Absence of Adverse Side Effects

Previous reports indicated that CCR5 is a redundant molecule in adults because CCR5-defective individuals have normal inflammatory and immune reactions [72]. On the other hand, studies with CXCR4 knockout mice revealed defects in B-cell lymphopoiesis and bone-marrow myelopoiesis. Moreover, mice lacking CXCR4 exhibit hematopoietic and cardiac defects identical to those observed in SDF-1-deficient mice, and die before birth [73-75]. However, all these deficits are developmental and it is not known whether SDF-1 and CXCR4 are essential for normal physiological processes after birth. Furthermore, although the safety of small-molecule CXCR4 inhibitors such as AMD3100 and ALX40-4C was investigated [76-78], it is still controversial. It is necessary to analyze the long-term safety of a novel approach targeting chemokine receptors under various conditions in human and nonhuman primates in more detail. However, no clinically significant adverse events were observed in all the monkeys immunized with cDDR5-MAP, cDDX4-MAP or cCD-MAP [65-67]. The previous pilot test demonstrated that the titer of anti-cDDR5 serum measured 4 weeks after the third immunization (10 wpim) was the highest in the vaccinated macaques. However, each macaque gained weight gradually during and after the immunization (Fig. (**8A**)). Furthermore, no significant changes in CD4+ cell/total PBMC (%) were observed in all the monkeys (monkeys nos. 11, 13, and 16 in Fig. (**6**)) immunized with cDDR5-MAP at 6, 10, and 12 wpim, as shown in Fig. (**8B**).

New Vaccine Strategy and Conclusions

We were interested in studying specific immune responses in HIV-1 infection in ESN individuals to reproduce the ESN immune status by a candidate vaccine. A small number of HIV-1-exposed individuals do not become infected, despite repeated and long-term exposures to the virus [79]. Interestingly, Lopalco *et al.* reported that anti-HLA class I, anti-CD4, and anti-CCR5 Abs are induced in ESN individuals [80-82]. In particular, the anti-CCR5 Ab seems to protect against R5 HIV-1 transmission as one of the "gatekeepers" as well as other restrictive barriers for HIV-1 transmission. Therefore, we created a cycloimmunogen (cDDR5-MAP) that mimics the deduced conformational epitope of UPA in CCR5 and immunized cynomolgus macaques. The results suggest that the high induction of the anti-CCR5 Ab can suppress viral propagation during acute HIV-1 transmission, but only a high induction of the anti-CCR5 Ab is not sufficient to clear detectable plasma-associated viruses because the anti-CCR5 Ab does not directly neutralize $SHIV_{SF162P3}$. It seems difficult to completely eliminate or inhibit HIV-1 acute infection *in vivo* when only the anti-CCR5 Ab delays viral propagation during the initial HIV-1 transmission. Lopalco *et al.* [83] have recently reported that anti-virus Abs such as IgA to gp41 are simultaneously induced with IgG to CCR5 and IgG to CD4 in some Italian ESN individuals. These humoral immune responses concomitantly seem to contribute to an extremely low level of viral replication below the detection limit of a standard assay in ESN individuals. Margolis and Shattok proposed that the field of vaccine development needs to establish strategies that target multiple arms of the immune system in the different stages of the viral life cycle [84]. Therefore, if the resistance to HIV-1 transmission is mediated by multiple mechanisms such as the simultaneous induction of the anti-CCR5 Ab and anti-HIV humoral responses (e.g., anti-gp41 Ab), the vaccine that targets multiple stages of mucosal transmission will most likely succeed in reproducing the ESN immune status, Fig. (**9**). We are developing a novel conjugate vaccine composed of cDDR5 and recombinant env protein.

Furthermore, a large fraction of patients progress to AIDS without the "phenotype switch", suggesting that in many patients, R5 viruses seem to be inherently more patho-

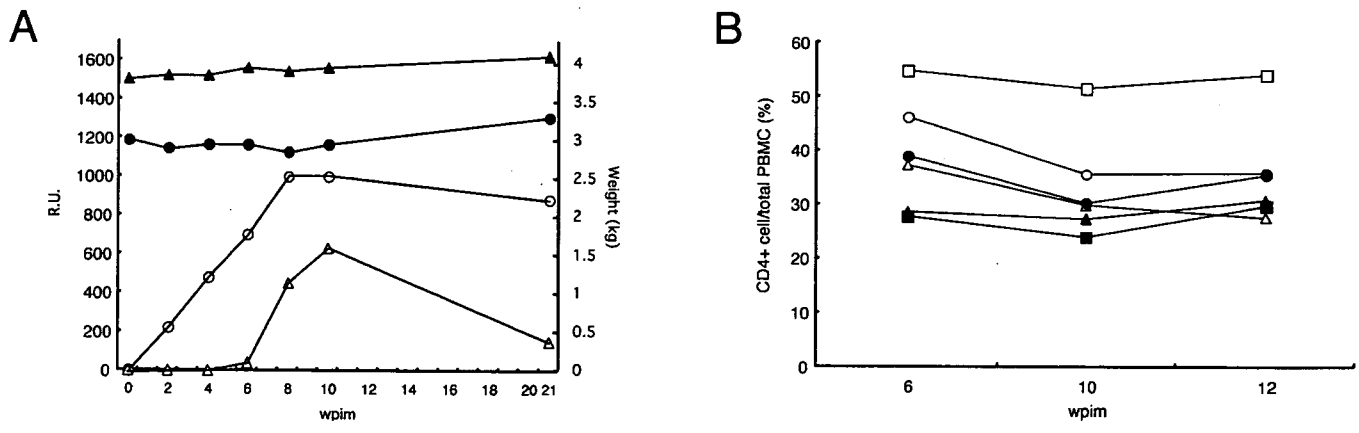

Fig. (8). Variations in weight and peripheral CD4+ T-cell number (%) after induction of cDDR5-specific Abs in cynomolgus macaques. (A) To determine whether the induction of cDDR5-specific Abs produces unwanted side effects, new serum samples obtained before and after immunization with cDDR5-MAP (at 0, 2, 4, 6, 8, 10, and 21 wpim) were collected. The antisera against the moiety of cDDR5 were detected by real-time biomolecular interaction analysis using surface plasmon resonance with a biotinylated-cDDR5-bound BIAcore biosensor (monkey No. 4: open circle; monkey No.5: open triangle). Simultaneously, the patterns of variation in/body weight were investigated in two macaques (monkey No. 4: closed circle; monkey No.5: closed triangle). (B) Whole-blood samples derived from the cDDR5-MAP-vaccinated (monkey No. 11 (open circle), monkey No.13 (open triangle), monkey No.16 (open square) in Fig. (**6**)) and control (monkey No. 7 (closed circle), monkey No. 8 (closed triangle), monkey No. 9 (closed square) in Fig. (**6**)) groups were collected at 6, 10 and 12 wpim, and the samples were examined for peripheral CD4+ T-cell count (%).

