Hepatic Expression of Secondary Lymphoid Chemokine (CCL21) Promotes the Development of Portal-Associated Lymphoid Tissue in Chronic Inflammatory Liver Disease

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The chronic inflammatory liver disease primary sclerosing cholangitis (PSC) is associated with portal inflammation and the development of neolymphoid tissue in the liver. More than 70% of patients with PSC have a history of inflammatory bowel disease and we have previously reported that mucosal addressin cell adhesion molecule-1 is induced on dendritic cells and portal vascular endothelium in PSC. We now show that the lymph node-associated chemokine, CCL21 or secondary lymphoid chemokine, is also strongly up-regulated on CD34⁺ vascular endothelium in portal associated lymphoid tissue in PSC. In contrast, CCL21 is absent from LYVE-1⁺ lymphatic vessel endothelium. Intrahepatic lymphocytes in PSC include a population of CCR7⁺ T cells only half of which express CD45RA and which respond to CCL21 in migration assays. The expression of CCL21 in association with mucosal addressin cell adhesion molecule-1 in portal tracts in PSC may promote the recruitment and retention of CCR7⁺ mucosal lymphocytes leading to the establishment of chronic portal inflammation and the expanded portal-associated lymphoid tissue. This study provides further evidence for the existence of portal-associated lymphoid tissue and is the first evidence that ectopic CCL21 is associated with lymphoid neogenesis in human inflammatory disease. (*Am J Pathol* 2002, 160:1445–1455)

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease in which bile ducts are damaged by a lymphocytic infiltrate.¹ The majority of patients

who develop the disease give a history of inflammatory bowel disease and we have proposed that the liver disease is a consequence of the inappropriate recruitment of mucosal lymphocytes into the liver.² In chronic inflammatory liver diseases, including PSC, portal tract infiltrates organize into lymphoid follicles containing B and T lymphocytes, dendritic cells (DCs), and new CD34⁺ mucosal addressin cell adhesion molecule-1 (MAdCAM-1)⁺ vessels with the morphology of high endothelial venules.^{2,3} Lymphoid neogenesis, the development of new lymphoid tissue in inflammatory sites at times when normal lymph node development is complete, has been reported in several chronic immune-mediated diseases including rheumatoid arthritis.^{4,5} Because these inflammatory lymphoid follicles provide a microenvironment for the recruitment and retention of lymphocytes at sites of chronic inflammation understanding the signals involved in lymphocyte recruitment to these sites will provide insights into the pathogenesis of chronic inflammation.⁶ The fibrous septa that are present in cirrhotic livers are rich in newly formed blood vessels and have been referred to as “fibrovascular membranes.”⁷ In addition to being an important component of evolving fibrosis they provide a potential pathway for lymphocyte recruitment and may be important for producing the lesion of interface hepatitis, which is seen at the periphery of portal tracts in many diseases, including PSC.

The chemokine secondary lymphoid chemokine (SLC), now designated CCL21, is expressed predominantly in lymphoid tissue where it recruits cells bearing its receptor CCR7.⁸ These cells include DCs, naïve T cells and the recently described central memory T cells that use CCR7 to home to lymphoid tissue, in distinction from effector memory T cells that are excluded from lymph nodes by their lack of CCR7.^{9–11} That CCL21 plays a critical role in the development and

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Table 1. Unconjugated and Conjugated and Isotype Control Antibodies Used in the Experiments

Antigen	Clone/name	Isotype	Supplier
Unconjugated antibodies			
SLC		Polyclonal	R&D Systems
CD11c	B-ly6	IgG ₁	PharMingen
CD31	JC/70A	IgG ₁	DAKO
CD34	BIRMA-K3	IgG ₁	DAKO
$\alpha 4\beta 7$	ACT-1	IgG ₁	Millenium
CCR7	7H12	IgG _{2b}	Millenium
CCR7	4H2	IgM	Millenium
CCR7	3D9	IgM	Millenium
LYVE-1	Rabbit	Polyclonal	D. Jackson
LYVE-1		IgG1	D. Jackson
Conjugated antibodies			
CD3 ECD	UCHT-1	IgG ₁	Coulter
CD8 CyChrome	RPA-T8	IgG ₁	PharMingen
CD45RA PE	HI100	IgG _{2b}	PharMingen
Isotype controls			
IgG ₁ ECD	679.1MC7	IgG ₁	Coulter
IgG ₁ CyChrome	MOPC-21	IgG ₁	PharMingen
IgG _{2b} PE	TEN/O	IgG _{2b}	Serotec

organization of lymph nodes¹² is shown by the failure of lymph node development in mice that are deficient in CCL21.¹³ Moreover, recent animal studies report that CCL21 expression is sufficient for lymphoid neogenesis because tissue-specific expression of a CCL21 transgene¹² or induction by lymphotoxin¹⁴ results in ectopic lymphoid neogenesis.

