Università degli Studi del Piemonte Orientale "Amedeo Avogadro" Dottorato di Ricerca in Medicina Molecolare

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Dott. Alfredo Amoroso

Evaluation of circulating CD4$^+$CD25$^+$ and liver-infiltrating Foxp3$^+$ cells in HCV-related hepatitis [SUBMITTED TO "*Immunological Investigations*"]

ABSTRACT
In HCV-related chronic hepatitis the immune system is unable to clear the viral infection. Recent studies have raised the possibility of an involvement of regulatory T cells (Tregs). In this study, we analysed the peripheral blood from thirty patients with HCV-related chronic hepatitis and twenty healthy controls by flow cytometry for the evaluation of the Treg population, (CD4$^+$CD25^{hi}Foxp3$^+$), as well as the activated/effector CD4$^+$ T cells, (CD4$^+$CD25^{low}), and IFN-γ-secreting cells. We also analysed liver biopsies of the patients by immunohistochemical evaluation of Foxp3$^+$ cells. Our results show higher proportions of CD4$^+$CD25^{low} and IFN-γ$^+$ cells in the patients. By contrast, the proportions of peripheral CD4$^+$CD25^{hi} cells did not significantly differ. The eleven patients displaying Foxp3$^+$ cells in the liver infiltrates showed significantly higher proportions of peripheral CD4$^+$CD25^{low} cells. Moreover, we found lower serum transaminase levels in the patients with Foxp3$^+$ immunohistochemistry, although only for ALT such difference resulted statistically significant. In conclusion, these data suggest that the presence of Tregs infiltrating the liver is associated with high levels of activated/effector T cells in the peripheral blood and lower activity of hepatitis. Therefore, liver infiltrating Tregs might play a role in limiting tissue damage and support an effective immune response against HCV.

INTRODUCTION
HCV-related chronic hepatitis is a chronic liver inflammation where the immune system is unable to efficiently clear the viral infection. Although viral factors are certainly involved, several pathophisiological alterations have also been claimed to be involved in the complex pathogenesis of this disease. One possibility is that the naturally tolerogenic microenvironment of the liver favours development of lymphocyte subsets not exerting the appropriate effector functions needed for virus clearance (Mengshol et al., 2007; Racanelli and Manigold, 2007).
Recent studies have raised the possibility that the chronic evolution of the HCV infection may involve *regulatory T cells* (Tregs) (Cabrera et al., 2004), exerting suppressive function on the anti-viral effector immune cells. Tregs have been shown to exhibit a relevant heterogeneity in their differentiation patterns, mechanisms of action, tissue distribution as well as phenotype presentation (Shevach, 2006; Taylor et al., 2006; Wilczynski et al., 2008). However, the main Treg subset is believed to be comprised in the CD4$^+$ T cell subset expressing high levels of CD25 (CD25^{hi}) and the transcription factor Foxp3 (*natural* Tregs). HCV-specific CD4$^+$ cells displaying this Treg phenotype have been identified in HCV-related chronic hepatitis (Ebinuma et al., 2008) and the proportions of Tregs have been correlated to the viral load in the PB. Therefore, these Tregs have been suggested to have a deleterious effect on the anti-viral immune response

impairing the ability to clear the infection and favouring the development of chronic hepatitis. In line with this possibility, HCV-specific Tregs have been shown to develop during the infection and to suppress the anti-viral cytotoxic CD8$^+$ cell response. However, the real impact of these cells on the disease's out-come is still debated (Dolganiuc and Szabo, 2008; Manigold and Racanelli, 2007).

Aim of this study was to analyse Treg and activated/effector cells in the peripheral blood (PB), and Treg cells in liver biopsies of patients with HCV-related chronic hepatitis and correlate these data with key disease parameters such as viremia, transaminase levels and histological activity, in order to provide further information about the role of these cells in this infection. Results showed that Tregs infiltrating the liver correlate with high levels of activated/effector cells in the PB and lower serum transaminase level, suggesting that these cells play an effective role in modulating the immune response to HCV while limiting damage to the liver.