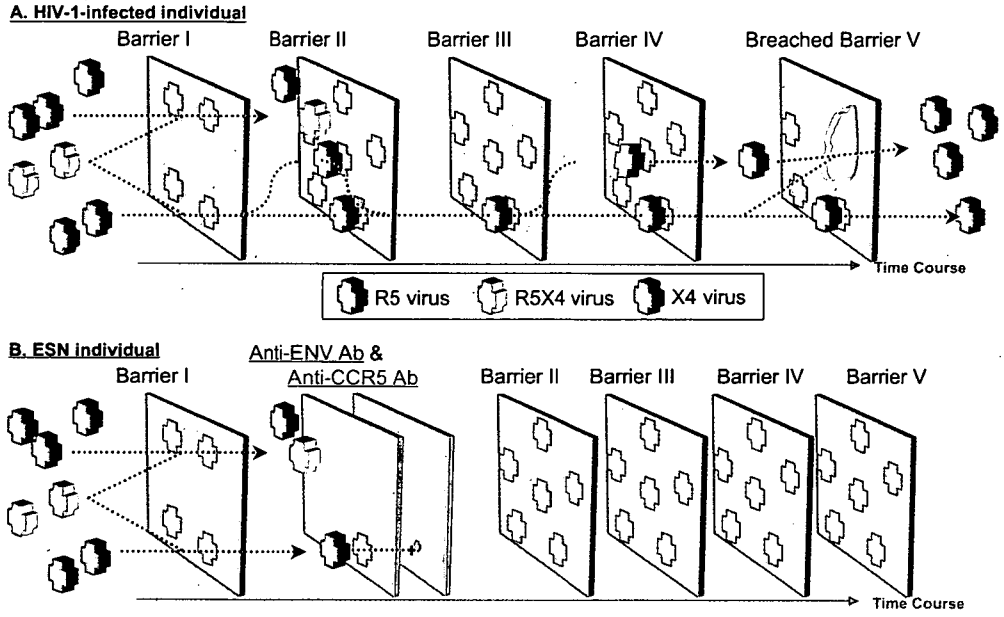

Fig. (9). Paradigm for the host's immune response to HIV and reproduction of ESN immuno status. (A) HIV-1-infected individual has a range of immune barriers (light gray squares edged with dark gray) that can potentially restrict the establishment of HIV-1 infection. Consequently, the HIV-1 strains that are most commonly responsible for transmission and dominate during the long asymptomatic phase are almost constantly restricted to CCR5 usage. A large fraction of patients progress to AIDS without the viral "phenotype switch" to CXCR4 usage. On the other hand, in some case, X4 virus emerges at the late phase of HIV-1 infection in the event that one of the barriers is breached (the barrier V in this figure), and its emergence is also associated with a rapid decline in the count of $CD4^+$ T cell and progression to AIDS. (B) In ESN individual, the resistance to HIV-1 transmission is mediated by multiple mechanisms such as the simultaneous induction of anti-CCR5 Ab (light gray square edged with green) and anti-HIV humoral responses (e.g. anti-gp41 Ab, light gray square edged with pink) in addition to potential immune barriers I-V. Therefore, we are developing a novel conjugate vaccine composed of cDDR5 and recombinant env protein to reproduce ESN immue status. Taken together, the vaccine that targets multiple stage of mucosal transmission could have the best of success.

genic at late phase of HIV-1 infection and X4 viruses appear only transiently on a background of sustained R5 dominance [85-87]. On the other hand, in some case, X4 viruses emerge during chronic infection, and its emergence is associated with a rapid decline in the count of $CD4^+$ T cell and progression to AIDS [88-90]. Therefore, the combination of cDDR5- and cDDX4-MAP, or cCD-MAP alone may provide a greater protection against HIV-1 infection than a single immunization of each cycloimmunogen.

ABBREVIATIONS

HIV-1 = Human immunodeficiency virus type 1

Env = Envelope

AIDS = Acquired immune deficiency syndrome

UPA = Undecapeptidyl arche

ESN = Exposed seronegative

Nt = *N*-terminus

ECL-2 = Second extracellular loop

MAP = Multiple-antigen peptide

MOE = Molecular Operating Environment

MAb = Monoclonal antibody

Ab = Antibody

SHIV = simian/human immunodeficiency virus

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Immunopathology and Infectious Disease

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-

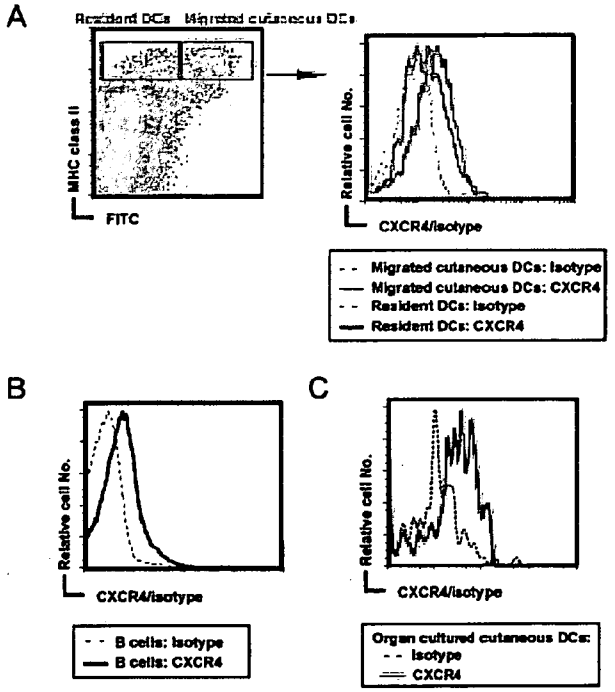

Figure 1. CXCR4 expression on resident DCs and migrated cutaneous DCs in lymph nodes. **A** and **B:** Draining lymph node cells were prepared from mice 24 hours after FITC painting on the abdomen. The profiles show flow cytometric analysis of the cells with the indicated markers. MHC class II$^+$ DCs 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

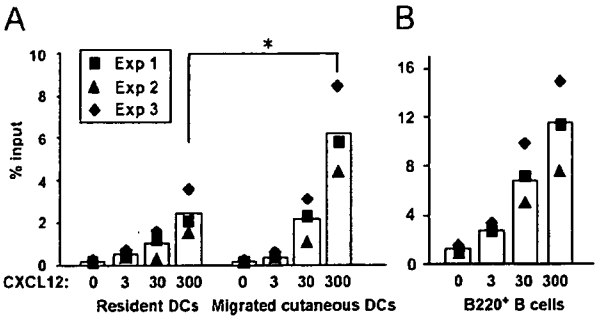

Figure 2. Chemotactic responses of resident DCs, migrated cutaneous DCs, and B cells to CXCL12. **A** and **B:** Draining lymph node cells were prepared from mice 24 hours after FITC application on the abdomen. Input cells and cells that migrated to the lower well of a transwell chamber in the absence 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).

