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CXCL12-CXCR4 Engagement Is Required for Migration of Cutaneous Dendritic Cells

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CCR7 is regarded as an essential chemokine receptor for cutaneous dendritic cell (DC) migration into the regional lymph nodes. However, complete migratory inhibition cannot be obtained in CCR7-deficient mice, suggesting that there exist other chemokine receptors involved in this process. Initially, we found that CXCR4 was highly expressed on migrated cutaneous DCs and that its ligand, CXCL12, was detected in the LYVE-1$^+$ lymphatic vessels in the skin. FITC-induced cutaneous DC migration into the draining lymph nodes was impaired by the specific CXCR4 antagonist 4-F-Benzoyl-TN14003. Among FITC$^+$ cells, Langerin$^+$ Langerhans cells and Langerin$^-$ (dermal) dDC subsets were detected as CD11c^{high+}CD11b^{int+} cells and CD11c^{high+}CD11b^{high+} plus CD11c^{low+}CD11b^{int+} cells, respectively, both of which were suppressed by CXCR4 antagonist. Moreover, *in vivo* contact hypersensitivity response was impaired by CXCR4 antagonist administered during the sensitization phase. The *in vitro* proliferative response to dinitrobenzene sulfonic acid of sensitized lymph node cells was inhibited by CXCR4 antagonist treatment. These findings demonstrated that CXCL12-CXCR4 engagement on cutaneous DCs plays a crucial role in the initiation of skin immune response by enhancing cutaneous DC migration. ***(Am J Pathol 2007, 171:1249–1257; DOI: 10.2353/ajpath.2007.070225)***

It is in the lymphoid organs that T lymphocytes and antigen-presenting cells such as dendritic cells (DCs) participate to generate adaptive immune responses.[1–3] There are two subsets of DCs in the skin, dermal DCs (dDCs) and epidermal Langerhans cells (LCs). The arrival of antigen-bearing DCs into lymph nodes from peripheral sites begins several hours after antigen exposure and reaches its peak for 1 to 3 days, depending on the type of antigen and DCs. However, the precise repertoire of signals that regulate these processes is not fully elucidated.[2–6] Recently, based on *in vitro* studies of chemotaxis and chemokine receptor expression[5,7,8] and *in vivo* studies using relevant rodent models, central roles for various chemokines and their receptors in DC migration have been identified.[2–6,9] Using human monocyte-derived DCs, it was reported that immature DCs express CCR1, CCR2, CCR5, and CXCR1 and that the induction of DC maturation by lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), or CD40L results in up-regulated expression of CCR7 and CXCR4.[8] CCR7 is a well-known chemokine receptor responsible for regulating DC function. CCR7 deficiency dramatically impairs migration of activated cutaneous DCs into draining lymph nodes 24 hours after fluorescein isothiocyanate (FITC) application, with profound morphological alterations in the architecture of secondary lymphoid organs.[10] However, it should be noted that this impairment of migration is incomplete. An another line of study using *plt* mice, which lack CCR7 ligands, has revealed that CCR7 ligand deficiency leads to an imperfect (approximately 70%) decrease in the number of FITC$^+$ migrated DCs in the draining lymph nodes.[11] These data have suggested that there should

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exist other chemokines/chemokine receptors responsible for cutaneous DCs migration into lymph nodes.

CXCR4 is a G-protein-coupled receptor expressed by a wide spectrum of cells. Its physiological importance in hematopoiesis and development of the vasculature and central nervous system has been emphasized by the lethal phenotype of its knockout mice. On the other hand, CXCR4 expression on monocyte-derived DCs is enhanced along with their activation, and DCs have chemotactic response to the CXCR4 ligand CXCL12 (stromal-cell derived factor-1) *in vitro*.[12] CXCR4 is also detected in human LCs, and its expression level is increased by granulocyte macrophage–colony-stimulating factor (GM-CSF).[13] Nevertheless, there is little knowledge about the function of CXCR4 in cutaneous DCs and its contribution to directional migration of DCs on skin inflammation *in vivo*. CXCL12 is expressed by murine stromal cells in the red pulp of spleen and the medulla of lymph nodes and by human skin endothelial cells.[5,14–16] CXCL12/CXCR4 interactions are largely unique and nonpromiscuous. In mice, CXCL12 or CXCR4 gene knockouts generate a similar phenotype, characterized by deficient B lymphopoiesis and myelopoiesis and abnormal neuronal and cardiovascular development.[17–19] Embryonic lethality associated with either CXCR4 or CXCL12 gene knockouts emphasizes the critical and unique role played by these gene products during development. This chemokine also plays a critical role in lymphocytic circulation and immune surveillance in the postnatal life. *In vitro*, CXCL12 has potent chemoattractant properties for cells expressing CXCR4, such as monocytes, lymphocytes, and $CD34^+$ hematopoietic stem cells.

In light of the emerging significance of various members of the chemokine system in DC biology, we tested the hypothesis that the chemokine receptor CXCR4 and its ligand CXCL12 influence cutaneous DC function and adaptive immune responses. We found that CXCR4 is highly expressed on activated cutaneous DCs and that CXCL12 is expressed in the lymphatic vessels of the skin. Mice treated with CXCR4 antagonist exhibited significantly impaired cutaneous DC migration and reduced contact hypersensitivity (CHS) response. These findings collectively provide evidence for an important role of CXCR4 in cutaneous DC functions.

Materials and Methods

Animals and Reagent

Female C57BL/6 (B6) mice at 8 weeks of age were purchased from Japan SLC (Hamamatsu, Japan). Mice were maintained on a 12-hour light/dark cycle under specific pathogen-free conditions. Protocols were approved by the Institutional Animal Care and Use Committee of the University of Occupational and Environmental Health.

