# An Immunomodulatory Role For $CD4^+CD25^+$ Regulatory T Lymphocytes in Hepatitis C Virus Infection

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**The $CD4^+CD25^+$ regulatory T lymphocytes have been implicated in suppressing T cell immune responses. Our aim was to characterize the frequency, phenotype, function, and specificity of $CD4^+CD25^+$ T cells in hepatitis C virus (HCV) infection. Peripheral $CD4^+CD25^+$ cells from recovered (n = 15), chronic infected (n = 30), and normal control (n = 15) subjects were analyzed *ex vivo* for quantitation, phenotype, and effect on HCV-specific interferon gamma production and proliferation. $CD4^+CD25^+$ specificity was determined by intracellular cytokine staining for interleukin 10 (IL-10). A higher proportion of $CD4^+CD25^+$ were found in chronic infection (mean, 3.02%) when compared with recovered (1.64%, $P = .001$) and normal controls (2.27%, $P = .02$). $CD4^+CD25^+$ cells display $CD45RO^{high}$, $CD45RA^{low}$, $CD28^{high}$, $CD62L^{high}$, and $CD95^{high}$ phenotype. HCV-specific interferon gamma activity was enhanced in peripheral blood mononuclear cells depleted of $CD4^+CD25^+$ and suppressed in peripheral blood mononuclear cells enriched with $CD4^+CD25^+$. Depletion of $CD4^+CD25^+$ cells also enhanced HCV-specific $CD4^+$ and $CD8^+$ T cell proliferation. Cytokine analysis suggested $CD4^+CD25^+$ cells secrete transforming growth factor beta (TGF-$\beta_1$) and IL-10. The inhibitory role for TGF-$\beta_1$ was confirmed by anti–TGF-$\beta_1$. Transwell studies showed $CD4^+CD25^+$ mediated suppression to be dose dependent and requiring cell contact. $CD4^+CD25^+$ cells showed HCV-specificity through IL-10 production, with a frequency ranging from 1.9% to 5.3%. A positive correlation was detected between $CD4^+CD25^+$ T cell frequency and HCV RNA titer, whereas an inverse relation was found with liver inflammatory activity. In conclusion, $CD4^+CD25^+$ T lymphocytes constitute a highly differentiated population and appear to play a role in viral persistence by suppressing HCV-specific T cell responses in a cell–cell contact manner.** (HEPATOLOGY **2004;40:1062–1071.**)

After hepatitis C virus (HCV) infection, interaction between the innate and adaptive immune response plays a pivotal role in perpetuation or clearance of HCV infection. After exposure to HCV, the quantity and breadth of interferon (IFN)-$\gamma$ HCV specific $CD8^+$ T cells has been directly associated with eradication in those patients with self-limited infection versus patients with a chronic course.[1] Furthermore, the differentiation of naïve $CD4^+$ T cell into HCV-specific inflammatory $CD4^+$ T cell ($T_H1$) or helper $CD4^+$ T cell ($T_H2$) with their respective, disparate cytokine profiles is also an integral part in determining the outcome of HCV infection.[2] The $T_H1$ proinflammatory cytokine profile (IFN-$\gamma$, tumor necrosis factor alpha [TNF-$\alpha$]) promote an antiviral immune response targeted at cytosolic and intracellular antigens by stimulating cytotoxic $CD8^+$ T cells and natural killer activation. The $T_H2$ anti-inflammatory cytokines (interleukin 10 [IL-10], transforming growth factor beta [TGF-$\beta$]) downregulate the $T_H1$ response. A strong $T_H1$ response has been observed in those who clear acute HCV infection, whereas a blunted $T_H1$ response with a more pronounced $T_H2$ response is seen in those with chronicity.[3]

Recently, much attention has focused on regulatory T cells (Tregs) and their contribution to disease states. The

*Abbreviations: HCV, hepatitis C virus; IFN, interferon; TNF-α, tumor necrosis factor alpha; IL-10, interleukin-10; TGF β1, transforming growth factor beta 1; Tregs, $CD4^+CD25^+$ regulatory T lymphocytes; PBMC, peripheral blood mononuclear cells; DC, dendritic cells; PHA, phytohemagglutinin antigen; HIV, human immunodeficiency virus; TT, tetanus toxoid.*

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*Received October 31, 2003; accepted July 13, 2004.*

*Supported by NIH grant DK2595, HL64817, and GCRC grant RR00082 (D.R.N.); an award from the American Liver Foundation and NIDDK grant DK02958 (C.L.); and NIH training grant T32 and LRP (R.C.).*

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*Published online in Wiley InterScience (www.interscience.wiley.com).*

*DOI 10.1002/hep.20454*

immune system employs naturally arising $CD4^+$ regulatory (or suppressor) T cells, which are engaged in the maintenance of self-tolerance by suppressing the activation and expansion of self-reactive lymphocytes that may cause autoimmune disease.[4] A controlled balance between initiation and downregulation of the immune response is vital in maintenance of immune homeostasis, because dysfunctional immune resolution may lead to chronic inflammation and/or autoimmunity.[5] Most regulatory cells constitutively express CD25 (the IL-2 receptor alpha-chain) in the physiological state. This Treg population, which constitutes 5% to 10% of peripheral $CD4^+$ T cells in humans, has a broad repertoire that recognizes various self and nonself antigens.[6] Evidence has accumulated that removal or reduction of this population can also enhance immune responses against infectious microbes.[7–14] More recently, Tregs in humans appear to suppress proliferation to self antigen as well as to foreign antigens.[15]

Because a hallmark of chronic HCV infection is impaired HCV-specific effector T cell responses,[16] it is intriguing to hypothesize that Tregs may play a role with long-term persistence of HCV infection. In this study, we show that Tregs are a highly differentiated population that suppress HCV-specific $CD4^+$ and $CD8^+$ T cell responses in a dose-dependent, cell contact manner involving TGF-$\beta_1$ and IL-10 and may play a role in viral persistence.

## Patients and Methods

***Patients and Controls.*** Sixty individuals were recruited using a protocol approved by the internal review board at the University of Florida. The study subjects included spontaneous recovered (n = 15), chronic HCV infection (n = 30), and healthy controls (n = 15). The recovered samples were from individuals with anti-HCV antibody, a history of acute HCV exposure with resolution, and negative HCV RNA by the transcription-mediated amplification qualitative assay.[17] The chronic group represents treatment-naïve subjects, all of whom had a liver biopsy, HCV RNA level, and serum alanine aminotransferase. In addition, 5 patients with chronic infection undergoing liver transplantation were used to evaluate compartmentalization of $CD4^+CD25^+$ cells.

