

## Regulatory T Cells Suppress In Vitro Proliferation of Virus-Specific CD8<sup>+</sup> T Cells during Persistent Hepatitis C Virus Infection

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Received 11 October 2004/Accepted 7 February 2005

**The basis of chronic infection following exposure to hepatitis C virus (HCV) infection is unexplained. One factor may be the low frequency and immature phenotype of virus-specific CD8<sup>+</sup> T cells. The role of CD4<sup>+</sup>CD25<sup>+</sup> T regulatory (T<sub>reg</sub>) cells in priming and expanding virus-specific CD8<sup>+</sup> T cells was investigated. Twenty HLA-A2-positive patients with persistent HCV infection and 46 healthy controls were studied. Virus-specific CD8<sup>+</sup> T-cell proliferation and gamma interferon (IFN- $\gamma$ ) frequency were analyzed with/without depletion of T<sub>reg</sub> cells, using peptides derived from HCV, Epstein-Barr virus (EBV), and cytomegalovirus (CMV). CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells inhibited anti-CD3/CD28 CD8<sup>+</sup> T-cell proliferation and perforin expression. Depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells from chronic HCV patients in vitro increased HCV and EBV peptide-driven expansion ( $P = 0.0005$  and  $P = 0.002$ , respectively) and also the number of HCV- and EBV-specific IFN- $\gamma$ -expressing CD8<sup>+</sup> T cells. Although stimulated CD8<sup>+</sup> T cells expressed receptors for transforming growth factor beta and interleukin-10, the presence of antibody to transforming growth factor beta and interleukin-10 had no effect on the suppressive effect of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells on CD8<sup>+</sup> T-cell proliferation. In conclusion, marked CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cell activity is present in patients with chronic HCV infection, which may contribute to weak HCV-specific CD8<sup>+</sup> T-cell responses and viral persistence.**

There are 170 million patients worldwide with persistent hepatitis C virus (HCV) infection who are at significant risk of progressive liver injury leading to cirrhosis, death from liver failure, and liver cancer (2, 11, 22). There is a crucial need to delineate the underlying pathogenesis. One important aspect of persistent HCV infection may be the low frequency of circulating HCV-specific CD8<sup>+</sup> T cells (13, 23), despite high-level viral replication and viral protein production. This is in marked contrast to acute infection, where the CD8<sup>+</sup> T-cell response may comprise 8% of total CD8<sup>+</sup> T cells and include responses to at least eight separate epitopes (24). If viremia persists after acute infection, CD8<sup>+</sup> T-cell responses wane rapidly (23).

It has been argued that T-cell exhaustion (20) or viral mutation (12, 20) may cause the low number of circulating antigen-specific CD8<sup>+</sup> T cells during HCV infection. The failure of HCV-specific CD8<sup>+</sup> T cells to expand may be important in viral persistence, as it has been recently shown that a strong CD8<sup>+</sup> T-cell response in the chimpanzee model correlates with protection against reinfection and is associated with viral clearance during primary infection (37). Furthermore, in nonvire-

mic HCV antibody-positive patients, HCV-specific CD8<sup>+</sup> T cells expand more readily compared to viremic patients (39).

Few studies have addressed the reasons for either the low frequency of HCV-specific CD8<sup>+</sup> T cells or the immature phenotype associated with persistent HCV viremia (27). One possible mechanism may be regulation by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (T<sub>reg</sub>) (38). A recent study has shown an increase in CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells during chronic HCV infection and that these cells may secrete interleukin (IL)-10 and transforming growth factor beta (TGF- $\beta$ ) (7). In addition, HCV-specific gamma interferon (IFN- $\gamma$ ) secretion by peripheral blood mononuclear cells (PBMC) could be recovered or increased by the addition of anti-TGF- $\beta$ .

Secondary memory CD8<sup>+</sup> T-cell responses in a murine model are controlled by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells (30). This is in contrast to primary immune responses, which are dependent on CD4<sup>+</sup> T-cell help (19). In murine and human studies, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells have been characterized by intracellular expression of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and have important roles in the prevention of autoimmune disease (29, 35, 36) and transplantation tolerance (9, 17, 33, 41). Secreted IL-10 and membrane-bound TGF- $\beta$  may both be critical to CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell function (14, 15), although in vitro data are inconclusive (8). We addressed the hypothesis that HCV-specific CD8<sup>+</sup> T-cell expansion in chronic HCV infection may be controlled by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells.

### MATERIALS AND METHODS

**Study subjects and samples.** Twenty HCV antibody-positive, HCV RNA-positive, HLA-A2-positive patients were selected from a cohort of outpatients in

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TABLE 1. Chronically HCV-infected patient data

Case no.	Sex	Genotype	Ethnicity	ALT <sup>a</sup> (IU/ml)	Fibrosis Score (0-6)
1	M	1	Caucasian	78	4
2	M	1	Caucasian	102	2
3	M	1	Caucasian	98	3
4	F	1	Caucasian	106	0
5	M	1	Caucasian	56	2
6	F	1	Caucasian	63	2
7	M	2	Caucasian	53	3
8	M	1	Asian	111	1
9	M	1	Caucasian	107	1
10	M	3	Caucasian	42	2
11	F	1	Asian	124	2
12	F	2	Caucasian	143	3
13	M	1	Caucasian	88	2
14	M	1	Caucasian	92	2
15	F	2	Caucasian	87	2
16	M	2	Caucasian	131	3
17	M	1	Caucasian	152	5
18	M	1	Caucasian	56	6
19	M	1	Caucasian	51	4
20	M	3	Caucasian	69	3

<sup>a</sup> ALT, alanine aminotransferase.

Cambridge, United Kingdom. Peripheral blood mononuclear cells (PBMC) were obtained from patients by centrifugation of heparinized blood over Lymphoprep (Nycomed, Roskilde, Denmark). PBMC were also isolated from six HLA-A2-positive healthy individuals with serological evidence of past cytomegalovirus (CMV) and/or Epstein-Barr virus (EBV) infection. All samples were obtained with approval of the Local Ethics Research Committee. Patient details are shown in Table 1.

