PD-1 modulates regulatory T cells and suppresses T-cell responses in HCV-associated lymphoma

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T regulatory (T<sub>R</sub>) cells suppress T-cell responses that are critical in the development of chronic viral infection and associated malignancies. Programmed death-1 (PD-1) also has a pivotal role in regulation of T-cell functions during chronic viral infection. To examine the role of PD-1 pathway in regulating T<sub>R</sub>-cell functions that inhibit T-cell responses during virus-associated malignancy, T<sub>R</sub> cells were investigated in the setting of hepatitis C virus-associated lymphoma (HCV-L), non-HCV-associated lymphoma (non-HCV-L), HCV infection alone and healthy subjects (HS). Relatively high numbers of CD4<sup>+</sup>CD25<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells, as well as high levels of PD-1 expressions on these T<sub>R</sub> cells were found in the peripheral blood of subjects with HCV-L compared with those from non-HCV-L or HCV alone or HS. T<sub>R</sub> cells from the HCV-L subjects were capable of suppressing the autologous lymphocyte response, and depletion of T<sub>R</sub> cells in peripheral blood mononuclear cells from HCV-L improved T-cell proliferation. Additionally, the suppressed T-cell activation and proliferation in HCV-L was partially restored by blocking the PD-1 pathway <em>ex vivo</em>, resulting in both a reduction in T<sub>R</sub>-cell number and the ability of T<sub>R</sub> to suppress the activity of effector T cells. This study suggests that the PD-1 pathway is involved in regulating T<sub>R</sub> cells that suppress T-cell functions in the setting of HCV-associated B-cell lymphoma.

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Hepatitis C virus (HCV) infects over 180 million people worldwide and exhibits a remarkable propensity toward chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Chronic HCV infection is also associated with B-cell lymphoproliferative disorders, including most notably mixed cryoglobulinemia and B-cell lymphomas. In chronically HCV-infected individuals, the frequencies of cytotoxic T lymphocytes are relatively low; similarly, the proliferative capacity as well as effector functions of HCV-specific CD4<sup>+</sup>/CD8<sup>+</sup> T cells are impaired, and the production of Th1-type cytokines (that is, interleukin-2 and interferon-γ) is dramatically suppressed.<sup>5–8</sup>

T regulatory (T<sub>R</sub>) cells, including CD4<sup>+</sup>CD25<sup>+</sup> as well as CD8<sup>+</sup>CD25<sup>+</sup> cell populations, suppress effector/memory T-cell responses that are critical in clearance of pathogens, thus having a pivotal role in the establishment and maintenance of chronic infections such as HCV.<sup>9–16</sup> Understanding the mechanisms underlying T-cell dysregulation in HCV infection has also been advanced since the identification of programmed death-1 (PD-1), an important inhibitory pathway in regulation of T-cell receptor signaling.<sup>17–20</sup> We have previously demonstrated that HCV core protein differentially regulates T- and B-lymphocyte functions through PD-1 and suppressor of cytokine signaling-1 negative signaling pathways.<sup>21–27</sup> We have recently observed that subjects with HCV-associated lymphoma (HCV-L) exhibit relatively higher PD-1 expression and more intense T-cell suppression than those with non-HCV-associated lymphoma (non-HCV-L) or HCV infection alone without lymphoma (HCV alone) or healthy subjects (HS). The relationship between PD-1 pathway and T<sub>R</sub> cells in regulation of T-cell responses in the setting of HCV-L, however, remain unknown. In this report, we describe relatively higher numbers of CD4<sup>+</sup>CD25<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells, as well as higher levels of PD-1 expression on these T<sub>R</sub> cells in the peripheral blood of subjects with HCV-L compared with those from non-HCV-L or HCV alone or HS. Interestingly, PD-1 appears to have a role in regulating T<sub>R</sub>-cell number as well as function in suppressing T-cell responses in the setting of chronically HCV-infected subjects with B-cell lymphoma.

