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Increased hepatitis C virus (HCV)-specific $CD4^+CD25^+$ regulatory T lymphocytes and reduced HCV-specific $CD4^+$ T cell response in HCV-infected patients with normal *versus* abnormal alanine aminotransferase levels

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Summary

$CD4^+CD25^+$ T regulatory cells may play a role in the different clinical presentations of chronic hepatitis C virus (HCV) infection by suppressing $CD4^+$ T cell responses. Peripheral $CD4^+CD25^+$ T cells from chronic HCV carriers with normal and abnormal alanine aminotransferase (ALT) were analysed for specificity and effect on HCV-specific $CD4^+$ T cell reactivity by flow cytometry for intracellular cytokine production and proliferation assay. HCV-specific $CD4^+CD25^{+high}$ T cells consistently produced transforming growth factor (TGF)-β but only limited amounts of interleukin (IL)-10 and no IL-2 and interferon (IFN)-γ. The HCV-specific TGF-β response by $CD4^+CD25^{+high}$ T cells was significantly greater in patients with normal ALT compared to patients with elevated ALT. In addition, a significant inverse correlation was found between the HCV-specific TGF-β response by $CD4^+CD25^{+high}$ T cells and liver inflammation. In peripheral blood mononuclear cells (PBMC), both HCV antigen-induced IFN-γ production and proliferation of $CD4^+$ T cells were greater in patients with elevated ALT compared with patients with normal ALT. Depletion of $CD4^+CD25^+$ cells from PBMC resulted in an increase of both IFN-γ production and proliferation of HCV-specific $CD4^+$ T cells that was significantly greater in patients with normal ALT levels compared with patients with elevated ALT. In addition, $CD4^+CD25^+$ T cells from patients with normal ALT levels proved to be significantly more potent to suppress $CD4^+$ T cell reactivity with respect to those from patients with elevated ALT. In conclusion, these data support the hypothesis that $CD4^+CD25^+$ cells may play a role in controlling chronic inflammatory response and hepatic damage in chronic HCV carriers.

Keywords: ALT, $CD4^+$ T cells, $CD4^+CD25^+$ T cells, HCV, IFN-γ, TGF-β

Introduction

Patients with chronic hepatitis C virus (HCV) infection with normal and elevated alanine aminotransferase (ALT) values show different clinical patterns [1–3]. Patients with normal ALT levels have a low activity grade and stage chronic hepatitis but similar serum and liver viral loads, compared with patients with elevated ALT levels [3]. T cells play a major role in the pathogenesis of chronic HCV infection, as they recognize virus-infected cells and respond either directly by lysis of the infected cell, or indirectly by secreting cytokines [4]. However, T cells may also cause hepatocellular injury in an effort to limit viral replication [5–9]. In particular, in a study in which $CD4^+$ T cell reactivity has been analysed in liver and peripheral blood from HCV patients segregated by their ALT levels, HCV-specific-induced T cell proliferation has been observed less often in patients with normal ALT, compared to patients with elevated ALT levels [10].

$CD4^+CD25^+$ regulatory T lymphocytes are a subset of circulating $CD4^+$ T cells with suppressive properties [11–14]. There are two general categories of $CD4^+CD25^+$ T cells. One $CD4^+CD25^+$ T cell subset develops during the process of T cell maturation in the thymus, resulting in the generation of a naturally occurring population of $CD4^+CD25^+$ T cells poised to prevent autoimmune responses by suppressing autoreactive T cells [15–17]. The second subset of $CD4^+CD25^+$ T cells develops as a consequence of *ex vivo* peripheral activation of naive $CD4^+CD25^-$ T cells [18]. These

induced CD4+CD25+ have been shown to suppress T cell responses to tumours and acute and chronic bacterial and viral infections [19–22]. In particular, it has been reported recently that CD4+CD25+ T cells suppress HCV-specific T cell responses and it has been suggested that they may play a role in viral persistence [23].

Because it is possible that a different host's CD4+ T cell reactivity could be the basis for the different clinical presentations of the HCV infection, it is intriguing to hypothesize that CD4+CD25+ T cells may play a role in controlling chronic inflammatory response and hepatic damage. Based on these considerations, the aim of the present study was to evaluate whether HCV carriers with normal and abnormal ALT may differ in the frequency of HCV-specific CD4+CD25+ T cells and whether this is associated with differences in the CD4+ T cell response.

Materials and methods

Patient population

Thirty-seven chronic hepatitis C patients without liver cirrhosis were included. Seven normal blood donors without a history of HCV infection served as controls. Other causes of liver disease were excluded. None of the patients had fever, evidence of other infectious diseases, inflammatory disorders or any kind of malignancy at the time the serum samples were obtained. No patients had received previous anti-viral or immunomodulatory treatment. Liver biopsies were graded and staged according to Ishak *et al.* [24,25]. Sixteen patients had persistently normal ALT levels for at least 12 months prior to study entry, as documented on four occasion 3 months apart. Twenty-one patients had persistently abnormal ALT levels during the same observation period. Table 1 shows the patients' characteristics. Informed consent was obtained from each patient included in the study.

Recombinant HCV proteins

The following fragments of HCV proteins (*Escherichia coli*-derived protein recombinant) were purchased from Virogen Corporation (Watertown, MA, USA): core [amino acids (aa) 1–119), NS3 (aa 1192–1459), NS4 (aa 1916–47) and NS5 (aa 2061–2302)].

