Pertussis Toxin-Sensitive Signal Controls the Trafficking of Thymocytes Across the Corticomedullary Junction in the Thymus

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We investigated a role of chemokines in thymocyte trafficking. Genes encoding stromal cell-derived factor-1 and its receptor CXCR4 were detected in the cortex by in situ hybridization. Early immigrant cells did not express CXCR4, whereas their descendant CD44-CD25+CD4-CD8- cells did. CXCR4 expression was down-modulated when CD4+CD8- double-positive cells became CD4+CD8+ or CD4-CD8+ single-positive (SP) cells. Positively selected CD69+CD3intermediate cells gained CXCR4, of which ligand, thymus activation-regulated chemokine, was expressed in the medulla. At the next developmental stage, CD69-CD3high cells lost CCR4 but gained CCR7. These results suggest that thymocytes use different chemokines along with their development. Blockade of chemokine receptor-mediated signaling by pertussis toxin perturbed the normal distribution of SP cells and resulted in the accumulation of SP cells in the cortex. Thus, a pertussis toxin-sensitive event controls the trafficking of SP cells across the corticomedullary junction. The Journal of Immunology, 1999, 162: 5981–5985.

Lymphocyte homing and recirculation comprise the physiological processes by which lymphocytes seek out and localize to particular tissues and specific microenvironments. The interaction of lymphocyte integrins with their counter-receptors on high endothelial venules are essential steps for facilitating lymphocyte extravasation (1, 2). Accumulating evidence has demonstrated that lymphocyte homing and recirculation are controlled by chemokines expressed in particular tissues (3–6). Chemokines induce the directional movement of leukocytes along a chemokine gradient and also control cell adhesion by activating adhesion molecules (7–10). However, it remains to be elucidated whether chemokines also control the distribution of lymphocytes within lymphoid tissues.

The thymus is anatomically divided into three regions, the subcapsular region, cortex and medulla. T cell development proceeds sequentially in the particular regions of the thymus. In mice, progenitors have been shown to enter the thymus at the corticomedullary junction through large venules in the medulla (11). Then, progenitors find their niche at the subcapsular region to proliferate there (11). After proliferation, they differentiate into DP cells that reside in the cortex of the thymus. Most DP cells die in the cortex, and only a small fraction is allowed to mature into CD4+8+ or CD4-8+ SP cells and migrate into the medulla. Mechanisms that control the trafficking or topological distribution of thymocytes within the thymus have not been elucidated.

Recently, a new category of chemokines has been found, which are constitutively expressed in lymphoid organs (3, 12–16). SDF-1 is known as pre-B cell-stimulating factor (17), but its mRNA was abundantly expressed in fetal and adult thymuses as well (18). CXCR4 is a unique receptor for SDF-1 and is known as a coreceptor for T-tropic HIV-1 entry (19). CXCR4 is expressed not only on mature T cells but also on thymocytes (18, 20). Thus, it has been speculated that SDF-1 and CXCR4 have a role in T cell development in the thymus. In the previous study, we demonstrated that most DP cells expressed CXCR4 and that the levels of CXCR4 were down-modulated after positive selection in the thymus (18). To further clarify this issue, we investigated the expression of mRNAs encoding SDF-1 and CXCR4 in the thymus by in situ hybridization. Moreover, the expression of other chemokine receptors in thymocyte subsets was investigated by RT-PCR. Finally, we investigated the effect of pertussis toxin (PT), an inhibitor of chemokine signaling, on the distribution of thymocytes by confocal fluorescence microscopy. The present result indicates that chemokines control the topological distribution and/or trafficking of thymocytes within the thymus.

Materials and Methods

Mice
C57BL/6 (B6), BALB/c, and (B6 × C3H)F1 mice were purchased from SLC, Shizuoka, Japan. The original founders of β2-microglobulin knock-out mice were obtained from M. Taniguchi, Chiba University, Chiba, Japan, and the mice were bred in our colony at the National Institute of Radiological Sciences, Chiba, Japan. In some experiments, mice received 15 μg of PT (Kaken Pharmaceutical, Shiga, Japan) i.p. All experiments

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1 Abbreviations used in this paper: SDF-1, stromal cell-derived factor-1; DN, double negative; DP, double positive; ELC, EB11 ligand chemokine; PT, pertussis toxin; SP, single positive; SLEC, secondary lymphoid tissue chemokine; TARC, thymus and activation-regulated chemokine; rh, recombinant human; DIG, digoxigenin; MACS, magnetic cell separation system.
were approved by and followed the instructions of the Animal Use-Committee of the National Institute of Radiological Sciences and the Animal Studies Committee of Hokkaido University School of Medicine.

