Hepatitis C Virus-Specific T-Cell Gamma Interferon and Proliferative Responses Are More Common in Perihepatic Lymph Nodes than in Peripheral Blood or Liver

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Received 29 May 2008/Accepted 8 August 2008

The activation state, differentiation state, and functions of liver lymphocytes and perihepatic lymph nodes during chronic hepatitis C virus (HCV) infection are not well understood. Here, we performed phenotypic and functional analyses of freshly prepared lymphocytes isolated from the livers, perihepatic lymph nodes, and peripheral blood compartments of chronic HCV-infected and disease control subjects with end-stage liver disease undergoing liver transplantation. We measured lymphocyte subset frequency and memory T-cell gamma interferon (IFN-γ) and proliferative responses to HCV peptide and control viral antigens in direct ex vivo assays. We found higher frequencies of CD4 cells in the lymph node compartment than in the other compartments for both HCV-infected and disease control subjects. Lymph node CD4 and CD8 cells less commonly expressed the terminal differentiation marker CD57, a finding consistent with an earlier differentiation state. In HCV-infected subjects, HCV-specific IFN-γ-producing and proliferative responses were commonly observed in the lymph node fraction, while they were uncommonly observed in the peripheral blood or liver fractions. In contrast, control viral CD4 protein antigen and CD8 peptide antigen-specific IFN-γ responses were commonly observed in the periphery and uncommonly observed in the lymph nodes of these same subjects. These findings are consistent with a selective defect in HCV-specific T-cell effector function or distribution in patients with advanced chronic HCV infection. The high frequency of HCV-reactive T cells in perihepatic lymph nodes indicates that a failure to generate or sustain T-lymphocyte HCV reactivity is not responsible for the paucity of functional cells even in end-stage liver disease.

During hepatitis C virus (HCV) infection, both CD4 and CD8 virus-specific lymphocytes are thought to play important roles both in control/clearance of viral infection and in hepatocellular damage (7, 9, 13, 23, 26, 32, 33, 37, 38, 40). Priming of these cells during acute infection and sustained activation of memory cells during chronic infection are thought to take place in regional lymph nodes that drain the tissue sites of infection. Chronic HCV infection is characterized by low frequencies of HCV-reactive T cells in circulation (16, 34, 42–44). While there are a number of potential explanations for these meager responses, the relative distribution levels of HCV-reactive T cells in lymph nodes, liver, and peripheral blood are not well recognized. We therefore examined the phenotype and function of HCV-specific T cells within the regional lymph nodes, livers, and peripheral blood of HCV-infected individuals, using freshly isolated tissue samples in order to obtain an improved understanding of the dominant T-cell dynamics during HCV infection and to establish a foundation for improved models of compartmentalized T-cell activation during chronic viral hepatitis. Results indicate similar paucities of functional HCV-specific T cells in the liver and periphery, while HCV-reactive T cells are commonly found in the perihepatic lymph node tissue, consistent with a model of ongoing T-cell accumulation or sequestration at this site.

MATERIALS AND METHODS

Study subjects and institutional review board approval. Subjects undergoing liver transplantation for end-stage disease included those with chronic HCV infection (HCV antibody and serum were RNA positive; n = 22), autoimmune hepatitis (n = 1), cryptogenic cirrhosis (n = 2), alpha-1-antitrypsin deficiency (n = 1), alcohol-related liver disease (n = 3), primary sclerosing cholangitis (n = 2), and nonalcoholic steatohepatitis (n = 4).

Subjects provided written informed consent for use of native liver explant and perihepatic lymph node tissues and peripheral blood sampling, which conformed to the ethical guidelines of the 1975 Declaration of Helsinki, with prior approval of the institutional review board for human studies at Henry Ford Health System and University Hospitals of Cleveland.

Lymphocyte preparation. Liver specimens (2 to 15 g) and perihepatic lymph node specimens (0.4 to 4 g) were washed with RPMI 1640 medium (GIBCO BRL, Grand Island, NY) with the addition of penicillin, streptomycin, L-glutamine, and 5% human AB serum (Gemini Bio-Products, Woodland, CA) within 2 h of harvest. Samples were then minced and homogenized in the absence of proteolytic enzymes within 16 h of harvest. Extracted intrahepatic lymphocyte (IHL) or lymph node lymphocyte (LNL) populations were centrifuged through an Isoprep cushion (Robbins Scientific Corp., Sunnyvale, CA) for isolation of lymphocytes. Peripheral blood lymphocyte (PBL) populations were prepared simultaneously by centrifugation through an Isoprep cushion.

