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Role for CXCR6 in Recruitment of Activated CD8+ Lymphocytes to Inflamed Liver

Tohru Sato,²,³†✦ Henrik Thorlacius,³,⁴†✦ Brent Johnston,⁵†✦ Tracy L. Staton,⁴† Wenkai Xiang,§ Dan R. Littman,§ and Eugene C. Butcher‡‡

Hepatic infiltration of activated CD8 lymphocytes is a major feature of graft-vs-host disease (GvHD). Chemotactic cytokines and their receptors are key regulators of lymphocyte trafficking, but the involvement of chemotactic receptors in the physiologic recruitment of cells into the inflamed liver has not been defined. The present study examines the role of the chemokine receptor CXCR6, which is highly expressed by liver-infiltrating CD8 T cells. Hepatic accumulation of donor CD8, but not donor CD4, lymphocytes was significantly reduced in GvHD induced by transfer of CXCR6−/−, H-2Db lymphocytes into BDF1, H-2Db recipients. To determine whether altered recruitment contributes to the reduced accumulation, CXCR6−/− or wild-type splenic lymphocytes participating in an active GvHD response were isolated and transferred i.v. into secondary recipients with active GvHD, and the short term (6-h) recruitment of transferred cells to the inflamed liver was assessed. CXCR6−/− CD8 (but not CD4) cells displayed a significant (33%) reduction in liver localization, whereas frequencies in blood of CXCR6−/− and wild-type CD8 cells were similar. Proliferation and apoptosis of liver-infiltrating donor CD8 cells were unaffected. We conclude that CXCR6 helps mediate the recruitment of activated CD8 lymphocytes in GvHD-induced hepatitis and may be a useful target to treat pathological inflammation in the liver. The Journal of Immunology, 2005, 174: 277–283.

Donor T cell recruitment into target tissues is a prominent component of graft-vs-host disease (GvHD), and a major complication in allogeneic bone marrow transplantation (1). Recruitment of activated lymphocytes is a multistep process in which the expression of specific adhesion molecules and chemokine receptors endows lymphocytes with the ability to access extravascular tissues (2). Chemokine receptors play a central role in lymphocyte recruitment by rapidly transmitting intracellular signals and triggering increased integrin affinity to adhere to endothelial molecule ligands displayed by endothelial cells (3). At present, >20 chemokine receptors have been described (4). The role of specific chemokine receptors in attracting activated lymphocytes appears to be both stimulus and organ dependent, contributing to inflammatory and homeostatic tissue localization of lymphocyte subsets (5). In GvHD the liver is a key target organ, characterized by massive lymphocyte accumulation (6), which may reflect enhanced recruitment and/or proliferation.

Acute GvHD is initiated by donor T cells recognizing and reacting to host allo-Ags and is characterized by a Th1-polarized response (7). Different combinations of chemokine receptors are associated with Th1- vs Th2-dependent immune reactions (8). Accordingly, it has been shown that the chemokine receptor CXCR6 (Bonzo/STRL33/TYMSTR) is expressed on T cells of Th1 phenotype (9–13). CXCR6, originally described as a novel simian immunodeficiency virus receptor (14) and fusion cofactor for HIV-1 strains (15), is expressed at very low levels in naive CD8 lymphocytes (but not CD4) and their receptors are key regulators of lymphocyte trafficking, but the involvement of chemotactic receptors in the physiologic recruitment of cells into the inflamed liver has not been defined. The present study examines the role of the chemokine receptor CXCR6, which is highly expressed by liver-infiltrating CD8 T cells. Hepatic accumulation of donor CD8, but not donor CD4, lymphocytes was significantly reduced in GvHD induced by transfer of CXCR6−/−, H-2Db lymphocytes into BDF1, H-2Db recipients. To determine whether altered recruitment contributes to the reduced accumulation, CXCR6−/− or wild-type splenic lymphocytes participating in an active GvHD response were isolated and transferred i.v. into secondary recipients with active GvHD, and the short term (6-h) recruitment of transferred cells to the inflamed liver was assessed. CXCR6−/− CD8 (but not CD4) cells displayed a significant (33%) reduction in liver localization, whereas frequencies in blood of CXCR6−/− and wild-type CD8 cells were similar. Proliferation and apoptosis of liver-infiltrating donor CD8 cells were unaffected. We conclude that CXCR6 helps mediate the recruitment of activated CD8 lymphocytes in GvHD-induced hepatitis and may be a useful target to treat pathological inflammation in the liver. The Journal of Immunology, 2005, 174: 277–283.