***Peripheral Blood Mononuclear Cells, Lymph Nodes, and Liver Preparation.*** Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood as described previously.[18] Liver and perihepatic lymph nodes obtained at the time of transplantation were processed with collagenase treatment. Tissue samples were dissected into 1-$mm^3$ pieces, added to cRPMI 1640 medium and collagenase type IV at 100 $\mu$L/mL, and the mixture incubated at 37°C for 60 minutes. The supernatant was removed and diluted in cRPMI 1640 medium, centrifuged at 125*g* for 10 minutes.

***HCV Peptide, Tetramers, and Antigen.*** HLA-A0201–restricted peptides NS3 (CINGVCWTV) and NS5B (GLQDCTMLV) synthesized at ICBR Protein Chemistry (Gainesville, FL), and HCV core antigen peptide (aa 2-122; Biodesign, Kennebunk, ME) were purified and processed as described previously.[18] PE-labeled HLA-A2 tetramer complexes were synthesized as described by Altman et al.[19] HCV antigens were obtained from commercial sources (Chiron, Emeryville, CA): Core, NS3, NS4, NS4/5, and NS5. These antigens were pooled at a final concentration of 10 $\mu$g/mL for use in some assays.

***Generation of Dendritic Cells.*** Dendritic cells (DCs) were generated from PBMC as described by Romani et al.[20]

***Preparation of PBMC, $CD8^+$, $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^-$.*** The effect of $CD4^+CD25^+$ cells on HCV-specific IFN-$\gamma$ responses was tested with 3 sets of PBMC: (1) PBMC; (2) PBMC depleted of $CD4^+CD25^+$ (PBMC – $CD4^+CD25^+$); and (3) PBMC supplemented with $CD4^+CD25^+$ (PBMC + $CD4^+CD25^+$). The PBMC – $CD4^+CD25^+$ subset was created by mixing CD4-depleted PBMC, obtained by negative $CD4^+$ cell selection from whole PBMC ($20–30 \times 10^6$) with CD4 microbeads (Miltenyi Biotec, Auburn, CA), and the $CD4^+CD25^-$ fraction, obtained by depleting the negatively selected $CD4^+$ cell fraction of $CD25^+$ cells (%$CD4^+CD25^+$ cells $< 10\%$), using positive selection beads (Miltenyi). The PBMC + $CD4^+CD25^+$ subset was established by adding the positively selected $CD25^+$ cells from the negatively selected $CD4^+$ cell fraction to PBMC at a 10:1 ratio ($0.2 \times 10^6$ PBMC: $0.02 \times 10^6$ $CD4^+CD25^+$). The final %$CD4^+CD25^+$ cells/well in the PBMC + $CD4^+CD25^+$ subset ranged between 8% and 18%.

The effect of $CD4^+CD25^+$ cells on HCV-specific T cell proliferation was examined by using $CD4^+$, $CD4^+CD25^-$, and negatively selected $CD8^+$ cells (Miltenyi) as effectors, when cocultured with $CD4^+CD25^+$ cells in CFSE and [$^3$H]thymidine assays. Purity of each fraction was $> 90\%$ by flow cytometry.

***CFSE Experiments.*** $CD8^+$ cells were incubated with 10 $\mu$mol/L CFSE (Molecular Probes, Eugene, OR) for 10 minutes at 37°C. Labeled $CD8^+$ cells were used as effectors in mixing culture experiments. Four groups of mixing experiments were analyzed to determine the suppressive effect of Tregs on HLA-A2–restricted HCV-cytotoxic T lymphocytes generated from pure $CD8^+$ cells as described previously.[18] CFSE-labeled $CD8^+$ cells were cocultured with DCs at a ratio of 20:1, 10 $\mu$g/mL pep-

tide, and 10 ng/mL IL-7 (B&D) as well as 1 of 3 fractions: (1 )$CD4^+CD25^+$; (2 ) $CD4^+CD25^-$; or (3) $CD4^+$ cells at a 10:1 ratio ($1 \times 10^6$ CD8:$0.1 \times 10^6$ CD4 fraction) . On day 7, cells were stained with PE-labeled HLA-2 tetramer complexes and analyzed by flow cytometry.

***ELISpot and Enzyme-Linked Immunosorbent Assay.*** An established ELISpot was used to evaluate IFN-$\gamma$ HCV-specific responses in the 3 PBMC subsets.[18] Enzyme-linked immunosorbent assays were used to measure TGF-$\beta_1$ (Promega, Madison, WI) and IL-10 (Endogen, Woburn, MA) in 48-hour culture supernatants. Neutralizing experiments were performed by adding 2 $\mu$g monoclonal anti-human TGF-$\beta_1$ (R&D Systems, Minneapolis, Minnesota) to 1 of the duplicate sets of the 3 PBMC subsets.

***Proliferation and Transwell Studies.*** The [$^3$H]thymidine protocol was performed as described previously.[18] In this assay, $CD4^+CD25^-$ cells served as effectors. They were cocultured with DCs in the presence of pooled HCV antigens (10 $\mu$g/mL), phytohemagglutinin antigen (PHA; 5 $\mu$g/mL), human immunodeficiency virus (HIV) antigens (10 $\mu$g/mL), or in the absence of antigens. Treg-mediated suppression of HCV-specific CD4 proliferation was examined by mixing effectors with $CD4^+CD25^+$ at a 10:1 ($2 \times 10^5$ effector: $0.2 \times 10^5$ Treg) and 2:1 ($2 \times 10^5$ effector:$1 \times 10^5$ Treg) ratio. The mean counts per minute (cpm) were calculated as mean cpm $\times 10^3$ after subtracting the cpm obtained in the absence of antigen.

Transwell experiments were conducted to determine whether $CD4^+CD25^+$-mediated suppression of $CD4^+CD25^-$ effectors requires cell contact. $CD4^+CD25^+$ cells ($2 \times 10^5$) were stimulated in the upper chamber with autologous DCs (20:1 ratio) and pooled HCV antigens, and $CD4^+CD25^-$ effectors ($2 \times 10^5$) were stimulated in the lower chamber with DCs (20:1 ratio) and pooled HCV antigens. After 72 hours, [$^3$H]thymidine was added to measure the responses of $CD4^+CD25^-$ effectors in the outer chambers.

***IL-10 Intracellular Cytokine Staining.*** Intracellular cytokine staining was performed by use of the BD Cytofix/Cytoperm kit (BD Biosciences, San Diego, CA). Briefly, $5 \times 10^5$ $CD4^+CD25^+$ cells, and DCs were incubated at a ratio of 10:1 with medium, 10 $\mu$g/mL HIV antigens, or 10 $\mu$g/mL HCV pooled antigens at 37°C for 2 days. After adding the transport inhibitor BD Golgistop at 0.67 $\mu$L/mL, the culture was incubated at 37°C for 5 hours. After blocking with 10% human antibody serum, cells were labeled with CD4–FITC, CD25-cychrome and permeabilized. Intracellular staining was performed with PE-labeled IL-10 antibodies or isotype control. $CD4^+$ and $CD4^+CD25^-$ cells cultured with DCs in medium or with pooled HCV antigens also served as controls.