For CXCR4 antagonist treatment, Alzet osmotic pumps (7-day duration, 0.5 μl per hour pumping rate; model 1007D; Durect Corporation, Cupertino, CA) were loaded with 40 mg/ml CXCR4 antagonist, 4F-benzoyl-TN14003,[20,21] in saline and were implanted subcutaneously to the back under intraperitoneal anesthesia according to the manufacturer's instructions. The administered dose was calculated to be 0.48 mg per kg body weight per day. No toxicity of CXCR4 antagonist was observed at 5 μmol/L *in vitro* as reported previously.[22] Moreover, the selectivity of the antagonist was confirmed by the finding that there was no significant inhibition against Ca^{2+} mobilization induced by MIP-1α stimulation through CCR5 (IC_{50} = 22 μmol/L) and against Ca^{2+} mobilization induced by sphingosine-1-phosphate stimulation through EDG3 ($IC_{50} > 30$ μmol/L) by the treatment of CXCR4 antagonist (data not shown). To characterize its specificity further, epidermal cell suspensions were applied to transwell for chemotaxis assay (see below for method). The chemotaxis of major histocompatibility complex (MHC) class II^+ LCs to CXCL12 was inhibited by CXCR4 antagonist, but such inhibitory effect was not observed toward CCR7 ligand, CXCL21 (data not shown).

Cell Preparation and Cultures

Complete RPMI (cRPMI), RPMI 1640 medium (Sigma, St. Louis, MO) containing 10% heat-inactivated fetal calf serum (Invitrogen, Carlsbad, CA), 5×10^{-5} mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 25 mmol/L HEPES (Cellgro, Herndon, VA), 1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin was used as culture medium. For depleting DCs, lymph node cells were dispersed and sorted to $CD11c^-$ population using CD11c microbeads with autoMACS per the manufacturers' protocol (Miltenyi Biotech, Gladbach, Germany). After depletion, the frequency of $CD11c^+$ DC fraction was less than 0.02%.

For organ culture assay, the skin of mouse ears were split along with cartilage, and the dorsal ear skin without cartilage was floated in a dermal side-down manner in 24-well tissue culture plates (Costar; Corning Life Sciences, Acton, MA) at 37°C. Twenty-four hours later, the cells in the wells were collected for analysis.[23]

Flow Cytometry and Immunohistochemistry

Cell suspensions were prepared from lymph nodes by mechanical disruption on 70-μm nylon cell strainers (BD Falcon, San Jose, CA). For flow cytometry, cells were plated at a density of 1×10^6 cells per well in 96-well U-bottomed plates (Falcon). They were stained for 20 minutes on ice with antibodies (Abs) in 25 μl of phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS), 1 mmol/L ethylenediamine tetraacetic acid (EDTA), and 0.1% NaN_3 and were washed twice with 200 μl of this buffer after each step. For staining with CXCR4, cells were preincubated with CD16/32 monoclonal Ab in 0.5% bovine serum albumin (BSA) containing RPMI 1640 medium for 30 minutes for resensitization and Fc receptor blocking and then stained as above. Data were collected on a FACSCanto or FACSCalibur (BD Biosciences, San Diego, CA) and analyzed with FlowJo software (TreeStar, San Carlos, CA).

Abs used were as follows: phycoerythrin (PE)-conjugated anti-CXCR4 (2B11; BD Biosciences) and isotype-matched control IgG2a, PE-Cy5-conjugated anti-MHC class II Ab, PE-Cy7-conjugated CD11b and B220 Ab, and allophycocyanin (APC)-conjugated anti-CD11c Ab (all from BD Biosciences). Langerin was detected using a specific Ab (929F3; kindly provided by Sem Saeland, Schering Plough) in permeabilized cell suspensions, followed by visualization with anti-rat Ig conjugated to PE.

For immunofluorescence analysis, the ears of B6 mice 24 hours after application with hapten were frozen in Tissue-Tek OCT compound 4583 (Sakura Finetechnical Co. Ltd., Tokyo, Japan). Cryostat sections (10 μm) were fixed in acetone and stained as described previously[24] with the following reagents: goat anti-mouse CXCL12 Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rat anti-mouse LYVE-1 Ab (R&D Systems, Minneapolis, MN). Goat anti-CXCL12 Ab, after incubation with CXCL12 blocking peptide (62.5 μg/1 mg of antibody; Santa Cruz Biotechnology, Inc.) for 1 hour on ice and was centrifuged at 13,000 rpm for 1 minute, and the supernatant was used for control staining. Goat and rat Abs were detected using Alexa Fluor 488 rabbit anti-goat IgG (H+L) (Invitrogen, Molecular Probes, Carlsbad, CA) and PE-conjugated donkey anti-rat IgG (H+L) (Jackson ImmunoResearch, West Grove, PA), respectively, mounted with Prolong Gold antifade reagent (Invitrogen, Molecular Probes), and viewed with a Zeiss Axioplan fluorescence microscopy. Images were acquired on a 600CL-CU cooled charge-coupled device video camera (Pixera, Los Gatos, CA) and were processed with InStudio 1.0.0 (Pixera).

