Reduced CC Chemokine Receptor (CCR) 1 and CCR5 Surface Expression on Peripheral Blood T Lymphocytes from Patients with Chronic Hepatitis C Infection

Mathias Lichterfeld,1 Ludger Leifeld,1 Hans Dieter Nischalke,1 Jürgen K. Rockstroh,1 Lothar Heß,2 Tilman Sauerbruch,1 and Ulrich Spengler1

T cell recruitment to the infected liver is an essential step for the efficient elimination of hepatitis viruses. The surface expression of CC chemokine receptor (CCR) 1, CCR4, and CCR5 on peripheral blood T lymphocytes and their responsiveness to the chemokines macrophage inflammatory proteins (MIP)–1α, MIP-1β, and RANTES (regulated on activation, normally T cell–expressed and secreted) was analyzed in patients with chronic hepatitis C and hepatitis B infection and compared with healthy subjects. Although CCR4 surface expression was not altered, hepatitis C virus (HCV)–infected patients had lower proportions of CD8 T cells with CCR1 and CCR5 surface expression (P < .05). Migration of CD8 T cells in response to MIP-1α, MIP-1β, and RANTES was significantly reduced in HCV-infected patients (P < .05). Intracellular CCR1 and CCR5 protein and messenger RNA levels in peripheral blood T cells did not indicate reduced chemokine receptor biosynthesis in hepatitis C infection. Thus, chronic hepatitis C, but not hepatitis B, infection alters surface expression of distinct CCRs, resulting in lower CC chemokine responsiveness.

The elimination of hepatitis C virus (HCV) requires a precisely tuned interplay of helper and cytotoxic T lymphocytes [1]. Because of the hepatic tropism of HCV, recruitment of virus-specific T cells to the liver represents a critical step for virus clearance. Chemokines and their receptors play a pivotal role in this process. Chemokines are small polypeptides that mediate directed leukocyte migration. Leukocytes sense chemokine concentration gradients and move toward increasing concentrations. Thus, both differential chemokine secretion in infected tissue and selective expression of different chemokine receptors on distinct leukocyte subsets regulate leukocyte trafficking [2].

The CC chemokines macrophage inflammatory protein (MIP)–1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) are important in hepatic immune surveillance because they are expressed on the portal vessel endothelium and trigger the invasion of macrophages and lymphocytes into the liver [3]. These chemokines bind to their corresponding receptors, CCR1 and CCR5. Both of these receptors are preferentially expressed on lymphocytes with a Th1 cytokine secretion pattern, which are pivotal for viral elimination, whereas lymphocytes with a Th2 cytokine profile predominantly express CCR4 [4]. A possible role of chemokine receptors in the pathogenesis of chronic hepatitis C infection is suggested by the recent finding that a genetically determined loss of CCR5 expression is linked to chronic hepatitis C infection and high-level viremia [5]. Here, we analyzed the expression of the chemokine receptors CCR1, CCR4, and CCR5 and the responsiveness to CC chemokines in peripheral blood T lymphocytes of patients with chronic HCV infection.

Patients and Methods

Patients. We studied serum and peripheral blood mononuclear cells (PBMC) of 12 patients (5 women and 7 men) with chronic replicative HCV infection (median age, 38 years [range, 20–68 years]; median duration of documented infection, 7 years [range, 2–16 years]; median HCV RNA load, 2.4 × 10⁶ copies/mL [range, 300–38,000,000 copies/mL]), 9 patients (3 women and 6 men) with chronic hepatitis B virus (HBV) infection, all of whom were hepatitis B surface antigen and hepatitis B e antigen positive (median age, 38 years [range, 26–57 years]; median duration of infection, 13 years [range, 2–28 years]), and 12 healthy volunteers (5 women and 7 men; median age, 30 years [range, 24–50 years]). All subjects were homozygous for the CCR5 wild-type gene. None of the patients received antiviral therapy at the time of the study.