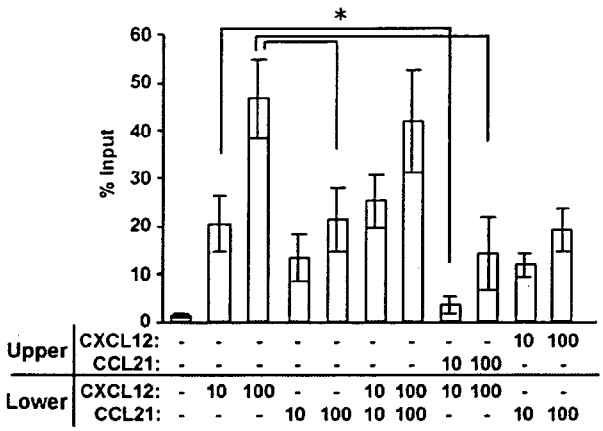

Figure 3. Epidermal LCs chemotactic activity to CXCL12 and CCL21. Epidermal cell suspensions (1×10^6) were incubated in cRPMI for 24 hours and applied to a transwell. Ten or 100 ng/ml CXCL12, CCL21, or CXCL12 and CCL21 in combination were administered to the upper or lower chamber. Migrated epidermal 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) × 100. Columns show the mean ± 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 ΔΔCT 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

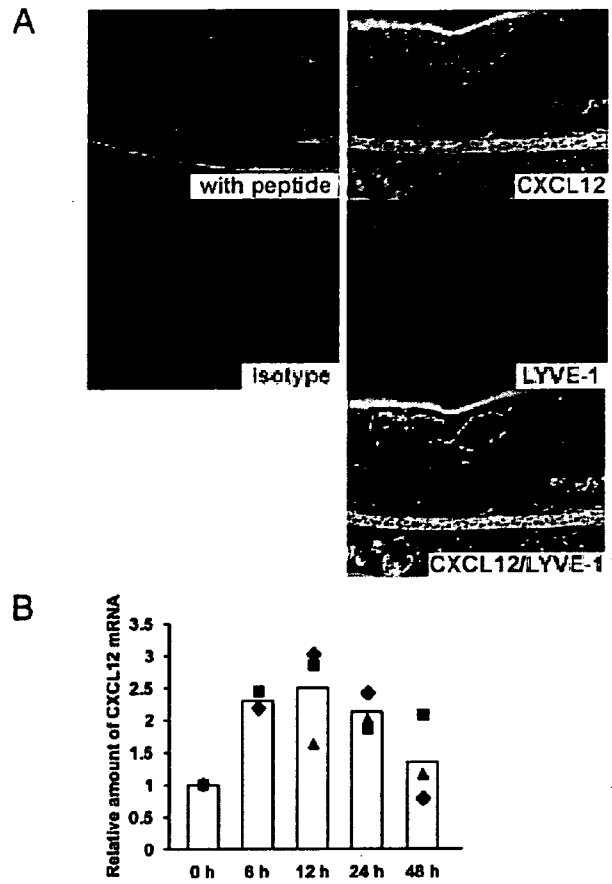

Figure 4. CXCL12 expression in lymphatic vessels of mouse skin. **A:** Skin sections from ears of mice treated with DNFB 24 hours prior were stained with goat anti-CXCL12 Ab with or without blocking peptide, and rat anti-LYVE-1 Ab or isotype control Ab, and sequentially immersed with Alexa Fluor 488 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-

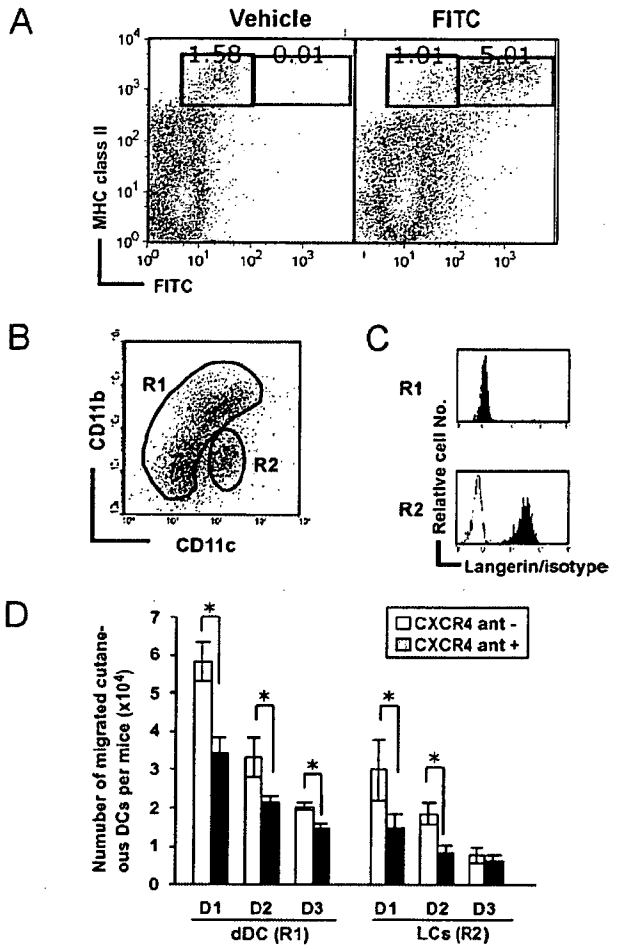

Figure 5. Impaired cutaneous DC accumulation in regional lymph nodes by CXCR4 antagonist. **A:** Flow cytometric analysis of MHC class II expression and FITC fluorescence in cells derived from the regional lymph nodes 24 hours after the application of 200 μl of 2% FITC or vehicle. **B:** Cervical lymph 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.

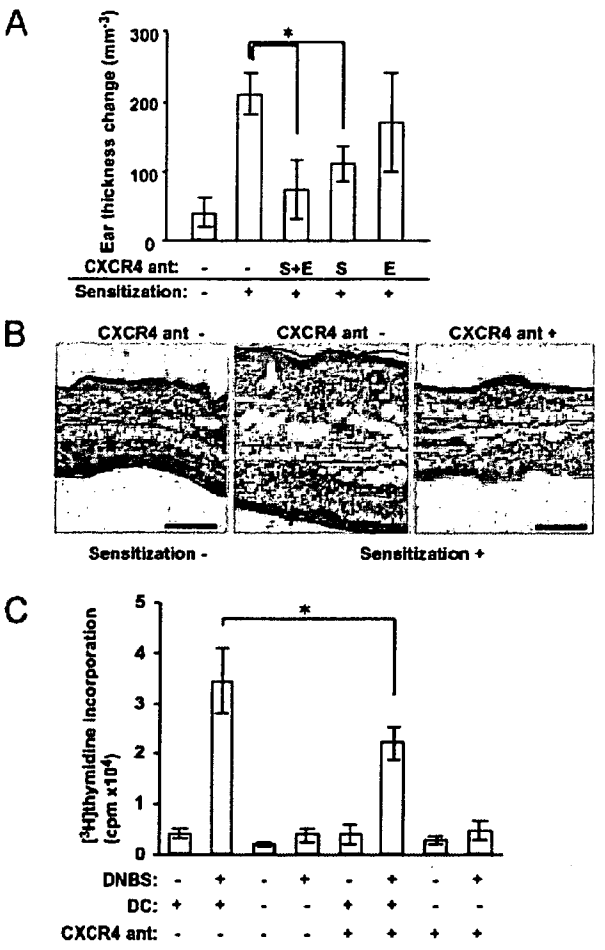

Figure 6. Perturbed initiation of skin immune response by blockade of CXCR4 engagement. **A:** Effect of CXCR4 antagonist on CHS response to DNFB. The ear thickness of DNFB-sensitized mice treated with either vehicle alone (−) or CXCR4 antagonist during sensitization (S), elicitation (E), or both (S+E) 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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Therapeutic Potential of the Chemokine Receptor CXCR4 Antagonists as Multifunctional Agents