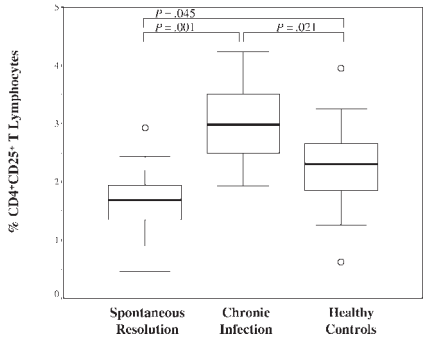


Fig. 1. An *ex vivo* comparison of peripheral blood $CD4^+CD25^+$ T cell frequency in spontaneous resolution (n = 15), chronic infection (n = 30), and normal control (n = 15) subjects. Data are expressed as box plots, in which the horizontal lines illustrate the 25th, 50th, and 75th percentiles of the frequencies of the $CD4^+CD25^+$ T lymphocytes as assessed by flow cytometry. The vertical lines represent the 10th and 90th percentiles, and circles denote values outside these percentiles. The *P* values were calculated by using the nonparametric Mann-Whitney *U* test.

***Flow Cytometry.*** The following monoclonal antibodies were used: anti-CD4, anti-CD25, anti-CD8, anti-CD45RO, anti-CD45RA, anti-CD27, anti-CD28, anti-CD95, anti-CD69, anti-CD38, anti-CCR5, anti-CD62L, and anti-Ki67 (BD PharMingen). Staining was performed with FITC, PE, Cychrome, and APC-coupled antibodies. Acquisitions and data analysis were performed with a FACSCalibur (BD Biosciences) and CellQuest software.

***Statistical Analysis.*** The Mann-Whitney *U* test and linear regression analysis using the Spearman correlation coefficient were performed with the SPSS 10.1 program. All *P* values are 2-tailed.

## Results

***$CD4^+CD25^+$ Frequency and Distribution.*** Prevalence of $CD4^+CD25^+$ cells in HCV infection was defined *ex vivo* from PBMC in chronic infection, self-limited infection, and normal controls by flow cytometry. A significantly higher proportion of $CD4^+CD25^+$ cells were found in those with chronic infection (median, 2.98%; mean, 3.02%; SE $\pm$ 0.11) when compared with recovered (1.68%, 1.64% $\pm$ 0.17, $P = .001$) and normal controls (2.30%, 2.27% $\pm$ 0.22, $P = .02$), suggesting a relationship with viral persistence (Fig. 1). Additionally, a lower prevalence of $CD4^+CD25^+$ cells was found in those with spontaneous resolution when compared with normal controls ($P = .04$). The distribution of $CD4^+CD25^+$ in PBMC, lymph node, and liver tissue was determined in 5 patients with chronic infection. The frequency of $CD4^+CD25^+$ cells was higher in the periph-

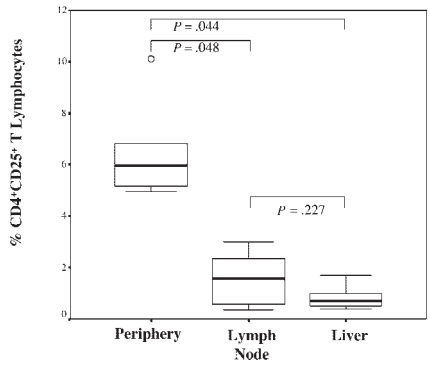


Fig. 2. Comparison of $CD4^+CD25^+$ T cell frequency in the liver, perihepatic lymph node, and peripheral blood of subjects with chronic infection at the time of liver transplantation (n = 5). Data are expressed as box plots, in which the horizontal lines illustrate the 25th, 50th, and 75th percentiles of the frequencies of the $CD4^+CD25^+$ T lymphocytes as assessed by flow cytometry. The vertical lines represent the 10th and 90th percentiles, and circles denote values outside these percentiles. The *P* values were calculated by using the nonparametric Mann-Whitney *U* test.

ery (median, 5.97%; mean, 6.59% ± 0.93), followed by perihepatic lymph nodes (1.55%, 1.56% ± 0.50); they were least abundant in liver parenchyma (0.69%, 0.84% ± 0.23) (Fig. 2).

***$CD4^+CD25^+$ Cells Display $CD45RO^{high}$, $CD45RA^{low}$, $CD27^{high}$, $CD28^{high}$, $CD62L^{high}$, $CD95^{high}$, $CD69^{low}$, and $CCR5^{low}$ Phenotype.*** Cell surface phenotypes of $CD4^+CD25^+$ in spontaneous resolution, chronic infection, and normal controls were compared by phenotype, level of activation, differentiation, memory, and homing marker with flow cytometry (Table 1). $CD4^+CD25^+$ cells display $CD45RO^{high}$, $CD45RA^{low}$, $CD27^{high}$, $CD28^{high}$, $CD62L^{high}$, $CD95^{high}$, $CD69^{low}$ and $CCR5^{low}$ phenotype in peripheral blood. No significant phenotypic differences were found when comparing recovered, chronic infected, and controls. In addition, the phenotypes of the $CD4^+CD25^+$ lymphocytes in peripheral blood, lymph node, and liver tissue from 5 patients with cirrhosis from chronic infection showed no compartmental differences (data not shown).

***Effect of $CD4^+CD25^+$ on HCV-specific $CD4^+$ T Cell Immune Responses.*** To demonstrate the effect of Tregs on HCV-specific $CD4^+$ responses, PBMC depleted or enriched with $CD4^+CD25^+$ was analyzed by IFN-γ ELISpot and compared with whole PBMC responses in subjects with chronic infection and spontaneous recovery. As shown in Fig. 3, depletion of $CD4^+CD25^+$ increased HCV-specific IFN-γ activity in chronic infection in response to core (mean, 108 vs. 84, $P < .01$), NS3 (37 vs. 26, $P < .01$), NS4 (29 vs. 9, $P < .01$), NS4/5 (100 vs. 64, $P < .01$), and NS5 (40 vs. 18, $P < .05$); and in recovered subjects in response to NS3 (645 vs. 295, $P < .01$), NS4/5 (591 vs. 468, $P < .05$) and NS5 (328 vs. 113, $P < .05$). Addition of $CD4^+CD25^+$ to PBMC at a 1:10 ratio (≈10% of $CD4^+CD25^+$) reversed the mean number of HCV-specific IFN-γ spots below baseline in response to HCV antigens. Overall, HCV-specific IFN-γ responses were enhanced by depletion of $CD4^+CD25^+$ and inhibited by the addition of these regulatory T lymphocytes.

