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Chemokine and Chemokine Receptor Interactions Provide a Mechanism for Selective T Cell Recruitment to Specific Liver Compartments Within Hepatitis C-Infected Liver[1]

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The role played by chemokines in regulating the selective recruitment of lymphocytes to different tissue compartments in disease is poorly characterized. In hepatitis C infection, inflammation confined to portal areas is associated with a less aggressive course, whereas T cell infiltration of the liver parenchyma is associated with progressive liver injury and cirrhosis. We propose a mechanism to explain how lymphocytes are recruited to hepatic lobules during bursts of necroinflammatory activity in chronic hepatitis C infection. We report here that lymphocytes infiltrating hepatitis C-infected liver express high levels of the chemokine receptors CCR5 and CXCR3. However, whereas the CCR5 ligands macrophage inflammatory protein-1α and -1β were largely confined to vessels within portal tracts, the CXCR3 ligands IFN-inducible protein-10 and monokine-induced by IFN-γ were selectively up-regulated on sinusoidal endothelium. In vitro, human hepatic sinusoidal endothelial cells secreted IFN-inducible protein-10 and monokine-induced by IFN-γ in response to stimulation with IFN-γ in combination with either IL-1 or TNF-α. This suggests that intrahepatic Th1 cytokines drive the increased expression of IFN-inducible protein-10 and monokine-induced by IFN-γ and thereby promote the continuing recruitment of CXCR3-expressing T cells into the hepatic lobule in chronic hepatitis C infection. ***The Journal of Immunology,*** **1999, 163: 6236–6243.**

The mechanisms that regulate lymphocyte recruitment to the liver in chronic hepatitis C infection are poorly understood but will be crucial in determining outcome. To mount a successful immune response against the virus, T cells must be able to enter liver tissue and recognize viral Ags on infected hepatocytes. These interactions may result either in clearance of the virus or, if the anti-viral response is ineffective, viral persistence and chronic hepatitis resulting in tissue damage, fibrosis, and cirrhosis (1). Lymphocytes must first recognize and then adhere to endothelium before they can extravasate from the circulation into tissue (2–4). The chemokine family of chemotactic cytokines plays a crucial role in this process by activating receptors on leukocytes that regulate leukocyte endothelial interactions and promote migration through the endothelium and into tissue (5–7). Chemokines activate β_1 and β_2 integrins on the leukocyte surface, thereby promoting arrest and firm adhesion to the vessel wall (8–10). The leukocyte then follows a hierarchy of chemokines that direct migration across the endothelium, through the extracellular matrix to the site of inflammation (11), where chemokines can also activate effector functions (6, 12, 13). The ability of chemokines to bind to proteoglycans allows them to be retained in the endothelial glycocalyx and the extracellular matrix, providing a mechanism for their immobilization at sites of inflammation (8, 14). Thus, lymphocytes will only be recruited to and retained in tissue if they express receptors that allow them to respond to locally presented chemokines (15). Chemokine-chemokine receptor interactions are likely to be particularly important in chronic hepatitis C virus (HCV)[3] infection, where T cells are recruited to the liver lobule to mediate clearance of virus-infected hepatocytes.

Chemokines can be classified according to their structure into four groups of which the largest subgroups are the CXC and CC families. These are defined by the presence (CXC) or absence (CC) of an amino acid between the first two cysteine residues in a conserved four-cysteine motif (11, 16). Most of the CXC family members, of which IL-8 is the best known, contain a glutamic acid-leucine-arginine (ELR) sequence near the N terminal and are potent chemoattractants for neutrophils (17), whereas IFN-inducible protein (IP-10) and monokine induced by IFN-γ (Mig) are members of a subset of CXC chemokines that do not contain the ELR motif and display potent lymphocyte chemotactic activity (18–20). The CC family includes macrophage chemotactic protein-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES, all of which have been reported to show chemotactic activity for T cells in vitro (7).

It has been proposed that the immune response in chronic hepatitis C is compartmentalized, with a predominantly Th2 or Th0 response in the periphery (21, 22) and a Th1 response in the liver (23, 24) associated with progressive liver injury. In contrast, patients who clear the virus have a peripheral Th1 response (25). Recent studies suggest that differences in chemokine receptor expression between Th1 and Th2 cells might explain their selective

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[3] Abbreviations used in this paper: HCV, hepatitis C virus; MIP, macrophage inflammatory protein; IP-10, IFN-inducible protein; Mig, monokine induced by IFN-γ; I-TAC, IFN-inducible T cell α chemoattractant; HSEC, human sinusoidal endothelial cells; MCF, median channel fluorescence.

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recruitment to tissue. In vitro, Th1 cells express CXCR3 and CCR5 and migrate to their respective chemokines, IP-10 and Mig (CXCR3 ligands) and RANTES, MIP-1α, and MIP-1β (CCR5 ligands) (26). CXCR3 and CCR5 have also been demonstrated on Th1 cells from rheumatoid synovial fluid, and these cells migrated to MIP-1α and IP-10 but not eotaxin (27, 28). CCR3 expression has been demonstrated on Th2 cells (26, 29). Thus, interactions between specific chemokines in tissue and their receptors on T cells may regulate the selective recruitment of primed Th1 cells to sites of inflammation. We examined the expression of chemokines and their receptors in hepatitis C-infected liver to determine the Th1/Th2 balance within the liver and if specific chemokine/chemokine receptor interaction are important for the selective recruitment of T cells to the liver in chronic hepatitis C.