**Cell isolation.** In the initial regulatory T-cell characterization experiments in Fig. 1 and 2, CD4<sup>+</sup>CD25<sup>+</sup> cells were first isolated by negative depletion of CD4<sup>+</sup> T cells from PBMC and the CD25<sup>+</sup> T cells were purified from this CD4<sup>+</sup> fraction by magnetic depletion using mouse anti-human CD25<sup>+</sup> beads as per the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) and separated into CD25<sup>+</sup> (referred to as CD4<sup>+</sup>CD25<sup>+</sup>) or CD25<sup>-</sup> cells (referred to as CD4<sup>+</sup>CD25<sup>-</sup>). All further experiments were performed using CD25<sup>+</sup> T cells isolated from PBMC (also referred to as CD4<sup>+</sup>CD25<sup>+</sup>) by magnetic depletion using mouse anti-human CD25<sup>+</sup> beads. Fluorescence-activated cell sorting analysis after depletion showed that over 85% of the isolated CD25<sup>+</sup> cells were CD4<sup>+</sup>. In the CD25<sup>-</sup>-depleted PBMC fraction, <5% of the original CD25<sup>+</sup> population was detectable. In addition, CD8<sup>+</sup> T cells were isolated using mouse anti-human CD8<sup>+</sup> beads (Miltenyi Biotec).

**Flow cytometric analysis.** The following conjugated antibodies and additional fluorometric reagents were used: CD4-fluorescein isothiocyanate (FITC)/Cy-Chrome, CD8-CyChrome, CD25-phycoerythrin (PE), CTLA-4-CyChrome, Ki-67-FITC, IL-10 receptor-PE (all from BD Pharmingen, Oxford, United Kingdom), TGF- $\beta$  receptor type II-FITC, active TGF- $\beta$ -biotinylated (R&D Systems, Minneapolis, MN), and streptavidin-PE (BD Pharmingen). HLA class I peptide tetramers were prepared as previously described (3) and included tetramers specific for five epitopes restricted by HLA-A2: HCV-NS3 peptides 1073 to 1081, CINGVCWTV; HCV-NS4 peptides 1406 to 1415, KLVALGINAV; HCV-NS5b peptides 2594 to 2603, ALYDVVTKL; CMV-pp65 peptides 495 to 503 NLPV-MVATV; and EBV-BMLF1 peptides 280 to 288, GLCTLVAML. All peptides were synthesized by Research Genetics (Invitrogen, Paisley, United Kingdom). Tetramer staining was performed as previously described (26). Cells were acquired on a FACSCalibur and analyzed using Cellquest software (BD Pharmingen). Intracellular staining was performed according to the manufacturers' instructions (Cytotif/Cytoperm; BD Pharmingen).

**Proliferation assays.** We plated 10<sup>5</sup> cells per well of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>, CD4<sup>+</sup>CD25<sup>-</sup> and CD8<sup>+</sup> T cells separately or in specific combinations in a 96-well round-bottom plate. Samples were prepared in triplicate, and anti-CD3 (1  $\mu$ g/ml) and anti-CD28 (1  $\mu$ g/ml) (BD Pharmingen) were added to each well to a total volume of 200  $\mu$ l. Proliferation was then determined on day 5 either by thymidine incorporation or by flow cytometric analysis using Ki-67 as a nuclear marker of cell proliferation. Proliferation was assessed by incorporation of 0.5  $\mu$ Ci/well [<sup>3</sup>H]thymidine (Amersham Pharmacia, Little Chalfont, United King-

dom) after culture and 6 h prior to harvesting. PBMC were collected onto filters using an automatic cell harvester (Wallac, Turku, Finland) and incorporated [<sup>3</sup>H]thymidine was measured using a  $\beta$ -counter (Wallac). The results were expressed as counts per minute (cpm) minus the mean cpm for the control wells. Cell counts did not differ by more than 10% ( $n = 6$ ) for the CD4<sup>+</sup>CD25<sup>+</sup> cells when performed on days 0 and 5 after anti-CD3/CD28 treatment.

**Peptide restimulation.** PBMC, depleted or not depleted of CD25<sup>+</sup> cells using CD25<sup>+</sup> beads (Miltenyi Biotec), were restimulated in 48-well plates (5  $\times$  10<sup>5</sup> cells per well) with 10  $\mu$ g/ml peptide in 1 ml of RPMI 1640, 10% human AB serum (First Link, United Kingdom), 100  $\mu$ g/ml streptomycin, 100 U/ml penicillin, and 2 mM glutamine (RPMI-10) and incubated at 37°C in a humidified CO<sub>2</sub> (5%) incubator. On days 3 to 4, 500  $\mu$ l of RPMI-10 containing 200 U/ml of recombinant interleukin-2 (Chiron, Emeryville, CA) was added, and further medium changes with 100 U/ml of recombinant interleukin-2 were performed every 3 to 4 days until they were analyzed on day 14. In parallel, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell-depleted PBMC were incubated with the CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells (termed repletion) at ratios of 1:2 and 1:10 (CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells:depleted PBMC). CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell repletion was also performed in the presence of 200 U/ml and 400 U/ml of IL-2 on day 0. After 14 days, the percentage of tetramer-specific CD8<sup>+</sup> T cells was determined. Cytokine blocking experiments were also performed using antibodies to TGF- $\beta$  or IL-10 (10  $\mu$ g/ml) (R&D Systems).

