Upregulation of PD-1 Expression on Circulating and Intrahepatic Hepatitis C Virus-Specific CD8⁺ T Cells Associated with Reversible Immune Dysfunction†‡

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Infection with hepatitis C virus (HCV) is associated with persistence in the majority of individuals. We demonstrate here that the inhibitory molecule programmed death-1 (PD-1) is significantly upregulated on total and HCV-specific CD8⁺ cytotoxic T lymphocytes (CTLs) in the peripheral blood and livers of patients with chronic infection compared to subjects with spontaneous HCV resolution, patients with nonviral liver disease, and normal controls. PD-1 expression on cytomegalovirus-specific CTLs also varies according to HCV status and is highest in patients with chronic infection. HCV-specific CTLs that are PD-1high express higher levels of the senescence marker CD57 than PD-1low CTLs, and CD57 expression is greater in chronic than in resolved infection. In vitro blockade of PD-1 by monoclonal antibodies specific to its ligands (PD-L1 and PD-L2) results in restoration of functional competence (proliferation and gamma interferon and interleukin-2 secretion) of HCV-specific CTLs, including those residing in the liver. This reversal of CTL exhaustion is evident even in individuals who lack HCV-specific CD4⁺ T-cell help. Our data indicate that the PD-1/PD-L pathway is critical in persistent HCV infection in humans and represents a potential novel target for restoring function of exhausted HCV-specific CTLs.

Chronic viral infections are frequently characterized by functional impairment of T cells. The activation of T cells via major histocompatibility complex-bound peptide on antigen-presenting cells (APCs) involves two signals. Signal 1 represents the T-cell receptor, and signal 2 represents signaling through a costimulatory molecule, the most predominant of which is the interaction of CD28 with its ligands, B7-1 (CD80) and B7-2 (CD86) on APCs (14). The ultimate fate of cellular immune responses is determined by the balance between positive and negative signals delivered by costimulatory molecules to T cells (20). The recently identified CD28 homolog and costimulatory molecule programmed death-1 (PD-1) is inducibly expressed on T cells, B cells, natural killer T cells, and monocytes upon activation (14, 21). The intracellular domain of PD-1 contains two tyrosine signaling motifs (ITIM and ITSM), and data indicate that PD-1 inhibits T-cell functions by recruiting intracellular phosphatase SHP2 (SRC homology 2 [SH2]-domain-containing protein tyrosine phosphatase 2) that dephosphorylates and deactivates downstream signal transducers (7).

Hepatitis C virus (HCV) is the major causative agent of chronic hepatitis and has an estimated global prevalence of 3% (31). It is not precisely understood why the majority of individuals exposed to HCV develop viral persistence and only a minority experience spontaneous resolution. Moreover, antiviral therapy is effective in only about half of chronically infected patients, and those who fail antiviral therapy are at increased risk of disease progression, including development of cirrhosis and end-stage liver disease (17). Chronic HCV infection is manifested by cytotoxic T lymphocytes (CTLs) that are functionally impaired or exhausted (decreased antiviral cytokine production, cytotoxicity, and proliferative capacity) (15, 30) and may exhibit phenotypic features of early stages of differentiation (1, 18). Recent reports indicate that PD-1 is markedly upregulated on surface of exhausted virus-specific CD8⁺ T cells in mice with lymphocytic choriomeningitis virus (3) and in humans with human immunodeficiency virus (HIV) infection (8, 24, 28), and emerging data indicate a significant role for this immunoreceptor in HCV infection (23, 25, 29). In this regard, hepatic expression of PD-1 mRNA recently was shown to be increased in four acutely infected chimpanzees that subsequently developed persistence in contrast to lower levels in the two animals who spontaneously resolved HCV (26).

In the present study, we report the expression of PD-1 on bulk and HCV-specific CTLs from patients with chronic infection and subjects with spontaneous recovery, as well as the consequences of manipulating PD-1/PD-L pathway on proliferation and effector cytokine production by these cells. We find that PD-1 is markedly upregulated in the peripheral and intrahepatic compartment of patients with chronic infection relative to subjects with spontaneous recovery and compared to expression of PD-1 on total CD8⁺ T cells in normal controls and patients with non-HCV liver disease. Moreover, PD-1 expression on CMVpp65-specific CTLs is higher in chronically in-

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affected patients than in normal controls, suggesting a global effect of HCV infection on the phenotype of T cells. In vitro blockade of PD-1 by monoclonal antibodies specific to its ligands (PD-L1 and PD-L2) results in significant enhancement of proliferation and antiviral cytokine (gamma interferon [IFN-γ] and interleukin-2 [IL-2]) secretion by HCV-specific CTLs, including those residing in the intrahepatic compartment and even in those individuals who lack CD4+ T-cell help. These results have significant implications for the development of novel immunotherapeutic approaches, e.g., blocking negative T-cell regulators, in order to restore T-cell function in this common disease.