CCL21 on high endothelial venules of mesenteric lymph nodes and Peyer's patches activates the $\alpha 4\beta 7$ integrin on CCR7⁺ T cells enabling them to bind to the mucosal addressin MAdCAM-1.^{8,15,16} MAdCAM-1 is critical for the homing of lymphocytes to mucosa-associated lymphoid tissues;^{17–19} and sites of mucosal inflammation where its expression increases promoting the lymphocytic infiltrate of inflammatory bowel disease.¹⁷ MAdCAM-1 has recently been detected *de novo* on portal vessels and in lymphoid aggregates in inflammatory liver diseases associated with inflammatory bowel disease^{2,20} suggesting that the recruitment of mucosal lymphocytes to the liver may be critical in the pathogenesis of these diseases.

Here we report the induction of CCL21 (previously considered to be a constitutive chemokine) on stromal tissues surrounding portal vessels and in lymphoid aggregates in chronic inflammatory liver disease. CCL21 was also expressed on the endothelium of CD34⁺ neovessels at the periphery of fibrous septa. Consistent with a role for CCL21 at this site, we detected significant numbers of both CD45RA⁺ and RA[–] CCR7⁺ T cells within the liver of patients with PSC and were able to show that these cells migrate to CCL21 *in vitro*. Thus increased expression of CCL21 in portal tracts in PSC may be important for the development of chronic inflammation by promoting the recruitment and retention in the liver of lymphocytes that are activated at mucosal sites.

Materials and Methods

Tissues, Immunohistochemistry, and Immunofluorescence/Confocal Microscopy

Diseased liver tissue and paired peripheral blood was obtained with consent at the time of liver transplantation. Surplus liver tissue removed from donor organs that had been reduced in size for use in pediatric recipients was used as a nondisease control. All tissues were snap-frozen in liquid nitrogen and stored at -70°C until use. Subsequently 6- μm cryostat sections of 1-cm³ liver blocks were cut for immunohistochemistry and immunofluorescence.²¹ These sections were air-dried on poly-L-lysine-coated slides (Sigma Chemical Co. Ltd., Poole, Dorset, UK), then fixed for 10 minutes in acetone before staining.

Antibodies

The antibodies used are listed in Table 1.

Immunohistochemistry

Dual-color immunohistochemistry was performed as described previously.²¹ Sections were incubated with all antibodies in Tris-buffered saline (TBS) in 20% normal swine serum. CCL21 was detected by incubating sections with polyclonal goat anti-human rhSLC at room temperature in a humidified container. A mouse anti-human monoclonal antibody to CD11c (a DC marker) incubated at room temperature for 1 hour was also used. Control sections were incubated without primary antibody and with an isotype-matched irrelevant control for the monoclonal antibodies, and with goat anti-rabbit antibody as a control for the polyclonal CCL21 antibody. Subsequently, sections were incubated for 30 to 45 minutes with rabbit anti-goat horseradish peroxidase and rabbit anti-mouse immunoglobulin followed by a wash. A further amplifica-

tion step using a 30-minute incubation with goat anti-rabbit horseradish peroxidase and mouse alkaline phosphatase anti-alkaline phosphatase was performed. To develop the chromagens, the two substrates were added separately, first, alkaline phosphatase substrate (Sigma Chemical Co. Ltd.) followed by a washing step in TBS for 5 minutes and then peroxidase substrate (diaminobenzidine substrate, Sigma) for 5 to 10 minutes. The sections were counterstained with hematoxylin. Positive CCL21 staining was identified by the presence of a dark brown reaction product whereas positive CD11c staining was identified by the presence of a red reaction product. All washes were performed with Tris-buffered saline.

Immunofluorescence/Confocal Microscopy

Six- μ m sections were incubated with goat anti-human CCL21 (as described above) and mouse anti-human CD31/CD34, PAL-E, and LYVE1. PAL-E stains vascular endothelium whereas LYVE-1 is absent from vascular endothelium being confined predominantly to lymphatic endothelium (Table 1).²²⁻²⁴ Sections were incubated with all antibodies diluted in TBS with 10% fetal calf serum and 1% sodium azide. After 1 hour, the sections were washed in TBS for 30 minutes in a water bath. Subsequently fluorescein isothiocyanate and Texas Red-labeled secondary antibodies (Cambridge Biosciences, Cambridge, UK) were added to the tissue sections and incubated for 60 minutes in darkness. After a final 30-minute wash in darkness, the sections were examined by immunofluorescence microscopy or confocal microscopy. All washes were performed with TBS.

Liver-Derived Lymphocyte Isolation

Liver-derived lymphocytes were isolated as previously described²⁵ using a combination of mechanical homogenization and enzymatic digestion (with 100U/ml collagenase 1A (Sigma, Poole, Dorset, UK) in RPMI 1640 for 90 minutes at 37°C) followed by density gradient centrifugation.

Peripheral Blood Lymphocytes

Lymphocytes were isolated from peripheral venous blood taken from patients with primary sclerosing cholangitis or from normal volunteers. Umbilical cord blood was also obtained postpartum from Birmingham Women's Hospital ($n = 3$). The blood was centrifuged over Lymphoprep (Life Technologies Ltd., Paisley, Scotland) for 30 minutes at 2000 rpm, the lymphocytes harvested, and washed in RPMI 1640 before use.

