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CXC Chemokine Ligand 16 Promotes Integrin-Mediated Adhesion of Liver-Infiltrating Lymphocytes to Cholangiocytes and Hepatocytes within the Inflamed Human Liver

Mathis Heydtmann,* Patricia F. Lalor,* J. Albertus Eksteen,* Stefan G. Hübischer,* Mike Briskin,† and David H. Adams2*}

Lympocyte recruitment to the liver is critical for viral clearance in acute hepatitis and in the pathogenesis of chronic inflammatory liver disease when persistent chronic inflammation leads to fibrosis and cirrhosis. Chemokines regulate leukocyte recruitment and positioning in tissues and are thus crucial regulators of chronic inflammation. The chemokine CXCL16, which is found in liver tissue, exists in a transmembrane as well as soluble form, providing a potential mechanism for localization to particular structures. We studied the role of CXCL16 and its receptor CXCR6 in lymphocyte recruitment and retention in the liver. A higher proportion of CXCR6+ T cells was detected in blood of hepatitis C virus patients compared with healthy subjects, and in chronic inflammatory liver disease >60% of intrahepatic T cells expressed CXCR6, including CD4, CD8, and CD56+ T cells compared with <30% in matched blood samples. CXCR6+ lymphocytes were found in association with CXCL16+ bile ducts in portal tracts and with hepatocytes at sites of interface hepatitis. Analysis of CXCL16 expression and subcellular distribution in cultured human cholangiocytes, sinusoidal endothelial cells, and hepatocytes revealed that all three cell types expressed CXCL16, with the strongest staining seen on cholangiocytes. CXCL16 on the cholangiocyte membrane was able to support lymphocyte adhesion by triggering conformational activation of β1 integrins and binding to VCAM-1. Thus, CXCL16 can promote lymphocyte adhesion to epithelial cells and may function to attract and retain effector cells that promote biliary and hepatic destruction in inflammatory liver disease. The Journal of Immunology, 2005, 174: 1055–1062.
an important role for CXCR6/CXCL16 in the positioning and retention of effector leukocytes in the human liver.

Materials and Methods

Patient characteristics and tissue studied

Ethics approval for the study was given by the South Birmingham Local Research Ethics Committee (Queen Elizabeth Hospital, Birmingham, U.K.) and the University Hospital Birmingham Trust (Queen Elizabeth Hospital, Birmingham, U.K.). All liver tissue and peripheral blood were collected with informed consent. Liver tissue from nondiseased liver and patients with end-stage hepatitis C cirrhosis as well as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) was used for immunohistochemical studies (five samples each) and isolation of liver-infiltrating lymphocytes (LIL) (six nondiseased, five HCV, and two PBC samples). Nondiseased liver was obtained either from patients undergoing hemi-hepatectomy to remove liver metastases or from organ donors in which the liver tissue was not used for transplantation. The tissues had no biochemical, virological, or clinical evidence of liver disease, and histology of the liver showed no significant abnormality. All patients with end-stage hepatitis C cirrhosis were undergoing transplantation for hepatitis C as the primary diagnosis and all were HCV-RNA positive by PCR. Diagnosis of PBC and PSC was made on clinical, immunological, and histological grounds before orthotopic liver transplantation. Samples of liver tissue (1 cm) from explanted livers or resected tissue were snap frozen in 2 l of dimethyl sulfoxide or resection for later immunohistochemistry. Tissue from donor organs had been perfused with University of Wisconsin (Madison, WI) preservation fluid and maintained at 4°C for 12–24 h before being processed. Matched peripheral blood was obtained from donors of liver tissue where possible. Peripheral blood was taken at the beginning of the operation before any blood transfusions. Peripheral blood was also collected from healthy donors and patients with chronic hepatitis C without cirrhosis.