MATERIALS AND METHODS

Study population. The study protocol was approved by the appropriate Institutional Review Boards. The study population recruited for the present study comprised four groups of individuals (Table 1). Group 1 consisted of 31 patients with longstanding (more than 10 years) HCV infection with evidence of persistent viremia, including 13 patients who subsequently underwent liver transplantation and from whom intrahepatic lymphocytes were studied directly ex vivo. None of the transplant recipients had received antiviral therapy within the 6 months prior to specimen collection; all of the other HCV patients were treatment naive. Twenty-seven recipients had received antiviral therapy within the 6 months prior to specimen collection; all of the other HCV patients were treatment naive. Twenty-seven patients had genotype 1 infection, two had genotype 2, and two had genotype 4. Group 2 was comprised of 11 subjects with spontaneous resolution of HCV infection and, as indicated by HCV antibody positivity and HCV RNA negativity by PCR and transcription-mediated amplification. Group 3 consisted of 12 patients with longstanding (more than 10 years) HCV infection with evidence of persistent viremia, including 13 individuals (Table 1). Group 1 consisted of 31 patients with longstanding (more than 10 years) HCV infection with evidence of persistent viremia, including 13 patients who subsequently underwent liver transplantation and from whom intrahepatic lymphocytes were studied directly ex vivo. None of the transplant recipients had received antiviral therapy within the 6 months prior to specimen collection; all of the other HCV patients were treatment naive. Twenty-seven recipients had received antiviral therapy within the 6 months prior to specimen collection; all of the other HCV patients were treatment naive. Twenty-seven patients had genotype 1 infection, two had genotype 2, and two had genotype 4. Group 2 was comprised of 11 subjects with spontaneous resolution of HCV infection, as indicated by HCV antibody positivity and HCV RNA negativity by PCR and transcription-mediated amplification. Group 3 consisted of 12 patients with liver disease unrelated to HCV; the etiologies included alcohol (5), nonalcoholic steatohepatitis (3), primary sclerosing cholangitis (2), FH (1), and alpha-1 antitrypsin deficiency (1). Ten healthy subjects without liver disease (all human leukocyte antigen [HLA] A2+) served as controls (group 4).

Sample preparation and storage. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll (Amershams Biosciences, Piscataway, NJ) density gradient centrifugation or cellular preparation tubes (Becton Dickinson, Franklin Lakes, NJ; anticoagulant sodium citrate). PBMC were viably frozen in 80% fetal bovine serum (BioWhittaker, Walkersville, MD), 10% dimethyl sulfoxide (DMSO), and 10% RPMI 1640 medium (Life Technologies, Grand Island, NY) in liquid nitrogen for subsequent analyses. Hepatic mononuclear cells were isolated from explanted liver tissue at time of liver transplantation for HCV-related liver disease. Tissue samples were dissected into 1-mm3 pieces, added to RPMI 1640 medium and 0.05% collagenase type IV (312 U/mg), and the mixture incubated at 37°C for 60 min. The supernatant was removed, diluted in RPMI 1640 medium, and centrifuged at 125 × g for 10 min. Plasma preparation tubes (BD Biosciences, San Jose, CA) were used to isolate plasma from whole blood, which was frozen and later thawed for viral load and genotype testing. HCV genotyping (LiPA) and viral level determination (HCV RNA 3.0 bDNA, lower limit of 615 copies/ml) were performed by Bayer Reference Testing Laboratory (Berkeley, CA).

Antibodies for analysis of cell surface antigen expression and fluorescence-activated cell sorting (FACS) analysis. Four-color multiparameter flow cytometry was performed by using a BD FACScalibur instrument (BD Biosciences) compensated with single fluorochromes and analyzed by using CellQuest software (BD Biosciences). Fluorochrome-labeled (fluorescein isothiocyanate [FITC];PerCP) monoclonal antibodies specific for CD3, CD4, CD8, CD27, CD28, CD37, CD45RA, CD45RO, CD62L, and CD69 were obtained from BD Biosciences. Anti-CD127 and anti-CCR7 antibodies were supplied by R&D Systems (Minneapolis, MN). Anti-CD-1-AF-647/FITC was obtained from eBioscience (San Diego, CA). Cryopreserved PBMC (1 × 106 to 2 × 106) were stained for cell surface antigen expression at 4°C in the dark for 30 min, washed twice in 2 ml of phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.01% sodium azide (Facs Wash), and then fixed in 200 ml of 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO). Isotype-matched control antibodies were used to determine background levels of staining.

HLA typing. HLA typing was performed by using PCR amplification with sequence-specific primers. HLA haplotypes (A2+ or A2−) were further confirmed by staining PBMC with monoclonal antibody MA2.1 (BD Biosciences). Additional screening for HLA A2 status was performed by flow cytometry using an anti-A2 antibody (BD Biosciences) according to the manufacturer’s instructions.

Analysis of antigen-specific CD8+ T-cell responses. Patients expressing the appropriate HLA class I alleles were assessed for antigen-specific responses to HCV by pentamer staining. PE-labeled Pro5 pentamers were supplied by Pro-Immune (Springfield, VA). HLA restriction and HCV peptide sequences are detailed in Table 2. PBMC were stained in conjunction with the antibodies detailed above according to the manufacturer’s instructions. The A2-restricted CMVpp65 was used as a non-HCV control. For flow cytometric analysis of antigen-specific cells, a minimum of 105 CD8+ events were acquired for each pentamer stain.

