The Journal of Immunology

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J Immunol 2003; 170:4649-4655; ;
doi: 10.4049/jimmunol.170.9.4649
http://www.jimmunol.org/content/170/9/4649

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

A Role of CXC Chemokine Ligand 12/Stromal Cell-Derived Factor-1/Pre-B Cell Growth Stimulating Factor and Its Receptor CXCR4 in Fetal and Adult T Cell Development in Vivo

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The functions of a chemokine CXC chemokine ligand (CXCL) 12/stromal cell-derived factor-1/pre-B cell growth stimulating factor and its physiologic receptor CXCR4 in T cell development are controversial. In this study, we have genetically further characterized their roles in fetal and adult T cell development using mutant and chimeric mice. In CXCL12$^{-/-}$ or CXCR4$^{-/-}$ embryos on a C57BL/6 background, accumulation of T cell progenitors in the outer mesenchymal layer of the thymus anlage during initial colonization of the fetal thymus was comparable with that seen in wild-type embryos. However, the expansion of CD3$^-$CD4$^-$CD8$^-$ triple-negative T cell precursors at the CD44$^-$CD25$^+$ and CD44$^-$CD25$^-$ stages, and CD4$^+$CD8$^+$ double-positive thymocytes was affected during embryogenesis in these mutants. In radiation chimeras competitively repopulated with CXCR4$^{-/-}$ fetal liver cells, the reduction in donor-derived thymocytes compared with wild-type chimeras was much more severe than the reduction in donor-derived myeloid lineage cells in bone marrow. Triple negative CD44$^+$CD25$^+$ T cell precursors exhibited survival response to CXCL12 in the presence of stem cell factor as well as migratory response to CXCL12. Thus, it may be that CXCL12 and CXCR4 are involved in the expansion of T cell precursors in both fetal and adult thymus in vivo. Finally, enforced expression of bcl-2 did not rescue impaired T cell development in CXCR4$^{-/-}$ embryos or impaired reconstitution of CXCR4$^{-/-}$ thymocytes in competitively repopulated mice, suggesting that defects in T cell development caused by CXCR4 mutation are not caused by reduced expression of bcl-2. ***The Journal of Immunology,*** **2003, 170: 4649–4655.**

The chemokine CXC chemokine ligand (CXCL)[5] 12/stromal cell-derived factor-1 (SDF-1)/pre-B cell growth stimulating factor (PBSF) was isolated from bone marrow stromal cells (1–3) and first characterized as a pre-B cell growth-stimulating factor (1, 3). CXCR4 has been shown to be a primary physiologic receptor for CXCL12 (4–10) and also function as an entry coreceptor for strains of HIV-1 (11). The multiple essential functions of CXCL12 and CXCR4 in development have been demonstrated using mutant mice with targeted gene disruption (7–10). CXCL12 and CXCR4 are essential for B cell development (7–10), colonization of bone marrow by hematopoietic progenitors (7–10), blood vessel formation in the gastrointestinal tract (8), cardiac ventricular septum formation (7–9), and cerebellar development (9, 10) as well as embryonic viability (7–10). In B cell development, it has been clearly shown that dependency on CXCL12 appeared at the earliest stages in both fetal liver and bone marrow (12). However, the roles of CXCL12 and CXCR4 in T cell development are controversial. No significant defect in T cell development in CXCL12$^{-/-}$ or CXCR4$^{-/-}$ embryos has been reported (7–10). Moreover, mature T cells developed normally in the thymus from CXCR4-deficient embryos engrafted under the kidney capsule of mice lacking TCR-α (9). These results suggest that the CXCL12/CXCR4 chemokine system is not required for T cell development in embryos. The role of CXCL12 and CXCR4 in adult hematopoiesis was examined using radiation chimeric mice reconstituted with fetal liver or bone marrow cells (13–15). Mice long-term reconstituted with CXCR4-deficient fetal liver or bone marrow cells have reduced donor-derived thymocytes (14, 15). These results suggest that CXCR4 is involved in adult T cell development. However, the possibility that the phenotype in the thymi in these mutant chimeric mice may be caused by the defects in multipotential hematopoietic progenitors cannot be excluded because the reductions in donor-derived thymocytes in mutant chimeras are in line with the reductions in donor-derived myeloid lineage cells in their bone marrow (14, 15). In contrast, it has been shown that CXCL12 and CXCR4 are expressed in both fetal and adult thymus, and T cell precursors exhibited marked chemotactic activity toward CXCL12, suggesting that CXCL12/CXCR4 may play a role in the migration of T cell precursors during development (16–22).

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Received for publication July 26, 2002. Accepted for publication March 3, 2003.

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[1] This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan and by the Future Program Grant of The Japanese Society for the Promotion of Science, Sports and Culture of Japan.

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[5] Abbrevations used in this paper: CXCL, CXC chemokine ligand; SDF-1, stromal cell-derived factor-1; PBSF, pre-B cell growth stimulating factor; FTOC, fetal thymus organ culture; E11.5, embryonic day 11.5; GFP, green fluorescent protein; TN, triple-negative; DP, double-positive; SP, single-positive; SCF, stem cell factor; ES, embryonic stem.