Flow Cytometry

Cells were stained for three-color analyses as previously described.²⁵ Where unconjugated antibody was used, 0.5×10^6 cells were incubated with cytomegalovirus immunoglobulin before incubation with primary unconjugated monoclonal antibody. Cells were then incubated with a goat anti-mouse fluorescein-isothiocyanate secondary antibody

(DAKO) for 30 to 45 minutes and then blocked for 10 minutes with 10% normal mouse serum (DAKO). Subsequently incubations with fluorescent-labeled CD3 (to detect T cells) and other fluorescein isothiocyanate-, phycoerythrin-, energy-coupled-dye (ECD)-, or CyChrome-conjugated antibodies (Table 1) were performed. All antibody incubations were performed at 4°C for 30 minutes and washes with phosphate-buffered saline (PBS) containing 1% heat-inactivated fetal calf serum. Cells were fixed in 1% paraformaldehyde before analysis on a Coulter XL flow cytometer (Coulter Electronics Ltd., Luton, Beds, UK).

Chemotaxis Assays

Microchemotaxis Chamber Assays of PSC Liver-Derived Lymphocytes

The migration of liver-derived lymphocytes to CCL21 and RANTES was assessed using a 48-well microchemotaxis chamber technique. Briefly, lymphocytes isolated from the explanted livers of patients with PSC were rested overnight in RPMI/10% fetal calf serum before being resuspended at 1.5×10^6 /ml in RPMI/0.1% bovine serum albumin. Subsequently, 29 μ l of chemokine in RPMI/0.1% bovine serum albumin was placed in the bottom chamber and separated from 50 μ l of liver-derived lymphocytes in the top chamber by a Nucleopore Track-Etch Membrane (Corning Costar Corp., Cambridge, MA) with 8- μ m pores. After 2 hours at 37°C and 5% CO₂, the chemotaxis membrane was removed, washed gently in PBS, and then fixed in methanol before staining with Diff-Quik (Dade Diagnostika GmbH, Munich, Germany). The chemotaxis membrane was then mounted on a slide in distyrene plasticiser and xylene mixture (DPX) before analysis. The numbers of cells on the underside of the chemotaxis membrane were counted. Five high-power fields ($\times 200$ magnification) per well and four wells per chemokine were analyzed and compared to control wells containing no chemokine. The average numbers of cells migrating were compared to the control wells and the results are expressed as a chemotactic index. Data represents six separate experiments.

Transwell Assays of PBL

Blood was depleted of monocytes by adding carbonyl iron (10 mg/ml) for 60 minutes at 37°C before removing cells using a Dynal Magnetic Particle Concentrator. The resulting cells were centrifuged over Lymphoprep (Life Technologies Ltd., Paisley, Scotland) harvested, and washed in RPMI 1640. The migration of different subtypes of lymphocyte was assessed using 6.5-mm diameter, 8- μ m pore size Transwell inserts (Corning Costar Corp.) as previously described.²⁶ Briefly, optimum titrations of the chemokines CCL21 (R&D Systems, Abingdon, Oxford, UK) and RANTES (Peprotech EC Ltd., London, UK) were prepared and prewarmed to 37°C in the bottom of the Transwell chamber. The lymphocytes were then resuspended in 0.5% Fraction V bovine serum albumin (Sigma)/RPMI 1640 and 5×10^5 cells were added to the upper chamber of each Transwell insert. After an 18-hour incubation at 37°C,

5% CO₂, cells were carefully resuspended from the upper and lower chambers into 250 μ l of 0.5% Fraction V bovine serum albumin (Sigma)/RPMI 1640. Control wells containing no chemokine were included in each assay.

Accurate counts of the number of resuspended cells in each chamber were obtained by diluting 50 μ l of the suspended cells in 250 μ l of 50% fetal calf serum/PBS. Shortly before analysis on the Coulter XL flow cytometer, 10 μ g of propidium iodide (Sigma) was added to each sample. The number of viable cells in a fixed volume of each cell suspension was determined based on cell size and propidium iodide exclusion. The remaining cells were stained for FACS analysis according to the protocol described above. The antibodies used for this were, unconjugated α 4 β 7 (ACT-1), goat anti-mouse fluorescein isothiocyanate, CD3-ECD, and CD8-CyChrome. The migration of each subset was determined as: specific cell migration equals the number of cells of a specific phenotype in the lower chamber divided by the total number of cells of that phenotype in both the upper and lower chambers combined. The results are expressed as chemotactic index that is the migration of cells relative to the negative control. Data represents four separate experiments.

Western Immunoblotting

Liver Homogenates and Protein Determination

Liver protein extracts were obtained by homogenization of 5-g pieces of liver tissue in a Teflon homogenizer in 5 to 6 ml of buffer (50 mmol/L HEPES, 100 mmol/L KCl, 3 mmol/L MgCl₂, 5 mmol/L ethylenediaminetetraacetic acid, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 10% v/v glycerol, 0.1% v/v Tween 20, 5 mmol/L dithiothreitol, 0.1 mmol/L phenylmethyl sulfonyl fluoride, 0.1 mg/ml pepstatin A, 30 μ g/ml leupeptin). Homogenates were centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatants collected in 1-ml aliquots and stored at -80°C. The protein content was determined by the BCA protein assay according to the manufacturer's instructions (Pierce, Rockford, IL).

