Differential Antigen Specificity of Hepatitis C Virus–Specific Interleukin 17– and Interferon γ–Producing CD8+ T Cells During Chronic Infection

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A subset of CD8+ T cells can secrete interleukin 17 (IL-17). However, very little information is currently available about their antigen specificity, tissue distribution, and biological relevance in chronic human viral infection. To address these issues, we comprehensively analyzed peripheral and intrahepatic CD8+ T-cell responses in a cohort of patients with chronic hepatitis C virus (HCV) infection for the antigenspecific production of IL-17 and interferon (IFN) γ. We found that HCV-specific IL-17–producing and retinoic acid receptor related orphan receptor γt-expressing CD8+ T cells are detectable in blood and liver and target different epitopes, compared with IFN-γ–producing CD8+ T cells. Their highest frequency was found in patients with low inflammatory activity, suggesting a protective role in chronic HCV infection.

CD8+ T cells play an important role in the outcome of human viral infections, including hepatitis C virus (HCV) infection [1]. Although most studies have focused on the role of interferon (IFN) γ–producing CD8+ T cells, it is increasingly becoming clear that a subset of CD8+ T cells can also secrete interleukin-17 (IL-17). For example, IL-17–producing CD8+ T cells have been observed in response to lymphocytic choriomeningitis virus infection in mice lacking both T-bet and eomesodermin [2]. In addition, IL-17–positive CD8+ T cells have been shown to have a protective role in mouse models of influenza virus and vaccinia virus infection [3, 4]. In rhesus macaques, Nigam et al reported the loss of IL-17–producing CD8+ T cells during the late chronic stage of pathogenic simian immunodeficiency virus infection [5]. Very little information is currently available about the role of IL-17–producing CD8+ T cells in humans. We have recently shown that a CD8+ T-cell lineage characterized by high CD161+ expression and coproduction of IL-17 and IFN-γ is enriched in the liver of chronically HCV-infected patients and linked to the control of HCV disease progression [6].

Taken together, there is growing evidence that IL-17–producing CD8+ T cells are involved in antiviral immunity and disease pathogenesis. However, several important questions have not been addressed to date. For example, very little information is currently available about the antigen specificity of human IL-17–positive CD8+ T cells, their relationship to IFN-γ–producing CD8+ T cells, their distribution in infected organs, and a possible association with clinical course. In this study, we addressed these important aspects in the context of chronic HCV infection.

MATERIALS AND METHODS

Participants and Study Samples
Twenty-two patients with chronic HCV genotype 1 infection (13 male; median age, 43.5 years; median alanine aminotransferase level, 66 U/l) were enrolled after informed consent and in agreement with the Declaration of Helsinki, federal guidelines, and the local ethics committee. Ethylenediaminetetraacetic acid anticoagulated blood was obtained, and peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Histopaque density gradient centrifugation (Pancoll; Pan Biotech). Fragments of diagnostic liver biopsy specimens were put into complete medium (Roswell Park Memorial Institute 1640 medium containing 10% fetal calf serum, 1% streptomycin-penicillin, and 1.5% HEPES buffer [1 mol/L]) and were homogenized using a 70-μm cell strainer (BD Biosciences).

Antibodies
Anti–CD8–peridinin-chlorophyll protein, anti–CD8–phycoerythrin (PE), and anti–IFN-γ–fluorescein isothiocyanate were obtained from BD Bioscience, and anti–IL-17–AlexaFluor647 and anti–IL-17–PE from eBioscience.

Peptides
Overlapping peptides derived from HCV strain H77 (genotype 1a) spanning the entire polyprotein (18 amino acids,
overlapping by 11) were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program.

Isolation and Polyclonal Expansion of Peripheral and Intrahepatic CD8+ T Cells

Procedures were performed as described elsewhere [7]. In brief, CD8+ T cells were isolated from homogenized liver tissue or PBMCs with use of anti-CD8 Dynabeads (Dynal). The isolated CD8+ T cells were then cultured antigen independently in the presence of 100 U/mL IL-2 (Hoffmann-La Roche), 0.04 μg/mL anti-human CD3 monoclonal antibody (ImmunoTech), and 2 × 10^6 irradiated autologous PBMCs as feeder cells for 2 weeks.

Enzyme-Linked Immunospot Assay

IL-17 (R&D Systems) and IFN-γ enzyme-linked immunospot (ELISPOT) kits (Diaclone Tepnel Research Products & Services) were used according to manufacturer’s instructions; 1 × 10^6 expanded intrahepatic and peripheral CD8+ T cells were plated per well. Peptides (final concentration, 10 μg/mL) were added in pools of 10 peptides, with each peptide contained in 2 different pools in a matrix setup, allowing identification of single positive peptides. Responses were considered positive if the number of spots per well was ≥3 times as high as the mean of negative controls (2 wells without peptides). The background level in the negative control of the IL-17 and IFN-γ ELISPOT assay was 0–3 spots/1 × 10^5 cells.

Intracellular Cytokine Staining

Peptides that tested positive in the matrix set-up ELISPOT assay were further tested in an intracellular cytokine staining using individual peptides, as described elsewhere [7].

