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PD-1 modulates Regulatory T cells and suppresses T cell responses in HCV-associated Lymphoma

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Abstract

T regulatory (T_R) cells suppress T cell responses that are critical in the development of chronic viral infection and associated malignancies. Programmed death-1 (PD-1) also plays a pivotal role in regulation of T cell functions during chronic viral infection. To examine the role of PD-1 pathway in regulating T_R cell functions that inhibit T cell responses during virus-associated malignancy, T_R 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 $CD4^+CD25^+$ and $CD8^+CD25^+$ T_R cells as well as high levels of PD-1 expressions on these T_R cells were found in the peripheral blood of subjects with HCV-L compared to those from non-HCV-L or HCV alone or HS. T_R cells from the HCV-L subjects were capable of suppressing the autogeneic lymphocyte response, and depletion of T_R cells in PBMC 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 *ex vivo*, resulting in both a reduction in T_R cell number and the ability of T_R to suppress the activity of effector T cells. This study suggests that the PD-1 pathway is involved in regulating T_R cells that suppress T cell functions in the setting of HCV-associated B cell lymphoma.

Keywords

T regulatory cells; PD-1; T cell suppression; HCV; lymphoma

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Introduction

Hepatitis C virus (HCV) infects over 180 million people worldwide and exhibits a remarkable propensity toward chronic hepatitis, liver cirrhosis and hepatocellular carcinoma[1]. Chronic HCV infection is also associated with B cell lymphoproliferative disorders[2], including most notably mixed cryoglobulinemia (MC) and B cell lymphomas. In chronically HCV-infected individuals, the frequencies of cytotoxic T lymphocytes (CTL) are relatively low; similarly, the proliferative capacity as well as effector functions of HCV-specific $CD4^+/CD8^+$ T cells are impaired, and the production of Th1-type cytokines (i.e., IL-2 and IFN-γ) is dramatically suppressed[3–8].

T regulatory (T_R) cells, including $CD4^+CD25^+$ as well as $CD8^+CD25^+$ cell populations, suppress effector/memory T cell responses that are critical in clearance of pathogens, thus playing a pivotal role in the establishment and maintenance of chronic infections such as HCV[9–16]. Understanding the mechanisms underlying T cell dysregulation in HCV infection has also been advanced since the identification of program death-1 (PD-1), an important inhibitory pathway in regulation of T cell receptor (TCR) signaling[17–20]. We have previously demonstrated that HCV core protein differentially regulates T and B lymphocyte functions through PD-1 and SOCS-1 negative signaling pathways[21–27]. 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) [Yao, et al. Immunological Investigation, in revision]. The relationship between PD-1 pathway and T_R 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 $CD4^+CD25^+$ and $CD8^+CD25^+$ T_R cells as well as higher levels of PD-1 expression on these T_R cells in the peripheral blood of subjects with HCV-L compared to those from non-HCV-L or HCV alone or HS. Interestingly, PD-1 appears to play a role in regulating T_R 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_R cells in regulation of T cell responses, we first determined the relative number of $CD4^+CD25^+$ and $CD8^+CD25^+$ T_R cell populations in the peripheral blood of subjects with HCV-L, and compared with those in non-HCV-L, HCV alone, or HS. As shown in Fig. 1A, both $CD4^+CD25^+$ and $CD8^+CD25^+$ T_R cells were found to be increased in the subjects with HCV-L when compared to those non-HCV-L, HCV alone, or HS. Similar results were obtained in repeated experiments using different antibody conjugates to compare $CD4^+CD25^+$ T_R cell numbers. Since the transcription factor Foxp3 has been accepted as a specific marker for T_R cells[28–29], intracellular FoxP3 expression was analyzed in $CD4^+CD25^+$ T_R obtained from a subject with HCV-L, three HCV alone, and a HS. As shown in figure 1B, $CD4^+CD25^+$ T_R cells were found to be higher in the subject with HCV-L compared to 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_R cell component in the setting of HCV-L.