Antibodies

The following monoclonal antibodies (mAbs) were used: anti-CD4, anti-CD25, anti-CD45RO, anti-CD45RA, anti-CD27, anti-CD28, anti-CD95, anti-CD69, anti-interleukin (IL)-2, anti-interferon (IFN)-γ, anti-IL-10, anti-IL-4 (all from PharMingen, San Diego, CA, USA) and anti-transforming growth factor (TGF)-β1 (from IQ Products, Groningen, the Netherlands). Staining was performed with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- and cychrome-coupled antibodies.

Cells

Peripheral blood mononuclear cells (PBMC) were separated using lymphoprep (Nycomed, Oslo, Norway). PBMC were resuspended in RPMI-1640 (Gibco, Grand Island, NY, USA) containing 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS) (complete medium). All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. To obtain CD4+CD25+-enriched cell populations we first obtained a purified population of CD4+ T cells by positive selection using CD4+ coated magnetic beads (Dynal Biotech, Lake Success, NY, USA). CD25+ cells were then selected positively using anti-CD25 dynabeads (Dynal). This procedure resulted in cell populations that were > 95% CD4+CD25+, as determined by flow cytometry analysis. CD4+CD25+-depleted PBMC were

Table 1. Clinical characteristics of patients.

Characteristics	Normal ALT ($n = 16$)	Elevated ALT ($n = 21$)	*P*-value
Age (years)*	48 ± 14	52 ± 10	n.s.
Sex (M/F)	7/9	8/13	n.s.
Known time of infection (months)*	301 ± 112	277 ± 89	n.s.
Risk factor			n.s.
IDU	2	4	n.s.
Blood transfusion	3	5	n.s.
Unknown	10	12	n.s.
ALT (IU/l)*	36 ± 7	134 ± 113	< 0·05
Histology*			n.s.
Grading	2·2	7·3	< 0·05
Staging	1	3·2	n.s.
HCV RNA (copies/ml × 10^3)*	68 ± 31	75 ± 38	n.s.
Genotypes (1b/non-1b)	13/16	17/21	n.s.

*Values are expressed as mean ± standard deviation. ALT, alanine aminotransferase; HCV: hepatitis C virus; IDU, intravenous drug users; n.s., non-significant.

obtained as follows: $CD4^+$ cells were purified as described above, then $CD25^+$ cells were removed using anti-CD25 dynabeads (Dynal). At the end of the procedure, the remaining $CD4^+CD25^-$ cells were returned to the $CD4^+$-depleted PBMC ($CD4^+CD25^+$-depleted PBMC contained less than 2% of $CD4^+CD25^+$ cells compared with non-$CD4^+CD25^+$-depleted PBMC, as determined by flow cytometry analysis).

Cell stimulation and intracellular cytokine staining

$CD4^+CD25^+$ T regulatory (T_{reg}) lymphocytes were defined as $CD4^+CD25^{high}$ according to Baecher-Allan *et al.* [26]. In a limited number of samples $CD4^+CD25^{+high}$ T cells were analysed for the forkhead-family transcription factor (FoxP3). In seven of seven sampled analysed almost all $CD4^+$ $CD25^{+high}$ T cells stained positive for FoxP3 (data not shown).To evaluate the HCV-specific cytokine profile of $CD4^+CD25^{+high}$ T cells, purified $CD4^+CD25^+$ T cells were cultured at a concentration of 2×10^5/well in 200 μl of complete medium in 96-well flat-bottomed cell culture plates (Corning Incorporated, Corning, NY, USA) with either 10 μg/ml of recombinant HCV proteins (this protein concentration was chosen on the basis of a dose titration in preliminary experiments; data not shown). Co-stimulatory mAbs to CD28 and CD49d (Becton Dickinson, San Jose, CA, USA) were added at 1 μg/ml each. This combination of co-stimulatory mAbs provides for optimal stimulation of T cell [27]. Phorbol myristate acetate (PMA) (20 ng/ml) plus ionomycin (IO) (500 ng/ml) and complete medium served as positive and negative controls, respectively. To confirm that the staining for TFG-β was working, we used American Type Culture Collection (ATCC) CRL-2159 (LS411N) cells, which constitutively secrete TGF-β. To evaluate IFN-γ production by effector T cells, whole or $CD4^+CD25^+$-depleted PBMC were cultured at a concentration of 2×10^5/well in 200 μl of complete medium in 96-well flat-bottomed cell culture plates and stimulated as described above, apart for the positive control, for which phytohaemagglutinin antigen (PHA), 5 μg/ml was used instead of PMA plus IO. The different cultures were kept for 2 h before the addition of brefeldin A (1 μg/ml; Sigma Aldrich). The incubation was then continued for an additional 4 h, as described previously [28,29]. After incubation, the cells were washed and stained for surface markers by incubation with the appropriate surface marker for 30 min in the dark on ice. Cells were then washed twice and resuspended in Cytofix/Cytoperm solution (Pharmingen) for 20 min in the dark on ice. The permeabilized cells were washed twice and stained for intracellular cytokines in the dark on ice for 30 min. After intracellular cytokine staining, the cells were washed and resuspended in 200 μl phosphate-buffered saline with 0·4% paraformaldehyde.