Blotting

Protein extracts (40 μ g) were resolved on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane (Hybond C-Extra, Amersham Pharmacia Biotech). The blotted membrane was blocked for 1 hour at room temperature in TBS containing 5% w/v of membrane-blocking reagent (nonfat dried milk), followed by an overnight incubation (18 hours) with the primary antibody to CCL21 (1:300 dilution) in TBS containing 1% of membrane-blocking reagent. After being washed three times with TBS containing 0.1% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated rabbit anti-goat at a dilution of 1:1000 for 1 hour. Protein bands were visualized using the enhanced chemiluminescent detection system (Amersham Pharmacia Biotech) followed by exposure of the membranes to Hyperfilm-ECL (Amersham Pharmacia Biotech) for 15 minutes. Quantification of the protein bands was performed using laser densitometry. Equal protein loading and transfer onto

membranes were checked by staining gels and membranes with Coomassie blue. The Western immunoblots were performed at least three times using cell lysates from different liver preparations for each liver type.

Results

CCL21 Expression in Normal Liver

In normal liver CCL21 staining was restricted to small vessels and occasional cells with a dendritic morphol-

Figure 1. SLC (CCL21) staining in human spleen and normal and diseased liver. Tissue was stained using a polyclonal goat anti-human antibody and an indirect peroxidase detection method (brown color when positive). **A** and **B**: Low- and high-power views of normal liver demonstrating small lymphatics and cells with DC morphology. **C**: Stromal staining in a perivascular area in a PBC liver. The vascular endothelium itself is negative. **D**: Small SLC⁺ vessels in an inflamed fibrous area. **E**: Portal lymphoid aggregate in a PSC liver containing scattered SLC⁺ cells with a dendritic morphology. **F**: Portal lymphoid aggregate in a PSC liver containing SLC⁺ cells with high endothelial vein morphology. **G**: PSC liver containing SLC⁺ cells in a portal tract; these include cells with the morphology of DCs as well as lymphatic and vascular channels. **H**: Staining in human spleen tissue as a positive control. **I** and **J**: Staining of an irrelevant goat control antibody on PSC tissue.

Figure 2. **A** and **B**: Dual-color immunohistochemistry of normal liver sections using a polyclonal goat anti-human antibody and a combination of an indirect peroxidase detection method (brown color when positive) and an indirect alkaline phosphatase anti-alkaline phosphatase (red when positive) were used. Dual immunostaining with the DC marker CD11c demonstrated co-localization of SLC with CD11c in a number of portal tract cells including lymphatics (**A**, arrow) and cells with the morphology of DCs. However, many CD11c⁺ cells were SLC-negative. Other structures within the liver, particularly vascular and sinusoidal endothelium were negative. To confirm the dual staining immunofluorescence labeling was used in which CD11c is stained green and SLC red. When the images are merged co-expression is detected by a yellow color. Co-expression in some CD11c⁺ cells is detected in both normal (**C**) and PSC liver tissue (**D**).

ogy within portal tracts (Figures 1, 2, and 3). To determine the nature of the CCL21-positive vessels we used immunofluorescence and confocal microscopy to co-localize the endothelial cell markers PAL-E, LYVE-1, CD31, and CD34 with CCL21 (Figure 3). CD31 is expressed on all endothelium including lymphatic endothelium whereas CD34 is primarily restricted to lymphatic endothelium and high endothelial venules and PAL-E is detected on vascular but not lymphatic endothelium. LYVE-1 has recently been shown to be expressed by lymphatic endothelium and sinusoidal endothelium in the liver (R Prevo, PH Weigel, and DG Jackson, Institute of Molecular Medicine, Oxford, unpublished observation).^{23,24,27} It is absent from vascular endothelium including portal and hepatic venous vascular endothelium in the liver. These experiments demonstrated that CCL21 was absent from PAL-E⁺ portal vessels in normal liver (Figure 3). Dual immunostaining and immunofluorescence with the DC marker CD11c demonstrated co-localization of CCL21 with CD11c in a number of portal tract DCs (Figure 2). However, many CD11c⁺ cells were CCL21-negative indicating that only a subset of DCs secretes CCL21 (Figure 2). Other structures within the normal liver, particularly vascular and sinusoidal endothelia were negative. These observations indicate that CCL21 in the normal liver is involved in the recruitment of DCs via lymphatics.

