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Reciprocal regulation of CXCR4 and CXCR7 in intestinal mucosal homeostasis and inflammatory bowel disease

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RECEIVED JANUARY 6, 2011; REVISED APRIL 10, 2011; ACCEPTED MAY 8, 2011. DOI: 10.1189/jlb.0111101

ABSTRACT

IBDs are characterized by increased influx of immune cells to the mucosa of genetically susceptible persons. Cellular migration to injury sites is mediated by chemokines. CXCL12 is a ubiquitous, constitutive chemokine that participates in stem cell proliferation and migration and mediates T lymphocyte migration to inflamed tissues. We have recently reported that CXCL12 and its receptor, CXCR4, are expressed in normal and more prominently, inflamed human intestinal mucosa. However, the interactions and roles of CXCL12 and its receptors, CXCR4 and the recently discovered CXCR7, in intestinal inflammation have not been defined. In the present study, we further dissected the effects of CXCL12 on lymphocytes in intestinal homeostasis and inflammation and delineated the interplay between CXCL12 and its receptors CXCR4 and CXCR7. To that end, fresh mononuclear cells were isolated from mucosa and PB of healthy or IBD patients. Phenotypical and functional assays were conducted using flow cytometry, Transwell migration chambers, and ELISA. The data show that CXCL12-mediated migration of T cells is CXCR4- but not CXCR7-dependent. T cell activation reciprocally regulates CXCR7 and CXCR4 expression and migratory capacity. IBD PBTs expressed more CXCR7 than normal PBTs. Finally, T cells attracted by CXCL12 are mostly of a memory phenotype. In conclusion, the present study suggests that the interplay between CXCL12 and its receptors affects homeostasis and inflammation in the intestinal mucosa. *J. Leukoc. Biol.* 90: 583–590; 2011.

Introduction

The IBDs, CD and UC, are characterized by augmented influx of $CD4^+$ T lymphocytes to the LP. Chemokines secreted to the ECM and chemokine receptors expressed by lymphocytes contribute extensively to this recruitment process [1, 2]. The chemokine CXCL12 (formerly, stromal cell-derived factor 1α) is ubiquitously secreted by hematopoietic and nonhematopoietic cells, including human IECs [3–5]. CXCL12 participates in stem cell proliferation and migration [6, 7] and mediates T lymphocyte migration to inflamed tissues [8]. Despite its original classification as a constitutive chemokine [4, 9, 10], CXCL12 has been recently suggested to have a role in inflammatory disorders [11–14]. For example, CXCL12 is up-regulated and redistributed across endothelial cells in multiple sclerosis [15], and in rheumatoid arthritis, enhanced secretion of CXCL12 from synoviocytes, mediated by inflammatory stimuli, such as IL-17, IL-1β, and CD40 ligand, has been shown recently [16]. In addition, an increase in the number of CXCL12-secreting cells in the submucosa of patients was reported in asthma [17]. These findings confirm the involvement of CXCL12 in Th1 and Th2 inflammatory responses.

We have reported recently that CXCL12 and its receptor CXCR4 are expressed in normal and more prominently, inflamed human intestinal mucosa [18]. We demonstrated that IBD and non-IBD mucosal lymphocytes are capable of migrating toward CXCL12 in vitro and that there was accumulation of $CXCR4^+$ cells in the vicinity of CXCL12-expressing IECs.

Recently, CXCR7 was reported as a receptor for CXCL12 [9]. However, the functional role of CXCR7 remains unknown. CXCR7 has been proposed to contribute to adhesion, cell survival, and transendothelial migration. It was also suggested that CXCR7 acts as a "decoy" receptor for the CXCL12–CXCR4 interaction [19–21], but the precise interactions of CXCL12 and its receptors CXCR4 and CXCR7 in the intestine have not been defined.

The aim of the present study, therefore, was to further investigate the effects of CXCL12 on lymphocyte migration in intestinal homeostasis and inflammation and to dissect the in-

Abbreviations: APC=antigen-presenting cell, CD=Crohn's disease, IBD=inflammatory bowel disease, IEC=intestinal epithelial cell, LP=lamina propria, LPMC=lamina propria mononuclear cell, LPT=lamina propria T cell, PB=peripheral blood, PBT=peripheral blood T cells, UC=ulcerative colitis

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terplay among CXCL12, CXCR4, and CXCR7, specifically asking whether these interactions have differential effects on lymphocyte function.

MATERIALS AND METHODS

Patients

Surgical specimens were taken from bowel resections performed at the Tel Aviv Sourasky Medical Center (Israel). Mucosa from IBD or non-IBD (control) samples was used, and IECs and LPMCs were freshly generated (see below). Normal control (noninflamed) mucosa was taken from resections performed as a result of colonic tumors and located at least 10 cm away from the tumor. In several cases, autologous PBMCs were generated as well. The Helsinki Committee at the Tel Aviv Sourasky Medical Center approved the study.

Mononuclear cell isolation

For PBMC isolation, heparanized blood from patients with active or inactive disease was isolated using Ficoll-Paque Plus (GE Healthcare, Otelfingen, Switzerland) density gradient. In brief, blood was diluted 1:3 with PBS and centrifuged through a Ficoll gradient for 30 min at 2000 rpm with the brake off. PBMCs were collected from the interface and washed with PBS. Cells were resuspended in culture medium (RPMI 1640, 10% FCS, 50 u/ml penicillin, 50 μg/ml streptomycin, 2 mmol/L glutamine, all from Biological Industries, Beit Haemek, Israel). For LPMC isolation, mucosa was separated from surgical specimens and washed with PBS (Sigma-Aldrich, St. Louis, MO, USA) and subsequently with RPMI 1640. After mincing, tissue was incubated for 15 min with 1 mM DTT (Sigma-Aldrich) at room temperature. Next, slices were incubated twice, each time for 30 min in 3 mg/ml Dispase II (Roche, Mannheim, Germany) in RPMI at 37°C. The mucosa was then incubated with 1 mg/ml collagenase (Sigma-Aldrich)/50 ml RPMI 1640 containing 5 μg/ml DNase (Sigma-Aldrich) for 45 min at 37°C. Medium containing LPMCs was collected and centrifuged for 7 min at 2000 RPM. Percoll gradient (GE Healthcare; 40%) was used for LPMC separation. LPMCs collected from the pellet were resuspended in RPMI 1640/10% FCS (Biological Industries) and incubated at 37°C, 5% CO_2.

T cell purification

LPMCs and PBMCs were incubated for 10 min at 4°C with magnetically labeled mAb against CD14, CD16, CD19, CD36, CD56, CD123, and glycophorin A. Afterward, cells were incubated with antibiotin microbeads for 15 min at 4°C. T cells were negatively selected using MACS (Miltenyi Biotec, Sunnyvale, CA, USA) magnetic separation columns. Purified $CD3^+$ lymphocytes (>90% $CD3^+$ purity, assessed by flow cytometry) were counted and resuspended in RPMI 1640/10% FCS.