Flow cytometry

Flow cytometry was performed using a fluorescence activated cell sorter (FACScan) flow cytometer and analysed with CellQuest software (Beckton Dickinson). For each analysis, 50 000–100 000 events were gated on CD3, CD4 and/or CD25 expression and a light-scatter gate designed to include only viable lymphocytes. Isotype-matched negative controls antibodies (PharMingen) were used to verify the staining specificity of anti-cytokine antibodies, and as a guide for setting markers to delineate positive and negative populations. Staining was considered positive if cytokine-positive cells formed a cluster distinct from the cytokine-negative cells and the difference between the frequency detected in stimulated and unstimulated cells was > 0·01% of the total population.

HCV-specific proliferative T cell response

$CD25^+$-depleted $CD4^+$ T cells (4×10^4) were plated in 150 μl complete medium in 96-well, U-bottomed plates (Costar) and stimulated with pooled recombinant HCV antigens (core, NS3, NS4, NS5: 2 μg/ml each) and anti-CD28 and anti-CD49d monoclonal antibodies. [^{3}H]-thymidine (2 μCi) (specific activity, 80 mCi/mmol; Amersham, Little Chalfont, UK) was added on day 6. Cultures were harvested on day 7 after 16 h of [^{3}H]-thymidine incorporation. Triplicate cultures were assayed routinely.

Statistical analysis

The Mann–Whitney non-parametric *U*-test and linear regression analysis using Spearman's correlation coefficient were performed (using SPSS version 10·1). For parameters such as the surface receptor/ligand expression, which is normalized as a percentage of positive cells, parametric statistical analysis was performed. All *P*-values are two-tailed. *P*-values < 0·05 were considered significant.

Results

Liver disease and viral load according to ALT

Among HCV patients, those with persistently elevated ALT values had significantly higher necroinflammatory activity and fibrosis grade compared with those with persistently normal ALT levels ($P < 0{\cdot}001$) (Table 1). Viraemia levels were similar independently of ALT levels.

Total $CD4^+CD25^+$ T cell frequency and phenotype

No significant differences were observed in the percentage of $CD4^+CD25^+$ T cells in peripheral blood in patients with normal ALT values (mean $3{\cdot}45 \pm 0{\cdot}34\%$) and elevated ALT (mean $3{\cdot}12 \pm 0{\cdot}31\%$, $P > 0{\cdot}05$). Cell surface phenotypes of $CD4^+CD25^+$ T cells in patients with normal and elevated ALT values were compared with flow cytometry (Table 2). $CD4^+CD25^+$ T cells display $CD45RO^{high}$, $CD45RA^{low}$, $CD27^{high}$, $CD28^{high}$, $CD69^{low}$ and $CD95^{high}$ phenotype in

Table 2. Phenotypic expression analysis of circulating $CD4^+$ $CD25^+$ T cells in patients with normal and elevated alanine aminotransferase (ALT) as determined by flow cytometry.

$CD4^+$ $CD25^+$ phenotype	Normal ALT	Elevated ALT	Note*
$CD45RO^+$	75 ± 12	77 ± 10	n.s.
$CD45RA^+$	19 ± 4	22 ± 4	n.s.
$CD27^+$	79 ± 9	83 ± 7	n.s.
$CD28^+$	83 ± 7	80 ± 9	n.s.
$CD95^+$	73 ± 10	70 ± 6	n.s.
$CD69^+$	1·2 ± 0·2	1·4 ± 0·1	n.s.

Peripheral blood mononuclear cells (PBMC) from patients with normal ALT levels and elevated ALT were stained with fluorochrome-labelled CD4, CD25, CD45RO, CD45RA, CD27, CD28, CD95 and CD69 surface molecules. Isotype antibodies served as controls; *n.s. = not significant.

peripheral blood. No significant phenotypic differences were found when comparing patients with normal and elevated ALT values.

Cytokine production profile of HCV-specific $CD4^+CD25^+$ T cells

$CD4^+CD25^+$ T cells from patients with normal and elevated ALT values were stimulated with HCV proteins and then analysed by flow cytometry for the production of IL-2, IFN-γ, IL-10 and TGF-β. $CD4^+CD25^{+high}$ T cells from HCV patients failed consistently to produce detectable amounts of IL-2, IFN-γ and IL-4 (data not shown). In most experiments, $CD4^+CD25^{+high}$ T cells also did not produce significant amounts of IL-10, both in patients with normal and elevated ALT values (4/16, 25% *versus* 6/21, 28%, $P > 0{\cdot}05$). In contrast, TGF-β production was detected in all experiments and the percentage of $CD4^+CD25^{+high}$ T cells expressing TGF-β was significantly higher in patients with normal ALT levels with respect to patients with elevated ALT (mean: 1·11% *versus* 0·57%, $P < 0{\cdot}001$) (Fig. 1). In addition, there was a negative correlation between the TGF-β response by $CD4^+CD25^{+high}$ T cells and histological inflammatory scores ($R = -0{\cdot}84$, $P < 0{\cdot}001$) (Fig. 2). No significant differences were detected between patients with normal and abnormal ALT values when $CD4^+CD25^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs (data not shown). Finally, the TGF-β response did not correlate with the viral load (data not shown). An example of the HCV-specific IL-10 and TGF-β production for one representative patient is shown in Fig. 3.