Proliferation assays. PBMC from patients with known pentamer reactivity were incubated with carboxyfluorescein diacetate succinimidy ester (CFSE; (10 μmol/liter; Molecular Probes, Eugene, OR) for 10 min at 37°C for use in proliferation assays. CFSE-labeled PBMC (107/ml) were incubated at 37°C in 5% CO2 and 95% air for 4-7 days. On day 6 of culture, the presence of HCV-specific peptides (10 μg/ml) was verified in sequence matched the pentamer peptide. Blocking antibodies against PD-L1 or PD-L2 (eBiosciences) or both were added to some of the cultures at a concentration of 1 μg/ml initially only. The addition of IL-2 at 5 ng/ml to peptide-stimulated wells on days 0, 2, 4, and 6 of culture served as a positive proliferation control. Cells were harvested and stained with anti-CD3, CD8, and isotype-matched pentamer. Loss of CFSE in pentamer-positive populations was analyzed by flow cytometry.

Enzyme-linked immunosorbent (ELISPOT) assays for HCV genome-wide analysis of CD4+ T-cell responses. Overlapping peptides (n = 750) were synthesized to span the complete amino acid sequence of the HCV polyprotein derived from HCV-1 (genotype 1a, accession no. M62321) and divided evenly into subgenomic peptide pools as described previously (27). The peptide compositions were confirmed by amino acid analysis. These pentadecamers (15-mer peptides) overlapping by 11 amino acids were resuspended at 20 ng/ml with DMSO and then concentrated so that the final volume of DMSO in the assay would not exceed 0.5%.

Cytokine analysis. On day 7 of the proliferation assay, supernatant was removed from each incubation tube and frozen for subsequent cytokine analysis. Thawed samples were transferred to MultiScreen filter plates (Millipore, Billerica, MA) and assayed with Beadlyte technology (Upstate, Charlottesville, VA) in conjunction with a Luminex100 IS System (Luminex Corp., Austin, TX) to determine the quantities of IFN-γ, IL-2, IL-10, and IL-13. Duplicate samples and standards were processed according to Multiple Cytokine Detection Protocol B (Upstate), opting for overnight incubation with Beadmates from Upstate’s Human Multi-Cytokine Flex kit, mixed, and serially diluted 1:2 in tissue culture medium for maximum detection range. The results were analyzed by using

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The study population recruited for the present study comprised four groups of individuals (Table 1). Group 1 consisted of 31 patients with longstanding (more than 10 years) HCV infection with evidence of persistent viremia, including 13 patients who subsequently underwent liver transplantation and from whom intrahepatic lymphocytes were studied directly ex vivo. None of the transplant recipients had received antiviral therapy within the 6 months prior to specimen collection; all of the other HCV patients were treatment naive. Twenty-seven recipients had received antiviral therapy within the 6 months prior to specimen collection; all of the other HCV patients were treatment naive. Twenty-seven patients had genotype 1 infection, two had genotype 2, and two had genotype 4. Group 2 was comprised of 11 subjects with spontaneous resolution of HCV infection, as indicated by HCV antibody positivity and HCV RNA negativity by PCR and transcription-mediated amplification. Group 3 consisted of 12 patients with liver disease unrelated to HCV; the etiologies included alcohol (5), nonalcoholic steatohepatitis (3), primary sclerosing cholangitis (2), FH (1), and alpha-1 antitrypsin deficiency (1). Ten healthy subjects without liver disease (all human leukocyte antigen [HLA] A2+) served as controls (group 4).
five-parameter logistic curves (fluorescence intensity versus pg/ml) generated by Luminex\textsuperscript{TM} IS software (versions 2.2 and 2.3).

**PD-L1 expression on DC subsets.** In order to quantify plasmacytoid dendritic cells (pDC) and myeloid DC (mDC), two million cryopreserved PBMC were thawed and stained with FITC-conjugated antibodies to BDCA2 and BDCA1, respectively (Miltenyi). PD-L1 surface expression was measured on DC subsets by using phycoerythrin-conjugated antibodies from eBioscience and a Becton Dickinson FACScan flow cytometer. PD-L1 staining was reported as the median fluorescence intensity and the percent positive, using appropriate isotype controls from eBioscience.

**Statistical analyses.** Results are expressed as medians. The two-tailed Wilcoxon rank-sum was used to compare differences between patient groups. Two-tailed Wilcoxon matched-pair signed-rank tests were used to determine the effects of blocking antibodies on cell cultures compared to cultures without the antibodies. A P value of <0.05 was considered significant. The JMP 6.0 (SAS Institute, Inc., Cary NC) statistical package and Prizm 4.03 (GraphPad Software, San Diego, CA) was used.

## RESULTS

**PD-1 is upregulated on bulk and virus-specific T cells in chronic HCV infection.** To examine potential mechanisms of T-cell dysfunction in HCV infection, we studied four groups of individuals: patients with chronic HCV infection (n = 31; 18 of these patients had peripheral lymphocytes and 13 patients had intrahepatic lymphocytes analyzed), patients with spontaneous resolution of HCV (n = 11), patients with liver disease not related to HCV (n = 12), and normal subjects (n = 10). The demographic and clinical features of the study groups are shown in Table 1. A panel of nine MHC class I pentamers containing frequently detected epitopes were synthesized and used to screen HCV responses, as well as an immunodominant HLA A2-restricted cytomegalovirus (CMV) epitope. The epitopes and their restricting alleles are shown in Table 2, as well as the frequency range of responses for the individual epitopes and their restricting alleles are shown in Table 2, as well as the frequency range of responses for the individual epitopes and their restricting alleles.