0022-1767/03/$02.00

Recently, the in vitro system using chimeric human-mouse fetal thymus organ culture (FTOC) seeded with $CD34^+$ juvenile thymic precursors and treated with neutralizing Abs against CXCL12 or CXCR4 revealed a significant reduction of the numbers of human thymocytes, suggesting that CXCL12/CXCR4 signaling plays an important role in T cell development in the juvenile human (23). Then, to determine the role of the CXCL12/CXCR4 chemokine system in fetal and adult T cell development in vivo, we examined further T cell development in $CXCL12^{-/-}$ or $CXCR4^{-/-}$ mice on a C57BL/6 background and determined the capacity of $CXCR4^{-/-}$ hematopoietic cells to compete with normal bone marrow cells for the long-term lymphomyeloid reconstitution. Although accumulation of T cell precursors observed in the outer mesenchymal layer of the thymus anlage during initial colonization of thymus at embryonic day 11.5 (E11.5) were normal, the expansion of T cell precursors in the thymus from E14.5 onward was impaired in the C57BL/6 mutant embryos. Moreover, chimeric mice competitively repopulated with $CXCR4^{-/-}$ fetal liver cells displayed markedly reduced donor-derived thymocytes, and the reduction was more severe than that observed for myeloid lineage cells, supporting the idea that CXCR4 plays a role in expansion of T cell precursors in thymus. In addition, enforced expression of bcl-2, an oncogene that prevents programmed cell death, did not rescue impaired T cell development in CXCR4-deficient embryos or impaired reconstitution of thymocytes in competitively repopulated $CXCR4^{-/-}$ chimeras, suggesting that defects caused by the lack of CXCR4 are not caused by reduced expression of bcl-2.

Materials and Methods

Mice

The generation of $CXCL12^{-/-}$ and $CXCR4^{-/-}$ mice has been previously described (7, 8). $CXCL12^{+/-}$ or $CXCR4^{+/-}$ mice were backcrossed at least seven times with C57BL/6-Ly5.2 mice. C57BL/6-Ly5.1 mice were a gift from Drs. M. Osawa and H. Nakauchi (University of Tsukuba, Tsukuba, Japan), and H2K-bcl-2 transgenic mice (24) were a gift from Drs. J. Domen and I. L. Weissman (Stanford University, Palo Alto, CA). The bcl-2/$CXCR4^{+/-}$ mice were generated by crossing with H2K-bcl-2 transgenic mice and $CXCR4^{+/-}$ mice.

To generate the mice in which the green fluorescent protein (*GFP*) gene was knocked into the CXCL12 locus (CXCL12/GFP knockin mice), exon 2 of the *CXCL12* gene was replaced by GFP expression cassette by homologous recombination in embryonic stem (ES) cells (7). Mutated ES colonies were used to produce mice hemizygous for the GFP insertion by blastocyst injection as described (7). Mice hemizygous for the GFP insertion, which have one functional *CXCL12* allele, are phenotypically normal and can be used for the analysis of CXCL12 expression.

Antibodies

Abs used in this study were as follows: FITC-conjugated anti-Ly5.2 (clone 104), anti-CD25 (7D4), anti-CXCR4 (2B11), and isotype control IgG2bκ (A95-1); PE-conjugated anti-CD4 (GK1.5), anti-CD25 (PC61), anti-CD44 (IM7), anti-Sca-1 (E13-161.7), anti-Gr-1 (RB6-8C5), and anti-CD19 (1D3); allophycocyanin-conjugated anti-CD8 (53-6.7), anti-B220 (RA3-6B2), anti-CD25, and anti-c-*kit* (2B8); biotinylated anti-CD3 (145-2C11), anti-CD4, anti-CD8, anti-Thy-1 (30-H12), anti-B220, anti-Mac-1 (M1/70), anti-Gr-1, and anti-erythroid lineage cells (TER119). All Abs were purchased from BD PharMingen (San Diego, CA). Biotinylated Abs were visualized with PerCP- or PharRed-conjugated streptavidin.

Immunohistiochemistry

Embryos at E11.5 and E12.5 were embedded in OCT compound and snap-frozen. They were cut into serial sections 5 μm thick using a cryostat and mounted onto 5% 3-amino propyltriethoxysilane (Sigma-Aldrich, St. Louis, MO)-coated slides. Freshly cut frozen sections were fixed with acetone at room temperature for 2 min. Primary Abs used in this study were rabbit anti-IKAROS Abs (25) and rabbit anti-keratin (wide spectrum screening; DAKO, Glostrup, Denmark). Staining was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Peroxidase activity was developed with 0.1% 3,3′-diaminobenzidine and 0.02% H_2O_2 in PBS. Sections were then counterstained with hematoxylin.

To examine GFP expression in CXCL12/GFP knockin mice, thymi were dissected, fixed in 4% paraformaldehyde overnight at 4°C, and frozen. Sections were mounted in Vectashield (Vector Laboratories) and viewed with a confocal microscope (LSM 5 PASCAL; Zeiss, Oberkochen, Germany).