Effect of $CD4^+CD25^+$ T cells on HCV-specific $CD4^+$ T cell immune response according to ALT

IFN-γ production by $CD4^+$ T cells was analysed in whole PBMC and in PBMC depleted of $CD4^+CD25^+$ T cells. In whole PBMC, the percentage of HCV-specific $CD4^+$ T cell was greater in patients with elevated ALT levels compared

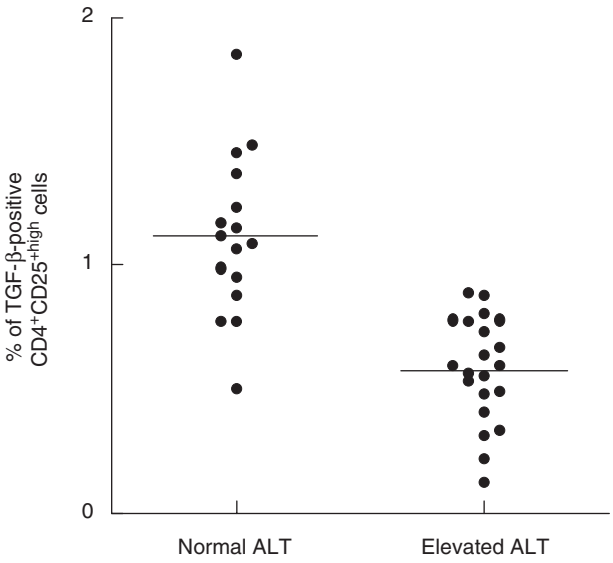

Fig. 1. Hepatitis C virus (HCV)-specific transforming growth factor (TGF)-β secretion by $CD4^+$ $CD25^+$ T cells in patients with normal and elevated ALT. Pure $CD4^+CD25^+$ T cells (1×10^5) were stimulated with pooled HCV antigens (core, NS3, NS4, NS5: 2 μg/ml each). The cells were stained with Cychrome-anti-CD4, fluorescein isothiocyanate (FITC)-anti-CD25 monoclonal antibody and phycoerythrin (PE)-anti-TGF-β monoclonal antibodies (mAbs). The cells were gated on $CD4^+$ and analysed on FL2 (FITC) *versus* FL1 (PE) two-dimensional plots to discriminate positive cells. The percentage of positive cells was calculated with respect to total $CD4^+CD25^+$ T cells. The number of positive events calculated in the same samples in the absence of antigen stimulation was constantly below 0·01%. No significant production of interleukin (IL)-2, interferon (IFN)-γ, IL-10 and TGF-β was detected in healthy controls.

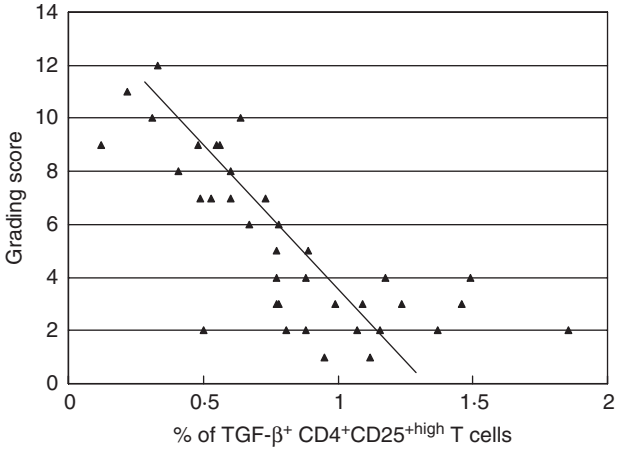

Fig. 2. Correlation analysis between hepatitis C virus (HCV)-specific transforming growth factor (TGF)-β response by $CD4^+CD25^+$ T cells and histological inflammatory grade. A significant negative correlation was observed ($r = -0{\cdot}84$, $P < 0{\cdot}001$) by the non-parametric Spearman's rank test.

