### Blockade of CXCL12/CXCR4 Axis Ameliorates Murine Experimental Colitis


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**ABSTRACT**

Recent studies indicate that the CXCL12/CXCR4 interaction is involved in several inflammatory conditions. However, it is unclear whether this interaction has a role in the pathophysiology of inflammatory bowel disease (IBD). We investigated the significance of this interaction in patients with IBD and in mice with dextran sulfate sodium (DSS)-induced colitis and the effect of a CXCR4 antagonist on experimental colitis. First, we measured CXCR4 expression on peripheral T cells in patients with IBD. Furthermore, we investigated CXCR4 expression on leukocytes and CXCL12 expression in the colonic tissue of mice with DSS-induced colitis, and we evaluated the effects of a CXCR4 antagonist on DSS-induced colitis and colonic inflammation of interleukin (IL)-10 knockout (KO) mice. Colonic inflammation was assessed both clinically and histologically. Cytokine production from mesenteric lymph node cells was also examined. CXCR4 expression on peripheral T cells was significantly higher in patients with active ulcerative colitis (UC) compared with normal controls, and CXCR4 expression levels of UC patients correlated with disease activity. Both CXCR4 expression on leukocytes and CXCL12 expression in colonic tissue were significantly increased in mice with DSS-induced colitis. Administration of a CXCR4 antagonist ameliorated colonic inflammation in DSS-induced colitis and IL-10 KO mice. CXCR4 antagonist reduced tumor necrosis factor-α and interferon-γ production from mesenteric lymph node cells, whereas it did not affect IL-10 production. The percentage of mesenteric Foxp3+ CD25+ T cells in DSS-induced colitis was not affected by CXCR4 antagonist. These results suggest that blockade of this chemokine axis might have potential as a therapeutic target for the treatment of IBD.

Inflammatory bowel disease (IBD), including Crohn’s disease (CD) and ulcerative colitis (UC), is a chronic, relapsing, and remitting condition with unknown etiology that exhibits various features of immunologic abnormality (Fiocchi, 1998; Blumberg et al., 1999). The pathogenesis of IBD involves the interplay of environmental, genetic, microbial, and immune factors, which result in chronic intestinal inflammation. Among these factors, immune cells, including CD4+ T cells, have crucial roles in the pathophysiology of IBD (Sartor, 1995). It is important to note that the expression of chemoattractive proteins (chemokines) and adhesion molecules expressed on various cells of the intestinal tissues regulate the recruitment of such inflammatory cells. Therefore, regulation of the migration of inflammatory leukocytes into the intestinal tissues is considered to be a therapeutic option for patients with IBD.

Chemokines are small cytokines exhibiting selective chemoattractive properties for targeting leukocytes. Based on

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**ABBREVIATIONS:** IBD, inflammatory bowel disease; CD, Crohn’s disease; UC, ulcerative colitis; DSS, dextran sulfate sodium; IL, interleukin; KO, knockout; Abs, antibodies; FACS, fluorescence-activated cell sorter; MTWSI, modified Truelove Witts severity index; GFP, green fluorescent protein; TF14016, 4-fluorobenzoyl-Arg-Arg-Nal-Cys-Tyr-Cit-Lys-D-Lys-Pro-Tyr-Cit-Cys-Arg-NH2, (knockout; Abs, antibodies; FACS, fluorescence-activated cell sorter; MTWSI, modified Truelove Witts severity index; GFP, green fluorescent protein; TF14016, 4-fluorobenzoyl-Arg-Arg-Nal-Cys-Tyr-Cit-Lys-D-Lys-Pro-Tyr-Cit-Cys-Arg-NH2, (knockout; Abs, antibodies; FACS, fluorescence-activated cell sorter; MTWSI, modified Truelove Witts severity index; GFP, green fluorescent protein; TF14016, 4-fluorobenzoyl-Arg-Arg-Nal-Cys-Tyr-Cit-Lys-D-Lys-Pro-Tyr-Cit-Cys-Arg-NH2,
the motif of the first two cysteines, chemokines are categorized into four major subfamilies: CC, CXC, C, and CX3C chemokines. The most important function of chemokines is their ability to regulate leukocyte recruitment, retention, and trafficking in inflamed tissues (Homay et al., 2002; Ogawa et al., 2004). The expression of several chemokines is increased in the colonic tissue of murine experimental colitis models (Andres et al., 2000; Ajubeor et al., 2004) as well as in colonic biopsy specimens from patients with IBD (Gjisebers et al., 2006). Therefore, much attention has been directed to such chemokines as one of the therapeutic targets for patients with IBD.