RNA Isolation, Complementary DNA Synthesis, and Quantitative Polymerase Chain Reaction

RNA was isolated from nonspecifically expanded intrahepatic and peripheral CD8+ T cells after 5 hours restimulation with peptide using the RNeasy Micro Kit (Qiagen). Complementary DNA synthesis was performed using a mixture of random primers and SuperScript III reverse-transcriptase (Invitrogen). Quantitative real-time polymerase chain reaction was performed using a LightCycler system (version 1.5; Roche Diagnostics) and a combination of preoptimized Qiagen QuantiTect Primer Assays (RORC [Hs_RORC_1_SG] and HPRT1 as internal control [Hs_HPRT1_1_SG]). LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) was used according to the manufacturer’s instructions. Fold expression changes were determined using the 2-DDCT method [8].

Statistical Analysis

Wilcoxon signed rank test and unpaired Student’s t test were performed using GraphPad Prism software, version 5 (GraphPad Software). Differences were considered statistically significant at P < .05.

RESULTS

Presence of Virus-Specific IL-17–Producing CD8+ T Cells in the Blood and Liver of Chronically HCV-Infected Patients

In a comprehensive analysis of peripheral and intrahepatic CD8+ T-cell responses with use of overlapping peptides spanning the complete HCV polyprotein in an IL-17 and IFN-γ ELISPOT assay, we were able to detect IL-17– and IFN-γ–producing HCV-specific CD8+ T cells in the majority of chronically HCV-infected patients. Indeed, as shown in Figure 1A, HCV-specific IL-17–producing CD8+ T cells were detectable in the blood of 6 and the liver of 9 of 22 patients. In most patients, a single epitope was targeted; however, 1 patient had an IL-17–producing intrahepatic HCV-specific CD8+ T cell response that targeted 5 different epitopes. IFN-γ–producing HCV-specific CD8+ T-cell responses were present in the blood of 6 and in the liver of 11 of 22 patients (Figure 1A). Of note, HCV-specific IFN-γ–producing CD8+ T cells derived from the intrahepatic compartment targeted significantly more epitopes, compared with the peripheral blood (mean, 1.3 vs. 0.4; P = .039) (Figure 1A). These results are in line with a recent comprehensive study from our group that showed that most HCV-specific IFN-γ–positive CD8+ T cell responses are indeed detectable in the liver only [9]. As shown in Figure 1B, of importance, in agreement with our previous study, IFN-γ–producing HCV-specific CD8+ T-cell responses were significantly stronger in the intrahepatic compartment (mean, 20.9 vs 2.0 spot-forming cells/10^5 CD8+ T cells; P = .003), thus suggesting enrichment in the liver. In contrast, IL-17–producing HCV-specific CD8+ T cells were equally distributed between the 2 compartments.

Targeting of HCV-Specific Epitopes by IL-17– and IFN-γ–Producing CD8+ T Cells

Next, we determined whether HCV-specific IL-17– and IFN-γ–producing CD8+ T cells target the same or different epitopes in our cohort of chronically HCV-infected patients. As shown in Figure 1C, IFN-γ–positive and IL-17–positive HCV-specific CD8+ T cell responses were distributed across the entire HCV polyprotein without a clear dominance of certain proteins. These results support the absence of a clear immunodominance of HCV-specific CD8+ T cell responses during chronic infection, as suggested elsewhere. Of interest, as also shown in Figure 1D for 2 representative patients, we found that specific epitopes were targeted by either IFN-γ– or IL-17–producing HCV-specific CD8+ T cells. Of note, 6 of the 33 epitopes with IFN-γ production, but none of those with IL-17 production, have been described elsewhere. This may reflect the fact that most HCV-specific epitopes described to date have been identified in assays using IFN-γ production as a readout. IL-17 and IFN-γ double-producing HCV-specific CD8+ T cell responses were not detectable, suggesting that
HCV-specific CD8\(^{+}\) T cells have either a cytotoxic T cell (TC)1-or a TC17-like cytokine profile. This is further confirmed by the detection of increased messenger RNA levels of retinoic acid receptor related orphan receptor (ROR)\(c\), a TC17-associated transcription factor, after peptide-specific stimulation (eg, shown for a representative patient in Figure 1E). Indeed, the antigen-specific IL-17 production correlated with the expression of ROR\(c\) after stimulation with peptide NS5A\(_{2043–2060}\).

**Association Between HCV-Specific IL-17–Producing CD8\(^{+}\) T Cells in the Liver and Milder Inflammatory Activity**

Next, we analyzed whether the number of epitopes targeted or the strength of intrahepatic HCV-specific CD8\(^{+}\) T cell responses correlated with the inflammatory activity or fibrosis level. Of importance, as shown in Figure 2, we found significantly more (1.3 vs. 0.08; \(P = .009\)) and higher (mean 13.4 vs. 0.67 spot-forming cells/\(10^{5}\) CD8\(^{+}\) T cells; \(P = .008\))
IL-17–producing HCV-specific CD8⁺ T cell responses in patients with a lower level of inflammatory activity (Metavir score, 0–1) than in those with a higher level (Metavir score, 2–3), suggesting that these cells may have a protective role in chronic HCV infection. Of note, for IFN-γ–producing HCV-specific CD8⁺ T cells, such a difference was not detectable. We did not find a differential IL-17 or IFN-γ HCV-specific CD8⁺ T cell response with respect to the fibrosis level (Figure 2C and 2D).