Flow cytometry

Lymphocyte surface marker expression by T lymphocytes was determined using flow cytometry (FACSCalibur, Becton Dickinson, San Diego, CA, USA), using CellQuest four-color analysis software. Briefly, pelleted lymphocytes were washed with PBS/1% BSA. Directly conjugated mAb against the following surface molecules and the relevant isotype controls were added: CD3-Per-CP, CD2-Per-CP, CXCR4 (CD184)-PE, CXCR7-PE, CD45RA-FITC, CD45RO-APC, or CD25-APC (all from Bactlab Diagnostics, Caesarea, Israel). Nonrelevant isotype antibodies (from Bactlab Diagnostics) were used as controls. Cells were incubated for 30 min at 4°C. After being washed with PBS/1% BSA, cells were fixed in 300 μl 1% paraformaldhyde (Sigma-Aldrich) and used for acquisition studies.

Transwell migration assays

In vitro migration of purified $CD3^+$ T cells was assessed using Costar Transwells (Corning, NY, USA). Transwells with 5 μm pore polycarbonate filters were precoated with fibronectin for 1 h at 37°C. Subsequently, cells ($2–5\times10^5$) in 100 μl PBS/0.1% BSA were placed in the upper chamber. The lower chamber contained 600 μl RPMI alone or RPMI with different chemokines. Cells were allowed to migrate for 3 h at 37°C. Afterward, cells that had transmigrated to the lower wells were collected and counted. The percentage of migrating cells was calculated by flow cytometry or a counting grid. For flow cytometry, cells were stained with fluorochrome-conjugated antibodies against the aforementioned surface molecules. Acquisition of cells was by a FACS for a fixed period of time (90 s). Percent migration was calculated as the number of migrating cells divided by the total number of cells placed in the upper wells, counted in the same time period, and multiplied by 100. In some experiments, lymphocytes were preincubated with antibodies against CXCR4-binding (clone 12G5, R&D Systems, Minneapolis, MN, USA) or CXCR7 (clone 11G8)- or CXCR7-binding compounds (CCX771 and CCX733, both kindly provided by ChemoCentryx (Mountain View, CA, USA) for 30 min, washed, and placed on the upper chambers of the Transwells for the migration experiments.

Lymphocyte-stimulation studies

Nontissue culture 24-well plates (Falcon, Franklin Lakes, NJ, USA) were coated with anti-CD3 (1 μg/ml), with or without anti-CD28 (2 μg/ml), or with 1 μg/ml anti-CD2 (all purchased from Serotec, Oxford, UK) in PBS. Plates were incubated overnight at 4°C. After blocking for 30 min with PBS/1% BSA at room temperature, 1×10^6 cells in 1 ml RPMI 1640/10% FCS were stimulated with the above-mentioned antibodies for 24 h at 37°C.

Cytokine secretion

Cytokine secretion was assessed using ELISA (R&D Systems), according to the manufacturer's instructions. Briefly, 96-well plates were coated with primary antibodies against IFN-γ, IL-4, IL-5, or IL-17. After inhibition of nonspecific binding (0.5% BSA), standards and samples were added for 2 h, followed by incubation with a secondary antibody and addition of streptavidin-HRP. Tetramethylbenzidine (DakoCytomation, Carpentaria, CA, USA) was used as the color substrate. The reaction was stopped using 2 N H_2SO_4. Samples were read at 450 nm using KC Junior ELISA analysis software (BioTek, Winooski, VT, USA).

Statistics

Statistical analysis was evaluated using the unpaired Student's t test. P values <0.05 were considered to indicate a significant difference.

RESULTS

Differential expression of the CXCL12Rs

We previously reported that in IBD, CXCR4 expression by T lymphocytes is high and comparable in the PB and LP. We now compared CXCR4 and CXCR7 expression in the PB and LP of IBD patients and controls. To that end, $CD3^+$ lymphocytes from IBD PB or LP were isolated, and receptor expression was assessed using flow cytometry. Supporting our previous report [18], >90% of PBTs and LPTs express CXCR4, without differences between IBD patients and controls (**Fig. 1**). By and large, the percentage of cells expressing CXCR7 was lower compared with CXCR4. However, although a significantly higher percentage of IBD PBTs expressed CXCR7 compared with controls (CD: 35.7±9.4%, UC: 18.2±5.8%, normal: 7.7±2.8%; $P \leq 0.05$ vs. normal PBTs), the percentage of CXCR7-expressing LPTs in IBD and normal control patients was comparable. These results suggest that the environment affects the expression of CXCR7: while in the PB, the normal PBTs had lower CXCR4 expression in the LP, stimulation and costimulation may have increased CXCR7 expres-

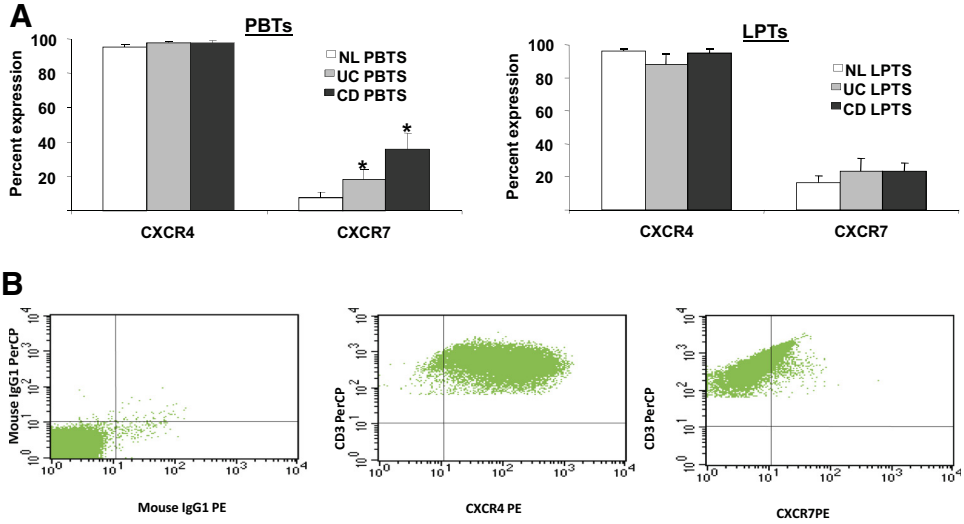

Figure 1. PBTs from IBD patients express more CXCR7 than normal PBTs. PBTs (upper panel) from normal (NL; n=7), CD (n=6), and UC (n=5) subjects and LPTs (lower panel) from normal (n=10), CD (n=9), and UC (n=5) subjects were isolated. CXCR4 and CXCR7 expressions were assessed using flow cytometry. (A) CXCR4 was highly expressed by PBTs and LPTs (>90% expression). CXCR7 was uniformly expressed by LPTs (~20% expression), but PBTs from IBD patients expressed more CXCR7 than normal PBTs (*P≤0.05 vs. normal PBTs). (B) Representative dot blot from PBTs of UC patient.

sion so that comparable CXCR7 expression in normal and IBD LPTs is noticed.