Adoptive transfer

Recipient C57BL/6-Ly5.1 mice were lethally irradiated with 1100 rad. A total of 2×10^6 E16.5 fetal liver cells ($CXCR4^{+/-}$ and $CXCR4^{-/-}$) were mixed with or without 2×10^5 $Ly5.1^+$ wild-type bone marrow cells and injected into recipient mice i.v. For bcl-2/$CXCR4^{+/-}$ or bcl-2/$CXCR4^{-/-}$ chimeric mice, 0.5×10^6 ($CXCR4^{+/-}$ and bcl-2/$CXCR4^{+/-}$) or 1×10^6 ($CXCR4^{-/-}$ and bcl-2/$CXCR4^{-/-}$) E16.5 fetal liver cells were transplanted into recipient mice with 2×10^6 $Ly5.1^+$ wild-type bone marrow cells. After transplantation, the animals were maintained in autoclaved cages on sterilized food and acidified sterile water. Donor hematopoietic repopulation was determined 16 wk after transplantation.

Flow cytometry

Flow cytometry analysis was performed on single-cell suspensions from fetal liver, fetal thymus, adult bone marrow, and thymus. Cells were stained with mAbs and secondary reagents, and analyzed with FACSCalibur and CellQuest software. Thymocytes were sorted using FACSVantage SE for chemotactic or survival assay. Dead cells were excluded by propidium iodide staining.

In vitro survival assay

Sorted cells from adult wild-type thymus were cultured in 96-well flat-bottom plates in a volume of 0.15 ml of RPMI 1640 with 5% FCS. CXCL12 was added at a concentration of 1 μg/ml and stem cell factor (SCF) at 100 ng/ml. After 72 h, cells were harvested, and the number of surviving T cell precursor was counted by flow cytometry.

Result

Impaired expansion of T cell precursors in the thymus from $CXCL12^{-/-}$ or $CXCR4^{-/-}$ mice on a C57BL/6 background during embryogenesis

First, we analyzed the thymi from $CXCL12^{-/-}$ or $CXCR4^{-/-}$ embryos on a C57BL/6 background. The colonization of thymus by hematopoietic progenitors or prothymocytes is the first step of intrathymic T cell development. Initial migration of hematopoietic progenitor cells into the thymus anlage occurs before vascularization of the organ. It has been thought that progenitors leave the adjacent blood vessels and reach the outer mesenchymal layer of the thymus anlage through the surrounding connective tissue (26). In E11.5 wild-type mice, many hematopoietic progenitors that express IKAROS protein were located in the outer mesenchymal layer of the thymus anlage and lined the border of the epithelial anlage (Fig. 1*A*) (27). The numbers of these cells in E11.5 $CXCL12^{-/-}$ mice on a C57BL/6 background were comparable to those seen in wild-type mice (Fig. 1*B*), suggesting that CXCL12/CXCR4 is not required for the migration of hematopoietic progenitors from blood vessels to the border of the thymic epithelial anlage. In E12.5 wild-type mice, a large number of $IKAROS^+$ cells were seen within the epithelial anlage. Those $IKAROS^+$ cells were also present in $CXCL12^{-/-}$ embryos (Fig. 1, *C* and *D*). The phenotype of the majority of the $IKAROS^+$ cells is thought to be $CD3^-CD4^-CD8^-$ triple-negative (TN) $CD44^+CD25^-$ (28). The TN $CD44^+CD25^-$ cells develop along the sequence TN $CD44^+CD25^+$, TN $CD44^-CD25^+$ to TN $CD44^-CD25^-$, and finally to $CD4^+CD8^+$ double-positive (DP) thymocytes, which were significantly detected on E16.5 (28, 29). As shown in Fig. 2*A*, thymi from $CXCL12^{-/-}$ embryos on a C57BL/6 background contained relatively normal or slightly reduced numbers of TN $CD44^+CD25^-$ and TN $CD44^+CD25^+$ cells but substantially reduced numbers of TN $CD44^-CD25^+$, TN $CD44^-CD25^-$, and DP cells from E13.5 onward ($n = 3$–5). The numbers of DP thymocytes were reduced ~5-fold in the C57BL/6 mutants at E18.5 ($n = 6$). Similar results were obtained when $CXCR4^{-/-}$ embryos were