Quantitative RT-PCR

Total mRNA was extracted from the mice ears with the SVTotal RNA Isolation system (Promega, Madison, WI) according to the manufacturer's protocol. Target gene expression was quantified in a two-step RT-PCR. cDNA was reverse transcribed from total RNA samples using the TaqMan RT reagents (Applied Biosystems, Foster City, CA). Murine CXCL12 (Assay ID: Mm00445552_m1) expression was quantified using TaqMan Gene Expression Assay (Applied Biosystems) in the ABI PRISM 7000 sequence detection system (Applied Biosystems). As an endogenous reference for these PCR quantification studies, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression was measured using the TaqMan rodent GAPDH control reagents (Applied Biosystems). The relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.[25] The expression of the target gene normalized to an endogenous reference and relative to calibrator is given by the formula $2^{-\Delta\Delta CT}$. Gene expression in untreated mice was used as a calibrator expression to calculate $\Delta\Delta C_T$.

Chemotaxis Assay and FITC-Induced Cutaneous DC Migration

Cells were tested for transmigration across uncoated 5-μm Transwell filters (Corning Costar Corp., Corning, NY) for 3 hours to CXCL12, CCL21 (R&D Systems), or medium in the upper or lower chamber and were enumerated by flow cytometry.[26] For FITC-induced cutaneous DC migration, mice were painted on the shaved abdomen with 200 μl of 2% FITC (Sigma) dissolved in a 1:1 (v/v) acetone/dibutyl phthalate (Sigma) mixture, and the number of migrated cutaneous DCs into draining inguinal and axillary lymph nodes was enumerated by flow cytometry. In some experiments, mice ears were painted with 20 μl of 0.5% FITC, and draining cervical lymph nodes were analyzed as above.

2,4-Dinitro-1-Fluorobenzene (DNFB)-Induced CHS Model

For CHS model, B6 mice were immunized by application of 25 μl of 0.5% DNFB in 4:1 (v/v) acetone/olive oil to their shaved abdomens on day 0. They were challenged on the right ear on day 5 with 20 μl of 0.3% (w/v) DNFB.[27] Ear thickness was measured before and 24 hours after challenge to assess inflammation. For treatment with CXCR4 antagonist 4F-benzoyl-TE14003, the compound was administered during the sensitization period (from 1 day before DNFB sensitization to 3 days after DNFB sensitization), elicitation period (from 1 day before challenge to 1 day after challenge), or both periods.

For 2,4-dinitrobenzene sulfonic acid (DNBS)-dependent *in vitro* proliferation of lymph node cells, cells were prepared from draining axillary and inguinal lymph nodes 5 days after the DNFB sensitization on the abdomen. CXCR4 antagonist was implanted subcutaneously to the backside of the skin from 1 day before DNFB sensitization to 5 days after. Cells (4×10^5) were cultured for 3 days with DNBS (50 μg/ml), a water-soluble compound with the same antigenicity as DNFB, and were pulsed with 1 μCi of [^{3}H]thymidine for the last 24 hours of culture.

Statistical Analysis

Data were analyzed using an unpaired two-tailed *t*-test. A P value of less than 0.05 was considered to be significant.

Results

CXCR4 Expression in Cutaneous DCs

Initially, we evaluated the expression levels of CXCR4 on migrated cutaneous DCs and resident DCs in the regional lymph nodes of mice by flow cytometry. FITC, known to induce DC maturation and mobilization,[28,29] was painted on the shaved abdomen, and the regional lymph node cells were isolated 24 hours later. After Fc receptor blocking with CD16/32 Ab (BD Biosciences) for 30 minutes, cells were incubated with PE-labeled CXCR4 or isotype-matched control Abs. Significant amounts of CXCR4 were detected in the MHC class II$^+$ DCs, and among them, the FITC$^+$ migrated cutaneous DC subset expressed a higher level of CXCR4 than the FITC$^-$ resi-

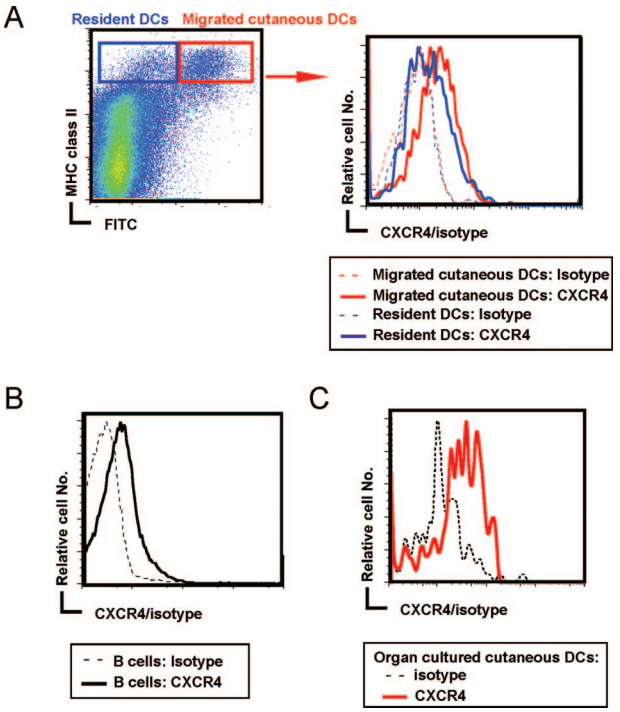

Figure 1. CXCR4 expression on resident DCs and migrated cutaneous DCs in lymph nodes. **A** and **B:** Draining lymph node cells were prepared from mice 24 hours after FITC painting on the abdomen. The profiles show flow cytometric analysis of the cells with the indicated markers. MHC class II^+ DCs were subdivided into $FITC^+$-migrated cutaneous DCs and $FITC^-$ resident DCs. The profiles show histograms of CXCR4 expression on MHC class II^+ $FITC^+$-migrated cutaneous DCs and MHC class II^+ $FITC^-$ resident DCs (**A**) and $B220^+$ B cells (**B**). Data are a representative of three independent experiments. **C:** Skin organ explants from the ears of the mice were incubated for 24 hours, and the expression of CXCR4 on the emigrated MHC class II^+ $CD11c^+$ cutaneous DCs was examined. Data are a representative of three independent experiments. As control, rat IgG2a isotype-matched control was used (**A–C**).