Blood samples were drawn in EDTA-treated tubes, and PBMC were prepared by ficoll-density gradient separation immediately after phlebotomy. Serum samples were stored at −80°C until analy-
sis. HCV and HBV antibodies were detected by commercial microparticle EIA (Axysm; Abbott). HCV and HBV viremia were identified by standard polymerase chain reaction (PCR) protocols, as described elsewhere [6]. HCV genotypes, determined with the INNOLIPA II line probe assay (Innogenetics), were as follows: 1a (n = 3), 1b (n = 5), 2a (n = 1), and undetermined (n = 2). We analyzed the CCR5-Δ32 gene polymorphism by real-time PCR on a LightCycler (Roche) by using primers flanking the Δ32 deletion site (5'-AAGGTCTTCATTACACCTGC-3' and 5'-GATGACCAGCAAGCAGCG-3'; thermal profile: 1 s at 60°C, 4 s at 72°C, and 1 s at 90°C). This allowed us to identify either CCR5 allele via amplified 50- and 82-kb fragments, respectively.

**Flow cytometry detection of chemokine receptor expression.** CCR1, CCR4, and CCR5 surface expression was studied by triple-color flow cytometry, using the following antibodies: anti–CD4 phycoerythrin (PE)/fluorescein isothiocyanate (FITC) (clone Leu-3a), anti–CD8-PE/FITC (clone Leu-2a), anti–CD3-PE/FITC (clone Leu-4) (all from Becton Dickinson), anti–CCR1-PE (clone 53504.111), and anti–CCR5-PE/FITC (clone sc6126; Santa Cruz Biotechnology). We mixed 5 μL of each monoclonal antibody with 10^6 PBMC in 50 μL of PBS and incubated the samples for 20 min. Unbound antibody was removed by 2 wash steps with PBS. Next, we analyzed 10^4 lymphocytes on a FACSort flow cytometer with the Cell Quest software package (Becton Dickinson). Intracellular chemokine receptor protein staining was done with the Fixperm kit (Caltag), according to the manufacturer’s instructions. Specificity was ascertained by including nonpermeabilized cells, in addition to isotype control antibodies. Results are reported as the proportion of cells with fluorescence signals above the cutoff, as defined by the isotype controls.

**Determination of chemokine receptor mRNA by real-time reverse-transcription PCR.** Total RNA was extracted from whole blood with the RNeasy extraction kit (Qiagen), reverse transcribed with Superscript reverse transcriptase (Promega), and amplified by real-time PCR on a LightCycler (Roche) by use of primer sequences for CCR1, CCR4, CCR5, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase), as described elsewhere [7]. The thermal profile was 1 s at 60°C, 15 s at 72°C, and 1 s at 90°C. Chemokine receptor mRNA levels were gauged relative to GAPDH mRNA and are reported as arbitrary units (AU).

**Analysis of migration in response to CC chemokines.** We seeded 6 × 10^5 freshly isolated PBMC into the upper compartment of a 5-μm nitrocellulose filter microchamber system (Neuroprobes). We added 300 μL of RPMI 1640 medium containing either MIP-1α, MIP-1β, or RANTES (PromoCell) at 500 ng/mL (the concentration that achieved maximal chemotactic effects) or FMLP (10^-8 M) or no chemotactic supplement to the lower compartment of the chemotaxis chamber. Cells were allowed to migrate through the filter for 5 h at 37°C. Migrated cells were harvested from the lower compartment and analyzed by flow cytometry. Results are reported as the migration index, which was calculated as the number of cells migrating toward the chemokine gradient divided by the number of cells migrating toward medium alone.

**Determination of chemokine serum levels.** Concentrations of MIP-1α and MIP-1β were measured in serum samples by commercial ELISA kits, according to the manufacturer’s instructions (R&D Systems). RANTES serum levels could not be measured because of its artificial release from platelets during centrifugation.

**Statistical analysis.** Results are given as median and range. Differences between groups were compared by the Mann-Whitney U test. P < .05 was considered to be statistically significant.