Materials and Methods

Tissue studied and patient characteristics

Liver was obtained within 1 h of explantation from patients with end-stage hepatitis C cirrhosis undergoing liver transplantation. Samples (1 cm^3) were snap frozen in liquid nitrogen for later immunohistochemical studies. Peripheral blood was also obtained from patients all of whom had evidence of active HCV infection (HCV RIBA +ve, HCV RNA +ve) by PCR (Roche amplicor assay), and biopsy confirmed cirrhosis. Normal donor liver was obtained from surplus liver tissue removed from surgically reduced grafts used for pediatric liver transplantation (30). Normal donors had no evidence of either hepatitis B or C infection (negative by HCV ELISA and HCV RIBA; hepatitis B sAg and hepatitis B core Ab negative). Donor organs had been perfused with University of Wisconsin preservation fluid and maintained at 4°C for 12–24 h before cell isolation. Material was collected in accordance with guidelines for human experimentation and approved by our local ethics committee.

Abs for flow cytometry and immunohistochemistry

The following primary Abs were used for immunohistochemistry and flow cytometry: CD3-PE (UCHT1) IgG1 1:20 (Becton Dickinson, San Diego, CA); CD45RO (UCHL1) IgG2a FITC 1/10 (a gift from Prof. P Beverley, UCH, London); CXCR3 IgG1 1:1000 (LeukoSite, Cambridge, MA); CCR5 IgG1 1:1000 (LeukoSite); CCR3 IgG1 1/500 (LeukoSite); MIP-1α rabbit polyclonal, MIP-1β rabbit polyclonal (gifts from Dr U. Siebenlist, National Institutes of Health, Bethesda, MD); IP-10 goat anti-human polyclonal 1:20 (R&D Systems, Abingdon, U.K.), IP-10 IgG1 mouse monoclonal 1:50 (R&D Systems), Mig goat anti-human polyclonal 1:20 (R&D Systems); IFN-inducible T cell α chemoattractant (I-TAC) rabbit anti-human polyclonal 1:50 (PeproTech, London, U.K.). HCV core Ab Mouse monoclonal 1:50 (a gift from Prof. S. H. Jap, University Hospital Gastuisberg, Leuven, Belgium). Secondary Abs used were rabbit anti-goat peroxidase 1:50 (Dako, Cambridge, U.K.); rabbit anti-mouse Igs 1:25 (Dako); alkaline phosphatase anti-alkaline phosphatase mouse monoclonal 1:50 (Dako); rabbit anti-mouse IgM FITC 1:20 (Dako).

Immunohistochemistry

The expression of chemokines (MIP-1α, MIP-1β, IP-10, and Mig), chemokine receptors (CXCR3, CCR5, and CCR3), and TNF-α were studied by immunohistochemistry on 6-μm cryostat sections as previously described (31). Briefly, sections were fixed in acetone for 10 min and then incubated with primary Ab followed by secondary rabbit anti-mouse Ab. Double stains were performed using primary Abs of different species and the relevant secondary Abs. These were detected by indirect alkaline phosphatase anti-alkaline phoshatase developed with napthol-AX and fast red TR or by an avidin-biotin complex peroxidase method. Endogenous peroxidase activity was blocked using sodium azide. Sections were counterstained with hematoxylin. All incubations were conducted at room temperature for 45 min, and sections were washed for 5 min with two changes of TBS, pH 7.4, buffer between incubations. Staining intensity of chemokines on each section was graded by two independent observers including a pathologist (S.G.H and P.L.S) from 0 to 3, where 0 = absent, 1 = weak, 2 = moderate, and 3 = strong staining using a scoring system previously validated by confocal microscopy (32). The mean staining intensity was calculated for all specimens analyzed. The distribution and intensity of staining in the following structures was recorded; portal tract venular and arterial endothelium, biliary epithelium and inflammatory infiltrate; hepatocytes, Kupffer cells, and sinusoidal and central vein endothelium.

Isolation of liver infiltrating lymphocytes

Hepatic T cells were isolated from fresh liver tissue collected into RPMI 1640 using two different methods, with or without collagenase digestion.

In the first method, tissue was diced using sterile blades into 1-mm^3 pieces in RPMI 1640 containing 1 mg/ml collagenase (type 1a; Sigma, Poole, U.K.) and incubated at 37°C for 2 h. After enzymatic digestion, tissue was passed through a nylon mesh filter (100 μm) to remove cell clumps and undissociated tissue. Cells were washed three times in PBS to remove collagenase, and the cell suspension was layered on a Percol density gradient (70/30%) and centrifuged for 30 min at 2000 rpm. The lymphocyte band was then removed from the interface between 30% and 70% Percoll and further washed three times in PBS. Greater than 95% of cells were viable by trypan blue exclusion.

In the second method, blocks of fresh liver tissue (2 $\times$ 2 cm) were perforated repeatedly with a 19-gauge needle (teabagging) and then injected with PBS to flush out cells from within the liver parenchyma. The resultant cell suspension was filtered through fine nylon mesh to remove tissue debris and used with no further preparation for Ab staining and FACS analysis. Cells obtained were compared directly with cells from the same liver isolated by the process of collagenase digestion and Percol gradient centrifugation.

Isolation of autologous PBL

Venous blood was collected into tubes containing EDTA before liver transplantation. PBL were separated by density centrifugation at 1500 rpm for 30 min at room temperature and washed twice with PBS.