Figure 3. Characterization of SLC (CCL21)-expressing endothelium by confocal microscopy. Dual immunostaining for SLC with the endothelial cell markers LYVE-1, PAL-E, CD31, and CD34 demonstrated that SLC stained a subset of CD34⁺ and CD31⁺ vessels. **A**: Staining in normal liver. There is faint SLC staining in the perivascular stroma in a portal tract but the PAL-E⁺ endothelium does not express SLC. In PSC SLC co-localizes with both CD34 (**B**) and PAL-E (**C**) suggesting that it is being expressed by neovessels within the inflamed portal tracts whereas LYVE-1 (**D**) that is expressed on lymphatic but not vascular endothelium does not co-localize with SLC in portal tracts in PSC.

CCL21 Expression Is Increased in Chronic Inflammatory Liver Disease

In chronic inflammatory liver disease, expression of CCL21 increased and this was particularly marked in PBC and PSC, two diseases associated with portal lymphoid infiltration and the formation of lymphoid aggregates (Table 2 and Figure 4).

In PSC and PBC the main site of CCL21 staining was in portal tracts where staining was detected on stroma surrounding portal veins and on endothelial cells in some vessels associated with inflamed portal tracts. However the most marked staining was seen on small vascular channels, frequently located at the periphery of portal tracts and fibrous septa, which stained strongly positive (Figure 1, C and D). Within portal lymphoid aggregates there were scattered cells with the morphology of DCs (Figure 1E) and CCL21-positive vessels. These vessels had the morphology of high endothelial venules. Dual immunostaining for CCL21 with the endothelial cell markers PAL-E, CD31, and CD34 demonstrated that CCL21 stained a subset of CD34⁺ and CD31⁺ vessels. These vessels also stained for PAL-E but not LYVE-1 indicating that they contain vascular rather than lymphatic endothelium (Figure 3). The morphology and CD34⁺ staining of some of the CCL21⁺ vessels is consistent with high endothelial venule-like neovessels, which develop in

Figure 4. Representative Western immunoblot showing levels of SLC (CCL21) protein in normal, PBC, PSC, and ALD liver cellular protein extracts. Aliquots of cellular protein extracts from normal, PBC, PSC, and ALD liver were subjected to Western Blot analysis (top). Quantification of the relative changes in SLC protein levels between the liver diseases as determined by densitometry is shown in the histogram.

chronic inflammation to promote the continuing entry of lymphocytes into areas of lymphoid neogenesis.²⁸ CCL21 was also demonstrable albeit weakly on sinusoidal endothelium in a number of cases. In cirrhosis associated with alcoholic liver disease endothelial and DC staining for CCL21 were seen much less frequently. CCL21 was undetectable on cholangiocytes or hepatocytes. In normal spleen tissue used as a positive control CCL21 was detected on cells in the T cell area of the periarteriolar sheath (Figure 1H).

We subsequently went on to corroborate the above findings by determining levels of CCL21 protein in normal, PBC, PSC, and alcoholic liver disease (ALD) liver tissue. Protein extracts were made from liver tissue homogenates and analyzed by Western blotting and densitometry. We were able to detect low levels of CCL21 protein in normal liver tissue and 8 to 10 times higher levels in PSC and PBC livers (Figure 4).

Expression of $\alpha 4\beta 7$ and CCR7 on Peripheral Blood and Liver-Derived Lymphocytes

CCR7, the receptor for CCL21, is expressed on naïve T cells including $\alpha 4\beta 7$ cells. CCL21 has been shown to activate both LFA-1 and $\alpha 4\beta 7$ integrins on naïve lymphocytes. We therefore analyzed the expression of $\alpha 4\beta 7$, CD45RA, and CCR7 on CD3⁺ lymphocytes from blood

and liver tissue. Analysis of truly naïve T cells obtained from umbilical vein blood revealed that 91% of the CD3⁺ T cells in the neonate were $\alpha 4\beta 7$ ⁺; the majority of these were also CD45RA⁺ (Figure 5). In normal peripheral blood, 59% of T cells were CD3⁺ $\alpha 4\beta 7$ ⁺ and in patients with PSC, 45% of T cells in blood were CD3⁺ $\alpha 4\beta 7$ ⁺ (Figure 5). A similar proportion of intrahepatic T cells from normal donor liver and PSC were $\alpha 4\beta 7$ ⁺ although the total number of lymphocytes in normal liver was much less than in PSC (data not shown). The liver was enriched for CD45RA⁺ $\alpha 4\beta 7$ ⁺ T cells in patients with PSC in whom 82% of the CD3⁺ $\alpha 4\beta 7$ ⁺ cells in peripheral blood were CD45RA⁺ compared with 51% of the CD3⁺ $\alpha 4\beta 7$ ⁺ cells in the liver (Figure 5).