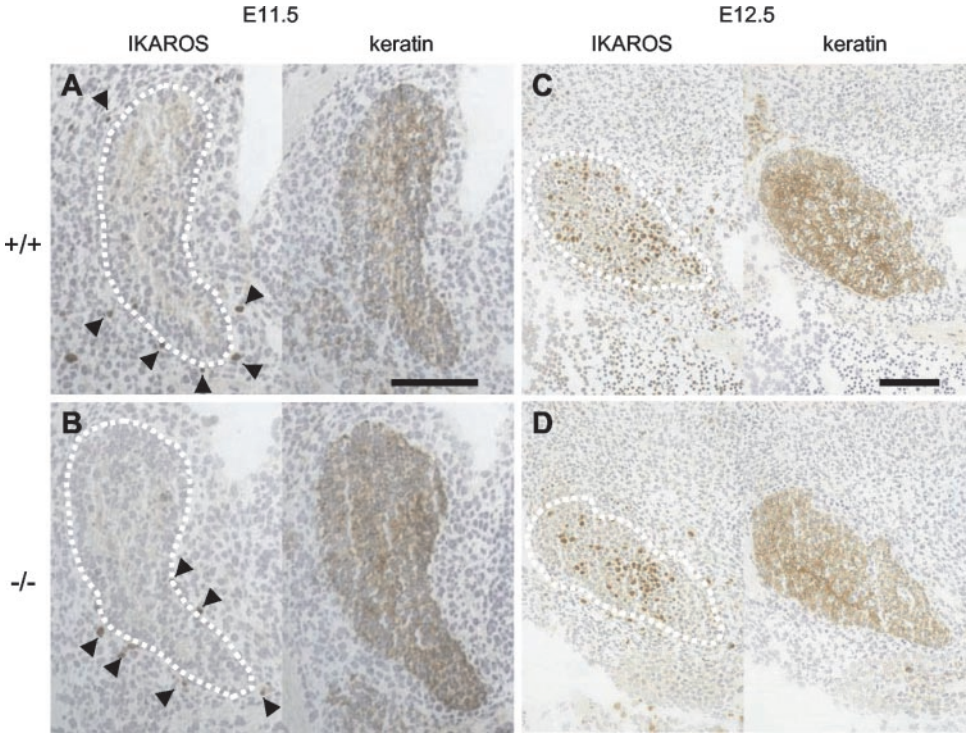

FIGURE 1. Initial migration of hematopoietic progenitor cells into the thymus anlage during ontogeny in CXCL12$^{-/-}$ mice on a C57BL/6 background. Serial frozen sections of wild-type (*A* and *C*) or mutant (*B* and *D*) embryos at E11.5 (*A* and *B*) or E12.5 (*C* and *D*) were stained with anti-IKAROS or anti-keratin Abs. Thymic epithelial cells are keratin^{+}. The white dotted lines indicate the border between the epithelial region and mesenchymal layer. IKAROS^{+} cells are indicated by arrowheads (*A* and *B*). Bars in *A* and *C* are 100 μm.

analyzed (data not shown). These results strongly suggest that CXCL12 and CXCR4 are required for the expansion of T cell precursors in fetal thymus. Flow cytometric analysis using anti-murine CXCR4 mAb has revealed that CXCR4 was strongly expressed in murine fetal thymocytes including TN CD44^{+}CD25^{-}, TN CD44^{+}CD25^{+}, TN CD44^{-}CD25^{+}, TN CD44^{-}CD25^{-}, and DP cells (Fig. 2*B*). These results are consistent with the previous studies using adult thymocytes that demonstrated that CXCR4 is expressed on TN and DP thymocytes (17–19). In addition, the study using the mice in which the *GFP* gene is knocked into the CXCL12 locus revealed that CXCL12 was expressed in spindle-shaped stromal cells, presumably thymic epithelial cells, ubiquitously distributed in fetal thymus (Fig. 2*C*). These results are also consistent with the previous study using juvenile human thymus (23). Finally, we examined the T lymphocytes in peripheral blood from wild-type or mutant embryos. Although the numbers of Gr-1^{+} myeloid cells were increased in peripheral blood from CXCR4$^{-/-}$ embryos (data not shown) (13), neither DP nor single-positive (SP) T cells were observed in peripheral blood from E18.5 wild-type or mutant embryos (data not shown).

The CXCR4 mutation affects the expansion of T cell precursors in adult thymi in the competitive reconstitution experiment

Previous studies have revealed that mice long-term reconstituted with CXCR4-deficient fetal liver cells or bone marrow cells have reduced donor-derived thymocytes (14, 15). However, the possibility that the phenotype in the thymi in these mutant chimeras may be caused by the defects in multipotential hematopoietic progenitors in bone marrow cannot be excluded, because the reductions in donor-derived thymocytes are in line with the reductions in donor-derived myeloid lineage cells in bone marrow (14, 15).

To further study the defect caused by CXCR4 mutation in adult thymus, we determined the capacity of CXCR4$^{-/-}$ hematopoietic cells to compete with normal bone marrow cells for the long-term lymphoid and myeloid reconstitution. Ly5.2^{+} fetal liver cells (CXCR4$^{+/-}$ or CXCR4$^{-/-}$) were mixed with or without Ly5.1^{+} wild-type bone marrow cells and transplanted into lethally irradiated normal Ly5.1^{+} wild-type recipients (test/competitor cell ratios of 10:1). At 16 wk after transplantation, the numbers of donor-derived thymocytes were decreased (~4-fold) in CXCR4$^{-/-}$ chimeric mice without competitors compared with CXCR4$^{+/-}$ chimeras, but the reductions were in line with the reductions in donor-derived Gr-1^{+} myeloid lineage cells (~4-fold) as shown previously (Fig. 3) (14). In contrast, CXCR4$^{-/-}$ chimeric mice with wild-type competitors displayed markedly reduced donor-derived thymocytes, including TN CD25^{+}c-*kit*$^{+}$ cells, which are thought to be the early T lineage committed precursor population, TN CD44^{-}CD25^{+}, DP, and SP thymocytes, compared with competitively repopulated CXCR4$^{+/-}$ chimeras (Fig. 3). Donor-derived DP thymocytes were reduced by ~80-fold, although donor-derived Gr-1^{+} cells were reduced by only ~5-fold in CXCR4$^{-/-}$ chimeras. Thus, the reduction in donor-derived thymocytes was more severe than the reduction in donor-derived myeloid lineage cells in the CXCR4$^{-/-}$ chimeras with competitors, suggesting that CXCR4 plays a role in the development of T cell precursors in adult thymi in vivo.