CXCR4 and CXCR7 expression is inversely regulated

We next examined CXCR4 and CXCR7 expression by stimulated T lymphocytes. As shown in **Fig. 2,** nearly all PBTs (93.9±1.4%, Fig. 2A, upper panel) and LPTs (94.6±1.9%, Fig. 2A, lower panel) expressed CXCR4, which decreased significantly following CD3/CD28 stimulation [PBTs (53.9±7.2%; P≤0.05 vs. no stimulation); LPTs (60.5±10.2%; P≤0.02 vs. no stimulation)]. In contrast, CD3/CD28 stimulation significantly increased the percentage of CXCR7-expressing PBTs

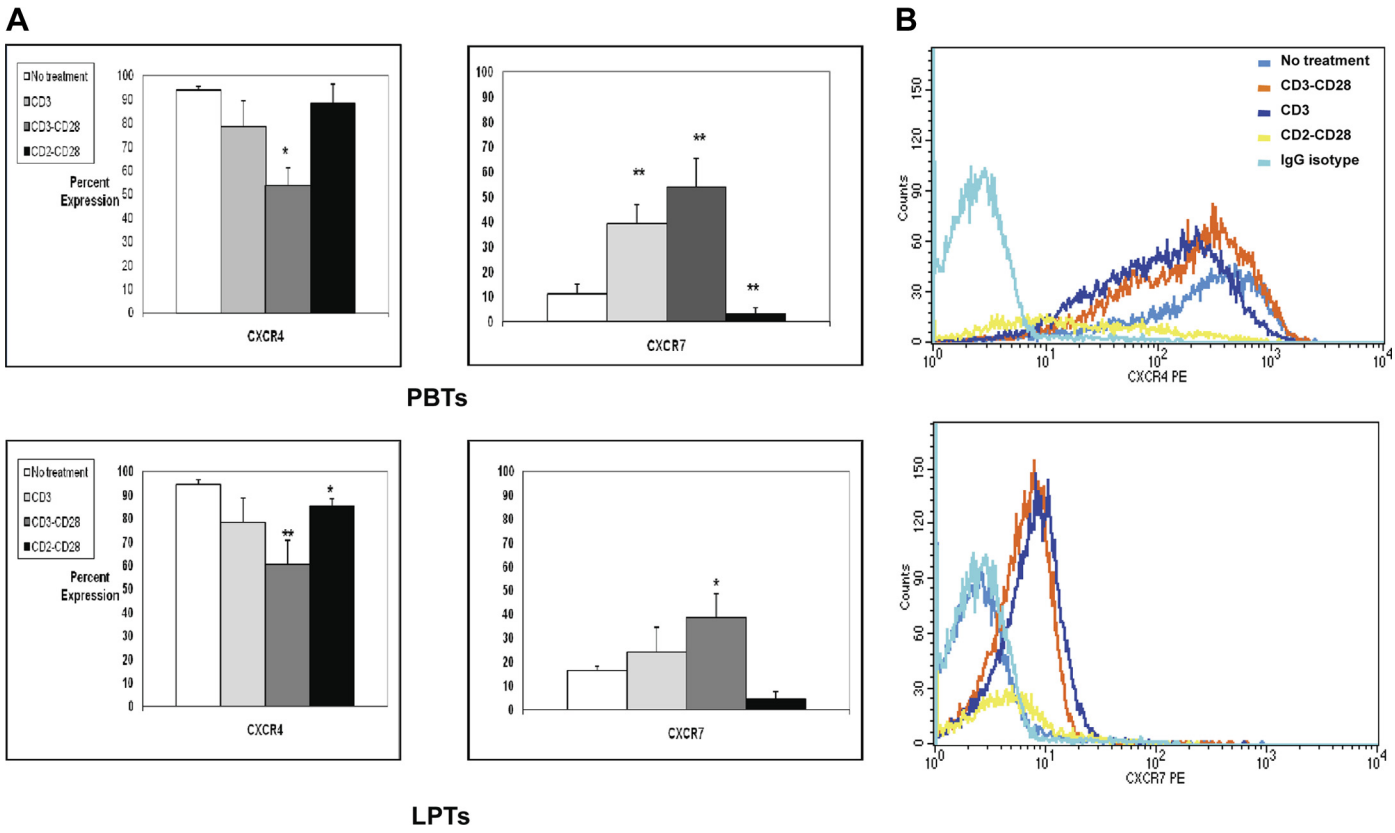

Figure 2. CXCR4 and CXCR7 are differentially expressed upon stimulation. Isolated CD3$^+$ PBTs (n=6, upper panels) or LPTs (n=6, lower panels) were stimulated for 24 h with antibodies against CD3 or CD2, with or without antibodies against CD28, using plate-bound antibodies. (A) CXCR4 and CXCR7 expression was assessed using flow cytometry (*P≤0.05 and **P≤0.02 vs. untreated cells). (B) Representative histogram depicting the influence of different stimulations on receptor expression.

(53.9±11.4%; $P \leq 0.008$ vs. unstimulated PBTs) and LPTs (38.5±4.3; $P \leq 0.05$ vs. unstimulated LPTs). Interestingly, CD2/CD28 stimulation had no effect on the percentage of CXCR4-expressing cells. However, it significantly decreased the percentage of CXCR7-expressing PBTs (3.2±2.5%; $P \leq 0.02$ vs. unstimulated PBTs) and LPTs (6.3±3.5%; P=NS vs. unstimulated LPTs). These results suggest that the expression of CXCR4 and CXCR7 depends on the activation status, as well as pathway, i.e., CD2/CD28 or CD3/CD28.

IBD PBTs are hyporesponsive to CD3/CD28 stimulation

To examine whether CXCL12R expression by IBD PBTs would be differentially affected by CD3/CD28 stimulation, we compared the stimulation-induced expression of CXCR4 and CXCR7 in IBD patients. As shown in **Fig. 3A**, the receptor expression upon CD3/CD28 stimulation was similarly regulated in IBD. However, response was decreased in UC and even more so in CD compared with normal control PBTs. Thus, the percentage of CXCR4-expressing cells decreased, whereas CXCR7-expressing cells increased to a lesser degree in IBD compared with normal controls. By analyzing the relative influence of CD3/CD28 stimulation in each experiment and calculating fold change in CXCR4- or CXCR7-expressing cells, we confirmed significant hyporesponsiveness of IBD PBTs to activation (Fig. 3B).

These results suggest that CXCR7 and CXCR4 expression by IBD PBTs is less affected by CD3/CD28 stimulation compared with normal PBTs. This may further suggest that the T cell response to CXCL12, after TCR triggering in IBD, is aberrant, contributing to lymphocyte accumulation in areas of high CXCL12 expression.

CD3/CD28 stimulation decreased T cell migration toward CXCL12

The results above suggest that T cell activation may be accompanied by decreased migration. Of note, chemokine receptors can be altered during inflammation, with consequent modification of chemokine-mediated migration as well as receptor expression [22, 23]. We thus examined how in vitro activation of T cells affects their migration toward CXCL12. As shown in **Fig. 4**, untreated PBTs and LPTs had a high migratory capacity toward CXCL12. Activation with anti CD3/CD28 significantly inhibited CXCL12-directed migration of PBTs and LPTs. This suggests that the activation status indeed affects migration. Interestingly, stimulation with anti-CD3 alone or anti-CD2/CD28 resulted in decreased migration of LPTs but not PBTs, suggesting that LPTs are more sensitive to activation-induced down-regulation of CXCL12-mediated migration. Of note, we were unable to discern different migratory capabilities in stimulated cells from IBD or normal cells (results not shown).