We then determined the numbers of CCR7/CD3⁺ lymphocytes in the blood and liver of normal donors and PSC patients (Figure 6). Normal PBL contained a mean of 50% CCR7⁺ cells whereas 76% of PBL in PSC were CCR7⁺. Only 9% of CD3⁺ lymphocytes isolated from normal liver were CCR7⁺ compared with 20% of intrahepatic T cells from patients with PSC (Figure 6C). CCR7⁺ T cells that are CD45RA⁺ represent a population of central memory cells that have the capability of homing to lymph nodes.¹¹ The percentage of blood CCR7⁺ cells that expressed CD45RA was higher in normal individuals (73%) but the proportions of intrahepatic T cells that were CCR7/CD45RA⁺ were similar (48% and 49%) in normal and PSC liver suggesting that both naïve and central memory type cells are being recruited during chronic inflammation (Figure 6). However our recent report of CD45RA⁺ memory T cells in human liver suggests that at least some of these naïve CD45RA⁺ intrahepatic T cells could be memory cells that have reverted to a CD45RA⁺ phenotype. Previous antigen priming in these cells can be determined by the expression of other markers the best of which is LFA-1. High levels of LFA-1 define memory (determined by tetramer staining) regardless of CD45RA status.²⁹ We therefore analyzed LFA-1 levels on the CD45RA⁺ cells (Figure 6). In blood CD45RA⁺ cells could be divided into an LFA-1^{high} population (revertants) and a larger LFA-1^{low} population (true naïve cells) in both patients [either PSC or ulcerative colitis (UC)] and organ donors. However when intrahepatic T cells were analyzed the majority of the CD45RA⁺ cells were LFA-1^{high} suggesting that they are antigen-primed or memory cells. We then attempted to localize CCR7⁺ lymphocytes within liver tissue by immunohistochemistry. Staining was weak but two different anti-CCR7 monoclonal antibody 3D9

Table 2. Staining of SLC (CCL21) in Liver Tissue

Liver disease (number)	Site of SLC (CCL21) Staining					
	Portal tracts*	Sinusoids	Lymphoid aggregates	Hepatocytes	Bile ducts	Dendritic cells
PSC (10)	++	+	++	—	—	+++
PBC (10)	+	+	++	—	—	++
Normal (8)	+/-	—	—	—	—	++
Other† (14)	+	—	—	—	—	+

*Staining of portal endothelial vessel and perivascular stroma.

†Includes patients with biliary atresia, cystic fibrosis, secondary biliary cirrhosis, and alcoholic cirrhosis.

Figure 5. Expression of $\alpha 4\beta 7^{+}$ on lymphocytes from the liver and peripheral blood of patients with PSC and organ donors. **A:** The phenotype of PSC peripheral blood- and liver-derived lymphocytes is demonstrated. Forty-five percent of PBLs were $CD3^{+}\alpha 4\beta 7^{+}$, and of these, 82% were $CD45RA^{+}$, whereas 23% of liver-derived lymphocytes were $CD3^{+}\alpha 4\beta 7^{+}$, and of these, 51% were $CD45RA^{+}$ ($n = 5$). Error bars represent SEM. **B:** Similar data for normal PBLs and liver-derived lymphocytes ($n = 5$) is demonstrated. Error bars represent SEM. **C:** Representative FACS contour plots demonstrating the phenotype of cord blood. **Circles** represent the percentage of $CD3^{+}CD45RA^{+}$ and $CD3^{+}\alpha 4\beta 7^{+}CD45RA^{+}$ T cells present in cord blood, respectively.

and 4H12 both detected occasional positive cells associated with areas of inflammation and within nodules (Figure 6F). Interestingly CCR7 staining was also seen on cells with the morphology of DCs and on endothelium in neovessels associated with inflamed stromal areas (Figure 6F).

Blood and Intrahepatic T Cells in PSC Migrate to CCL21 in Vitro

To determine whether CCR7 on circulating and intrahepatic $CD3^{+}$ T cells is functionally active we tested the ability of CCL21 to stimulate the migration of blood and liver-derived lymphocytes *in vitro*. Because we had limited numbers of intrahepatic T cells, chemotaxis experi-

ments were done with two concentrations of CCL21 previously shown to stimulate optimal migration in PBL (data not shown). We used responses to RANTES as a positive control because we have previously shown that large numbers of intrahepatic T cells express CCR5 and migrate to RANTES *in vitro*.^{25,30} Lymphocytes derived from the liver of patients with PSC were able to migrate to CCL21 *in vitro* and the magnitude of the response to CCL21 was comparable to that seen with RANTES (Figure 7a).

We then analyzed which lymphocyte subsets were migrating to CCL21 using a Transwell chemotaxis assay from which we could retrieve migrated cells for phenotypic analysis. It was not possible to use liver-derived lymphocytes in this assay because the numbers of cells available were too small, therefore normal lymphocytes and lymphocytes from patients with UC were tested for their ability to migrate to CCL21. Using this technique we were able to demonstrate that CCL21 attracted $CD45RA^{+}$ and $\alpha 4\beta 7^{+}$ T cells from both normal and UC blood but significantly higher rates of migration were observed with lymphocytes from patients with UC (Figure 7b). The ability of $CD3^{+}\alpha 4\beta 7^{+}$ cells from patients with UC to respond to CCL21 provides a potential mechanism for the recruitment of these cells via MAdCAM-1-expressing vessels in PSC.¹⁶