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ABSTRACT:

The chemokine receptor CXCR4 possesses multiple critical functions in normal and pathologic physiology. CXCR4 is a G-protein-coupled receptor that transduces signals of its endogenous ligand, the chemokine CXCL12 (stromal cell-derived factor-1, SDF-1). The interaction between CXCL12 and CXCR4 plays an important role in the migration of progenitors during embryologic development of the cardiovascular, hemopoietic, central nervous systems, and so on. This interaction is also known to be involved in several intractable disease processes, including HIV infection, cancer cell metastasis, leukemia cell progression, rheumatoid arthritis (RA), and pulmonary fibrosis. It is conjectured that this interaction may be a critical therapeutic target in all of these diseases, and several CXCR4 antagonists have been proposed as potential drugs. Fourteen-mer peptides, T140 and its analogues, were previously developed in our laboratory as specific CXCR4 antagonists that were identified as HIV-entry inhibitors, anti-cancer-metastatic agents, anti-chronic lymphocytic/acute lymphoblastic leukemia agents, and anti-RA agents. Cyclic pentapeptides, such as FC131 [cyclo(D-Tyr-Arg-Arg-L-3-(2-naphthyl)alanine-Gly)], were also previously found as CXCR4 antagonist leads based on pharmacophores of T140. This review article describes the elucidation of multiple functions of CXCR4 antagonists and the development of a number of low-molecular weight CXCR4 antagonists involving FC131 analogues and other compounds with different scaffolds including linear-type structures. © 2006 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 88: 279–289, 2007.

Keywords: cancer metastasis; chemokine receptor; HIV infection; leukemia cell progression; low-molecular weight CXCR4 antagonist; rheumatoid arthritis

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INTRODUCTION

In a postgenome era, proteomics has been prosperous in biology and life science, and artificial functional molecules involving selective agonists and antagonists are highly useful for studies of chemical biology. Transmembrane proteins are attractive targets for chemical biology and drug discovery. Chemokine receptors are classified into G-protein-coupled receptor families, which transduce signals of the corresponding chemokines. Specific probes for chemokine receptors can accelerate studies of proteomics and

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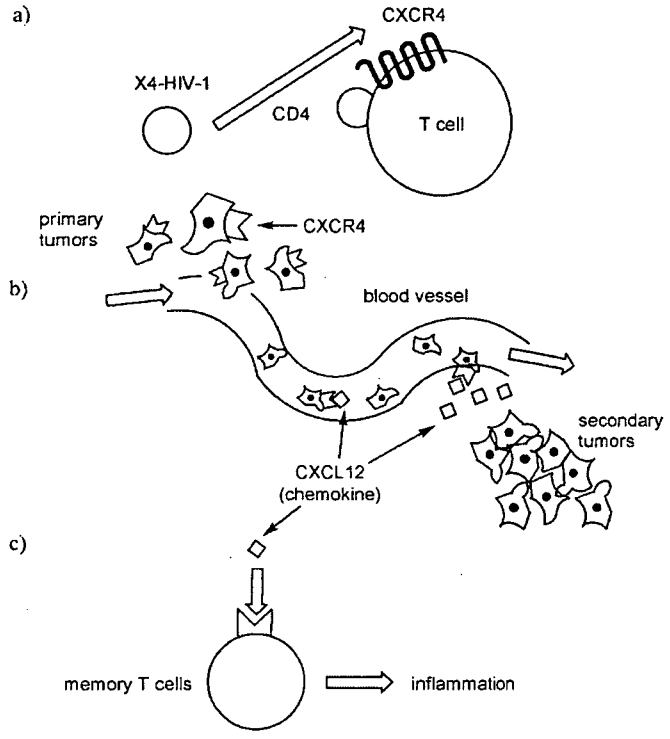


FIGURE 1 Brief presentation of various diseases, which are relevant to the CXCR4-CXCL12 axis: (a) HIV infection (AIDS), (b) cancer metastasis, and (c) rheumatoid arthritis (RA).

chemical biology.[1] Chemokines belong to a chemotactic cytokine family that attracts and induces migration of leukocytes. The relationships between chemokines and their receptors are highly interconnected and complicated: a single chemokine recognizes a plurality of receptors, while one chemokine receptor recognizes several chemokines. Numerous chemokines lack receptor selectivity. However, the chemokine receptor CXCR4 possesses the chemokine CXCL12/stromal cell-derived factor-1 (SDF-1) as its unique ligand.[2–5] The interaction between CXCR4 and CXCL12 is essential to the migration of progenitor cells during embryonic development of the cardiovascular, hemopoietic, and central nervous systems. However, its physiological roles in adults remain poorly disclosed. On the other hand, it is known that the CXCR4-CXCL12 pair is involved in various disease processes such as HIV infection,[6] cancer cell metastasis,[7–24] leukemia cell progression,[25–27] and rheumatoid arthritis (RA) (Figure 1).[28] First, CXCR4 was identified as a second cellular receptor (coreceptor) of T cell line-tropic (X4-) HIV-1 entry through its association with the first receptor CD4.[6] Macrophage-tropic (R5-) HIV-1 strains, which use the chemokine receptor CCR5 as another coreceptor, are major in early stages of HIV infection,[29–33] while in the late stages, X4 HIV-1 strains become dominant. The molecular mechanism of HIV-1 replication has been elucidated in detail, especially for a dynamic supramolecular mechanism relevant to HIV entry/fusion process, suggesting that blocking of HIV entry/fusion is a more effective strategy for HIV infection/AIDS therapy. At the first stage, an envelope protein gp120 on the viral surface binds to a cell surface protein CD4. This induces a conformational change of gp120 and its subsequent binding to a coreceptor, CCR5[29–33] or CXCR4.[6] The interaction between gp120 and each of the coreceptors leads to penetration of another envelope subunit, gp41, which anchors the HIV envelope from the N-terminus end on the membrane. Finally, the formation of a "trimer-of-hairpins" structure of gp41 causes membrane fusion of HIV to the cells, resulting in completion of the infection.[34] Elucidation of the above dynamic molecular mechanism of the fusion process has encouraged many researchers to develop inhibitors aimed at blocking HIV-entry or fusion. These generally target the coreceptor, CCR5 or CXCR4, and the dynamic process involving formation of the gp41 trimer-of-hairpins structure. Second, it has also been reported that CXCR4 is expressed on the surfaces of several types of cancer cell, and that CXCL12 is highly expressed in internal organs that are the primary goals of cancer cell metastasis.[7–13] It has been shown that the CXCL12-CXCR4 axis is associated with metastasis of several types of cancer, including cancer of the breast, kidney, prostate, lung, and pancreas, and melanoma, neuroblastoma, non-Hodgkin's lymphoma, multiple myeloma, ovarian cancer, and malignant brain tumors.[7–24] Third, this axis is also correlated with the progression of precursor-B (pre-B) acute lymphoblastic leukemia (ALL) cells and chronic lymphocytic leukemia (CLL) B-cells.[25–27] Fourth, RA is caused mainly by $CD4^+$ memory T cell accumulation in the inflamed synovium. CXCR4 is highly expressed on the surface of memory T cells and CXCL12 concentration is extremely elevated in the synovium of RA patients. Further, CXCL12 stimulates migration of the memory T cells and thereby inhibits T cell apoptosis. This indicates that the CXCR4-CXCL12 interaction also plays a crucial role in the accumulation of T cells in the RA synovium.[28] Thus, the chemokine receptor CXCR4 appears to be an attractive therapeutic target for these diseases in the light of these observations, and our recent research concerning the development of CXCR4 antagonists including low-molecular weight compounds and structure tuning of these antagonists is discussed in this review article.