CXCL12 was first characterized as pre-B cell growth stimulating factor (Nagasawa et al., 1996a) and is constitutively expressed in stromal cells within the bone marrow (Tokoyoda et al., 2004). Its primary physiologic receptor is CXCR4, which also functions as an entry receptor for strains of human immunodeficiency virus (Bleul et al., 1996). The CXCL12/CXCR4 chemokine axis has an important role in the migration of progenitors during embryonic development of the cardiovascular, hematopoietic, and central nervous systems (Nagasawa et al., 1996b; Tachibana et al., 1998; Zou et al., 1998). Thus, this chemokine axis is considered to serve as a homing beacon during differentiation.

Recent studies showed that this chemokine axis is also involved in several inflammatory diseases, including rheumatoid arthritis (Nanki et al., 2000; Tamamura et al., 2004; Haas et al., 2005), inflammatory liver diseases (Terada et al., 2003; Wald et al., 2004), uveitis (Curnow et al., 2004), and pulmonary fibrosis (Phillips et al., 2004). Nanki et al. (2000) reported that memory T cells highly express CXCR4, and the CXCL12 concentration is extremely high in the synovial fluid of patients with rheumatoid arthritis. Furthermore, Wald et al. (2004) reported that CXCL12 is up-regulated in the endothelium of neovessels of chronically inflamed liver tissues, and CXCR4+ lymphocytes are increased in hepatitis C virus-infected liver tissues with chronic hepatitis. These findings suggest that the CXCL12/CXCR4 axis has an important role in cell trafficking not only in the homeostatic state but also under inflammatory conditions. However, it is not clear whether this chemokine axis is involved in the pathophysiology of IBD.

To elucidate the role of the CXCL12/CXCR4 interaction in colonic inflammation, we first investigated CXCR4 expression on peripheral T cells in patients with IBD. Next, we investigated the expression of both CXCR4 in peripheral T cells and its ligand CXCL12 in the colonic tissue in a dextran sulfate sodium (DSS)-induced colitis mouse model, and then we examined the effect of a CXCR4 antagonist on DSS-induced colitis and interleukin (IL)-10 knockout (KO) mice.

Materials and Methods

**Human Samples of Peripheral Blood Cells.** Peripheral blood cells were obtained from the following: 17 patients (7 men, 10 women) with active UC; 9 patients (2 men, 7 women) with inactive UC; 8 patients (6 men, 2 women) with active CD; 16 patients (13 men, 3 women) with inactive CD; 6 patients (4 men, 2 women) with infectious colitis; and 5 patients (5 men) as normal controls. Lymphocytes of blood samples were separated by Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). Cells were stained with appropriate fluorochrome-conjugated antibodies (Abs) and were incubated for 30 min at 4°C. Monoclonal antibody against human CD3 (UCHT1) was obtained from eBioscience (San Diego, CA), and anti-human CXCR4 (12G5) and IgG isotype control were obtained from Dako Denmark A/S (Glostrup, Denmark). Stained cells were analyzed with a fluorescence-activated cell sorter (FACS) (Beckman Coulter, Fullerton, CA). To determine disease activity, modified Truelove Witts severity index (MTWSI) was used for patients with UC and CD activity index was used for patients with CD. UC patients who scored >4 on the MTWSI and CD patients who scored >150 on CD activity index were classified to have active disease. Informed consent was obtained from each patient, and the experimental designs of these studies were approved by the Kyoto University Hospital Ethics Committee.

**Animals.** Female C57BL/6 mice (8–10 weeks of age, weighing 17–20 g) obtained from Japan SLIC Inc. (Shizuoka, Japan) and CXCL12/green fluorescent protein (GFP) knockin mice (Tokoyoda et al., 2004) were used for the experiments. They were fed with standard laboratory chow and tap water ad libitum. All mice were housed in specific pathogen-free conditions in the animal facility of Kyoto University. The studies were approved by the animal protection committee of our institution.

**Experimental Design of DSS-Induced Colitis.** For the induction of colitis, C57BL/6 mice (wild-type and CXCL12/GFP knockin mice) were given 2.5% DSS (molecular mass, 36–50 kDa; MP Biomedicals, Irvine, CA) in their drinking water for 5 days (from day 0 to 4). On day 5, they were switched to regular drinking water. Normal control mice received regular drinking water throughout the experiment.

**CXCR4 Antagonist TF14016.** CXCR4 antagonist TF14016 was obtained from Prof. N. Fuji (Kyoto University, Kyoto, Japan) (Tamamura et al., 2003, 2004). One hundred micrograms of TF14016 dissolved with 200 μl of phosphate-buffered saline (PBS) or 200 μl of PBS alone was administered intraperitoneally once a day during the study period (from day 0 to day 10). Body weight was measured daily throughout the experiment, and mice were killed by cervical dislocation at 10 days after the start of DSS administration. The colonic tissues and mesenteric lymph nodes (MLNs) were removed from each mouse and examined as described below. At necropsy, the length from the ileoceleal junction to the anal verge was measured as the colonic length.