dent DC subset (Figure 1A). As a comparison, we monitored the expression level of CXCR4 in $B220^+$ B cells where CXCR4 was also expressed (Figure 1B). It is worth noting that the level of CXCR4 expression on the migrated cutaneous DCs was comparable or even higher than that on B cells. Then, we performed a skin explant culture assay and analyzed the cells that migrated into the culture medium 24 hours after incubation. We found that MHC class II^+ $CD11c^+$ cutaneous DCs were already positive for CXCR4 (Figure 1C), suggesting the precedent up-regulation of CXCR4 on cutaneous DCs in the skin, where DCs are ready to migrate toward lymphatic vessels.

CXCL12 Responsiveness of Resident and Migrated DCs

To assess the chemotactic activity of resident and migrated cutaneous DCs to CXCL12, we prepared draining lymph node cells 24 hours after FITC application and applied them on chemotaxis assay using transwells. Both $FITC^-$ MHC class II^+ resident DCs and $FITC^+$ MHC class II^+ migrated DCs showed chemotactic response to CXCL12 in a dose-dependent manner (Figure 2A). The response was more pronounced in $FITC^+$ MHC class

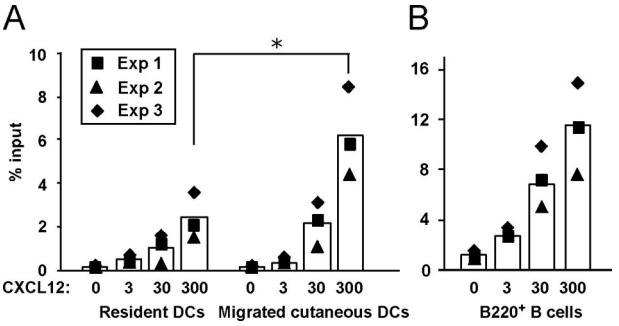

Figure 2. Chemotactic responses of resident DCs, migrated cutaneous DCs, and B cells to CXCL12. **A** and **B:** Draining lymph node cells were prepared from mice 24 hours after FITC application on the abdomen. Input cells and cells that migrated to the lower well of a transwell chamber in the absence of CXCL12 (0) or in response to 3, 30, or 300 ng/ml CXCL12 were analyzed by flow cytometry to detect MHC class II^+ $FITC^+$-migrated cutaneous DCs, MHC class II^+ $FITC^-$ resident DCs (**A**), and $B220^+$ B cells (**B**). **Filled symbols** indicate three independent experiments, and columns represent the average. A Student's *t*-test was performed between the indicated groups, and an **asterisk** indicates $P < 0.05$.

II^+-migrated DCs (Figure 2A). As a positive control, the chemotaxis test of $B220^+$ B cells to CXCL12 was simultaneously performed in parallel with DCs (Figure 2B).

CXCL12 and CCL21 Responsiveness of Epidermal LCs

To evaluate whether CXCL12-CXCR4 interactions could serve as an optional backup to CCL21-CCR7 interactions or coordinated interplay between them, we examined the chemotactic activity of LCs to CXCL12 and CCL21. Epidermal cell suspensions were incubated in cRPMI for 24 hours and applied to transwells with or without CXCL12, CCL21, or both in combination of the upper and lower chambers. The migrated epidermal LCs were identified as MHC class II^+ cells in the lower chamber. When CXCL12 or CCL21 was added to the lower chamber, LCs had a good chemotactic response to either of them, but the additional effect was not observed with CXCL12 or CCL21 combinatorially administered to the lower chamber (Figure 3). Interestingly, when CCL21 was added to the upper chamber, the chemotactic response to CXCL12 was significantly abrogated, but such an effect was not observed in the chemotaxis to CCL21 with CXCL12 added to the upper chamber (Figure 3). These data suggest that CXCL12-CXCR4 interactions can interplay coordinately with CCL21-CCR7 interactions and implicate that when CXCL12 and CCL21 coexist, LCs preferentially migrate into CCL21-producing sites.

In addition, the finding that CCL21 added to the upper chamber abrogated the chemotactic response of LCs to CXCL12 and CXCL12 added to the upper chamber unaffected the response to CCL21 raised a possibility that CCL21 down-regulates the expression of CXCR4 and CXCL12 does not affect CCR7 expression. We thus stained MHC class II^+ LC in the epidermal cell suspensions for CXCR4 and CCR7 5 hours after incubation with CCL21 or CXCL12. The treatments, however, did not alter the chemokine receptor expression levels at all (data not shown).