RESULTS AND DISCUSSION

As an initial approach to characterize the role of T<sub>R</sub> cells in regulation of T-cell responses, we first determined the relative number of CD4<sup>+</sup>CD25<sup>+</sup> and CD8<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub>-cell populations in the peripheral blood of subjects (Table 1) with HCV-L, and compared with those in non-HCV-L, HCV alone, or HS. As shown in Figure 1a, both

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CD4+CD25+ and CD8+CD25+ T<sub>R</sub> cells were found to be increased in the subjects with HCV-L when compared with that non-HCV-L, HCV alone or HS. Similar results were obtained in repeated experiments using different antibody conjugates to compare CD4+CD25+ T<sub>R</sub>-cell numbers. As the transcription factor Foxp3 has been accepted as a specific marker for T<sub>R</sub> cells, intracellular Foxp3 expression was analyzed in CD4+CD25+ T<sub>R</sub> cells obtained from a subject with HCV-L, three HCV alone and a HS. As shown in Figure 1b, CD4+CD25+ T<sub>R</sub> cells were found to be higher in the subject with HCV-L compared with HCV subjects without lymphoma and a HS. Correspondingly, Foxp3 expression was found to be highest in the subject with HCV-L, followed by HCV alone and the lowest in the HS. These results confirm the finding of an increased T<sub>R</sub>-cell component in the setting of HCV-L.

As CD25 (interleukin-2R α-chain) is an early T-cell activation marker, higher levels of CD25 expression on CD4+ T cells might simply be because of T-cell activation rather than secondary to the development of T<sub>R</sub> cells in subjects with HCV-L. To further elucidate this concern, we stimulated peripheral blood mononuclear cells (PBMC) isolated from HCV-L, HCV alone and HS with phytohaemagglutinin (PHA) for 24 h, and then evaluated CD69 cells (PBMC) isolated from HCV-L, HCV alone and HS with HCV-L, HCV alone and HS. These results suggest that blocking the PD-1 pathway may regulate T<sub>R</sub>-cell development and rescue T-cell activation in the setting of HCV-L.

To determine the effect of blocking the PD-1 pathway on T-cell proliferation in HCV-L, we next preincubated carboxyfluorescein succinimidyl ester (CFSE)-labeled PBMC with anti-PDL-1 or control antibody following stimulation with either anti-CD3/CD28 or autogeneic healthy PBMC for 5 days. T-cell proliferation as examined by CFSE dilution was analyzed by flow cytometry after double staining and gating on T-cell populations. As shown in Figure 2a, treated the PBMC isolated from an HCV-L subject with anti-PDL-1 or control antibody overnight, followed by incubation with HCV peptides ex vivo for 5 days, and then examined the total number of CD4+CD25+ T<sub>R</sub> cells and CD69 expression on CD4+ T cells. As shown in Figure 2a, compared with treatment by the control antibody, blocking the PD-1 pathway by treating the cells with PDL-1 antibody reduced the total number of CD4+CD25+ T<sub>R</sub> cells and CD69 expression on CD4+ T cells. These results suggest that blocking the PD-1 pathway may regulate T<sub>R</sub>-cell development and rescue T-cell activation in the setting of HCV-L.