Enforced expression of bcl-2 does not rescue impaired T cell or B cell development in CXCR4$^{-/-}$ embryos or CXCR4$^{-/-}$ radiation chimeras

Next, to elucidate the molecular mechanism that is responsible for the defects of T cell development caused by CXCL12 or CXCR4 mutation, we analyzed whether bcl-2, an inhibitor of apoptosis, is responsible for the defect caused by CXCR4 mutation in T cell development. Recently, it has been reported that CXCL12 increases the viability of T cell precursors, probably because of an up-regulation of bcl-2 expression and down-regulation of bax expression (23). First, CXCR4$^{+/-}$ mice were crossed with H2K-bcl-2 transgenic mice that express a human bcl-2 cDNA constitutively in all hematopoietic cells (24, 30). Resultant progeny (bcl-2/CXCR4$^{+/-}$) were backcrossed with CXCR4$^{+/-}$ mice. Lymphocyte development in fetal livers and thymi from litters, including CXCR4$^{+/-}$, bcl-2/CXCR4$^{+/-}$, CXCR4$^{-/-}$, and bcl-2/CXCR4$^{-/-}$ embryos was analyzed by flow cytometry. There was no significant difference in the numbers of

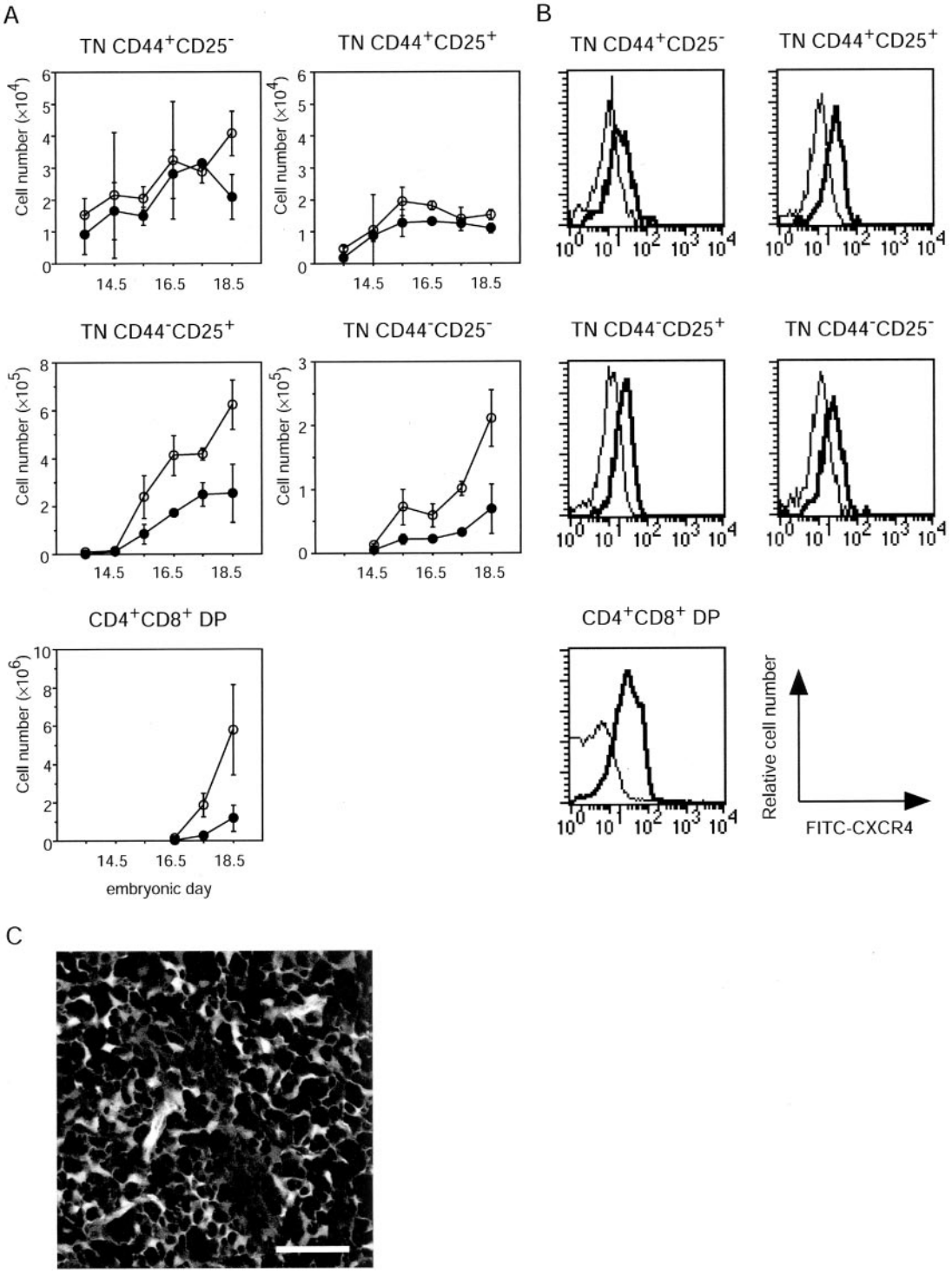

FIGURE 2. *A*, The numbers of T cell precursors in CXCL12$^{-/-}$ mice on a C57BL/6 background during embryogenesis. The numbers of T cell precursors, including CD3$^-$CD4$^-$CD8$^-$ (TN) CD44$^+$CD25$^-$, TN CD44$^+$CD25$^+$, TN CD44$^-$CD25$^+$, TN CD44$^-$CD25$^-$, and CD4$^+$CD8$^+$ (DP) cells found in wild-type (○) and CXCL12$^{-/-}$ (●) thymi are represented at different ages of embryogenesis. *B*, Expression of CXCR4 in murine fetal thymocytes. TN thymocytes were prepared from E14.5 thymi, and DP thymocytes were prepared from E18.5 thymi. Bold lines represent the expression of CXCR4, and thin lines indicate negative staining with isotype-matched control IgG. *C*, The expression of CXCL12 in the E14.5 fetal thymus from the mice in which the *GFP* gene is knocked into the CXCL12 locus. Bar is 50 μm.