DISCOVERY OF CXCR4 ANTAGONISTS DERIVED FROM T140 AND INHIBITION OF X4-HIV-1 ENTRY

As a clinical treatment for HIV-1-infected patients, highly active anti-retroviral therapy (HAART) is frequently used. In

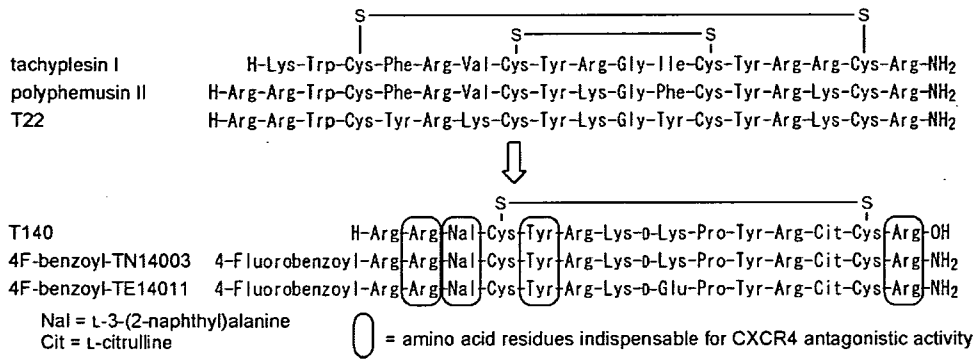


FIGURE 2 Sequences of horseshoe crab peptides and CXCR4 antagonists, T22 and T140, and its biologically stable analogues, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011.

this therapy, a combination of two or three different agents comprising reverse transcriptase inhibitors and protease inhibitors is used. HAART shows significant positive effects, but fails to resolve some serious problems including considerable adverse effects, the high cost, and the emergence of the viral strains with multiple drug resistance (MDR).[35,36] An ideal therapeutic approach would suppress the generation of drug-resistant strains of HIV. Enfuvirtide (DP-178, T-20, Fuzeon, Trimeris and Roche), a 36-mer peptide derived from the C-terminal helical region of gp41, has been used clinically as a fusion inhibitor targeting MDR HIV-1 strains.[37] Our research has focused on a search for drugs that target CXCR4. Antagonists of another coreceptor, CCR5, and fusion inhibitors targeting gp41 have been reviewed elsewhere.[38]

Antibacterial and antiviral peptides, tachyplesins and polyphemusins, were isolated from the hemocyte debris of the Japanese horseshoe crab (*Tachypleus tridentatus*) and the American horseshoe crab (*Limulus polyphemus*), which are 17-mer and 18-mer peptides, respectively (Figure 1).[39,40] Our earlier research on these peptides led to the development of T22 ([$Tyr^{5,12}$, Lys^{7}]-polyphemusin II)[41,42] and its shorter 14-mer peptide, T140, which possesses strong anti-HIV activity (Figure 2).[43] Both peptides block X4-HIV-1 entry to the cell effectively by binding specifically to CXCR4, and also inhibit Ca^{2+} mobilization induced by CXCL12 stimulation through CXCR4.[44–46] The T140 analogue exhibited a remarkable delaying of the appearance of drug-resistant strains in in vitro passage experiments using cell cultures,[47] and it was presumed that the development of T140 analogues would be useful for its suppressive effect against drug-resistant strains. T140 forms an antiparallel β-sheet structure supported by a disulfide bridge between Cys^{4} and Cys^{13} and connected by a type II' β-turn.[48] Four amino acids in T140, Arg^{2}, L-3-(2-naphthyl)alanine (Nal),[3] Tyr^{5}, and Arg^{14}, are residues crucial for significant activity.[49]

T140 is not stable in mouse/feline serum or in rat liver homogenate.[50,51] When deletion of indispensable residues (Arg^{14} in serum; Arg^{2}, Nal^{3}, and Arg^{14} in liver homogenate) from the N- or the C-terminus occurs, the efficacy of degraded peptides is drastically diminished. Modification of T140 analogues at each terminus suppresses the biodegradations and leads to development of novel and effective compounds, which shows high activity and increased biological stability. It is also noticeable that the N-terminal modification studies found an electron-deficient aromatic ring such as a 4-fluorobenzoyl moiety at the N-terminus to constitute a new pharmacophore for strong anti-HIV activity. The T140 analogues, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, which contain an N-terminal 4-fluorobenzoyl moiety, have anti-HIV activity two orders of magnitude higher than that of the original T140 peptide and enhanced stability in serum/liver homogenates (Figure 2).[52]

EFFECT OF T140 ANALOGUES AGAINST PANCREATIC CANCER, BREAST CANCER, MELANOMA, SMALL CELL LUNG CANCER, AND MULTIPLE MYELOMA

CXCR4 mRNA is expressed both in pancreatic cancer cell lines (AsPC-1, BxPC-3, CFPAC-1, HPAC, and PANC-1) and in pancreatic cancer tissues.[10] CXCL12 mRNA is expressed in pancreatic cancer tissues. Stimulation by CXCL12 induces both migration and invasion of AsPC-1 and PANC-1 in dose-dependent manners in vitro, suggesting that the interaction between CXCL12 and CXCR4 is involved in progression and metastasis of pancreatic cancer cells. The cell migration and invasion caused by CXCL12 stimulation are suppressed by T140 analogues.[11] Treatment of PANC-1 cells with CXCL12 induces a drastic increase in actin cytoskeleton (polymerization), leading to the invasion and subsequent metastasis of malignant cells into tissues. T140 analogues effectively inhibit this phenomenon.