**Enzyme-linked immunospot assays.** CD4<sup>+</sup> T-cell IFN- $\gamma$  ELISpot assays were performed on 2  $\times$  10<sup>5</sup> PBMC with or without CD25<sup>+</sup> cells ( $n = 6$ ). Cells were preincubated for 24 h in the presence of a pool of three HCV peptides, an EBV peptide (10  $\mu$ g/ml), or phytohemagglutinin (at 1  $\mu$ g/ml; Sigma, Dorset, United Kingdom). In parallel, 96-well Immobilon-P membrane microtiter plates (Millipore, Bedford, MA) were coated with 100  $\mu$ l of 5  $\mu$ g/ml anti-IFN- $\gamma$  monoclonal antibody in a human IFN- $\gamma$  enzyme-linked immunospot (ELISpot kit; BD Biosciences). These plates were incubated for 16 h at 4°C, after which they were washed and then blocked with RPMI-10 for 2 h. The medium was removed and the preincubated cell cultures were transferred to the Immobilon-P plates and incubated for a further 24 h. Following this, plates were washed with water and then with phosphate-buffered saline (PBS)/0.5% Tween-20; 100  $\mu$ l of 2  $\mu$ g/ml detection biotinylated anti-IFN- $\gamma$  was added to each well and incubated for 2 h at room temperature. The plates were washed again, and 100  $\mu$ l of avidin-horseradish peroxidase conjugate was added to each well and incubated for 1 h at room temperature. Unbound conjugate was removed by thorough washing, and finally 100  $\mu$ l of AEC (3-amino-9-ethylcarbazole) substrate was added to each well for exactly 15 min. The reaction was stopped with extensive washing with water. The plates were dried overnight at room temperature and the spots were counted by the AID ELISpot reader system (AID Diagnostika GmbH, Strassberg, Germany). The number of specific spot-forming cells was determined as the mean number of spots in the presence of an antigen minus the mean number of spots in the wells with medium only and expressed per 10<sup>6</sup> PBMC.

**Isolation of intrahepatic lymphocytes.** Fresh liver tissue was obtained from six viremic patients at diagnostic biopsy. Samples were placed in RPMI supplemented with 10% fetal bovine serum and 0.02% EDTA, disaggregated using 21-gauge needles, and the cell suspension was filtered through nylon mesh. Hepatocytes were removed by centrifugation at 50  $\times$  g. The supernatant was centrifuged at 2,000  $\times$  g and the pellet was washed twice in PBS and suspended in RPMI. The yield of mononuclear cells was 1.5 to 5  $\times$  10<sup>5</sup>.

**Immunohistochemistry of intrahepatic lymphocytes.** Formalin-fixed, paraffin-embedded sections (5  $\mu$ m) on 3-aminopropyltriethoxysilane (APES)-coated slides were dewaxed in xylene and taken through a series of ethanol washes. The tissues were pressure-cooked in 0.08 M citrate buffer (pH 6.0) for 3 min to facilitate antigen retrieval. Following washes in Tris-buffered saline (TBS), endogenous peroxidase activity was quenched by incubation in 0.6% hydrogen peroxide in TBS and blocked with appropriate serum (10% rabbit) in TBS for 2 h (DAKO, United Kingdom). A goat polyclonal Foxp3 (ABCAM, United Kingdom) was applied (100  $\mu$ l) in a humidified chamber at 4°C overnight with gentle shaking in 1% bovine serum albumin/TBS with 0.1% Triton X-100. The slides were washed in TBS containing 0.025% Triton X-100 and incubated for 1 h with rabbit anti-goat Foxp3. A streptavidin-horseradish peroxidase system (DAKO, United Kingdom) with the substrate diaminobenzidine was used for development. Slides were counterstained with Harris hematoxylin, dehydrated in ethanol, and cleared in xylene. Coverslips were applied with DPX mounting medium (Gurr, United Kingdom). Omission of the primary antibody served as a negative control.

**Statistical analysis.** In paired experiments, the distribution of the data was assumed to be nonparametric. The medians of the data sets were then compared using a Wilcoxon signed rank test. Statistical analysis was performed on SPSS version 11.5. Significant values ( $P < 0.05$ ) are indicated.