MATERIALS AND METHODS

Patients

Thirty patients with HCV-related chronic hepatitis were recruited at the Department of Internal Medicine of P.O. "G. Rodolico", Azienda Ospedaliero-Universitaria "Policlinico-Vittorio Emanuele", Catania, Italy. They were followed up by clinical examination, measurement of transaminase plasma levels and circulating viral load, and ultra-sound exploration of the liver. They were also subjected to liver biopsies to assess the hepatitis activity and the fibrosis stage.

They were 12 men and 18 women, with an age comprised between 39 and 71 years. The modality of infection was in all cases considered as *community acquired* or *undetermined*. The HCV genotype was 1b in all patients. Only two patients were receiving therapy when analyzed (*Rebetol* 1200 mg and *PegIntron* 135).

Controls were healthy volunteers, 13 men and 7 women, with an age comprised between 28 and 61 years. The study was approved by the local Ethical Committee.

Sample collection and tissue processing

After having obtained the informed consent, venous PB drawings were collected from the patients and from the controls, in heparin; needle liver biopsies were obtained percutaneously from the patients.

Clinical laboratory analyses

Serum transaminase levels were measured by using a *Modular Analytics* instrument (Roche).

Detection of the HCV RNA was performed by using the quantitative *cobas TaqMan 48 HCV 2.0* Real Time PCR test, on a *COBAS® TaqMan® 48* lightcycler (Roche).

Histological evaluation

Liver specimens were fixed in 10% neutral buffered formalin and processed for embedding in paraffin wax. Sections of tissue were stained with hematoxylin and eosin for standard light microscopic evaluation using standard methods. The biopsies were classified according to the Sheuer score (portal activity/lobular activity and fibrosis, from 0 to 4).

Immunohistochemical analysis

Tissue sections (5 µm) were microwave-heated for 15 min in 10 mM citrate buffer (pH 6.0) (Millipore, Billerica. USA). Then, they were treated with 1% hydrogen peroxide for 15 min and subsequently blocked with prediluted normal goat serum (SantaCruz

Biotechnology Inc., SantaCruz, USA) for 20 min at room temperature. Sections were then incubated with anti-FOXP3 antibodies (dilution 1:100, sc-80792, SantaCruz, USA) and stained with the streptavidin–biotin–peroxidase complex system (ImmunoCruz[TM] Staining System; SantaCruz Biotechnology Inc., SantaCruz, USA). Finally, sections were counterstained with Mayer's hematoxylin. Slides were dehydrated, mounted and observed by conventional light microscopy. Negative control staining were performed by substituting the primary antibody with non-immune serum.

Flow cytometry analysis
Whole blood samples were surface-stained using FITC-conjugated CD4, PE-conjugated CD8, and PECy5-conjugated CD25 monoclonal antibodies (mAb) (Becton Dickinson). Then, cells were permeabilized with *Foxp3 Buffer Set* (eBioscience), stained with APC-conjugated anti-Foxp3 mAb (Becton Dickinson), and fixed in PBS containing 1% of paraformaldehyde. Control stainings were performed with the appropriate isotype matched Ab.
For detection of IFN-γ producing cells, 500 µl of whole blood were mixed with 500 µl of RPMI 1640 medium with glutamine and stimulated with 25 ng/ml of PMA (phorbol 12-myristate,13-acetate, Sigma) and 1 µg/ml of ionomycin (Sigma) in the presence of Brefeldin (Becton Dickinson). The cells were then incubated in 15 ml tubes at 37°C in 5% CO_2, humidified atmosphere for 4 hours. After incubation, surface and intracellular staining were performed as indicated above using the anti-CD4 mAb and anti-Foxp3 mAb plus a PE-conjugated anti-IFN-γ mAb (Becton Dickinson); these cells were not stained for CD25, since preliminary experiments showed a substantial downmodulation of this marker upon the treatment with PMA+ionomycin. The samples were analyzed in a *FACScalibur* cytometer (Becton Dickinson).