B220$^+$CD19$^+$ B cell precursors in fetal liver and DP thymocytes in thymus between E16.5 CXCR4$^{-/-}$ and bcl-2/CXCR4$^{-/-}$ embryos. Thus, overexpression of bcl-2 cannot rescue impaired B cell and T cell development in CXCR4-deficient embryos (Fig. 4*A*).

Second, Ly5.2$^+$ fetal liver cells (CXCR4$^{+/-}$, bcl-2/CXCR4$^{+/-}$, CXCR4$^{-/-}$, or bcl-2/CXCR4$^{-/-}$) were mixed with Ly5.1$^+$ wild-type bone marrow cells and transplanted into lethally irradiated normal Ly5.1$^+$ wild-type recipients to make radiation chimeras. In bcl-2/CXCR4$^{-/-}$ chimeric mice, the proportion of donor (Ly5.2$^+$)-derived thymocytes relative to competitor (Ly5.1$^+$)-derived thymocytes (Ly5.2:Ly5.1 ratio) in thymus was increased (~30-fold) compared with that in CXCR4$^{-/-}$ chimeras (Fig. 4*B*). However, the

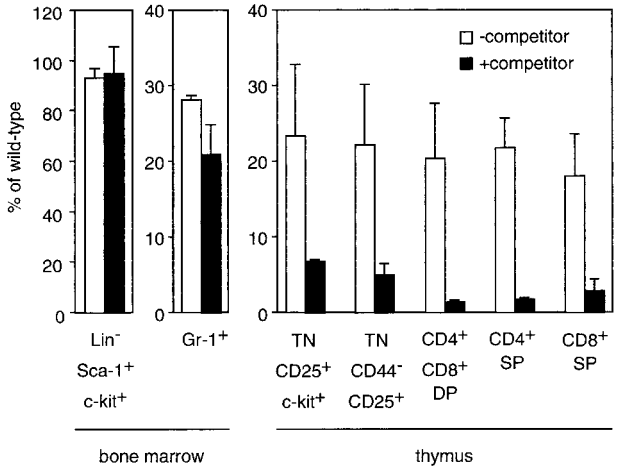

FIGURE 3. A progressive reduction in donor-derived T lymphocytes in adult thymi from competitively repopulated CXCR4$^{-/-}$ chimeras. Ly5.2^{+} fetal liver cells (CXCR4$^{+/-}$ or CXCR4$^{-/-}$) were mixed with or without Ly5.1^{+} wild-type bone marrow cells and transplanted into lethally irradiated normal Ly5.1^{+} wild-type recipients (test/competitor cell ratios of 10:1). At 16 wk after transplantation, recipient mice were analyzed for hematopoietic contribution to bone marrow cells and thymocytes by flow cytometric analysis. The proportion of the numbers of donor-derived (Ly5.2^{+}) cells from CXCR4$^{-/-}$ chimeras relative to CXCR4$^{+/-}$ chimeras is shown. □, Chimeras without competitors; ■, chimeras with competitors.

Ly5.2:Ly5.1 ratio for thymocytes in bcl-2/CXCR4$^{+/-}$ chimeras were also increased (~70-fold) in thymus compared with that in CXCR4$^{+/-}$ chimeras, and the increase in bcl-2/CXCR4$^{+/-}$ chimeras compared with CXCR4$^{+/-}$ chimeras was stronger than that in bcl-2/CXCR4$^{-/-}$ chimeras (Fig. 4*B*). Thus, enforced expression of bcl-2 does not rescue the impaired T cell reconstitution in competitively repopulated CXCR4$^{-/-}$ chimeras.