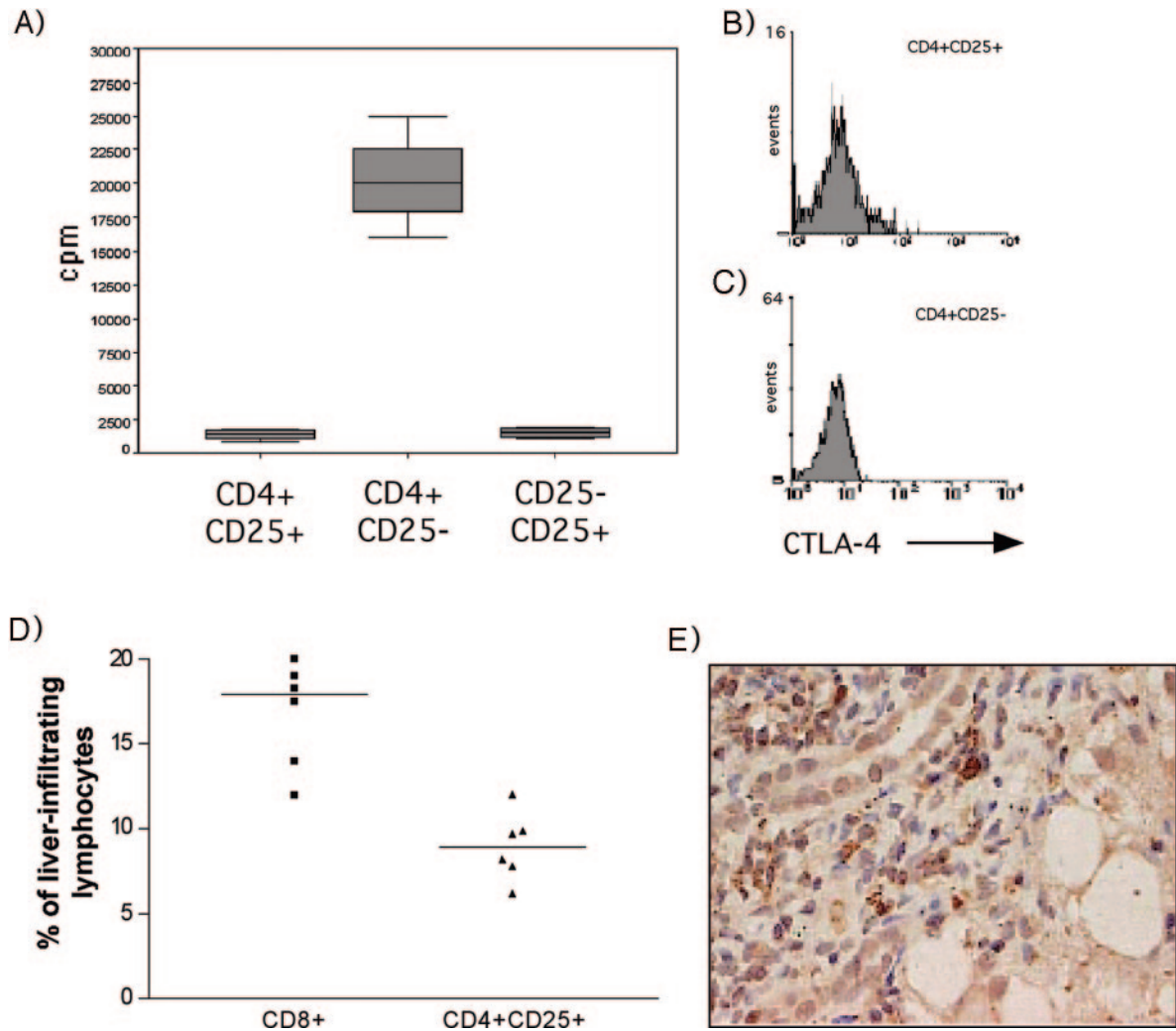


FIG. 1. Function and phenotype of  $CD4^+CD25^+$  cells from patients with persistent HCV infection. (A)  $CD4^+CD25^+$   $T_{reg}$  cells,  $CD4^+CD25^-$  T cells, or a combination of these at a ratio of 1:1 were stimulated with anti-CD3 and cell proliferation was measured using [ $^3$ H]thymidine incorporation. (B and C) Intracellular CTLA-4 expression of (B)  $CD4^+CD25^+$  cells and (C)  $CD4^+CD25^-$  cells. (D) Percentage of  $CD8^+$  and  $CD4^+CD25^+$  cells within the intrahepatic lymphocyte population. (E) Histological staining for Foxp3 within the liver of an HCV-infected individual.

## RESULTS

**$CD4^+CD25^+$   $T_{reg}$  cells from patients with HCV do not proliferate and regulate both  $CD4^+CD25^-$  and  $CD8^+$  T-cell proliferation.** To determine whether the  $CD4^+CD25^+$  T-cell population had suppressor function in persistent HCV infection and whether the cells isolated in these experiments were comparable with those in other studies, classical T-cell suppression assays were performed in six patients. Isolated  $CD4^+CD25^+$   $T_{reg}$  cells did not proliferate after nonspecific soluble anti-CD3 stimulation. In contrast,  $CD4^+CD25^-$  T cells proliferated, and this was abrogated in coculture with  $CD4^+CD25^+$   $T_{reg}$  cells at a 1:1 ratio. (Fig. 1A). In addition, increased expression of intracellular CTLA-4 was present in  $CD4^+CD25^+$   $T_{reg}$  cells (Fig. 1B) compared to  $CD4^+CD25^-$  T cells (Fig. 1C).

To examine the frequencies of  $CD25^+$  T cells in liver (a likely site of activity), the percentage of  $CD4^+CD25^+$   $T_{reg}$  cells (median 8.95%) and  $CD8^+$  T cells (median 17.9%) was deter-

mined from liver biopsies of 6 chronic HCV patients (Fig. 1D). Therefore an approximate ratio within the liver of  $CD4^+CD25^+$   $T_{reg}$  cell:  $CD8^+$  T cells is 1:2. Foxp3 stained liver histology samples were also analyzed (Fig. 1E), which indicated that  $CD4^+CD25^+$   $T_{reg}$  cells appear to predominate around the portal tract. Therefore, the  $CD4^+CD25^+$   $T_{reg}$  cell:  $CD8^+$  T-cell ratio may vary significantly within various compartments of the liver.

To determine whether the  $CD4^+CD25^+$   $T_{reg}$  cells could also suppress the proliferation of  $CD8^+$  T cells during persistent HCV infection, the latter were stimulated with anti-CD3/anti-CD28. Proliferation of  $CD8^+$  T cells, measured by nuclear Ki-67 expression, was inhibited by coculture with  $CD4^+CD25^+$   $T_{reg}$  cells in a 1:1 ratio ( $P = <0.05$ ,  $n = 6$ ; Fig. 2A-B).  $CD4^+CD25^+$   $T_{reg}$  cells also suppressed the expression of intracellular perforin in activated  $CD8^+$  T cells ( $P = <0.05$ ;  $n = 6$ ; Fig. 2D). Group data of Ki-67 and perforin expression are