| Subject | Diagnosis<sup>a</sup> | Age | Gender | GT | Viral load (IU/ml<sup>-1</sup>) | Lymphocyte count | Treatment<sup>b</sup> |
|---------|------------------------|-----|--------|----|-------------------------------|------------------|-------------------|
| 1       | HCV-NHL                | 56  | M      | 1a | 97 765                        | 1.6 x 10<sup>8</sup> | s/p chemotherapy  |
| 2       | HCV-NHL                | 62  | M      | 1b | 1 260 000                     | 0.9 x 10<sup>8</sup> | s/p stem cell transplant |
| 3       | HCV-NHL                | 59  | M      | 1a | 8 730 000                     | 1.8 x 10<sup>8</sup> | s/p chemotherapy  |
| 4       | HCV alone              | 65  | M      | 1a | 5 000 000                     | 1.6 x 10<sup>8</sup> | Before pegIFN+RBV  |
| 5       | HCV alone              | 51  | M      | 1a | 2 110 000                     | 2.4 x 10<sup>8</sup> | Before pegIFN+RBV  |
| 6       | HCV alone              | 63  | M      | 1b | 530 590                       | 1.7 x 10<sup>8</sup> | Before pegIFN+RBV  |
| 7       | NHL alone              | 58  | M      | NA | 18 x 10<sup>8</sup>           | 1.3 x 10<sup>8</sup> | s/p chemotherapy  |
| 8       | NHL alone              | 61  | M      | NA | 18 x 10<sup>8</sup>           | 1.8 x 10<sup>8</sup> | s/p chemotherapy  |
| 9       | NHL alone              | 59  | M      | NA | 18 x 10<sup>8</sup>           | 1.2 x 10<sup>8</sup> | s/p chemotherapy  |
| 10      | Healthy                | 51  | M      | NA | 1.9 x 10<sup>7</sup>          | NA               | NA                |
| 11      | Healthy                | 49  | M      | NA | 2.5 x 10<sup>7</sup>          | NA               | NA                |
| 12      | Healthy                | 43  | M      | NA | 1.8 x 10<sup>7</sup>          | NA               | NA                |

Abbreviations: HCV, hepatitis C virus; HCV-NHL, HCV-associated lymphoma; GT, genotype; NA, not applicable; NHL, non-Hodgkin’s lymphoma; pegIFN, pegylated interferon; RBV, ribavirin; s/p, status/post.

<sup>a</sup> All the NHL patients were diagnosed by clinical and histological features, and confirmed by immunohistochemistry or flow cytometry studies revealing that tumor cells were positive for B-cell markers.

<sup>b</sup> Subject 1 was diagnosed as follicular lymphoma grade-2, immunohistochecmical stains positive for L26, CD10, CD20, Bcl-2 and Bcl-6. Subject 7 and 8 were diagnosed as follicular lymphoma grade-1a. Subject 2 was diagnosed as follicular lymphoma grade-1b, and CD30. Subject 9 was diagnosed as diffuse large B-cell lymphoma, immunochemistry showed large atypical lymphoid cells that stained strongly for CD20.
ex vivo, the effect on rescuing T-cell function by PD-1 blocking may be secondary to the reduced T<sub>R</sub> cells in the bulk PBMC or the result of directly blocking PD-1 signaling on effector T cells, or both. Thus, we further examined the role of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>R</sub> cells in suppressing T-cell proliferation by mixed lymphocyte culture. To this end, we separated the CD25<sup>+</sup> cells and CD25<sup>-</sup> cells from a subject with HCV-L, incubated with CFSE-labeled healthy PBMC and analyzed by CFSE dilution to determine the ability of these two cell populations to suppress healthy PBMC proliferation. Gating strategy is shown above and intracellular expression of FoxP3 in the gated CD4<sup>+</sup>CD25<sup>+</sup> cell population is shown in the histogram. (

Because in vitro depletion of CD25<sup>+</sup> cells results in increased responsiveness of the HCV-specific effector cells, it has been suggested that induction of T<sub>R</sub> cells have a causal role in the establishment of chronic HCV infection. To further elucidate the role of T<sub>R</sub> cells and PD-1 in suppressing T-cell proliferation, we compared the proliferative ability of CD25<sup>-</sup>-depleted cells versus bulk PBMC from a subject with HCV-L, in the presence of anti-PD-L1 or control antibody, by CFSE dilution and flow cytometry analysis. As shown in Figure 2d, CD25<sup>-</sup>-depleted cells treated with anti-PD-L1 proliferated better compared with bulk PBMC treated with anti-PD-L1 or CD25<sup>-</sup>-depleted cells treated with the control antibody. The least proliferation was observed in the setting of bulk PBMC treated with the control antibody (Figure 2d, lower right panel). These results suggest that both T<sub>R</sub> and PD-1 signaling have a role in suppressing T-cell proliferation in the setting of HCV-L.