CXCR4 and another chemokine receptor, CCR7, are highly expressed on the surface of human breast cancer cells, while CXCL12 and a CCR7 ligand, CCL21, are highly expressed in lymph nodes, bone marrow, lung, and liver, the common metastatic destinations of breast cancer. This suggests that the CXCL12-CXCR4/CCR7-CXCL12 axis might determine the metastatic destination of tumor cells.[7] Neutralizing CXCR4 with anti-CXCR4 antibodies can inhibit metastasis of breast cancer cells to the lung in mice. Practically, to evaluate the potency of CXCR4 antagonists as antimetastatic agents, we investigated the inhibitory activity of our T140 analogues against the migration of breast cancer cells in vitro and experimental metastasis of breast cancer cells in vivo.[23] The T140 analogues in dose-dependent manners inhibit the migration of a CXCR4-positive human breast carcinoma cell line MDA-MB-231 induced by CXCL12 in cell migration assays using cell culture chambers. The antimetastasis activity of the biostable CXCR4 antagonist, 4F-benzoyl-TN14003, was confirmed using experimental metastasis models of breast cancer, in which MDA-MB-231 cells were injected intravenously into the tail vein of SCID mice and trapped in the lung to which they migrated through the heart and the pulmonary artery. An effective suppression of tumor accumulation on the lung surface as a result of MDA-MB-231 metastasis can be shown in mice subcutaneously injected with an Alzet osmotic pump (DURECT, Cupertino, CA) with 4F-benzoyl-TN14003. This suggests that CXCR4 antagonists, such as T140 analogues, could replace anti-CXCR4 antibodies as neutralizers of cancer metastasis.

An excessive expression of CXCR4 enhances the metastatic accumulation of B16 melanoma cells in mouse lung, and T22 blocks pulmonary metastasis in mice injected with CXCR4-transduced B16 cells.[53] Whether T140 analogues inhibit pulmonary metastasis in mice injected with B16 cells, which are not transduced with CXCR4, was investigated.[9] Poly D,L-lactic acid (PLA) microcapsules containing 4F-benzoyl-TE14011 was adopted in experimental metastatic models of CXCR4-positive B16-BL6 melanoma cells.[9] From the PLA microcapsules this T140 analogue is released in a controlled fashion for a long period in vivo, maintaining the level of the 4F-benzoyl-TE14011 concentration in bloods. A subcutaneous administration of 4F-benzoyl-TE14011-PLA significantly decreases the number of colonies ascribed to pulmonary metastasis of B16-BL6 cells. This suggests that a controlled release of CXCR4 antagonists might lead to effective suppression of cancer metastasis.

In Western countries, small cell lung cancer (SCLC) which comprises four-fifth of lung cancers is the leading cause of death.[54] CXCR4 is highly expressed on the surface of primary tumor cells isolated from SCLC patients. CXCL12 is constitutively secreted by marrow stromal cells and plays an important role in homing of hematopoietic cells to the marrow. CXCL12 stimulates both SCLC cell invasion into the extracellular matrix, and firm adhesion to marrow stromal cells. T140 effectively suppress invasion and adhesion in vitro, confirming the involvement of the CXCL12-CXCR4 interaction in SCLC metastasis.[18] Cell adhesion-mediated drug resistance (CAM-DR) is caused by adhesion of SCLC cells to the extracellular matrix or to accessory cells within the tumor microenvironment via integrin signaling. CXCL12 induces activation of α_2, α_4, α_5, and β_1 integrins through CXCR4, and in turn the T140 analogue inhibits this activation. It was shown that stromal cells protect SCLC cells from apoptosis induced by an anticancer drug (etoposide), and that the T140 analogue inhibits this protection.[55] Thus, CXCL12-mediated CAM-DR in SCLC might be overcome by the use of T140 analogues in combination with anticancer drugs.

CXCL12 might play a potential role in the recruitment and activation of osteoclast precursors to the bone marrow because the CXCL12 level is correlated to the expression of multiple radiological bone lesions in individuals with multiple myeloma. It was shown that 4F-benzoyl-TE14011 significantly inhibits CXCL12-mediated osteoclast activity in addition to the osteoclast activity stimulated by the medium conditioned by myeloma plasma cell line (RPMI-8226) in vitro,[16] suggesting that blockade of the CXCL12-CXCR4 axis might be an effective remedy against osteolysis in multiple myeloma patients.

Taken together, CXCR4 is expressed in malignant cells in at least 23 different types of cancers,[56] including those discussed earlier. Antagonists of CXCR4 such as the T140 analogues might be useful lead compounds for the development of antimetastatic agents in several types of cancer.

ANTI-ALL AND -CLL ACTIVITY OF T140 ANALOGUES

Growth and survival of pre-B cell ALL might be caused by intimate contact with bone marrow stromal layers using the β_1 integrins. CXCR4 has been uniformly and highly expressed in the cells. CXCL12 regulates migration of these pre-B cells into stromal layers. T140 blocks CXCL12-induced chemotaxis and attenuates the migration of pre-B ALL cells into bone marrow stromal layers. Furthermore, T140 analogues enhance the antiproliferative and cytotoxic effects of other anticancer agents such as dexamethasone and vincristine. This suggests that T140 analogues might overcome CAM-DR in ALL chemotherapy.[27]

On the other hand, B cell CLL, the most common leukemia in adults in Western countries, is caused by the accumu-