<table>
<thead>
<tr>
<th>Restriction</th>
<th>HLA restriction</th>
<th>Protein</th>
<th>Amino acids</th>
<th>Epitope sequence</th>
<th>Median response (% CD8\textsuperscript{+} T cells)\textsuperscript{*a}</th>
<th>No. of patients PD1 stained</th>
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<tr>
<td>A1</td>
<td>A1</td>
<td>NS3-5H</td>
<td>1436–1444</td>
<td>ATDALMTGY</td>
<td>0.47 (0.13–7.30)</td>
<td>9</td>
</tr>
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<td>A2</td>
<td>A2</td>
<td>Core B</td>
<td>132–140</td>
<td>DLMGYIPAV</td>
<td>0.26 (0.10–5.91)</td>
<td>14</td>
</tr>
<tr>
<td>A2</td>
<td>A2</td>
<td>NS3-1P</td>
<td>1073–1081</td>
<td>CINGVCVTW</td>
<td>0.55 (0.21–10.58)</td>
<td>16</td>
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<tr>
<td>A2</td>
<td>A2</td>
<td>NS3-5H</td>
<td>1406–1415</td>
<td>KLVALGINAV</td>
<td>0.36 (0.10–5.95)</td>
<td>16</td>
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<tr>
<td>A2</td>
<td>A2</td>
<td>NS5-A1</td>
<td>1987–1995</td>
<td>VLSDFKTWL</td>
<td>0.55 (0.21–5.96)</td>
<td>14</td>
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<tr>
<td>A2</td>
<td>A2</td>
<td>NS5-B2</td>
<td>2594–2602</td>
<td>ALYDVVTKL</td>
<td>0.28 (0.11–0.88)</td>
<td>13</td>
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<tr>
<td>A3</td>
<td>A3</td>
<td>NS5-B2</td>
<td>2588–2596</td>
<td>RVCEKMAKY</td>
<td>0.50 (0.16–1.15)</td>
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<tr>
<td>B7</td>
<td>B7</td>
<td>Core B</td>
<td>111–119</td>
<td>DPRRRSRNL</td>
<td>0.55 (0.19–0.60)</td>
<td>2</td>
</tr>
<tr>
<td>B8</td>
<td>B8</td>
<td>NS3-5H</td>
<td>1395–1403</td>
<td>HSKKKCDDEL</td>
<td>0.53 (0.22–0.94)</td>
<td>3</td>
</tr>
<tr>
<td>A2</td>
<td>A2</td>
<td>CMVpp65</td>
<td>495–503</td>
<td>NLVPMVATV</td>
<td>0.43 (0.07–15.08)</td>
<td>24</td>
</tr>
</tbody>
</table>

\textsuperscript{*a} The range is indicated in parentheses.

We used flow cytometry to examine PD-1 expression on bulk (total) and virus-specific CD8\textsuperscript{+} T cells directly ex vivo from the four groups of subjects (representative dot plots shown in Fig. 1A). PD-1 expression was significantly higher on bulk CD8\textsuperscript{+} T cells from patients with chronic HCV compared to those with spontaneous viral eradication, patients with non-HCV liver disease, and normal controls (Fig. 1B). In total, 95 individual HCV-specific pentamer responses were examined. HCV-specific CTLs displayed median (% positive and median fluorescence intensity [MFI]) levels of PD-1 that were markedly elevated among patients with chronic HCV (Fig. 1D) relative to subjects who had spontaneously resolved HCV infection. Statistical comparison of the data displayed in Fig. 1C and D reveals significantly higher PD-1 expression on HCV-specific CTLs compared to total CTLs in chronic infection (P < 0.0001).

Because HCV preferentially replicates within the liver, we hypothesized that PD-1 expression might be upregulated on HCV-specific CTLs in hepatic tissue to a greater extent than on T cells circulating in the blood. After mechanical and collagenase digestion of explanted livers from patients who subsequently underwent transplantation, we examined the direct ex vivo expression of PD-1. As shown in Fig. 1B (last panel) and Fig. 1D, the MFI, which correlates directly with the number of molecules expressed on a per-cell basis, was significantly higher for PD-1 on HCV-specific CTLs within the intrahepatic compartment compared to peripheral blood. Thus, a higher level of viral antigen within the hepatic microenvironment likely leads to greater PD-1 upregulation on virus-specific CTLs.

Next, we examined whether there were HCV epitope-related differences in PD-1 expression. As shown in Fig. 1E, irrespective of the pentamer used, PD-1 expression was consistently higher in chronic infection (in the peripheral and intrahepatic compartments) than in resolved infection. On average, the percentage of pentamer-positive cells expressing PD-1 ranged from 66.87 to 81.42% in chronic infections and from 10.04 to 68.70% in resolved infections.

CMV-specific CTL responses in the peripheral blood of healthy controls were characterized by significantly lower PD-1 expression (median = 8.8%) than HCV-specific responses in chronically infected patients (P < 0.0001). Moreover, as shown in Fig. 2, CMV-specific CTLs exhibited significantly higher PD-1 levels in patients with chronic infection than in patients with non-HCV liver disease and normal controls. Patients with resolved infection (n = 3) demonstrated intermediate levels of PD-1 (median = 24%).