Flow cytometry

Two- and three-color flow cytometry was performed on freshly isolated autologous PBL and liver-infiltrating cells from patients with hepatitis C cirrhosis. Cell staining for flow cytometry was conducted using standard techniques (33). Briefly, cells were incubated with normal human Ig to block Fc receptors, washed with PBS, and then incubated on ice for 1 h with primary mouse anti-chemokine receptor mAb at predetermined optimal concentrations in 100 μl of PBS with 0.2% BSA. Cells were washed twice with cold PBS and further incubated on ice for 45 min with FITC-conjugated $F(ab')_2$ of rabbit anti-mouse Ig. Cells were washed twice and incubated with normal mouse serum to saturate free binding sites on the FITC-conjugated $F(ab')_2$. PE-conjugated anti-CD3 (Dako) was used as a second primary Ab to detect T cells in two-color analysis. In three-color analysis, T cells were incubated with PE-conjugated CD45RO and biotin-conjugated CD3 labeled with Red670 (Life Technologies, Paisley, U.K.). After final incubation, cells were washed twice and fixed with 1% paraformaldehyde. The lymphocyte population was gated using forward and side scatter parameters to exclude debris and dead cells and by back gating on CD3-positive cells. Two- and three-color analysis was performed using a Coulter flow cytometer (Coulter, Palo Alto, CA), and the data were analyzed using WinMdi software (Scripps Research Institute, La Jolla, CA).

The effect of collagenase isolation method on chemokine receptor expression by T cells

PBMC were cultured in RPMI 1640 with or without PHA (10 μg/ml) for 24 h and then with collagenase 1A (1 mg/ml; Sigma) for 1 h. Cells were washed once in PBS/50% FCS and then twice in PBS before being stained with a panel of mAb and analyzed by FACS. Median channel fluorescence (MCF) and percentage positive cells were calculated using WinMdi software. Expression of T cell markers and chemokine receptors was also examined on freshly isolated T cells obtained by the collagenase method and by the "teabagging" method, which did not involve the use of collagenase or density gradient centrifugation.

Culture of intrahepatic human sinusoidal endothelial cells (HSEC)

Intrahepatic HSEC were isolated from human livers as described previously (34, 35) by mechanical and enzymatic digestion of liver tissue followed by immunomagnetic purification using Abs to the endothelial adhesion molecule CD31. Cells were established in culture in endothelial basal medium containing 20% human AB serum, 10 ng/ml of vascular endothelial growth factor (Bachem, Saffron Walden, Essex, U.K.) and 10ng/ml of hepatocyte growth factor (Bachem). The cells grew to confluence with the classical cobblestone appearance of endothelial cells and expressed CD31 (>95%). HSEC were grown to confluence in 24-well plates and stimulated for 24 h with human recombinant TNF-α (R&D Systems), IFN-γ (R&D Systems), and IL-1α (Genzyme, West Malling,

Table I. *Chemokine expression by liver vascular endothelium in normal and hepatitis C cirrhotic liver*[a]

	MIP-1α	MIP-1β	Mig	IP-10
Normal liver	$n = 7$	$n = 8$	$n = 8$	$n = 8$
Sinusoidal endothelium	0.42 (0–1)	1.0 (0–2)	1.4 (1–2)	0.38 (0–1)
Portal vein	2.43 (2–3)	2.50 (2–3)	0.87 (0–2)	0 (0)
Hepatic vein	0.57 (0–1)	0.75 (0–1)	0.57 (0–1)	0 (0)
Hepatitis C cirrhotic liver	$n = 7$	$n = 14$	$n = 14$	$n = 16$
Sinusoidal endothelium	0.86 (0–1)	1.1 (0–2)	2.6 (2–3)*	1.4 (0–3)**
Portal vein	2.7 (2–3)	2.7 (2–3)	1.5 (0–2)	0 (0)
Hepatic vein	0.7 (0–1)	0.85 (0–2)	0.4 (0–1)	0 (0)

[a] Table summarizes results of immunohistochemistry with staining intensity scored as follows: 0 = absent, 1 = weak, 2 = moderate, and 3 = strong. Values represent overall mean staining intensity with range of individual scores in parentheses. Significant differences were seen between hepatitis C cirrhotic and normal liver for Mig (*, $p = 0.002$, Mann-Whitney *U* test) and IP-10 expression (**, $p = 0.015$) on sinusoidal endothelium.

U.K.), and combinations thereof, at final concentrations of 10 ng/ml, 100 U/ml, and 10 U/ml, respectively.

ELISA for IP-10 and Mig

Extracellular IP-10 and Mig levels in culture supernatants were measured via a sandwich-type ELISA using a monoclonal anti-human IP-10 or Mig capture Ab and a matched biotinylated polyclonal detection Ab (Ab pairs from R&D Systems). The substrate was 3,5,2′,5′-tetramethylbenzidine (Sigma), the color reaction was stopped with 2.5 M H_2SO_4, and absorbance was determined at 450 nm. The detection limit of these assays was 100 pg/ml.

RT-PCR

IP-10 and Mig chemokine gene expression was analyzed by RT-PCR amplification of RNA from HSEC, comparing stimulated and unstimulated cells. Total RNA was extracted from cells using RNAzol extraction (Biogenesis, Bournemouth, U.K.), and single-stranded cDNA synthesis was conducted as previously described (36). The PCR reaction was conducted using 1 μl of cDNA per reaction. Reaction conditions were 1× (95°C, 5 min), 1× (55°C, 5 min), 35× (94°C, 30 s; 55°C, 30 s; 72°C, 2 min), and 1× (72°C, 5 min). Primers were designed from GenBank sequences for β-actin, IP-10, and Mig: β-actin forward, 5′-CAT CAC CAT TGG CAA TGA GC-3′; β-actin reverse, 5′-CGA TCC ACA CGG AGT ACT TG-3′; IP-10 forward, 5′-TCT AGA ACC GTA CGC TGT ACC TGC-3′; IP-10 reverse, 5′-CTG GTT TTA AGG AGA TCT-3′; Mig forward, 5′-GGA ACT CCA TTC TAT CAC-3′; Mig reverse, 5′-TAT TGG TGA AGT GGT CTC-3′. Product size for each set of primers were: β-actin, 284 bp; IP-10, 231 bp; and Mig, 414 bp. Positive and negative controls were included in each assay. Amplified products were analyzed on 2% agarose gel containing ethidium bromide.