**Results**

**CC chemokine receptor surface expression.** The surface expression of CCR1, CCR4, and CCR5 on CD4 and CD8 T lymphocytes is summarized in figure 1A. We detected 3–4-fold more CD8 T cells with CCR1 and CCR5 surface expression in healthy subjects and patients with chronic hepatits B infection than in patients with chronic hepatitis C infection. Likewise, compared with HCV-infected patients, an ~2–3-fold higher proportion of CCR5-expressing CD4 cells was found in healthy volunteers and in patients with hepatitis B infection. Unlike CD8 T cells, CCR1 surface expression on CD4 T cells did not reveal any significant differences among our study groups. Moreover, there was no substantial difference in CCR4 surface expression on CD4 and CD8 T cells between patients with hepatitis C infection and the 2 other groups (figure 1A).

**Chemokine responsiveness.** Figure 2 shows the chemokine responsiveness of peripheral blood lymphocytes. After stimulation with MIP-1α, MIP-1β, and RANTES, significantly fewer CD8 T cells were attracted in patients with hepatitis C infection than in healthy control subjects and in patients with hepatitis B infection. Migration of CD4 T cells was also reduced in patients with hepatitis C infection, but the differences did not reach statistical significance. No major differences in migratory responses to FMLP were found among the study groups, indicating that migration was not generally impaired in patients with hepatitis C infection.

**Intracellular chemokine receptor expression.** After permeabilization, the proportion of CD4 and CD8 cells with detectable intracellular CCR5 expression was significantly higher in patients with hepatitis C infection than in healthy control subjects and patients with hepatitis B infection (P < .05; figure 1B). Intracellular CCR1 expression was also more frequent in lymphocytes from HCV-infected patients, but statistical significance was only reached for CD4 cells. CCR1 and CCR5 mRNA levels (median [range]) tended also to be higher in patients with hepatitis C infection (CCR1, 3.02 AU [0.54–8.01 AU]; CCR5, 1.61 AU [0.52–5.81 AU]) than in healthy control subjects (CCR1, 1.7 AU [0.25–3.56 AU]; CCR5, 1.13 AU [0.87–2.79 AU]) and patients with hepatitis B infection (P < .05; figure 1C). Levels of CCR4 mRNA were not different among the study groups (data not shown).

**Chemokine serum levels.** MIP-1α and MIP-1β serum levels (median [range]) were elevated in the HCV-infected patients (MIP-1α, 8 pg/mL [0–30 pg/mL]; MIP-1β, 44 pg/mL [26–81 pg/mL]) and HBV-infected patients (MIP-1α, 5 pg/mL [0–24 pg/mL]; MIP-1β, 44 pg/mL [16–62 pg/mL]) to the same extent as in healthy control subjects (MIP-1α, 2 pg/mL [0–25 pg/mL],
Discussion

In this cross-sectional study, we demonstrated reduced surface expression of CCR1 and CCR5 on peripheral blood T lymphocytes from patients with chronic hepatitis C infection. Chemokine receptor surface expression was decreased to a greater extent on CD8 lymphocytes than on CD4 lymphocytes, probably because of a higher physiologic CCR1 and CCR5 expression on CD8 lymphocytes. Reduced chemokine receptor expression in hepatitis C infection corresponded to decreased T lymphocyte migration in response to MIP-1α, MIP-1β, and RANTES in chemotaxis assays. Unlike CCR1 and CCR5, expression of the chemokine receptor CCR4 was not altered. Because normal chemokine receptor expression and function were observed in chronic hepatitis B infection, our data suggest that chemokine-mediated trafficking and recruitment of T lymphocytes is specifically altered in chronic hepatitis C infection.