**Chemotaxis assay**

In the in vitro migration of cells in response to recombinant human SDF-1β/pre-B cell stimulating factor (R&D Systems, Minneapolis, MN) was assessed in 5-μm pore size Transwell plates (Corning Costar, Cambridge, MA). In brief, 3.5 × 10⁶ thymocytes in 100 μl of FCS-free α-MEM were added to upper chambers. To observe chemotaxis, rhSDF-1β was added to lower chambers containing 600 μl of 1% FCS-containing α-MEM. To observe chemokinesis, rhSDF-1β was added to upper chambers and incubated at 4°C for 4 h. The sections were then incubated with 0.2 M HCl for 10 min, treated with 0.25% acetic anhydride in 0.1 M triethanolamine, and gradually dehydrated in ethanol. For immunofluorescence staining, frozen sections of thymus were fixed with diethyl ether. Perfusion fixation was performed in vivo via the left ventricle, and blood was withdrawn from the right atrium. Perfusion media consisted of physiological saline followed by ice-cold 4% paraformaldehyde in 0.02 M sodium phosphate buffer, pH 7.3. Tissues were harvested, dissected, incubated in the same fixative overnight, and embedded in paraffin blocks. Tissue sections (6 μm) on silane-coated glass slides were deparaffinized with xylene and then gradually hydrated in ethanol. After washing with 0.1 M phosphate buffer, pH 7.4, tissue sections were treated for 30 min at 37°C with 10 μg/ml proteinase K (in 10 mM Tris-HCl, 1 mM EDTA) and then deparaffinized with xylene and then gradually hydrated in ethanol. The sections were then incubated with 0.2 M HCl for 10 min, treated with 0.25% acetic anhydride in 0.1 M triethanolamine, and gradually dehydrated in ethanol.

**Preparation of cRNA probe for in situ hybridization**

The cDNA clones encoding mSDF-1α and mCXCR4 have been reported (18, 21). PstI fragment of SDF-1α cDNA (DBJ accession no. D43804; PstI fragment; 50–512) and BamHI fragment of CXCR4 cDNA (DBJ accession no. AB000803; BamHI fragment; 1–558) were subcloned into pBluescript II SK(+) vector (Stratagene, La Jolla, CA). The direction of each fragment was determined by restriction enzyme digestion and direct sequencing. For construction of the SDF-1α cRNA probe, SDF-1α cDNA-inserted plasmid was linearized with EcoRI or XbaI (for antisense and sense, respectively) and transcribed with T3 and T7 RNA polymerase in the presence of DIG-labeled UTP. For the CXCR4 cRNA probe, CXCR4 cDNA-inserted plasmid was linearized by restriction digestion with XbaI or EcoRI (for antisense and sense, respectively) and transcribed with T3 and T7 RNA polymerase.

**In situ hybridization**

In situ hybridization was performed as previously described (22, 23). In brief, hybridization was conducted for 20 h at 50°C using DIG-labeled cRNA probe in hybridization buffer (50% formamide, 0.75 M NaCl, 10 mM PIPES (pH 6.8), 1 mM EDTA, 100 μg/ml RNA, 0.05% heparin, 0.1% BSA, and 1% SDS). The sections were subsequently washed with 2× SSC, 50% formamide for 30 min at 60°C and treated with 10 μg/ml RNase A. After elimination of the remaining RNAase A, the slides were treated with anti-DIG Ab (×1000) following the manufacturer’s protocol (Boehringer Mannheim, Mannheim, Germany). Each slide was then examined by three experienced pathologists. Some of the slides were immunohistochemically investigated using specific Ab to define the cell character.

**Chemokine-Ig fusion proteins**

A fusion gene, pSDF-1α/Cysteine-rich Modular Immunoglobulin Superfamily, was constructed between murine SDF-1α cDNA and genomic gene coding for the human constant region (18, 24). The fusion gene in BCMGSneo vector was transfected into Ag8.653 B cell tumors, and stable transfectants were cloned by G418 selection and limiting dilution.