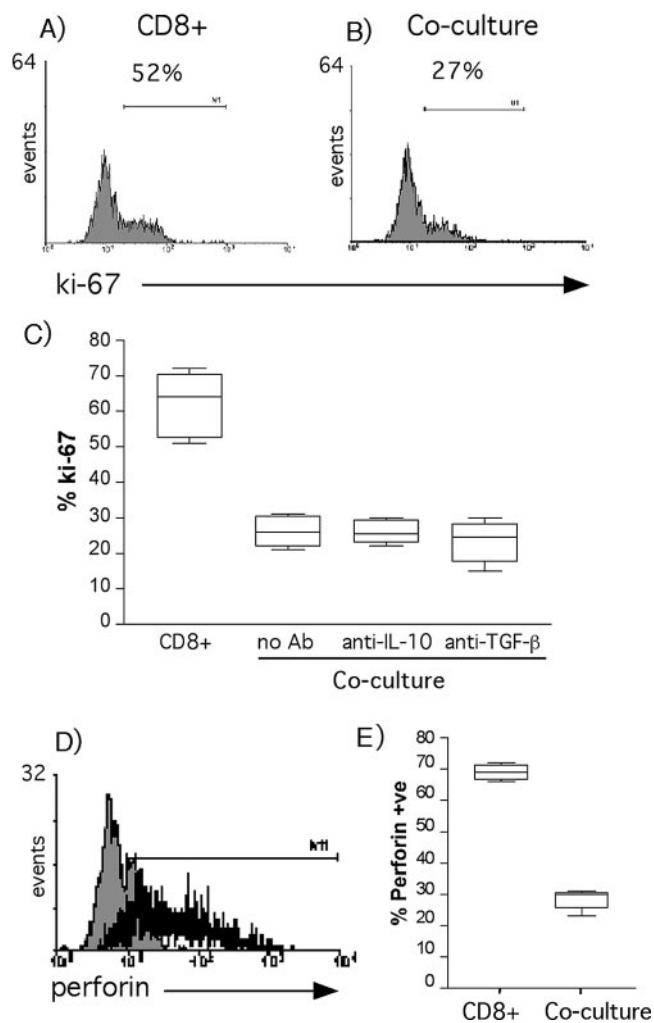


FIG. 2. Inhibition of CD8<sup>+</sup> T-cell proliferation and perforin expression by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells. (A) CD8<sup>+</sup> T cells or (B) CD8<sup>+</sup> T cells in the presence of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells were stimulated with anti-CD3/CD28 and stained with anti-Ki-67, a nuclear marker of proliferation. Histograms are gated on CD8<sup>+</sup> T cells. (C) The proportion of Ki-67-positive CD8<sup>+</sup> T-cell was reduced in the presence of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells (coculture; *n* = 6) and was not restored upon addition of anti-IL-10 or anti-TGF-β antibodies. (D) Intracellular perforin expression of anti-CD3/CD28 activated CD8<sup>+</sup> T cells with (grey filled) or without CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells (black line) at a 1:1 ratio. (E) The proportion of intracellular perforin-positive CD8<sup>+</sup> T cells was reduced in the presence of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells (coculture; *n* = 6). Horizontal bars represent median values, the box represents the interquartile range, and the error bars represent the range.

shown in Fig. 2C and E, respectively. The inhibitory effect of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells on CD8<sup>+</sup> T-cell expansion could not be reversed by culture with antibody to either TGF-β or anti-IL-10 (Fig. 2C).

**CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells regulate proliferation of HCV-specific CD8<sup>+</sup> T cells.** The frequency of HCV-specific CD8<sup>+</sup> T cells after in vitro expansion with peptide increased with depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells (*P* = 0.0005, Fig. 3A, top panel; Fig. 3B, top panel summarizes the 12 individual responses). In 12/20 patients studied, there was a significant increase (as determined by the size of the tetramer response)

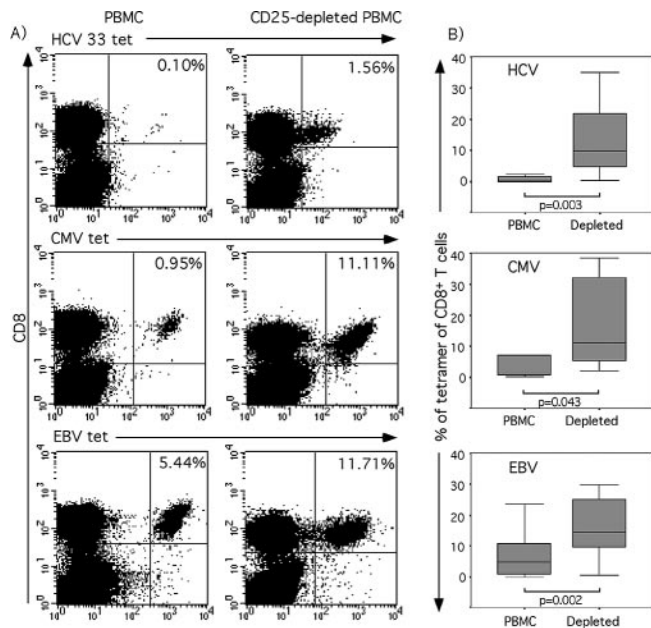


FIG. 3. CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells suppress the expansion of virus-specific CD8<sup>+</sup> T cells during HCV infection. Proliferation in response to peptide stimulation was measured by tetramer frequency. Examples of individual patients are shown: (A) PBMC alone (left-hand panels) and CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>-depleted PBMC (right-hand panels) were restimulated with HCV-specific (top panels), CMV-specific (middle panels), or EBV-specific (bottom panels) peptides, and the extent of proliferation was measured by tetramer frequency. (B) Overall results are shown for HCV (top panel), CMV (middle panel), and EBV (bottom panel). The left-hand box represents the responses in unfractionated PBMC, the right-hand box represents the responses following depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells. Horizontal bars represent median values, the box represents the interquartile range, and the error bars represent the range.

in antigen-specific CD8<sup>+</sup> T-cell expansion to at least one of the three HCV peptides studied following depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells. In 5 of those 12 patients, the HCV-specific CD8<sup>+</sup> T-cell response was undetectable after restimulation of PBMC until depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells. For the 12 individual peptide responses there was a median 43-fold increase in the size of the tetramer response following depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells (*P* = 0.0005).

To determine if this effect was HCV specific or extended to other virus-specific CD8<sup>+</sup> T-cell responses, the effect of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell depletion on CMV- and EBV-specific CD8<sup>+</sup> T-cell responses was studied. Five of the patients had serological evidence of previous CMV infection and 15 had serological evidence of previous EBV infection.

The tetramer frequency of CMV-specific CD8<sup>+</sup> T cells also increased (median, sixfold) in all five CMV-positive individuals following depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells (Fig. 3A, middle panel; Fig. 3B, middle panel summarizes group data) (*P* = 0.043). The frequency of EBV-specific CD8<sup>+</sup> T cells also increased (median 3 fold) in 14/15 patients following depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells (Fig. 3A, bottom panel; Fig. 3B, bottom panel summarizes group data) (*P* = 0.002).

**Addition of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells suppresses antigen-specific CD8<sup>+</sup> T-cell proliferation.** Isolated CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>

cells were added back to the depleted PBMC population prior to restimulation. Almost total abolition of HCV-specific CD8<sup>+</sup> T-cell responses was seen in 11/12 patients at ratios of 1:2 and 1:10 (CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells: CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>-depleted PBMC) (Fig. 4A, left-hand side panels; Fig. 4B). All 5 CMV-specific CD8<sup>+</sup> T-cell responses were suppressed by repletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells. (Fig. 4, right-hand side panels). The effect of adding back CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells (repletion) could not be overcome by supplementation with IL-2 (Fig. 4B).