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Abbreviations used in this paper: GvHD, graft-vs-host disease; 7-AAD, 7-aminoactinomycin D; CXCL, CXC ligand; DC, dendritic cell; LIL, liver-infiltrating lymphocyte; WT, wild type.
vs CXCR6<sup>−/−</sup> lymphocytes. Our results show that CXCR6 has a significant role in recruiting activated CD8, but not CD4, lymphocytes to the infiltrated liver in GVHD-induced hepatitis.

Materials and Methods

**Animal model of GVHD**

C57BL/6J (B6, H-2<sup>b</sup>; The Jackson Laboratory), C57BL/6J-Igh<sup>Thy1.1</sup>Gpi1<sup>Thy1.1</sup> (Thy1.1, H-2<sup>b</sup>; The Jackson Laboratory), and CXCR6-GFP knockin mice (CXCR6<sup>+/+</sup>, backcrossed onto C57BL/6 more than eight generations, H-2<sup>b</sup>) were used as donors. Donor CXCR6-GFP mice have the CXCR6-coding sequence replaced with a sequence for enhanced GFP, which is regulated through the allogeneic CXCR6 promoter (17). B6D2F1j mice (BDF<sub>1</sub>, H-2<sup>md</sup>-<sup>md</sup>; The Jackson Laboratory) were used as recipients or as donors for syngeneic control experiments. All mice were 8–12 wk old and housed under conventional conditions in the animal facility at the Veterans Affairs Palo Alto Health Care System. All experiments were approved by the local ethics committee at Stanford University. Nonlethal GVHD was induced as previously described (20). Briefly, 50 × 10<sup>6</sup> donor lymphocytes isolated from the spleen were transferred i.v. into recipient mice on day 0.

The splenocytes from C57BL/6J and CXCR6<sup>−/−</sup> were similar with respect to the expression of activation markers (CD44, CD25, CD44, and L-selectin), which is regulated through the allogeneic CXCR6 promoter (17). B6D2F1j mice (BDF<sub>1</sub>, H-2<sup>md</sup>-<sup>md</sup>; The Jackson Laboratory) were used as recipients or as donors for syngeneic control experiments. All mice were 8–12 wk old and housed under conventional conditions in the animal facility at the Veterans Affairs Palo Alto Health Care System. All experiments were approved by the local ethics committee at Stanford University. Nonlethal GVHD was induced as previously described (20). Briefly, 50 × 10<sup>6</sup> donor lymphocytes isolated from the spleen were transferred i.v. into recipient mice on day 0.

The expression of activation markers, adhesion molecules, and cell subset distribution (CD4, CD8, NK, NKT, and B cells; data not shown). On day 7, recipient mice were killed by CO<sub>2</sub> inhalation, and the liver, spleen, and blood were harvested. In indicated experiments, syngeneic lymphocytes were labeled with 0.5 μM CFSE (Molecular Probes) at 37°C for 10 min before injection.