CXCL12-mediated migration is CXCR7-independent

The migration studies suggest that migration is CXCR4- but not CXCR7-dependent, as CXCR4 expression decreases in parallel to the decreased migration, and CXCR7 expression and migration are discordant. We now wished to directly assess the role of CXCR4 and CXCR7 in CXCL12-directed migration. To this end, we examined the effect of CXCR4 and CXCR7 blocking on lymphocyte migration. As shown in **Fig. 5**, antibodies against CXCR4 significantly inhibited PBT migration. In contrast, neither mAb against CXCR7 nor small molecule CXCR7 inhibitors (all generously supplied by ChemoCentryx) affected CXCL12-mediated migration of PBTs. Thus, the results so far suggest that the decreased lymphocyte migration shown is CXCR4- but not CXCR7-dependent.

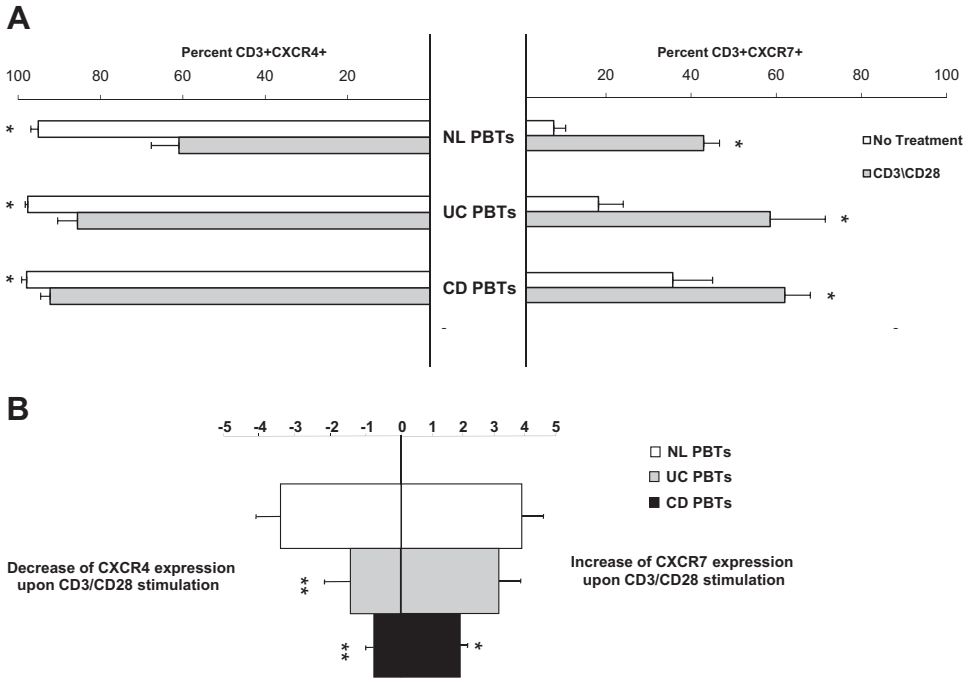

Figure 3. CXCR4 and CXCR7 expression by IBD PBTs is less influenced by CD3/CD28 stimulation. PBTs from normal (n=10), CD (n=6), and UC (n=5) subjects were left untreated or stimulated for 24 h with antibodies against CD3/CD28. (A) The cells were then collected and examined for the CXCL12 receptors using flow cytometry. (B) The results are analyzed for relative influence by fold increase or fold decrease of receptor expression after stimulation (% expression before stimulation/% expression after stimulation×100; $^{**}P \leq 0.005$ and $^{*}P \leq 0.03$ vs. normal PBTs).

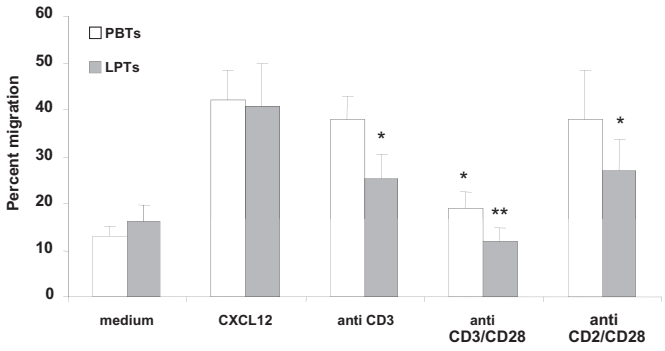

Figure 4. Migration to CXCL12 is abolished by activation with CD3/CD28. PBTs (n=8) and LPTs (n=4) were isolated, and $CD3^+$ lymphocytes were untreated or stimulated with antibodies against CD2 or CD3, with or without CD28 for 24 h. Using the Transwell assay, cells in the upper well were allowed to migrate toward CXCL12 in the lower well. Results are represented as percentage of migrating cells out of the total number in the upper well (*P≤0.05 and **P≤0.02 vs. migration toward CXCL12).

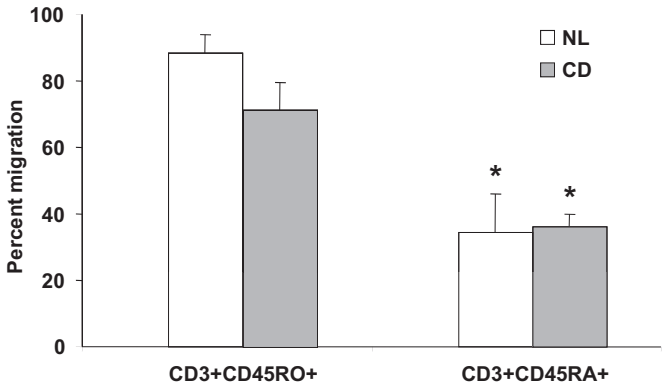

Figure 6. CXCL12 preferentially attracts $CD3^+CD45RO^+$ cells. PBTs (n=6–8) migrated toward CXCL12, as described in Materials and Methods. Migrating cells were collected and examined for surface marker expression using flow cytometry. The percentage of $CD3^+CD45RO^+$ cells migrating toward CXCL12 was significantly higher compared with migrating $CD3^+CD45RA^+$ (*P≤0.002) in normal and CD. The results were calculated by gating on a migrating subpopulation compared with the total from the same subpopulation.

CXCL12 preferentially attracts memory T lymphocytes

To examine whether CXCL12 attracts specific T cell subsets, we compared characteristics of CXCL12-attracted T cells. As shown in **Fig. 6**, significantly more $CD3^+CD45RO^+$ PBTs migrated toward CXCL12 compared with $CD3^+CD45RA^+$ T cells (P≤0.002). A similar pattern was observed in PBTs obtained from CD patients (P≤0.05). Thus, CXCL12 preferentially attracts memory T cells in normal controls and IBD patients.