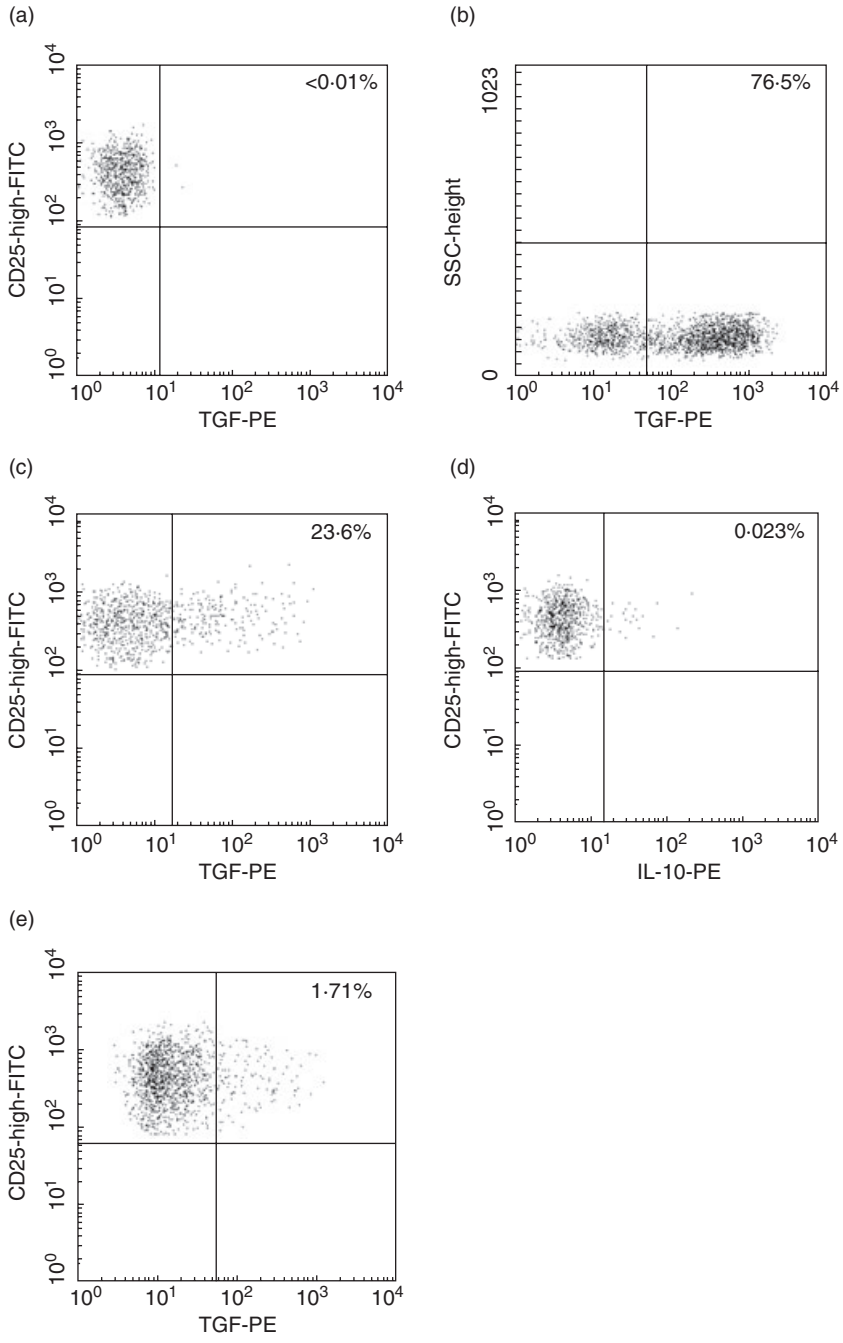

Fig. 3. Hepatitis C virus (HCV)-specific transforming growth factor (TGF)-β and interleukin (IL)-10 production by $CD25^{+high}$ T cells from a single patient. (a) $CD4^+CD25^+$ T cells, stimulated with medium alone, gated on $CD25^{high}$ population. (b) American Type Culture Collection CRL-2159 cells (positive staining for TGF-β). (c) $CD4^+CD25^+$ T cells, stimulated with phorbol myristate acetate (PMA) plus ionomycin (IO) (positive staining for IL-10). (d) $CD4^+CD25^+$ T cells, stimulated with pooled HCV antigens (core, NS3, NS4, NS5: 2 μg/ml each) and stained with phycoerythrin (PE)-anti-IL-10 monoclonal antibodies (mAbs), gated on $CD25^{high}$ population. (e) $CD4^+CD25^+$ T cells, stimulated with pooled HCV antigens (core, NS3, NS4, NS5: 2 μg/ml each) and stained with PE-anti-TGF-β mAbs, gated on $CD25^{high}$ population.

with patients with normal ALT, in response to core (mean, 0·087 *versus* 0·048, $P < 0·01$), NS3 (0·045 *versus* 0·018, $P < 0·01$), NS4 (0·039 *versus* 0·016, $P < 0·01$) and NS5 (0·051 *versus* 0·023, $P < 0·01$) (Fig. 4). No HCV-specific $CD4^+$ T cell IFN-γ production was found in HCV-antigen-stimulated PBMC from healthy controls (data not shown). In contrast, the percentage of HCV-specific $CD4^+$ T cell was not different between elevated and normal ALT patients after depletion of $CD4^+CD25^+$ T cells, in response to core (mean, 0·11 *versus* 0·13, $P > 0·05$), NS3 (0·049 *versus* 0·058, $P > 0·05$), NS4 (0·055 *versus* 0·047, $P > 0·05$) and NS5 (0·075 *versus* 0·061, $P > 0·05$) (Fig. 5). This was due to a greater increase of HCV-specific IFN-γ activity in patients with normal ALT levels compared with patients with elevated ALT.

The regulatory properties of $CD4^+CD25^+$ T cells were also examined by [^{3}H]-thymidine proliferation. It is noteworthy that the [^{3}H]-thymidine proliferation results parallel those of IFN-γ experiments (Fig. 6). Indeed, in purified total $CD4^+$ T cell the mean counts per minute (cpm) (calculated after subtracting the cpm in the absence of the antigen) in HCV-stimulated cells was: 31×10^3 in patients with elevated ALT and 17×10^3 in patients with normal ALT ($P < 0·01$). In contrast,