Statistical analysis
The percentages of T cell subsets in examined groups were expressed as median and interquartile ranges (IR). The statistical significance was assessed by the Mann-Whitney U-test. Differences were considered to be statistically significant at a level of $p < 0.05$.

RESULTS
The histological evaluation was performed by an expert histopathologist using the Sheuer score system (portal activity/lobular activity and fibrosis, from 0 to 4 points each). The patients displayed a grading ranging from 1/0, with 1 point for fibrosis, to 3/2 with 3

Sheuer's score	No. of specimens (n=30)	Foxp3[+]	Foxp3[-]	p[1]
1/0/1	2	0	2	
2/1/1	1	0	1	
2/1/2	3	1	2	
2/2/1	4	0	4	0.72
2/2/2	3	3	0	
3/1/2	4	2	2	
3/2/2	8	3	5	
3/2/3	5	2	3	
[1]Yates' chi square test.				

points for fibrosis, in the distribution resumed in table 1.

Table 1. Association of Foxp3 expression with Sheuer's score in HCV-affected liver sections

The immunohistochemical analysis for Foxp3 detected positive cells in 11 patients out of

30 (table 1), and showed that Foxp3$^+$ cells were found among the lymphocytes infiltrating the portal area (fig. 1).
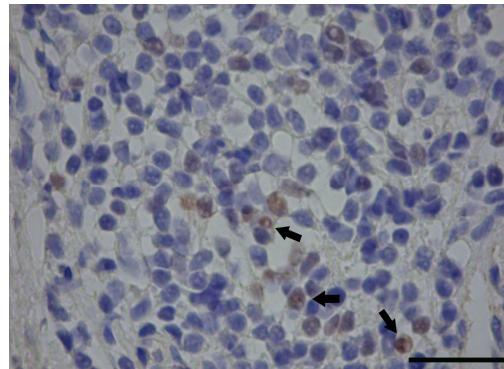

Figure 1. Representative micrograph of liver section from a HCV affected patient. Some lymphocytes are immunolabelled for Foxp3 (arrows) (bar: 60 µm).

Transaminase levels were elevated in most patients: 29 and 19 patients for ALT and AST, respectively. The mean ALT serum level was 97.27 ± 32.09 U/I in Foxp3$^+$ patients vs 127 ± 33.22 in Foxp3$^-$ patients; the mean AST serum level was 56.73 ±28.09 U/I in Foxp3$^+$ patients vs 76.21 ± 32.21 in Foxp3$^-$ patients. Such difference between the mean values of Foxp3$^+$ and Foxp3$^-$ patients resulted statistical significance for ALT (p=0,03)

	Foxp3$^-$ *(n=19)*	*Foxp3$^+$* *(n=11)*
ALT (U/L)		
mean value (SD)	127 (33.22)	97.27 (32.09)
median (IQR)	128 (106-156)	102 (74-119)
p		**0.03**
AST (U/L)		
mean value (SD)	76.21 (32.21)	56.73 (28.09)
median (IQR)	72 (53-102)	57 (27-69)
p		0.08

(table2).**Table2**: Mean ALT and AST serum concentrations in the patients subgrouped by Foxp3 liver immunohistochemistry. The difference between patients with Foxp3$^+$ and Foxp3$^-$ histology is statistically significant for ALT (p= 0,03).

Flow cytometry analysis of the PB samples showed a relative increase of total lymphocytes in the patients compared to the controls; the median percentage of circulating lymphocytes was 27.85% (IR: 25.82% - 30.91%) in the patients and 25.02% (IR: 17.08% - 28.54%) in the controls (p=0.02). Within the lymphocyte population, the median percentages of T helper CD4$^+$ cells were 44.20% (IR: 40.06% - 49.87%) in the patients and 37.20% (IR: 31.41% - 42.52%) in the controls (p=0.0002); the median percentage of CD8$^+$ T cells were 38.63% (IR: 34.70% - 39.77%) in the patients and 23.87% (IR: 19.45% - 25.28%) in the controls (p<0.0001). In CD4$^+$ lymphocytes, expression of CD25 distinguished three subsets: CD4$^+$CD25$^-$, CD4$^+$CD25^{low} and CD4$^+$CD25^{hi}. The marker separating the CD25^{low} and CD25^{hi} subset was set at one logarithmic decade from the CD25$^-$/CD25^{low} cut-off (fig 2a). Within CD4$^+$ cells, median percentages of CD25$^-$, CD25^{low}, and CD25^{hi} cells were 47.13% (IR: 42.27% - 60.58%), 49.59% (IR: 38.57% - 53.84%) and 2.47% (IR: 1.67% - 3.44%) in the patients, and 64.39% (IR: 56.71% - 72.59%), 32.13% (IR: 24.45% - 39.42%), and 3.01% (IR: 2.32% - 3.29%) in the controls, respectively. Proportions of CD25^{low} cells were significantly higher and those of CD25$^-$ cells significantly lower in the patients than in the controls (p<0.0001), whereas those of