**Phenotypic differences of PD-1\textsuperscript{high} versus PD-1\textsuperscript{low} CTLs relative to HCV outcome and intrahepatic versus peripheral CTLs.** The phenotypic profile of PD-1\textsuperscript{high} and PD-1\textsuperscript{low} cells was assessed by using a wide profile of markers, including CCR7, CD27, CD28, CD45RA, CD57, and CD62L on HCV-specific CTLs derived from patients with chronicity and those...
FIG. 1. PD-1 expression increased in chronic HCV infection. Representative dot plots and histograms of PD-1 expression on total CD8+ T cells (A) and HCV-specific CTLs (B), including patients with chronic and resolved HCV infection. (C) PD-1 expression on peripheral CD8+ T cells from patients with chronic infection (n = 18), subjects with spontaneously resolved infection (n = 11), subjects with non-HCV liver disease (n = 12), and healthy control subjects (n = 10), as well as intrahepatic lymphocytes (n = 13) from patients with chronic infection, is shown. Horizontal bars represent the medians. The PD-1 MFI on CD8+ T cells is higher in chronic infection than in resolved infection, non-HCV liver disease, or in healthy controls, with no difference observed between the peripheral and intrahepatic compartments. PD-1 expression is also higher in resolved infection than in healthy controls. (D) Total HCV-specific PD-1 expressed as a percentage of pentamer+ CD8+ T cells positive for PD-1 and as the MFI. Chronic HCV infection is associated with a greater percentage of PD-1+ HCV-specific CTLs in the periphery (n = 17 patients, 45 pentamers responses), as well as in the liver (n = 9 patients, 29 pentamers). The data are shown for seven patients (21 pentamers) with resolved infection. The intensity of PD-1 staining is also higher on the cells of chronically infected patients, showing the most concentrated PD-1 expression in the liver. (E) Breakdown of HCV-specific PD-1 expression by epitope displaying higher PD-1 in chronic infection compared to resolved for all...
with spontaneous resolution. Irrespective of virologic outcome, PD-1\textsuperscript{high} HCV-specific CTLs in the peripheral blood expressed significantly higher levels of CCR7, CD27, CD57, and CD62L but lower levels of CD45RA (Table 3); virtually all pentamer-positive cells were CD45RO\textsuperscript{+}. Furthermore, the levels of the senescence marker CD57 were greater in chronic infections than in resolved infections for both PD-1\textsuperscript{high} and PD-1\textsuperscript{low} fractions. The relatively higher expression of CD45RA and CD57 on HCV-specific CTLs compared to their reported expression on HIV-specific CTLs suggests that these cells are more differentiated in HCV than reported in HIV (1, 8, 24, 28). However, although it might be expected that these cells would also express lower levels of CCR7, a previous report (18) indicated that chronic HCV is characterized by skewed maturation and inability to downregulate CCR7. Taken together, these data indicate that in HCV infection, the phenotypic expression of maturational markers is generally associated with PD-1 expression but is independent of virologic outcome. Intrahepatic CTLs that specifically targeted HCV epitopes expressed an activated memory phenotype (CD69\textsuperscript{+} CD45RO\textsuperscript{+}) compared to peripheral CTLs (Table 4). Furthermore, we found that HCV-specific CTLs in the liver expressed significantly higher levels of PD-1 (MFI) and lower levels of CD127, confirming and expanding results from a recent study of smaller sample size (25). In this regard, we have recently shown that loss of CD127 (IL-7 receptor \alpha) expression favors the development of viral persistence in patients with acute infection (13).

PD-L1 and PD-L2 blockade restores HCV-specific T-cell effector function in chronic HCV infection. PD-1 has two ligands: PD-L1 (B7-H1), expressed on hepatocytes (19), hematopoietic and parenchymal cells (9), and PD-L2 (B7-DC), predominantly expressed on macrophages and DCs (14). In order to dissect the potential pathways mediating PD-1-associated T-cell exhaustion, we stimulated PBMC in culture with cognate HCV peptide alone or in the presence of antibodies to PD-L1, PD-L2, both PD-L1 and PD-L2, and IL-2. We used CFSE to monitor the proliferation of pentamer-positive HCV-specific T cells after 7 days. As shown in Fig. 3, in a chronic infection, the addition of either blocking antibody (anti-PD-L1 and anti-PD-L2) or IL-2 significantly augments the number of proliferating (CFSE\textsuperscript{low}) HCV-specific CTLs. On average, in chronic HCV, blockade with either the anti-PD-L1 or anti-PD-L2 antibody enhanced proliferation 2-fold, whereas dual-ligand blockade increased the frequency of proliferating HCV-specific CTLs 3.4-fold. Of note, in all of the cultures derived from chronic patients, dual blockade increased CTL proliferation, including those from the liver (Fig. 3C). As expected, in patients with resolved HCV infection, the level of proliferation after stimulation with peptide alone was greater than in patients with chronic HCV infection. Although blockade of the PD-1 pathway did increase proliferation in some subjects with resolved infection, the difference was not statistically significant compared to stimulation with peptide alone (Fig. 3B, bottom panel).