The regulatory properties of $CD4^+CD25^+$ T cells were also examined with 2 fractions created from total $CD4^+$, $CD4^+CD25^+$, and $CD4^+CD25^-$, by [$^3$H]thymidine proliferation in chronic infection (n = 30). In the first series of tests, $CD4^+CD25^-$ cells were used as effectors and cultured with autologous DCs and pooled HCV antigens (Fig. 4). $CD4^+CD25^-$ cells were then mixed with $CD4^+CD25^+$ cells at indicated ratios (10:1, 2:1) and stimulated with pooled HCV antigens and DCs. $CD4^+CD25^+$ cells significantly inhibited HCV-specific $CD4^+CD25^-$ proliferation when added at a 10:1 (mean $18 \times 10^3$ cpm vs. 30, $P < .05$) and 2:1 ratio (7.5 vs. 30, $P < .01$) when compared with the control $CD4^+CD25^-$ cell culture in the absence of Treg cells. The degree of suppression increased with $CD4^+CD25^+$ cell number added to the coculture, suggesting a dose-dependent relationship.

***Effect of $CD4^+CD25^+$ on HCV-Specific $CD8^+$ T Cell Immune Responses.*** Two HLA-A2–restricted-peptides (NS3 and NS5B) and autologous DCs were used to

**Table 1. Phenotypic Expression Analysis of Circulating CD4+CD25+ T Cell in Spontaneous Recovered, Chronic Infected, and Normal Control Subjects as Determined by 4-Color Flow Cytometry**

| CD4+CD25+ Phenotype | Normal Controls | Spontaneous Resolution | Chronic Infection | Note |
|---|---|---|---|---|
| CD45RO+/CD45RA+ | 9.08 | 9.16 | 9.71 | NS |
| CD45RO+/CD45RA− | 67.47 | 67.60 | 68.11 | NS |
| CD45RO−/CD45RA+ | 20.60 | 21.61 | 19.74 | NS |
| CD45RO−/CD45RA− | 3.09 | 1.64 | 2.44 | NS |
| CD27+/CD28+ | 69.47 | 75.23 | 70.72 | NS |
| CD27+/CD28− | 13.53 | 16.52 | 15.11 | NS |
| CD27−/CD28+ | 15.51 | 7.43 | 12.75 | NS |
| CD27−/CD28− | 1.49 | 0.82 | 1.42 | NS |
| CD95+/CD69+ | 0.65 | 1.76 | 1.27 | NS |
| CD95+/CD69− | 69.54 | 76.51 | 71.10 | NS |
| CD95−/CD69+ | 0.67 | 0.61 | 0.98 | NS |
| CD95−/CD69− | 29.14 | 21.13 | 26.65 | NS |
| CCR5+/CD62L+ | 3.22 | 1.56 | 2.47 | NS |
| CCR5+/CD62L− | 2.00 | 1.75 | 1.76 | NS |
| CCR5−/CD62L+ | 72.68 | 73.53 | 83.71 | NS |
| CCR5−/CD62L− | 22.10 | 24.15 | 13.06 | NS |

NOTE. PBMCs from recovered, chronic, and control subjects were stained with fluorochrome-labeled CD4, CD25, CD45RO, CD45RA, CD27, CD28, CD95, CD69, CCR5, and CD62L surface molecules. Isotype antibodies served as controls. No significant phenotypic differences are noted when comparing the 3 groups.

Abbreviation: NS, not significant.

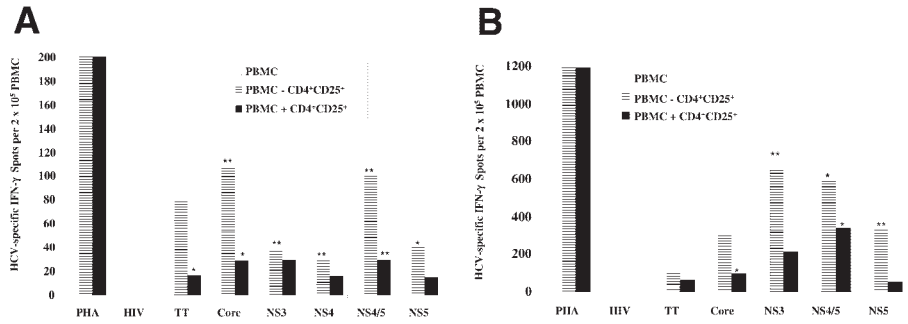


Fig. 3. $CD4^+CD25^+$ T cells suppress HCV-specific IFN-$\gamma$ secretion. HCV-specific IFN-$\gamma$ responses were assessed with the IFN-$\gamma$ ELISpot using 3 PBMC cultures (PBMC, PBM $-$ $CD4^+CD25^+$; PBMC $+$ $CD4^+CD25^+$) that were mixed with HCV antigens, positive controls (PHA, TT), or negative controls (medium, HIV) in subjects with (A) chronic infection (n = 30) and (B) spontaneous recovery (n = 15). The magnitude of the bars represent the mean number of IFN-$\gamma$ spots per $2 \times 10^5$ mononuclear cells from the 3 PBMC cultures after subtracting the number of IFN-$\gamma$ spots from their negative control wells. Although depletion of $CD4^+CD25^+$ T cells (PBMC $-$ $CD4^+CD25^+$ T cells) enhances HCV-specific IFN-$\gamma$ responses, supplementation of whole PBMC with $CD4^+CD25^+$ T cells (PBMC $+$ $CD4^+CD25^+$ T cells) suppresses HCV-specific IFN-$\gamma$ responses when compared with whole PBMC. $*P < .05$ and $**P < .001$ calculated with the Mann Whitney *U* test. PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin antigen; HIV, human immunodeficiency virus; TT, tetanus toxoid.

generate cytotoxic T lymphocytes from pure CD8 cells of $HLA\text{-}A2^+$ chronic patients. The CFSE dye was used to assess the proliferative capacity of HCV-specific $CD8^+$ T cells.[21] $CD8^+$ T cells were stained with CFSE, and HCV-specific $CD8^+$ T cells were quantified by determining the CFSE content of tetramer-positive cells after 7 days of *in vitro* stimulation with peptide and coculture DCs as well as $CD4^+$ T cell fractions ($CD4^+$, $CD4^+CD25^-$, or $CD4^+CD25^+$). The results of a representative patient with chronic infection are shown in Fig. 5. After 7 days of coculture, NS3- and NS5-specific $CD8^+$ T cell proliferation are 16.0% and 16.4% in the presence of a normal CD4 cell population. Addition of $CD4^+CD25^+$ cells decreased $CD8^+$ T cell NS3-specific proliferation to 10.7% and NS5B-specific proliferation to 12.1%. Coculturing with $CD4^+CD25^-$ cells increased $CD8^+$ T cell NS3-specific proliferation to 27.2% and NS5B-specific proliferation to 28.8%. No NS3 or NS4 tetramer-positive proliferation was observed with HIV peptide (0.5%-1.2% CFSE signal in the left upper quadrant) when used as a negative control.