DISCUSSION

Here, we report the presence of HCV-specific IL-17–producing CD8⁺ T cells in the blood and liver of chronically HCV-infected patients. Indeed, HCV-specific IL-17–producing CD8⁺ T cells are present in almost half of chronically HCV-infected patients. Of interest, HCV NS4-specific IL-17–producing CD4⁺ T cells have also been shown to be present in chronically HCV-infected patients [10], suggesting that HCV-specific IL-17–producing T cells are not restricted to the CD8⁺ T cell subset. In contrast to IFN-γ–producing HCV-specific CD8⁺ T cells, however, IL-17–producing CD8⁺ T cells are not enriched in the liver, suggesting that they are either not specifically recruited to the site of inflammation, become exhausted, or are rapidly deleted after they arrive. Of interest, HCV-specific IL-17– and IFN-γ–producing CD8⁺ T cells target different epitopes and do not overlap in their epitope repertoire. Most likely, this can be explained by differential T cell lineage commitment by which antigen-specific T cells may differentiate into either IFN-γ– or IL-17–producing effector cells. This is supported by our finding that IL-17–producing CD8⁺ T cells showed a high expression of RORγt, the master transcription factor of Tc17-differentiated cells. Mechanisms contributing to the differential commitment of antigen-specific T cells might include the cytokine milieu present during priming [11], the type of antigen-presenting cell, the place of priming, or specific antigenic properties.

However, after antigen-nonspecific stimulation with PMA-ionomycin, IL-17–secreting human T lymphocytes may also cosecrete IFN-γ. For example, we reported elsewhere the intrahepatic accumulation of IL-17– and IFN-γ–coproducing CD8⁺ T cells in the livers of chronically HCV-infected patients that was linked to the control of disease progression [6]. Thus, the clear distinction between IL-17– or IFN-γ–producing cells are not enriched in the liver, suggesting that they are either not specifically recruited to the site of inflammation, become exhausted, or are rapidly deleted after they arrive. Of interest, HCV-specific IL-17– and IFN-γ–producing CD8⁺ T cells target different epitopes and do not overlap in their epitope repertoire. Most likely, this can be explained by differential T cell lineage commitment by which antigen-specific T cells may differentiate into either IFN-γ– or IL-17–producing effector cells. This is supported by our finding that IL-17–producing CD8⁺ T cells showed a high expression of RORγt, the master transcription factor of Tc17-differentiated cells. Mechanisms contributing to the differential commitment of antigen-specific T cells might include the cytokine milieu present during priming [11], the type of antigen-presenting cell, the place of priming, or specific antigenic properties.

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Figure 2. Correlations with clinical parameters, including correlations between the number and strength (mean spot-forming cells [SFCs]) of intrahepatic interleukin 17 (IL-17) and interferon (IFN) γ responses and inflammatory activity (A, B) or fibrosis level (C, D) in the liver of 22 patients with chronic hepatitis C virus (HCV) infection. Higher inflammatory activity relates to a low number of IL-17 responses. Statistical comparisons were made with unpaired Student’s t test.
CD8+ T cells may be present only on a strict antigen-specific level, as also shown in Figure 1D. These results suggest that T-cell receptor-mediated antigen-specific stimulation induces different signaling pathways, compared with strong antigen-nonspecific stimulation.

Of note, HCV-specific IL-17–producing CD8+ T cells are primarily detectable in the liver of patients with mild inflammation, pointing to a protective role of IL-17–producing CD8+ T cells in chronic infection, as has been suggested in mouse models [3, 4]. The responsible mechanisms are unclear; however, of note, a previous study in a mouse model of liver injury has suggested that IL-22 (which is coexpressed with IL-17) has a protective role in virus-induced inflammation [13]. From our human study, however, it is not possible to formally prove a causal link between the presence of higher IL-17–producing CD8+ T cell responses and low inflammation. Of interest, CD8+ T cells producing both IL-17 and IFN-γ after stimulation with PMA-ionomycin were associated with lower fibrosis stage in our previous study [6], whereas in our current study, antigen-specific IL-17–producing CD8+ T cells were associated with lower inflammation activity. This suggests a differential impact of these IL-17–producing cell subsets on disease progression (inflammation vs fibrosis). Of note, in the case of chronic hepatitis B virus infection, IL-17–producing CD4+ T cells have been suggested to contribute to disease pathogenesis and inflammation [13].

In summary, our results show the presence of virus-specific IL-17–producing CD8+ T cells in the blood and liver of chronically HCV-infected patients that show a different antigen specificity, compared with IFN-γ–producing CD8+ T cells, and may protect from hepatic inflammation in chronically HCV-infected patients. Thus, these results add another HCV-specific cytokine-producing T cell population to the field of HCV immunobiology that needs to be considered in future studies analyzing HCV-specific immunity.

**Notes**

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