Since CD25 (IL-2R α chain) is an early T cell activation marker, higher levels of CD25 expression on $CD4^+$ T cells might simply be due to T cell activation rather than secondary to the development of T_R cells in subjects with HCV-L. To further elucidate this concern, we stimulated PBMC isolated from HCV-L, HCV alone, and HS with PHA for 24 h, and then evaluated CD69 expression, a marker of T cell activation, on $CD4^+CD25^+$ T_R cell populations. As shown in figure 1C, CD69 expression on $CD4^+CD25^+$ T cell populations in the setting of HCV-L was lower than in subjects with HCV alone or HS, suggesting that the increased CD25 marker is secondary to the increased number of T_R rather than T cell activation. Additionally, CD69 expressions were found to be lower on $CD4^+CD25^-$ T cell populations as well as on $CD8^+CD25^-$ T cells and HCV-specific $CD8^+$ T cells in HCV-L subjects when compared with HCV alone or HS, which is consistent with the finding that T cell activation is more severely suppressed in the setting of HCV-associated lymphoma.

It has been previously reported that PD-1 is highly expressed on $CD4^+CD25^+$ T_R cells[30–31], although its role in regulating T_R cell number and activity remains less clear. Here we measured PD-1 expression on $CD4^+CD25^+$ and $CD8^+CD25^+$ T_R cells obtained from the subjects with HCV-L, Non-HCV-L, and HCV alone without lymphoma by three-color-labeling FACS analysis and also found this high level of expression to be the case on T_R cells in all groups, i.e., $CD4^+CD25^+$ and $CD8^+CD25^+$ T_R cells exhibit relative higher levels of PD-1 expression versus $CD4^+CD25^-$ and $CD8^+CD25^-$ effector T cells, with the highest PD-1 level in the setting of HCV-L (fig. 1D).

Because we had found increased numbers of T_R cells and higher level of PD-1 expression on these cells in the setting of HCV-L, we next examined the possibility that the PD-1 pathway regulates T_R cells' ability to inhibit T cell activation and proliferation. To this end, we 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_R cells and CD69 expression on $CD4^+$ T cells. As shown in Fig. 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_R cells (top), and increased CD69 expression (bottom) on the suppressed $CD4^+$ T cells. These results suggest that blocking the PD-1 pathway may regulate T_R 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 pre-incubated CFSE-labeled PBMC with anti-PDL-1 or control antibody followed by 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 Fig. 2B, the proliferating T cells (shown in the M1 and M2 gates) were detected more frequently in the setting of anti-PDL-1 treatment compared to the control antibody-treated cells when PBMC were stimulated with anti-CD3/CD28 antibodies (upper panel). This difference was more significant when the cells were stimulated with autogeneic healthy PBMC (lower panel), in which case 47% and

23% of proliferating T cells were detected in the M1 and M2 gates, respectively, upon blocking with PD-1 signaling versus 28% and 22% cells detected with control antibody treatment. Correspondingly, the resting T cells in M3 gating following anti-PD-L1 treatment were observed at 29%, while 48% were detected in the control group. These results suggest that blocking the PD-1 pathway may rescue impaired T cell function in HCV-L.

Even though blocking PD-1 signaling can reduce the number of T_R cells and rescue the suppressed T cell activation and proliferation *ex vivo*, the effect on rescuing T cell function by PD-1 blocking may be secondary to the reduced T_R 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 $CD4^+CD25^+$ T_R cells in suppressing T cell proliferation by mixed lymphocyte culture (MLC). To this end, we separated the $CD25^+$ cells and $CD25^-$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 from the subject to suppress healthy PBMC proliferation by MLC. As shown in Fig. 2C, healthy PBMC co-cultured with $CD25^+$ T_R cells from the HCV-L subject proliferated poorly when compared to those co-cultured with $CD25^-$ cells (upper panel). Repeated experiments using effector cells from another healthy subject were similar (lower panel). These results suggest that T_R cells functionally suppress autogeneic healthy T cell proliferation.