As another measure of CTL effector function, we assessed antiviral effector cytokine production from the same culture medium as the proliferation studies. Dual blockade with
PD-L1 and PD-L2 induced production of IFN-γ and IL-2; coculture with either antibody alone significantly enhances IL-2 production (Fig. 4). Higher PD-1 expression on HCV-specific CTLs (MFI) was associated with a greater fold increase in IL-2 (P = 0.04). IL-13, which has been shown to have direct antiviral effects and be associated with improved liver disease status in HIV infection (2), but not previously characterized in HCV, was elevated in cultures with dual PD-1 blockade. In contrast, the production of the immunosuppressive cytokine IL-10 was not significantly affected by blocking the PD-1 pathway.

Next, we explored whether blockade of the PD-1/PD-L1/PD-L2 pathway could restore CD8+ T-cell responses in the absence of virus-specific CD4+ T-cell help. As previously described in the lymphocytic choriomeningitis virus model (3), “helpless” CTLs demonstrate even more profound functional defects. We comprehensively assessed CD4+ T-cell help by synthesizing overlapping peptides that spanned the entire HCV polyprotein and, for all of the patients studied in Fig. 3, we performed an IFN-γ ELISPOT assay using CD8-depleted PBMC (5 × 10^5/well) that were stimulated with 33 peptide pools spanning distinct subgenomic regions, as detailed previously (for CD4+ ELISPOT results, see Fig. S1 in the supplemental material). Even patients lacking HCV-specific CD4+ T cells demonstrated increased CTL proliferation and ability to produce cytokines after PD-1 blockade (see Fig. S2 in the supplemental material).

Taken together, these data indicate that in patients with longstanding chronic HCV infection, blocking the PD-1/PD-L1/PD-L2 pathway restores the capacity of peripheral and intrahepatic HCV-specific CTLs to proliferate and secrete antiviral cytokines, even profoundly exhausted CTLs without CD4+ T-cell help.

**PD-1 expression on mDC and pDC.** It is conceivable that different viruses induce unique combinations of costimulatory ligands that determine the fate of antiviral immunity (16). In this regard, viruses that specifically induce expression of PD-L1 and PD-L2 in APCs might downregulate the adaptive immune response and favor persistence. In order to determine whether the higher expression of PD-1 on CTLs in chronic infection is related to differential PD-L1 on APCs, we measured its expression on mDC and pDC; moreover, we recognize these ligands are broadly expressed on different cell types.

As shown in Fig. 5, we found that a higher percentage of mDCs expressed PD-L1 compared to pDCs, but within groups there was only statistical significance between chronic pDCs and mDCs (P = 0.007). However, the number of PD-L1 molecules expressed on a per-cell basis as measured by the MFI was greater for pDCs than for mDCs. Although there was no appreciable difference in PD-L1 expression between patients with viral persistence and subjects with spontaneous recovery that might explain differences in PD-1 expression, PD-L1 MFI was higher on mDC derived from patients with chronic infection compared to non-HCV (P = 0.014) and normal healthy controls (P = 0.070).

**DISCUSSION**

Impaired proliferative, cytokine, and cytotoxic effector functions of HCV-specific CTLs are a hallmark of chronic HCV infection (4), likely contributing to the development of viral persistence in the majority of exposed individuals. The precise mechanisms governing dysfunction of the antiviral CTL effector response remain poorly defined. Putative explanations include induction of anergy by high antigen levels, impaired CD4 helper functions, the suppressive activity of regulatory T cells, or HCV escape variants with altered epitope ligands (4, 5, 6).

The PD-1 inhibitory pathway has been shown in both mouse and human models (3, 8, 23, 24, 25, 26, 28, 29) to induce functional impairment or exhaustion of CTLs. Our results expand considerably the understanding of its potential role in chronic and resolved HCV infection. In the first study to characterize PD-1 in HCV, Urbani et al. (29) demonstrated that PD-1 expression was upregulated on CTLs specific for two tetramers in patients with acute HCV who subsequently developed persistence, whereas its expression declined in patients

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**TABLE 3. Phenotype of PD-1^{high} and PD-1^{low} CTLs that specifically target HCV in chronic and resolved infection (peripheral blood)^a**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Chronic</th>
<th>Resolved</th>
<th>Chronic vs resolved</th>
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<tbody>
<tr>
<td></td>
<td>PD-1^{high}</td>
<td>PD-1^{low}</td>
<td>PD-1^{high}</td>
</tr>
<tr>
<td>CCR7</td>
<td>88.99 (18.29–100)</td>
<td>57.81 (37.93–76.19)</td>
<td>0.0002</td>
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<tr>
<td>CD27</td>
<td>92.54 (42.28–100)</td>
<td>85.33 (7.84–95.24)</td>
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<tr>
<td>CD28</td>
<td>85.00 (37.61–98.41)</td>
<td>52.63 (24.18–83.08)</td>
<td>0.0008</td>
</tr>
<tr>
<td>CD45RA</td>
<td>91.77 (14.78–100)</td>
<td>76.67 (58.33–100)</td>
<td>0.0406</td>
</tr>
<tr>
<td>CD57</td>
<td>70.59 (16.05–100)</td>
<td>37.01 (12.99–55.55)</td>
<td>0.0329</td>
</tr>
<tr>
<td>CD62L</td>
<td>68.12 (7.88–88.88)</td>
<td>31.25 (12.5–71.43)</td>
<td>0.0028</td>
</tr>
</tbody>
</table>

^a Values (other than P values) are expressed as median percentages of HCV-specific PD-1^{high} or PD-1^{low} cells coexpressing antigen. Ranges are indicated in parentheses. P values were calculated using a nonparametric Mann-Whitney U test. NS, not significant.