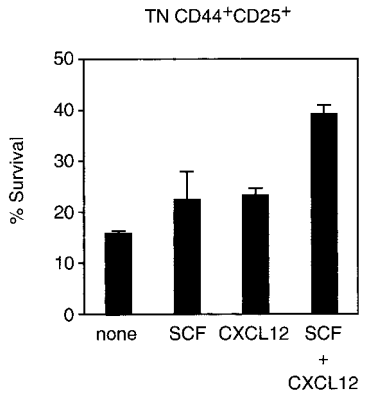

FIGURE 5. Survival-promoting effects of CXCL12 in combination with SCF on T cell precursors in liquid culture. The sorted TN CD44^{+}CD25^{+} T cell precursors were cultured with the indicated cytokines, and the numbers of surviving cells were measured at 72 h.

CXCL12 exhibited migratory- and survival-promoting effects on murine T cell precursors

To understand the mechanism by which CXCL12 functions in T cell development, we analyzed cellular functions of CXCL12 on T cell precursors. It has been shown that CXCL12 stimulated the proliferation of human T cell precursors in the presence of IL-7 (23). We sorted murine T cell precursors TN CD44^{+}CD25^{-} and TN CD44^{+}CD25^{+} cells in adult thymus and analyzed migratory- and survival-promoting effects of CXCL12 on these cells. In a transwell migration assay, CXCL12 exhibited migratory effects on TN CD44^{+}CD25^{-} and TN CD44^{+}CD25^{+} cells (data not shown) as shown previously (16, 22). Next, the sorted T cell precursors were cultured in the presence of SCF, and the numbers of surviving cells were measured at 72 h. CXCL12 exhibited survival-promoting effects on TN CD44^{+}CD25^{+} cells in the presence of SCF, but not on TN CD44^{+}CD25^{-} cells (Fig. 5, data not shown).

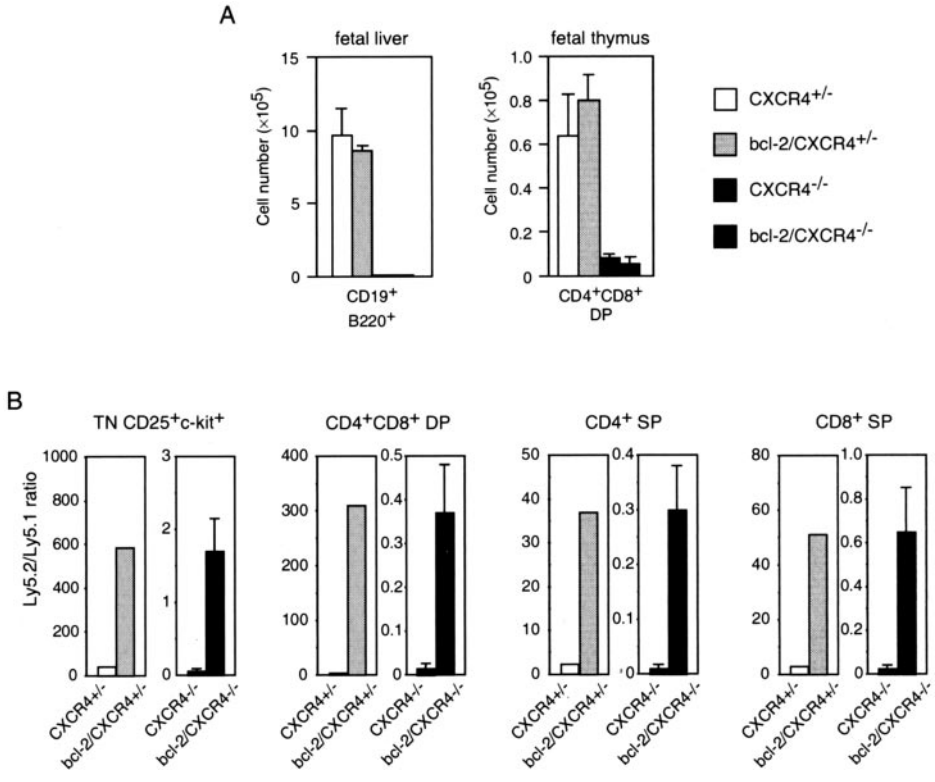

FIGURE 4. Enforced expression of bcl-2 does not rescue impaired T cell development in CXCR4$^{-/-}$ embryos or CXCR4$^{-/-}$ radiation chimeras. *A*, Enforced expression of bcl-2 does not rescue B cell and T cell development in CXCR4$^{-/-}$ mice. The numbers of B220^{+}CD19^{+} cells in fetal liver or CD4^{+}CD8^{+} DP cells in E16.5 fetal thymus are shown. *B*, Ly5.2^{+} fetal liver cells (CXCR4$^{+/-}$, bcl-2/CXCR4$^{+/-}$, CXCR4$^{-/-}$, or bcl-2/CXCR4$^{-/-}$) were mixed with Ly5.1^{+} wild-type bone marrow cells and transplanted into lethally irradiated normal Ly5.1^{+} wild-type recipients to make radiation chimeras. At 16 wk after transplantation, recipient mice were analyzed for hematopoietic contribution to bone marrow cells and thymocytes by flow cytometric analysis. The proportion of donor (Ly5.2^{+})-derived cells relative to competitor (Ly5.1^{+})-derived cells (Ly5.2:Ly5.1 ratio) is shown.