Overall, depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells led to a significant increase in the expansion of HCV-specific CD8<sup>+</sup> T cells in HCV-infected individuals (Fig. 5A). A consistent but less marked increase in CMV- and EBV-specific responses after restimulation was also seen in HCV infected patients. No significant effect was seen on either EBV- or CMV-specific CD8<sup>+</sup> T-cell expansion following depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells in six healthy individuals (Fig. 5A).

**CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells control HCV CD8<sup>+</sup> T-cell IFN- $\gamma$  secretion.** An ELISpot to quantify the number of IFN- $\gamma$ -secreting HCV-specific CD8<sup>+</sup> T cells was used to analyze the effects of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells in short-term assays. Six patients from the original cohort of 20 were selected such that three had increased HCV tetramer frequency following depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells, while 3 did not (negative control) and all six had detectable EBV responses. Increased numbers of IFN- $\gamma$ <sup>+</sup> HCV-specific CD8<sup>+</sup> T cells were seen with 3 pooled HCV peptides following CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell depletion in all three responders (Fig. 5B). IFN- $\gamma$ <sup>+</sup> HCV-specific CD8<sup>+</sup> T cells were not detected in nonresponders pre- or post-CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell depletion. However, increased numbers of IFN- $\gamma$ <sup>+</sup> EBV-specific CD8<sup>+</sup> T cells were seen following CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell depletion in all 6 patients (Fig. 5C).

**HCV-specific CD8<sup>+</sup> T cells ex vivo do not express TGF- $\beta$  and IL-10 receptors.** Activated CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells express membranous TGF- $\beta$  and secrete IL-10 (6, 14, 25). TGF- $\beta$  and IL-10 receptor expression on virus-specific CD8<sup>+</sup> T cells was therefore determined. HCV-specific CD8<sup>+</sup> T cells ( $n = 4$ ) revealed no expression of TGF- $\beta$  and IL-10 receptors (Fig. 6A and B). Similarly, CMV-specific CD8<sup>+</sup> T cells revealed little expression of either the TGF- $\beta$  receptor (Fig. 6C) or IL-10 receptor ex vivo (Fig. 6D). Expression of both receptors on tetramer-positive cells was observed, however, after in vitro peptide stimulation (Fig. 6E and F). CMV- and EBV-specific CD8<sup>+</sup> T cells from healthy individuals had similar levels of IL-10 and TGF- $\beta$  receptor expression ex vivo (i.e., <3%;  $n = 3$ ) which, also increased after peptide stimulation (data not shown).

## DISCUSSION

Hepatitis C is a major pandemic that affects more than 170 million people worldwide. Persistent infection is associated with ineffective CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses. The causes of T-cell failure are unclear but may include viral variation, T-cell exhaustion, dysfunction of dendritic cells, modulation by specific viral proteins such as core, and intrahepatic death of T cells (6, 40). Another hallmark of the poor response is lack of CD8<sup>+</sup> T-cell maturation despite initial priming and continued antigen exposure (4, 16). We proposed that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>

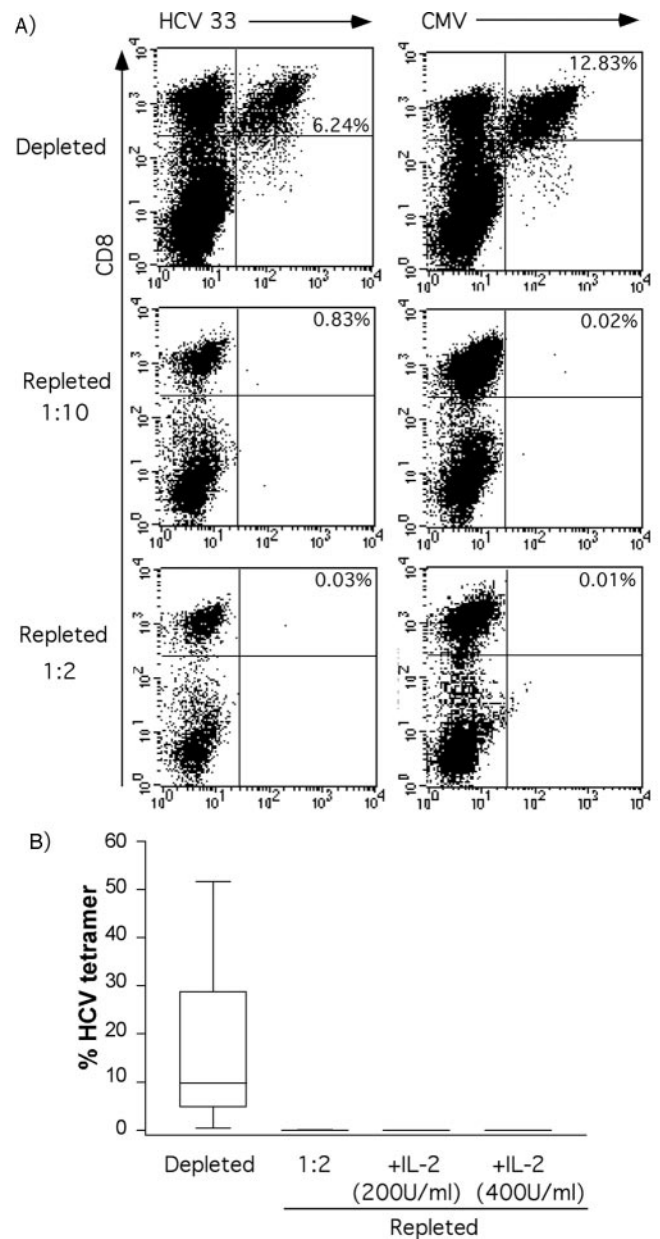


FIG. 4. Repletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells to CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell-depleted PBMC inhibits CD8<sup>+</sup> T-cell peptide-induced proliferation. Proliferation in response to peptide stimulation was measured by tetramer frequency. Examples are shown with HCV peptide-specific (left-hand panels) or CMV peptide-specific (right-hand panels) expansions. CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>-depleted PBMC (top panels), repletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>-depleted PBMC with CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells at 1:10 (middle panels), or 1:2 (CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell: PBMC) (bottom panels). (B) Group data ( $n = 12$ ) of HCV-specific tetramer frequency following peptide restimulation with or without the addition of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells. Repletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells was also performed with the addition, on day 0, of IL-2 at concentrations of 200 U/ml and 400 U/ml.

cells, which regulate human and mouse CD4<sup>+</sup> and CD8<sup>+</sup> T-cell function, could modulate HCV-specific CD8<sup>+</sup> T-cell responses.

CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells isolated from patients with persis-

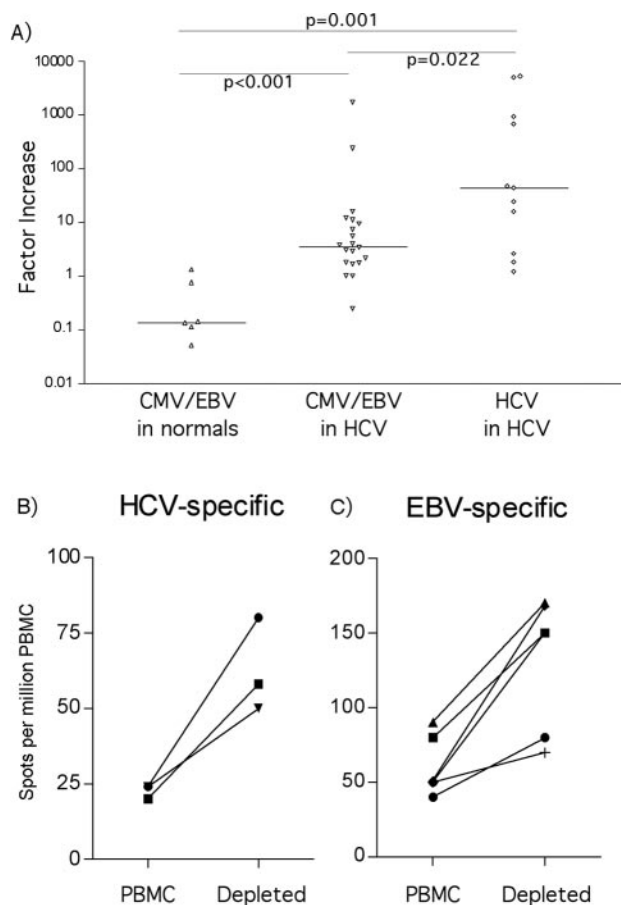


FIG. 5. Virus-specific responses are suppressed by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells in HCV patients compared to healthy individuals. (A) The ratio of the specific CD8<sup>+</sup> T-cell increase following CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell depletion over PBMC alone of all patients studied. The EBV- and CMV-specific responses in healthy individuals are in the left column, the EBV- and CMV-specific responses in HCV-infected patients are in the middle column, and the HCV-specific responses in HCV-infected patients are in the right column. The horizontal line in each column represents the median. (B) Depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells enhances IFN-γ secretion by virus-specific CD8<sup>+</sup> T-cell in HCV-infected patients. PBMC alone (PBMC) and following depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells (Depleted) were used in an IFN-γ ELISpot assay using a pool of three HCV peptides or (C) the EBV peptide.

tent HCV infection were hypo-proliferative and expressed intracellular CTLA-4. They inhibited anti-CD3 stimulated CD4<sup>+</sup>CD25<sup>-</sup> proliferation, anti-CD3/anti-CD28 CD8<sup>+</sup> T-cell proliferation, and HCV peptide-driven T-cell proliferation, consistent with functional human and murine studies of the CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell subset. CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells also inhibited acquisition of CD8<sup>+</sup> T-cell perforin and IFN-γ expression.

The CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub>:CD8<sup>+</sup> T-cell ratio used in these coculture experiments was high considering ratios of cells in peripheral blood. However, the ratio of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells to CD8<sup>+</sup> T cells, as we show, may be relatively high, and particularly within portal tracts, where their effects may be relevant. The percentage of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells within the liver (≈9%) differs significantly from that of another recent study (7). However, the percentage within this study was de-

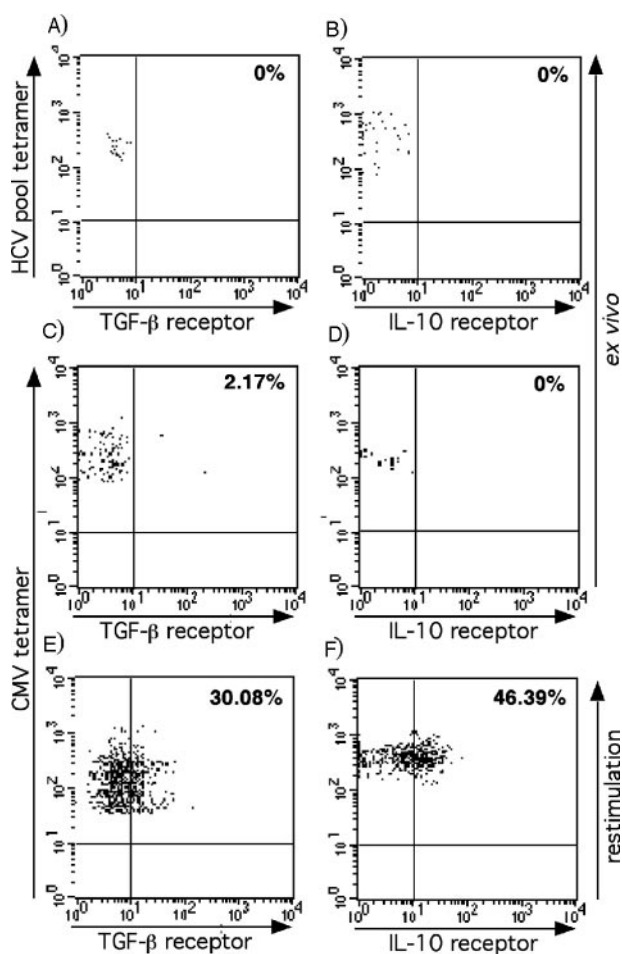


FIG. 6. Expression of the TGF-β receptor type II and IL-10 receptors on virus-specific CD8<sup>+</sup> T cells. (A) TGF-β receptor type II and (B) IL-10 receptor expression on HCV-specific CD8<sup>+</sup> T cells ex vivo. (C) TGF-β receptor type II and (D) IL-10 receptor expression on CMV-specific CD8<sup>+</sup> T cells ex vivo. (E) TGF-β receptor type II and (F) IL-10 receptor expression on CMV-specific CD8<sup>+</sup> T cells after peptide restimulation.

termined in livers at the time of transplantation, which indicates that these livers may be cirrhotic and thus differ from livers studied at earlier stages of disease.