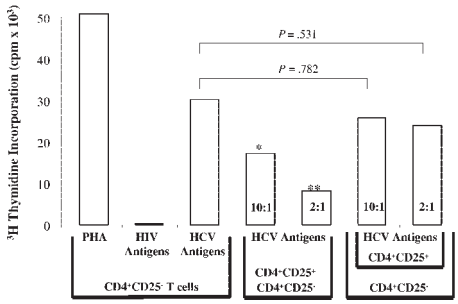


Fig. 4. $CD4^+CD25^+$ T cell immunosuppression of HCV-specific $CD4^+$ T cell proliferation is dose dependent. $CD4^+CD25^-$ T cells from subjects with chronic infection (n = 30) were used as effector cells to examine HCV-specific $CD4^+$ T cell proliferation as measured by $^3$H-thymidine incorporation in response to pooled HCV antigens in coculture with $CD4^+CD25^+$ T cells at various ratios (10:0, 10:1, and 2:1). Positive (PHA) and negative controls (HIV antigens) are included. Results are expressed as mean counts per minute (cpm $\times 10^3$) after subtracting the cpm in the absence of antigen. $CD4^+CD25^+$ T cells suppress HCV-specific $CD4^+CD25^-$ proliferation in a dose-dependent manner. To determine whether the suppression was cytokine mediated and/or cell-contact requiring, transwell studies were performed. Suppression of HCV-specific $CD4^+CD25^-$ proliferation was abolished after separating the 2 cell fractions ($CD4^+CD25^-$ and $CD4^+CD25^+$) with a transwell insert, suggesting that suppression is cell–cell contact dependent. $*P < .05$, $**P < .01$ by the Mann-Whitney *U* test. PHA, phytohemagglutinin antigen ; HIV, human immunodeficiency virus; HCV, hepatitis C virus.

***$CD4^+CD25^+$ Secrete TGF-$\beta_1$ and IL-10.*** To evaluate for cytokine release from Tregs, ELISA was performed for TGF-$\beta_1$ and IL-10 by using the supernatants from the 3 PBMC cultures (PBMC, PBMC $-$ $CD4^+CD25^+$, and PBMC $+$ $CD4^+CD25^+$) in chronically infected subjects (n = 30). As shown in Fig. 6, cytokine analysis showed lower levels of TGF-$\beta_1$ and IL-10 in the supernatants of PBMC depleted of $CD4^+CD25^+$ versus baseline PBMC (for TGF-$\beta_1$: core 75 vs. 173, $P < .05$; NS4/5 139 vs. 272, $P < .01$; NS5 114 vs. 270, $P < .01$; PHA 264 vs. 289, HIV 18 vs. 19; for IL-10: core 38 vs. 57, $P < .01$; NS3 37 vs. 59, $P < .05$; NS4/5 30 vs. 44, $P < .05$, NS5 23 vs. 38 $P < .01$; PHA 60 vs. 75, HIV 6 vs. 8). In addition, significantly higher levels of both cytokines were noted in PBMC enriched with $CD4^+CD25^+$ on comparison with their respective whole PBMC. These results suggest that $CD4^+CD25^+$ regulatory T cells secrete significant amounts of TGF-$\beta_1$ and IL-10.

***Neutralization of $CD4^+CD25^+$ Suppression with Anti-TGF-$\beta_1$.*** To determine whether TGF-$\beta_1$ was an immunoregulatory cytokine involved in the suppressive

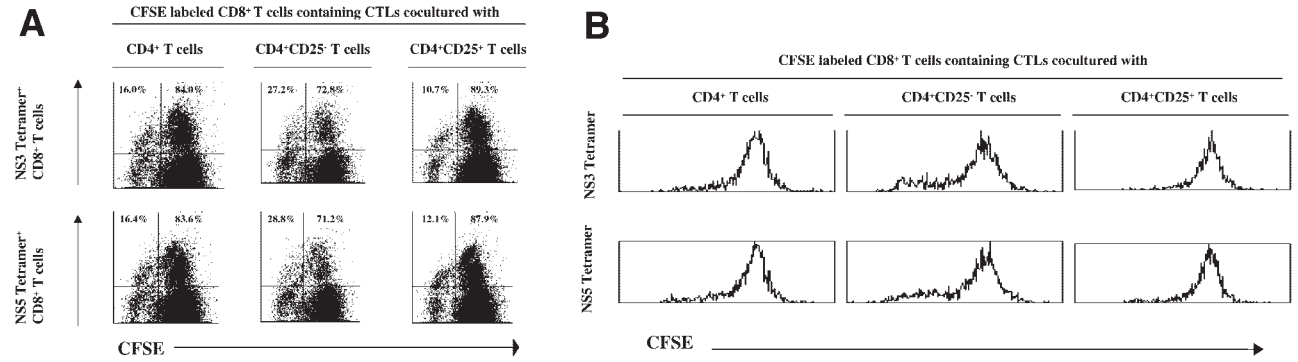


Fig. 5. $CD4^+CD25^+$ T cells suppress HCV-specific $CD8^+$ T cell proliferation. CFSE assay for a representative patient with chronic infection. $CD4^+CD25^+$ T cells suppress proliferation of HCV-specific $tetramer^+$ CTLs. Two HLA-A2–restricted peptides (NS3 and NS5B) were used to generate CTLs from pure $CD8^+$ cells of $HLA\text{-}A2^+$ subjects. HCV-specific $CD8^+$ T cells proliferation were evaluated with CFSE, anti-CD8, and PE-HLA-A2 tetramers. (A) CFSE-labeled $tetramer^+$ CTLs as detected after a 7-day culture; with unlabeled $CD4^+$ T cells, with unlabeled $CD4^+CD25^-$ T cells and with unlabeled $CD4^+CD25^+$ T cells. $CD8^+$ T cells were cocultured with the various CD4 fractions at 10:1 ratio ($1 \times 10^6$ CD8:0.1 $\times$ $10^6$ CD4 fraction).The CFSE signal in the left upper quadrant represents the percentage of HCV-specific CTLs that have proliferated during the 7-day culture. Dot plots were generated by gating for lymphocytes. (B) Histogram plot showing the CFSE signal intensity of the CD8 T cells in the presence of $CD4^+$, $CD4^+CD25^-$, and $CD4^+CD25^+$ T cells when using the NS3 and NS4 tetramers.