CD25^{hi} cells were not different in the two groups (p=0.08) (fig. 2b).
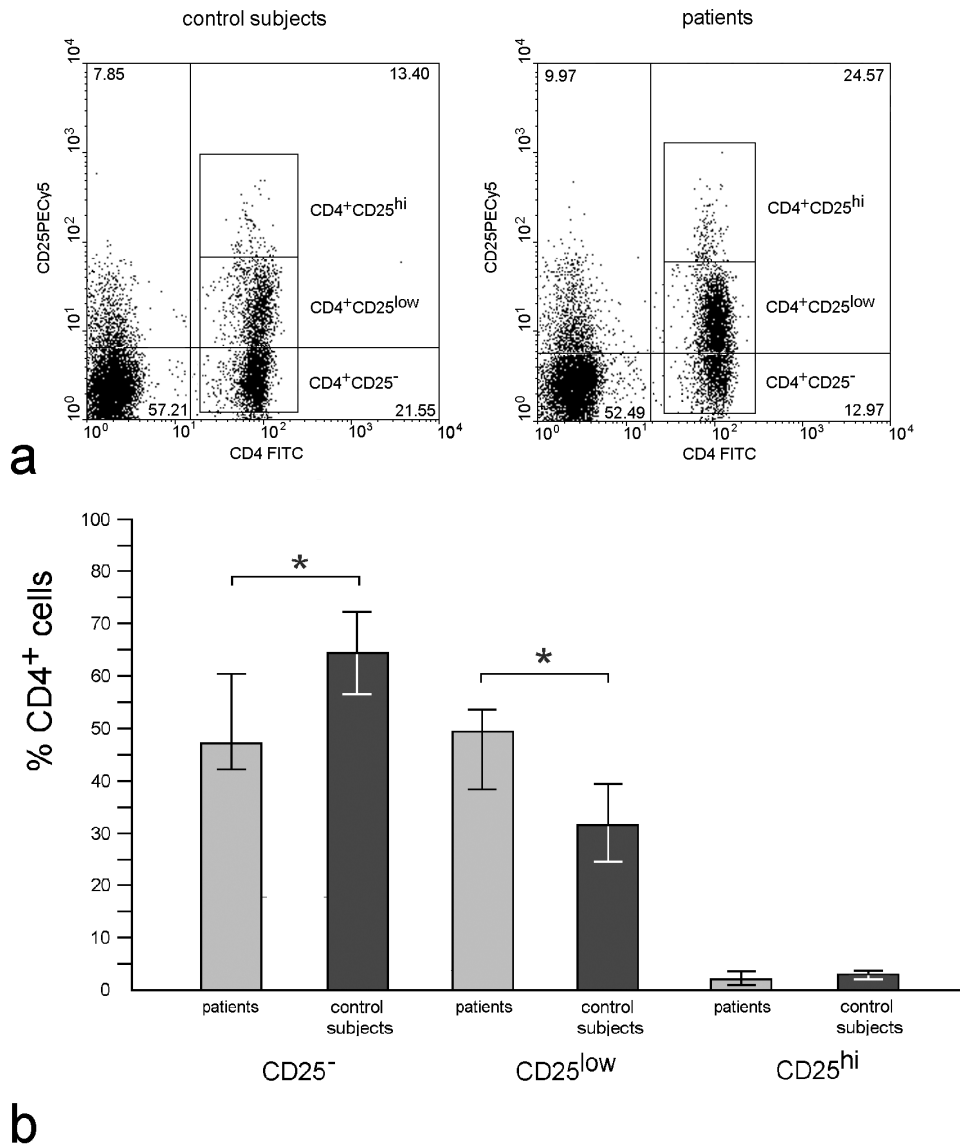