Analysis of cytokine secretion by T cells migrating toward CXCL12

As previous reports suggested that CXCL12 may contribute to Th1- and Th2-mediated [15–17, 24, 25], as well as Th17-mediated, inflammatory processes [26], we further examined whether T cells migrating toward CXCL12 are skewed toward a Th1, Th2, or Th17 response. To this end, we performed further Transwell migration assays toward CXCL12. $CD3^+$ T lymphocytes that migrated toward CXCL12 were collected from the lower wells, and cytokine secretion was compared with that of equal cell numbers of lymphocytes randomly migrating toward medium. Supernatant was collected after 24 h of CD3/CD28 stimulation for ELISA assays. As seen in **Fig. 7**, although IFN-γ secretion was comparable among normal and IBD PBTs, LPTs generated from the small bowel of CD patients secreted significantly higher levels of IFN-γ (1466.0±356 pg/ml) than all other LPT groups examined (CD-colon LPTs: 288.8±218.2 pg/ml, UC LPTs: 346.1±94.7 pg/ml, normal control LPTs: 77.1±35.0 pg/ml; P≤0.03 vs. small bowel CD). Differences in IL-4 secretion between PBTs and LPTs were also observed: normal CXCL12-directed PBTs secreted significantly higher levels of IL-4 compared with randomly migrating PBTs (15.6±3.3 vs. 5.6±2.2 pg/ml, respectively; P≤0.01). In contrast, CXCL12-directed CD PBTs secreted comparable IL-4 levels with randomly migrating CD PBTs (7.7±2.2 vs. 10.4±2.1 pg/ml; P=NS). IL-4 secretion, by CXCL12-directed CD LPTs (both from the small bowel and the colon), was significantly lower compared with that from randomly migrating CD LPTs (5.3±1.9 vs. 14.8±3.5 pg/ml; P≤0.03), suggesting that CXCL12-directed CD LPTs are not Th2-skewed. IL-4 levels in UC LPTs were below detection levels. IL-17 secretion by CXCL12-attracted and nonattracted PBTs and LPTs was comparably high (data not shown), without differences between IBD and normal controls. IL-5 secretion was below detection levels for all samples tested (data not shown). Thus, the cytokine secretion patterns suggest that T cells migrating toward CXCL12 in controls and IBD are not Th1-, Th2-, or Th17-specific. However, CXCL12-directed CD LPTs are skewed toward a higher IFN-γ and lower IL-4 secretion pattern.

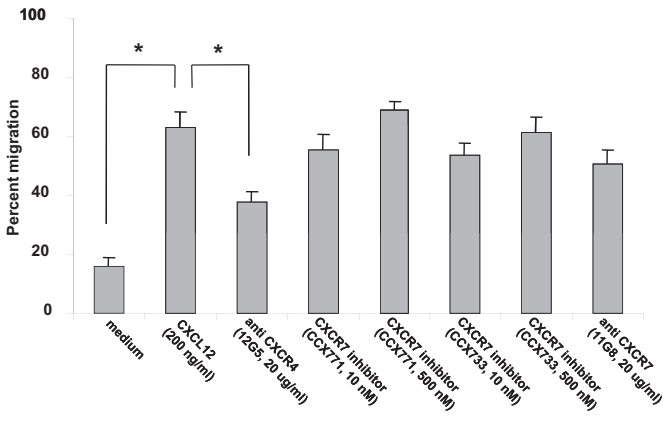

Figure 5. CXCL12-mediated migration is CXCR4- but not CXCR7-dependent. PBTs (n=6) were preincubated for 30 min with anti-CXCR4 antibodies, anti-CXCR7 (11G8) antibodies, or CXCR7 inhibitor compounds (CCX771 and CCX733), followed by Transwell migrations toward CXCL12 (*P≤0.02 vs. migration toward CXCL12 without pretreatment).

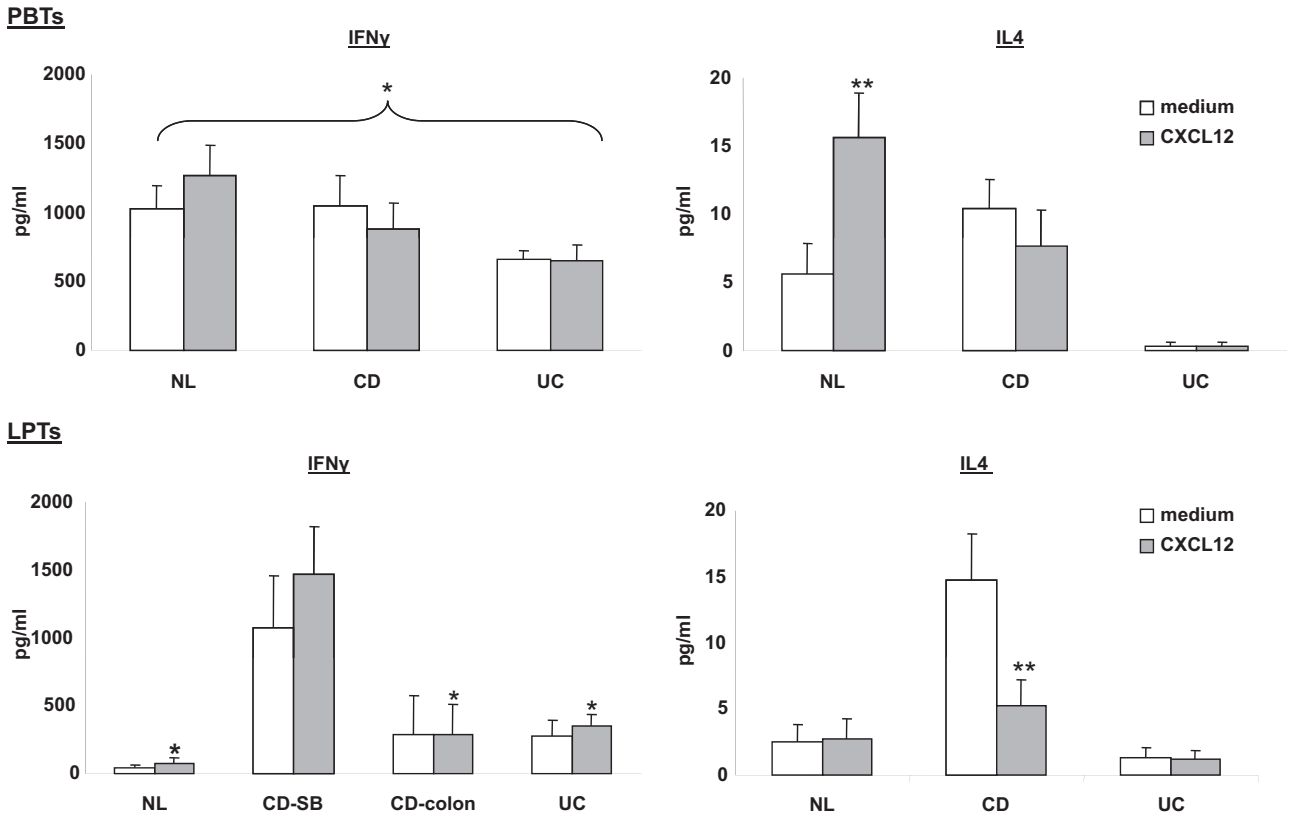

Figure 7. CXCL12 attracts non-Th2-secreting CD T cells. Isolated PBTs (n=6 in each group) and LPTs from normal (n=8), CD (n=5), and UC patients (n=5) were subject to Transwell assay, as mentioned above. Migrating T cells, to medium or CXCL12, were collected and stimulated with CD3/CD28. After 24 h, the cells were harvested, and the supernatant was collected for ELISA assays. All stimulated PBTs produced high levels of IFN-γ. UC PBTs secreted less IFN-γ compared with normal PBTs (upper left, *$P \leq 0.03$). IL-4 was secreted from normal, migrating PBTs (upper right, **$P \leq 0.02$ compared with nonmigrating, normal PBTs) and from CD PBTs. IFN-γ secretion, by migrating CD LPTs generated from the small bowel (CD-SB), was significantly higher compared with all other groups tested (lower left, *$P \leq 0.04$). IL-4 secretion by migrating CD LPTs was significantly lower compared with nonmigrating CD LPTs (lower right, **$P \leq 0.03$ vs. CD LPTs migrating toward medium).