**Microscopic Assessment of Colonic Damage.** The distal third of the colon was evaluated because this segment is most severely affected in DSS-induced colitis (Okayasu et al., 1990). The entire colon was removed, opened longitudinally, and washed with PBS. The distal third of the colon was dissected and then the longitudinal section (1.5 cm from the anal verge) was prepared. For section staining, samples were fixed in acetone and stained with hematoxylin and eosin, and histologically was analyzed in a blind manner. Histological damage was quantified by the histological scoring system described by Williams et al. (2001). In brief, the sections were graded to access inflammation severity, inflammation extent, and crypt damage. The grading index for inflammation severity was as follows: 0, none; 1, mild; 2, moderate; and 3, severe. The grading index for inflammation extent was as follows: 0, none; 1, mucosa; 2, mucosa and submucosa, and transmural. The grading index for crypt damage was as follows: 0, none; 1, basal one-third damaged; 2, basal two-thirds damaged; 3, crypts lost but surface epithelium present; and 4, crypts and surface epithelium lost. Each of these grades was also scored according to the percentage of involvement (0%, 1, 1–25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%). Each sub-score (inflammation severity score, inflammation extent score, and crypt damage score) was the product of the grade multiplied by the percentage of involvement. The total colitis score was the sum of the three subscores.

**Flow Cytometry Analysis.** For analyzing changes of CXCR4 expression on leukocytes, peripheral blood was taken by tail cutting at day 0, 3, 7, and 10 after start of DSS administration. Erythrocytes were removed using lysis buffer (0.16 M NH₄Cl and 0.017 M Tris, adjusted to pH 7.2), and leukocyte population was resuspended in Dulbecco’s modified Eagle’s medium containing 2% fetal calf serum. Cells (1 × 10⁵) were stained with the appropriate fluorochrome-
conjugated Abs and were incubated for 30 min at 4°C. Monoclonal Abs against granulocyte-differentiation antigen-1 (Gr-1) (RB6-8C5), macrophage adhesion molecule-1 (Mac-1) (M1/70), CXCR4, CD4 (L3T4), CD8 (Ly-2), CD25 (T4), and rat IgG isotype control were obtained from BD Pharmingen (San Diego, CA). Regulatory T cells (Foxp3+ cells) were stained with the Mouse Regulatory T cell Staining Kit (eBioscience). Stained cells were analyzed with FACSCalibur (BD Biosciences, San Jose, CA). Dead cells were excluded by propidium iodide staining. The data are presented as relative fluorescence intensity or geometric mean fluorescence intensity depicting the degree of the expression of the surface molecule on the cell.

**Immunohistochemical Staining and Confocal Microscopy.**

For section staining, samples were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose/PBS or fixed with acetone for 2 min. Cryostat sections of colonic tissues were stained and mounted with Permafluor (Beckman Coulter). All confocal microscopy was carried out on a LSM 510 META (Carl Zeiss Inc., Thornwood, NY). Monoclonal Abs against α-smooth muscle actin (α-SMA), platelet endothelial cell adhesion molecule-1 (PECAM-1), CD4, CD8, Gr-1, Mac-1 (BD Pharmingen), and mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) were used. For secondary antibodies, Alexa Fluor 546 goat anti-rat or rabbit IgG and Cy5 donkey anti-rat IgG (Jackson ImmunoResearch Laboratories Inc.) were used. Biotinylated antibodies were visualized with streptavidin-Alexa Fluor 546 (Invitrogen, Carlsbad, CA) or streptavidin-Cy5 (Jackson ImmunoResearch Laboratories Inc.).

**Isolation and Stimulation of Mesenteric Lymph Node Cells.**

Mesenteric lymph node cells were collected under sterile conditions in ice-cold medium, and lymph nodes were mechanically disrupted and filtered through a cell strainer (70 μm). Cells (2 × 10^6/well) were incubated with immobilized anti-CD3 (5 μg/ml, anti-mouse CD3ε; BD Pharmingen) in 200 μl of culture medium for 72 h at 37°C in 5% CO₂ air. Cytokine levels in the supernatant of the culture medium were measured by enzyme-linked immunosorbent assay kit (eBioscience).