Figure 2. (**a**) Representative dot plots from peripheral blood. The cytograms show the expression of CD4 and CD25 in the peripheral blood lymphocyte population of healthy controls and patients. (**b**) Bar histogram representing the mean percentages of CD25$^-$, CD25^{low} and CD25^{hi} cell populations among the CD4$^+$ cells in peripheral blood lymphocytes, in patients and controls. The percentage of CD4$^+$CD25^{low} cells is increased in the patients, compared to controls. [a]P<0.0001

Foxp3 staining was positive at low intensity in the CD4$^+$CD25^{hi} cells only (fig.3), and the Foxp3 mean fluorescence in this population was not significantly different in the patients and the controls (21.61 ± 9.39 vs 20.34 ± 10.21, p=0.65). Intriguingly, the proportion of PB CD4$^+$CD25^{low} cells was significantly higher in the patients displaying a Foxp3$^+$ immunohistochemistry than in those displaying a Foxp3$^-$ immunohistochemistry (52.87%, IR: 41.80% - 57.73% vs 41.26, IR: 37.54% - 50.24%); p=0.04) (fig. 4). Both patients receiving therapy were positive for Foxp3 in the biopsies. No differences were found in the CD4$^+$C25^{hi} and CD4$^+$CD25$^-$ cell proportions between the patients Foxp3$^+$ and Foxp3$^-$ at the histology.
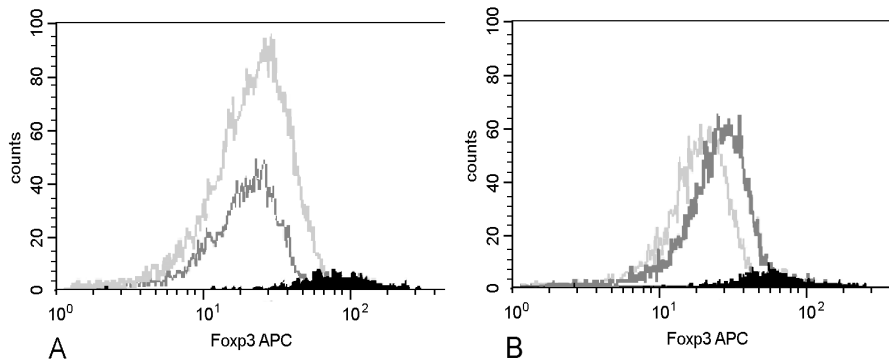
Figure 3. Histograms representing Foxp3 expression intensity among the CD25⁻, CD25^{low} (empty histograms) and CD25^{hi} (filled histograms) cell populations in controls (A) and patients (B). The CD4^{+}CD25^{hi} cells are Foxp3^{+} at low intensity, while the CD4^{+}CD25^{-} and the CD4^{+}CD25^{low} cells are Foxp3^{-}. The fluorescence intensity of the isotype control resulted similar to the intensity of the anti-Foxp3 antibody in the CD4^{+}CD25^{-} and CD4^{+}CD25^{low} cells (data not shown).
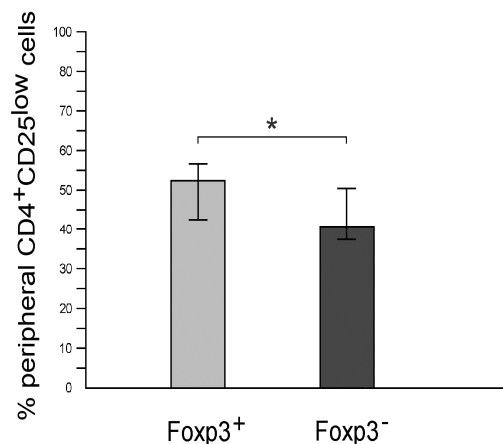