DISCUSSION

CXCL12 is a ubiquitous chemokine with pleiotropic, physiological effects in numerous tissues, expressed by a variety of cells, including epithelial cells [27–29]. Although CXCL12 was long-considered as being a constitutive chemokine, recent reports have implicated a putative role for CXCL12 in joint, lung, and brain inflammatory processes [8, 11, 15–17, 24, 25]. We recently reported up-regulation of CXCL12 in intestinal inflammation and its ability to attract PBTs and LPTs in vitro and in vivo [18]. Moreover, Mikami et al. [30] used small molecule inhibitors of the interaction of CXCL12 with its receptor CXCR4 to ameliorate experimental colitis. In the present study, the interactions of CXCL12 with lymphocyte subsets and the mechanistic role of CXCL12Rs, CXCR4 and CXCR7, in IBD and normal control patients was investigated.

Our main findings were that CXCL12-mediated migration of T cells is CXCR4- but not CXCR7-dependent. Accordingly, CXCL12-mediated migration is abrogated upon T cell activation, and it correlates with decreased CXCR4 expression. Interestingly, the same T cell activation mediates up-regulation of CXCR7 expression, strengthening the finding that the main role of CXCR7 is not mediation of migration. Noteworthy, CXCL12-attracted T cells were mostly of a memory phenotype.

CXCL12 was documented to be up-regulated in the mucosa of IBD patients compared with normal controls [18, 31, 32]. Yet, the functional role and expression of its receptors, CXCR4 and CXCR7, in mucosal and peripheral lymphocytes in IBD and normal mucosa have eluded full delineation.

In the current study, we show that CXCR4 is ubiquitously expressed on T cells, whereas CXCR7 expression is restrained to a small population of $CD3^+$ LPTs and PBTs. Our novel finding that CXCR7 expression by IBD PBTs is increased suggests an involvement of this receptor in intracompartment migration in IBD. CXCR7 was reported to regulate CXCL12-mediated transendothelial migration [20, 33]. As such, it is reasonable to consider that increased expression of CXCR7 in the PB of IBD patients could foster increased influx of T cells to sites of mucosal inflammation. Published reports of CXCR7 expression on leukocytes are contrasting and range from nonexistent [19, 34] to high [9]. Contrasting results could pertain to cell manipulations or source of cells. Importantly, our results are specifically relevant to IBD, as CXCR7 expression by freshly isolated human T cells from the PB or LP of IBD patients and normal controls was assessed. Thus, further scrutiny of this unique receptor is still expected.

It should be borne in mind that CXCR7 is also a receptor for CXCL11. Thus, our results might pertain to an involvement of CXCL11 in IBD. Although there are few reports implicating CXCL11 in IBD, IFN-γ was shown to be capable of inducing CXCL11 in epithelial cells [35] and in T cells from a mouse model of colitis [36]. Thus, the differences in CXCR7 expression observed in the current investigation could suggest that there are separate pathways of lymphocyte migration and activation for CXCL12 and CXCL11.

When examining the influence of T cell stimulation, we found that CD3/CD28 stimulation had a sensitizing effect on migratory capacity of T cells, and migration of PBTs toward CXCL12 was abrogated to levels similar to random migration. This was reinforced by the finding that CD3/CD28 stimulations significantly decrease the percentage of CXCR4-expressing cells and concurrently increase the percentage of CXCR7-expressing cells. The reciprocal regulation of expression by the TCR suggests discordant roles for CXCR4 and CXCR7 in T lymphocyte behavior. Interestingly, when responses of IBD PBTs to CD3/CD28 stimulation were compared with normal PBTs, a significantly less-prominent effect of CD3/CD28 on receptor-expressing cells was observed in IBD. This may suggest that in IBD, in contrast to normal, PBTs are less-responsive to TCR engagement. However, this did not correlate with differences in migration between stimulated IBD and normal PBTs (data not shown). It is thus possible that in IBD, there is a positive-feedback loop, where increased CXCL12 expression and cell stimulation are not associated with significantly decreased receptor expression, consequently contributing to T cell accumulation in the inflamed intestine.

This might also suggest that the effect of CD3/CD28 on CXCL12 receptors might be related to additional functional tasks, such as enhanced adhesion [37], which might enable retention of reactive T cells in the vicinity of soluble CXCL12, a process that is up-regulated in IBD [18]. Of note, in contrast to CD3 stimulation, which strongly decreased CXCR4 and increased CXCR7, CD2/CD28 stimulation modestly reduced CXCR4 expression and considerably decreased CXCR7 expression in PBTs and LPTs. This behavior could point to a different functional task for CXCR7 upon CD2/CD28 stimulation.

Last, activation of LPTs but not of PBTs, via CD3 alone or CD2/CD28, impaired the chemotactic potential toward CXCL12. This suggests that LPTs are more sensitive to submaximal stimulations, or as previously reported, LPTs are responsive to CD2 ligation, in contrast to PBTs [38–41].

Given that the use of the small molecule CXCR7 compounds and the anti-CXCR7 antibodies did not affect CXCL12-mediated migration, our findings suggest that CXCR7 is not essential for CXCL12-mediated migration. Further support for this contention is that CD3 stimulation increased CXCR7 expression, and migration decreased.

As advocated by others [19, 42], our results suggest that CXCR7 is not involved in CXCL12-mediated migration. Thus, as CXCR7 does not mediate cell signaling, calcium influx, or chemotaxis, its role may be that of a "silent receptor", as hypothesized previously [9, 37, 43].

Regarding the functional effect of CXCL12 on migrating lymphocyte characteristics, our hypothesis was that CXCL12 would attract memory T cells with a specific proinflammatory cytokine-secretion pattern. Such an effect would be expected in light of our recent findings of increased expression of CXCL12 and CXCR4 in IBD [18].

As shown, CXCL12 is a potent chemoattractant of memory T cells. Given that memory $CD45RO^+$ LPTs predominate in the LP, this could implicate CXCL12 as a contributor to mucosal deregulation.

CXCL12 has been recently reported to be induced in inflammatory disorders of Th1 and Th2 phenotypes. Thus, in IBD, T cells migrating toward CXCL12 might be distinguished according to their cytokine profile. IFN-γ secretion from stimulated PBTs was comparable, whether migrating or not toward CXCL12. Stimulated, normal PBTs, which were CXCL12-attracted, secreted significantly more IL-4 compared with nonattracted PBTs. This finding, reflecting the proinflammatory nature of the LP, is supported by Fuss et al. [44], who showed an increase of IFN-γ production in LPMCs from CD patients. Other investigators also suggested an increase of IL-4 or IFN-γ production from $CD3^+$ LPTs of IBD patients [44, 45] and that IBD IECs may promote such a reaction [46]. Our findings strengthen the latter concept of a dominating, Th1-skewed response in CD.

In conclusion, we demonstrate that stimulated PBTs and LPTs are desensitized to CXCL12-induced migration; this migration is CXCR4- but not CXCR7-dependent; and CXCL12 is an attractant of memory, Th1-biased lymphocytes. Desensitization mechanisms were less prominent in IBD compared with the normal mucosa. Taken together, our results further support a role for CXCL12 and its receptors in the intestinal mucosa in homeostasis and inflammation, thereby designating CXCL12-CXCR4 as a potential target for therapeutic interventions in the setting of IBD.