**TABLE 4. Phenotype of HCV-specific CTLs in peripheral and intrahepatic compartments of chronically infected patients**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Median % tetramer (range)</th>
<th>Peripheral</th>
<th>Intrahepatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-1</td>
<td>60.91 (33.76–161.42)</td>
<td>90.19 (42.9–178.53)</td>
<td>0.0017</td>
</tr>
<tr>
<td>CD28</td>
<td>74.51 (40.79–94.2)</td>
<td>65 (1.16–97.14)</td>
<td>NS</td>
</tr>
<tr>
<td>CD27</td>
<td>92 (22.5–98.02)</td>
<td>93.16 (79.51–98.28)</td>
<td>NS</td>
</tr>
<tr>
<td>CCR7</td>
<td>75.72 (24.79–96.47)</td>
<td>65.88 (8.62–100)</td>
<td>NS</td>
</tr>
<tr>
<td>CD45RA</td>
<td>86.27 (24.66–100)</td>
<td>84.54 (13.99–100)</td>
<td>NS</td>
</tr>
<tr>
<td>CD57</td>
<td>52.83 (21.7–99.56)</td>
<td>64.38 (10.85–94.12)</td>
<td>0.0119</td>
</tr>
<tr>
<td>CD62L</td>
<td>50.72 (13.86–76.67)</td>
<td>43.34 (1.92–89.36)</td>
<td>NS</td>
</tr>
<tr>
<td>CD69</td>
<td>39.62 (9.48–100)</td>
<td>96.61 (81.56–100)</td>
<td>0.0006</td>
</tr>
<tr>
<td>CD127</td>
<td>33.33 (2.94–90)</td>
<td>6.85 (2.76–33)</td>
<td>0.0169</td>
</tr>
<tr>
<td>CD45RO</td>
<td>89.66 (72.9–100)</td>
<td>99.46 (88.52–100)</td>
<td>0.0027</td>
</tr>
</tbody>
</table>

^a Values for HCV-specific CTL (PD-1) are indicated as the MFI. All other values were calculated using the nonparametric Mann-Whitney U test. NS, not significant.

^b P values were calculated using the nonparametric Mann-Whitney U test.
FIG. 3. The proliferation of HCV-specific CTLs is enhanced in response to PD-1 ligand blockade. PBMC from patients with chronic and spontaneously resolved HCV infection were cultured for 7 days in the presence of either viral peptide alone or peptide plus anti-PD-L1, anti-PD-L2, both anti-PD-L1 and anti-PD-L2, or IL-2 (as a positive control). The HCV peptide concentration was 10 μg/ml. PD-L1 and PD-L2 were added at 10 μl/ml only initially; IL-2 was added at 5 ng/ml on days 0, 2, 4, and 6 of culture. Cells were gated on total CD8+ lymphocytes and analyzed for expression of CFSE and peptide-matched pentamer. The peptides and/or pentamers used were NS3 1436, core 132, NS3 1073, NS3 1406, NS5 1987, and NS5 2594 (see also Table 2). Experiments included seven patients with chronic HCV (12 pentamer responses) and three subjects with resolved HCV (5 pentamer responses). (A) The upper left quadrant of representative dot plots illustrate HCV-specific, proliferating CTLs in chronically infected patients (top row) and in patients who spontaneously resolved infection (bottom row). (B) In chronic infection, PBMC cultures with peptide plus IL-2, anti-PD-L1 and/or anti-PD-L2 exhibited significantly greater expansion of HCV-specific CTLs compared to stimulation with peptide alone. As expected, resolved patient cultures showed greater proliferation with peptide alone than cultures derived from chronic patients; although there were some increases in HCV-specific CD8 expansion with PD-1 ligand blockade and IL-2, they were not statistically significant. Comparisons were made by using the Wilcoxon signed-rank (matched pairs) test. (C) Intrahepatic lymphocytes stimulated in the presence of anti-PD-L1 and anti-PD-L2 demonstrated greater proliferation than intrahepatic lymphocytes culture with peptide NS5 1987 alone; CFSE comparisons are shown for three patients studied (see also Fig. S2 in the supplemental material).
who spontaneously cleared HCV. Moreover, Radziewicz et al. (25) found that PD-1 was upregulated and CD127 was downregulated for the CD8\(^+\) T-cell population within the liver relative to peripheral blood; however, these authors were able to evaluate HCV-specific CTLs in only two livers. Our data, derived from a larger group of patients, confirm and significantly extend these results. We found increased PD-1 expression on total and HCV-specific CTLs in both the peripheral and the intrahepatic compartments of patients with longstanding HCV infection relative to those with spontaneous resolution, nonviral liver disease, and normal controls. The fact that PD-1 expression was higher on the total CD8\(^+\) T-cell population in chronic HCV infection adds to the growing literature that HCV has a global effect on the phenotype of T cells, beyond those that are HCV specific (18). Accordingly, we found that PD-1 expression on CMVpp65-specific CTLs was significantly higher in chronically HCV-infected patients (median = 78%) than in normal subjects (8%) or patients with non-HCV liver disease (17%). Our findings provide a plausible mechanistic basis for the observation that patients with HCV have significantly higher prevalence of other infections, including CMV and herpes simplex virus infections (10).