The cause for altered chemokine receptor expression and chemokine responsiveness in hepatitis C infection remains unclear at present. Reduced chemokine receptor expression due to the CCR5-D32 mutation, which has been found more frequently in hemophilic patients with hepatitis C infection [5], can be excluded, since all the subjects were homozygous for the CCR5 wild-type gene. Preferential hepatic compartmentalization of CXCR3+/CCR5+ lymphocytes recently has been proposed for the immune response against HCV [3].

Of note, CCR1/5 surface expression might favor an intrahepatic accumulation of the respective lymphocytes in the portal region.
Migration of CD4 and CD8 T lymphocytes in a chemotaxis assay after stimulation of peripheral blood mononuclear cells with the CC chemokines macrophage inflammatory protein (MIP)–1α, MIP-1β, and RANTES. Stimulation with FMLP served as a control. Results are given as the migration index, calculated as the number of cells migrating toward the chemokine concentration gradient divided by the number of cells migrating toward medium alone. Columns indicate mean migration indices; error bars show corresponding SD in patients with chronic hepatitis C virus (HCV; n = 12) or hepatitis B virus (HBV; n = 9) infection and healthy control subjects (Healthy; n = 12). *p < .05.

tracts of HCV-infected livers, whereas CXCR3+ lymphocytes are probably directed to the hepatic sinusoids, where interferon-inducible protein 10 (CXCL10), an important ligand for CXCR3, is mainly released [3]. CXCR3+/CCR5+ lymphocytes may therefore be redistributed from the peripheral blood into the liver, resulting in reduced proportions of these cells in the peripheral blood. However, in our experiments, intracellular CCR1 and CCR5 expression were detected in higher proportions of peripheral blood T cells from HCV-infected patients. Thus, it is unlikely that depletion of the CCR5+ cells from the peripheral circulation can account exclusively for our findings. Alternatively, intracellular receptor trafficking and cycling may be altered in hepatitis C infection, possibly because of an interaction of HCV proteins with either the receptors themselves or their putative escort proteins [8]. Moreover, chemokine receptors are rapidly internalized into the cytoplasm after exposure to their ligands, thus inducing desensitization of the cells [9, 10]. Indeed, we found higher serum concentrations of MIP-1α and MIP-1β in patients with hepatitis C infection than in healthy control subjects. However, chemokine serum levels likewise were elevated in patients with chronic hepatitis B infection, which was not associated with decreased CCR1 and CCR5 surface expression on T cells. Thus, the reduced lymphocellular CCR1 and CCR5 surface expression in patients with HCV cannot be attributed to increased ligand-induced endocytosis of the receptor proteins alone. Finally, CCR5 function is directly inactivated by interleukin (IL)–10 [11], the increased production of which has been reported in patients with hepatitis C infection [12]. However, although IL-10 secretion could induce chemokine unresponsiveness, it does not down-regulate CCR5 surface expression [11].

Decreased CCR1 and CCR5 surface expression and chemokine responsiveness in hepatitis C infection might have important consequences for the host’s immune defense. Early after HCV infection, prominent CD8 T cell responses are observed involving transient up-regulation of CCR5 expression [13]. In chronic hepatitis C infection, however, CD8 lymphocytes show sustained dysfunction, with poor type 1 cytokine secretion in response to HCV antigens [13, 14]. Because CCR1 and CCR5 expression correspond to functional differentiation toward a type 1 cytokine secretion pattern [4], decreased CCR1 and CCR5 expression in hepatitis C infection might reflect diminished lymphocellular type 1 cytokine responses, as reported elsewhere [12, 14]. Type 2 responses—commonly associated with CCR4+ lymphocytes—however, are apparently preserved. Moreover, recruitment of CD8 T cells into the liver might become more ineffective as more CCR1 and CCR5 expression is reduced on lymphocytes. Of interest, sequential liver biopsies in patients with hepatitis C infection demonstrated steadily declining numbers of intrahepatic CD8 T cells over the course of chronic infection [15]. Thus, reduced CCR1 and CCR5 expression and signaling may contribute to the sustained lymphocyte dysfunction favoring viral persistence in hepatitis C infection.

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