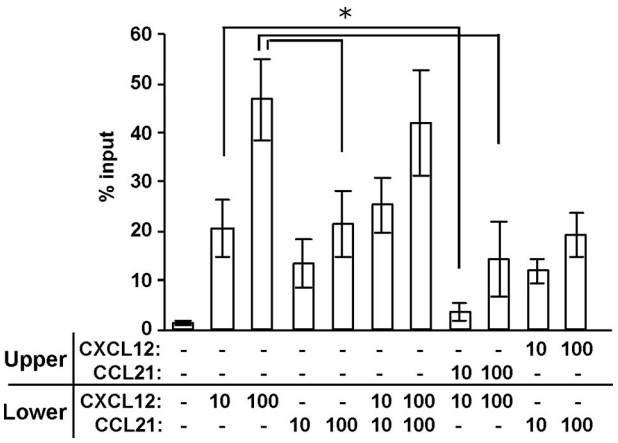

Figure 3. Epidermal LCs chemotactic activity to CXCL12 and CCL21. Epidermal cell suspensions (1×10^6) were incubated in cRPMI for 24 hours and applied to a transwell. Ten or 100 ng/ml CXCL12, CCL21, or CXCL12 and CCL21 in combination were administered to the upper or lower chamber. Migrated epidermal LCs were identified as MHC class II^+ subset in the lower chamber. The % input was calculated as follows: (the number of LCs migrated into the lower chamber)/(the number of LCs applied into the upper chamber) $\times$ 100. Columns show the mean $\pm$ SD from three independent experiments. Student's *t*-test was performed between the indicated groups, and an **asterisk** indicates $P < 0.05$.

Localization of CXCL12 in the Skin

It has been demonstrated that CXCL12 is expressed in the medullary cords of regional lymph nodes and human skin endothelial cells,[14–16] and its expression level is increased by skin wounding.[16] However, the role of CXCL12 in the context of antigen exposure remains unknown. We performed an immunohistochemical analysis on CXCL12 expression in the mouse skin and detected a significant amount of CXCL12 signal in the dermis 24 hours after epicutaneous immunization with DNFB (Figure 4A, right top). In addition, we observed that the CXCL12-expressing cells were tightly associated with LYVE-1^+ lymphatic vessels, whereas CXCL12-expressing cells were sparse in other areas of the skin (Figure 4A, right middle and bottom). The specificity of this staining was confirmed by the blocking peptide treatment or isotype-matched Ab staining (Figure 4A, left). On the other hand, the expression level of CXCL12 was less significant in the steady state (data not shown).

We then examined CXCL12 mRNA levels in the skin of ears treated with 20 μl of 0.2% DNFB ears for 6, 12, 24, and 48 hours. The intensities of CXCL12 probes were normalized against GAPDH as an endogenous control. The amount of CXCL12 mRNA in the DNFB-treated skin was expressed as the mean relative to that in non-DNFB-treated skin using the $\Delta\Delta$CT method. Its expression was induced 6, 12, 24, and 48 hours after hapten application with peak expression at 12 to 24 hours (Figure 4B).

Impairment of Cutaneous DC Accumulation in Regional Lymph Nodes by CXCR4 Antagonist Treatment

To investigate the functions and *in vivo* significance of CXCR4 in cutaneous DCs, we performed FITC-induced

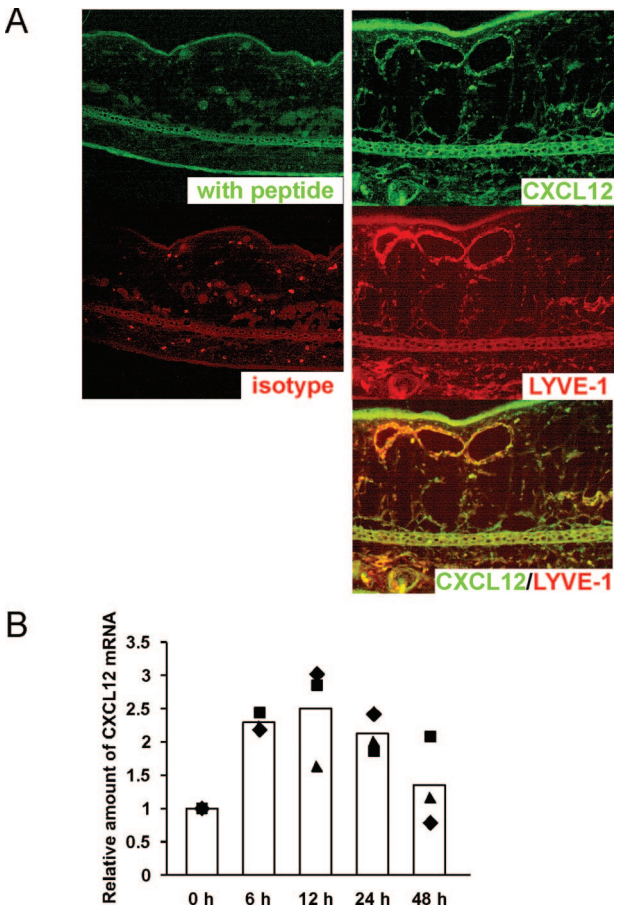

Figure 4. CXCL12 expression in lymphatic vessels of mouse skin. **A:** Skin sections from ears of mice treated with DNFB 24 hours prior were stained with goat anti-CXCL12 Ab with or without blocking peptide, and rat anti-LYVE-1 Ab or isotype control Ab, and sequentially immersed with Alexa Fluor 488 rabbit anti-goat IgG and PE-conjugated donkey anti-rat IgG, respectively (the labels are the same color as the reaction product). **B:** The ears of mice treated with 20 μl of 0.3% DNFB for 6, 12, 24, and 48 hours were isolated. The levels of CXCL12 mRNA were normalized against GAPDH as an endogenous control. The CXCL12 mRNA amounts in the skin from DNFB-treated mice relative to that from non-DNFB-treated mice (0 hours) were induced 6, 12, and 24 hours after hapten application. **Filled symbols** indicate three independent experiments, and columns represent the average.