Because *in vitro* depletion of $CD25^+$ cells results in increased responsiveness of the HCV-specific effector cells[9–11], it has been suggested that induction of T_R cells play a causal role in the establishment of chronic HCV infection. To further elucidate the role of T_R cells and PD-1 in suppressing T cell proliferation, we compared the proliferative ability of $CD25^+$-depleted cells versus bulk PBMC from a subject with HCV-L, in the presence of anti-PDL-1 or control antibody, by CFSE dilution and flow cytometry analysis. As shown in Fig. 2D, $CD25^+$-depleted cells treated with anti-PDL-1 proliferated better compared to bulk PBMC treated with anti-PD-L1 or $CD25^+$-depleted cells treated with the control antibody. The least proliferation was observed in the setting of bulk PBMC treated with the control antibody (Fig 2D, lower right panel). These results suggest that both T_R and PD-1 signaling play a role in suppressing T cell proliferation in the setting of HCV-L.

Although several biomarkers have previously been recognized to play a role in defining T_R cells in disease conditions[30–31], the discovery of high expression of PD-1 in high number of T_R 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 T_R cells as a means of suppressing T cell responses in the context of chronically HCV-infected subjects with B cell lymphoma. It remains unclear as to whether the increased T_R cells are a consequence of or contribute to the development of HCV-L.

Materials and Methods

Subjects

An institutional review board (IRB)-approved protocol at James H. Quillen VA Medical Center and East Tennessee State University (Johnson City, TN) 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 healthy subjects as normal controls are included in this study. All HCV subjects, either genotype 1a or 1b, with HCV RNA levels range from 97,765~50,000,000 IU/ml, were selected prior to peg-interferon 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 post-antitumor therapy for at least six months.

Cell isolation and culture

Human peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood of subjects by Ficoll density centrifugation with lympholyte-H (Cedarlane Labs, Ontario, Canada). If indicated, $CD25^+$ 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). Cells were cultured as described previously[25].