Figure 4. Median percentages of CD25^{low} cells in peripheral CD4^{+} T cell population in patients subgrouped for liver Foxp3 expression. [b]$P<0.05$

After stimulation with PMA and ionomycin, analysis of IFN-γ expression showed higher median percentages of IFN-γ^{+} cells in the patients (34.25%, IR: 30.85% - 39.37%) than in the controls (17.13%, IR: 13.91% - 20.40%) (p<0.0001); this difference was significant in both the CD4^{+} (12.61%, IR: 10.38% - 17.42% vs 4.84%, IR: 3.49% - 7.23%) and CD4^{-} (20.65%, IR: 18.71% - 25.02%, vs 10.51%, IR: 7.37% - 14.89%) subpopulations (p<0.0001) (fig. 5a). After the in vitro stimulation, the proportion of Foxp3^{+} cells was still not significantly different between patients and controls: 1.21% (IR: 0.15%-2.87%) in control subjects, 1.27 % (IR: 0.24%-3.11%) in patients. As expected, Foxp3 was expressed only in CD4^{+} cells (data not shown) and double-positive IFN-γ/Foxp3^{+} cells were substantially absent (fig. 5b).
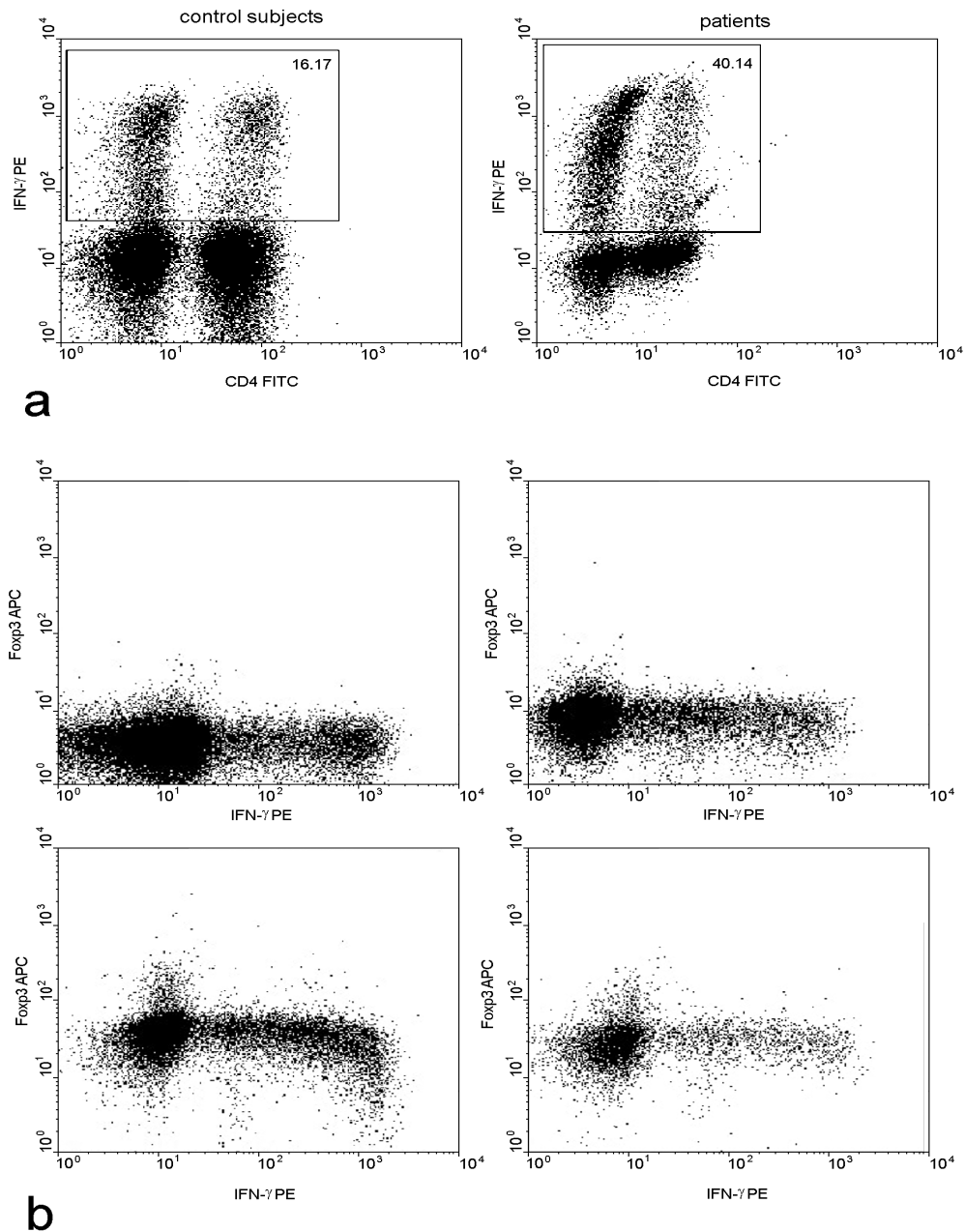
Figure 5. (a) Representative dot plot cytograms showing the expression of CD4 and IFN-γ after stimulation with 25 ng of PMA and 1 μg of ionomycin, in the peripheral blood lymphocyte population, in the healthy controls and in the patients. The percentage of IFN-γ$^+$ cells is increased in the patients, both from the CD4$^+$ and CD4$^-$ subsets. **(b)** Representative dot plot cytograms showing the expression of IFN-γ and Foxp3 after stimulation with 25 ng of PMA and 1 μg of ionomycin, in the peripheral blood lymphocyte population in the patients. No populations are identifiable showing a double positivity for the two markers.

