dorshkind, 1990). The marrow vasculature contains an extensive sinusoidal network. Hematopoiesis occurs in the intersinusoidal space with maturation of different lineages in distinct but overlapping locations. For instance, early B lymphopoiesis is more abundant in the subendosteal reticulum (Kincade et al., 1989; Kee and Paige, 1995). Myeloid elements are often seen in close association with paratrabecular stromal cells with maturing

Figure 7. Lymphocyte Localization in Lymph Node and Spleen from Mice 4 Months Post Transplant with CXCR4-Deficient Fetal Liver Cells

(A–C) Sections of lymph node stained with hematoxylin/eosin (A), B220 mAb (B), and CD3 mAb (C). (D and E) Sections of spleen stained with hematoxylin/eosin (D) and B220 mAb (E). (F) Section of Peyer's patch stained with hematoxylin/eosin. M, mantle zone of follicle; GC, germinal center; Foll, follicle; PALS, periarteriolar lymphoid sheath; and RP, red pulp.

granulocytes migrating toward the central venous sinus to enter the bloodstream (Westen and Bainton, 1979). Hematopoietic precursors need to be localized in close contact with stromal cells for the extensive interactions necessary for guiding proper growth and maturation.

Little is known about the molecular mechanisms for retaining hematopoietic precursors within the appropriate niches. Adhesion molecules, such as VLA-4, have been implicated in mediating adhesion of the B cell precursors to bone marrow stromal cells in vitro (Miyake et al., 1991; Ryan and Tang, 1995). Chemokines and their receptors are important in directing the migration of leukocytes and activation of integrin adhesiveness (Springer, 1995). As a chemokine receptor, CXCR4 might direct migration of hematopoietic precursors toward stromal cells that produce SDF-1. In the absence of CXCR4, B cell and myeloid precursors may fail to localize properly in association with stromal cells, and exit bone marrow prematurely before full differentiation and proliferation has taken place. This demonstrates a retention signal for hematopoietic precursor cells.

Chemokines and their receptors are important in regulating the trafficking patterns of recirculating lymphocytes (Baggiolini, 1998; Luster, 1998). Previously, CXCR4 and SDF-1 have been shown to mediate chemotaxis of both T and B lymphocytes (Bleul et al., 1996b, 1997, 1998). SDF-1 has been also demonstrated to induce the arrest of rolling lymphocytes under flow conditions, which is critical for recruitment (Campbell et al., 1998). However, we find that CXCR4-deficient lymphocytes in reconstituted mice can migrate to secondary lymphoid organs and repopulate their appropriate architectural niches efficiently. Thus, CXCR4 does not appear to be required for the trafficking and appropriate localization of mature lymphocytes in secondary lymphoid organ in vivo. In addition, although $CD34^+$ hematopoietic progenitors express CXCR4 (Deichmann et al., 1997) and can be mobilized by its ligand SDF-1 (Aiuti et al., 1997), CXCR4-deficient stem cells can successfully seed bone marrow and give rise to all hematopoietic lineages.

Our data show that CXCR4 is an important factor for retaining B lineage and granulocytic precursors within the bone marrow microenvironment. The precise role of CXCR4 in localization of hematopoietic precursors within bone marrow niches remains to be explored. Interestingly, both erythroid and megakaryocytic maturation are unaffected in the CXCR4-deficient embryos and reconstituted mice, suggesting that other factors might be involved in regulating the retention of these lineages within the medullary space. Further studies will be required to determine whether other chemokine receptors regulate hematopoiesis of other cell lineages by similar mechanisms.

Experimental Procedures

Adoptive Transfer of Fetal Liver Cells

Six- to eight-week-old mice of C57BL/6.SJL (CD45.1) congenic strain (Taconic) were lethally irradiated using ^{137}Cs source with doses of 800 and 400 rad separated by 3 hr. Fetal livers were harvested from E15.5 embryos generated by breeding of CXCR4-deficient heterozygous mice on a C57BL/6 and 129 mixed background. Single cell suspensions of individual fetal livers were prepared in Hanks' balanced salt solution, and 5×10^5 cells in 300 μl medium were injected intravenously into recipient mice. Tails from embryos were saved and were used to determine genotype by PCR (Ma et al., 1998) subsequent to fetal liver transplantation. Mice were maintained on acidified water and kept under pathogen-free conditions.

Hematological Analysis

For collecting fetal blood, E18.5 embryos with attached placentas were washed free of maternal blood and the cord was cut without damaging the embryos. Blood was collected by putting bleeding embryos in medium on ice until they were completely pale.