**Short term recruitment assay**

GVHD was induced in BDF<sub>1</sub> mice by transferring lymphocytes from B6-Thy1.2, congenic B6-Thy1.1, or CXCR6<sup>−/−</sup>/CXCR6<sup>−/−</sup> mice as described above. This congeneric model for identification of donor lymphocytes was chosen because using CXCR6<sup>−/−</sup> mice expressing GFP as donors excluded CFSE labeling of donor cells. On day 8 after transfer, lymphocytes were isolated from the spleen of GVHD mice induced by Thy1.2 cells (wild-type (WT) or CXCR6<sup>−/−</sup>), and 20 × 10<sup>6</sup> cells were subsequently transferred to GVHD mice induced by Thy1.1 cells (short recruitment mice). Six hours after the second transfer, the short term recruitment mice were killed, and cells from the liver, spleen, and blood were harvested. This 6-h window allowed us to compare the recruitment of Thy1.2 cells with or without CXCR6<sup>−/−</sup> into infiltrated livers. The phenotypes of the 20 × 10<sup>6</sup> lymphocytes injected from WT and CXCR6<sup>−/−</sup> mice were similar with respect to the expression of activation markers, adhesion molecules, and cell subset distribution described above (data not shown). A liver recruitment ratio was calculated as the absolute number of the number of cells recruited to the liver divided by the number of the number of injected cells. Short term recruitment was determined for CD<sup>4</sup>-CD<sup>8</sup>-CD<sup>45</sup>R<sup>−</sup>Thy1.2<sup>+</sup>, CD<sup>4</sup>-CD<sup>8</sup>-CD<sup>45</sup>R<sup>−</sup>Thy1.1<sup>+</sup>, and CD<sup>19</sup>-H<sup>2</sup>-I<sup>g<sub>d</sub></i>T<sup>+</sup>CD<sup>45</sup>R<sup>−</sup> cell subsets. Additionally, the cell subset of donor cells settling in the blood was analyzed.

**Isolation of liver-infiltrating lymphocytes (LIL)**

After mice were killed, the liver was perfused through the portal vein with 5 ml of PBS. The liver was harvested, dispersed through a stainless steel mesh (70-μm pore size), and washed with HBSS containing 10% bovine calf serum. The dispersed cells were mixed with a 33% Percoll (Amersham Biosciences) solution containing 100 U/ml heparin (Sigma-Aldrich) and centrifuged at 800 × g at room temperature. Next, the top pellet was resuspended in double-distilled water for 20 s to lyse RBC, and the lysis was stopped with 2 × PBS. The isolated lymphocytes were then resuspended in HBSS (2% bovine calf serum), counted in a hemocytometer, and kept on ice until staining for flow cytometric analysis.

**RT-PCR and real-time PCR**

Total RNA was extracted from whole liver tissue using an RNaseasy kit according to the manufacturer’s instructions (QIAGEN). RNA concentration and purity were determined by measuring absorbance spectrophotometrically at 260 and 280 nm. RT-PCR was performed with ThermoScript RT-PCR System (Invitrogen Life Technologies). Mouse β-actin served as an internal control gene. The PCR profile was 40 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final extension of 7 min at 72°C. The primer sequences of CXCR6 are: forward, 5′-GCC ACT TAC GAG GGA GGA G-3′; reverse, 5′-CATTGGTGA3′; and CXCL16 (forward, 5′-GGCTCGCAGGGATG-3′; reverse, 5′-CCCTGACTTTAATGCCTTTG-3′; CXCR6 (forward, 5′-GCCATGGTATTCCGATCC-3′; reverse, 5′-GGTGTTCTGCCATTTCTCTAGGT-3′). The chemokine or chemokine receptor expression in normal liver was given an arbitrary value of 1, and their expression in GVHD liver was expressed as fold up-regulation from normal.

**Flow cytometry**

Isolated cells (1–2 × 10<sup>6</sup>) were stained with the following rat anti-mouse Abs conjugated to FITC, PE, PerCP, PE-indotricarbocyanine, allophycocyanin, allophycocyanin-indotriscarbocyanine, or biotin: H-2<sup>Dd</sup> (clone 24-3-14) (BD Biosciences), D<sup>4</sup> (clone 53-2.1), CD<sup>45</sup>R<sup>B</sup> (clone 53-2.1), CD<sup>19</sup> (clone 1D3), Ig<sup>D</sup> (clone 217-170), and NK1.1 (clone 53K16). The above-mentioned Abs were obtained from BD Pharmingen. NeutrAvidin Cascade Blue was purchased from Molecular Probes. To study proliferation, we used a BrdU Flow Kit (BD Biosciences, San Jose, CA) following the manufacturer’s instructions. Briefly, 1 mg of BrdU was injected i.p. 2 h before death, and the isolated lymphocytes were subsequently permeabilized for detection of intracellular BrdU. Four-color flow cytometry was performed using a FACScalibur (BD Biosciences), and seven-color flow cytometry was performed with LSR II (BD Biosciences). The data were acquired and analyzed on CellQuest software (version 3.3) for FACScanCalibur and on LSR II (version 2.1) and Six-color flow cytometry (version 4.0) for LSR II. Data are presented as the mean ± SEM. Student’s t test was used for between-group comparisons, and significance was set at p < 0.05.