cutaneous DC migration assay. FITC applied to the skin is taken up by cutaneous DCs, which then migrate to the draining lymph nodes as $FITC^+$ MHC class II^+ cells.[23] We isolated cervical lymph node cells 24 hours after FITC application to ears and characterized $FITC^+$ MHC class II^+ cutaneous DCs (Figure 5A) therein by flow cytometry. Among $FITC^+$ MHC class II^+ cutaneous DCs, two subsets, R1 ($CD11c^{high+}$ $CD11b^{high+}$ and $CD11c^{low+}$ $CD11b^{int+}$) and R2 ($CD11c^{high+}$ $CD11b^{int+}$), were detected when they were stained with CD11c and CD11b (Figure 5B). In these populations, only the R2 subset expressed Langerin, a marker for LCs (Figure 5C). Therefore, the R2 subset originates from LCs, and the R1 subset is from dDCs. It has been shown that after FITC painting on the skin, a rapid influx of $FITC^+$ cutaneous DCs into the draining lymph nodes occurs at a peak time of 24 hours, and FITC-labeled DCs remain elevated in number for another 2 days and then decline to the normal level by day 6.[30] We injected CXCR4 antagonist subcu-

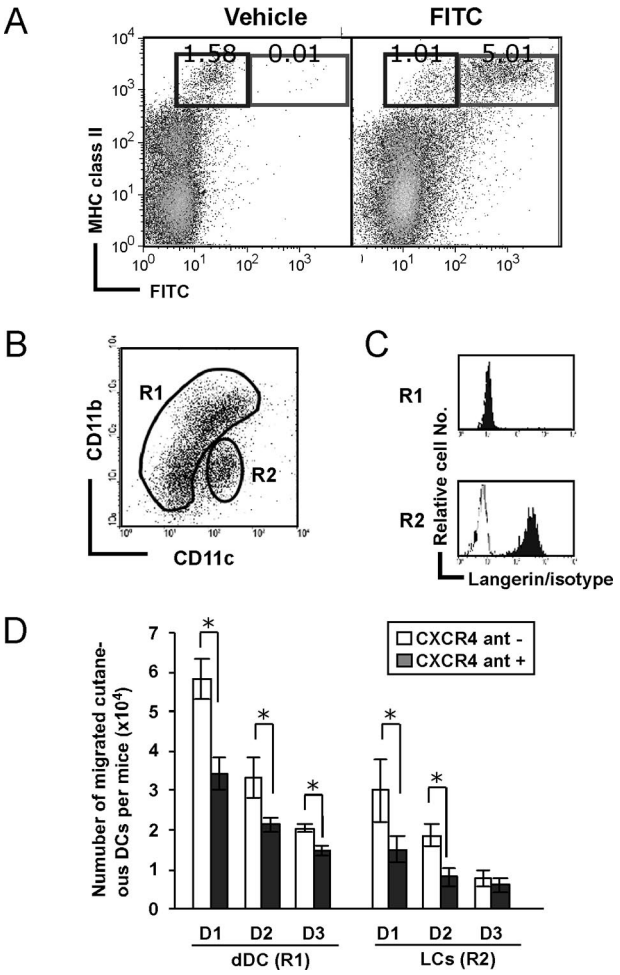

Figure 5. Impaired cutaneous DC accumulation in regional lymph nodes by CXCR4 antagonist. **A:** Flow cytometric analysis of MHC class II expression and FITC fluorescence in cells derived from the regional lymph nodes 24 hours after the application of 200 μl of 2% FITC or vehicle. **B:** Cervical lymph node cells were prepared from mice 24 hours after 10 μl of 0.5% FITC painting on the ears. Among FITC$^+$ cutaneous DCs, two subsets, R1 (CD11c^{high+} CD11b^{high+} and CD11c^{low+}CD11b^{int+}) and R2 (CD11c^{high+} CD11b^{int+}) were identified. **C:** The histogram of Langerin is shown in each subset. Note that only R2 subset expresses LC marker Langerin. **D:** The numbers of migrated dermal DCs (dDCs) and LCs 24, 48, and 72 hours after FITC painting are calculated. Columns show the mean ± SD from at least four mice per group. Student's *t*-test was performed between the indicated groups, and an **asterisk** indicates $P < 0.05$. Data are a representative of three independent experiments.