Isolation of cells

PBL were isolated from venous blood collected into tubes containing EDTA and diluted 1:1 with PBS. Lymphocytes were isolated by centrifugation at 403 x g for 30 min at room temperature on a Ficoll (Lymphoprep; Nycomed Pharma) gradient and washed twice with PBS. LIL were isolated, as described before (18), within 24 h of harvesting. Liver tissue collected into RPMI 1640 medium (Invitrogen Life Technologies) was diced into 1-mm³ pieces and digested in RPMI 1640 containing collagenase type 1a (Sigma-Aldrich) at 37°C for 1.5 h. The digested tissue was washed through a nylon mesh filter (100 collagens type 1a (Sigma-Aldrich) at 37°C for 1.5 h. The digested tissue was washed through a nylon mesh filter (100 µm) to remove debris and washed three times in PBS with FCS (Invitrogen Life Technologies) to remove collagenase. The cell suspension was layered on a Percoll (Amer Sham Biosciences) density gradient (30/70%) and centrifuged for 30 min at 717 x g. The lymphocyte band was removed from the interface between 30/70% Percoll, and further washed three times in PBS before resus- pension in FCS containing 10% DMSO (Sigma-Aldrich) for storage in liquid nitrogen using temperature-controlled freezing containers (Nalgene). Cells were rapidly thawed at 37°C and rested for 2 h before they were used for adhesion assays or staining for cytometry. Previous experiments comparing fresh lymphocytes with thawed, rested cells showed no difference in the functional behavior of these cells in a number of different adhesion assays. It has been previously reported (11) that CXCR6 expression is modulated by the cell isolation protocol used, and so we investigated the effect of different lymphocyte isolation protocols on CXCR6 expression. We compared CXCR6 expression on Ficoll-isolated PBLs, lymphocytes isolated by centrifugation over a Percoll gradient, and cells directly isolated from liver using the Cellpop method at the same concentration used in our isolation protocol for LIL. Again, CXCR6 expression was found to be unchanged. Finally, we used a nonenzymatic technique described before (18) to isolate lymphocytes from liver tissue, and again detected no effect on CXCR6 expression compared with the collagenase technique (data not shown).

Human cholangiocytes were isolated according to Joplin (19). The isolation is similar to the isolation of LIL, with an additional step of positive selection using magnetic beads (Dynal Biotech) coated with HEA-125 Ab (PROGEN Biotechnik) after centrifugation over a Percoll density gradient (30/70%). Cells were cultured until confluent on coverslips (Merck) in cholangiocyte culture medium consisting of 45% HAM’s F12, 45% DMEM (both from Invitrogen Life Technologies), and 10% heat-inactivated FCS (Invitrogen Life Technologies) with glutamine (2 mM/ml; Invitrogen Life Technologies), penicillin (100 U/ml; Britannia Pharmaceuticals), streptomycin (100 µg/ml; Celltech Group), epidermal growth factor (10 ng/ml; Sigma-Aldrich), insulin (0.124 IU/ml; Eli Lilly), hydrocortisone (2 µg/ml; Pharmacia), cholina toxin (10 ng/ml; Sigma-Aldrich), and Tri-iodothyronin (2 nM/ml; Sigma-Aldrich). Adherent cells were used either for immunohistochemical staining or for adhesion assays. For some experiments, the human cholangiocyte cell line AKN-1 was used (a kind gift of A. Nussler, Department of General Surgery, University of Ulm, Ulm, Germany). AKN-1 cells were initially cultured in the medium described by Nussler et al. (20), and adhesion experiments were compared with AKN-1 cells cultured in our cholangiocyte medium. No differences were seen in the adhesion results between the two media, and for all subsequent experiments cells were cultured in cholangiocyte culture medium until confluent on glass coverslips in 48-well plates and used immediately for adhesion assays.

![Image](http://www.jimmunol.org/Downloadedfrom)
Human sinusoidal endothelial cells (SEC) were isolated using the same method as for cholangiocytes, but substituting CD31 Ab (10 μg/ml; DakoCytomation) during the magnetic bead selection step. Cells were then cultured in SEC medium containing 10 ng/ml vascular endothelial growth factor and 10 ng/ml HGF (R&D Systems), as described before (21).

