Hepatitis C Virus–Induced Secretion of Inflammatory Chemokines Preferentially Recruits NKG2A⁺CD8⁺ T Cells

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In patients with hepatitis C, a loss-of-function mutation of chemokine receptor CCR5 (CCR5Δ32) has been shown to be associated with spontaneous viral clearance and lower levels of hepatic inflammation. In the present study, we show that CCR5 is coexpressed with the inhibitory NKG2A receptor on CD8⁺ T cells. Consequently, CCR5⁺ T cells were highly susceptible to NKG2A-mediated inhibition of cytotoxic activity and NKG2A⁺ lymphocytes were preferentially attracted by CCR5 ligands induced by hepatitis C virus E2 antigen. Thus, CCR5 is likely to exert immunoregulatory effects in hepatitis C virus infection by preferentially recruiting CD8⁺ T cells bearing the inhibitory NKG2A receptor to the liver.

Infection with the hepatitis C virus (HCV) often results in chronic liver disease with variable progression toward cirrhosis. Many aspects of the immune response to HCV infection are poorly understood, including the reasons for spontaneous clearance, chronic infection, and the wide variation in disease severity. However, there is increasing evidence suggesting that virus-specific CD8⁺ T cell responses play a critical role in determining the outcome of infection [1].

CCR5 belongs to the family of CC chemokine receptors, which are importantly involved in the regulation of cell migration. Expression of CCR5 is acquired by activated CD8⁺ T cells, especially under conditions of type-1 cytokine polarization. The CCR5 gene is subject to several mutations, including a 32-bp deletion in the coding region (CCR5Δ32). This mutation leads to a frame shift, which results in a truncated protein that is not expressed on the cell surface. Heterozygosity for this deletion (CCR5 WT/Δ32) is found in about 10% of white individuals. Of note, individuals with a CCR5 WT/Δ32 genotype have significantly lower cell surface levels of CCR5 than do individuals who are homozygous for the wild type.

One striking feature of CCR5 deficiency in mice is a more robust T cell response toward various infectious agents, including Listeria species and lymphocytic choriomeningitis virus [2, 3]. With respect to hepatitis C, Goulding et al. demonstrated that the CCR5Δ32 mutation was associated with spontaneous viral clearance. In addition, they found a trend toward less-severe hepatic inflammatory scores in CCR5 WT/Δ32 subjects, compared with CCR5WT/WT subjects [4], and similar observations have been made for hepatitis B [5]. However, it remains unclear how CCR5 restrains T lymphocyte responses.

In general, the activation of T cells is induced by signals generated by direct interaction between the T cell receptor (TCR) and peptide antigens presented via major histocompatibility complex class I (MHC-I) molecules [6]. Importantly, subsets of CD8⁺ T cells also express NK cell receptors (NKRs), including inhibitory NKG2A receptors and activating NKG2C receptors [7]. NKG2A and NKG2C are members of the C-type lectin domain family and specifically interact with the nonclassical MHC-I molecule HLA-E. Increasing evidence suggests that inhibitory NKRs such as NKG2A are importantly involved in regulation of cytotoxic T lymphocyte (CTL) functionality [7–9]. In chronic hepatitis C, the expression of NKG2A on NK cells and CD8⁺ T lymphocytes is increased and NKG2A⁺ lymphocytes have been shown to accumulate in the liver in infected individuals. Of note, increased NKG2A expression resulted in impaired cytotoxic T cell activity in vitro and might, therefore, be involved in the impaired T cell function observed in patients with hepatitis C. This assumption is supported by our recent finding that HCV infection is associated with enhanced intrahepatic expression of HLA-E. In the present study, we describe the fact that NKG2A is preferentially expressed on CCR5⁺CD8⁺ T cells, which might explain some of the functional characteristics of CCR5⁺ T cells and their potential role in hepatitis C and other inflammatory conditions.