Although several biomarkers have previously been recognized to have a role in defining T<sub>R</sub> cells in disease conditions, the discovery of high expression of PD-1 in high number of T<sub>R</sub> cells in the setting of HCV-L represents a novel finding, most notably in that the function of these cells appears to be regulated via PD-1 signaling. These findings suggest that the PD-1 pathway is involved in the regulation of TR cells and CD8<sup>+</sup>CD25<sup>-</sup> effector T cells. Representative dot plot with percentage of PD-1 expression levels are shown above and the data from multiple subjects are summarized as bar figure below.
whether the increased T<sub>Reg</sub> cells are a consequence of or contribute to the development of HCV-L.

**METHODS**

**Subjects**

An institutional review board-approved protocol at James H Quillen VA Medical Center and East Tennessee State University (Johnson City, TN, USA) has contributed to a database for the storage of blood samples from HCV-infected individuals. Three HCV subjects with B-cell lymphoma, three with non-HCV-associated B-cell lymphoma, three HCV-infected individuals without lymphoma and three HS as normal controls are included in this study. All HCV subjects, either genotype 1a or 1b, with HCV RNA levels range from 97,765~500,000 IU/ml~1, were selected before peginterferon and ribavirin treatment. HCV-L or non-HCV-L subjects were diagnosed by clinical and histological features, confirmed by immunochemistry and flow cytometry and were at post-antitumor therapy for at least 6 months.

**Cell isolation and culture**

Human PBMCs were isolated from the peripheral blood of subjects by Ficoll density centrifugation with lymphocyte-H (Cedarlane Labs, Ontario, Canada). If indicated, CD25<sup>+</sup> T cells were further purified or deleted from isolated PBMC by incubation with a magnetic beads-conjugated anti-CD25 antibody, followed by positive or negative selection per the manufacturer's instruction (Miltenyi Biotec., Auburn, CA, USA). Cells were cultured as described previously.  

**Flow cytometry**

To determine cell-surface marker expression on T lymphocytes, 1 x 10<sup>6</sup> purified PBMCs were stimulated with either 1 µg/ml~1 PHA (Sigma, St Louis, MI, USA), or 10 µg HCV peptides (HLA-A-0201/NS3/1073-1081/CINGVCWTV; HLA-A-0201/NS3/1073-1081/GKVALGINAV; synthesized by GenScript Corporation, Piscataway, NJ, USA), for 24 h and washed as described. The following antibody conjugates, purchased from either BD Pharmingen (Franklin Lakes, NJ, USA) or eBioscience (San Diego, CA, USA), were used in
the double or triple staining as described. PE-anti-CD4, CD25, CD69, PD-1, PD-L1, FoxP3 transcription factor; FITC-anti-CD4, CD8, CD45RA, CD45RO; and APC-CD25, PD-1, PD-L1. The primary PE-, FITC-, APC-isotype controls were used to determine the level of background staining. In triple staining, gated lymphocytes were further gated on CD4+ or CD8+ cells; then analyzed for the expression of cell surface markers in the double-positive cell populations. For intracellular FoxP3 staining, a human Treg staining kit (eBioscience) was employed and the staining was performed according to the manufacturer’s instruction.

Cell proliferation
PBMCs isolated from subjects who were prelabeled with CFSE according to the instructions per CellTrace Kit (Molecular Probes, Eugene, OR, USA). To determine the role of PD-1 pathway in regulation of T-cell proliferation, the CFSE-labeled cells were incubated with anti-PD-1 or a control isotype IgG antibody (10 μg/ml, eBioscience) overnight then stimulated the cells with PHA (1 μg/ml), Sigma) or anti-CD3/CD28 or autologous PBMC for 5 days. After double staining and gating on T lymphocytes, the cell proliferation, represented as CFSE dilution from M4/M3 (resting cells) to M2/M1 (proliferating cells), was analyzed by flow cytometry with 488 nm excitation and emission filters appropriate for CFSE fluorescein. Percentage of cells as detected in the M1-, M2-, M3- and M4-gating was shown on the histogram of the gated T-cell populations.

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