**Quantitative Analysis of RNA Expressions.**

Samples of colonic tissue for mRNA isolation were removed from the distal third of the colon at 10 days after the start of DSS administration. Total RNA was extracted using the guanidium isothiocyanate-phenol-chloroform method. RNA (1 μg) was reverse transcribed with MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA), and the resulting complementary DNAs (50 ng/reaction mixture) were analyzed for CXCL12 mRNA expression by real-time polymerase chain reaction using an ABI Prism 7700 sequence detection system (Applied Biosystems). The reaction mixtures were incubated for 2 min at 50°C, denatured for 10 min at 95°C, and subjected to 45 amplification cycles consisting of annealing and extension at 60°C for 1 min and 1 cycle followed by denaturation at 95°C for 15 s. The primers and probes used for this experiment were obtained from Applied Biosystems. To quantify isolated RNA and to measure cDNA synthesis efficiency, target cDNAs were normalized to the expression levels of the endogenous reference housekeeping gene, 18S ribosomal RNA (rRNA). The oligonucleotide primers used for CXCL12 rRNA amplification and detection were 5′-CAG CAT GCC ACG TCA AGT-3′ (forward) and 5′-CGC CCG TGC AAC AAT CGT AA-3′ (reverse). The oligonucleotide primers used for 18S rRNA amplification and detection were 5′-TAGAGTGGTTTCAAGCAGGCCC-3′ (forward) and 5′-CCACACAAATAGAACCGGGTG-3′ (reverse). For simplicity, the expression level of the target gene was expressed as values relative to the control in the experiment.

**Migration Assay.**

Fresh bone marrow cells and MLN cells from C57BL/6 mice were preincubated with or without 1 μM of TF14016 for 30 min at 37°C. Then they were transferred to the upper layer of 3- or 5-μm pore polycarbonate membrane (Transwell; Corning Inc., Corning, NY) overlaid with collagen-coated 100 ng/ml CXCL12. After 2 h, a fraction of the cells that migrated to the lower chamber was stained and analyzed by flow cytometry.

**Experimental Design of IL-10 KO Mice.**

Fifty milligrams/mice per day of CXCR4 antagonist, TF14016, or PBS alone was administered intraperitoneally to IL-10 KO mice at 6 weeks of age. Twenty-eight days (4 weeks) after the start of treatment, TF14016 or PBS-treated mice were killed for histological analysis of colonic tissue.

**Microscopic Assessment of Chronic Damage in IL-10 KO Mice.**

Mice were monitored for clinical signs of colitis, including diarrhea and weight loss. At necropsy, samples of the colon (transverse, distal, and proximal) and the rectum were collected and histopathologically examined as described previously. For each section, inflammation (macrophage, lymphocyte, and neutrophil infiltration in the lamina propria or submucosa) was scored for severity according to the following criteria: normal, 0; minimal, 1; mild, 2; moderate, 3; marked, 4; and severe, 5. Gland loss and epithelial hyperplasia were scored by percentage of area involved: none, 0; 1–10% of the mucosa affected; 2, 11–25% affected; 3, 26–50% affected; 4, 51–75% affected; and 5, 76–100% affected. The summed scores for inflammation (lamina propria or submucosa), gland loss, and gland hyperplasia were then determined for each animal. One pathologist without knowledge of this study scored the sections according to standard criteria.

**Statistical Analysis.**

All normalized data were represented as mean ± S.D. In human experiments assessing CXCR4 expression on T cells, the Kruskal-Wallis test with Bonferroni/Dunn analysis was used. A linear regression analysis was used to access the quantitative relation between the intensity of CXCR4 expression on T cells and disease activity. In animal experiments, for two-group comparisons, the Student’s unpaired t and Mann-Whitney U tests were used. For multiple comparisons, the Kruskal-Wallis test with Bonferroni/Dunn analysis for nonparametric analysis or two-way analysis of variance with Bonferroni/Dunn ad hoc analyses for parametric analysis was performed. A repeated analysis of variance was performed assess the effect of TF14016 treatment on changes in body weight after an induction of DSS-induced colitis. In experiments using TF14016 treatments, because control mice showed a negligible level of inflammatory cytokine in tissue, one comparison using the unpaired t test was performed. Any significant interaction was detected in multiple comparisons (data not shown). A two-tailed p value of <0.05 was considered statistically significant. The SPSS software package for Windows (version 10; SPSS, Tokyo, Japan) was used.

**Results**

**CXCR4 Expression on Peripheral T Cells Was Increased in Patients with Active UC.**

First, we examined whether this chemokine axis is involved in human IBD. We investigated CXCR4 expression on peripheral T cells from patients with IBD and with acute infectious colitis compared with those from healthy controls. CXCR4 expression on peripheral T cells in patients with active UC was significantly higher than that in inactive UC and controls, whereas this expression in patients with active CD, inactive CD, inactive UC, and infectious colitis was not different from that in controls (Fig. 1a). Furthermore, there was a significant correlation between CXCR4 expression on peripheral T cells and disease activity (MTWSI) in patients with active UC (Fig. 1b).