**Migration assay**

LIL were isolated from GVHD livers induced by WT, CXCR6<sup>+/+</sup>, and CXCR6<sup>−/−</sup> spleen cells at day 7 of disease. LIL (0.5–1 × 10<sup>6</sup>) were placed in the upper chamber of Transwell inserts (5-μm pore size; Corning Costar) and migrated to chemokines at their optimal concentrations for 2 h at 37°C as described previously (21). After migration, polystyrene beads (Polysciences) were added to each bottom well as an internal standard, and an aliquot was collected for analysis by flow cytometry for calculation of the percent migrated. Triplicate wells were pooled and stained with Abs to H-2<sup>Dd</sup> and CD8 for calculating donor CD8 migration. The data presented are the percent migration of the indicated subsets, determined by measuring the number of cells of each subset in the starting and migrated populations. The chemokine CXCL12 was purchased from PeproTech, and CXCL16 was obtained from R&D Systems.

**Results**

CXCR6 is up-regulated in GVHD hepatitis

We initially asked whether CXCR6 and CXCL16 are relevant candidates to study in liver homing by examining their expression under normal and inflamed conditions. We examined livers from control and/or GVHD mice 7 days after lymphocyte transfer. CXCR6 and CXCL16 mRNA were amplified by PCR from total RNA, and we found clear evidence of CXCR6 and CXCL16 mRNA expression in both healthy controls and GVHD animals (data not shown). We also quantitatively analyzed the expression of CXCR6 and CXCL16 and found an 1–2-fold increase in mRNA expression in GVHD-infamed liver compared with normal liver, as assessed by real-time PCR (mean ± SEM fold increase: CXCR6, 1.6 ± 0.1; CXCL16, 1.9 ± 0.1; Fig. 1a; n = 4). Next, we asked whether CXCR6 was expressed on transferred lymphocytes infiltrating the liver. For this purpose, we used heterozygous GFP
knockin mice, in which one allele of the CXCR6 gene has been replaced by enhanced GFP, to examine the expression of CXCR6 in normal (syngeneic transfer) and inflamed (allogeneic transfer) liver (17). We analyzed GFP expression in LIL from day 7 of GvHD induced by CXCR6-GFP (CXCR6<sup>+/−</sup>, H-2<sup>b</sup>) mice. The same splenocytes were transferred into Thy1.1 (syngeneic, H-2<sup>b</sup>) mice as a negative control. GFP expression as a marker for CXCR6 expression was assessed by flow cytometry on donor LIL subsets. Representative histograms are shown. Histograms are scaled to more easily compare the percentage of positive lymphocytes. c, GFP (CXCR6) expression on donor lymphocyte subsets is compared in liver (∆) and spleen (□). d, CXCR6 message was amplified from C57BL/6 (B6) and CXCR6-GFP (CXCR6<sup>+/−</sup>) livers. β-Actin message was amplified as a control. The primers used are described in Materials and Methods. e, Liver-infiltrating lymphocytes from day 7 of GVHD hepatitis induced by WT, CXCR6<sup>+/+</sup>, or CXCR6<sup>−/−</sup> donor cells were migrated to CXCL16 and CXCL12. A representative experiment of three independent experiments is shown.