Subjects, materials and methods. A total of 56 white individuals were enrolled in this study, including 21 HCV RNA–positive individuals and 35 healthy HCV RNA–negative blood
donors. Venous blood was drawn from each subject for the isolation of peripheral blood mononuclear cells (PBMCs) by Ficoll-Paque density gradient centrifugation. The study conformed to the guidelines of the Declaration of Helsinki and was approved by our ethics committee. CD8+ T cell lymphocytes were separated from total PBMCs using MACS cell separation kits in accordance with the manufacturer’s recommendations (Miltenyi Biotec).

The following antibodies were used for fluorescence-activated cell sorter (FACS) analysis: anti-CD56, anti-CD8, anti-CD3, anti-NKG2A, anti-NKG2C, anti-CCR7, and anti-CCR5 (all obtained from BD Bioscience). After incubation of the cells with 10 μL of antibody for 20 min and washing with PBS, samples were analyzed on a FACScalibur flow cytometer with CellQuest software.

Immobilization of HCV E2 and HCV core proteins (gift of Dr. M. Houghton; Chiron) on 96-well plates (Greiner, Germany) was performed as described elsewhere [10]. In brief, monoclonal antibody (MAb) was diluted in carbonate buffer (15 mmol/L Na2CO3 and 35 mmol/L NaHCO3; pH 9.6) and incubated overnight at 4°C. Next, plates were washed and saturated for 30 min at 37°C with fetal calf serum prior to the addition of cells. After further washing, cells (PBMCs or purified subpopulations) were added in complete medium. The resulting supernatant was collected after overnight incubation.

To analyze cell migration, 6 × 10^5 PBMCs were seeded into the upper compartment of a 5-μm-pore nitrocellulose filter microchamber system (Neuroprobes). The lower compartment was filled with 300 μL of RPMI 1640 medium containing RANTES (25 ng) (PromoCell) or no chemotactic supplement. Where indicated, the supernatants obtained from the HCV E2 stimulation experiments were added to the lower compartment. After 5 h at 37°C, migrated cells were harvested from the lower compartment of the chemotaxis chamber and analyzed by flow cytometry. The migration index was defined as the number of cells migrating toward supernatants from stimulation experiments divided by the number of cells migrating toward medium alone.

Cytotoxic activity of CD8+ T lymphocytes was determined in accordance with the method described by Betts et al. In brief, 2 × 10^5 purified CD8+ T cells were cultured in RPMI medium that contained 1 μL of 2 mmol/L monensin (Sigma) and 1 μL of fluorescein isothiocyanate–conjugated anti-CD107a MAb. The cells were then stimulated with anti-CD3 (OKT3) or pooled 10-mer HCV core peptides (EMC). After 5 h of incubation, cells were washed, stained with anti-CCR1, anti-CCR5, and anti-CD8, and analyzed by FACS. To determine the functional role of NK cell receptor expression on T cells, the cells were costimulated using immobilized anti-NKG2A. Immobilization of anti-NKG2A on 96-well plates was performed as described above [8].

**Results.** First, we analyzed the expression of NK cell receptors NKG2A and NKG2C on CCR5+CD8+ T cells in patients with chronic hepatitis C. As a control, we studied T cells positive for CCR7, another member of the CC chemokine family. Flow cytometric analysis revealed that CCR5+ T cells encompassed a significantly higher percentage of NKG2A+ lymphocytes, compared with CCR5− T cells (figure 1A and 1B). In contrast, expression of the activating NKG2C receptor was significantly higher on CCR5−CD8+ T cells, with CCR5+ lymphocytes. A different pattern of NKG2 expression was observed with respect to CCR7, because we found that CCR7+CD8+ T cells expressed only low levels of both NKG2A and NKG2C (figure 1A). Accordingly, NKG2A+CD8+ T cells displayed high levels of CCR5, whereas levels of CCR7 expression were low (figure 1B). Importantly, differential expression of NKG2A and NKG2C on CCR5+CD8+ and CCR7+CD8+ T cells was also seen among healthy control subjects (data not shown).