**Cxcl12 Expression Was Increased in the Colonic Tissue of DSS-Induced Colitis Mice.**

Next, we investigated the changes of CXCL12 expression in colonic tissue before and after DSS administration using CXCL12/GFP knockin mice. Confocal microscopic analysis was performed before and at 10 days after the start of DSS administration. CXCL12-expressing cells were mainly observed in the perivascular sites of the normal colonic mucosa (Fig. 2, a–d).
The CXCL12-expressing cells were morphologically considered to be reticular cells adjacent to the endothelial (PECAM-1⁺ and/or α-SMA⁺) cells because they were negative for PECAM-1, α-SMA, and other blood cell marker’s staining.

At 10 days after the start of DSS administration, the number of CXCL12-expressing cells was increased in the inflamed colonic mucosa compared with normal colonic tissues (Fig. 2, e and f). Expression of CXCL12 mRNA was also significantly higher in the colonic tissue of mice with DSS-induced colitis (at 10 days after DSS administration) than that of normal mice (Fig. 2g). These results suggest that enhanced CXCL12 expression in the colonic mucosa might induce the migration of inflammatory cells into the inflamed colonic tissues of mice with DSS-induced colitis.

**CXCR4 Expression on Peripheral T Cells Is Increased in Mice with DSS-Induced Colitis.** To investigate whether the CXCL12/CXCR4 chemotactic axis is involved in DSS-induced colonic inflammation, we analyzed CXCR4 expression on peripheral blood cells from mice with DSS-induced colitis. Serial changes in CXCR4 expression in the peripheral blood cells of these mice were evaluated by flow cytometry. FACS analysis revealed that CXCR4 expression on peripheral granulocytes (Gr-1⁺ cells) was significantly increased at 7 days and normalized at 10 days after the start of DSS administration. CXCR4 expression on both peripheral CD4⁺ and CD8⁺ cells from mice with DSS-induced colitis was also significantly increased at 7 and 10 days (CD4⁺), and at 3, 7, and 10 days (CD8⁺) after the start of DSS administration, compared with the levels before DSS administration (day 0) (Fig. 2h).

**A CXCR4 Antagonist Efficiently Inhibits Leukocyte Migration to CXCL12 in Vitro.** To evaluate whether a CXCR4 antagonist, TF14016, efficiently blocks leukocyte migration toward CXCL12, we performed an in vitro leukocyte chemotaxis assay. Migration analysis showed that the CXCL12-induced chemotactic responses of bone marrow granulocytes and mesenteric CD4⁺ as well as CD8⁺ T cells were significantly inhibited by TF14016 (Fig. 3).

**Effect of a CXCR4 Antagonist on DSS-Induced Colitis.** To investigate whether the blockade of the CXCL12/CXCR4 chemotactic axis attenuates inflammation in DSS-induced colitis, we intraperitoneally administered a CXCR4 antagonist, TF14016, to mice with DSS-induced colitis. During DSS administration, the percentage of body weight in control mice (DSS alone) decreased. The body weight loss of mice with DSS-induced colitis treated with TF14016, however, was significantly lower than that of nontreated mice from 7 to 10 days after the start of DSS administration (Fig. 4a). There was also a significant difference in colonic length between nontreated and TF14016-treated mice with DSS-induced colitis (Fig. 4b).

In mice with DSS-induced colitis, the histologic findings demonstrated epithelial destruction, remarkable inflammatory cell infiltration with lymphoid aggregation, and submucosal edema (Fig. 5a). In contrast, in TF14016-treated mice with DSS-induced colitis, epithelial destruction and inflammatory cell infiltration, including lymphoid aggregation, were obviously reduced compared with nontreated mice (Fig. 5b). As a result, the total colitis score in TF14016-treated mice with DSS-induced colitis was significantly lower than that in the nontreated mice with DSS-induced colitis (Fig. 5c). Immunohistochemical analysis revealed that the number of CD4⁺ and CD8⁺ T cells and lymphocyte aggregation in the lamina propria were remarkably decreased in TF14016-treated mice with DSS-induced colitis compared with the nontreated mice with DSS-induced colitis (Fig. 6, a and b).

**Blockade of the CXCL12/CXCR4 Axis Reduces the Production of Proinflammatory Cytokines, but Not IL-10 Production, from MLN Cells in Mice with DSS-Induced Colitis.** We also measured the cytokine production from unseparated MLN cells from mice with DSS-induced colitis treated with TF14016. The production of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and IL-10 from unseparated MLN cells was significantly increased in mice with DSS-induced colitis. However, TF14016 treatment significantly reduced the increased production of TNF-α and IFN-γ. In contrast, TF14016 treatment had no effect on the production of IL-10 in mice with DSS-induced colitis (Fig. 7).