FIGURE 1. CXCR6/CXCL16 expression and in vitro migration to CXCL16. a, GVHD was induced in BDF<sub>1</sub> (H-2<sup>b+bd</sup>) mice by the transfer of splenocytes from C57BL/6 (H-2<sup>b</sup>) mice. On day 7 of disease, livers were harvested from GvHD and normal BDF<sub>1</sub> mice, mRNA was isolated, and real-time PCR was performed. Samples were normalized to 15Sa rRNA, and chemokine and chemokine receptor expression from normal liver was assigned a relative value of 1.0. Data are expressed as the mean ± SEM (n = 4). b, GVHD was induced in BDF<sub>1</sub> (allogeneic) mice by transfer of splenocytes from heterozygous CXCR6-GFP (CXCR6<sup>+/−</sup>, H-2<sup>b</sup>) mice. The same splenocytes were transferred into Thy1.1 (syngeneic, H-2<sup>b</sup>) mice as a negative control. GFP expression as a marker for CXCR6 expression was assessed by flow cytometry on donor LIL subsets. Representative histograms are shown. Histograms are scaled to more easily compare the percentage of positive lymphocytes. c, GFP (CXCR6) expression on donor lymphocyte subsets is compared in liver (∆) and spleen (□). d, CXCR6 message was amplified from C57BL/6 (B6) and CXCR6-GFP (CXCR6<sup>+/−</sup>) livers. β-Actin message was amplified as a control. The primers used are described in Materials and Methods. e, Liver-infiltrating lymphocytes from day 7 of GVHD hepatitis induced by WT, CXCR6<sup>+/+</sup>, or CXCR6<sup>−/−</sup> donor cells were migrated to CXCL16 and CXCL12. A representative experiment of three independent experiments is shown.

absence of CXCR6 results in reduced effector CD8 cell accumulation in liver

We next asked whether the absence of CXCR6 would influence the efficiency of transferred effector T cells to accumulate in the liver in a model of GvHD. Liver infiltration of donor T cells in GvHD was induced by transfer of WT (B6 into BDF<sub>1</sub>) or CXCR6-deficient (CXCR6<sup>−/−</sup> into BDF<sub>1</sub>) donor lymphocytes. We confirmed that these CXCR6 gene-targeted mice do not express CXCR6, as shown by RT-PCR (Fig. 1d), and thus constitute an ideal tool for investigating the role of CXCR6. We also confirmed in vitro that activated CXCR6<sup>−/−</sup> CD8 lymphocytes fail to migrate to the CXCR6 ligand CXCL16, but are still able to migrate to another chemokine, CXCL12, which was used as a positive control (Fig. 1e). CXCR6<sup>−/−</sup> mice have an intermediate phenotype (Fig. 1e). Subsequent comparisons were made between GvHD induced by
WT and CXCR6−/− splenocytes. In our in vivo analysis on day 7, both groups of mice displayed marked hepatitis; the numbers of LIL were 6.1 ± 1.1 × 10^6 and 6.8 ± 1.9 × 10^6 cells/liver in GvHD generated by WT and CXCR6−/− lymphocytes, respectively. In comparison, syngeneic controls had 1.9 ± 0.3 × 10^6 cells/liver. Importantly, there were far fewer donor CD8 T cells in GvHD induced by CXCR6−/− cells (54% decrease in donor-derived CD8+ lymphocytes compared with WT GvHD, 172 ± 18 × 10^3 vs 79 ± 3 × 10^3 (mean ± SEM); n = 7; p < 0.05; Fig. 2a). No difference was observed in the number of donor cells infiltrating the spleen (B6, 1.7 ± 0.3 × 10^6; CXCR6−/−, 1.7 ± 0.4 × 10^6; Fig. 2b), suggesting that this reduction in liver CD8 cells is not a generalized phenomenon characterized by decreased donor cells in all compartments. The difference in liver extended to a reduction in the percentage of donor CD8 cells (39% reduction) among total CD8 cells (i.e., (donor CD8)/(total CD8) × 100; B6, 38 ± 5%; CXCR6−/−, 23 ± 4%; Fig. 2c; p < 0.05). However, no statistically significant difference was found in donor CD4 cells (Fig. 2d). Considered together, the decrease in donor CXCR6−/− CD8 lymphocytes in percentage and absolute numbers indicate that there was a selective decrease in the CXCR6−/− cell subset, not only an overall decrease in total LIL. These findings stress the importance of CXCR6 in the accumulation of pathological antihost CD8 T cells to a target organ of GvHD.