**Flow cytometry analysis and cell sorting**

To purify CD4⁺ CD8⁻ cells, thymocytes were reacted with both Dynabeads mouse CD4 (L3T4) and Dynabeads mouse CD8 (Ly2) (Dynal, Oslo, Norway) and negatively separated by MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). For CXCR4 staining, cells were pretreated with 10% normal goat serum. Then, cells were reacted with Ag8.653 supernatant or SDF-1α-containing supernatant at 4°C for 30 min in PBS containing 0.1% sodium azide and 1% FCS, washed once, and further reacted with FITC-anti-human IgG goat (Fab)₂ Ab (Cappel, Aurora, OH) for 20 min. In some experiments, biotin-anti-human IgG goat Ab and APC-streptavidin (PharMingen) were used instead of the FITC-anti-human IgG. After washing, cells were further reacted with normal human serum to saturate human IgG activity, and the following combinations of mAbs: PE-anti-Cd4 and Cy-Chrome-anti-Cd8; biotin-anti-CD25 and Cy-Chrome-anti-Cd44 followed by PE-streptavidin (Biomeda, Foster City, CA); FITC-anti-NK1.1 and Cy-Chrome-anti-TCRβ; FITC-anti-NK1.1 and PE-anti-TCRγδ. Cells were analyzed by FACSCalibur (Becton Dickinson, Mountain View, CA). mAbs coupled with fluorescent reagents were from PharMingen.

For cell sorting, whole thymocytes were stained with PE-anti-CD4 and FITC-anti-CD8, and DP and CD4⁻²SP cells were sorted by FACSStar (Becton Dickinson). To sort CD8 SP cells, CD4⁺ cells were depleted by MACs sorting, and residual thymocytes were stained with FITC-anti-CD8 and PE-anti-CD4. To sort CD4⁺ CD8⁻⁴CD3 cells, CD4⁺ and CD8⁻ cells were depleted by MACs sorting. Residual cells were stained with PE-anti-CD3e and FITC-anti-NK1.1, and CD4⁺ NK1.1⁺ cells were sorted. To sort CD69⁺CD4⁻Low intermediate, CD69⁺CD8⁻High, and CD69⁺CD8⁺ high cells, whole thymocytes were stained with PE-anti-CD69 (PharMingen) and biotin-anti-Cd3e (PharMingen) followed by fluorescein-avidin DCS (Vector). Cell sorting was repeated once to improve cell purity if necessary. The purity of the sorted cells was more than 99% in all thymocyte subsets.

**Results and Discussion**

**SDF-1 and CXCR4 mRNAs are expressed in the cortex region of murine thymus**

SDF-1 and CXCR4 mRNAs were abundantly expressed in fetal and adult thymus (18). In situ hybridization was used to localize the place of gene expression in the thymus (Fig. 1). CXCR4 mRNA was predominantly detected in the cortex of the thymus, indicating that cortical immature thymocytes bore CXCR4 receptors. SDF-1 mRNA was also detected in the cortex, and especially in the subcapsular region. This, thus indicates that SDF-1 may have a role in the chemotraction of thymocytes in the cortex but not in the medulla.

**DN and DP thymocytes are chemoattracted by rhSDF-1β**

SDF-1 showed strong chemotaxis activity on pre-B cells (17), CD34⁺ hematopoietic precursor cells (25), and mature T cells (24, 26). To investigate the functional expression of CXCR4 on thymocytes, a cell migration test was performed in vitro. As shown in Fig. 2A, murine thymocytes migrated dose dependently in response to rhSDF-1β. This migration was chemotaxis but not chemokinesis, because the addition of rhSDF-1β in the upper chamber failed to induce cell migration. In agreement with the result of in situ hybridization, migrated thymocytes were CD4⁻ CD8⁻ DN and DP cells (Fig. 2B). A chemotaxis assay showed that this was not due to any killing of SP thymocytes by rhSDF-1β; SP cells did not
die in suspension culture at 37°C for 4 h in rhSDF-1β- and 1% FCS-containing medium (data not shown). Thus, rhSDF-1β predominantly induced the chemotaxis of DN and DP thymocytes. A similar finding was recently reported by others (27).