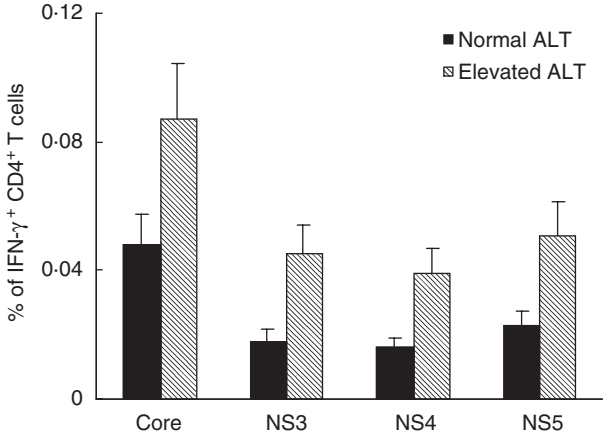

Fig. 4. Hepatitis C virus (HCV)-specific interferon (IFN)-γ secretion in patients with elevated and normal alanine aminotransferase (ALT). HCV-specific IFN-γ responses by $CD4^+$ T cells were assessed by flow cytometry in whole peripheral blood mononuclear cells (PBMC) that were mixed with HCV antigens (core, NS3, NS4, NS5: 2 μg/ml each) and anti-CD28 and anti-CD49d monoclonal antibodies or positive controls [phytohaemagglutinin (PHA)] or negative controls (medium). The percentage of IFN-γ-$CD4^+$ T cells in PHA-stimulated controls was 12·2% and 14·5% in patients with elevated and normal ALT, respectively, $P > 0{\cdot}05$. The number of positive events in the absence of antigen stimulation was constantly below 0·01%.

in $CD25^+$ depleted $CD4^+$ T cell the cpm in HCV-stimulated cells was: 56×10^3 in patients with elevated ALT and 53×10^3 in patients with normal ALT ($P > 0{\cdot}05$). Thus, depletion of $CD4^+CD25^+$ T cells resulted in a greater increase of the proliferative response in patients with normal ALT *versus* patients with elevated ALT levels.

Finally, $CD4^+$ $CD25^-$ T cells were mixed with $CD4^+CD25^+$ T cells at the ratios indicated (10 : 1, 5 : 1, 2·5 : 1) and stimulated with pooled HCV antigens. $CD4^+CD25^+$ T cells significantly inhibited HCV-specific IFN-γ production by $CD4^+CD25^-$ T cells when compared with the control $CD4^+CD25^-$ T cell cultured in the absence of $CD4^+CD25^+$ T cells (100% $CD25^-$ T cells, $P > 0{\cdot}05$, $CD25^-/CD25^+$ 10 : 1, $P < 0{\cdot}01$, $CD25^-/CD25^+$ 5 : 1, $P < 0{\cdot}01$, $CD25^-/CD25^+$ 2·5 : 1, $P < 0{\cdot}01$, normal *versus* elevated ALT, respectively) (Fig. 7). In contrast, the inhibition by $CD4^+CD25^+$ T cells on PHA-induced IFN-γ production was not different at any ratio between normal and elevated ALT patients (data not shown).

Discussion

In this study we have assessed the phenotype, frequency and function of $CD4^+CD25^+$ T cells and the $CD4^+$ T cell response in peripheral blood of HCV-infected patients with persistently normal and abnormal ALT levels. We postulate that $CD4^+CD25^+$ T cells play a role with the different clinical presentation of chronic HCV infection by suppressing $CD4^+$ T cell reactivity. In support of this hypothesis, we found that a subpopulation of $CD4^+CD25^{+high}$ T cells, which is activated specifically by HCV-antigen via T cell receptor (TCR) engagement, is expanded in peripheral blood of HCV-infected patients with normal ALT values and minimal HCV-related liver disease with respect to patients with abnormal ALT values and advanced hepatitis. Antigen-specific

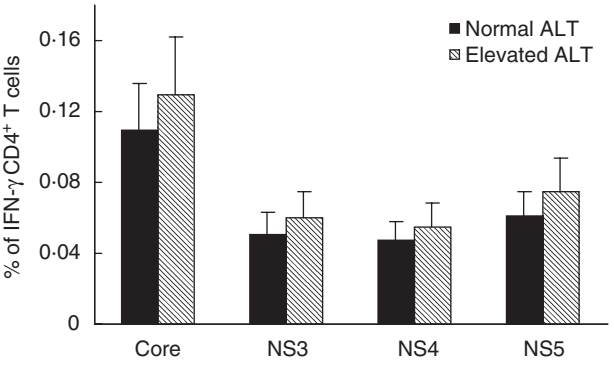

Fig. 5. $CD4^+CD25^+$ T cell suppression of hepatitis C virus (HCV)-specific interferon (IFN)-γ secretion in patients with elevated and normal alanine aminotransferase (ALT). HCV-specific IFN-γ responses by $CD4^+$ T cells were assessed by flow cytometry in peripheral blood mononuclear cells (PBMC) depleted of $CD4^+CD25^+$ T cells that were mixed with HCV antigens (core, NS3, NS4, NS5: 2 μg/ml each) and positive controls (PHA) or negative controls (medium) and anti-CD28 and anti-CD49d monoclonal antibodies. The percentage of IFN-γ-$CD4^+$ T cells in PHA-stimulated controls was 24·3% and 26·4% in patients with elevated and normal ALT, respectively, $P > 0{\cdot}05$. The number of positive events in the absence of antigen stimulation was constantly below 0·01%.