AUTHORSHIP

L.W. designed the study, as well as the generation, collection, assembly, analysis, and/or interpretation of data and drafting of the manuscript. H.E. generated and collected the data. E.B. and H.T. generated, collected, and assembled the data and provided analysis and/or interpretation of the data. S.V. and U.K. generated and collected the data. Z.H. approved the final version of the manuscript. H.G-G. designed the study and approved the final version of the manuscript. I.D. designed the study and provided assembly, analysis, and interpretation of data, as well as drafting and approval of the manuscript.

ACKNOWLEDGMENTS

We thank Esther Eshkol for editing assistance and the Division of Surgery for the consistent collaboration.

REFERENCES

1. Zimmerman, N. P., Vongsa, R., Wendt, M. K., Dwilnell, M. B. (2008) Chemokines and chemokine receptors in mucosal homeostasis at the intestinal epithelial barrier in inflammatory bowel disease. *Inflamm. Bowel Dis.* **14,** 1000–1011.
2. Papadakis, K. A. (2000) The role of chemokines and chemokine receptors in mucosal inflammation. *Inflamm. Bowel Dis.* **6,** 303–313.
3. Heidemann, J. (2004) Mucosal angiogenesis regulation by CXCR4 and its ligand CXCL12 expressed by human intestinal microvascular endothelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **286,** G1059–G1068.