**FIGURE 2.** Lymphocyte accumulation in tissues. The absolute numbers of donor CD8 lymphocytes (CD8+H-2Dd+ in liver (a) and spleen (b) 7 days after adoptive transfer are shown. GvHD was induced in BDF1 mice by transfer of splenocytes from WT and CXCR6-deficient (CXCR6−/−) C57BL/6 mice. Transfer of syngeneic CFSE-labeled BDF1 splenocytes into BDF1 mice served as the negative control (Control). The absolute number of donor lymphocytes was calculated by multiplying the percentage of CD8+H-2Dd+ (allogeeneic) and CD8+ CFSE+ (syngeneic) cells in the small lymphocyte gate by the absolute number of lymphocytes recovered. The percentages of CD8 (c) and CD4 (d) lymphocytes of donor origin in liver and spleen were calculated by dividing the number of CD8+H-2Dd+ and CD4+H-2Dd+ lymphocytes by the total number of CD8+ and CD4+ lymphocytes in the respective tissues. The percentage of syngeneic donor CD8 lymphocytes was calculated by dividing the number of CD8+ CFSE+ by the total number of CD8+ lymphocytes in the respective tissues. Data are shown as the mean ± SEM (n = 7), p < 0.05, WT vs CXCR6−/− groups.

Decreased accumulation of CXCR6−/− cells in liver is due to decreased recruitment

The decreased accumulation of antihost CXCR6−/− CD8 T cells could reflect decreased recruitment to the liver; however, altered proliferation or survival could also play a role. To differentiate between these possibilities, we developed a short term recruitment assay that minimizes the potential for proliferative or survival signals to influence the accumulation of donor lymphocytes in the liver. With this assay we were able to track donor cells infiltrating liver and lymphoid organs during a 6-h period after injection by determining the number of donor H-2Dd+Thy1.2+ cells in various compartments. We chose 6 h for the short term recruitment assay based on initial experiments that demonstrated the ability to detect migrating, short term recruitment donor cells in the liver in the midst of on-going GvHD hepatitis (data not shown). Importantly, essentially all donor CD8 T cells (H-2Db), whether CXCR6−/− or WT, were of the previously activated phenotype, as shown by the expression of (CD44high, CD45RBlow, and L-selectinlow); thus, the donor CD8 T cells appeared to share a similar phenotype and state of activation. Spleen cells from day 8 GvHD mice were injected i.v. into recipients (H-2Dbh+) also undergoing day 8 GvHD. After collecting cells from various tissues, we calculated a liver recruitment ratio, defined as the absolute number of CD8 or CD4 short
term recruitment donor cells (CD8+H-2Dd-Thy1.2+ or CD4+H-2Dd-Thy1.2+) harvested from a specific compartment divided by the absolute number of such donor cells injected multiplied by 100. By performing this short term recruitment assay using donor lymphocytes from WT GvHD and CXCR6-deficient GvHD, we found that the liver recruitment ratio of donor CXCR6−/− CD8 cells was decreased by 33% (B6, 12 ± 2%; CXCR6−/−, 8 ± 1%; n = 8; p < 0.05; Fig. 3a), which corresponds well with the 39% decrease in the percentage of donor CD8 lymphocytes infiltrating the liver as described above (Fig. 2c). These findings suggest that CXCR6 supports recruitment, rather than proliferation, of activated CD8 cells in GvHD. It is noteworthy that the liver recruitment ratios of described above (Fig. 2).

Reduced hepatic recruitment of CXCR6−/− CD8 lymphocytes could also reflect decreased availability in the blood. To address this, we examined the percentage of relevant cells circulating in the blood and found that the levels of CXCR6−/− vs WT CD8 cells (CD8 donor cells from the second transfer, i.e., CD8+H-2Dd-Thy1.2+; Fig. 3b; n = 8) in the blood of recipient mice 6 h after injection (i.e., at the end of the short term recruitment assays) were similar, suggesting that CXCR6 deficiency did not significantly alter availability in the blood.