Next, we studied the comparative functional relevance of NKG2A expression on CCR5+CD8+ and CCR7+CD8+ T cells using CD107a expression. Stimulation of the T cell receptor with anti-CD3 alone did not result in significantly different CD107a expression for CCR5+ CD8+ T lymphocytes, compared with CCR7+ T cells. More importantly, costimulation of the TCR together with NKG2A resulted in markedly reduced cytotoxic activity in CCR5+CD8+ T cells. In contrast, only negligible effects were observed when CCR7+CD8+ cells were analyzed in TCR-NKG2A costimulation experiments (figure 1C). Accordingly, we found a significant reduction of HCV core antigen–specific secretion of interferon (IFN)–γ and CD107a expression on CCR5+CD8+ T cells from HCV-infected patients, whereas only slight effects were observed for CCR7+ cells (figure 1D).

Chronic hepatitis C is associated with significantly enhanced intrahepatic expression of the inflammatory chemokine RANTES (CCL5). Recently, we showed that incubation of circulating and intrahepatic PBMCs with HCV E2, but not with HCV core antigen, resulted in enhanced secretion of RANTES [11]. Because RANTES represents a potent chemoattractant for CCR5+ lymphocytes, we speculated that the interactions of HCV E2 with CD8+ T lymphocytes should preferentially attract NKG2A+ T cells, due to their coexpression of CCR5. Figure 2A illustrates NKG2A expression on CD3+CD8+ lymphocytes following migration toward supernatant of CD8+ T cells incubated with or without immobilized recombinant HCV E2. We found that NKG2A+ T cells were preferentially attracted by supernatants obtained from HCV E2–stimulated cells, compared with the supernatants of cells cultured with HCV core antigen or in the absence of any HCV proteins (figure 2A and 2B). In contrast, migration toward CCL21 (Exodus) decreased the proportion of NKG2A+ T cells. This differential migratory behavior was specific for NKG2A+ T cells because NKG2C+ cells were mainly attracted by CCL21 but not by CCL5 (figure 2C and 2D). Double staining experiments confirmed coexpression of CCR5 and NKG2A on cells that were recruited by CCL5 (figure 2E).

The concept of differential migration in relationship to CCR5 expression was further confirmed by the supplemen-
tary observation that NKG2A+/CD8+ T cells displayed significantly stronger migration toward the inflammatory chemokine CCL5 (RANTES) than did NKG2A− lymphocytes (migration index, 3.9 ± 0.2 vs. 2.5 ± 0.2; P = .002). In contrast, when lymphocyte migration toward the lymphoid chemokine CCL21 was tested, the migratory capacity of NKG2A− T cells exceeded that of NKG2A+/CD8+ T lymphocytes (P = .046) (figure 2F).

Discussion. It has been suggested that the chemokine receptor CCR5 is involved in the regulation of immune responses. On the basis of data from CCR5−/− mouse models, in which CCR5-deficient mice were found to have increased CD8+ T cell responses [2, 3], CCR5 has been suggested as a negative regulator of T cells in an immune response. Alternatively, CCR5 expression might define a subset of functionally distinct CD8+ T cells, rather than directly affecting T cell function(s).

In the present study we demonstrated that CCR5+CD8+ T cells express a specific pattern of NK cell receptors, with high levels of inhibitory NKG2A receptors but low levels of activating NKG2C receptors. In contrast, CCR7+ T cells displayed low expression of both receptors, indicating that high NKG2A expression is a distinguishing feature of CCR5+ T cells. Of note, coexpression of NKG2A and CCR5 is not specific to patients with chronic hepatitis C because this relationship was also seen in healthy subjects and thus might also be relevant during acute HCV infection.