Blood samples were obtained from the tail vein of adult mice using heparinized microcapillary tubes (Baxter). Complete blood counts were determined by automatic cell counter (Coulter). Differential counts were assessed based on cellular morphology of blood smears by Wright-Giemsa stain (Sigma). For preparation of cytospins from bone marrow or peripheral blood, erythrocytes were depleted by lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA), and leukocyte suspensions were cytocentrifuged onto glass slides according to the manufacturer's instructions (Shandon).

Flow Cytometry and Cell Sorting

Single cell suspensions were prepared from tissues and peripheral blood after erythrocyte depletion. Cells were stained with antibodies as recommended by the manufacturer (Pharmingen). The monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), R-Phycoerythrin-RM4-5 (PE), or Cy-Chrome (CyC) were anti-CD45.2-FITC, anti-CD45.1-FITC, anti-B220-CyC, anti-CD43-PE, anti-IgM^-FITC, anti-CD4-PE, anti-CD8-CyC, anti-CD3-FITC, and anti-Gr-1-FITC. Data were collected on FACScan and analyzed by CELLQuest software (Becton Dickinson). Cell sorting was carried out on EPICS Elite ESP using an agon laser (488 nm) (Coulter). Single cell suspensions stained with B220 and IgM mAbs were held on ice during sorting. The selected $B220^+IgM^-$ population was sorted directly into tubes with RPMI 1640 medium.

In Vitro Bone Marrow Culture

Femur bone marrow was collected and resuspended at a concentration of 1×10^6 cells/ml in RPMI 1640 medium supplemented with 5% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin (Whitlock and Witte, 1982). Recombinant murine IL-7 (R&D Systems) was used at a concentration of 10 ng/ml. Bone marrow cell suspensions were plated (2 ml per well of 24-well dishes) and cultured for 5 days. The nonadherent cells were collected for flow cytometry analysis.

One thousand $B220^+IgM^-$ cells purified by cell sorting from peripheral blood were plated per well onto confluent monolayers of ST2 stromal cells in a 24-well dish. Cells were cultured under the same conditions as above for 10 days. The nonadherent cells were collected for flow cytometry.

Histology and Immunohistochemistry

Histologic analysis was performed on formalin-fixed paraffin-embedded sections of secondary lymphoid organs and lightly decalcified bone marrow. For cytochemical staining, naphthol AS-D chloroacetate ester was used for specific esterase activity (Sigma). Immunohistochemical staining of lymphoid organs was performed on cryostat sections using a rat mAb against the B cell marker CD45/B220, a hamster mAb against the T cell marker CD3e and a directly biotinylated mouse mAb against the CD45.2 allele of leukocyte common antigen (Pharmingen). Appropriate biotinylated secondary antibodies and the Elite Vectastain avidin-biotin-horseradish peroxidase reagent were used with diaminobenzidine tetrahydrochloride as chromogenic substrate (Vector Laboratories).

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References

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

Baggiolini, M. (1998). Chemokines and leukocyte traffic. Nature *392*, 565–568.

Berliner, N. (1998). Molecular biology of neutrophil differentiation. Curr. Opin. Hematol. *5*, 49–53.

Bleul, C.C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T.A. (1996a). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature *382*, 829–833.

Bleul, C.C., Fuhlbrigge, R.C., Casasnovas, J.M., Aiuti, A., and Springer, T.A. (1996b). A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J. Exp. Med. *184*, 1101–1110.

Bleul, C.C., Wu, L., Hoxie, J.A., Springer, T.A., and Mackay, C.R. (1997). The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc. Natl. Acad. Sci. USA *94*, 1925–1930.

Bleul, C.C., Schultze, J.L., and Springer, T.A. (1998). B lymphocyte chemotaxis regulated in association with microanatomic localization, differentiation state, and B cell receptor engagement. J. Exp. Med. *187*, 753–762.

Campbell, J.J., Hedrick, J., Zlotnik, A., Siani, M.A., Thompson, D.A., and Butcher, E.C. (1998). Chemokines and the arrest of lymphoyctes rolling under flow conditions. Science *279*, 381–384.

D'Apuzzo, M., Rolink, A., Loetscher, M., Hoxie, J.A., Clark-Lewis, I., Melchers, F., Baggiolini, M., and Moser, B. (1997). The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor. Eur. J. Immunol. *27*, 1788–1793.