PB viral load ranged from 12 x 10^4 to 2.2 x 10^6 genome equivalents/ml (geq/ml), except for the two patients receiving therapy, whose viral load was substantially lower (5 x 10^4 and 8 x 10^4 geq/ml). A correlation was found with the proportion of PB CD4$^+$CD25^{low} cells (fig. 6).
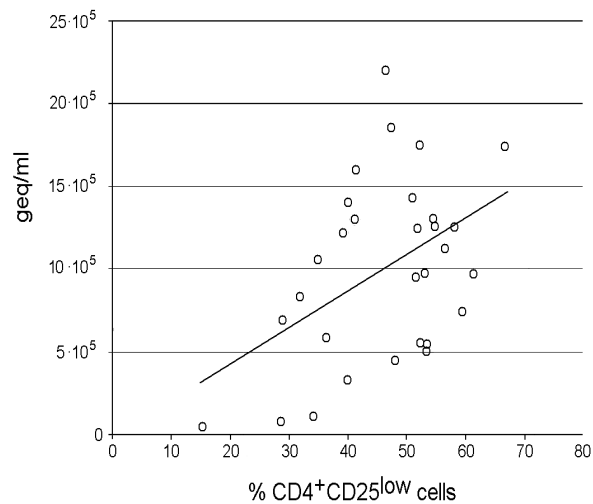
Figure 6. Diagram representing the percentage of peripheral blood $CD4^+CD25^{low}$ cells related to the circulating viral load in the patients.

DISCUSSION

In HCV-related chronic hepatitis, the immune system is unable to effectively clear the viral infection, despite effective activation of the humoural and cellular immune response (Bode et al., 2008). Viral factors are involved such as the ability of HCV to modify its surface antigens and to exploit different entry mechanisms to infect the target (Flint and Tscherne, 2009). However, a role is also played by the liver microenvironment that may inhibit mounting of an effective immune response. The highly tolerogenic liver microenvironment, rich in TGF-β and other profibrotic factors, has been shown to favour an immune response different from the Th1 response expected to be effective against the viral infection (Erhardt et al., 2007; Manigold and Racanelli, 2007; Hall et al., 2010).