Expression of NKG2A on CD8+ T cells has been proposed as a mechanism for fine-tuning T cell responses by raising the threshold for TCR triggering [12], thereby serving as a mechanism for peripheral tolerance [7]. In line with this idea, NKG2A has been shown to inhibit cytokine production and cytolytic activity in antigen-specific T cells [9, 13], changes which are
Figure 2. Migratory capacity of NKG2A⁺CD8⁺ T cells. A, Purified CD8⁺ T lymphocytes were tested in a chemotaxis assay by using the supernatants from CD8⁺ T cells cultured with or without immobilized hepatitis C virus (HCV) E2 or HCV core antigen as stimuli. Migration toward supernatant of HCV E2–stimulated CD8⁺ lymphocytes resulted in an accumulation of NKG2A⁺ cells. B, Mean NKG2A expression on CD8⁺ T cells before and after migration, respectively. Results are given as mean migration indices from 3 independent experiments, calculated as described in Methods. Error bars, corresponding standard error of the mean (SEM). *P < .05. C, Representative flow cytometric analysis of NKG2A expression on CD8⁺ T cells that were attracted either by CCL5 or by CCL21, compared with lymphocytes prior to migration. D, Mean NKG2A expression on CD8⁺ T cells before and after migration, respectively. Error bars, corresponding SEM. *P < .05. E, Representative flow cytometric analysis of NKG2A and CCR5 coexpression on CD8⁺ T cells that were recruited by CCL5, clearly demonstrating that cells coexpressing these molecules migrated through the nitrocellulose filter. F, Isolated CD8⁺ T lymphocytes were tested in a chemotaxis assay toward either CCL5 or CCL21 and migratory capacity of NKG2A⁺ and NKG2A⁻ lymphocytes was analyzed separately. NKG2A⁺ T cells were efficiently recruited by CCL5 but not by CCL21, whereas the opposite was true for NKG2A⁻ T cells. Results are given as mean migration indices from 3 independent experiments, calculated as described in Methods. Error bars, corresponding SEM. *P < .05.
likely to contribute to viral persistence [8, 9]. Accordingly, we found that NKG2A-CCR5 coexpression rendered CCR5+CD8+ T cells more susceptible to NKG2A-mediated inhibition of cytolytic activity and IFN-γ secretion, compared with CCR7+ cells.

Usually, the inhibitory function of CD94-NKG2A is rather weak in T cells. However, chronic hepatitis C is associated with upregulated HLA-E expression on a great variety of different intrahepatic cells [14, 15]. As a potential underlying mechanism, we demonstrated the binding of HCV peptides to HLA-E and subsequent stabilization of HLA-E expression [14], which may allow more efficient interaction between HLA-E and CD94-NKG2A, leading to inhibition of CTL function. In support of this hypothesis, we recently demonstrated that enhanced NKG2A expression on CD8+ T cells in patients with chronic hepatitis C was associated with impairment of cytotoxic function in T cells when NKG2A was costimulated via the TCR. Moreover, the cytolytic activity of CD8+ T cells against HLA-E–expressing cells was reduced in patients with hepatitis C, compared to that observed in healthy patients [15].

Coexpression of NKG2A and CCR5 could provide a plausible mechanism to explain the observed accumulation of NKG2A-positive lymphocytes observed in the livers of HCV–infected individuals, particularly because chronic hepatitis C is associated with enhanced intrahepatic levels of CCR5 ligands such as RANTES. Indeed, we found that NKG2A+CD8+ T cells displayed preferential migration toward RANTES and supernatants from HCV E2–stimulated lymphocytes, resulting in an accumulation of NKG2A+ cells.

In conclusion, we found expression of NKG2A on CD8+ T cells to be associated with expression of CCR5, rendering CCR5+CD8+ T cells highly susceptible to NKG2A-mediated inhibition of cytolytic activity. These findings have important implications, as the failure to generate a sufficient cytolytic activity in the infected liver is quite likely to contribute to viral persistence in chronic hepatitis C.

References