Flow cytometric analysis. Phenotypes of freshly isolated PBLs, LNLs, and IHLs were determined by flow cytometry to identify frequencies of the CD4 and CD8 T cells and the proportions of T cells that express the terminal differentiation marker CD57 after the lymphocytes were stained with conjugated monoclonal antibodies. Antibodies were obtained from Becton Dickinson (Mountain View, CA) and included CD3 peridinin chlorophyll protein (clone SK7), CD4

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17 Published ahead of print on 20 August 2008.
RESULTS

Clinical characteristics of subjects. The clinical characteristics of subjects undergoing liver transplantation are shown in Table 1. The proportion of women tended to be lower in the HCV-infected group than in the disease control group (P = 0.06), while the proportion of subjects with hepatocellular carcinoma was greater in the HCV-infected group than in the disease control group (P = 0.01). Bilirubin levels were higher in the disease control group (P = 0.03), while the calculated model for end-stage liver disease scores, ages, creatinine levels, and international normalized ratio did not differ significantly between groups. Additional characteristics include 23% of HCV-infected subjects having genotype 2 infections and 77% having genotype 1 infections. HCV plasma levels ranged from 82,290 to 1,440,000 IU/ml. No subject was on immunosuppressive therapy at the time of research sampling. Eight HCV-infected subjects had received prior HCV therapy (six with no response to HCV therapy and two with response and then relapse), though no subject was on HCV therapy at the time of transplant.

CD4 cell frequency is greater in the lymph node than in the peripheral blood mononuclear cell or liver compartments of HCV-infected and disease control subjects, and these cells are in an earlier differentiation state. To understand the frequency and differentiation state of T cells within the PBL, LNL, and IHL compartments, we performed flow cytometric analysis of freshly prepared cells. CD56 was used as a marker to exclude NK cells in our analysis. The proportion of lymphocyte preparations that were T cells (CD3+ CD56−) was greater in the LNL compartment than in the PBL compartment (mean of 31.1% versus 15.6%; P value of 0.002 for all subjects) (Fig. 1A). This difference was present for both HCV-infected and disease control groups (P values of 0.01 and 0.05, respectively). T-cell subset analysis revealed that this increased T-cell frequency was within the CD4 cell subset (P values of 0.001 for all subjects, 0.002 for HCV-infected subjects, and 0.09 for disease control subjects) (Fig. 1B) and not the CD8 subset (Fig. 1D).

CD57 is a marker expressed by terminally differentiated T cells incapable of additional proliferation (8, 17, 22). We measured CD57 expression to determine whether LNLs differ from PBLs and IHLs in terms of differentiation state and specifically whether IHLs might comprise a selective accumulation of terminally differentiated T cells that had trafficked to the liver, as...
has been previously proposed (5, 18, 31). CD4 cells from the LNL compartment expressed CD57 less commonly than did those found in the PBL compartment (mean of 3.9% versus 7.8%; P value of 0.01 for all subjects) (Fig. 1C). This difference was significant in the HCV-infected group (P = 0.02) and not in the disease control group (P = 0.4). Similarly, CD8 cells from the LNL compartment less commonly expressed CD57 than CD8 cells from the PBL compartment (mean of 7.5% versus 39.8%; P value of <0.001 for all subjects) (Fig. 1E), and this difference was significant in both HCV-infected (P = 0.001) and disease control (P = 0.02) groups. Contrary to expectation, CD8 cells from the IHL compartment less commonly expressed CD57 than did CD8 cells from the PBL compartment (mean of 17.6% versus 39.8%; P value of 0.03 for all subjects), and this difference was significant in the HCV-infected group (P = 0.05) but not in the disease control group (P = 0.3).