effect by $CD4^+CD25^+$ T cells, neutralizing experiments using anti-TGF-$\beta_1$ antibody were performed in the chronically infected subjects (n = 15). The effect of introducing anti-TGF-$\beta_1$ on the mean number of HCV-specific IFN-$\gamma$ spots was assessed by using HCV-stimulated PBMC, PBMC - $CD4^+CD25^+$, and PBMC + $CD4^+CD25^+$ cell cultures and compared with their respective subsets in the absence of anti–TGF-$\beta_1$ (Fig. 7). Addition of anti–TGF-$\beta_1$ increased HCV-specific IFN-$\gamma$ responses in the stimulated PBMC (29 vs. 15, $P < .01$) and PBMC + $CD4^+CD25^+$ (35 vs. 13, $P < .01$) cultures when compared with stimulated subsets in the absence of anti–TGF-$\beta_1$. Although addition of anti–TGF-$\beta_1$ abrogated $CD4^+CD25^+$-mediated suppression in these cultures, no reversal was found with an isotype antibody ($P > .87$), confirming that TGF-$\beta_1$ was a critical mediator of this suppressive effect. Introduction of anti–TGF-$\beta_1$ into the mononuclear cells depleted of $CD4^+CD25^+$ did not elicit any significant change in HCV-specific IFN-$\gamma$ response when compared with the PBMC − $CD4^+CD25^+$ culture in the absence of anti–TGF-$\beta_1$ ($P > .65$)

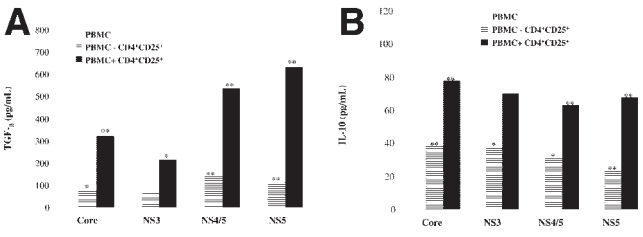


Fig. 6. $CD4^+CD25^+$ T cells in chronic infection (n = 30) produce TGF-$\beta_1$ and IL-10. Enzyme-linked immunosorbent assay cytokine analysis for TGF-$\beta_1$ and IL-10 was performed on culture supernatants from the 3 PBMC cultures representing whole PBMC, PBMC depleted of $CD4^+CD25^+$ cells (PBMC − $CD4^+CD25^+$), and PBMC supplemented with $CD4^+CD25^+$ cells (PBMC + $CD4^+CD25^+$). Bars represent the difference in cytokine production after HCV antigen and negative control antigen stimulation. (A) Lower levels of TGF-$\beta_1$ are found in $CD4^+CD25^+$ depleted PBMC cultures and higher levels noted in PBMC cultures supplemented with $CD4^+CD25^+$ T cells in comparison with whole PBMC cultures in subjects with chronic infection. (B) IL-10 levels are also lower in PBMC − $CD4^+CD25^+$ T cell cultures and higher in PBMC + $CD4^+CD25^+$ T cell cultures when compared with whole PBMC. *$P < .05$ and **$P < .01$ calculated using the Mann-Whitney $U$ test. TGF-$\beta$, transforming growth factor beta; PBMC, peripheral blood mononuclear cells; IL, interleukin.

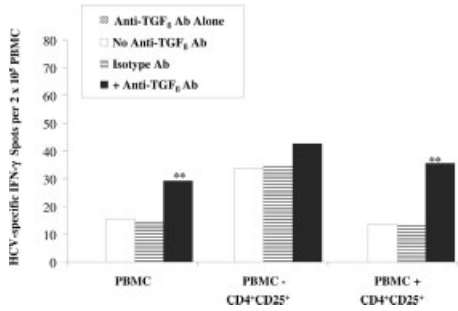


Fig. 7. TGF-$\beta_1$ neutralization reverses $CD4^+CD25^+$–mediated suppression in chronic infection (n = 15). HCV-specific IFN-$\gamma$ response in the IFN-$\gamma$ ELISpot by whole PBMC, PBMC − $CD4^+CD25^+$, and PBMC + $CD4^+CD25^+$ cultures in response to anti-TGF-$\beta_1$ antibody alone (diagonal striped column), pooled HCV antigens in the absence of a blocking antibody (white columns), presence of an isotype antibody (horizontal striped column), and after introduction of anti-TGF-$\beta_1$ antibody (black column) in chronic infection. Introduction of anti-TGF-$\beta_1$ antibody significantly enhances HCV-specific IFN-$\gamma$ responses in PBMC and PBMC + $CD4^+CD25$ cultures when compared with those observed in PBMC and PBMC + $CD4^+CD25^+$ cultures in the absence of a neutralizing antibody. HCV-specific response is not increased by anti–TGF-$\beta_1$ antibody in PBMC cultures depleted of $CD4^+CD25^+$. **$P < .01$ by the Mann-Whitney $U$ test. HCV, hepatitis C virus; IFN-$\gamma$ interferon gamma; PBMC, peripheral blood mononuclear cells.

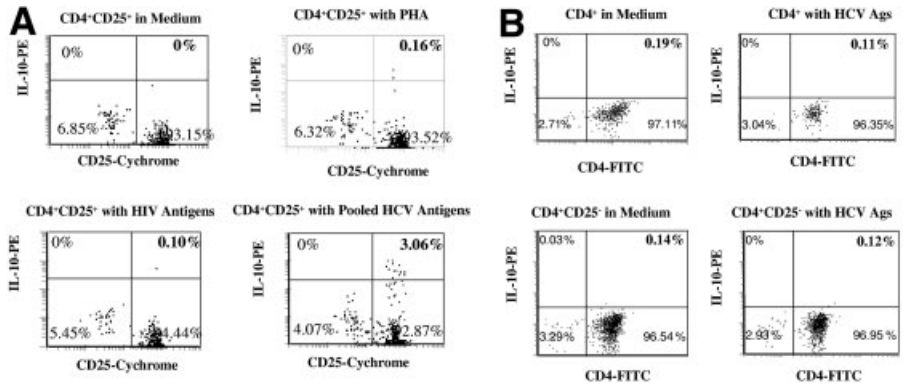


Fig. 8. A minority of $CD4^+CD25^+$ regulatory T cells in chronic infection exhibit HCV-specificity. Pure $CD4^+CD25^+$ T cells cocultured with autologous monocyte-derived dendritic cells produce IL-10 in response to pooled HCV antigens by intracellular cytokine staining. (A) Intracellular cytokine staining of $CD4^+CD25^+$ regulatory T cells for IL-10 in a subject with chronic infection. Pure $CD4^+CD25^+$ T cells cultured with PHA, HIV antigens and in the absence of antigen showed no IL-10 production highlighting their HCV-specificity. (B) Coculture of $CD4^+$ and $CD4^+CD25^+$ fractions with pooled HCV antigens did not elicit IL-10 production. IL, interleukin; PE, ; PHA, phytohemagglutinin antigen; HIV, human immunodeficiency virus; HCV, hepatitis C virus.