Results

Chemokines are expressed in normal and hepatitis C-infected liver

MIP-1α and MIP-1β were detected on vascular endothelium, particularly portal vein endothelium within portal tracts, both in normal and hepatitis C cirrhotic liver with similar staining intensity (results are shown in Table I and Fig. 1, *a–h*). Sinusoidal endothelium stained weakly for MIP-1α and MIP-1β both in nondiseased liver and hepatitis C cirrhotic liver. Mig was detected on sinusoidal endothelium in normal and hepatitis C cirrhotic liver, but staining intensity was increased markedly in hepatitis C ($p = 0.002$; Mann-Whitney *U* test). Mig was also detected weakly on portal venules within normal and hepatitis C-infected liver. In arterioles, Mig was detected within the vessel wall, staining elastic and smooth muscle fibers, but not in the endothelium. IP-10 was selectively expressed by sinusoidal endothelium and was absent from vascular endothelium in portal tracts both in normal and hepatitis C cirrhotic liver. IP-10 staining of sinusoidal endothelium within hepatitis C cirrhotic liver was increased compared with normal liver ($p = 0.015$; Mann-Whitney *U* test). The more recently characterized chemokine I-TAC has also been recognized as a ligand of the CXCR3 receptor. We found I-TAC in normal ($n = 5$) and HCV cirrhotic liver ($n = 8$) showing a similar distribution to IP-10 with expression by sinusoidal endothelial cells within hepatocyte lobules but not portal vascular endothelium.

TNF-α was expressed within the liver in chronic hepatitis C by portal vascular endothelial cells and by mononuclear cells within inflamed portal tracts (Fig. 1*g*). Staining within the liver lobules was mainly localized to Kupffer cells within the sinusoidal spaces, although there was patchy and weak staining of hepatocytes (Fig. 1*h*). An immunohistochemical stain for HCV core Ag using an Ab previously validated by Yap et al. (37) demonstrated localization of virus protein within hepatocytes and in sinusoidal endothelial cells within the liver lobules (data not shown).

Liver-infiltrating lymphocytes show increased expression of CXCR3 and CCR5

Immunohistochemistry. CXCR3 staining was strong on the majority of infiltrating lymphocytes within portal tracts and lobules in hepatitis C cirrhotic liver (Fig. 1*f*). In nondiseased livers from organ donors ($n = 6$) and in biopsy samples from patients with histologically mild chronic hepatitis C ($n = 5$), the few mononuclear cells infiltrating portal tracts and hepatic parenchyma were also positive for CXCR3. No other structures within liver expressed CXCR3. CCR5 was also detected on the majority of mononuclear cells infiltrating hepatitis C and normal liver, although the staining was weaker than for CXCR3. CCR5 was absent from other structures. CCR3 was strongly positive on a few cells that had the morphology of eosinophils and monocytes in HCV liver, both in portal tracts and hepatic lobules. CCR3 was absent from T cells as demonstrated by double immunostaining with CD3 and CCR3, which failed to show co-localization.

Flow cytometry. Freshly isolated liver-infiltrating T cells from patients with hepatitis C cirrhosis ($n = 9$) were all CXCR3 positive (MCF (±SEM), 12.03 ± 4.9) with increased expression compared with autologous peripheral blood T cells (2.82 ± 0.47, $p = 0.0039$) and in normal donors ($n = 6$; 8.83 ± 1.81 vs 2.79 ± 0.44, $p = 0.03$) (Fig. 2*A*). CCR5 expression on peripheral blood T cells revealed two distinct populations of CCR5-positive and CCR5-negative cells. This heterogeneity was lost on liver-infiltrating cells, the majority of which were positive compared with autologous peripheral blood. In hepatitis C-infected patients ($n = 9$), the mean percentage of positive liver-derived T cells was 76.2 ± 3.1% vs 29.75 ± 2.9% on autologous peripheral blood T cells ($p = 0.004$). In normal donors ($n = 6$), a greater proportion of liver T cells expressed CCR5 than peripheral blood T cells (58.6 ± 9.3% vs 27.4 ± 3.5%, $p = 0.009$) (Fig. 2*B*). CCR5 expression was increased on freshly isolated $CD45RO^+$ cells from HCV liver compared with autologous peripheral blood $CD45RO^+$ cells (Fig.