Human hepatocytes were isolated from an encapsulated liver wedge and cultured overnight in serum-free, arginine-free medium, as described before (22). They were used immediately for immunocytochemistry.

**Immunohistochemistry**

Expression of CXCL16 and CXCR6 in liver tissue was studied by immunohistochemistry, as previously described (18). Briefly, sections of snap-frozen liver tissue were fixed in acetone for 10 min, and incubated with primary mouse anti-human Abs (SD7, anti-CXCL16 IgG1 used 1/10, or 7A2, anti-CXCR6 IgG2a, 1/100) or isotype-matched control for 45 min, followed by incubation with secondary Ab (rabbit anti-mouse; DakoCytomation). The secondary Ab was detected by indirect alkaline phosphatase anti-alkaline phosphatase method as for cholangiocytes, but substituting CD31 Ab (10 μg/ml; DakoCytomation) during the magnetic bead selection step. Cells were then incubating on ice for 45 min with the IgG2a primary mouse Ab against CXCR6 at predetermined optimal concentrations (1/500 of clone 7F3; 1/50 clone 7A2) in 100 μl of PBS. Cells were washed with cold PBS and further incubated on ice for 45 min with FITC-conjugated F(ab)2 of goat anti-mouse Ig (DakoCytomation). Cells were again washed with PBS and incubated with mouse IgG (1 mg/ml; Sigma-Aldrich) to saturate nonspecific binding sites on the FITC-conjugated F(ab)2. Up to two other Abs (PE-conjugated CD8, CD4, CD56 (all from BD Biosciences); PE-conjugated CD3 (DakoCytomation); PE-conjugated CD11a, and ECD (PE-Texas Red)-conjugated CD8, CD3 (from Beckman Coulter) were used in a further incubation step. After final incubation, cells were washed with PBS and incubated with 2% paraformaldehyde (Sigma-Aldrich) before analysis in an Epics XL Coulter flow cytometer (Beckman Coulter) with WinMDi software (The Scripps Research Institute, La Jolla, CA). The lymphocyte population was gated using forward and side scatter parameters to exclude debris and dead cells, and staining on labeled cells was compared with staining using matched control Ab. A negative population was gated at staining intensity of 98% of negative control cells.

**Adhesion assays**

Cholangiocytes and AKN-1 cells isolated and cultured as described above were used for the adhesion assays because immunocytochemistry and confocal microscopy had shown the strongest staining of CXCL16 on these cells (Fig. 4). To investigate the CXCR6/CXCL16 interaction, blocking Abs were added to lymphocytes (anti-CXCR6, anti-CD18) and/or biliary epithelial cells (anti-CXCL16, anti-VCAM-1) before adhesion. Confluent cell layers were incubated for 30 min at 37°C in 100 μl of the appropriate medium with anti-CXCL16 (dilutions of a stock solution of 4.33 mg/ml), anti-VCAM-1 (1/100, clone BBIG-V1(4B2) from R&D Systems), or isotype-matched control Ab, as indicated. A total of 1 × 10^5 normal PBLs or LIL from undiseased liver was incubated with anti-CXCR6 (dilutions of a stock
solution of 1.8 ng/ml or anti-CD18 (1/100) for 30 min at 37°C in 100 μl of PBS. After incubation of the lymphocytes and the cell layers separately, lymphocytes were added to the biliary cells for a further 30 min of incubation. G protein-mediated signaling was blocked in some assays by adding pertussis toxin (Sigma-Aldrich; 100 ng/ml) to the lymphocytes for 90 min at 37°C before the assay. After incubation, nonadherent lymphocytes were washed off using PBS, and the cell layers with adherent lymphocytes were fixed in 70% ethanol. For each experiment, adherent lymphocytes in at least 10 high power fields of view (surface area of 1 high power field = 0.14 mm²) were counted using a phase contrast microscope. To validate the method, an initial series of experiments was counted by a blinded investigator (P. Lalor). Flow assays were performed, as described before (21). In brief, microslides used for lymphocyte perfusion were coated with VCAM-1 at the concentrations indicated with or without CXCL16 at a method, an initial series of experiments was counted by a blinded inves- tigator (P. Lalor). Flow assays were performed, as described before (21). In brief, microslides used for lymphocyte perfusion were coated with VCAM-1 at the concentrations indicated with or without CXCL16 at a method, an initial series of experiments was counted by a blinded inves- tigator (P. Lalor). Flow assays were performed, as described before (21). In brief, microslides used for lymphocyte perfusion were coated with VCAM-1 at the concentrations indicated with or without CXCL16 at a method, an initial series of experiments was counted by a blinded inves- tigator (P. Lalor). Flow assays were performed, as described before (21). In brief, microslides used for lymphocyte perfusion were coated with VCAM-1 at the concentrations indicated with or without CXCL16 at a method, an initial series of experiments was counted by a blinded inves- 