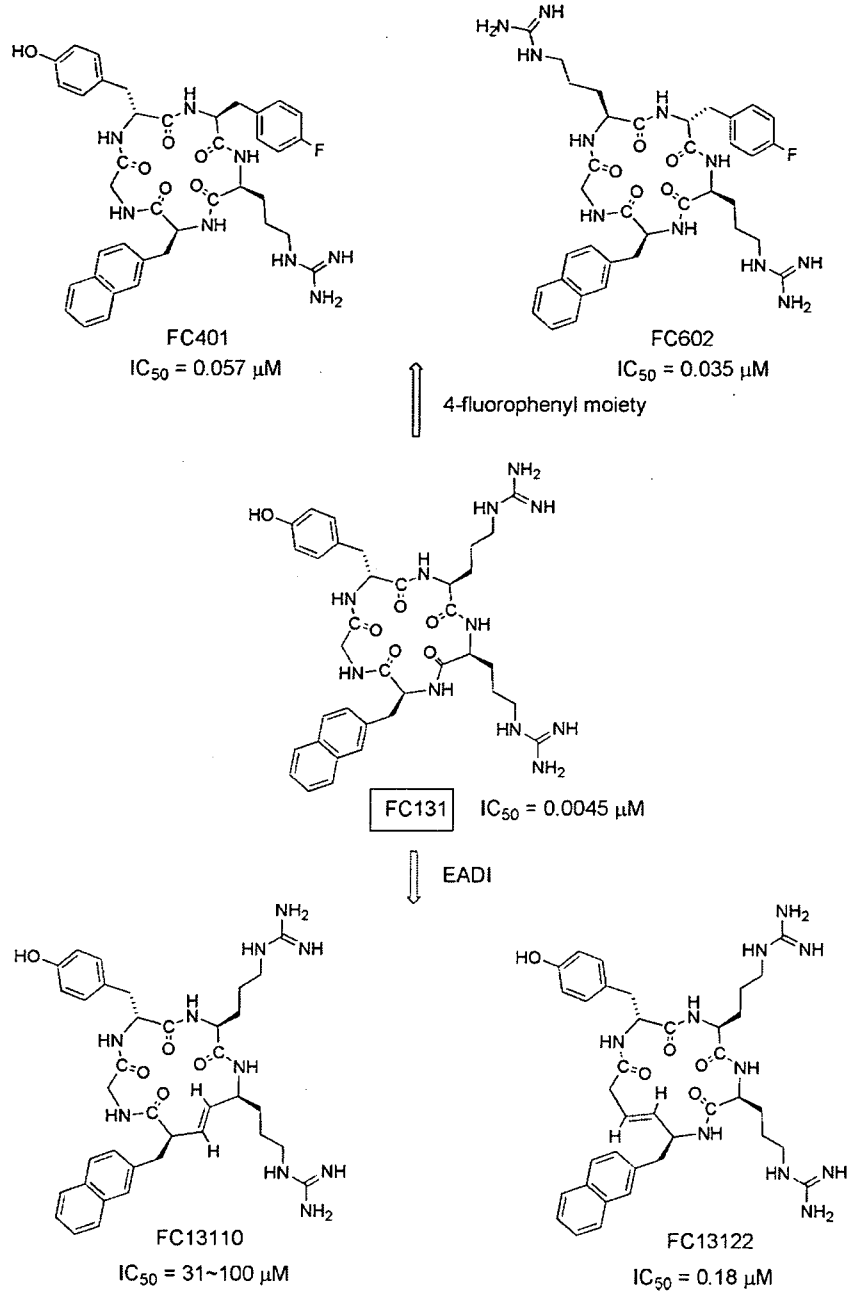


FIGURE 3 Structures of a cyclic pentapeptide FC131, L/D-Phe(4-F)-containing FC131 analogues, FC401 and FC602, and EADI-containing analogues, FC13110 and FC13122. IC_{50} values are based on the inhibition of [^{125}I]-CXCL12 binding to CXCR4 transfectants of CHO cells.

lation of long-lived, monoclonal B malignant cells in blood, secondary lymphoid organs, and bone marrow. CXCL12 released from marrow stromal cells or nurselike cells activates CLL B cells that highly express CXCR4. CXCL12 rescues the CLL B cells from apoptosis and contributes to their accumulation. Thus, the CXCL12-CXCR4 system might also be a therapeutic target in B cell CLL.[25] Practically, the T140 analogues inhibit chemotaxis of CLL cells induced by CXCL12, their migration beneath marrow stromal cells, and actin polymerization in dose-dependent manners.[26] Furthermore, the T140 analogues reduce the antiapoptotic effect of CXCL12 and then prevent stromal cells from protecting against spontaneous apoptosis of CLL cells. CLL cells are protected by cocultures with marrow stromal cells from apoptosis induced by a drug (fludarabine), followed by causing stromal CAM-DR. Treatment with T140 analogues resensitizes these CLL cells to fludarabine-induced

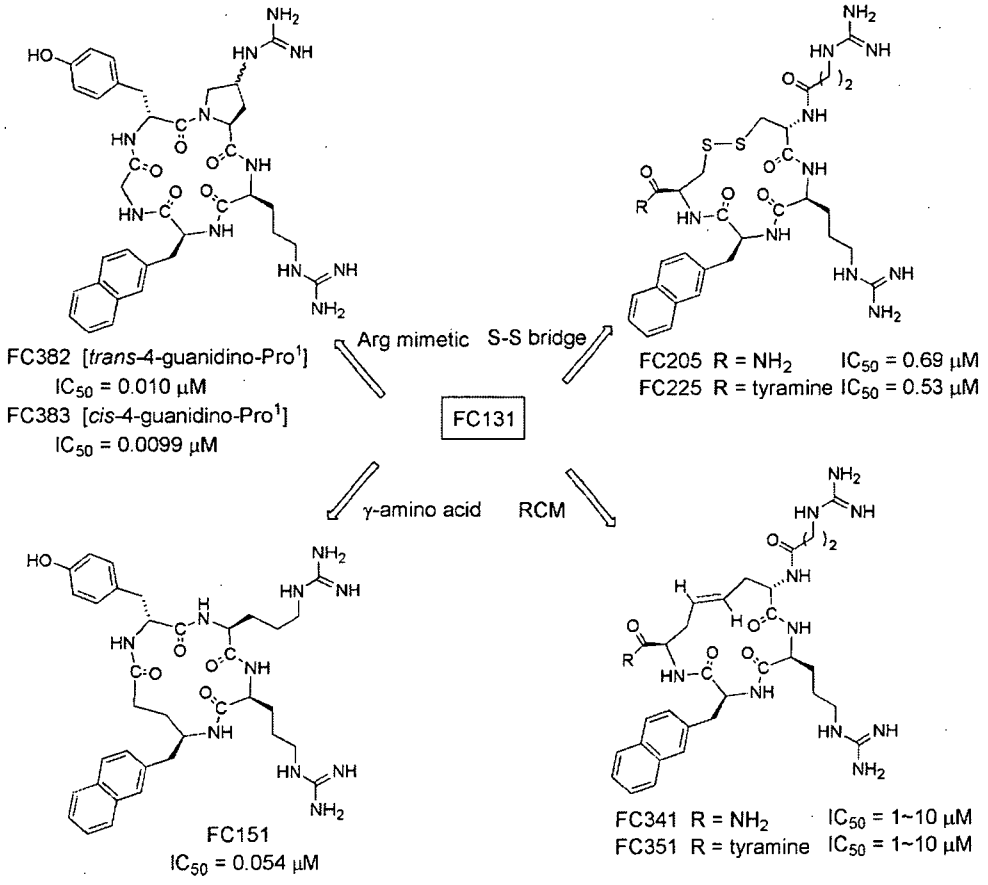


FIGURE 4 Structures of cyclic peptides containing conformationally constrained Arg mimetics, FC382 and FC383, a γ-amino acid-containing cyclic peptide FC151 and cyclic peptides bridged by a disulfide bond, FC205 and FC225, and by an olefin using RCM, FC341, and FC351. IC_{50} values are based on the inhibition of [^{125}I]-CXCL12 binding to CXCR4 transfectants of CHO cells.

apoptosis. Consequently, the T140 analogues might be able to overcome CAM-DR which is a serious problem in the clinical chemotherapy of CLL.