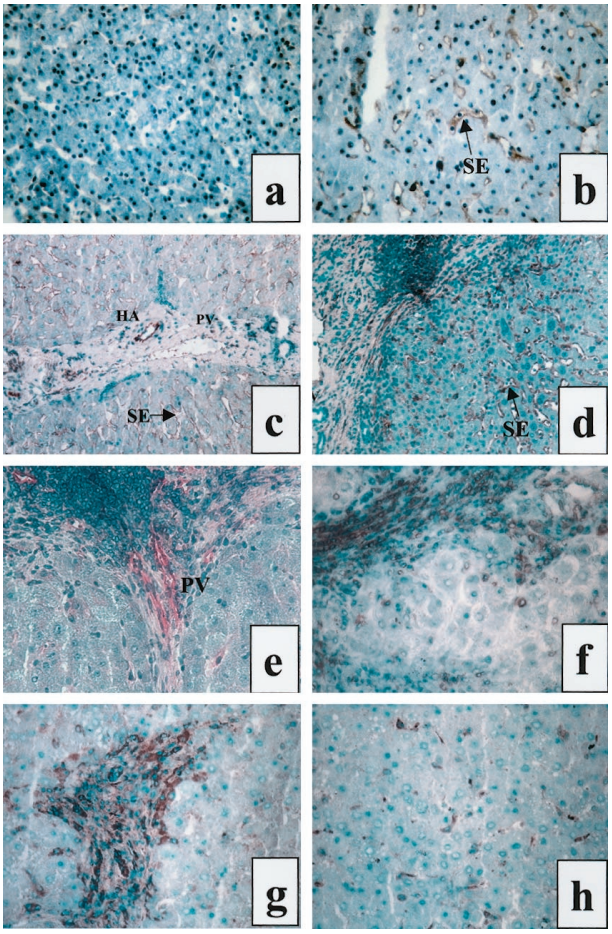

FIGURE 1. Chemokine and chemokine receptor expression in the liver. Immunohistochemical peroxidase stains for IP-10 in both normal (*a*) and hepatitis C cirrhotic liver (*b*) (magnification, ×100) showing expression within sinusoidal endothelium (SE) in the hepatocyte lobule. Staining of sinusoidal endothelium is increased in the hepatitis C-infected liver compared with normal. IP-10 is selectively expressed on sinusoidal endothelium, and its expression is increased in the hepatitis C-infected liver. Mig is expressed by vascular endothelium in normal liver (*c*) and in hepatitis C cirrhotic liver (*d*) (magnification, ×100), where its expression on sinusoidal endothelium is increased. As well as expression on portal vein (PV), it is expressed within the wall (subendothelial layer) of small arteries (HA) within the liver. MIP-1β is expressed mainly by portal vein endothelium within the portal tracts (*e*; alkaline phosphatase anti-alkaline phosphatase fast red stain; magnification, ×200). The chemokine receptor CXCR3 (for IP-10 and Mig) is expressed on the majority of liver infiltrating lymphocytes, here shown within hepatitis C cirrhotic liver (magnification, ×200) using a peroxidase method (*f*). TNF-α is detected in mononuclear cells in portal tracts within hepatitis C-infected liver, and there is also weak staining in hepatocytes (*g*). Kupffer cells in the sinusoids in hepatic lobules were strongly positive for TNF-α in hepatitis C-infected liver (*h*).

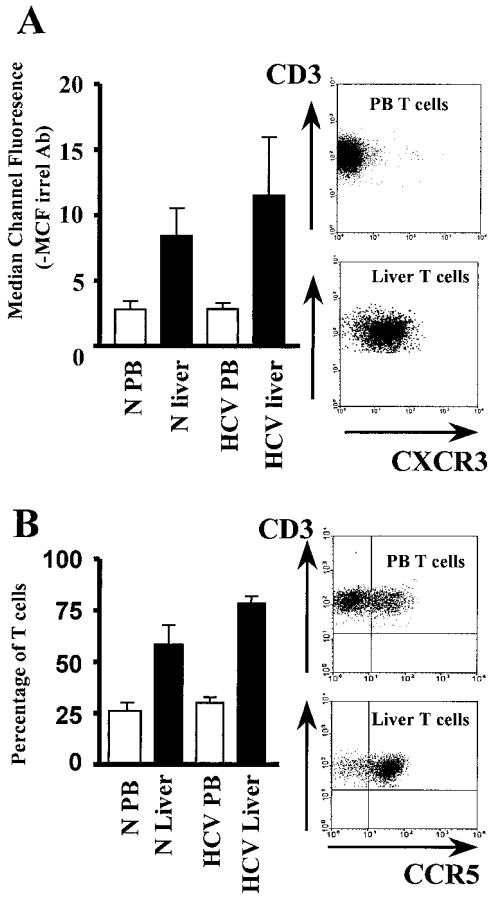

FIGURE 2. Chemokine receptor expression by peripheral blood- and liver-derived T cells. Two-color flow cytometry of T cells from normal (N) liver ($n = 6$) and normal peripheral blood (N PB, $n = 6$) and hepatitis C cirrhotic (HCV) liver ($n = 8$) compared with autologous peripheral blood (HCV PB) T cells ($n = 8$). Data shown for liver-derived T cells is that using the collagenase digestion method. T cells were gated by forward and side scatter characteristics and by their expression of CD3. *A,* Mean CXCR3 staining intensity (MCF − MCF of irrelevant Ab ± SEM) on T cells and flow cytometry data (dot plot) for an individual patient's peripheral blood- and liver-derived T cells is representative of all the patients studied. Liver-derived T cells expressed higher levels of CXCR3 than peripheral blood T cells ($p = 0.0039$ for hepatitis C patients, $p = 0.03$ for normal donors). *B,* The percentage of T cells expressing chemokine receptor CCR5 (mean ± SEM) and data for a single representative patient. A higher percentage of liver T cells expressed CCR5 than peripheral blood T cells ($p = 0.004$ for HCV patients, $p = 0.009$ for normal donors).

3). There was low level expression of CCR3 on both peripheral blood- and liver-derived T cells (Fig. 4). Incubation with collagenase 1a in vitro had no significant effect on the percentage of lymphocytes staining positively or the MCF intensity for CD3 or CD45RO. Chemokine receptor expression of CCR3 and CCR5 were not greatly affected by collagenase, but CXCR3 expression was reduced on PHA blasts after incubation with collagenase 1a (MCF, 23.7 (control) vs 8.0 (collagenase 1a)). Comparison of the collagenase digestion/Percol density gradient isolation technique with the "teabagging technique" where collagenase was not used revealed no significant change for the majority of markers but a significant reduction in median fluorescence intensity from 72.3 to 14.6 for the chemokine receptor CXCR3.

Human hepatic endothelial cells secrete IP-10 and Mig in response to stimulation with IFN-γ and TNF-α in vitro

Culture supernatants from HSEC incubated in fresh complete medium for 24 h contained undetectable levels of IP-10 and Mig. Incubation of HSEC with TNF-α (10 ng/ml) or IFN-γ (100 U/ml) for 24 h significantly increased secretion of IP-10 but not Mig (Fig. 5*A*). Combinations of IFN-γ with either TNF-α or IL-1 synergistically increased IP-10 compared with each cytokine alone and induced Mig secretion although levels of Mig were consistently lower than for IP-10. RT-PCR amplification of total RNA gave PCR products of predicted size on ethidium bromide-stained gels. RNA from LPS-stimulated PBMC was used as a positive control throughout. A negative control (no template) was included in all

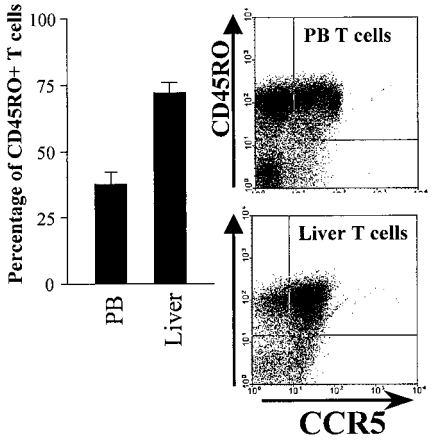

FIGURE 3. CCR5 expression on memory ($CD45RO^+$) T cells from peripheral blood (PB) and liver of patient with hepatitis C cirrhosis ($n = 6$). Three-color flow cytometry was performed by gating on CD3-positive cells. Tissue infiltrating $CD45RO^+$ cells show increased expression of CCR5 than peripheral blood $CD45RO^+$ cells ($p = 0.03$).

amplification runs. Levels of IP-10 and Mig mRNA were undetectable in unstimulated HSEC. IP-10 was detected at low levels after IFN-γ and TNF-α stimulation alone, whereas Mig mRNA was undetectable. IFN-γ plus TNF-α and IFN-γ plus IL-1 stimulation resulted in expression of both IP-10 and Mig mRNA (Fig. 5*B*). Actin expression was similar for all samples measured, and, bearing in mind that the mRNA levels were measured at one time point (24 h) and the protein levels were accumulated over the 24 h, these data for mRNA expression are consistent with the secretion data.

Discussion

The present study provides evidence that specific chemokine/chemokine receptor interactions regulate the recruitment of primed T cells to different compartments of the liver in chronic hepatitis C infection. Furthermore, we propose that the pattern of chemokine secretion in the liver will determine the distribution and severity of the T cell inflammatory infiltrate. We studied two C-C and two CXC chemokines that have been reported to have activity for T cells and detected all four chemokines in hepatitis C-infected liver. The C-C chemokines MIP-1α and MIP-1β were expressed predominantly by vascular endothelium within portal tracts in both normal and hepatitis C liver, whereas the CXC chemokines Mig and IP-10 were preferentially expressed by sinusoidal endothelium. Although Mig was detected in normal liver, the strongest

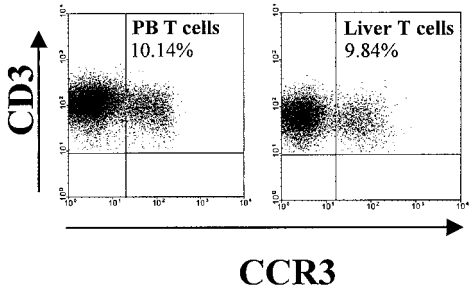

FIGURE 4. CCR3 expression on peripheral blood (PB)- and liver-derived T cells from a patient with hepatitis C cirrhosis. Two-color flow cytometry was performed by gating on CD3-positive cells. Figures in quadrants represent percentage of T cells expressing CCR3. Data is representative of three paired patient samples studied.

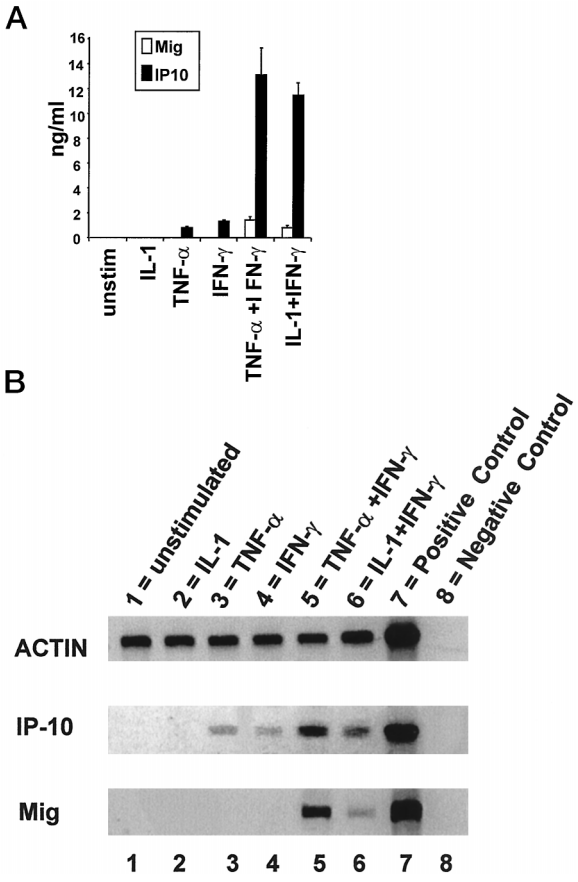

FIGURE 5. IP-10 and Mig expression and secretion from HSEC isolated from normal liver. Cells were cultured until confluent and then stimulated for 24 h in media containing no cytokines or proinflammatory cytokines either alone or in combination (*lanes 1–8*). *A*, IP-10 and Mig protein was measured by ELISA, and the graph represents mean data of three replicates ($\pm$SD). One experiment representative of three separate experiments is shown. *B*, IP-10 and Mig gene expression (mRNA) in HSEC measured by RT-PCR.

expression was on sinusoidal endothelium in hepatitis C, and IP-10 expression was restricted to sinusoidal endothelium within hepatocyte lobules and was up-regulated within inflamed hepatitis C-infected liver compared with normal. Thus, it seems likely that IP-10 and Mig play a specific role in the recruitment of T cells to the hepatic parenchyma via the sinusoids and may thereby determine the outcome of chronic hepatitis. We also have preliminary data on a more recently characterized chemokine I-TAC, a ligand for the receptor CXCR3, which appears to be expressed in both normal and inflamed HCV-infected liver. I-TAC has a similar distribution to IP-10 in that the protein is predominantly seen within sinusoidal endothelial cells in liver lobules and is absent from portal vascular endothelium.

Infiltration of the parenchyma determines the severity of chronic viral hepatitis but may have a dualistic effect. On one hand, lobular inflammation is associated with liver damage and progression to cirrhosis, while on the other, cytolytic T cells must enter the lobules if they are to destroy hepatitis virus within infected hepatocytes (38).

We suggest that hepatic sinusoidal endothelial cells are an important source of IP-10 and Mig during hepatitis C infection. It has been shown recently that HUVEC can secrete IP-10 (39) and Mig (40) and that both IP-10 and Mig can induce adhesion of activated T cells to endothelium via activation of the CXCR3 receptor (40).

Thus, expression of CXC chemokines on endothelium at sites of inflammation may promote the recruitment of CXCR3-bearing effector T cells. In our study, IP-10 and Mig staining was largely restricted to sinusoidal endothelial cells, and we were able to induce secretion from isolated HSEC in vitro. Unstimulated HSEC isolated from normal donor liver failed to secrete IP-10 or Mig. IL-1 alone failed to induce chemokine expression, although IP-10 was produced after stimulation with TNF-α or IFN-γ. The combination of IFN-γ with either IL-1 or TNF-α induced expression of IP-10 and Mig mRNA and protein secretion into HSEC culture supernatants. We detected higher levels of IP-10 than Mig protein, and further studies will be required to address the significance of this finding. It is known that expression of Th1 cytokines, including IFN-γ, are increased in the hepatitis C-infected liver and that these levels correlate with liver injury (23). Furthermore, the TNF system is activated in chronic hepatitis C with increased levels of TNF-α (41, 42) and its receptor (43, 44) in the serum and elevated levels of TNF-α within the liver (43) compared with controls. We demonstrated strong TNF-α expression by Kupffer cells within liver lobules but more patchy expression by hepatocytes in hepatitis C patients. TNF protein was also expressed by a small population of mononuclear cells within T cell areas in portal tracts and by portal vascular endothelial cells. The expression of TNF-α protein within the liver in chronic hepatitis B (although not chronic hepatitis C) has been localized to mononuclear cells and hepatocytes in one previous study (45). Thus, local Th1 cytokines produced by infiltrating mononuclear cells and Kupffer cells could induce IP-10 and Mig expression by sinusoidal endothelium in vivo, thereby promoting lymphocyte recruitment to the parenchyma. Further support for the role of IP-10 in hepatitis C infection comes from a recent study (46) in which serum levels of IP-10 correlated with histological activity in chronic hepatitis C. It is unclear why IP-10 is not induced on vascular endothelium in inflamed portal tracts. This may represent different sensitivities for IP-10 induction by endothelium at the two sites or differences in the local cytokine milieu.

To determine whether the chemokines studied might have a functional role in lymphocyte recruitment to the liver, we looked for expression of their respective receptors on liver-infiltrating lymphocytes using both immunohistochemistry and flow cytometry of freshly isolated liver-infiltrating T cells. CXCR3 was detected on all liver-infiltrating T cells by immunohistochemistry, and flow cytometry revealed that CXCR3 receptor density was consistently higher on liver-infiltrating compared with autologous peripheral blood T cells both in normal donor and hepatitis C cirrhotic liver. CCR5 was also detected on the majority of liver-infiltrating T cells by immunohistochemistry, although staining was weaker than for CXCR3. Staining for CCR5 by flow cytometry revealed two populations of T cells in peripheral blood that could be differentiated by whether or not they expressed CCR5, whereas nearly all liver-infiltrating T cells were $CCR5^{high}$. In contrast, there were no differences in the level of expression of CCR3 between peripheral blood- and liver-derived T cells, and very few T cells within the liver stained for CCR3. This suggests that both CXCR3 and CCR5 are important for T cell recruitment to the inflamed hepatitis C liver. Because both CXCR3 and CCR5 can be up-regulated on lymphocytes by activation in vitro, it was important that we used freshly isolated cells that had not been activated or expanded ex vivo. Using freshly isolated T cells, we directly compared levels of CCR5 on tissue-infiltrating T cells with circulating memory T cells. It has been reported that circulating memory T cells express higher levels of CCR5 than naive T cells (27), and we now demonstrate that levels of CCR5 are higher on tissue-infiltrating memory T cells when compared with memory cells in the circulation. Greater than 70% of liver infiltrating $CD45RO^+$ T cells were $CCR5^{high}$, compared with $<40\%$ of circulating $CD45RO^+$ cells. Thus, there is heterogeneity of CCR5 expression within $CD45RO^+$ memory T cells, and we propose that high levels of CCR5 expression denote a tissue-infiltrating phenotype. The levels of CXCR3 on the liver-derived T cells are likely to be an underestimate given that collagenase, which we used to isolate T cells from liver tissue, resulted in reduced staining for CXCR3. T cells isolated using the collagenase-independent method showed very high levels of CXCR3. CCR5, CCR3, and other markers were not affected by collagenase (47).