Although little proliferation is likely to occur during the 6-h assay, to rule out any possible effect of proliferation on cell numbers, we compared the proliferation of CXCR6−/− vs WT donor T cell populations by measuring the incorporation of BrdU into LIL. We injected BrdU i.p. 2 h before harvesting LIL from day 7 WT and CXCR6-deficient GvHD mice. As shown above (Fig. 2a), the absolute number of CXCR6−/− donor lymphocytes infiltrating the liver was again decreased (Fig. 4a), but the percentage of WT donor vs CXCR6−/− CD8 lymphocytes incorporating BrdU was almost identical (WT B6, 14 ± 1%; CXCR6−/−, 16 ± 3%; Fig. 4b; n = 4–5), suggesting that the absence of CXCR6 does not affect the proliferative capacity of the donor CD8 cells in the liver.

A third potential contributing factor to the accumulation of cells in the liver is apoptosis. It has been well established that a large number of activated cells programmed for apoptosis accumulate in the liver (22). To assess the possibility that CXCR6/CXCL16 may be solely providing a survival signal in the liver and thus accumulating more cells in WT GvHD, we examined donor LIL for annexin V and 7-aminoactinomycin D (7-AAD) staining to assess the number of cells undergoing apoptosis. With our method of isolating LIL, we found few of the harvested cells to be undergoing apoptosis or necrosis and little difference between the WT and CXCR6-deficient donor cells in terms of their apoptotic markers (annexin V or CD19+ IgG, 3 ± 0.6% of WT B6; 2 ± 0.5% of CXCR6−/− (p = 0.17); 7-AAD, 2 ± 0.2% of WT B6 vs 2 ± 0.4% of CXCR6−/− (p = 0.87); Fig. 4c; n = 4–8). These data thus support an important role for CXCR6 in CD8 cells homing into the liver.

**Discussion**

In this study we examined the in vivo role of the chemokine receptor CXCR6 in a model of lymphocyte recruitment to the liver during GvHD hepatitis. First, we examined the expression of CXCR6 on LIL using mice heterozygous for a GFP knockin mutation; the endogenous CXCR6 promoter drives the expression of GFP, allowing the detection of CXCR6+ cells by green fluorescence (17). Using these splenocytes as donors, we found few T cells infiltrating the liver under normal conditions over a 1-wk period, in contrast to the significant increase observed under inflamed conditions, concomitant with an increase in the percentage of lymphocytes expressing CXCR6. The increase in frequency of CXCR6+ T cells was most prominent in target tissues of inflammation (i.e., liver) compared with secondary lymphoid organs (i.e., spleen). This is consistent with past findings showing that CXCR6 is up-regulated upon activation and is found in abundance on T cells in Th1-mediated inflammation as in hepatitis C-induced (9, 18) and alcohol-induced cirrhosis and rheumatoid arthritis (9).

Next, we examined expression of the ligand, CXCL16, and found it under both normal and inflamed conditions in the liver by RT-PCR. Our finding of CXCL16 up-regulation of mRNA expression during GvHD is consistent with the findings of others who have reported up-regulation during inflammation in the liver (16, 19) and in heart valves (23). CXCL16 is also observed in normal bone

![FIGURE 3. Short term recruitment of donor lymphocytes in the liver.](image-url)
marrow, where it may participate in the homing of plasma cells to the bone marrow (24).

After confirming the expression of the receptor-ligand pair in GVHD hepatitis, we examined the functional significance of the pair by focusing on the natural course of GVHD in mice induced by transferred CCRX6-deficient cells. Transferring splenocytes from homozygous CCRX6-GFP mice, we examined the severity of disease by analyzing the frequency of donor cells infiltrating the liver. We found that the lack of CCRX6 on donor cells resulted in fewer CD8 LIL, in both percentage and absolute numbers, suggesting a specific decrease in the pathologically relevant cell population. Because this observation was made 1 wk after donor cell transfer, we could not exclude the possibility that CCRX6 was required for lymphocyte proliferation or amplification of the inflammatory reaction. We therefore examined the role of CCRX6 in recruitment more closely using a short term recruitment assay. By focusing our attention on a 6-h window, we were able to minimize the effect of proliferation and survival while concentrating on actual homing. With this short term recruitment assay we again found a subset-specific decrease in the donor CD8 T cells, demonstrating a specific role for CCRX6 in activated CD8 T cell localization from blood into the inflamed liver.