The high expression of PD-1 on HCV-specific CTLs is comparable to recent reports (8, 28) of various persistent human viruses, e.g., HIV and Epstein-Barr virus. In HIV infection, it has been shown that the viral level correlates directly with PD-1 expression on viral specific CTLs (8), although this finding is not consistent (24). In the present study, significantly
higher median PD-1 expression (i.e., the MFI) was found on the HCV-specific CTLs within the intrahepatic compartment, where presumably viral replication and antigen expression is more robust than the peripheral compartment. This concept is in keeping with the recent demonstration by Penna et al. of a positive correlation between frequency (%) and the surface expression (MFI) of PD-1 and the circulating levels of hepatitis C viremia (23).

We characterized various functional aspects of CTL impairment in patients with longstanding infection and, of considerable interest, demonstrated that the proliferative capacity and antiviral cytokine secretion can be restored by manipulation of the PD-1/PD-L pathway. In the recent study by Radziejewicz et al. (25), the addition of IL-2 and anti-PD-L1 to PBMC from two patients with chronic HCV infection resulted in enhanced proliferation. As shown in Fig. 3 and previously reported for HIV (24), we show that IL-2 alone can increase proliferation (as well as effector cytokine secretion), underscoring the importance of excluding it in cultures in order to assess the contribution of PD-1 blockade alone. We demonstrate that in vitro blockade with monoclonal antibodies to each PD-1 ligand (PD-L1 and PD-L2) in the absence of IL-2 enhances proliferation in the chronic disease state, restoring antiviral effector function in exhausted HCV-specific T cells. Considerable evidence supports a predominant role for cytokines in viral replication in infected cells (12, 16); in accord with the study by Penna et al. (23), we found enhancement of HCV-specific IL-2 and IFN-γ secretion after PD-1 blockade (we examined the effects of anti-PD-L1, anti-PD-L2, and their combination). Moreover, importantly, the rescue effect was demonstrable even in cultures derived from patients who lack HCV-specific CD4+ T-cell help, i.e., so-called “helpless CTLs” (27) (see Fig. S2 in the supplemental material). Furthermore, intrahepatic lymphocytes are particularly exhausted, as indicated by their phenotypic profile (PD-1high CD57high CD127low), and we demonstrate for the first time that their function is restored by blocking PD-1 (Fig. 3C).

This reversal of exhausted function of HCV-specific CTLs implicates PD-1 as a potential therapeutic target. However, because the PD-1/PD-L pathway has important roles in regulating the balance between T-cell activation and tolerance (11), it might be critical to limiting detrimental inflammatory consequences of anti-HCV effector responses within the liver. Such a protective role has been indicated in studies of PD-1−/− knockout mice that more rapidly clear adenovirus infection but develop more severe hepatocellular injury than wild-type mice (11, 22). Thus, it will be necessary to identify the optimal patient characteristics, including the severity of liver disease, in order to minimize the risk of immunopathology or autoimmunity in patients with chronic HCV infection (11).

In summary, our data indicate that HCV uses the PD-1 inhibitory pathway to inhibit antimicrobial immunity. Expression of PD-1 is upregulated on virus-specific CTLs of patients with chronic HCV (particularly within the intrahepatic compartment) relative to subjects with spontaneous resolution of HCV and normal controls. PD-L1 is associated with the phenotypic expression of maturational markers, and the levels of the senescence marker CD57 are greater in chronic HCV infections than in resolved HCV infections. Manipulation of the PD-1/PD-L pathway with monoclonal antibodies restores the functional competence of HCV-specific CTLs, including those that reside within the hepatic compartment and those lacking CD4+ T-cell help. Future studies blocking single or multiple components of this pathway, perhaps in combination with IFN-based therapies, should be considered in selected patients with chronic HCV.

ACKNOWLEDGMENTS

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REFERENCES


FIG. 5. PD-L1 levels in peripheral pDC and mDC. PBMC from patients with chronic HCV (n = 12) or resolved HCV (n = 8) and from normal patients (n = 8) and patients with non-HCV liver disease (n = 12) were stained and analyzed by flow cytometry to separate mDC (BDCA1 positive) and pDC (BDCA2 positive). PD-L1 was measured on each group and reported as a percentage (A) and as the MFI (B). The horizontal bar represents the median of the group. There was no appreciable difference in PD-L1 expression between patients with viral persistence and subjects with spontaneous recovery, but PD-L1 MFI was higher for pDC derived from patients with chronic infection compared to non-HCV patients (P = 0.01) and normal healthy controls (P = 0.07). PD-L1 MFI was also higher for mDC derived from patients with viral persistence compared to healthy controls (P = 0.04). In addition, in the chronic group the percentage of mDC expressing PD-L1 was higher than that for the pDCs (P = 0.007).