**CD25**DN thymocytes start to express CXCR4

In the previous study, we found that DN thymocytes in fetal thymus expressed low levels of CXCR4 by flow cytometry using SDF-1-Ig fusion protein (18). The migratory path of progenitor thymocytes might be different between fetus and adult mice. Therefore, we investigated where CXCR4 was expressed on immature thymocytes in adult mice. The CD44\(^{+}\)CD25\(^{-}\)DN subset contains progenitor thymocytes and NK T cells (28, 29). Since the latter cells were CXCR4\(^{+}\) (data not shown), we investigated the CXCR4 expression on CD44\(^{-}\)CD25\(^{-}\)DN thymocytes in β₂-microglobulin KO mice that were devoid of NK1.1\(^{+}\) DN cells (30).

As shown in Fig. 3A, early immigrant cells, CD44\(^{-}\)CD25\(^{-}\)lo DN cells, were CXCR4\(^{+}\). Their descendant CD44\(^{-}\)CD25\(^{-}\)hi DN cells were CXCR4\(^{+}\). We confirmed that CD44\(^{-}\)CD25\(^{-}\)NK1.1\(^{+}\)DN cells from B6 mice were CXCR4\(^{+}\) (data not shown). Since early immigrant CD44\(^{-}\)CD25\(^{-}\)DN cells are CXCR4\(^{+}\), it is suggested that they do not use SDF-1 for immigration into the thymus. This notion is consistent with the successful early thymopoiesis in SDF-1- and CXCR4-deficient mice (31, 32). Rather, this suggests that SDF-1 may be important for intrathymic trafficking of CD44\(^{-}\)CD25\(^{-}\)DN cells to the subcapsular region where SDF-1 mRNA is abundantly expressed (Fig. 1).

**CCR4 is expressed in CD69\(^{+}\)CD3\(^{intermediate}\) cells while CCR7 is expressed in CD69\(^{-}\)CD3\(^{high}\) SP cells**

In situ hybridization demonstrated that the levels of CXCR4 mRNA decreased in the medulla (Fig. 1). In agreement with this, CXCR4 expression decreased on CD4 and CD8 SP thymocytes (Fig. 3B). Thus, CXCR4 is down-modulated in SP cells, at least in part, by transcriptional regulation. Assuming that chemokines are essential for thymocyte trafficking, the medulla must express chemokines other than SDF-1 and SP cells must express receptors for these chemokines. Several chemokines besides SDF-1 are constitutively expressed in the thymus. Thymus-expressed chemokine (15) and TARC (13) were expressed in the medulla. ELC and pulmonary and activation-regulated chemokine were also expressed in human thymus (14).

We investigated the gene expression of CCR4, CCR7, and CXCR4 in thymocyte subpopulations by RT-PCR. The CXCR4 gene was expressed in DN and DP cells. The CCR4 gene encoding
CD69 is an activation marker that is up-regulated on positive selection in DP cells, and CD3 intermediate(high) cells but not in CD69 intermediate SP cells. In contrast, levels of CCR7 gene expression increased along with the development and CD69 intermediate(high) SP cells expressed at the highest level. In the previous study, we demonstrated that the down-modulation of CXCR4 occurred at the CD69 intermediate maturational stage (18). It is of interest that TARC mRNA is expressed in interdigitating cells in the medulla near the corticomedullary junction (T. Imai, Shionogi Science Institute, Osaka, Japan, personal communication). Since the positively selected DP cells in the cortex are ready to emigrate to the medulla, the present result suggests that cortical chemokine SDF-1 and medullary chemokine TARC may control the trafficking of nonselected and positively selected DP cells.

The CCR7 gene was expressed in CD69(high)CD3(intermediate) cells at low levels and in mature SP cells at high levels (Fig. 4). Kim et al. (27) also reported that CCR7 was predominantly expressed in murine CD4 SP cells. The ligands of CCR7 are ELC and SLC. It is not determined the type of ELC-producing cells in the thymus. In peripheral lymphoid tissues, SLC is expressed in high endothelial venules and controls the transmigration of lymphocytes across high endothelial venule (35). It is to be determined in future works whether ELC controls the emigration of SP cells from thymus.