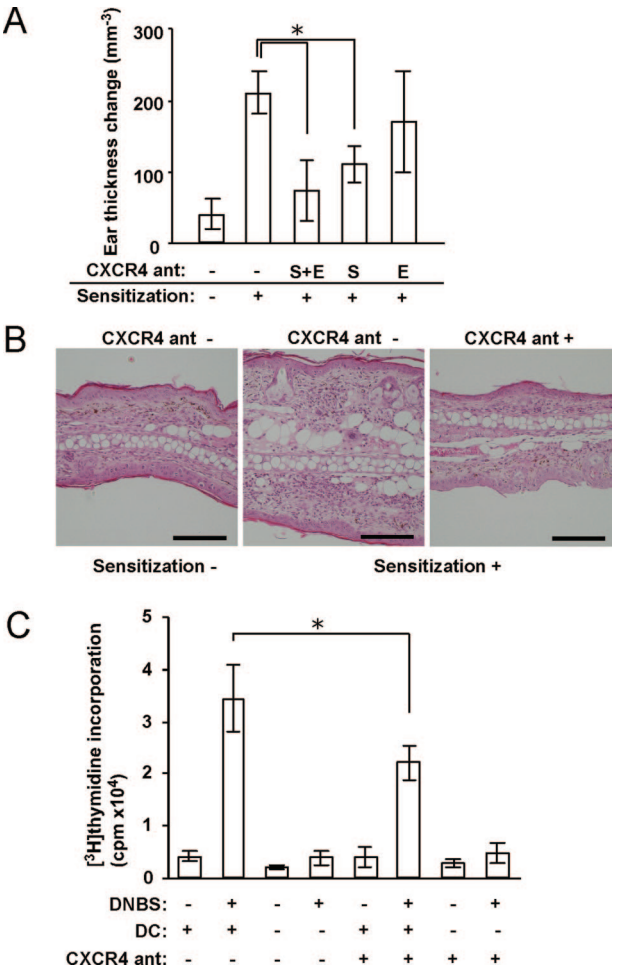

Figure 6. Perturbed initiation of skin immune response by blockade of CXCR4 engagement. **A:** Effect of CXCR4 antagonist on CHS response to DNFB. The ear thickness of DNFB-sensitized mice treated with either vehicle alone (−) or CXCR4 antagonist during sensitization (S), elicitation (E), or both (S+E) was measured after challenge with DNFB. *$P < 0.05$ versus vehicle-treated mice ($n = 6$ per group). **B:** H&E staining of ears from mice treated with (CXCR4 ant$^+$) or without (CXCR4 ant-) CXCR4 antagonist 24 hours after challenge with DNFB. Scale bar = 100 μm. **C:** The proliferative response of DNFB-immune lymphocytes to DNBS. The lymph node cells from mice sensitized by DNFB with or without CXCR4 antagonist (CXCR4 ant) treatment were stimulated with DNBS for 72 hours. Lymph node cells eliminated by CD11c$^+$ DCs were also prepared using autoMACS. The proliferative response was measured in triplicate. Columns show the mean ± SD from triplicate wells. Student's *t*-test was performed between the indicated groups, and an **asterisk** indicates $P < 0.05$. Results are representative of three independent experiments. cpm, counts per minute.

taneously into FITC-treated mice. The numbers of accumulated LCs and dDCs (represented by R2 and R1, respectively, in Figure 5B) 24 and 48 hours after FITC application was significantly reduced (Figure 5D). Therefore, loss of CXCL12-CXCR4 signaling resulted in impaired lymph node accumulation of cutaneous DCs in response to antigen exposure to the skin.

Perturbed Initiation of CHS by CXCR4 Antagonist

The relevance of the observed CXCR4-mediated regulation of cutaneous DC function to *in vivo* immune responses is a matter to be clarified. With CHS as an *in vivo* model, we investigated whether inhibition of CXCR4 signaling affected an immune response to an exogenous antigen. B6 mice were sensitized by DNFB as a hapten to the abdomen. In mice treated with CXCR4 antagonist, challenge of ears 5 days later disclosed a significant ($P < 0.05$) decrease in ear swelling compared with the nontreated control mice (Figure 6A). A histological analysis of the nontreated mice revealed pronounced spongiosis and extensive infiltration of lymphocytes in the edematous dermis, whereas the extent of such changes was markedly reduced in mice treated with CXCR4 antagonist (Figure 6B). We next administered CXCR4 antagonist either throughout the experimental protocol or selectively during the sensitization or elicitation period. Treatment with CXCR4 antagonist during the sensitization phase,

but not the elicitation phase, resulted in a significant ($P < 0.05$) decrease of ear swelling. The extent of the former's inhibition was comparable to that of the administration of the antagonist throughout CHS period (Figure 6A). These results indicated that CXCL12-CXCR4 signaling is important in the priming of T cells, which is in accordance with the notion that cutaneous DCs play a pivotal role in sensitization. We also isolated the regional lymph node cells 5 days after DNFB sensitization and examined the responsiveness of immune T cells to DNBS, a water-soluble compound with the same antigenicity as DNFB, in the presence or absence of recombinant murine CXCL12. The proliferative response of cells was significantly enhanced by the addition of DNBS, but when $CD11c^+$ cells were depleted, this proliferative response was markedly attenuated (Figure 6C), suggesting that this response is dependent on DCs. Moreover, this DNBS-induced proliferative response was inhibited by CXCR4 antagonist treatment (Figure 6C). However, such an antiproliferative effect by CXCR4 antagonist was not shown when 2×10^5 $CD4^+$ cells were stimulated with 10 ng/ml phorbol myristate acetate (Sigma Chemical) in combination with 1 μmol/L ionomycin (Wako, Osaka, Japan), which are independent of DC function (data not shown). This is interpreted as an indication that CXCL12-CXCR4 interactions are important for initiation of skin immune response by acting cutaneous DC.

Discussion

The above findings demonstrated that CXCR4 was highly expressed on cutaneous DCs, and its ligand CXCL12 was produced by the lymphatic vessels of the skin after antigen exposure. Cutaneous DCs in the regional lymph nodes had a stronger chemotactic response to CXCL12 than resident DCs, suggesting that activated DCs are attracted to the lymphatic vessels by virtue of CXCR4 and CXCL12. Consistently, FITC-induced DC migration into the lymph nodes was partially but substantially impaired by CXCR4 signaling blockade. These results suggest that CXCL12-CXCR4 interaction is important for the migration of cutaneous DCs.