HCV-reactive T cells capable of proliferation and IFN-γ production are most commonly observed in the lymph nodes, while CMV- and CEF-specific IFN-γ-producing T cells are most commonly identified in the periphery. To examine the compartmentalization of the virus-specific immune response, we measured the antigen-specific functions of PBL, LNL, and IHL T cells. HCV peptide antigen (10 separate pools of HCV overlapping peptides spanning the entire HCV genotype 1a proteome), CMV protein antigen known to elicit CD4 T-cell responses, and control CD8 cell viral peptide antigens (CEF CMV, Epstein-Barr virus, and influenza virus peptide pool) specific to IFN-γ-producing cell frequency were measured by ELISPOT assay. Proliferative function was measured by thymidine incorporation. The use of 10 pools of HCV peptides allows for analysis of the HCV-specific immune response to 441 possible 18-mer peptides with a limited number of cells (47). Mitogen-induced IFN-γ and proliferation responses were measured to ensure cell viability. HCV-specific IFN-γ responses were not commonly found in the PBL or IHL compartments (responses were found in 17% and 25% of HCV-infected subjects, respectively), while they tended to be more commonly found in the LNL compartment (43% of HCV-infected subjects) (PBL versus LNL, P = 0.18; PBL versus IHL, P = 0.125) (Fig. 2A). In contrast, when CMV protein antigen-specific responses were analyzed in CMV-seropositive HCV-infected subjects, IFN-γ responses were quite commonly found in the periphery (58% of CMV-seropositive HCV-infected subjects) and less commonly found in the LNL and IHL compartments (30% and 40%, respectively) (PBL versus LNL, P = 0.25). CMV-specific proliferative responses were not observed in CMV-seronegative subjects, while a CMV-specific IFN-γ response was observed in the PBL compartment of one of eight CMV-seronegative subjects. CEF-specific CD8 IFN-γ responses were also quite commonly observed in the PBL (60% of HCV-infected subjects) and LNL (43% of HCV-infected subjects) compartments but not in the IHL compartment (0% of HCV-infected subjects). Data from a smaller number of disease control subjects indicate similar patterns of compartmentalized responses directed at CMV and CEF viral antigens, except for a CEF-specific response that was identified in the IHL compartment of one disease control subject (not

![FIG. 1. T-cell subset frequencies and differentiation states in peripheral blood, lymph nodes, and livers of HCV-infected and disease control subjects. PBL, LNL, and IHL populations were analyzed by flow cytometry for the frequencies of T (CD3+CD56-), CD4 (CD3+CD4+), and CD8 (CD3+CD8+) cells and the proportions of CD4 and CD8 cells that express the terminal differentiation marker CD57. Bars represent median values.](http://jvi.asm.org/Downloaded from http://jvi.asm.org)
terized by the number of HCV peptide pools targeted. Zero to
breadth of the cellular immune response to HCV was charac-
tersized, while the compartmentalization of responses to CMV
antigen-specific responses were also measured (Fig. 2B). The case of proliferative function, all three viral
antigen-specific proliferative responses were also measured
to compare, though the response magnitudes do appear to be
comparable in the LNL and PBL compartments. Finally, the magnitude of the PBL compart-
ment response to HCV was nearly 10-fold less than that di-
rected at CMV or CEF. Therefore, due to the paucity of HCV-specific IHL responses, the magnitudes of the responses
in the IHL compartment and other compartments are difficult
to compare, though the response magnitudes do appear to be
comparable in the LNL and PBL compartments and appear to
be reduced in comparison to those directed at other viral
antigens in the same hosts.

Relationships among IHL phenotype, IFN-γ secretion, HCV, and alanine aminotransferase levels. Neither tissue
compartment T-cell frequency nor HCV-specific IFN-γ-produc-
ting cell frequency in any of the compartments analyzed was
associated with any of the clinical variables prior to transplant,
including bilirubin level, prior HCV therapy response, HCV
asymptomatic genotype, HCV plasma level, and the presence of hepatocel-
lar carcinoma. An attempt was made to correlate the HCV-
specific IHL responses, the magnitudes of the responses
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DISCUSSION

While the phenotypic distribution of human IHLs (11, 30,
35) and LNLs (27) is known to differ from that in the periph-
ery, little is known about the compartmentalization of func-
tional HCV-specific T cells in the setting of chronic HCV
10 peptide pools were targeted in this assay system. As shown
in Fig. 3A, the IFN-γ responses were similar in breadth in the
PBL, LNL, and IHL compartments (when a response was present, a median of 3, 3.5, and 2 pools were targeted in the
PBL, LNL and IHL compartments, respectively). Similarly, the proliferative responses were comparable in breadth among
compartments when a response was present (Fig. 3B). Because
each pool of peptides represented a discrete antigenic region
of the HCV protein sequence, the specificity of the response
was characterized as the number of times a particular pool of
HCV peptides was targeted. As shown in Fig. 3C and D, there
was no particular viral region targeted by the IFN-γ or prolifer-
ative cellular immune response. Therefore, the breadth and
specificity of the HCV-specific responses did not differ remark-
ably among PBL, LNL, and IHL compartments.