**Blockade of the CXCL12/CXCR4 Axis Did Not Block the Migration of Foxp3⁺ Regulatory T Cells to MLN.** To elucidate why IL-10 production in MLN cells was not
changed by TF14016 treatment despite the decrease in the severity of colitis, we focused on the effect of TF14016 on regulatory T cells, one of MLN's IL-10-producing cells. First, we analyzed CXCR4 expression on regulatory T cells in MLN. In mice with DSS-induced colitis, CXCR4 expression on mesenteric CD4<sup>+</sup>CD25<sup>+</sup> T cells (nonregulatory T cells) was significantly elevated compared with that of normal mice. In contrast, there was no significant difference in CXCR4 expression on mesenteric CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells between DSS-induced colitis mice and normal mice (Fig. 8a).

We then analyzed the population of Foxp3<sup>+</sup> regulatory T cells in MLN. FACS analysis revealed that although the percentage of Foxp3<sup>+</sup> CD25<sup>+</sup> regulatory T cells in MLN was significantly increased in mice with DSS-induced colitis com-

Fig. 2. CXCL12 expression in the colonic tissue and serial changes in CXCR4 expression on peripheral blood cells of mice with DSS-induced colitis. a to d, colonic tissue sections from CXCL12/GFP knockin mice were stained with antibodies against PECAM-1 (blue) and α-SMA (red) (b–d, magnified; b and c, vertical section; d, horizontal section). White bars indicate 20 μm (a) and 10 μm (b–d). e to g, the change of CXCL12 expression was analyzed in CXCL12/GFP knockin mice before (e) and 10 days after the start of DSS administration (f, g). CXCL12 mRNA expression in colonic tissues was quantified by quantitative real-time polymerase chain reaction. Results are presented as means ± S.D. (n = 7 in each group). * p < 0.05 compared with control mice without DSS. h, Peripheral blood cells were obtained from mice with DSS-induced colitis before and 3, 7, and 10 days after the start of DSS administration. Serial changes of CXCR4 expression on peripheral granulocytes (Gr-1<sup>+</sup> cells) and T cells (CD4<sup>+</sup> and CD8<sup>+</sup> cells) were evaluated by FACS. Results are presented as means ± S.D. (n = 5 in each group). * p < 0.05 compared with normal mice (day 0).

Fig. 3. TF14016 inhibits chemotactic responses to CXCL12 in vitro. CXCL12-induced chemotactic responses of bone marrow granulocytes (Gr-1<sup>+</sup> cells) and mesenteric T cells (CD4<sup>+</sup> and CD8<sup>+</sup> cells) were assessed in the presence (f) or absence (e) of TF14016. Data are expressed as percentage (%) of input of the loaded cells to the lower chamber. Results are presented as means ± S.D. (n = 4 in each group). * p < 0.05 compared with leukocytes in the absence of TF14016.
pared with normal mice, TF14016 treatment did not affect the percentage of Foxp3+/CD25+ regulatory T cells in mice with DSS-induced colitis (Fig. 8b).

**Effect of a CXCR4 Antagonist on IL-10 KO Mice.** All IL-10 KO mice treated with TF14016 or PBS survived throughout the study period. Histologic examination of colonic tissue from PBS-treated IL-10 KO mice demonstrated epithelial hyperplasia, crypt abscess, and severe acute and chronic cellular infiltration and lymphoid aggregation in lamina propria (Fig. 9a). In contrast, colonic inflammation and amount of lymphocyte aggregation were significantly decreased in IL-10 KO mice treated with a CXCR4 antagonist (Fig. 9b). As shown in Fig. 9c, colonic histological scores in IL-10 KO mice treated with TF14016 were significantly lower than in those treated with PBS alone.

**Discussion**

The present study demonstrated that CXCR4 expressions on peripheral T cells of active UC patients were significantly higher than those of normal controls, and they were significantly correlated with disease activity. Furthermore, the expression of both CXCR4 on peripheral T cells and CXCL12 in the colonic tissue of mice with DSS-induced colitis were significantly increased compared with normal mice. More importantly, the administration of a CXCR4 antagonist decreased the severity of DSS-induced colitis and colonic inflammation of IL-10 KO mice, as assessed by clinical, histologic, and immunologic parameters. These results strongly suggest that the CXCL12/CXCR4 chemokine axis has an important role in the pathophysiology of IBD, particularly in UC, and that antagonist has a therapeutic effect on experimental colitis.