Discussion

CCL21 is considered to be a constitutive chemokine the expression of which is limited to lymphoid tissue where it acts to promote the recruitment of DCs and naïve T cells.⁸ However, some chronic inflammatory diseases are associated with the development of secondary or inflammatory lymphoid aggregates that resemble lymph nodes.^{31,32} These structures develop in response to local cytokines such as tumor necrosis factor- α and - β and act as a focus for sustained lymphocyte recruitment and retention via vessels that resemble the high endothelial venules of lymph nodes.^{33,34} Primary sclerosing cholangitis is a chronic inflammatory disease in which lymphocyte-mediated damage of bile ducts is associated with expanded portal tracts and the presence of portal lymphoid aggregates. The disease is strongly associated with inflammatory bowel disease. We have shown previously that $\alpha 4\beta 7^{+}$ T cells activated in mucosal sites can bind to inflamed portal vessels in PSC via MAdCAM-1, which is induced on these vessels in PSC.² However, MAdCAM-1 expression alone is probably insufficient to maintain the chronic lymphocytic infiltrate in PSC. Recent evidence suggests that overexpression of CCL21 in non-lymphoid tissues can drive neolymphoid development¹² and in light of this and the ability of CCL21 to activate $\alpha 4\beta 7^{+}$ adhesion to MAdCAM-1, we investigated the expression and function of CCL21 in PSC.

In normal liver CCL21 was restricted to a few small vessels with the morphology of lymphatics and occasional $CD11c^{+}$ DCs within portal tracts. CCL21 has been detected on lymphatic vessels in lymph node where it is critical for recruiting DCs from tissue.^{8,35} Thus CCL21 on

Figure 6. Expression of CCR7 on liver-derived and peripheral blood lymphocytes. **A:** CCR7 expression on peripheral blood and liver-derived lymphocytes from an organ donor without liver disease. **B:** CCR7 expression on peripheral blood and liver-derived lymphocytes from a patient with PSC. A significant proportion of intrahepatic lymphocytes are CCR7⁺. **C:** The percentage of CD3⁺ T cells that express CCR7 is shown for peripheral blood and liver-derived lymphocytes from organ donors without liver disease (normal) and patients with PSC. **D:** The proportion of CD3⁺/CCR7⁺ T cells that express CD45RA for normal and PSC T cells is shown. **E:** To determine the nature of the CD45RA⁺ T cells in the liver fresh liver-derived lymphocytes were gated on CD3 and analyzed for expression of LFA-1. In blood (**top**) the majority of CD45RA⁺ T cells are LFA-1^{low} confirming that they are naïve cells whereas in liver-derived cells the majority are LFA-1^{high} and thus likely to be primed/memory cells (**bottom**). Representative staining is shown for matched blood and liver-derived lymphocytes from a patient with PSC, an organ donor (normal), and blood from a patient with UC. Cells are gated on lymphocytes by forward/side scatter and on CD3 staining. **F:** Immunostaining for CCR7⁺ lymphocytes within PSC liver tissue revealed occasional lymphocytes associated with areas of inflammation (**white arrows**) as well as positive cells with the morphology of DCs (**black arrow**). Staining was weak but two different anti-CCR7 monoclonal antibody 3D9 and 4H12 both detected positive cells.

lymphatic vessels in normal liver could regulate the trafficking of DCs from the liver to the portal tract, an important pathway for DC emigration from the liver to the draining lymph node.^{36,37}

However, the most striking expression of CCL21 was detected in patients with chronic inflammatory liver disease, particularly PSC in which there is an associated expansion of portal tracts with secondary lymphoid aggregates. In these conditions we detected increased expression of CCL21 in portal tracts where not only DCs but also PAL-E-expressing vascular endothelium stained strongly. Further evidence that these vessels were vascular endothelium is provided by their failure to stain with the lymphatic endothelial marker LYVE-1. Many of the CCL21⁺ PAL-E⁺ vessels were small neovessels, frequently located at the periphery of portal tracts and fibrous septa. In addition to being an important component of evolving fibrosis these small neovessels provide a

potential pathway for the recruitment of T lymphocytes, which are often present within areas of fibrosis and may be associated with foci of interface hepatitis extending into the adjacent liver parenchyma.³⁸ Staining was also detected on CD34⁺ vessels, some of which had the morphology of high endothelial venules. CD34 is characteristically expressed by high endothelial venules in lymph nodes and by neovessels at sites of chronic inflammation.²⁸ In some chronic inflammatory diseases lymphoid neogenesis occurs in response to local cytokines and provides a site for continued lymphocyte recruitment to tissue.^{28,39,40} The presence of CCL21 within these aggregates could promote the recruitment of CCR7⁺ DCs and lymphocytes including CD45RA⁺ and α 4 β 7⁺ cells.^{8,16} In support of this we detected a high proportion of CCR7⁺ T cells within the liver in PSC, 50% of which were CD45RA⁺. To demonstrate that CCR7 on these cells is functional we performed chemotaxis assays