To characterize the magnitude of the cellular immune re-
sponse, we measured the cumulative frequency of IFN-γ-produc-
ting cells (the sum of responses to all peptide pools tar-
ged; for CMV and CEF, only one protein antigen or peptide
pool was tested). Because T-cell frequency varied among com-
partments and T cells are primarily responsible for IFN-γ in
these assays, we normalized the response frequency to the
number of T cells plated in each assay (as measured by flow
cytometric analysis). As shown in Fig. 3E to G, the magnitude
of the IFN-γ response to HCV tended to be greater in the IHL
than LNL compartment (P = 0.08), though there are notably
only three responses identified in the IHL compartment of this
group, making it difficult to interpret this finding. Similarly,
due to the paucity of CMV responses in the LNL compart-
ment, it is difficult to compare the magnitudes of the IFN-γ
responses to CMV among compartments. The magnitudes of
the responses to CEF were comparable in the PBL and LNL
compartments. Finally, the magnitude of the PBL compart-
ment response to HCV was nearly 10-fold less than that di-
rected at CMV or CEF. Therefore, due to the paucity of
HCV-specific IHL responses, the magnitudes of the responses
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FIG. 2. HCV-, CMV-, and CEF-specific viral cellular immune re-
sponses in PBL, LNL, and IHL compartments of HCV-infected sub-
jects. HCV peptide pool-, CMV protein antigen-, and CEF peptide
pool-specific IFN-γ-producing responses were identified by ELISPOT
assay, and proliferation responses were identified by a tritiated-thym-
dine incorporation assay. Proportions of HCV-infected subjects with a
response in each compartment (PBL, LNL, and IHL) to each of the
viral antigens (HCV, CMV, and CEF) are shown for IFN-γ (A) and
proliferation (B) activities. For response to HCV, a response to any of
the HCV peptide pools was interpreted as a response.
infection based on direct ex vivo assays. In this study, we used tissues made available as a result of organ transplantation for end-stage liver disease to begin to address this question. Cells were freshly prepared and analyzed without in vitro expansion. Our data indicate that HCV-specific IFN-γ-producing T cells are common in the LNL compartment and uncommon in the PBL and IHL compartments. This may be related to ongoing T-cell activation in the lymph node, even in the setting of end-stage liver disease. Notably, we did not distinguish between CD4 and CD8 responses in these assays, so we cannot comment on whether this compartmentalization encompasses CD4, CD8, or both subsets of T cells. Our demonstration of HCV-specific T-cell responses within the IHL and PBL compartments in under half of the subjects is similar to responses previously found in the PBL compartment of chronic HCV-infected patients with compensated liver function, using the same assay method (47). This underscores the paucity of IFN-γ-producing T cells previously described for the PBL compartment (16, 24, 25, 42–44) and extends the observation to the IHL compartment in the setting of direct ex vivo assays. Importantly, this lack of IFN-γ-secreting effector function in the IHL and PBL compartments appears to be specific to HCV in that T cells with effector function are readily demonstrable at these sites in response to CMV protein antigen and CD8 CEF peptide antigens.

One possible explanation for a paucity of functional T cells in the IHL compartment is a suboptimal environment for T-cell activation. This may be the result of soluble inhibitory factors or inadequate antigen-presenting cell (APC) function. Here, we assayed IHLs in the presence and absence of CD3-depleted PBLs to ascertain if exogenous APCs could correct any identified defect. We found no difference in IFN-γ-producing frequency, proliferation index, or specificity by comparing assays performed in the presence or absence of additional APCs (not shown). This suggests that peripheral APCs are not sufficient to restore any possible IHL compartment APC defect. Notably, we did not perform assays in the presence of LNLs depleted of CD3 cells, which may be more functional than the peripherally derived APCs that have been described as defective in a number of studies (4, 19, 20, 39, 41, 45, 46).

FIG. 3. Breadth, specificity, and cumulative frequency of HCV-reactive IFN-γ and proliferative responses. The breadth of IFN-γ (A) and proliferation (B) responses directed at HCV are represented as the numbers of HCV peptide pools targeted by each HCV-infected subject. The specificities of the HCV-specific IFN-γ (C) and proliferation (D) responses are represented as the number of times each HCV antigenic region (represented by each peptide pool) was targeted in the HCV-infected group. The cumulative frequency or magnitude of the virus-specific response is shown for the HCV (E)-, CMV (F)-, and CEF (G)-specific IFN-γ responses within each compartment of HCV-infected subjects. The cumulative frequency represents the sum of frequencies for each IFN-γ-producing response. sfu, spot-forming cells.
Another possible explanation for the paucity of functional HCV-specific T cells within the liver is that liver tissue from subjects with end-stage liver disease may not be reflective of the immune response during earlier stage liver disease. While the pathogenesis of HCV during end-stage liver disease may differ from that during earlier stage disease, assays performed here indicate that functional HCV-specific T cells can be found in perihepatic lymph nodes even in advanced disease. Additionally, the phenotype of IHLs with regard to CD57 expression in earlier stage liver disease (less than grade 4 fibrosis) is similar to that in end-stage disease when liver biopsy specimen lymphocytes are analyzed in comparison to PBLs (data not shown).

