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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.
Recently, the in vitro system using chimeric human-mouse fetal thymus organ culture (FTOC) seeded with CD34−/CD4−/CD8−/CD25−/bcl-2/CXCR4−/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/-/
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. 2B). 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. 2C). 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 thymus 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 thymus in these mutant chimeras may be caused by the defects in multipotent 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 thymus 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
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. 4A).

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. 4B). However, the
Ly5.2:Ly5.1 ratio for thymocytes in bcl-2/CXCR4+/−/H11001/H11002 chimeras were also increased (~70-fold) in thymus compared with that in CXCR4+/−/H11001/H11002 chimeras, and the increase in bcl-2/CXCR4+/−/H11001/H11002 chimeras compared with CXCR4+/−/H11001/H11002 chimeras was stronger than that in bcl-2/CXCR4+/−/H11002 chimeras (Fig. 4B). Thus, enforced expression of bcl-2 does not rescue the impaired T cell reconstitution in competitively repopulated CXCR4+/−/H11001/H11002 chimeras.

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+/−/H11001 CD25+ and TN CD44+/−/H11001 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+/−/H11001 CD25+ and TN CD44+/−/H11001 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+/−/H11001 CD25+ cells in the presence of SCF, but not on TN CD44+/−/H11001 CD25− cells (Fig. 5, data not 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 thymus, the expansion of TN CD44⁺CD25⁺, TN CD44⁺CD25⁻, and DP cells was impaired in CXCL12⁻/⁻ or CXCR4⁻/⁻ embryos on a C57Bl/6J 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 more modest than the defects in B cell development in CXCL12⁻/⁻ or CXCR4⁻/⁻ embryos (Fig. 2A) (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-intrakinase revealed impaired T cell maturation into SP T cells in adult thymus (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 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. 2C) 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-7Rα⁻/⁻ or IL-7Rγc⁻/⁻ 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.

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