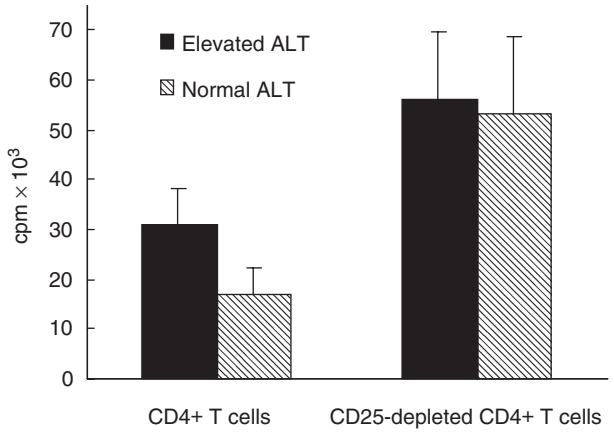

Fig. 6. Inhibition of $CD4^+$ T cell proliferation by $CD4^+CD25^+$ T cell in patients with normal and elevated alanine aminotransferase (ALT). $CD25^+$ depleted $CD4^+$ T cells (4×10^4) were stimulated with pooled recombinant hepatitis C virus (HCV) antigens (core, NS3, NS4, NS5: 2 μg/ml each) and anti-CD28 and anti-CD49d monoclonal antibodies. HCV-specific cell proliferation was measured by [^{3}H]-thymidine incorporation. The counts per minute (cpm) ($\times 10^3$) in phytohaemagglutinin (PHA)-stimulated controls were $323{\cdot}9 \times 10^3$ and $354{\cdot}1 \times 10^3$ in patients with elevated and normal ALT, respectively, $P > 0{\cdot}05$. In the absence of stimuli and in normal subjects stimulated with HCV antigens the cpm was constantly below 10^3.

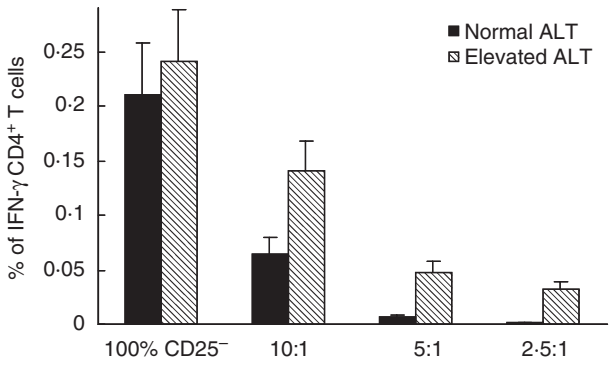

Fig. 7. Dose–response inhibition of hepatitis C virus (HCV)-specific interferon (IFN)-γ production by $CD4^+CD25^+$ T cell in patients with normal and elevated alanine aminotransferase (ALT). $CD4^+$ T cells were used as effector cells to examine HCV-specific IFN-γ activity as measured by flow cytometry in response to pooled HCV antigens (core, NS3, NS4, NS5: 2 μg/ml each) and anti-CD28 and anti-CD49d monoclonal antibodies in co-culture with $CD4^+CD25^+$ T cells at various ratios. Duplicate wells without stimuli were also included to determine the background level of cytokine production. The number of positive events in the absence of antigen stimulation was constantly below 0·01%.

$CD4^+CD25^+$ T cells can be generated from mature $CD4^+CD25^-$ T cell population as a consequence of exposure to antigen in a distinct immunological context, such as exposure to antigens in the presence of IL-10, low-affinity antigens or altered TCR signal transduction [30–32]. Thus it is possible that host and/or viral factors may regulate the intensity of the expansion of suppressive $CD4^+CD25^+$ T cells upon HCV infection. Expression of CD25 is used as a marker of suppressive $CD4^+CD25^+$ T cells. However, CD25 is also expressed by non-suppressive, activated $CD4^+$ T cells [33]. Because the expression of CD25 occurs early after T cell activation, the use of this marker to select suppressive $CD4^+CD25^+$ T cells after T cell activation may be misleading. For this reason, in this study purified $CD4^+CD25^+$ T cells were obtained from PBMC before any T cell stimulation. Despite the limitations of the use of CD25 as a marker for regulatory T cells, no completely satisfactory marker for regulatory T cells has yet been found. $CD4^+CD25^+$ T cells were shown to constitutively express cytotoxic T lymphocyte-associated antigen (CTLA)-4 [34]. However, similar to CD25, CTLA-4 surface expression is up-regulated by activation of conventional T cells. Regardless of the functional meaning of CTLA-4 expression on $CD4^+CD25^+$ regulatory T cells, the use of CTLA-4 as a marker presents little advantage over the use of CD25. OX40 (TNFRSF4), 4-1BB (TNFRSF9) and glucocorticoid-induced TNF receptor superfamily member 18 (GITR, also known as TNFRSF18) are expressed at higher levels by unstimulated $CD4^+CD25^+$ regulatory T cells than by conventional T cells; however, similar to expression of CD25 and CTLA-4, expression of OX40, 4-1BB and GITR is up-regulated in conventional T cells upon activation [35]. Foxp3 is a transcriptional repressor [36] and is expressed specifically by T cells with regulatory functions [37]. The literature suggests that Foxp3 can represent the best marker for regulatory T cell activity. However, Foxp3 has been shown recently to be up-regulated in activated human $CD4^+$ cells, thus potentially complicating the use of this marker. This cell type appears to be better distinguishable based on its cytokine production profile and its ability to suppress immune responses. Moreover, the identification of a subset within the $CD4^+CD25^+$ T cells in the circulation of normal humans has been reported recently [26] that exhibit strong *in vitro* regulatory function (> 95% inhibition of anti-CD3-induced proliferation) with characteristics similar to those of murine $CD4^+CD25^+$ regulatory cells. This $CD4^+CD25^{+high}$ T cell subset in humans comprises 1–2% of circulating $CD4^+$ T cells, unlike that in rodents where 6–10% of $CD4^+$ T cells demonstrate regulatory function. Whereas the entire population of $CD4^+CD25^+$ T cells expressing both low and high CD25 levels exhibit regulatory function in the mouse, only the $CD4^+CD25^{high}$ population ($CD4^+CD25^{+high}$) exhibits a similarly strong regulatory function in humans. These $CD4^+CD25^{+high}$ cells inhibit proliferation and cytokine secretion induced by TCR cross-linking of $CD4^+CD25^-$ responder T cells in a contact-dependent manner. Here we found that the cytokine production profile of HCV-antigen-activated $CD4^+CD25^{+high}$ T cells is remarkably similar to that of suppressive $CD4^+CD25^+$ T cell clones [38] and is distinct from that of IL-10-producing regulatory T cells [39] (which produce high levels of IL-10 and moderate amounts of TGF-β and IFN-γ) and of Th3 cells [40]. In contrast to data obtained with HCV-specific $CD4^+CD25^{+high}$ T cells, we did not detect significant differences in the frequency of total $CD4^+CD25^+$ T cells and in their phenotype between patients with normal and elevated ALT levels. This suggests that the steady-state level of $CD4^+CD25^+$ T cells before infection may not determine whether individuals will develop liver disease.