Flow cytometry

To determine cell surface marker expression on T lymphocytes, 1×10^6 purified PBMCs were stimulated with either 1 μg/ml PHA (Sigma, Saint Louis, MI), or 10 μg HCV peptides (HLA-A-0201/NS3/1073-1081/CINGVCWTV; HLA-A-0201/NS3/1406-1415/KLVALGINAV; synthesized by GenScript Corporation, Piscataway, NJ), for 24 h and washed as described[24–25]. The following antibody conjugates, purchased from either BD Pharmingen or eBioscience, were used in the double or triple staining as described[25–26]: 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 were pre-labeled with CFSE according to the instructions per CellTrace Kit (Molecular Probes, Inc., Eugene, OR). To determine the role of PD-1 pathway in regulation of T cell proliferation, the CFSE-labeled cells were incubated with anti-PD-L1 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 autogenic PBMC for 5 days. After double staining and gating on T lymphocytes, the cell proliferation, represented as CSFE 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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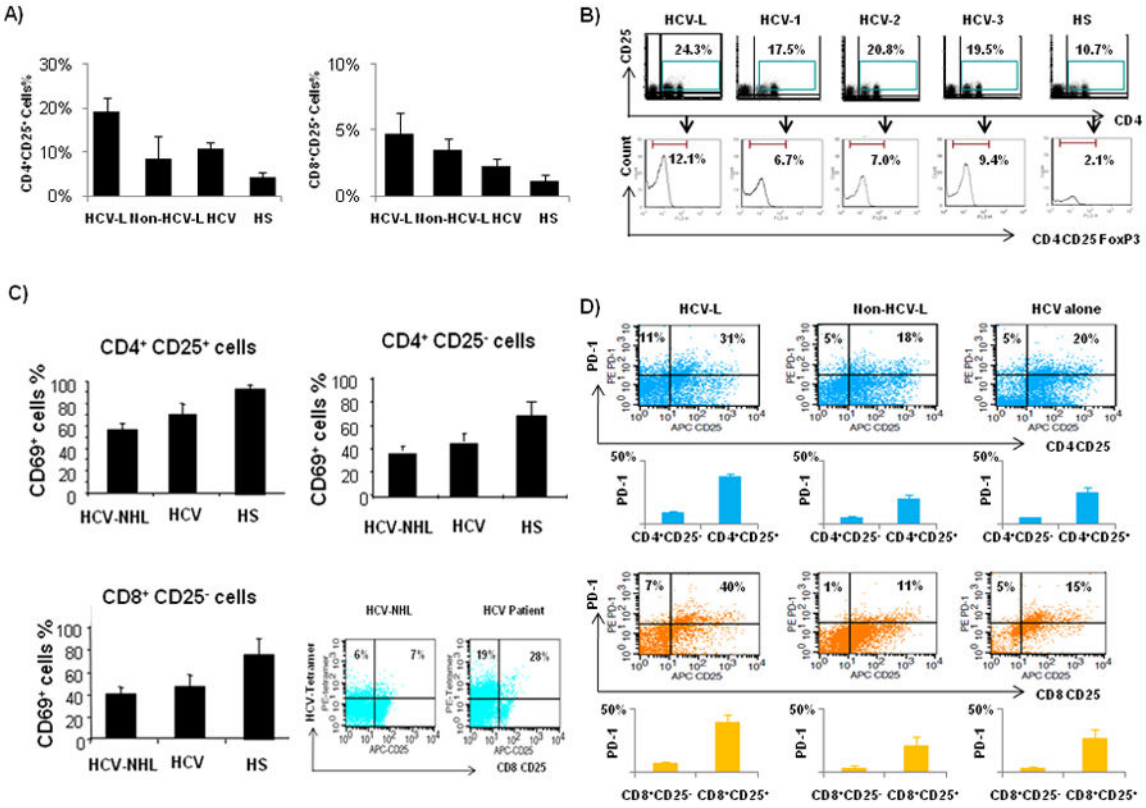

Fig. 1. T_R cells and PD-1 expression are increased in HCV-L

A) Increased $CD4^+CD25^+$ and $CD8^+CD25^+$ T_R cells in HCV lymphoma. PBMC, isolated from three subjects with HCV-L, three non-HCV-L, three HCV subjects without lymphoma, and three healthy subjects were subjected to flow cytometric analysis following double staining with PE-anti-human CD25 and FITC-anti-CD4 or CD8 antibodies. Summary of the percentages of $CD25^+$ cells in the gated $CD4^+$ or $CD8^+$ T cell populations are shown. Similar results were obtained in repeated experiments using different antibody conjugates to compare $CD4^+CD25^+$ T_R cell numbers. B) Increase in $CD4^+CD25^+FoxP3^+$ T_R cells in HCV-L. PBMC, isolated from a subject with HCV-L, HCV subjects without lymphoma, or a healthy subject, were subjected to flow cytometric analysis following triple staining with FITC-anti-CD4, PE-anti-human CD25, and APC-anti-FoxP3 antibodies. Gating strategy is shown above and intracellular expression of FoxP3 in the gated $CD4^+CD25^+$ cell population is shown in the histogram. C) CD69 expression on T_R cells in HCV-L. PBMC isolated from HCV-L, HCV alone, and healthy individuals were treated with PHA for 24 h, and then evaluated for CD69 expression, a marker for T cell activation, on $CD4^+CD25^+$ T_R cell populations via flow cytometry. Mean ± SD of the CD69 expressions on $CD4^+CD25^+$, $CD4^+/CD25^-$, and $CD8^+CD25^-$ cells in three HCV-L subjects versus three HCV alone and three healthy subjects are shown. HCV-tetramer staining was also carried out in $CD8^+CD25^+$ and $CD8^+$ $CD25^-$ T cells stimulated with HCV NS3 peptides in subjects with HCV-L vers HCV without lymphoma. D) PD-1 expression is high on T_R cells in HCV-L $CD8^+CD25^-$ effector T cells. PBMC isolated from subjects with HCV-L, non-HCV-L, or HCV alone were treated with PHA for 24 h, and then evaluated for PD-1 expression on $CD4^+CD25^+$ and $CD8^+CD25^+$ T_R cells versus $CD4^+CD25^-$ or $CD8^+CD25^-$ 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.