Accumulating evidence has shown an essential role for chemokine system in the migration of cutaneous DCs and the maintenance of the microanatomic environment of secondary lymphoid organs.[10,11] It is well known that the migration of DCs from peripheral tissues into lymphatic vessels requires CCR7 and most likely occurs in response to CCL21, which is released from lymphatic endothelium and lymph nodes.[3,6] In contrast to CCL21 expression in lymphoid organs, its expression in lymphatic vessels is largely independent of lymphotoxin $\alpha 1\beta 2$, which is also important for DC homeostasis in the secondary lymphoid organs.[24,31,32] Thus, the lymphatic vessel seems to have some specialized system of chemokine production. In addition, complete migratory inhibition has not been achieved in CCR7-deficient mice, suggesting that there exist other factors than CCR7. One candidate is CCR2, which has already been implicated in DC homing from the skin to the lymph nodes, although the precise mechanism is unclear.[33] On the other hand, DCs have a chemotactic response to CXCL12, but its *in vivo* significance in cutaneous DC migration has not been elucidated.[12] The present study revealed that CXCL12 and CXCR4 play a key role for cutaneous DC migration into the draining lymph nodes *in vivo*.

The present study cannot clarify the respective characteristic role of CCR7 and CXCR4 in the *in vivo* migration of dDCs and LCs. This should be addressed in future studies using CCR7-deficient mice treated with or without CXCR4 antagonist. From our *in vivo* study, the extent of impairment of cutaneous DC migration into regional lymph nodes is more significant in CCR7-deficient mice[10] than in mice treated with CXCR4 antagonist. However, epidermal LCs had better chemotactic activity to CXCL12 rather than CCL21. Moreover, no additional effect was observed when both chemokines were added to the lower chamber. These data suggest that CXCL12-CXCR4 interactions interplay coordinately with CCL21-CCR7 interactions rather than backup optionally for CCL21-CCR7 signaling. Moreover, the chemotactic activity to CXCL12 was completely abrogated with CCL21 added to the upper chamber, whereas the activity to CXCL12 retained when CCL21 was added to the upper chamber. These results suggest that when both chemokines coexist, LCs preferentially migrate to CCL21-producing sites.

On the other hand, recent studies have revealed that CCR7 expression level or signaling sensitivity can be modulated by several factors,[34,35] raising a possibility that signaling via one chemokine receptor affects another receptor signaling. However, the level of CCR7 expression on LCs was not changed by CXCR4 antagonist when epidermal cell suspensions were incubated with this blocker for 24 hours in the setting of culturing without FCS as previously reported[36] (Supplemental Figure 1; see *http//ajp.amjpathol.org*).

Because of their location in the epidermis, LCs have previously been considered to initiate and control skin immune responses. For example, in a model of allogeneic graft-*versus*-host disease, LCs are sufficient for the development of cutaneous immune response.[37] However, this concept has been challenged by the findings that dDCs, but not LCs, initiate protective T-cell responses to certain epidermal viral antigens.[38] Recently, three groups of investigators have independently generated LC-deficient mice, and each type of the modified mice showed different manifestations, as they exhibited impaired,[39] not affected,[40] and enhanced CHS responses.[41] Different cutaneous DC subsets may play their own roles in the generation and regulation of immune responses.[42] In our experiments, both the cutaneous DC migration and the CHS response were partially but significantly impaired by CXCR4 blockade. Moreover, there was no difference in the antagonist inhibition of migratory activity between LCs and dDCs. Therefore, CXCR4 usage in these cell types seems to be virtually the same, and we could not further address the respective roles of LCs and dDCs in our system.

In this investigation, it was suggested that CXCL12 was expressed in the lymphatic vessels and engaged with CXCR4 on cutaneous DCs to change their function.

It should be noted that CXCR4 was already expressed in the skin when we did the organ skin culture assay and examined the expression level of CXCR4 on migrated cutaneous DCs in the culture medium 24 hours after incubation (data not shown), suggesting that CXCR4 on skin DCs is already up-regulated in the skin, where it is needed to migrate toward lymphatic vessels. On the other hand, it is known that human DCs themselves express CXCL12, suggesting that DCs attract naive T cells.[1,43,44] Rolling B cells can be induced to arrest in high endothelial venules either by CCR7 agonists or by CXCL12.[45] These findings suggest that the CXCL12-CXCR4 interaction may play critical roles in lymphocytic circulation and immune surveillance in the postnatal life. Although the number and localization of T and B cells in the regional lymph nodes were not apparently affected by CXCR4 antagonist (data not shown), this cannot negate the possibility that cells other than DCs are involved in this process. A study using mice with conditional CXCR4 depletion on DCs might clarify this issue in future.

Interactions between CXCL12 and CXCR4 are largely unique and nonpromiscuous. In mice, CXCL12 or CXCR4 gene knockouts generate a similar phenotype, and embryonic lethality associated with either CXCR4 or CXCL12 gene knockouts emphasizes the critical and unique role played by their products during development.[17–19] Because it has not been established that LCs are reconstituted efficiently in fetal liver chimeric mice, the CXCR4 antagonist is a useful chemical probe to evaluate the role of CXCR4 on cutaneous DC function. CXCR4 was considered to be the single receptor for CXCL12, but another chemokine receptor, CXCR7, has recently been identified.[46] CXCR7 is expressed in tumor cell lines, activated endothelial cells, and fetal liver cells. At present, CXCR7 was not found in immune cells, but we cannot exclude the possibility that CXCR7 may be involved in DC function. In human skin inflammatory diseases, CXCL12-positive cells, including endothelial cells and pericytes of adult small capillary blood vessels, are colocalized with CXCR4-positive inflammatory cells, such as DCs,[14] suggesting that CXCL12-CXCR4 interaction may be involved not only in mice but also in the formation of human skin immune response. Understanding of factors that determine cutaneous DC trafficking and function might offer new opportunities for therapeutic intervention to suppress or stimulate the immune response.

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