Deichmann, M., Kronenwett, R., and Haas, R. (1997). Expression of the human immunodeficiency virus type-1 coreceptors CXCR-4 (fusin, LESTR) and CKR-5 in $CD34^+$ hematopoietic progenitor cells. Blood *89*, 3522–3528.

Dorshkind, K. (1990). Regulation of hemopoiesis by bone marrow stromal cells and their products. Annu. Rev. Immunol. *8*, 111–137.

Feng, Y., Broder, C.C., Kennedy, P.E., and Berger, E.A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science *272*, 872–877.

Hori, T., Sakaida, H., Sato, A., Nakajima, T., Shida, H., Yoshie, O., and Uchiyama, T. (1998). Detection and delineation of CXCR-4 (Fusin) as an entry and fusion cofactor for T cell-tropic HIV-1 by three different monoclonal antibodies. J. Immunol. *160*, 180–188.

Ikuta, K., Uchida, N., Friedman, J., and Weissman, I.L. (1992). Lymphocyte development from stem cells. Annu. Rev. Immunol. *10*, 759–783.

Kee, B.L., and Paige, C.J. (1995). Murine B cell development: commitment and progression from multipotential progenitors to mature B lymphocytes. Int. Rev. Cytol. *157*, 129.

Kincade, P.W., Lee, G., Pietrangeli, C.E., Hayashi, S.-I., and Gimble, J.M. (1989). Cells and molecules that regulate B lymphopoiesis in bone marrow. Annu. Rev. Immunol. *7*, 111–143.

Luster, A.D. (1998). Chemokines—chemotactic cytokines that mediate inflammation. New Eng. J. Med. *338*, 436–445.

Ma, Q., Jones, D., Borghesan, P.R., Segal, R.A., Nagasawa, T., Kishimoto, T., Bronson, R.T., and Springer, T.A. (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc. Natl. Acad. Sci. USA *95*, 9448–9453.

Marmont, A.M., Damasio, E., and Zucker-Franklin, D. (1988). Neutrophils. In Atlas of Blood Cells: Function and Pathology, D. Zucker-Franklin, M.F. Greaves, C.E. Grossi, and A.M. Marmont, eds. (Philadelphia: Lea and Febigen), pp. 159–190.

Miyake, K., Weissman, I.L., Greenberger, J.S., and Kincade, P.W. (1991). Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. J. Exp. Med. *173*, 599–607.

Nagasawa, T., Kikutani, H., and Kishimoto, T. (1994). Molecular cloning and structure of a pre-B-cell growth-stimulating factor. Proc. Natl. Acad. Sci. USA *91*, 2305–2309.

Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S.-I., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature *382*, 635–638.

Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Virelizier, J.-L., Arenzana-Seisdedos, A., Schwartz, O., Heard, J.-M., Clark-Lewis, I., Legler, D.F., et al. (1996). The CXC chemokine, stromal cell derived factor 1 (SDF-1), is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. Nature *382*, 833–835.

Otten, G., and Yokoyama, W.M. (1992). Flow Cytometry. In Current Protocols in Immunology, J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober, eds. (New York: John Wiley and Sons, Inc.), pp. 5.4.1–5.4.19.

Rolink, A., and Melchers, F. (1993). B lymphopoiesis in the mouse. Adv. Immunol. *53*, 123–156.

Ryan, D.H., and Tang, J. (1995). Regulation of human B cell lymphopoiesis by adhesion molecules and cytokines. Leuk. Lymphoma *17*, 375–389.

Springer, T.A. (1995). Signals on endothelium for lymphocyte recirculation and leukocyte emigration: the area code paradigm. In The Harvey Lectures, Series 89. The Harvey Society of New York, ed. (New York: Wiley-Liss, Inc.), pp. 53–103.

Tachibana, K., Hirota, S., Lizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S-I., et al. (1998). The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature *393*, 591–594.

Tashiro, K., Tada, H., Heilker, R., Shirozu, M., Nakano, T., and Honjo, T. (1993). Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. Science *261*, 600–603.

Tenen, D.G., Hromas, R., Licht, J.D., and Zhang, D-E. (1997). Transcription factors, normal myeloid development, and leukemia. Blood *90*, 489–519.

Westen, H., and Bainton, D.F. (1979). Association of alkaline-phosphatase-positive reticulum cells in bone marrow with granulocytic precursors. J. Exp. Med. *150*, 919–937.

Whitlock, C.A., and Witte, O.N. (1982). Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc. Natl. Acad. Sci. USA *79*, 3608–3612.

Zou, Y-R., Kottmann, A.H., Kuroda, M., Taniuchi, I., and Littman, D.R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature *393*, 595–599.