In general, chemokines and chemokine receptor systems are highly redundant. One chemokine reacts with its unique receptor but sometimes also cross-reacts with other chemokine receptors (36, 37). Moreover, a subset of leukocytes expresses several different chemokine receptors at the same time (3, 37). In CD69(intermediate)(high) cells, CCR4 and CCR7 seemed to be coexpressed (Fig. 4). In human, CXCR4 and CCR5 are coexpressed on immature thymocytes, although CCR5 is expressed only marginally (38). Dairaghi et al. (39) also reported the CCR5 expression on immature thymocytes. T cell development is normal in SDF-1(−/−) and CXCR4(−/−) mice (31, 32). Thus, SDF-1 may not be the only chemokine that controls the trafficking of immature thymocytes in the cortex. Moreover, CXCR4 is not detected on early immigrant cells in the thymus, indicating that a chemokine other than SDF-1 is responsible for immigration of progenitor cells into the thymus. Further studies are needed to clarify the set of chemokine receptors expressed in these cells.

**PT perturbs thymocyte distribution within thymus**

An anatomical border exists between the cortex and medulla, which is called the corticomedullary junction where many interdigitating reticulums and macrophages harbor (40). It is believed that interdigitating cells present self Ags and induce apoptosis of self-reactive T cells at the corticomedullary junction. In addition, there must be a kind of gate at the corticomedullary junction through which only positively selected SP cells can pass. Since DP cells and SP cells expressed different set of chemokine receptors, and because the cortex and medulla produced different chemokines, we hypothesized that chemokines may control the trafficking across the corticomedullary junction. To test this assumption, we utilized PT to block trafficking.

Chemokine receptor is coupled with PT-sensitive G protein that is irreversibly inhibited by PT (41, 42). Therefore, PT can block the chemokine-mediated migration of cells irrespective of the redundancy of chemokines and chemokine receptor systems. PT

![FIGURE 4](attachment:image1.png)

**FIGURE 4.** CXCR4, CCR4, and CCR7 are expressed in different thymocyte subsets. Thymocytes were separated into CD3−CD4−CD8− NK1.1− (DN), CD4−CD8+ (DP), CD4+CD8+ (CD4), and CD4+CD8− (CD8) cells in A, and into CD69−CD3(intermediate) (1, 4, 7), CD69+CD3(high) (2, 5, 8) and CD69−CD3(high) (3, 6, 9) cells in B by FACS sorting. The purity of each thymocyte subset, and RT-PCR was performed using 12.5 ng template or 3-fold diluted template per tube. Representative results of two experiments.

![FIGURE 5](attachment:image2.png)

**FIGURE 5.** PT perturbs SP cell trafficking across the corticomedullary junction. The thymus was removed for immunohistochemistry 2.5 days after PT injection. Thymus sections from normal (A) and PT-treated (B) mice were double-stained with reagents specific for CD4 (Texas Red) and CD8 (FITC) and analyzed by confocal fluorescence microscopy. DP cells were stained greenish yellow, while CD4 and CD8 SP were stained red and green, respectively. In PT-treated mice, SP cells accumulated in the cortex. Representative results of four mice.
treatment in vivo decreased the thymus volume and reduced the number of thymocytes by 37% in 2.5 days in PT-treated mice. Immunohistochemical analysis of the thymus demonstrated that PT treatment decreased the cellularity of thymus especially in the cortex. Moreover, it perturbed the normal distribution of SP cells (Fig. 5). In control mice, DP and SP cells were restricted to the cortex and medulla, respectively, whereas in PT-treated mice, SP cells were not restricted to the medulla but were also seen in the cortex. Even after PT treatment, CD4- SP cells bore a decreased level of CXCR4 and a high level of CCR7 gene expression (data not shown). This result was consistent with the observation in lck-PT transgenic mice, in which the PT gene was expressed under the control of lck promoter (43). In those transgenic mice, SP cells accumulated in the thymus, but the exact site of SP cell accumulation, the corticomedullary junction or the medullary-venule junction, was not determined. In the PT-treated mice, positively selected SP cells (or their precursors) were unable to cross the corticomedullary junction and remained in the cortex (Fig. 5). Thus, it is suggested that a PT-sensitive process probably induced by chemokines is indispensable for trafficking across the corticomedullary junction.

Since SDF-1 was expressed in the subcapsular region (Fig. 1) and because CXCR4 was coupled with PT-sensitive G-protein (24), it was speculated that the decreased cellularity of thymus in PT-treated mice was due either to the decreased influx of DN cells into the subcapsular region or to the decreased outflow of DN cells from the subcapsular region. However, our assay system was not sensitive enough to assess the issue. Further studies on both the topological distribution of chemokines in the thymus and the regulation of chemokine receptor expression during development may help our understanding of the thymocyte trafficking within the thymus.

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