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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

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Lymphocyte 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 critical 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 β3 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 hepatocyte destruction in inflammatory liver disease. The Journal of Immunology, 2005, 174: 1055–1062.

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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 orthotopic 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) taken during explant or resection tissue were snap frozen in 2-3 ml of explantation 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 × 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 (Inveritgen Life Technologies) was diced into 1-mm³ pieces and digested in RPMI 1640 containing collagenase type Ia (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 (Amerham Biosciences) density gradient (30/70%) and centrifuged for 30 min at 717 × g. The lymphocyte band was removed from the interface between 30 and 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 three 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 labeled in whole blood. We did not detect any changes in CXCR6 expression when using these methods. We then incubated PBLs for up to 2.3 h of explantation 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.

FIGURE 1. CXCR6⁺ cells are enriched in the liver. A, Percentages of CXCR6⁺ positive cells in LIL compared with PBL in donors of undiseased (normal) liver and patients with end-stage chronic HCV. Each point represents a single sample; matched samples from the same donor are linked with a line. For comparison, values from healthy donors (normal PBL, left scatter plot) and values for CXCR6 in the blood of HCV⁺ patients without significant liver disease (HCV PBL) are also shown. B, Percentage of CXCR6⁺ positive cells in the patients from A, in which matched samples were available. Values given are mean ± SEM, and p values are given for statistical comparison between peripheral blood and LIL. C and D, Immunohistochemical expression of CXCR6 in the liver of a normal liver donor (C) and a patient with end-stage PSC (D). Positive cells stain red with the APAAP technique (see Materials and Methods). C, In normal liver, occasional CXCR6⁺ lymphoid cells are seen in portal tracts (arrows). D, In chronic hepatitis associated with PSC, many CXCR6⁺ lymphocytes are seen infiltrating the portal tracts and some are gathered around bile ducts (BD). E and F, Histograms of two representative patient samples gated for Ag-experienced (CD11a⁺ high) CD8⁺ T cells. PBL are shown in red, and LIL in black lines and IMC in blue. E, PBL and LIL from a donor of undiseased liver; F, PBL and LIL from a hepatitis C-infected patient with end-stage liver disease undergoing liver transplantation. A higher proportion of CD11a⁺ high (Ag-experienced) CD8⁺ T cells in the liver expresses CXCR6 compared with the same subset in blood.
Human sinusoidal endothelial cells (SEC) were isolated using the same method as for cholangiocytes, but substituting CD31 Ab (10 μg/ml; DakoCytonation) 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; DakoCytonation). The secondary Ab was detected by indirect alkaline phosphatase-anti-alkaline phosphatase method as for cholangiocytes, but substituting CD31 Ab (Sigma-Aldrich; 1 mg/ml), washed with PBS, and then incubated with anti-alkaline phosphatase developed with naphthol-AX and fast red (APAAP) or with an avidin-biotin complex and peroxidase. All incubations were conducted at room temperature for 45 min, and sections were washed for 5 min with two changes of PBS, pH 7.4, buffer between incubations. Endogenous peroxidase activity was blocked using sodium azide (Sigma-Aldrich). Sections were counterstained with hematoxylin (BDH), and the staining intensity of CXCL16 and CXCR6 was graded by two observers (M. Heydtmann and S. Hübser) using a system previously validated by confocal microscopy (23). The distribution and intensity of staining in the following structures were recorded: portal tract, venular and arterial endothelium, biliary epithelium and inflammatory infiltrate, hepatocytes, Kupffer cells, and sinusoidal and central vein endothelium.

We also assessed the expression of CXCL16 on isolated cholangiocytes, SEC, and hepatocytes. The cells were cultured overnight on coverslips, fixed with acetone for 5 min, and stained as described for tissue sections. Tissue sections and cultured cells stained using APAAP and fast red were also analyzed using confocal microscopy to determine subcellular localization.

**Western immunoblotting**

Protein extracts from liver tissue were blotted on PBL and LIL, using standard techniques (18). Cells were incubated with goat IgG to block Fc receptors (Sigma-Aldrich; 1 mg/ml), washed with PBS, and then incubated 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 (DakoCytonation). 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 (DakoCytonation); 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 fixed 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 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 BB1G-V1(4B2) from R&D Systems), or isotype-matched control Ab, as indicated. A total of 1 x 10^5 normal PBLs or LIL from undiseased liver was incubated with anti-CXCR6 (dilutions of a stock

**FIGURE 2.** CXCR6 is present on all of the main subtypes of T cells. CXCR6 is present on the main subtypes of T cells (CD4 and CD8 T cells) as well as CD56+ NK/NKT T cells with enrichment in LIL for all subtypes. A, Each panel shows the percentages of CXCR6+ cells in lymphocytes gated, as indicated on CD3, CD4, CD8, or CD56. LIL are compared with peripheral blood in normal blood donors (n), donors of undiseased liver (‘n”), and end-stage chronic HCV patients. B, Mean percentages ± SEM of CXCR6-positive cells in the patients from A, in which matched samples were available (pooled samples for donors of undiseased liver and HCV patients). Values of p indicate significance of differences in CXCR6 expression between peripheral blood and LIL.