Activation of VLA-4 integrin as a result of CXCR6-mediated intracellular signaling

To determine whether occupancy of CXCR6 influenced function of the integrin VLA-4, CXCR6 was engaged using purified CXCL16 protein (gift from Millennium Pharmaceuticals). Titration of concentrations between 0.02 and 20 μg/ml determined the optimum concentration for these assays. VLA-4 activation by manganese (2 mM MnCl₂; Sigma-Aldrich) was used as a positive control and BSA as a negative control. To detect changes in VLA-4 activation on mouse and human lymphocytes, Abs that recognize conformation-dependent epitopes on the activated integrin were used: 12G10 (24) (IgG1, mouse anti-human; gift from M. Humphries, School of Biological Sciences, University of Manchester, Manchester, U.K.) to detect activated human VLA-4 on LIL, and 9EG7 (25) (IgG2a, rat anti-mouse; gift from D. Vestweber, Max Planck Institute for Immunology, Freiburg, Germany) to detect activated mouse VLA-4 on L1.2 cells. Incubation with the species-specific primary Ab or isotype-matched control (IMC) was performed for 45 min at room temperature and at the same time as CXCR6 engagement. In some experiments, lympho- cytes were preincubated with pertussis toxin (100 ng/ml) for 90 min immediately before addition of ligand to inhibit Gi protein-mediated sig- naling. In a second step, species-specific FITC-conjugated secondary Ab (goat anti-mouse or goat anti-rat; Southern Biotechnology Associates) was used to detect 12G10 or 9EG7, and samples were analyzed by flow cy- tometry, as described above.

Statistical analysis

For comparison of matched samples, Student’s paired samples t test was used on the raw numbers where possible, and a probability of <0.05 (two tailed) was regarded as significant. For comparison of unpaired results and means, the nonparametric Mann-Whitney U test was used with the same significance levels.

Results

CXCR6 expression is increased on LIL

The comparison of CXCR6 levels by flow cytometry on LIL and matched PBL from organ donors and HCV patients revealed that more LIL expressed CXCR6 than matched PBL and that levels of receptor were higher on LIL (Fig. 1). Overall 61.5% of LIL stained with CD11a<sup>high</sup>CD8<sup>+</sup>T cells, and CD56<sup>+</sup> cells demonstrated an enrichment of CXCR6 cells within all major subtypes of lymphocytes in the liver (Fig. 2). Although the percentages are similar between LIL from diseased and nondiseased liver, absolute numbers of lymphocytes are several steps higher in the HCV livers, and the amount of CXCR6 per cell, determined by analysis of mean channel fluorescence, was also in- creased (Fig. 1, D and E). Lymphocytes isolated from the livers of two patients with PBC were analyzed in the same way, and a similar enrichment of CXCR6-positive cells was found. Sections of undis- eased liver, end-stage hepatitis C-infected liver, and livers from pa- tients with PBC and PSC were stained with anti-CXCR6 Abs. In the normal liver, CXCR6 staining was generally weak and confined to occasional leukocytes and dendritic-like cells in portal tracts (Fig. 1C). In contrast, strong staining of CXCR6 was seen in the lymphocyte-rich areas of inflammatory infiltrates in all of the chronic inflam- matory liver diseases (Fig. 1D).