Our results also indicate that in total PBMC, both IFN-γ production and proliferation of HCV-specific $CD4^+$ T cells were significantly greater in patients with elevated ALT levels in patients with normal ALT. Thus, there is an inverse correlation between $CD4^+$ reactivity and the frequency of suppressive HCV-specific $CD4^+CD25^{+high}$ T cells. More importantly, a significant inverse correlation was found between the amount of the HCV-specific TGF-β response by $CD4^+CD25^{+high}$ T cells and the grade of liver inflammation. In addition, in $CD4^+CD25^+$ T cell-depleted PBMC, a significantly higher increase of both IFN-γ production and proliferation of HCV-specific $CD4^+$ T cells was documented in patients with normal ALT levels compared with patients with elevated ALT. This indicates that the degree of suppression of $CD4^+$ T cell reactivity by $CD4^+CD25^+$ T cells is enhanced in patients with normal ALT levels in patients with elevated ALT. These data are strengthened further by the finding that a significantly greater suppression of $CD4^+$ T cell reactivity could be obtained in patients with normal ALT values with respect to patients with elevated ALT with a similar number

of $CD4^+CD25^+$ T cells. $CD4^+CD25^+$ population can contain other cell types in addition to $CD4^+CD25^+$ T_{reg} cells; as such, it is possible that the observed loss of regulatory function seen in elevated ALT patients reflects a decrease in regulatory activity on a per cell basis or a loss of antigen-specific $CD4^+CD25^+$ T_{reg} cells from within this population. Indeed it cannot be excluded that $CD4^+CD25^-$ cells from elevated ALT patients are less susceptible to $CD4^+CD25^+$ T_{reg} cell-mediated suppression.

Taken together, the data presented here strongly support the possibility that in patients with chronic HCV infection and normal ALT values, $CD4^+CD25^+$ T cells suppress $CD4^+$ T cell reactivity and T cell-mediated liver disease. This mechanism might be relevant because $CD4^+$ T cell-mediated cytokine responses have been associated with progressive liver injury in chronic hepatitis [5–10]. Furthermore, evidence is accumulating of abnormal dendritic cell function in chronic HCV infection [41,42]. In the presence of a weak $CD4^+$ T cell response, dendritic cells might not be sufficiently stimulated and, in turn, might not activate appropriately HCV-specific cytotoxic T lymphocytes (CTL), which have also been suggested to mediate hepatic damage [43,44].

$CD4^+CD25^+$ T cells control the intensity of T cell immune responses at the infection site [20]. Another role of $CD4^+CD25^+$ T cells in controlling immunity is to suppress pathological anti-microbial immune responses that cause tissue damage [45–47]. In patients with chronic HCV infection, HCV-specific $CD4^+CD25^+$ T cells may be redistributed to the liver, the principal site of antigen presentation and inflammation, where they may establish a status of long-lasting low level inflammation critical to the survival of both HCV and the host. However, Cabrera *et al.* [23] have reported recently that $CD4^+CD25^+$ T cells accumulate in peripheral blood and not the liver. An important limitation of that study is that total, and not HCV-specific, $CD4^+CD25^+$ T cells were studied and that the population sampled represented a selected group with heavily fibrosed liver tissue. Such distorted liver architectures are well known to have lower numbers of intrahepatic lymphocytes.

It remains to be determined whether T_{regs} play a protective or detrimental role in chronic HCV infection. Effective immune responses against pathogens are sometimes accompanied by strong inflammatory reactions. To minimize damage to self, the activation of the immune system also triggers anti-inflammatory circuits. Both inflammatory and anti-inflammatory reactions are normal components of the same immune response which, together, fight infections while preventing immune pathology. Expansion of adaptive HCV-specific $CD4^+CD25^+$ T_{reg} cells could hypothetically decrease the magnitude of T cell responses in viraemic patients and contribute to prevent clearing of the infection. Alternatively, $CD4^+CD25^+$ T_{reg} cells may have a protective effect and limit the massive immunopathology that could be caused by high-level viraemia. In agreement with the latter, the data presented here strongly support the hypothesis that $CD4^+CD25^+$ T cells play a role with the different clinical presentation of chronic HCV infection by suppressing the $CD4^+$ T cell response. A longitudinal study examining $CD4^+CD25^+$ T_{reg} cells depletion and clinical progression will most probably address this issue.

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