ANTI-RA ACTIVITY OF A BIOSTABLE T140 ANALOGUE

RA is an annoying disease, which requires novel chemotherapy. Inflammatory cytokines, such as tumor necrosis factor, (TNF)-α, interferon, IFN-γ, and the interleukins, IL-1 and IL-6, play an important role in RA.[28] The development of biological drugs such as monoclonal antibodies, which target these cytokines, has produced useful results in clinical RA therapy; however, complete curative effects have not yet been achieved. To improve chemotherapy for RA, other drugs that are independent of the functions of these cytokines are required. Collagen-induced arthritis in mice was adopted as the first in vivo experimental RA model for evaluation of the 4F-benzoyl-TN14003 activity. It was observed that several symptoms of arthritis (score increase, body weight loss, ankle swelling, and limb weight gain) were markedly suppressed in mice treated with 4F-benzoyl-TN14003 subcutaneously using an Alzet osmotic pump after treatment with the bovine type II collagen (CII) emulsion booster. In addition, the increase in levels of serum anti-bovine CII IgG2a antibody was apparently suppressed. 4F-benzoyl-TN14003 shows a blockade effect to the humoral immune response to CII. As an in vivo experimental model of the cellular immune response, delayed-type hypersensitivity (DTH) reaction induced by sheep red blood cells was performed.[57] Subcutaneous injection of 4F-benzoyl-TN14003 using an Alzet osmotic pump significantly suppressed the footpad swelling (the DTH response) in a dose-dependent manner. These results suggest that CXCR4 antagonists such as T140 analogues should also be useful leads for anti-RA agents.

CXCR4 ANTAGONISTIC ACTIVITY OF CYCLIC PEPTIDES DERIVED FROM T140

Arg^2, Nal^3, Tyr^5, and Arg^{14} of T140 are essential to high antagonistic activity toward CXCR4, as described previ-

ously.[49] These four amino acid residues are located in close proximity to each other in an antiparallel β-sheet structure. Downsizing of T140 analogues was attempted by a pharmacophore-guided approach using cyclic pentapeptide libraries involving two L/D-Arg, L/D-Nal, and L/D-Tyr in addition to Gly as a spacer. As a result, FC131 [*cyclo*(-Arg^1-Arg^2-Nal^3-Gly^4-D-Tyr^5-)], which showed strong CXCR4-antagonistic activity comparable to that of T140 (Figure 3), was obtained.[58] NMR and simulated annealing molecular dynamics revealed the near-symmetrical pentagonal backbone structure of FC131.

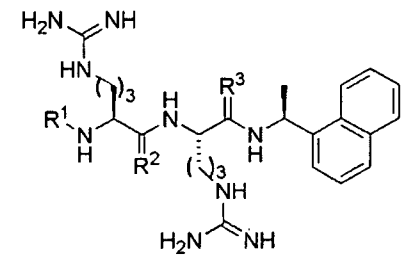
The 4-fluorophenyl moiety identified as a pharmacophoric element through the N-terminal modification study of T140 analogues[52] was introduced into cyclic pentapeptides. Since the phenol group of D-Tyr^5 could not be replaced by the 4-fluorophenyl group, the 4-fluorophenyl group was incorporated into position 1 and the resulting compound FC401 ([Phe(4-F)^1]-FC131) shows significant CXCR4-binding activity.[59] Since a second Arg residue is thought to be indispensable for high activity and an aromatic residue [L/D-Phe(4-F)] has been incorporated into position 1, D-Tyr^5 was replaced by L/D-Arg^5. Among four analogues [L/D-Phe(4-F)^1, L/D-Arg^5]-FC131, FC602, which is [D-Phe(4-F)^1, Arg^5]-FC131, shows the most potent activity, which is one order of magnitude greater than that of [D-Tyr^1, Arg^5]-FC131.[59] Thus, FC602 may be useful as a novel lead different from FC131.

In the interests of bioavailability, reduction of the peptide character of the obtained cyclic pentapeptides was pursued by introduction of (*E*)-alkene dipeptide isosteres (EADIs).[60–63] Substitution of EADIs for constituent backbone amide bonds is a common strategy for this purpose. Several FC131 analogues, such as FC13110 and FC13122, in which such isosteres were substituted for the amide bond backbones, were developed on the basis of the synthetic strategy reported previously, which utilizes the combination of stereoselective aziridinyl ring-opening reactions and organozinc-copper-mediated reactions.[64–66] Structure–activity relationship studies of the isostere-substituted compounds provided useful information concerning the reduction of peptide character.[67]

With the idea of fixing the backbone and side chain, conformationally constrained Arg mimetics, *trans*-4-guanidino-Pro and *cis*-4-guanidino-Pro, were incorporated into Arg^1 at position 1 in FC131. FC382 ([*trans*-4-Guanidino-Pro^1]-FC131) and FC383 ([*cis*-4-guanidino-Pro^1]-FC131) showed high CXCR4-antagonistic activities that were twice as strong as that of [g-Dab^1]-FC131 (g-Dab = γ-*N*-amidino-L-2,4-diaminobutyric acid), bearing the same length of the linear-type side chain of the amino acid at the position 1, while [Pro^1]-FC131 did not show so high activity (Figure 4).[68] In consideration of the fact that the introduction of a pyrrolidinyl ring caused a significant reduction of potency, it is thought that fixing the side chain effectively increased potency. NMR analysis showed similar dispositions of guanidino groups of the Arg mimetics of FC382 and FC383 in space,[68] which might be the reason for essentially no difference in potency of these peptides.

Several cyclic tetrapeptide-scaffolds have been prepared and investigated with a view to tuning the spatial arrangement of pharmacophores. FC151, a γ-amino acid-containing peptide containing 4-amino-5-naphthalen-2-yl-pentanoic acid in the place of the Nal-Gly sequence, various disulfide-bridged cyclic peptides, FC205 [*N*-3-guanidinopropanoyl-Cys(*S*-)-Arg-Nal-D-Cys(*S*-)-NH_2] and FC225 [*N*-3-guanidinopropanoyl-Cys(*S*-)-Arg-Nal-D-Cys(*S*-)-tyramine], all showed significant CXCR4-antagonistic activity (Figure 4). The cyclic compounds FC341 and FC351, which contain an olefin bridge formed by ring-closing metathesis (RCM) exhibited moderate CXCR4-antagonistic activity.[68] Attempts at further downsizing and structural tuning are currently in progress.

Next, a linear type of several low molecular weight CXCR4 antagonists were developed based on T140 analogues. Several compounds that were synthesized based on pharmacophores of T140 analogues showed significant anti-HIV activity and binding affinity for CXCR4. First, three tripeptide mimetics containing amide bonds and/or reduced amide bonds were designed based on the sequence of Arg^1-Arg^2-Nal^3 in the N-terminal region of T140 (Figures 2 and 5) and synthesized using solution-phase techniques involving



1 R^1 = H; R^2 = R^3 = O IC_{50} > 100 μM
2 R^1 = H; R^2 = O; R^3 = H, H IC_{50} = 52 μM
3 R^1 = R^2 = H, H; R^3 = O IC_{50} = 46 μM

⇩

4 R^1 = H-Tyr; R^2 = O; R^3 = H, H IC_{50} = 22 μM
5 R^1 = H-Tyr; R^2 = H, H; R^3 = O IC_{50} = 26 μM
6 R^1 = 4-fluorobenzoyl; R^2 = O; R^3 = H, H IC_{50} = 11 μM
7 R^1 = 4-fluorobenzoyl; R^2 = H, H; R^3 = O IC_{50} = 1.7 μM

FIGURE 5 Tri- and tetrapeptide mimetics with CXCR4-antagonitic activity based on the sequence of Arg^1-Arg^2-Nal^3. IC_{50} values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells.