Expression of Chemokine Receptors on Intrahepatic and Peripheral Lymphocytes in Chronic Hepatitis C Infection: Its Relationship to Liver Inflammation

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Background. Intrahepatic lymphocytes are believed to be directly involved in the immunopathogenesis of chronic liver diseases. Little is known about the trafficking of lymphocytes into the liver and their role in chronic hepatitis C infection.

Methods. The expression of 4 chemokine receptors and an activation marker on multiple lymphocyte subsets in paired liver biopsy and peripheral blood specimens from 23 patients with chronic hepatitis C infection were analyzed by a 6-color flow-cytometric assay.

Results. CCR5, CXCR3, and CXCR6 were expressed on intrahepatic CD4+ and CD8+ T cells, natural killer (NK) T cells, NK cells, and B cells at significantly higher frequencies than on peripheral lymphocyte subsets. The expression of these receptors and the activation marker CD38 tended to increase with the severity of liver inflammation. This increase was significant for several intrahepatic lymphocyte subsets. Correlations in expression differed among pairs of these extralymphoid homing receptors on the intrahepatic T cells.

Conclusions. The homing program for intrahepatic lymphocytes involves multiple extralymphoid chemokine receptors that are regulated by >1 pathway. The expression of homing receptors on intrahepatic lymphocytes is associated with the immunopathogenesis of chronic hepatitis C disease. These preliminary results indicate that confirmational studies with larger sample sizes are warranted.

Hepatitis C virus (HCV) is a major cause of chronic liver disease. It is estimated that 5%–20% of cases of chronic hepatitis C infection will progress to cirrhosis, hepatocellular carcinoma, and liver failure [1, 2]. The mechanisms that underlie hepatocyte injury in HCV infection are poorly understood. However, immune responses, including T, NK, and NKT lymphocyte–mediated cellular immune responses, are thought to be involved in the immunopathogenesis of HCV infection [3–5].

The primary site of HCV replication and inflammatory response is the liver, where most immunopathologic events associated with the infection are likely to occur. The infiltration of lymphocytes increases with disease progression, which suggests that infiltrating lymphocytes, and activated lymphocytes in particular, play a role in the pathogenesis of chronic hepatitis. The recruitment of lymphocytes into target organs or tissues is mediated by the interaction between chemokines and their cognate receptors [6]. Chemokines are potent activators and chemoattractants for leukocyte subpopulations. Their actions are mediated by a large family of chemokine receptors. These receptors are expressed on different cell types, and their binding and response to specific chemokines are highly variable [7, 8].

Using explanted end-stage HCV-infected liver [9], we previously demonstrated that intrahepatic T, NK, and NKT cells express high levels of the inflammation-related chemokine receptors CCR5, CXCR3, and CXCR6 but not tissue-specific chemokine receptors, including CCR7 (lymph node), CCR4 (skin/lung), CCR10 (intestine/skin), and CCR9 (small intestine) [10–16]. Taking advantage of newly developed polychromatic flow-cytometric technology, we designed a 6-color staining strategy that enabled the analysis of multiple cell-surface markers on different
subsets of intrahepatic lymphocytes simultaneously, with use of percutaneous liver-biopsy specimens from subjects with chronic hepatitis C infection. The major goals of the study were to (1) compare the expression of extralymphoid tissue–trafficking chemokine receptors CCR5, CXCR3, and CXCR6 and the lymph node–trafficking chemokine receptor CCR7 on multiple subsets of lymphocytes in the liver and peripheral blood during various stages of mild to moderate chronic hepatitis C disease; (2) characterize patterns of coexpression of multiple chemokine on intrahepatic lymphocyte subsets; and (3) examine the relationship, if any, between the expression of chemokine receptors on intrahepatic lymphocytes and the degree of liver inflammation.

PATIENTS, MATERIALS, AND METHODS

Patients and specimens. We enrolled 23 patients (table 1) with chronic HCV infection who were undergoing liver biopsy for diagnostic purposes. Biopsy specimens were evaluated by a pathologist for severity of necroinflammation activity grade and fibrosis stage on a scale of 0–4 by use of a modification of the Knodell score [17, 18]. Excess tissue from histological testing was used for the preparation of intrahepatic lymphocytes. A venous blood sample was also collected from each patient and mixed with sodium heparin on the day of biopsy. The study protocol was approved by the institutional review board at Stanford University. Informed consent was obtained from the study subjects.

Preparation of intrahepatic lymphocytes and peripheral blood mononuclear cells (PBMCs). Intrahepatic lymphocytes and PBMCs were isolated as described elsewhere [19]. In brief, liver-biopsy samples were washed with 10 mL of RPMI 1640 medium that contained 10% fetal calf serum (FCS) and were gently ground with the small pestle of a Dounce tissue grinder filled with 5 mL of RPMI 1640 medium and 10% FCS, to release the intrahepatic lymphocytes. The cell suspension was centrifuged at 300 g for 10 min, to pellet the cells. The cells were resuspended in RPMI 1640 and 10% FCS and then counted. PBMCs were isolated from venous blood by standard Ficoll-Hypaque gradient centrifugation.

Cell staining and flow-cytometric analysis. Freshly prepared intrahepatic lymphocytes (0.1 × 10⁹–0.6 × 10⁹) or PBMCs (1 × 10⁹) were resuspended in 30 μL of FACS buffer (0.5% bovine serum albumin and 0.05% NaN₃, in PBS) and stained with combinations of fluorescence-conjugated or unconjugated mouse anti-human monoclonal antibodies or their isotype controls at predetermined concentrations. All antibodies were purchased from BD Pharmingen, unless otherwise specified. The antibodies used were CD4 phycoerythrin (PE)–Cy7 (SK3 or IgG₁), CD8 allophycocyanin (APC)–Cy7 (SK1 or IgG₁), CD19 PE-Cy7 (4G7 or IgG₁), CD3 APC-Cy7 (SK7 or IgG₁), CD56 fluorescein isothiocyanate (FITC; NCAM16.2 or IgG₂b), CD56 APC (B159 or IgG₁), CCR5/CDC195 FITC (2D7/CDC5 or IgG₂a), CXCR3/CDC183 PE (1C6/CXCR3 or IgG₁), CCR7 PE (3D12 or IgG₂a), CD38 APC (HI12 or IgG₁), mouse IgG₂a–isotype FITC (G155.178), mouse IgG₁–isotype PE (MOPC-21), mouse IgG₂a–isotype APC (MOPC-21), CXCR6 (5611.111 or IgG₂a) (R&D Systems), and mouse IgG₂a–isotype antibody. If the unconjugated anti-CXCR6 antibody or its isotype control was included in the combination, cells were first stained with this antibody, followed by staining with biotinylated horse anti–mouse IgG (Vector Laboratories) and then with streptavidin–peridinin-chlorophyll-protein complex–Cy5.5 (BD Biosciences). The cells were finally stained with a mixture of all the conjugated antibodies. All staining reactions were done at room temperature for 30 min, followed by washing with FACS buffer. The stained cells were fixed with 1% paraformaldehyde and analyzed on a LSR II 3-laser flow cytometer (BD Biosciences), by use of FACSDiVa software (version 2.2.1; BD Biosciences). The lymphocyte population in the liver or peripheral blood was defined by gating based on forward and side scattering. CD4⁺ T cells were defined by gating on the CD4⁺CD8⁻ lymphocyte population, CD8⁺ T cells were defined by gating on the CD4⁻CD8⁺ lymphocyte population, NK cells were defined by gating on the CD3⁻CD56⁺ lymphocyte population, and CD14⁺CD16⁻ monocytes were defined by gating on the CD14⁺CD16⁻ monocyte population.

Table 1. Characteristics of patients studied.

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NOTE. F, female; HCV, hepatitis C virus; M, male; NT, not tested; sALT, serum alanine aminotransferase.

* All patients tested positive for serum HCV RNA, according to the results of qualitative reverse-transcription polymerase chain reaction.
...and B cells were defined by gating on the CD3−, CD19+ lymphocyte population.

**Statistical analysis.** The effect of inflammation or fibrosis scores across subjects and the effects of site (intrahepatic vs. peripheral) or subset (CD4 vs. CD8) within subjects were tested in combination by fitting multiple-regression models to the frequency outcomes by use of generalized estimating equations (GEE) [20]. These regression models included an “interaction” term that allowed the fitted line across inflammation or fibrosis scores to differ in slope between liver and peripheral blood or between CD4 and CD8 cells. This permitted the estimated difference in frequency between liver and peripheral blood or between CD4 and CD8 cells to increase or decrease across increasing inflammation or fibrosis scores. For these regression analyses, the value of 2 was subtracted from the inflammation or fibrosis score for each subject. This “centering” enabled tests of group differences (e.g., between intrahepatic and peripheral lymphocyte subsets or between CD4+ and CD8+ T cell subsets) to be made consistently at the midrange score of 2. Because this comparison used the fitted lines from the regression model, comparison at the midrange score of 2 was based on the data from all subjects, rather than just those with a score of 2. Without this centering, comparisons of frequencies between groups would have been made at the extreme score of 0. GEE fitting used a Gaussian distribution with an identity link or a γ-distribution with a natural-logarithm link. Working correlation structures were either compound symmetric or unstructured.

Polychromatic flow cytometry allowed us to quantify co-occurrence patterns of pairs of markers on cell surfaces separately within each patient. To examine the coexpression pattern of the chemokine receptors CCR3, CXCR3, and CXCR6, 2 × 2 frequency tables of gated cell counts were created for each patient—1 table for each pair of receptors, for a total of 3 tables. A φ-coefficient [21] was calculated from each frequency table. The φ-coefficient measures the degree of co-occurrence (correlation) and ranges from −1.0, if one chemokine receptor never occurs when the other chemokine receptor is present, to +1.0, when both chemokine receptors only occur together on the cell surface. The average correlation was estimated across patients for each pair of receptors. To account for the possibility that average correlation might vary with inflammation grade, regression analysis was performed with φ-coefficients as the dependent variables and inflammation scores as the independent variable. A quadratic term allowed for curvature in response and was centered around 2, to reduce collinearity [22, 23]. Regression used weighted least squares [22, 23], with total quantities of gated cells serving as weights so that biopsy samples that provided larger cell counts were weighted more heavily in the fit.

Throughout the results presented below, hypothesis tests are considered to be statistically significant for significance levels of $P \leq .05$. To conserve the power of individual tests, no corrections for compounding type I errors across multiple hypothesis tests were made [24]. Given the limited sample size of the study, the results presented here should be viewed as preliminary.

**RESULTS**

**Lymphocyte subsets in the liver and blood of patients with chronic HCV infection.** The frequencies of CD4+, CD8+, NK, and B cells in the liver and blood of patients with chronic HCV infection were analyzed. Our goal was to compare the average frequency of each subset in the intrahepatic lymphocytes against that in the peripheral blood and to examine whether average frequencies vary with the degree of liver fibrosis or inflammation. Multiple-regression models were fitted by use of GEEs (see statistical analysis in Patients, Materials, and Methods for details).

The frequencies of 5 intrahepatic and peripheral lymphocyte subsets across fibrosis stages are summarized in figure 1. Overall, CD8+ T cells (20%) and CD4+ T cells (14%) were the most abundant lymphocytes in the liver, followed by NK cells (8.2%), NK cells (7.8%), and B cells (3.9%). CD4+ T cells (41%) were the most abundant subset in the blood, followed by CD8+ T cells (16%), NK cells (15%), B cells (7.7%), and NK cells (3.7%). The ratios of CD8+ and CD4+ T cells in liver and blood were ∼1:1–1:5:1 and 1:2–1:3, respectively, which agrees with the results of a previous study [9]. At fibrosis stage 2, the average frequencies of CD8+ T cells and NK cells were significantly higher in the liver, and average frequencies of CD4+ T cells, NK cells, and B cells were significantly higher in the blood. For CD4+ T cells, the slope of subset frequency across fibrosis stage differed significantly between liver and blood ($P = .01$), which suggests that the difference in average frequencies between the liver and blood narrowed at higher stages of fibrosis (figure 1). However, no significant changes in average frequency were observed across fibrosis stages for any subset in the liver or peripheral blood except for CD4+ T cells in the blood, in which case the percentage of CD4+ T cells decreased significantly with increasing fibrosis stage ($P = .03$). None of the average frequencies of the 5 subsets in the liver or blood changed significantly with increasing inflammation grade (data not shown).

**Expression of chemokine receptors and activation markers on lymphocyte subsets and their relationship to liver inflammation.** We also examined the expression of chemokine receptors CCR5, CXCR3, CXCR6, and CCR7 and the activation marker CD38 on liver and blood lymphocyte subsets. For each of the markers expressed on each lymphocyte subset, a multiple-regression model was fitted by use of GEE (see statistical analysis in Patients, Materials, and Methods for details). The results are shown in figure 2.

1. As indicated by the $P$ values for liver versus blood, the
Figure 1. Percentage of lymphocyte subsets in paired liver (L) and blood (B) specimens from patients with chronic hepatitis C virus (HCV) infection across fibrosis stages. Twenty-three subjects were studied. Because of the smaller size of some biopsy specimens that did not allow all the staining combinations, only 19 subjects were analyzed for NK and NKT cells, and only 20 were analyzed for B cells. Regression models were fitted with generalized estimating equations. P values for L, B, and L vs. B (LvsB) are the attained significance levels for the linear effect of fibrosis score on the frequencies of intrahepatic and peripheral lymphocyte subsets and for the average difference between the frequencies of the intrahepatic and peripheral lymphocyte subsets at fibrosis stage 2, respectively. See statistical analysis in Patients, Materials, and Methods for details. Underlined P values (\( \leq .05 \)) are considered to be significant.

average expression frequencies of the extralymphoid chemokine receptors CCR5, CXCR3, and CXCR6 at inflammation grade 2 were significantly higher for all the intrahepatic lymphocyte subsets than for their counterparts in the blood. In contrast, the average expression frequencies of the lymph-node homing receptor CCR7 were significantly higher for peripheral CD4+ and CD8+ T cells and NKT cells, compared with those for their corresponding intrahepatic subsets. Of note, the slope of expression frequency over inflammation grade differed significantly (\( P \leq .05 \)) between liver and blood for some receptors in certain lymphocyte subsets. In particular, as illustrated in figure 2, the difference between the average expression levels in liver and blood tended to widen with increasing inflammation grade for CXCR6 on CD8+ T cells, CXCR3 on NK cells, and CCR5 on B cells \( (P \leq .02) \), whereas this difference narrowed with increasing inflammation grade for CXCR6 on B cells \( (P = .04) \).

A recent publication reported that the isolation of PBMCs with Ficoll gradient centrifugation resulted in a loss of CCR5 staining on CD4+ and CD8+ T cells \[25\]. To evaluate the effect of this finding on our conclusions on the basis of the CCR5 staining of Ficoll-purified PBMCs, we compared CCR5 staining results of CD4+ and CD8+ T cells, CXCR3 on NK cells, and CCR5 on B cells \( (P \leq .02) \), whereas this difference narrowed with increasing inflammation grade for CXCR6 on B cells \( (P = .04) \).

1. The average expression frequencies of CCR5, CXCR3, and CXCR6 on intrahepatic lymphocyte subsets tended to increase with inflammation grade for most of the lymphocyte subsets. This increase was significant for all 3 receptors on CD8+ and CD4+ T cells, for CCR5 and CXCR6 on NK T cells, and for CXCR3 on NK T cells. For the intrahepatic B cells, the average frequency of CCR5 increased significantly with inflammation grade, whereas the frequency of CXCR6 declined significantly with liver inflammation. Of note, CCR5 was only expressed on a minor fraction of B cells in the liver and peripheral blood, whereas the majority of intrahepatic B cells expressed CXCR6 across all inflammation grades.

2. The average expression frequency of the activation marker CD38 on the intrahepatic CD8+ T cells was significantly higher than that on peripheral CD8+ T cells. In addition, the average expression frequency of CD38 on intrahepatic T cells tended to increase with inflammation score, and the increase was significant for CD8+ T cells.

3. Average expression frequencies of certain chemokine receptors on peripheral lymphocyte subsets also increased significantly with increasing inflammation in the liver. These included CCR5 and CXCR3 on CD8+ T cells and CCR5 on NKT cells. This result suggests
Figure 2. Frequencies of intrahepatic and peripheral lymphocyte subsets expressing chemokine receptors and CD38 across liver (L) inflammation grades. Twenty-one subjects with data available were studied. Because of the smaller size of some biopsy specimens that did not allow all of the staining combinations, only 18 subjects were analyzed for NK and NKT cells, and only 20 were analyzed for B cells. Regression models were fitted with generalized estimating equations. \( P \) values for L, blood (B), and L vs. B (LvsB) are the attained significance levels for the linear effect of inflammation score on the frequencies of each marker expression on intrahepatic and peripheral lymphocyte subsets and for the average difference between the intrahepatic and peripheral lymphocyte subsets at inflammation grade 2, respectively. See statistical analysis in Patients, Materials, and Methods for details. Underlined \( P \) values (\( \leq .05 \)) are considered to be significant. Expression frequencies of CD38 on NKT, NK, and B cells were not assessed.
a relationship between peripheral blood markers and the severity of liver inflammation.

5. An analysis was conducted to examine the relationship between the expression levels of CCR5, CXCR3, CXCR6, and CD38 on intrahepatic lymphocytes and the serum alanine aminotransferase (ALT) level of each patient. No change in the expression of these markers with increasing ALT level was detected statistically for any of the lymphocyte subsets (data not shown).

Coexpression of chemokine receptors on T cells in liver and blood. To examine the profile of multiple chemokine receptor coexpression in the liver and blood, we classified lymphocytes into 8 phenotypes on the basis of the expression patterns of CCR5, CXCR3, and CXCR6. For this analysis, we focused on CD4+ and CD8+ T cells, the 2 most abundant subsets identified in the liver and peripheral blood. The average frequency for each of the 8 phenotypes is categorized by subset and site in table 2. Using GEEs, we tested for differences in average phenotype frequencies between the intrahepatic and peripheral lymphocyte subsets and between CD4+ and CD8+ T cells at inflammation grade 2 (see statistical analysis in Patients, Materials, and Methods for details). The main results are summarized as follows.

1. Regarding the chemokine receptor phenotypes of T cells in liver versus blood, average frequencies of CD4+ and CD8+ T cells expressing 2 or 3 of these receptors were significantly higher in the liver than in the blood, whereas frequencies of T cells expressing none of these markers were significantly higher in the blood than in the liver. This result indicates that the homing of T cells to the liver is associated with the expression of multiple chemokine receptors.

2. Regarding the chemokine receptor phenotypes of CD4+ versus CD8+ T cells, the average frequencies of all phenotypes differed significantly between CD4+ and CD8+ T cells in both the liver and the blood, except for CCR5+CXCR3+CXCR6− and CCR5+CXCR3−CXCR6+. The difference between blood and liver in cells expressing CCR5 alone was significantly greater for CD8+ (8.57% vs. 4.53%) than for CD4+ T cells (6.50% vs. 6.23%), whereas the difference between blood and liver in cells expressing CXCR3 alone was significantly greater for CD4+ (13.8% vs. 34.4%) than for CD8+ (12.4% vs. 9.0%) T cells. The difference between blood and liver in cells expressing none of these chemokine receptors was significantly greater for CD4+ (76.5% vs. 13.4%) than for CD8+ (51.7% vs. 10.5%) T cells, with the average frequency of this negative phenotype being highest for CD4+ T cells in the blood (76.5%). Taken together, these results suggest that the potential contribution of these receptors for homing to the liver differs between the CD4+ and CD8+ T cell subsets.

To determine whether the expression of each of these chemokine receptors correlated with each other at the single-cell level, we estimated the mean correlation coefficient between each of the 3 pairs of markers for intrahepatic CD4+ and CD8+ T cells. As shown in table 3, the correlation between CXCR3 and CXCR6 and between CXCR3 and CCR5 was weak (near zero) for CD8+ T cells and was weak to modest for CD4+ T cells. In contrast, the correlation was moderately strong between CCR5 and CXCR6 for both CD4+ and CD8+ T cells. These analyses suggest that the expression of homing receptors on liver-infiltrating lymphocytes, especially for CD8+ T cells, is regulated by at least 2 nearly independent regulatory pathways—one that regulates the expression of CXCR3 and another that regulates a degree of the coexpression of CCR5 and CXCR6. Of note, the average correlation between CCR5 and CXCR6 declined significantly with increasing inflammation grade for intrahepatic CD8+ T cells but not CD4+ T cells, which suggests that the regulation of chemokine receptor expression differs between CD4+ and CD8+ T cells. We did not detect any curvature (the quadratic term in the regression model) in the relationship between average correlation and inflammation grade for any of the pairs of markers (P > .08), which indicates that the rate of decline in correlation between CCR5 and CXCR6 was constant across increasing inflammation grades.

### Table 2. Mean percentage (± SD) of each of 8 phenotypes for CD4+ and CD8+ T cells in peripheral blood and liver at inflammation grade 2 (see statistical analysis in Patients, Materials, and Methods for details) (n = 21).

<table>
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<tr>
<th>Sample, phenotype</th>
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<th>CCR5−CXCR3+CXCR6−</th>
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<td>0.93 (0.85)</td>
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DISCUSSION

A comprehensive characterization of different immune-cell subsets is crucial for understanding the complex functions of the immune system. Newly introduced polychromatic flow-cytometric technology enables analyses encompassing ≥6 colors, so that multiple phenotypic markers on several subsets of immune cells can be assessed simultaneously [26, 27]. This allows the multivariate analysis of multiple cellular characteristics on a single-cell basis, which may reveal biologically interesting relationships. Such a capability is especially important for studying lymphocytes isolated from biopsy samples, because of the very limited number of cells available for analysis. Hence, this new technology has great potential for generating a more complete and integrated picture for intrahepatic lymphocytes, which are likely to be directly involved in the immunopathogenesis of HCV infection.

We used a 6-color flow-cytometric analysis to investigate the expression of multiple chemokine receptors and an activation marker on 5 lymphocyte subsets in liver and blood samples from patients with chronic HCV infection. Our results demonstrate that, in general, lymphocyte subsets expressing the extralymphoid homing receptors CCR5, CXCR3, and CXCR6 were enriched in the liver, compared with the peripheral blood, of patients who had mild to moderately severe chronic HCV infection. In addition, CD8+ and CD4+ T cells, NK cells, and B cells are less frequent in the liver than in the blood. However, no significant changes in the average frequency of intrahepatic lymphocyte subsets were found across fibrosis stages 0–4. These 2 complementary studies suggest that the enrichment of NK cells in end-stage HCV liver disease, NK cells comprise the major lymphocyte subset, followed by T and B cells [9]. In the present study, we found that, during the earlier stages of chronic HCV infection, CD8+ and CD4+ T cells are the most abundant subsets in the liver, whereas CD4+ T cells, NK cells, and B cells are less frequent in the liver than in the blood. However, no significant changes in the average frequency of intrahepatic lymphocyte subsets were found across fibrosis stages 0–4. These 2 complementary studies suggest that the enrichment of NK cells in end-stage HCV liver disease may occur only at the highly advanced cirrhosis stage. Of note, a sizable portion of the intrahepatic lymphocyte population could not be defined with the antibodies used in the present study. Unconventional lymphocytes, including CD4 and CD8 double-positive and double-negative T cells, have been identified at high frequencies in the liver [42]. The origin, phenotype, and function, as well as the role of these cells in the immunopathogenesis of HCV disease, need to be examined in future studies.

Information regarding the trafficking of lymphocyte subsets into the liver is scarce in the literature [32–35]. Using polychromatic flow-cytometric analysis, we were able to analyze the coexpression of multiple chemokine receptors with small numbers of intrahepatic lymphocytes obtained from needle-biopsy specimens. Our results indicate that the expression of CXCR3 and CXCR6 may be relatively independent of each other, whereas the expression of CCR5 and CXCR6 were correlated to a certain extent. Although these 3 chemokine receptors have been proposed to be involved in the homing of lymphocytes to inflamed tissues, the present results suggest that the expression of these receptors is regulated by multiple pathways.

The liver is an organ with unique immunological characteristics, including a tolerant nature and the existence of early lymphoid progenitors, which suggests a possible hematopoietic role [36–38]. It remains unclear as to which subset of intrahepatic lymphocytes predominates in chronic HCV infection [39–43]. Our previous work has shown that, in patients with end-stage HCV liver disease, NK cells comprise the major lymphocyte subset, followed by T and B cells [9]. In the present study, we found that, during the earlier stages of chronic HCV infection, CD8+ and CD4+ T cells are the most abundant subsets in the liver, whereas CD4+ T cells, NK cells, and B cells are less frequent in the liver than in the blood. However, no significant changes in the average frequency of intrahepatic lymphocyte subsets were found across fibrosis stages 0–4. These 2 complementary studies suggest that the enrichment of NK cells in end-stage HCV liver disease may occur only at the highly advanced cirrhosis stage.

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Table 3. Correlation coefficient between expression of pairs of markers on intrahepatic T cells at each inflammation grade (n = 21).

<table>
<thead>
<tr>
<th>T cell subset, pair of markers</th>
<th>Inflammation grade, mean (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td></td>
</tr>
<tr>
<td>CXCR3 vs. CCR5</td>
<td>0.102 (0.059–0.144)</td>
</tr>
<tr>
<td>CXCR3 vs. CXCR6</td>
<td>0.112 (0.070–0.159)</td>
</tr>
<tr>
<td>CCR5 vs. CXCR6</td>
<td>0.534 (0.491–0.576)</td>
</tr>
<tr>
<td>CD8</td>
<td></td>
</tr>
<tr>
<td>CXCR3 vs. CCR5</td>
<td>−0.077 (−0.117 to −0.037)</td>
</tr>
<tr>
<td>CXCR3 vs. CXCR6</td>
<td>−0.023 (−0.063 to 0.017)</td>
</tr>
<tr>
<td>CCR5 vs. CXCR6</td>
<td>0.510 (0.470–0.550)</td>
</tr>
</tbody>
</table>

* Statistically significant decrease (P = .03) in correlation with increasing inflammation grade.
results suggest that the progression of HCV-related chronic hepatitis to end-stage liver disease is accompanied by changes in the composition of the intrahepatic lymphocyte population.

Our results also reveal different patterns of chemokine receptor expression on distinct intrahepatic lymphocyte subsets. For example, CD8+ T cells in the liver showed a higher average coexpression of CCR5, CXCR3, and CXCR6 at inflammation grade 2 than did the intrahepatic CD4+ T cells (table 2). In fact, less than one-half of the intrahepatic CD4+ T cells expressed ≥2 of these chemokine receptors. These results suggest that the hepatic trafficking of various intrahepatic lymphocyte subsets may be controlled by different combinations of homing receptors and/or that homing receptors other than CCR5, CXCR3, and CXCR6 may be involved in the homing of intrahepatic lymphocytes.

Another finding of the present study was the relationship between the expression of CCR5, CXCR3, and CXCR6 on intrahepatic T, NK, and NK cells and the necroinflammation activity in the liver with chronic HCV infection, which suggests a role for both the innate and the adaptive cellular immune responses in the immunopathology of chronic HCV infection. It has been reported that the chemokines macrophage inflammatory protein (MIP)-1b, RANTES, MIP-1, and monocyte chemoattractant protein–2 are ligands for CCR5; that inflammatory protein–10 and Mig are ligands for CXCR3; and that CXCL16 is a ligand for CXCR6 [6, 44–46]. Several previous studies have shown that the expression levels of these chemokines in the liver are positively related to the severity of hepatic inflammation in chronic HCV infection [32, 33, 35]. Our data, which focus on the receptors for these chemokines, demonstrate that the average expression levels of the chemokine receptors CCR5, CXCR3, and CXCR6 on intrahepatic lymphocytes may increase with the severity of liver inflammation. The elevated expression of the chemokines in severely inflamed liver may more efficiently attract lymphocytes that express the relevant receptors to the liver [6, 35, 47]. Alternatively, the presence of higher levels of the chemokines in a severely inflamed liver may enhance the expression of their cognate receptors on the lymphocytes circulating through the liver, which increases their chance of being recruited or retained in the liver. In either case, the expression levels of chemokine receptors on the intrahepatic lymphocyte subsets will reflect the severity of liver inflammation.

In addition to the intrahepatic lymphocyte subsets, the expression levels of some chemokine receptors on PBMC subsets were found to increase with the degree of liver inflammation. This observation suggests that the severity of liver inflammation might be assessable by use of a panel of markers on peripheral lymphocyte subsets.

Our results indicate that multiple extralymphoid tissue-trafficking inflammation chemokine receptors mediate the homing of lymphocyte subsets to the liver, which contributes to liver pathogenesis during chronic HCV infection. Further studies with larger sample sizes should be conducted to confirm and expand these observations. The characterization of the expression patterns of these homing receptors on intrahepatic lymphocyte subsets is important for understanding the mechanism by which these cells migrate to the liver and their roles in the immunopathogenesis of HCV infection. Such knowledge may lead to new therapeutic interventions based on the role of the homing receptors in chronic HCV infection.

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References

16. Kunkel EJ, Campbell JJ, Haraldsen G, et al. Lymphocyte CC chemokine receptor 9 and epithelial thymus–expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: epi-