CXCL16 is expressed in normal and chronically inflamed liver tissue, including chronic hepatitis C-infected liver

We demonstrated the presence of CXCL16 protein in normal, HCV, and other end-stage cirrhotic livers by Western blotting and densitometry. CXCL16 was detected in all liver samples studied, including liver from organ donors and from patients with chronic inflam- matory liver disease. Levels in the liver were much higher than those detected in other tissues, although densitometry of Western blots suggested that the total amount of CXCL16 was similar in nondiseased and diseased liver tissue (Fig. 3). We subse- quently used immunohistochemistry to determine the distribu- tion of CXCL16 staining in the chronic inflammatory liver dis- eases PSC, PBC, and end-stage hepatitis C-infected liver. In normal liver, CXCL16 was detected on hepatocytes, bile ducts, and lymphocytes in portal tracts and on sinusoidal cells and occa- sional cells with a dendritic morphology in the liver parenchyma. It was absent from portal vessels and central veins (Fig. 4). The overall staining patterns were similar in the chronic inflammatory liver diseases, although there was increased staining of sinusoids in HCV and of bile ducts and ductules in PBC and PSC and increased focal expression of CXCL16 on periportal hepatocytes at sites of interface hepatitis (Table I and Fig. 4, D and E).

CXCL16 is expressed on hepatocytes, cholangiocytes, and sinusoidal endothelial cells

We investigated subcellular localization of CXCL16 on primary cultures of different human hepatic cell types. CXCL16 was strongly expressed on cholangiocytes with moderate expression on hepatocytes and weak expression on sinusoidal endothelial cells in culture (Fig. 5). Confocal imaging showed the highest staining intensity on cholangiocytes, with a membranous pattern, whereas in hepatocytes staining was overall weaker and present in the cytoplasm (Fig. 5). This led us to use cholangiocytes for the sub- sequent studies of lymphocyte binding.
CXCL16 on cholangiocytes promotes the adhesion of CXCR6+ lymphocytes

Having demonstrated high expression of CXCR6 on LIL and CXCL16 on target cells within the liver, we investigated the ability of CXCL16 to promote the adhesion of either LIL or PBL to liver cells using both primary human cholangiocytes and the biliary epithelial cell line AKN-1 (20). These cells were used because of high staining intensity and membranous expression detected by confocal immunohistochemistry. A significantly higher proportion of LIL adhered to cholangiocytes compared with PBL (Fig. 6A). Binding of both PBL and LIL was inhibited by the presence of blocking mAb against CXCL16 or CXCR6. Lymphocyte adhesion was inhibited by 61 and 47% with anti-CXCL16 and anti-CXCR6, respectively (Fig. 6B). Adhesion blockade was dose dependent, with a maximum effect

Table I. Immunohistochemical staining of CXCL16 in undiseased liver, liver from HCV-infected transplant patients, and patients transplanted for PBC and PSCa

<table>
<thead>
<tr>
<th>Portal Fields</th>
<th>Parenchyma</th>
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<tr>
<td>Bile Duct</td>
<td>Portal Vessels</td>
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<tr>
<td>Normal</td>
<td>0.5 (0.35)</td>
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<tr>
<td>HCV</td>
<td>0.5 (0.5)</td>
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<tr>
<td>PBC</td>
<td>0.7 (0.45)**</td>
</tr>
<tr>
<td>PSC</td>
<td>0.6 (0.55)**</td>
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*a Staining was reported by two investigators (M. Heydtmann and S. Hubscher) in portal fields and parenchyma and graded semiquantitatively according to intensity. 0, No staining seen; 1, weak staining; 2, moderate staining; 3, strong staining, as described and validated before (23). In addition to endothelial staining of the sinusoids, there were occasional intrasinusoidal cells seen with a distribution and morphology of Kupffer cells. * Focal increase at sites of interface hepatitis. ** Focal increase on inflamed bile ducts.
at a concentration of 45 μg/ml CXCL16 or 20 μg/ml CXCR6 (Fig. 6C), and no additional effect was gained by combining optimal concentrations of both Abs (53% inhibition with a mixture of anti-CXCR6 and anti-CXCL16). Inhibition of CXCR6/CXCL16 had a similar effect on adhesion as blockade of CD18 integrin-mediated binding (58% inhibition), but there was no additive effect when blockade of CXCR6/CXCL16 was combined with anti-CD18 (data not shown). Lymphocytes were preincubated with anti-CXCR6 Abs or anti-CD18 (blocking adhesion to ICAM), or cholangiocytes were preincubated with anti-CXCL16. L1.2, LIL, five or more experiments each, *p < 0.025 of combined L1.2 and LIL experiments vs control experiment without blocking Ab (Student’s t tests on raw counts). C, Similar levels of adhesion were seen with PBL binding to primary human cholangiocytes and the cholangiocyte cell line AKN-1. Inhibition of adhesion was conducted as in B using different concentrations of either anti-CXCL16 or anti-CXCR6.