A role for CCRX6 and CXL16 in cell recruitment from blood has been suggested previously based on their ability to mediate chemotaxis (13, 16) and firm adhesion (24, 25) of different cell populations in vitro. In its soluble form, CXL16 could be transported and presented luminally by endothelial cells to mediate chemokine-dependent activation of surface integrins on lymphocytes. Indeed, CXL16-dependent firm adhesion of αβ+ lymphocytes on VCAM-1+ endothelial cells has been suggested to be important in firm adhesion of lymphocytes to neocapillaries of infected cardiac heart valves (23). In its transmembrane form, CXL16 may be able to function as a structural adhesion molecule, anchoring activated CD8 lymphocytes, which may promote their retention in the site of inflammation (24, 25). CXL16 joins fractalkine (CX3C ligand 1) as the only other transmembrane chemokine that can function in both its soluble and its membrane-bound form to regulate cell migration and adhesion (26, 27).

A role for CCRX6/CXL16 in providing a survival signal could also be put forth, because CXL16 is up-regulated on DC under activating conditions (16) with a concomitant increase in CCRX6 on cocultured T cells (9). Other chemokines, such as stromal cell-derived factor-1, have been shown to be survival factors for CD4 T cells (28). However, in our analysis of markers of proliferation (BrdU incorporation) and apoptosis (annexin V and 7-AAD) on donor CD8 LIL, we found no significant difference in the percentage of positive cells, suggesting no proliferative or survival advantage of WT cells over CCRX6-deficient cells. Our current studies have concentrated on short term recruitment, but additional studies will be necessary to critically examine possible additional roles of CCRX6/CXL16 in the liver. One factor we have not been able to address directly in the current studies is the rate of exit of homed cells from the liver. Detailed studies conducted in sheep estimate the total output of lymphocytes from the liver through the afferent lymphatics to be ~1 × 10^6 cells/h under normal conditions (29). However, under activated conditions (i.e., after the administration of i.v. endotoxin), afferent lymphatic flow from the liver to the liver-draining lymph node peaked at 6 h, but cellular output decreased until 6 h and subsequently peaked by 18 h. Furthermore, cell transfer experiments of alloantigen-stimulated lymphocytes into normal rats showed progressive accumulation of cells in the liver from 6–9 h, but peak accumulation in the liver-draining lymph node occurred later, at 15 h (30). The results of both studies suggest that at 6 h after injection, the liver is rapidly accumulating cells, and cellular exit is likely to have little effect on cell numbers at this early time point.

The residual accumulation of CCRX6−/− CD8 cells in the liver indicates that other mechanisms compensate for the CCRX6 deficit. Other investigators have suggested a role of CCR5 in mediating recruitment of CD8 donor T lymphocytes to the liver in GVHD (31–33). Their studies examined total lymphocyte accumulation at different time points, but did not exclude a potential contribution of concomitant effects on proliferation; nevertheless, CCR5 and CCRX6 may both participate in the accumulation of...
CD8 lymphocytes in GvHD-induced hepatitis. Indeed, CCR5-, CXCR3-, and CXCR6-expressing lymphocytes have been found in liver infiltrates of patients with hepatitis C (18, 34), and combinations of these three chemokine receptors can be coexpressed on activated T cells (8, 17). Experiments have also supported an important role of CXCR3 in many target organs of GvHD, including the liver, as shown by improved liver histology in GvHD induced by CXCR3-deficient lymphocytes compared with WT (35). Being partially redundant for recruitment into the liver, multiple chemokine receptors may also play a role in navigation within the liver. Others have stressed the concept of a "synergy" of chemokines mediating an inflammatory process (36) and have emphasized that combinations of chemokine receptors, rather than individual receptors, act together to define the trafficking of differentiated effector/memory lymphocyte populations (8).

In conclusion, our novel data demonstrate that CXCR6 is an important mediator of the accumulation of activated CD8 T cells in the liver in GVHD-induced hepatitis. The reduced accumulation of CXCR6-/- CD8 cells appears to reflect, at least in large part, a significant defect in their ability to migrate into inflamed liver from blood. Thus, we conclude that CXCR6 plays an important role in the hepatic homing and accumulation of activated CD8 T lymphocytes and may act in concert with other chemokine receptors to orchestrate pathologic inflammation in the liver.

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References