In this study, we showed that proportions of PB T cells expressing the $CD4^+CD25^{low}$ phenotype, comprising the activated/effector compartments of T helper (Th) cells, were increased in the patients' PB, compared to the controls. The proportion of these cells correlated with the PB viral load, which supports their possible involvement in the anti-viral response. In line with these findings, also proportions of IFN-γ-secreting cells were increased in both $CD4^+$ and $CD4^-$ lymphocyte cell subsets as expected in a viral infection.

By contrast, the proportions of PB T cells expressing the $CD4^+CD25^{hi}Foxp3^+$ phenotype, comprising cells with Treg activity (referred to as *natural* Tregs), were not significantly different in the patients and the controls and did not correlate with the viral load.

No double-positive $IFN-γ^+Foxp3^+$ cells were found in our experiments, which makes unlikely that $Foxp3^+$ cells were simply activated effector cells, as suggested by other authors (Morgan et al., 2004; Wang et al, 2007).

An intriguing data came out from the immunohistochemistry analysis which shows that the concentrations of serum transaminases are lower in the patients displaying $Foxp3^+$ cells in the liver inflammatory infiltrates than in those lacking these cells, although this difference resulted statistical significant only for ALT. Moreover, the proportion of PB $CD4^+CD25^{low}$ cells is significantly higher in the same patients.

Although the observation showing a lack of correlation between PB $CD4^+CD25^{hi}Foxp3^+$ cells and the circulating viral load disagrees with other reports showing a correlation between these cells and the viral load in the PB (Wilczynski et al., 2008), our findings are consistent with the study of Ward and colleagues (Ward et al., 2007) in the idea that the main effect of Treg presence and activation within the liver parenchyma during HCV infection leads to a relative reduction of liver inflammation while they might favour fibrogenesis by the production of TGF-β. However, since the correlation with the fibrosis still remains unclear, analysis of the TGF- β distribution pattern in relation to the presence

of Foxp3^{+} cells within the liver should be taken into consideration as an important goal in further immunihistochemistry experiments.

Our findings are in line with the notion that Treg cells help to direct the anti-viral response towards the appropriate exogenous targets, limiting the damage to tissues due to either exaggerated effector responses, or autoreactive responses secondary to molecular mimicry. Moreover, suppression may even prolong the immune response by preventing the massive activation and the consequent exhaustion of effector cells (Rouse and Suvas, 2004), as recent studies have pointed out how Tregs can play a key role in allowing the establishment of a long term immunity to Leishmania infection (Belkaid et al., 2002). They suggested that presence of Tregs within the inflammatory infiltrates has a double purpose: from the one hand prevents the exhaustion of effector cells, while from the other hand, by preventing a complete pathogen clearance, allows constitution of an antigen reservoir that chronically stimulates the immune system and create a long term immunity.

It must be underlined that other subsets of Tregs can be involved in the disease, but have not been evaluated in this work since their detection is elusive (Wilczynski et al., 2008); these other subsets (indicated as *peripherally-induced* Tregs), which can be either CD4^{+} or CD8^{+}, are often Foxp3^{-} and may differentiate from inappropriately activated naïve T cells, such as those activated in the absence of costimulatory signals or in the presence of high levels of TGF-β or IL-10 which are abundant in the liver (Vieira et al., 2004). Alternatively, they may differentiate from exhausted effector lymphocytes.

In conclusion, it is possible to assume that Treg cells are likely to exert a control on HCV-specific cell-mediated immune response. Such control, however, would function as a general mechanism to limit damage to tissues and to prevent the exhaustion of effector cells. It is also reasonable, however, that because of their great heterogeneity and plasticity, some liver-infiltrating T cells, conditioned by the local cytokine milieu, can develop phenotypes favouring fibrogenesis. Moreover, as a result of an aberrant activation mechanism during HCV-related chronic inflammation, some HCV-specific T cells displaying a regulatory phenotype can arise in the liver, being able to inappropriately inhibit the anti-viral immune response (Wilczynski et al., 2008; Ebinuma et al., 2008). Further studies will also have to be addressed in investigating on the interplay between thymus-derived *natural* Tregs and *peripherally-induced* Tregs.

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