4. Agace, W. W., Amara, A., Roberts, A. I., Pablos, J. L., Thelen, J. L., Li, X. Y., Marsal, J., Ebert, E. C., Moser, B., Parker, C. M. (2000) Constitutive expression of SDF1α by mucosal epithelia and its role in HIV transmission and propagation. *Curr. Biol.* **10,** 325–328.
5. Katsuta, T., Lim, C., Shimoda, K., Shibuta, K., Banner, B. F., Mori, M., Barnard, G. F. (2000) Interleukin-8 and SDF1-α mRNA expression in colonic biopsies from patients with inflammatory bowel disease. *Am. J. Gastroenterol.* **95,** 3157–3164.
6. Peled, A., Petit, I., Kollet, O., Magid, M., Nagler, A., Ben Hur, H., Many, A., Lider, O., Alon, R., Zipori, D., Lapidot, T. (1999) Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science* **283,** 848–852.
7. Wright, D. E., Bowman, E. P., Wagers, A. J., Butcher, E. C., Weissman, I. L. (2002) Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. *J. Exp. Med.* **195,** 1145–1154.
8. Smith, J. M., Johansen, P. A., Wendt, M. K., Binion, D. G., Dwinell, M. B. (2005) CXCL12 activation of CXCR4 regulated mucosal host defense through stimulation of epithelial cell migration and promotion of intestinal barrier integrity. *Am. J. Physiol. Gastrointest. Liver Physiol.* **288,** G316–G326.
9. Balabanian, K., Lagane, B., Infantino, S., Chow, K. Y., Moepps, B., Thelen, M., Bachelerie, F. (2005) The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J. Biol. Chem.* **280,** 35760–35766.
10. Patterson, B. K., Czerniewski, M., Andersson, J., Sullivan, Y., Su, F., Jiyamapa, D., Burki, Z., Landay, A. (1999) Regulation of CCR5 and CXCR4 expression by type 1 and type 2 cytokines: CCR5 expression is downregulated by IL-10 in CD4-positive lymphocytes. *Clin. Immunol.* **91,** 254–262.
11. García-Vicuña, R., Gomez-Gaviro, M. V., Dominguez-Luis, M. J., Pec, M. C., Alvaro-Garcia, J. M., Diaz-Gonzalez, F. (2004) CC and CXC chemokine receptors mediate migration, proliferation, and matrix metalloproteinase production by fibroblast-like synoviocytes from rheumatoid arthritis patients. *Arthritis Rheum.* **50,** 3866–3877.
12. Arms, L., Girard, B. M., Vizzard, M. A. (2010) Expression and function of CXCL12/CXCR4 in rat urinary bladder with cyclophosphamide-induced cystitis. *Am. J. Physiol. Renal. Physiol.* **298,** F589–F600.
13. Liu, K. K., Dorovini-Zis, K. (2009) Regulation of CXCL12 and CXCR4 expression by human brain endothelial cells and their role in CD4+ and CD8+ T cell adhesion and transendothelial migration. *J. Neuroimmunol.* **215,** 49–64.
14. Lukacs, N. W., Berlin, A., Schols, D., Skerlj, R. T., Bridger, G. J. (2002) AMD3100, a CXCR4 antagonist, attenuates allergic lung inflammation and airway hyperreactivity. *Am. J. Pathol.* **160,** 1353–1360.
15. McCandless, E. E., Piccio, L., Woerner, B. M., Schmidt, R. E., Rubin, J. B., Cross, A. H., Klein, R. S. (2008) Pathological expression of CXCL12 at the blood-brain barrier correlates with severity of multiple sclerosis. *Am. J. Pathol.* **172,** 799–808.
16. Kim, K. W., Mi-La, C., Kim, H., Ju, J., Park, M., Oh, H., Kim, J., Park, S., Lee, S., Kim, H. (2007) Up-regulation of CXCL12 production in rheumatoid synovial fibroblasts through interactions with T lymphocytes. Role of interleukin-17 and CD40L-CD40 interaction. *Arthritis Rheum.* **56,** 1076–1086.
17. Hoshino, M., Aoike, N., Takahashi, M., Nakamura, Y., Nakagawa, T. (2003) Increased immunoreactivity of SDF1α and angiogenesis in asthma. *Eur. Respir. J.* **21,** 804–809.
18. Dotan, I., Werner, L., Vigodman, S., Weiss, S., Brazowski, E., Maharshak, N., Chen, O., Tulchinsky, H., Halpern, Z., Guzner-Gur, H. (2010) CXCL12 Is a constitutive and inflammatory chemokine in the intestinal immune system. *Inflamm. Bowel Dis.* **16,** 583–592.
19. Hartmann, T. N., Grabovsky, V., Pasvolsky, R., Shulman, Z., Buss, E. C., Spiegel, A., Nagler, A., Lapidot, T., Thelen, M., Alon, R. (2008) A cross-talk between intracellular CXCR7 and CXCR4 involved in rapid CXCL12-triggered integrin activation but not in chemokine-triggered motility of human T lymphocytes and CD34+ cells. *J. Leukoc. Biol.* **84,** 1130–1140.
20. Mazzinghi, B., Ronconi, E., Lazzeri, E., Sagrinati, C., Ballerini, L., Parente, E., Mancina, R., Stefano, G., Becherucci, F., Gacci, M., Carini, M., Gesualdo, L., Rotondi, M., Maggi, E., Lasagni, L., Serion, M., Romagnani, S., Romagnani, P. (2008) Essential but differential role for CXCR4 and CXCR7 in the therapeutic homing of human renal progenitor cells. *J. Exp. Med.* **205,** 479–490.
21. Rajagopal, S., Kim, J., Ahn, S., Craig, S., Lam, C. M., Gerard, N. P., Gerard, C., Lefkowitz, R. J. (2010) β-Arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proc. Natl. Acad. Sci. USA* **107,** 628–632.
22. Peacock, J. W., Jirik, F. R. (1999) TCR activation inhibits chemotaxis toward stromal cell-derived factor-1: evidence for reciprocal regulation between CXCR4 and the TCR. *J. Immunol.* **162,** 215–223.
23. Bermejo, M., Martin-Serrano, J., Oberlin, E., Serrano, A., Santiago, B., Caruz, A., Loetscher, P., Arenzana, S. F., Alcami, J. (1998) Activation of blood T lymphocytes down-regulates CXCR4 expression and interferes with propagation of X4 HIV strains. *Eur. J. Immunol.* **28,** 3192–3204.
24. Shadidi, K. R., Aarvak, T., Henriksen, J. E., Natvig, J. B., Thompson, K. M. (2003) The chemokines CCL5, CCL2 and CXCL12 play significant roles in the migration of Th1 cells into rheumatoid synovial tissue. *Scand. J. Immunol.* **57,** 192–198.
25. Gonzalo, J. A., Lloyd, C. M., Peled, A., Delaney, T., Coyle, A. J., Ramos, J. C. (2000) Critical involvement of the chemotactic axis CXCR4/SDF-1α in the inflammatory component of allergic airway disease. *J. Immunol.* **165,** 499–508.
26. Hsu, H. C., Yang, P., Wang, P., Wu, Q., Myers, R., Chen, J., Yi, J., Tousson, A., Stanus, A. L., Le, T. V., Carter, R. H., Chaplin, D. D., Williams, R. W., Mountz, J. D. (2008) IL17-producing T helper cells and IL-17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. *Nat. Immunol.* **9,** 166–175.
27. Moser, B., Loetscher, P. (2001) Lymphocyte traffic control by chemokines. *Nat. Immunol.* **2,** 123–128.
28. Murdoch, C. (2000) CXCR4: chemokine receptor extraordinaire. *Immunol. Rev.* **177,** 175–184.
29. Kucia, M., Jankowski, K., Reca, R., Wysoczynski, M., Bandura, L., Allendorf, D. J., Zhang, J., Ratajczak, J., Ratajczak, M. Z. (2004) CXCR4-SDF-1 signaling, locomotion, chemotaxis and adhesion. *J. Mol. Histol.* **35,** 233–245.
30. Mikami, S., Nakase, H., Yamamoto, S., Takeda, Y., Ueno, S., Uza, N., Oishi, S., Fujii, N., Nagasawa, T., Chiba, T. (2008) Blockade of CXCL12/CXCR4 axis ameliorates murine experimental colitis. *J. Pharmacol. Exp. Ther.* **327,** 383–392.
31. Olsson, J., Poles, M., Spetz, A., Elliott, J., Hultin, L., Giorgi, J., Andersson, J., Anton, P. (2000) Human immunodeficiency virus type 1 infection is associated with significant mucosal inflammation characterized by increased expression of CCR5, CXCR4, and β-chemokines. *J. Infect. Dis.* **182,** 1625–1635.
32. Struyf, S., Noppen, S., Loos, T., Mortier, A., Gouwy, M., Verbeke, H., Huskens, D., Parmentier, M., Geboes, K., Schols, D., Van Damme, J., Proost, P. (2009) Citrullination of CXCL12 differentially reduces CXCR4 and CXCR7 binding with loss of inflammatory and anti-HIV-1 activity via CXCR4. *J. Immunol.* **182,** 666–674.
33. Zabel, B. A., Wang, Y., Lewen, S., Penfold, M. E., Zhang, P., Powers, J., Summers, B. C., Miao, Z., Zhao, B., Jalili, A., Janowska-Wieczorek, A., Jaen, J. C., Schall, T. J. (2009) Elucidation of CXCR7-mediated signaling events and inhibition of CXCR4-mediated tumor cell transendothelial migration by CXCR7 ligands. *J. Immunol.* **183,** 3204–3211.
34. Berahovich, R. D., Zabel, B. A., Penfold, M. E. T., Lewen, S., Wang, Y., Miao, Z., Gan, L., Pereda, J., Dias, J., McGrath, K. E., Jaen, J. C., Schall, T. J. (2010) CXCR7 protein is not expressed on human or mouse leukocytes. *J. Immunol.* **185,** 5130–5139.
35. Dwinell, M. B., Lugering, N., Eckmann, L., Kagnoff, M. F. (2001) Regulated production of interferon-inducible T-cell chemoattractants by human intestinal epithelial cells. *Gastroenterology* **120,** 49–59.
36. Singh, U. P., Singh, S., Iqbal, N., Weaver, C. T., McGhee, J. R., Lillard, J. W. (2003) IFN-γ-inducible chemokines enhance adaptive immunity and colitis. *J. Interferon Cytokine Res.* **23,** 591–600.
37. Boldajipour, B., Mahabaleshwar, H., Kardash, E., Reichman-Fried, M., Blaser, H., Minina, S., Wilson, D., Xu, Q., Raz, E. (2008) Control of chemokine-guided cell migration by ligand sequestration. *Cell* **132,** 463–473.
38. Qiao, L., Schurmann, G., Betzler, M., Meuer, S. C. (1991) Activation and signaling status of human lamina propria T lymphocytes. *Gastroenterology* **101,** 1529–1536.
39. Targan, S. R., Deem, R. L., Liu, M., Wang, S., Nel, A. (1995) Definition of a lamina propria T cell responsive state. *J. Immunol.* **154,** 664–675.
40. Ebert, E. C. (2006) CD2 activation of human lamina propria lymphocytes reduces CD3 responsiveness. *Immunology* **117,** 71–77.
41. Secchiero, P., Zella, D., Curreli, S., Mirandola, P., Capitani, S., Gallo, R. C., Zauli, G. (2000) Engagement of CD28 modulates CXC chemokine receptor 4 surface expression in both resting and CD3-stimulated CD4+ T cells. *J. Immunol.* **164,** 4018–4024.
42. Zhang, Z., Zhong, W., Hall, M. J., Kurre, P., Spencer, D., Skinner, A., O'Neill, S., Xia, Z., Rosenbaum, J. T. (2009) CXCR4 but not CXCR7 is mainly implicated in ocular leukocyte trafficking during ovalbumin-induced acute uveitis. *Exp. Eye Res.* **89,** 522–531.
43. Patel, M., McInnes, I. B., Graham, G. (2009) Atypical chemokine receptors in inflammatory disease. *Curr. Mol. Med.* **9,** 86–93.
44. Fuss, I. J., Heller, F., Boirivant, M., Leon, F., Yoshida, M., Yang, Z., Exley, M., Kitani, A., Blumberg, R. S., Mannon, P., Strober, W. (2004) Nonclassical CD1d-restricted NK T cells that produce IL-13 characterize an atypical Th2 response in ulcerative colitis. *J. Clin. Invest.* **113,** 1490–1497.
45. Rogler, G., Andus, T. (1998) Cytokines in inflammatory bowel disease. *World J. Surg.* **22,** 382–389.
46. Dotan, I., Allez, M., Nakazawa, A., Brimnes, J., Schulder-Katz, M., Mayer, L. (2007) Intestinal epithelial cells from inflammatory bowel disease patients preferentially stimulate CD4+ T cells to proliferate and secrete interferon-γ. *Am. J. Physiol. Gastrointest. Liver Physiol.* **292,** G1630–G1640.

KEY WORDS:
Crohn's disease · ulcerative colitis · chemokines · CXCL12