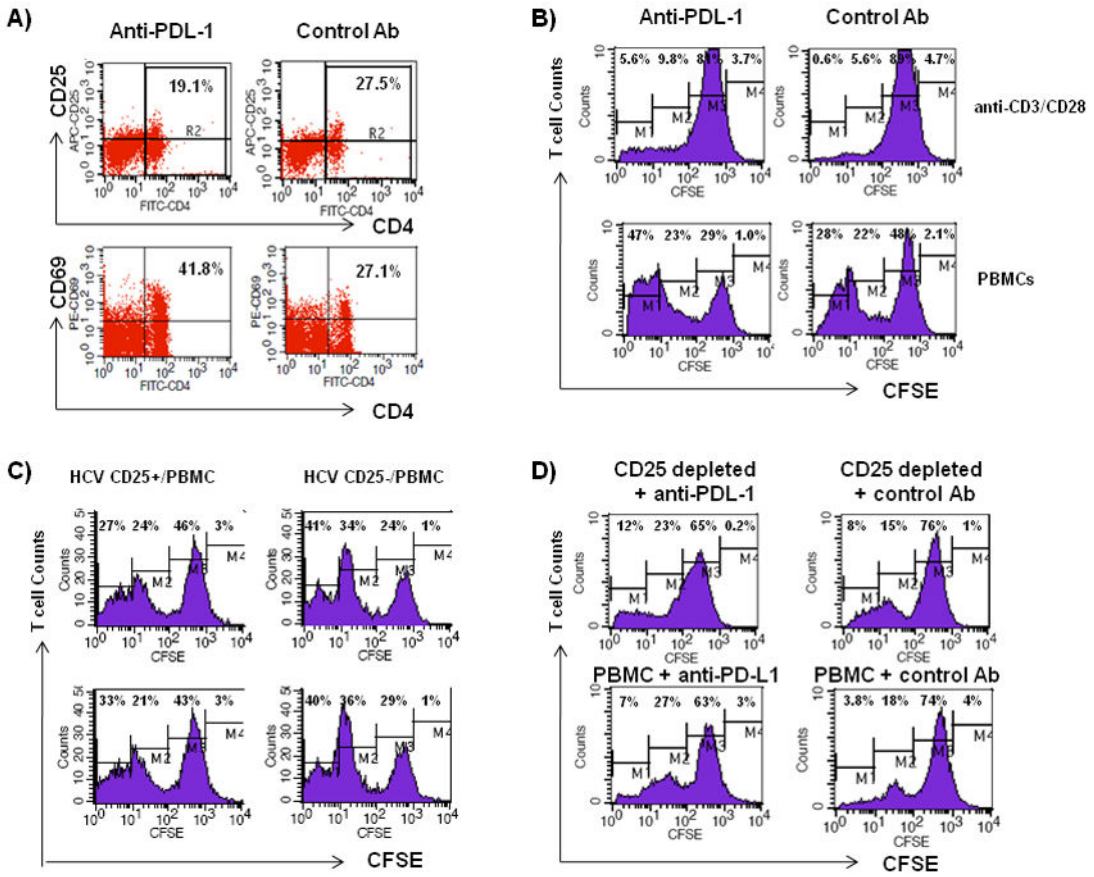

Fig. 2. PD-1 signaling regulates T_R cells to suppress T cell activation and proliferation