Previous reports demonstrated that CXCL12 mRNA expression ratio in biopsy specimens from the colonic mucosa of
patients with UC was significantly higher than that in patients with CD (Katsuta et al., 2000). In addition, IL-4 induces surface CXCR4 expression on human T cells, suggesting that this receptor might be associated with T helper (Th)2 cells (Jourdan et al., 1998; Annunziato et al., 1999). On the contrary, CXCR4/CXCL12 axis is associated with several inflammatory diseases such as rheumatoid arthritis, in which IL-6 and TNF-α are mainly involved. Our data also showed that CXCR4 expression in peripheral T cells is likely to be increased in patients with active CD compared with inactive CD, although there was no significant difference. These data may suggest that Th1 immune response is involved in CXCR4 expression. Fuss et al. (1996) reported that lamina propria CD4+ T lymphocytes from UC patients produce both Th1 cytokine IFN-γ and Th2 cytokine IL-5. Several reports showed that anti-TNF-α antibody administration reduced intestinal inflammation in patients with UC (Rutgeerts et al., 2005). Taken together, the immune response in acute flare of UC is very complicated because both Th1 and Th2 immune response and several proinflammatory cytokines are involved. Considering these data, Th2 immune response (IL-4 and IL-5) might augment CXCR4 expression on peripheral T cells under the condition of enhanced Th1 immune response. Accordingly, CXCR4 expression on peripheral T cells was strongly increased in active UC patients and that its expression level was proportional to the disease activity. In this regard, CXCR4 expression on peripheral T cells of patients with UC could be a good marker of their disease activity. However, further investigation will be needed to elucidate the exact mechanism of significant up-regulation of CXCR4 in UC patients compared with CD patients.

In animal models, we investigated CXCL12 expression in colonic tissues using CXCL12/GFP knockin mice because its expression was hardly observed by immunohistochemistry. In the steady state, CXCL12 expression was observed in submucosal lesions, mainly adjacent to the vascular endothelial cells. Based on their location, the majority of CXCL12-expressing cells are considered to be pericytes, mesenchymal-like cells located close to small blood vessel walls. Previous studies (Imai et al., 1999a,b; Peled et al., 1999a; Ponomaryov et al., 2000) reported that human and murine endothelial cell lines express CXCL12 mRNA and produce CXCL12 protein. CXCL12 is expressed on neoblood vessel endothelial cells in the portal tracts and on active lymphoid follicles, suggesting the involvement of CXCL12 in the initial entry of cells into the liver during chronic hepatitis B and hepatitis C virus infection (Wald et al., 2004). In contrast to those previous reports, we found that endothelial cells did not express CXCL12 in colonic tissues, although the reason for the discrepant results is not known. We also revealed that CXCL12 expression in the inflamed colonic tissue of mice with DSS-induced colitis was significantly increased compared with normal mice. These data might suggest that circulating CXCR4+ leukocytes are attracted to inflamed colonic tissues by the increased number of CXCL12-expressing cells at the perivascular sites.

To further clarify the role of the CXCL12/CXCR4 chemokine axis, we next observed CXCR4 expression on peripheral leukocytes in mice with DSS-induced colitis and found that CXCR4 expression on granulocytes and CD4+ and CD8+ lymphocytes is increased in mice with DSS-induced colitis compared with wild-type mice. Thus, it may be reasonable to speculate that those CXCR4-expressing T cells are recruited to the inflamed mucosa of DSS-induced colitis by enhanced expression of CXCL12. The fact that the CXCR4 antagonist ameliorates DSS-induced colitis may further support such possibility. Recent studies have indicated that various cytokines and growth factors, including IL-2, IL-4 (Jourdan et al., 1998), IL-6, stem cell factor (Peled et al., 1999b), vascular endothelial growth factor, basic fibroblastic growth factor, and transforming growth factor-β (Buckley et al., 2000), can enhance CXCR4 expression on a number of cell types. In DSS-induced colitis, the production of various cytokines and growth factors is increased (Dieleman et al., 1994; Matsuura et al., 2005). Accordingly, these cytokines or growth factors might be involved in the induction of CXCR4 expression on granulocytes and T cells in DSS-induced colitis.

CXCR4 expression on T cells was significantly higher at 7 and 10 days after DSS administration than before DSS administration, whereas CXCR4 expression on granulocytes was increased only at day 7. In general, colonic inflammation induced by DSS administration is considered to be mainly caused by direct chemical injury to colonic epithelial cells and activation of resident macrophages and neutrophils (Okyasu et al., 1990; Cooper et al., 1993; Dieleman et al., 1994). However, we found little difference in the infiltration of mononuclear cells or neutrophils in colonic mucosa between mice with DSS-induced colitis and normal mice at 10 days.
after starting DSS administration (data not shown). These data suggest that granulocytes are attracted to the inflamed colonic tissue soon after DSS administration by other chemokines like CXCL8/IL-8 and CXCL10/inducible protein-10, the expressions of which are increased during the early phase of DSS-induced colitis (Murano et al., 2000; Melgar et al., 2006). On the other hand, the sustained increase of CXCR4 expression on peripheral T cells at the late phase of DSS-induced colitis, as observed in the present study, suggests that T cells are involved in sustained colonic inflammation after DSS administration in C57BL/6 mice. Melgar et al. (2006) reported that only 5-day administration of DSS induces chronic inflammation of the colon in C57BL/6 mice. Taken together, the increased expression of CXCR4 on T cells might mainly contribute to the development of chronic colitis induced by DSS administration.