CXCL16 does not activate adhesion to immobilized VCAM-1 under conditions of fluid flow

To study the role of CXCR6 on adhesion of lymphocytes to immobilized VCAM-1 under fluid conditions, we compared adhesion of L1.2 wild-type and CXCR6-transfected cells with different concentrations of VCAM-1 in the presence or absence of coimmobilized CXCL16. In all experiments, the adhesion of L1.2 cells increased with increasing VCAM-1 concentrations (Fig. 7B). For CXCR6-transfected L1.2 cells, coimmobilization of CXCL16 and VCAM-1 did not lead to an increase in lymphocyte adhesion compared with VCAM-1 alone (Fig. 7B). To minimize the possibility

CXCR16 AND CXCR6 IN THE LIVER

FIGURE 6. Lymphocyte binding to cholangiocytes is reduced by inhibition of CXCR6/CXCL16. A, LIL show increased adhesion compared with PBL to cholangiocytes (+, p = 0.014). B, Adhesion of both PBL and LIL to cholangiocytes was reduced using anti-CXCL16 or CXCR6 mAb. In each experiment, at least 10 high powered fields were counted, and the result was expressed as percentage of control (addition of an isotype-matched irrelevant Ab). Lymphocytes were preincubated with anti-CXCR6 Abs or anti-CD18 (blocking adhesion to ICAM), or cholangiocytes were preincubated with anti-CXCL16. L1.2, LIL, five or more experiments each, *p < 0.025 of combined L1.2 and LIL experiments vs control experiment without blocking Ab (Student’s t tests on raw counts). C, Similar levels of adhesion were seen with PBL binding to primary human cholangiocytes and the cholangiocyte cell line AKN-1. Inhibition of adhesion was conducted as in B using different concentrations of either anti-CXCL16 or anti-CXCR6.

CXCR6-transfected L1.2 cells were unable to adhere to CXCL16 under static conditions or under low blood flow rates of 0.05 Pa (data not shown). The experiments were then repeated using immobilized VCAM-1. There was little spontaneous static adhesion of L1.2 cells to VCAM-1-coated glass slides, but static adhesion of CXCR6-transfected L1.2 cells to VCAM-1 was activated by the presence of CXCL16 at 2 μg/ml in a time-dependent manner. Wild-type L1.2 cells lacking CXCR6 did not show CXCL16-mediated adhesion to VCAM-1 (Fig. 7A).

CXCL16 does not activate adhesion to immobilized VCAM-1 under conditions of fluid flow

To study the role of CXCR6 on adhesion of lymphocytes to immobilized VCAM-1 under fluid conditions, we compared adhesion of L1.2 wild-type and CXCR6-transfected cells with different concentrations of VCAM-1 in the presence or absence of coimmobilized CXCL16. In all experiments, the adhesion of L1.2 cells increased with increasing VCAM-1 concentrations (Fig. 7B). For CXCR6-transfected L1.2 cells, coimmobilization of CXCL16 and VCAM-1 did not lead to an increase in lymphocyte adhesion compared with VCAM-1 alone (Fig. 7B). To minimize the possibility