Figure 7. Liver-derived lymphocytes migrate to SLC (CCL21) *in vitro*. **A:** Freshly isolated liver-derived lymphocytes were tested for their ability to migrate to SLC or RANTES in migration assays as described in Materials and Methods. Migration in the absence of chemokine is normalized to 1. Both SLC and RANTES induced dose-dependent migration of intrahepatic T cells. **B:** Migration of peripheral blood lymphocytes from patients with UC or normal volunteers to SLC (CCL21). Analysis of the lymphocyte subsets migrating to SLC was studied using peripheral blood lymphocytes from healthy volunteers (normal) and patients with UC. SLCs attracted CD45RA⁺ and $\alpha 4\beta 7$ ⁺ T cells from both normal and UC blood but significantly higher rates of migration were observed with $\alpha 4\beta 7$ ⁺CD3⁺ lymphocytes from patients with UC. *, *P* < 0.05 compared with control migration.

with intrahepatic T cells from patients with PSC and demonstrated that they migrated efficiently to CCL21 *in vitro*. Furthermore we were able to show that CCL21 can preferentially attract $\alpha 4\beta 7$ ⁺ lymphocytes from PBL of patients with UC suggesting that overexpression of CCL21 in the liver could recruit $\alpha 4\beta 7$ ⁺ mucosal lymphocytes to chronically inflamed portal tracts in PSC.

In a previous study we showed that the endothelium of larger native portal vessels expressed MAdCAM-1 in cases of PSC and occasionally in other chronic inflammatory liver diseases. This provides a pathway for the recruitment of $\alpha 4\beta 7$ ⁺ T cells. In the present study we were unable to detect CCL21 in the endothelium of larger portal vessels, but found strong expression of CCL21 in the smaller neovessels and in surrounding stromal tissues. Because CCL21 contains a glycosaminoglycan-binding domain it is possible that it is sequestered in the stromal matrix where it could provide a mechanism for retaining T cells that have migrated across vascular endothelium thereby promoting the development of perivascular portal-associated lymphoid tissue.^{5,41}

Our findings in a chronic inflammatory human disease are supported by recent studies in an animal model of chronic hepatic inflammation. Yoneyama and colleagues⁴² demonstrated the induction of endothelial CCL21 in a murine model of granulomatous hepatitis and reported the development of lymphoid aggregates in response to *Propionibacterium*. The functional importance of CCL21 in this setting was confirmed because the portal-associated lymphoid tissue diminished after treatment with an anti-CCL21 antibody. The same group have demonstrated similar findings in *P. acnes*-induced lung inflammation indicating that this might be a common mechanism of neolymphoid tissue development at mucosal sites.⁴³

When CCL21 binds to CCR7 it can activate $\alpha 4\beta 7$ binding to MAdCAM-1.¹⁶ CCR7 is expressed at high levels on naïve T cells⁴⁴ and on a subset of so-called central memory T cells that lack immediate effector functions but which can traffic to the lymph node and efficiently stimulate DCs.^{11,45} The proportion of circulating T cells expressing CCR7 was increased in PSC and up to 25% of intrahepatic T cells were CCR7⁺. These intrahepatic CCR7⁺ T cells were equally divided between CD45RA⁺ and CD45RA⁻ cells suggesting that they include a population of central memory cells (CCR7⁺/CD45RA⁻) that have undergone differentiation in response to antigen. Whether this antigen is encountered in the portal-associated lymphoid tissue or in draining portal lymph nodes is unclear. However, the increased numbers of CCR7⁺ CD45RA⁻ cells in blood in PSC suggests that these cells recirculate.¹¹ The proportion of CCR7⁺ cells retrieved from inflamed liver is likely to be less than those entering tissue because CCR7⁺ memory cells lose CCR7 on differentiation into effector cells in response to antigen, which could occur within portal-associated lymphoid tissue.⁴⁵

A high proportion of T cells in blood and liver tissue in PSC were CD45RA⁺. This was particularly true for the CD3⁺ $\alpha 4\beta 7$ ⁺ cells of which 82% were CD45RA⁺ in blood and 51% in liver. Thus naïve T cells may also be recruited to the inflamed liver in response to CCL21. However, the majority of CD45RA⁺ cells within the liver were LFA-1^{high}, which defines a primed/memory population. Thus the ability of CCL21 to activate integrin binding to MAdCAM-1, ICAM-1, and VCAM-1, all of which are increased on endothelium within portal tracts, could result in the retention of CCR7⁺ T cells within the liver.^{2,16} Whether this results in the recruitment of true naïve T cells that are then fully activated within portal-associated lymphoid tissue is unknown. It is more likely that the CCR7⁺ cells recruited are either a population of central memory T cells, memory cells, or primed cells that have reverted to a CD45RA⁺ phenotype. In either case they may be important for sustaining chronic inflammatory responses in the liver.⁴⁶

These findings suggest that CCL21 expressed during chronic inflammatory liver disease promotes the recruitment of both CD45RA⁺ and CD45RA⁻ T cells. Together with the induction of functionally active MAdCAM-1 in chronic inflammatory liver disease,^{2,20} and the development of organized lymphoid aggregates these data imply

a role for CCL21 in driving the development of organized lymphoid aggregates within the chronically inflamed liver.

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