Another possible explanation for the paucity of functional HCV-specific T cells in PBL and IHL compartments is terminal differentiation due to chronic antigen exposure (perhaps HCV-specific T cells are present but not detectable in direct ex vivo assays). Notably, based upon in vitro T-cell expansion data, HCV-specific T cells are thought to compartmentalize to the liver, existing there in greater frequency than in the circulation (1, 15). Perhaps these cells have a reversible state of dysfunction. Our data evaluating the magnitude of the HCV-specific response in the IHL compartment are inadequate for us to comment on the clonal frequencies of HCV-specific T cells in this compartment compared to those in the LNL or PBL compartment, though of the responses identified in this compartment, the magnitude may be greater than that of the responses identified in other compartments. We additionally attempted liver lymphocyte expansions (data not shown) using the method described by Erickson et al. (12) with samples from five HCV-infected and one disease control subject. While background IFN-γ spot-forming units prohibited interpretation for a number of these samples, we were able to expand cells that were reactive to regions of HCV not identified in the direct ex vivo assays in two instances, consistent with the concept of compartmentalization of dysfunctional cells discussed above.

To investigate the origins of PBL, IHL, and LNL compartmentalization of the viral antigen-specific immune response, we evaluated the lymphocyte phenotypes in parallel. The liver is thought to participate in T-cell trafficking by trapping activated CD8 cells (5, 6, 18, 31). Therefore, some of the IHL CD8 population present in chronic HCV-infected patients may reflect the non-antigen-specific trapping of activated and differentiated circulating CD8 cells. Alternatively, CD8 T-cell activation and terminal differentiation of HCV-specific T cells may occur within the hepatic parenchyma. To examine the differentiation states of IHLs versus PBLs and LNLs, we measured surface CD57 expression as a marker of terminally differentiated CD8 cells incapable of additional proliferation (8, 17, 22). We observed reduced expression of CD57 on CD4 and CD8 cells in the LNL compartment compared to that in the PBL compartment. This finding, in combination with our finding of enhanced CMV-, CEF-, and HCV-specific proliferative activities within the LNL compartment, suggests an earlier differentiation state (CD57-negative) central memory cell population existing in the LNL compartment, consistent with normal lymph node biology.

We further found reduced expression of CD57 on cells in the IHL compartment compared to that of CD8 cells in the PBL compartment. This latter finding suggests that either CD57 expression is diminished due to the liver environment itself, a possibility shown in other systems where memory CD8 phenotype has been shown to change in a manner related to tissue localization (28), or that a portion of intrahepatic CD8 T cells are not terminally differentiated effector CD8 cells, as has been previously proposed (14). In mouse model systems of prototypical antigen-specific immunity, intrahepatic (as opposed to extrahepatic) antigen presentation results in increased accumulation of memory T cells in the liver, providing direct evidence of intrahepatic T-cell activation (29). It has therefore been proposed that a suboptimal intrahepatic costimulatory environment for T-cell activation may result in the presence of intrahepatic T cells with differing functions and phenotypes (30). Our phenotypic data are consistent with such a model.

HCV-specific T-cell immunity has been implicated both in viral replication control/clearance and in hepatocellular damage during HCV infection (9, 23, 26, 33, 40). Conceivably, the compartmentalization of these lymphocyte subsets within the liver impacts the host response to HCV infection. Our data here indicate that HCV-specific cells with direct ex vivo effector function activity are likely activated or sequestered in the perihepatic lymph node yet may not localize to the IHL compartment or within the context of the liver may be rendered dysfunctional. Future investigation is warranted to confirm that the HCV-specific effector function of intrahepatic T lymphocytes is poor and to identify potential mechanisms.

ACKNOWLEDGMENTS

This work was supported by VA Advanced Research Career Development, VA Merit, NIH R01 DK068361, and the CWRU Center for AIDS Research Core facilities (AI 36219).

We thank the study subjects for their participation. We also thank Chris Walker and members of his laboratory for assistance with in vitro expansion assay methodology.

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