***$CD4^+CD25^+$-Mediated Suppression Is Cell-Contact Dependent.*** Having established that $CD4^+CD25^+$ T cells mediate their suppressive effect through TGF-$\beta_1$ secretion, we then investigated whether such a suppression requires cell contact between $CD4^+CD25^+$ cells and their targets in chronic infection. Using a transwell culture system, $CD4^+CD25^+$ cells in the inner well and $CD4^+CD25^-$ targets cells in the outer well were both cultured with pooled HCV antigens and autologous DCs. Suppressor function of the $CD4^+CD25^+$ was measured after a 72-hour incubation by assessing $CD4^+CD25^-$ $[^3H]$thymidine incorporation. Whereas coculture of $CD4^+CD25^-$ and $CD4^+CD25^+$ cells at increasing ratios (10:1, 2:1) resulted in significant suppression of HCV-specific $CD4^+CD25^-$ proliferation, $CD4^+CD25^+$ cells failed to suppress through a membrane (Fig. 4). This finding suggests that $CD4^+CD25^+$ T cell immunosuppression is cell–cell contact dependent.

***$CD4^+CD25^+$ Respond to HCV Antigens via IL-10 Secretion.*** To evaluate for viral specificity, we performed intracellular cytokine staining for IL-10 on $CD4^+CD25^+$ T cells (>95% purity by flow) that were cocultured with autologous DCs and either pooled HCV antigens or HIV antigens (negative control). Intracellular cytokine staining results showed HCV-specific IL-10 production that ranged between 1.9% and 5.3% compared with no spontaneous, PHA-, or HIV-stimulated response in the 5 chronically infected patients evaluated. An example of the specificity of the HCV-specific IL-10 production for a representative patient is shown in Fig 8. The absence of detectable IL-10 by intracellular cytokine staining in the total $CD4^+$ and $CD4^+CD25^-$ fractions support the existence of $CD4^+CD25^+$ cells responding directly to HCV antigens by IL-10 production. The failure of PHA alone to stimulate the $CD4^+CD25^+$ IL-10 production further illustrates its HCV specificity and is in agreement with studies classifying Treg as unresponsive (anergic) to nonspecific stimulation.[22,23]

***Correlation With Clinical Parameters.*** Analysis of chronic infected patients (n = 30) showed no correlation between $CD4^+CD25^+$ T cell frequency and ALT level and fibrosis scores (Fig. 9). However, there was a positive correlation between $CD4^+CD25^+$ Treg frequency and HCV RNA titer ($R = 0.457$, $P = .011$) and an inverse relationship with histologic inflammatory scores ($R = -0.67$, $P = .001$) by the nonparametric Spearman rank.

## Discussion

In this study, we provide an extensive analysis of the frequency, phenotype, and function of $CD4^+CD25^+$ T lymphocytes in HCV infection. We postulate that $CD4^+CD25^+$ T cells play a role in suppressing $CD4^+$ and $CD8^+$ T cell responses to HCV, and thereby promote HCV persistence. In support of this hypothesis, we found a significantly higher proportion of $CD4^+CD25^+$ T cells in chronically infected patients compared with spontaneously recovered and normal controls. This finding also raises the possibility that the steady-state level of regulatory $CD4^+CD25^+$ cells before infection may predetermine and/or predict whether individuals will clear or develop chronic infection. Furthermore, our results indicate that $CD4^+CD25^+$ T cells suppress IFN-$\gamma$ secretion and proliferation of HCV-specific T cells in a dose-dependent and cell-contact fashion. Surprisingly, a small number of $CD4^+CD25^+$ T cells can respond directly to HCV antigens through cytokine production. The demonstration of "suppressive" HCV antigens is novel and implies a

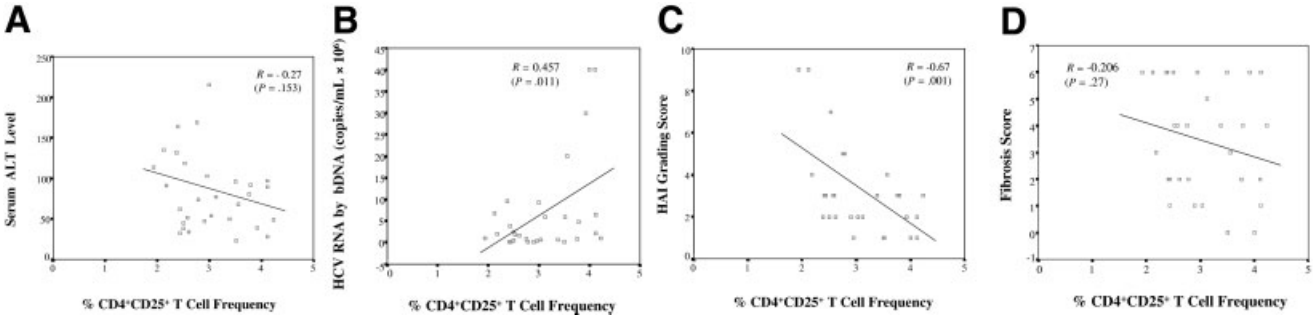


Fig. 9. Correlation analysis between $CD4^+CD25^+$ regulatory T cell frequency in chronic infection and (A) serum alanine aminotransferase level , (B) HCV RNA viral load, (C) histological inflammatory grade, and (D) fibrosis score. A significant positive correlation is found between $CD4^+CD25^+$ regulatory T cell frequency and HCV RNA titer ($R = 0.457$, $P = .011$) , and a negative correlation is observed with inflammatory scores ($R = -0.67$, $P = .001$) by the nonparametric Spearman rank. ALT, alanine aminotransferase; HCV, hepatitis C virus; bDNA, branched DNA; HAI, histological activity index.

viral-specific Treg response. These data implicate the involvement of $CD4^+CD25^+$ T cells in maintenance of chronic HCV infection.