A) Blocking the PD-1 pathway regulates T_R cell number and rejuvenates $CD4^+$ T cell activation. PBMC isolated from an HCV-L subject were treated with anti-PDL-1 (10 μg/ml, eBiosciences, San Diego), or control antibody overnight, followed by incubation with HCV peptides (10 μg/ml, GenScript, Piscataway, NJ) *ex vivo* for 5 days; total number of $CD4^+CD25^+$ T_R cells and CD69 expression on $CD4^+$ T cells were examined by flow cytometry. B) Blocking PD-1 pathway partially restores the proliferation of T cells isolated from HCV-L. CFSE-labeled PBMC were incubated with anti-PDL-1 or a control antibody overnight, then stimulated with either anti-CD3/CD28 (upper panels, 1 μg/ml each) or healthy PBMC (lower panels, with the HCV-L: healthy PBMC ratio = 5:1) 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. C) $CD25^+$ T cells isolated from HCV-L inhibit healthy T cell proliferation. $CD25^+$ cells and $CD25^-$ cells were isolated from a subject with HCV-L, incubated with CFSE-labeled healthy PBMC (with the HCV-L: healthy PBMC ratio = 1:5) for 5 days, and then examined by CSFE dilution using flow cytometry. Upper and lower panels represent PBMC from two different healthy subjects. D) $CD25^+$ T_R cell depletion and PD-1 blockade improve T cell proliferation in HCV-L. CSFE-labeled, $CD25^+$-depleted cells (upper panels) or bulk PBMC (lower panels) isolated from a subject with HCV-L were treated with anti-PDL-1 or control antibody, followed by anti-CD3/CD28 (1 μg/ml each) and subsequent CFSE dilution analysis by flow cytometry.

Table 1

Clinical characteristics of the subjects included in the study.

Subject	Diagnosis*	Age	Gender	GT	Viral load (IU/ml)	Lymphocyte count	Treatment**
1	HCV-NHL	56	M	1a	97,765	1.6×10^3	s/p chemotherapy
2	HCV-NHL	62	M	1b	1,260,000	0.9×10^3	s/p stem cell transplant
3	HCV-NHL	59	M	1a	8,730,000	1.8×10^3	s/p chemotherapy
4	HCV alone	65	M	1a	50,000,000	1.6×10^3	prior to pegIFN + RBV
5	HCV alone	51	M	1a	2,110,000	2.4×10^3	prior to pegIFN + RBV
6	HCV alone	63	M	1b	530,590	1.7×10^3	prior to pegIFN + RBV
7	NHL alone	58	M	N/A	N/A	1.3×10^3	s/p chemotherapy
8	NHL alone	61	M	N/A	N/A	1.8×10^3	s/p chemotherapy
9	NHL alone	59	M	N/A	N/A	1.2×10^3	s/p chemotherapy
10	Healthy	51	M	N/A	N/A	1.9×10^3	N/A
11	Healthy	49	M	N/A	N/A	2.5×10^3	N/A
12	Healthy	43	M	N/A	N/A	1.8×10^3	N/A

*All the NHL patients were diagnosed by clinical and histological features, and confirmed by immunochemistry or flow cytometry studies revealing that tumor cells were positive for B cell markers. Subject 1 was diagnosed as follicular lymphoma grade 2, immunohistochemical stains positive for L26, CD10, CD20, Bcl-2, and Bcl-6. Subject 2 was diagnosed as follicular lymphoma grade 3a, immunohistochemical features positive for L26, CD10, CD20, Bcl-2 and Bcl-6. Subject 3 was diagnosed as diffuse large B cell lymphoma, immunohistochemical stains positive for CD10, CD20, and CD30. Subject 7 and 8 was diagnosed as follicular lymphoma grade 2. Subject 9 was diagnosed as diffuse large B cell lymphoma, immunochemistry showed large atypical lymphoid cells that stained strongly for CD20.

**pegIFN + RBV = pegylated interferon and ribavirin therapy. GT=genotype.