The mechanism of action of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells was not addressed directly. It is thought that TGF-β and IL-10 play important roles in CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell control in autoimmune disease (5, 28, 32). Circulating HCV-specific CD8<sup>+</sup> T cells exhibit no expression of TGF-β and IL-10 receptors, and only weak expression was seen on EBV- or CMV-specific CD8<sup>+</sup> T cells. However, both receptors were readily up-regulated upon stimulation, which indicates that TGF-β and IL-10 may have an alternate suppressive role on activated CD8<sup>+</sup> T cells.

To date, in vitro experiments have failed to conclusively define a role for TGF-β or IL-10 in human CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell function (8). However, this does not exclude a role in vivo. A recent study has shown an effect of TGF-β but not IL-10 on HCV-specific CD8<sup>+</sup> T cells during chronic HCV infection (7). This study identified TGF-β-secreting antigen-specific CD4<sup>+</sup> T

cells and analyzed the effect of anti-TGF- $\beta$  antibody on IFN- $\gamma$  expression by PBMC. It also showed that the suppression of proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells was contact dependent. Our data, together with those of Boettler et al. (6a), also suggest that the suppression of proliferation (in the case of CD8<sup>+</sup> T cells) is contact dependent. However, in both of these studies proliferation was not inhibited by anti-TGF- $\beta$  antibody. These data potentially suggest that assays of different CD8<sup>+</sup> T-cell functions may identify distinct activities of membrane-bound and secreted TGF- $\beta$ . Potential differences in activity of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells on HCV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells requires further investigation.

The significant increase in proliferation for all virus-specific CD8<sup>+</sup> T cells in the absence of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells in patients with chronic HCV infection suggests that these cells are capable of controlling expansion of CD8<sup>+</sup> T cells during HCV infection. Furthermore when CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells were added back to depleted PBMCs, proliferation was reduced. These findings are consistent with a murine study in which depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells enhanced secondary proliferation of CD8<sup>+</sup> T-cell memory responses (30). In addition, we have shown that the suppression is not due to removal of IL-2 by CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells as has previously been suggested (10). High doses of IL-2 added at the time of peptide stimulation or thereafter had no effect on the lack of proliferation seen in the repletion experiments and, together with the cell-cell contact dependent mechanism of inhibition, indicate that IL-2 has no role in suppression.

The finding that the depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells in patients with chronic HCV also enhanced expansion of EBV- and CMV-specific CD8<sup>+</sup> T cells was not mirrored in healthy controls. This may suggest that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells are more active in patients with chronic HCV and have effects on non-HCV-specific CD8<sup>+</sup> T cells. These data are also consistent with recent evidence of inhibition of maturation of CMV-specific CD8<sup>+</sup> T cells in persistent HCV infection (27). Our own and previous published data have also indicated that CD8<sup>+</sup> T-cell maturation is inhibited in chronic HCV infection, as measured by reduced perforin expression (27, 31).

Therefore, a proposed mechanism by which suppression of proliferation and differentiation of CD8<sup>+</sup> T cells during HCV infection occurs at the immature central memory T-cell phase could contribute to the apparent immaturity of CD8<sup>+</sup> T cells in addition to the low frequency of HCV-specific CD8<sup>+</sup> T cells. The frequency of other virus-specific CD8<sup>+</sup> T-cell responses (such as CMV) has not been shown to alter during HCV infection (18, 34), although these populations are very variable between individuals and may change with age (21). It is likely that such T-cell populations are established prior to HCV infection. Thus, the overall consequences may be an impact of HCV persistence on the maturation state of CMV and bulk CD8<sup>+</sup> T cells rather than a decrease in the precursor pool. Additionally, the suppressive effect on EBV- and CMV-specific CD8<sup>+</sup> T-cell responses was not as high as the effect on the HCV response (Fig. 5A), which may allow those former populations to maintain a relatively higher frequency.

These data confirm and extend recent studies of the role of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells in chronic human virus infections. One study of HCV, based upon depletion of CD4<sup>+</sup> T cells, revealed increased numbers of IFN- $\gamma$ -secreting cells (38). A further

study in human immunodeficiency virus infection also suggested CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell activity in chronic infection (1). Neither of these studies addressed the influence of such cells on proliferation and maturation. The inhibitory effect of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells on CD8<sup>+</sup> T-cell expansion and function may be one mechanism whereby chronic HCV infection is associated with limited CD8<sup>+</sup> T-cell expansion and maturation. It may represent a direct effect of the virus or be part of the normal response to chronic inflammation, either hepatic or systemic.

Our results complement and are reinforced by another group (6a) that used CD4<sup>+</sup> depleted PBMC to show the same suppression of virus-specific CD8<sup>+</sup> T-cell proliferation in both chronic and resolved HCV patients but not in healthy individuals. Their results also show similar suppression of virus-specific CD8<sup>+</sup> T-cell proliferation (HCV- and influenza virus-specific responses), compared to our HCV-, EBV- and CMV-specific responses) and were also able to demonstrate that this effect was cell-contact dependent and showed similar potency in titration experiments. Similarly, they were also able to show that this effect was TGF- $\beta$  and IL-10 independent.

These results provide a plausible explanation for the low frequencies and retarded maturation state (low perforin and IFN- $\gamma$  expression) of HCV-specific CD8<sup>+</sup> T cells seen in persistently infected patients and indicate that modulation of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cell function may be one therapeutic strategy for the treatment of chronic HCV.

#### ACKNOWLEDGMENTS

This study was supported by the Digestive Disorders Foundation (S.M.R. and G.J.M.A.), the Wellcome Trust (S.M.W. and P.K.), and EU grant QLK2-CT-1999-00356 (M.L.).

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