The progressive liver injury in chronic hepatitis C is associated with up-regulation of intrahepatic Th1 cytokines (IFN-γ, IL-2) and down regulation of Th2 cytokines (IL-10) (23) and a reduced Th1 response in the periphery (21, 22). This suggests that the peripheral blood pool is depleted of Th1 cells as they are recruited to the liver. Recent evidence has shed light on the potential mechanisms of Th1 recruitment to tissue. In vitro studies show that CCR5 and CXCR3 are preferentially expressed on Th1 lymphocyte clones, and these cells migrate in response to CCR5 and CXCR3 ligands (26). Furthermore, CCR5 and CXCR3 have been associated with tissue infiltration by T cells into rheumatoid synovium, multiple sclerosis (28), and hepatocellular carcinoma (60). Conversely, it has been reported that CCR3 is up-regulated on Th2 cells (26, 48). Our study provides the first in vivo evidence in humans that tissue-infiltrating T cells in a chronic viral infection in which Th1 responses predominate are $CXCR3^{high}$ and $CCR5^{high}$ and $CCR3^{low}$.

Our findings suggest that CCR5 and CXCR3 are important, not only for the recruitment of T cells to inflamed hepatitis C liver but also to normal 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. However, there was no biochemical or histological evidence of pathological inflammation. T cells can be detected in most nondiseased livers by immunohistochemistry, where they are probably patrolling the tissue as part of the continual process of physiological immune surveillance (49). An alternative theory has been proposed in the mouse suggesting that the liver is a major site of T cell clearance via apoptosis (50), although we have failed to detect large numbers of apoptotic T cells in normal human liver (51). Most T cells in normal livers are detected in the portal tracts, and this might be a consequence of the reduced expression of chemokines IP-10 and Mig on noninflamed sinusoidal endothelium.

The different patterns of chemokine expression that were observed within portal tracts and the liver parenchyma provide further evidence for different pathways of lymphocyte recruitment to the liver (52). Portal inflammation is a frequent finding in many inflammatory liver diseases and is the predominant pattern of inflammation seen in cases of chronic viral hepatitis, including hepatitis C (53). Portal tracts are also the main site in which lymphocytes are found in the normal liver (54). The present study suggests that interaction between the CC chemokines MIP-1α and MIP-1β and CCR5 receptor may be involved in the recruitment of T cells to portal areas. Because these CCR5 ligands are constitutively expressed on portal vessels, they provide a pathway whereby $CCR5^{high}$ memory T cells migrate into portal areas in normal livers during immune surveillance. The same pathway may also be used for recruiting T cells to portal areas in other inflammatory liver diseases (55). Cases of chronic hepatitis in which inflammation is confined to portal areas ("chronic persistent hepatitis") are generally associated with a favorable outcome. In contrast, extension of the inflammatory process into the adjacent liver parenchyma in the form of interface hepatitis ("piecemeal necrosis") may be associated with destruction of periportal hepatocytes and

progressive periportal fibrosis ("chronic active hepatitis") (56). Little is known about the factors that promote migration of lymphocytes from portal areas into the adjacent liver parenchyma. However, there is increasing evidence to suggest that bursts of necroinflammatory activity within liver lobules may be important in the pathogenesis of progressive liver injury (57–59). This pattern of damage may be particularly relevant in cases of chronic hepatitis C infection, which despite being characterized by relatively mild changes in portal/periportal areas, with little or no interface activity, has a high risk of progression to chronic liver damage and cirrhosis. The present study suggests that the expression of the CXCR3 ligands IP-10 and Mig on sinusoidal endothelium may be an important mechanism for the direct recruitment of $CXCR3^{high}$ T cells to the liver parenchyma. The fact that expression of IP-10 and Mig increased in HCV-positive livers compared with normal controls supports a role for this pathway in the recruitment of lymphocytes to the inflamed liver. Clearly in hepatitis C, where the majority of the viral load is within the lobules, in hepatocytes and sinusoidal cells (37), T cell access to the parenchyma will be crucial for the effective clearance of virus-infected hepatocytes.

These findings have potential therapeutic implications. Progression of hepatitis to cirrhosis is related to the severity of inflammatory activity and particularly the presence of parenchymal inflammation. Thus, specific inhibitors of CXCR3 interactions may be effective in selectively reducing recruitment into the parenchyma, thereby slowing progression to cirrhosis. Such a strategy might be of benefit in autoimmune hepatitis but in chronic viral hepatitis will have the disadvantage of reducing access of lymphocytes to infected hepatocytes. Thus, in adoptive immunotherapy for chronic viral hepatitis, it may be equally important to promote IP-10 and Mig/CXCR3 interactions so that effector T cells have access to the parenchyma.

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