The distribution of $CD4^+CD25^+$ T cells suggest that these cells accumulate mainly in peripheral blood and not the actual site of infection, the liver. Typically, HCV-specific $CD4^+$ and $CD8^+$ T cells are compartmentalized to the liver and not the periphery.[24,25] Thus, we had anticipated that these peripherally induced regulatory T cells would be concentrated at the site of inflammation or antigen presentation. This discrepancy is difficult to explain in the context of our current understanding of HCV and regulatory T cell homeostasis and suggests a potential interfering role for HCV. It is possible that an intrahepatic deficiency in this regulatory cell leads to progressive liver injury. An important limitation of the data is that the population sampled represents a selected group with heavily fibrosed liver tissue obtained at the time of liver transplantation. Such distorted liver architectures are well known to have lower numbers of intrahepatic lymphocytes, which may contribute to the lower percentage of $CD4^+CD25^+$ T cells in the liver parenchyma of this group.

Phenotypic analysis of $CD4^+CD25^+$ T cells showed that that they are $CD27^{high}$, $CD28^{high}$ $CD45RO^{high}$, $CD45RA^{low}$ $CD62L^{high}$ $CD95^{high}$, $CD69^{low}$ and $CCR5^{low}$. This suggests that $CD4^+CD25^+$ T cells are an early antigen-primed memory T cell population and susceptible to apoptosis. In T cell activation and clonal expansion, the costimulatory receptor CD28 and its closest structural relative, the de-activating receptor cytotoxic T lymphocyte antigen-4 (CTLA-4), are of central importance.[26] CTLA-4 expression is primarily restricted to $CD4^+CD25^+$ T cells, where it is constitutively expressed, serving the functional role of the primary costimulatory signal.[27] Indeed, blockade studies of CTLA-4 abrogating the ability of $CD4^+CD25^+$ T cells to suppress T cell–mediated immune responses reveal its critical role in regulating T cell responses.[28] Lin et al. found that the response of regulatory T cells to conventional costimulation was ineffective in comparison with conventional T cells, but readily expanded *in vitro* with "superagonistic" CD28-specific monoclonal antibody without the need for T cell receptor engagement.[29] Therefore, costimulatory receptors CD28 and CD27 expression on $CD4^+CD25^+$ regulatory T cells is probably not a reflection of function as observed on general $CD4^+$ T cells. Most of the $CD4^+CD25^+$ T cells express the CD45RO isoform, which is characteristic of memory T cells, and few express CD45RA, a marker for naïve T cells. The upregulation of CD95, a Fas apoptotic receptor or late activation marker, and CD62L, a lymph node homing marker, support that they are a differentiated population. Lastly, we found that very few cells express CCR5, which is preferentially expressed on lymphocytes with a $T_H1$ cytokine profile.

Current data suggest that once Tregs are activated, they inhibit both $CD4^+$ and $CD8^+$ T cell responses in an antigen-nonspecific manner.[30,31] Our results indicate that $CD4^+CD25^+$ cells secrete IL-10 and TGF-$\beta_1$, and that anti–TGF-$\beta_1$ reverses their suppressive effect. This confirms an inhibitory role for TGF-$\beta_1$. Furthermore, our findings suggest $CD4^+CD25^+$ T cell–mediated suppression of HCV-specific T cell response is dose-dependent and requires cell contact. Recent mechanistic investigations have shed some light on this juxtaposition requirement by finding expression of the cell surface form of TGF-$\beta_1$ by regulatory $CD4^+CD25^+$ T cell and expression of the TGF-$\beta$ receptor by target CD4 and CD8 T cells.[32] Importantly, a minority of Treg display HCV specificity to IL-10 secretion, which may play a regulatory role in promoting viral persistence. This is consistent with the findings of Belkaid et al., who detected a key role for IL-10 in their model.[7] Their use of IL-10–deficient mice or blockade of IL-10 resulted in a sterilizing cure of *Leish-*

*mania* infection. This indicates that IL-10 secreted by $CD4^+CD25^+$ regulatory T cells may attenuate the function of macrophages to kill the intracellular parasite. IL-10 has also been shown to inhibit HCV-specific immunity when administered exogenously in patients with chronic HCV.[18] Although the observed percentage of IL-10 producing Tregs is low, this finding may provide a novel mechanism used by HCV to evade the immune system and have a significant implication in the arena of vaccine development.

Expression of CD25 has been used as a useful marker of $CD4^+CD25^+$ regulatory T cells. An important caveat is that this immunophenotype $CD4^+CD25^+$ is also observed on activated $CD4^+$ T cells, including those that are pathogen specific. This represents the major limitation of this study. The expression of CD25 occurs early during T cell stimulation, and its up-regulation has been used as a sensitive technique to detect HCV-specific T cells during *in vitro* HCV antigen stimulation.[33] Thus, CD25 is a poor marker of Treg cells after T cell activation and is best used on a naïve T cell repertoire in an *ex vivo* fashion before any T cell stimulation as performed in this study and others.[34] Although the surface expression of other molecules such as the TNF family molecule glucocorticoid-induced TNF receptor and CTLA-4 may serve as markers for Treg cells, an exclusive marker to identify this population of T cells remains elusive. Recently, foxp3 was reported potentially as an exclusive marker for $CD4^+CD25^+$ regulatory T cells in mice, but its relevance in human $CD4^+CD25^+$ Treg cells is not clear.[23] Until an exclusive marker is found to identify $CD4^+CD25^+$ regulatory T cells, the best marker for their presence is demonstrating their suppressive properties on CD4 and CD8 T cell proliferation and cytokine production.

In conclusion, our results indicate that $CD4^+CD25^+$ T cells can respond directly to HCV antigens and suppress the HCV-specific immune response in a dose-dependent cell contact manner, which may promote HCV survival within the host. The higher frequency of $CD4^+CD25^+$ regulatory T cell in chronicity when compared with recovered subjects and its positive correlation with HCV viral load further supports its association with HCV persistence. Current investigation is underway to clarify the specificity of the antigenic determinants and the mechanism of their action. It is likely that antiviral vaccine candidates would want to avoid epitopes that lead to strong regulatory T cell stimulation. Conversely, the finding that $CD4^+CD25^+$ T cell frequency is inversely related to histological inflammatory activity underscores the potential role in suppressing pathological T cell–mediated immunological responses. Future investigation needs to be considered on how to manipulate the function of these regulatory T cells in the clinical setting. Possible manipulation strategies to enhance immune responses may include altering $CD4^+CD25^+$ Treg cell activity by means of cell depletion, CTLA-4 blockade, glucocorticoid-induced TNF receptor signaling, or blocking effector cytokine function.[35]

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