FIGURE 7. CXCR6 promotes adhesion of lymphocytes to VCAM-1 in static conditions, but not under conditions of flow. A, Static adhesion: CXCL16 rapidly triggers static adhesion of CXCR6-transfected L1.2 cells to VCAM-1. A total of 2 ng/ml CXCL16 was added to L1.2 cells (WT = wild type; B = CXCR6 transfectants) on dishes coated with 1 μg/ml VCAM-1 for 0 (control) to 10 min. Manganese chloride was added as a positive control and induced similar levels of adhesion of wild-type and CXCR6 transfectants. Only the CXCR6-positive transfectants responded to CXCL16 with a marked increase in adhesion, which peaked at 2 min. B, Adhesion of lymphocytes to VCAM-1 under flow. Wild-type and CXCR6-transfected L1.2 cells were perfused over VCAM-1 at 0.05 Pa. Adhesion to VCAM-1 increased with increasing concentration. Coincubation of VCAM-1 and CXCL16 had no additive effect on the number of lymphocyte binding. Immobilization of CXCL16 in the microslide in the absence of VCAM-1 was unable to support adhesion of either lymphocyte population.
of competition between CXCL16 and VCAM-1 for immobilization to the glass surface, we coated with either VCAM-1 and CXCL16 together (Fig. 7B) or in two separate stages (VCAM-1 as the first step or CXCL16 as first step). There was no difference in either adhesion of lymphocytes or the amount of immobilized VCAM-1 bound (detected using Abs) when we examined glass coated in these different ways (data not shown).

**Signaling from CXCR6 leads to activation of the α₄β₁ integrin VLA-4**

To demonstrate that occupancy of CXCR6 can activate VLA-4, leading to increased binding to VCAM-1, we activated CXCR6 on human LIL and CXCR6-transfected murine L1.2 cells using rCXCL16. Conformational activation of VLA-4 on human lymphocytes was detected by staining with mAb 12G10, which recognizes an activation-induced epitope on the β1 A-domain. Induction of this epitope parallels the activation state of the integrin (24). The mAb 9EG7, which recognizes an activation-inducible β1 neoepitope on murine VLA-4, was used to detect activation on L1.2 cells (25). Incubation of the CXCR6-transfected mouse cell line L1.2 with CXCL16 led to an increase in the VLA-4 activation epitope detected by staining with 9EG7 (Fig. 8, A and B). This peaked at 25 min and was maximal with a concentration of 2 μg/ml CXCL16 (Fig. 8A). The 9EG7 staining was prevented by preincubating transfected cells with pertussis toxin (Fig. 8B). The fact that staining with 9EG7 peaked at only 25 min is consistent with a conformational activation of the integrin rather than increased protein expression, and suggests a role for CXCR6 in the activation of lymphocyte adhesion via VLA-4. We next tested the ability of LIL to respond to CXCL16. A small number of LIL were 12G10 positive in the absence of stimulation, but the majority expressed 12G10 only after activation with 2 μg/ml CXCL16. This was a similar magnitude response to that seen following treatment of the cells with manganese (Fig. 8C).

**Discussion**

Chronic inflammation is characterized by the accumulation of lymphocytes as a consequence of increased recruitment from blood and retention within the tissue at sites of infection or target cell damage. Previous studies from our group and others suggest that specific chemokine/chemokine receptor interactions are involved in the recruitment of lymphocytes into the liver via either portal or sinusoidal endothelium, but little is known about the signals that position and retain lymphocytes at epithelial surfaces within the liver (18, 26). CXCR6 expression has been reported on activated extralymphoid lymphocytes, particularly those polarized for Th1 responses (13–15), and we now confirm that CXCR6⁺ cells are enriched in several major lymphocyte subsets within the liver, suggesting that CXCR6 is associated with liver infiltration. Because naïve T cells are largely excluded from the liver, an alternative explanation is that the enrichment of CXCR6⁺ cells in the liver is a consequence of CXCR6 being increased on primed/Ag-experienced T cells compared with naïve T cells. To address this, we used high levels of CD11a expression to discriminate between primed and naïve CD8 T cells and compared expression of CXCR6 on CD8⁺ cells with similarly high levels of CD11a from the liver and blood. The expression of CXCR6 was markedly higher on primed (CD11a⇑) CD8 T cells from the liver compared with primed (CD11a⇑) T cells in blood, suggesting that CXCR6 expression is associated with tissue infiltration rather than merely reflecting compartmentalization of primed T cells in the liver.