An important finding of our study is that blocking the CXCL12/CXCR4 chemotaxis axis significantly ameliorated DSS-induced colitis and colonic inflammation of IL-10 KO mice. Indeed, administration of a CXCR4 antagonist, TF14016, significantly reduced body weight loss of mice with DSS-induced colitis. Moreover, histologic findings revealed that the number of infiltrated lymphocytes and amount of lymphocyte aggregation in both DSS-induced colitis mice and IL-10 KO mice were significantly decreased when treated by the administration of a CXCR4 antagonist. These data strongly support an idea that the CXCL12/CXCR4 chemokine interaction has an important role in the development of experimental colitis, probably through the recruitment of CXCR4-positive lymphocytes to the inflamed colonic mucosa.

It should be noted that although the CXCR4 antagonist significantly reduced the expressions of TNF-α and IFN-γ, it did not alter IL-10 production from MLN cells in mice with DSS-induced colitis, despite the amelioration of colonic inflammation. To clarify the reason for the lack of the effect of the CXCR4 antagonist on IL-10 production, we observed the effect on regulatory T cells in MLN. We first found that although CXCR4 expression on mesenteric CD4⁺CD25⁺ cells

![Fig. 7. The cytokine production of MLN cells from DSS-induced colitis mice treated with or without TF14016. MLN cells from mice 10 days after the start of DSS administration were cultured with immobilized anti-CD3. Supernatants were collected after 72 h, and TNF-α (a), IFN-γ (b), and IL-10 (c) were tested by enzyme-linked immunosorbent assay. Results are presented as means ± S.D. (n = 8 in each group). *, p < 0.05 compared with DSS-induced colitis mice without TF14016 treatment. n.s., not significant.](image)

![Fig. 8. CXCR4 expression on mesenteric regulatory T cells and the population of mesenteric Foxp3⁺ regulatory T cells in mice with DSS-induced colitis. a, CXCR4 expression on mesenteric CD4⁺CD25⁺ and CD4⁺CD25⁻ cells was analyzed before (white bar) and 10 days after starting DSS administration (black bar). b, the population of mesenteric Foxp3⁺ regulatory T cells from TF14016-treated and the nontreated mice with DSS-induced colitis were analyzed by FACS. Results are presented as means ± S.D. (n = 5 in each group). *, p < 0.05 compared with normal mice. n.s., not significant.](image)
The Role of CXCL12/CXCR4 Axis in Inflammatory Bowel Disease

Colonic inflammation is mainly attributed to its increased expression on CD4⁺CD25⁺ T cells. Taken together, the present data suggested that the ameliorating action of the CXCR4 antagonist on DSS-induced colitis is mainly due to its inhibitory effect on migration of CD4⁺CD25⁺ T cells with increased CXCR4 expression that seem to be involved in exacerbation of intestinal inflammation in IL-10 KO mice that lack the function of regulatory T cells.

In conclusion, taken together with human and mouse studies, CXCL12/CXCR4 chemokine axis seems to be involved in the pathophysiology of IBD, especially ulcerative colitis. Considering the potent anti-inflammatory effect of the CXCR4 antagonist on experimental colitis, the CXCR4 antagonist might be one of the therapeutic options for patients with IBD. However, further clinical trials will be needed to assess this possibility.

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References


Katayama T, Lim C, Shimoda K, Shibata K, Mitra P, Banner FP, Mori M, and Barnard was increased in DSS-induced colitis, there was no significant change in CXCR4 expression on mesenteric CD4⁺CD25⁺ cells. Furthermore, although the percentage of Foxp3⁺CD25⁺ cells in mice with DSS-colitis was significantly higher than normal mice, administration of the CXCR4 antagonist did not affect the percentage of Foxp3⁺CD25⁺ cells. These data indicated that the lack of the effect of CXCR4 antagonist on IL-10 production seems to result from both no increase of CXCR4 expression on regulatory T cells in mice with DSS-induced colitis and no change of the percentage of regulatory T cells by the CXCR4 antagonist administration. Moreover, our data showed that the increased CXCR4 expression on CD4⁺ T cells in mice with