Discussion

Although it has been shown previously that CXCL12 and CXCR4 are responsible for fetal and adult B cell development (1, 3, 7–10, 12), their roles in T cell development are controversial. In this study we genetically analyzed further the functions of CXCL12 and CXCR4 in T cell development using mutant and chimeric mice. In fetal thymi, the expansion of TN $CD44^{-}CD25^{+}$, TN $CD44^{-}CD25^{-}$, and DP cells was impaired in $CXCL12^{-/-}$ or $CXCR4^{-/-}$ embryos on a C57BL/6 background. In contrast, the development of lineage Ag-negative (Lin^{-}) primitive unipotential T cell precursors in fetal liver (31) was relatively normal in $CXCL12^{-/-}$ embryos (12). In addition, accumulation of T cell progenitors in the outer mesenchymal layer of the thymus anlage during initial colonization of the fetal thymus in the E11.5 mutants was comparable with that seen in wild-type embryos. Thus CXCL12 and CXCR4 may play a role in later stages of T cell development in fetal thymus, although these molecules are essential for the earliest stages of B cell development in fetal liver. We note that our results do not exclude the possibility that cell targets for CXCL12 are more primitive T cell precursors including TN $CD44^{+}CD25^{-}$ cells or TN $CD44^{+}CD25^{+}$ cells, which are important in generating more mature thymocytes. In addition, the defects in thymocyte development are much more modest than the defects in B cell development in $CXCL12^{-/-}$ or $CXCR4^{-/-}$ embryos (Fig. 2*A*) (12). Other chemokines may compensate part of the defects in T cell development in the mutants (22).

In the mice long-term reconstituted with CXCR4-deficient fetal liver cells, the numbers of donor-derived thymocytes have been reduced, and their reductions are in line with the reductions of donor-derived myeloid lineage cells in bone marrow (Fig. 3) (14). In contrast, the reconstitution of donor-derived DP thymocytes with $CXCR4^{-/-}$ fetal liver cells was more severely affected (~80-fold) than that of donor-derived myeloid lineage cells (~5-fold) when competed against wild-type bone marrow cells at a ratio of 10:1, suggesting that CXCR4 is involved in the expansion of thymocytes in adult thymus in vivo. Although the mice in which the functions of CXCR4 were suppressed by the expression of CXCL12-intrakine revealed impaired T cell maturation into SP T cells in adult thymi (15), $CD4^{+}$ and $CD8^{+}$ SP T cells developed normally in the thymus from $CXCR4^{-/-}$ chimeric mice. Together, our results suggest that CXCR4 plays a role in the expansion of T cell precursors in both fetal and adult thymus in vivo. This is consistent with the results seen in the in vitro system using chimeric human-mouse FTOC seeded with $CD34^{+}$ juvenile thymic precursors and treated with neutralizing Abs against CXCL12 or CXCR4 where CXCL12 was involved in survival and expansion of T cell precursors but not in their migration into the thymus (23). It has been shown that CXCL12/CXCR4-mediated signaling promoted the survival or proliferation of hematopoietic cells, including T lymphocytes (3, 12, 23, 32–36), and CXCL12 has been shown to stimulate the prolonged activation of proteins, including protein kinase B and extracellular signal-related kinase 2, that are implicated in cell survival and proliferation in T lymphocytes (37). Thus, CXCL12 and CXCR4 may ensure proper survival or proliferation of T cell precursors during development. However, considering that CXCL12 has shown low survival-promoting activity but high chemotactic activity on the immature thymocytes (Fig. 5, data not shown) (16–18, 20–22), the major role of CXCL12 in T cell development might be attracting and tethering thymocytes in a thymic microenvironment in the vicinity of the CXCL12-expressing stromal cells (Fig. 2*C*) where early T cell precursors receive the antiapoptotic or proliferative signal from other cytokines such as SCF or IL-7.

It has been reported that CXCL12 increased the viability of human early T cell precursors, up-regulating the expression of bcl-2 (23). However, overexpression of bcl-2 did not rescue the defects in the number of DP thymocytes caused by the deletion of the *CXCR4* gene during embryogenesis or the defects in the numbers of donor-derived thymocytes in radiation chimeras competitively repopulated with $CXCR4^{-/-}$ fetal liver cells. Thus, a distinct signaling pathway, insensitive to bcl-2 overexpression, may be affected in the CXCR4-deficient T cell precursors. Because IL-7 induced high level expression of bcl-2 in T cell precursors (38) and bcl-2 transgene rescued impaired T cell development in IL-$7R\alpha^{-/-}$ or IL-$7R\gamma^{-/-}$ mice (39, 40), it is less likely that CXCL12 may work upstream of IL-7R signaling.

Together, this study has shown that CXCL12 and its receptor CXCR4 were involved in regulating fetal and adult T cell development in vivo.

Acknowledgments

We thank Dr. H. Kawamoto (Kyoto University, Kyoto, Japan) for helpful comments. We also thank J. Yoshida, K. Matsumoto, and E. Yoshimura for technical assistance and Drs. J. Domen and I. L. Weissman for H2K-bcl-2 transgenic mice.

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