We detected CXCL16 in primary human cholangiocytes and hepatocytes and used cholangiocytes because of their high CXCL16 expression to investigate whether CXCL16 can act directly as an adhesion receptor. Direct adhesion has been described to the other transmembrane chemokine, fractalkine, and more recently to CXCL16 transfected into COS cells (10, 27, 28). We were able to demonstrate CXCR6/CXCL16-dependent adhesion of lymphocytes to human cholangiocytes in vitro, although in contrast to the studies with COS cells, adhesion to CXCL16⁺ cholangiocytes was inhibited by pertussis toxin, suggesting that in these human cells signaling via CXCR6 is required for adhesion rather than a direct adhesive interaction between CXCR6 and CXCL16. There may be several explanations for the differences between our experiments with primary human cells and the transfected COS cells. One obvious difference is the use of murine lymphocytes with primate epithelial cells in the Shimaoka paper (27). This system does not provide murine VCAM-1 or ICAM-1 for the murine lymphocytes to bind.
We used functional binding assays and reporter Abs to show that activation of CXCR6 on LIL triggers β1 integrin-mediated adhesion to VCAM-1. Thus, in vivo CXCL16 could trigger adhesion to VCAM-1, which is strongly expressed on inflamed bile ducts (29). To investigate the mechanism of CXCL16-mediated adhesion, we activated either L1.2 CXCR6 transfectants or CXCR6high LIL with CXCL16 and studied activation of VLA-4. Occupancy of CXCR6 by ligand resulted in the detection of activation-dependent epitopes on VLA-4, confirming that CXCL16 can induce conformational activation of lymphocyte integrins. However, immobilized rCXCL16 had no effect on lymphocyte adhesion in flow-based adhesion assays, and this together with the higher levels of CXCL16 detected on epithelial cells compared with vascular endothelium suggests that CXCR6/CXCL16 interactions may be more important for maintaining stable adhesion to epithelial cells. CXCR6-expressing murine NK-T cells fail to migrate to soluble CXCL16 (30), and it has been postulated that membranous CXCL16 may be required for direct cell-cell adhesion and the facilitation of migration on or through epithelium or endothelium. Our data suggest that adhesion of effector lymphocytes to cholangiocytes will require not only CXCL16 expression, but the presence of VCAM-1 to support integrin-mediated adhesion. Thus, the lack of VCAM-1 on non diseased bile ducts might prevent the development of bile duct damage even in the presence of constitutive CXCL16 expression.

The fact that many of the lymphocytes isolated from the livers of organ donors were CXCR6high suggests that CXCR6/CXCL16 interactions may be involved in the positioning and retention of lymphocytes in normal liver. Normal liver contains a large number of conventional T cells, which include a population of CD8 T cells closely associated with bile ducts (31) as well as NK and NK T cells (32, 33). The high levels of CXCR6 expressed by NK T cells and some other effector cells in blood may be an important factor in the recruitment and retention of these cells within the liver. The normal livers we studied were obtained from organ donors, and we cannot exclude the possibility that T cells are recruited to these livers as part of a low grade inflammatory response (34). However, there was no biochemical or histological evidence of pathological inflammation in any of the normal specimens.

In summary, we propose an important role for CXCL16 in the recruitment and retention of CXCR6+ effector cells in the human liver during inflammatory liver disease, and suggest that this chemokine/receptor pair is a potential therapeutic target to prevent inflammatory damage in chronic hepatitis.

Acknowledgments

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References