**FIGURE 3.** CXCL16 is present in undiseased and chronic HCV-infected liver. A, Western blot of CXCL16 protein in liver samples from patients transplanted for hepatitis C (HC), alcoholic liver disease (ALD), PBC, or PSC, and in normal (undiseased) liver (NL). For comparison, samples from spleen and resected colon (normal) and colon (HCV) were also shown. CXCL16 was detected using anti-CXCL16 Abs (1/250) in all liver samples. Actin is shown as a control for protein loading. B, Mean OD units of five Western blots for each of hepatitis C, normal, PBC, PSC liver (measured by Bio-Rad gel doc), and SEM. For comparison, mean and SEM for colon and spleen tissue (five samples each) are also given. Expression in the liver is similar in normal liver compared with any of the liver diseases. All liver samples have significantly higher CXCL16 RNA expression than colon or spleen samples, which is highly significant (p < 0.001 for each comparison).
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 experiments 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 concentration of 10 μg/ml for 2 h and were blocked using BSA immediately before the assay. PBL or LIL was perfused through the microslides at a shear stress of 0.05 Pascal (Pa), which is comparable to sinusoidal wall shear stress, and phase contrast video recordings were used to analyze percentage of rolling and statically adherent cells. Rolling cells moved slowly, but steadily, over the surface during 5–10 s of observation, while stationary adherent cells did not make any detectable movement over the same period.

**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 2 μg/ml as 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 isotope-matched control (IMC) was performed for 45 min at room temperature and at the same time as CXCR6 engagement. In some experiments, lymphocytes were preincubated with pertussis toxin (100 ng/ml) for 90 min immediately before addition of ligand to inhibit Gα protein-mediated signaling. 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 cytometry, 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**

**CXCL16 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 CXCL16 than matched PBL, and that levels of receptor were higher on LIL (Fig. 1). Overall 61.5% of LIL stained for CXCR6 vs 32.8% of matched PBL (Fig. 1; eight matched samples, p = 0.004). Increased levels of CXCR6 on LIL were confirmed by analysis of mean channel fluorescence: 18.4 ± 3.8 for LIL vs 9.7 ± 1.7 for matched PBL. To see whether these differences merely reflect an enrichment of Ag-experienced T cells in the liver, we compared CXCR6 in blood and liver T cells gated on CD11a\textsuperscript{high}CD8\textsuperscript{+}. High expression of CD11a discriminates between Ag-experienced and naive CD8 T cells. The levels of CXCR6 and proportion of CXCR6\textsuperscript{+} cells were higher in the CD11a\textsuperscript{high} LIL than CD11a\textsuperscript{high} PBL in both undiseased liver and HCV, suggesting that CXCR6 is associated with tissue infiltration (Fig. 1, D and E). Analysis of CD3\textsuperscript{+} T cells, CD8\textsuperscript{+} T cells, CD4\textsuperscript{+} T cells, and CD56\textsuperscript{+} 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 log higher in the HCV livers, and the amount of CXCR6 per cell, determined by analysis of mean channel fluorescence, was also increased (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 undiseased liver, end-stage hepatitis C-infected liver, and livers from patients 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 inflammatory liver diseases (Fig. 1D).

CXCR6\textsuperscript{+} cells are increased in peripheral blood of patients with end-stage liver disease due to hepatitis C

CXCR6 was expressed on 34.7% (±6.5) of PBL in end-stage HCV cirrhosis vs 11.4% (±1.6) in normal controls (p vs cirrhotic patients = 0.004) and 12.1% (±3.0) of precirrhotic chronic HCV patients (p vs cirrhotic patients = 0.005). Again, increased percentages of CXCR6\textsuperscript{+} cells were seen in CD3\textsuperscript{+}, CD8\textsuperscript{+}, CD4\textsuperscript{+}, and CD56\textsuperscript{+} subsets (Fig. 2).

**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 inflammatory 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 subsequently used immunohistochemistry to determine the distribution of CXCL16 staining in the chronic inflammatory liver diseases 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 occasional 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 strongest in the cytoplasm (Fig. 5). This led us to use cholangiocytes for the subsequent 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

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

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<td>Bile Duct</td>
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<tr>
<td>Normal</td>
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<tr>
<td>PBC</td>
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<td>0.2 (0.3)</td>
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<tr>
<td>PSC</td>
<td>0.6 (0.55)**</td>
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* 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.
CXCL16 and CXCR6 in the Liver

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 flow 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

CXCL16 does not support adhesion in isolation, but triggers integrin-mediated adhesion to VCAM-1

To determine whether CXCL16 can directly support adhesion, we conducted static and flow-based adhesion assays using glass microcapillary slides onto which CXCL16 was immobilized.

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). Incubation of lymphocytes with pertussis toxin inhibited lymphocyte adhesion by 80% (data not shown). Combining anti-CXCR6 or CXCL16 Abs with pertussis toxin did not increase inhibition over that seen with pertussis toxin alone, implying a major role for G protein-linked signaling.

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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 α₄β₇ 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⁺) CD8 T cells from the liver compared with primed (CD11a⁺CD8⁺) 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 Shi-maoka 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 CXCR6(+) LIL with CXCL16 and studied activation of VLA-4. Occupancy of CXR6 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 rCXCL6 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. CXR6-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 also the presence of VCAM-1 to support integrin-mediated adhesion. Thus, the lack of VCAM-1 on nondoised 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 CXCR6(+) 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 CXR6(+) effectors 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.

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References

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