Evaluation of circulating $CD4^+CD25^+$ and liver-infiltrating $Foxp3^+$ cells in HCV-associated liver disease

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Abstract. In hepatitis C virus (HCV)-associated liver disease, the immune system is unable to clear the viral infection. Previous studies have raised the possibility of an involvement of regulatory T cells (Tregs). In this study, we analysed the peripheral blood from 30 patients with HCV-associated chronic liver disease and 20 healthy controls by flow cytometry for the evaluation of the Treg population [$CD4^+CD25^{hi}$ forkhead box protein 3 $(Foxp3)^+$], 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 showed higher proportions of $CD4^+CD25^{low}$ and IFN-γ^+ cells in the patients than in the controls. By contrast, the proportions of peripheral $CD4^+CD25^{hi}$ cells did not significantly differ. The 11 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 than in the controls, as shown by $Foxp3^+$ immunohistochemistry, although these results were only statistically significant as regards alanine transaminase (ALT). 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 may play a role in limiting tissue damage and may thus support an effective immune response against HCV.

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Introduction

Hepatitis C virus (HCV)-associated chronic liver disease is a chronic liver inflammation where the immune system is unable to efficiently clear the viral infection. Although viral factors are certainly involved, several pathophysiological alterations have also been suggested to be involved in the complex pathogenesis of this disease. One possibility is that the naturally tolerogenic microenvironment of the liver favours the development of lymphocyte subsets not exerting the appropriate effector functions needed for clearance of the virus (1,2).

Previous studies have raised the possibility that the chronic evolution of HCV infection may involve regulatory T cells (Tregs) (3), 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 (4-6). However, the main Treg subset is believed to be comprised of the $CD4^+$ T cell subset expressing high levels of CD25 ($CD25^{hi}$) and the transcription factor forkhead box protein 3 (Foxp3) (natural Tregs). HCV-specific $CD4^+$ cells displaying this Treg phenotype have been identified in HCV-associated chronic liver disease (7) and the proportions of Tregs have been correlated with the viral load in the peripheral blood (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 outcome of the disease is still under debate (8,9).

The aim of this study was to analyse Treg and activated/effector cells in PB, and Treg cells in liver biopsies of patients with HCV-associated chronic liver disease and correlate these data with transaminase levels and histological activity, in order to provide further information as regards the role of these cells in this infection. The results showed that Tregs infiltrating the liver correlate with high levels of activated/effector cells in the PB and lower serum transaminase levels, 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 ultrasound exploration of the liver. They were also subjected to liver biopsies to assess the hepatitis activity and the fibrosis stage.

The patients included 12 males and 18 females, aged 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 2 patients were already receiving therapy at the time of analysis (Rebetol 1200 mg and PegIntron 135 mg). The controls comprised healthy volunteers, 13 males and 7 females, aged between 28 and 61 years. The study was approved by the local ethics committee.

Sample and tissue collection. After having obtained informed consent from all patients, PB specimens were drawn from the patients and control subjects and collected in heparin collection tubes. Needle liver biopsies were obtained percutaneously from the patients.

Clinical laboratory analyses. Serum transaminase levels were measured by using a Modular Analytics instrument (Roche, Diagnostics, Indianapolis, IN, USA).

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, MA, USA). They were then treated with 1% hydrogen peroxide for 15 min and subsequently blocked with pre-diluted normal goat serum (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 20 min at room temperature. Sections were then incubated with anti-Foxp3 antibodies (dilution 1:100, sc-80792; Santa Cruz Biotechnology Inc.) and stained with the streptavidin-biotin-peroxidase complex system (ImmunoCruz™ Staining System; Santa Cruz Biotechnology Inc.). Finally, the sections were counterstained with Mayer's hematoxylin. Slides were dehydrated, mounted and observed by conventional light microscopy. Negative control staining was 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, Franklin Lakes, NJ, USA). The cells were then permeabilized with the Foxp3 Buffer Set (eBioscience, San Diego, CA, USA), 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 antibody.

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 phorbol 12-myristate, 13-acetate (PMA; Sigma Chemical Co., St. Louis, MO, USA) 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 h. 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 treatment with PMA + ionomycin. The samples were analysed in a FACSCalibur cytometer (Becton-Dickinson).

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

Sheuer's score	No. of specimens (n=30)	Foxp3+	Foxp3-	p-value[a]
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	

[a]Yates' Chi-square test.

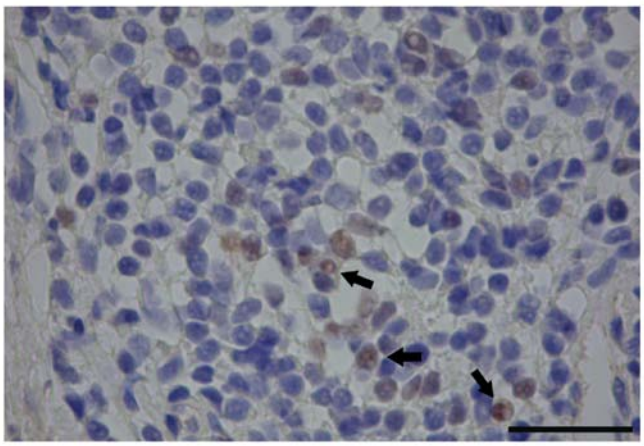

Figure 1. Representative micrograph of a liver section from an HCV-affected patient. Some lymphocytes are immunolabelled for Foxp3 (arrows) (bar, 60 μm).

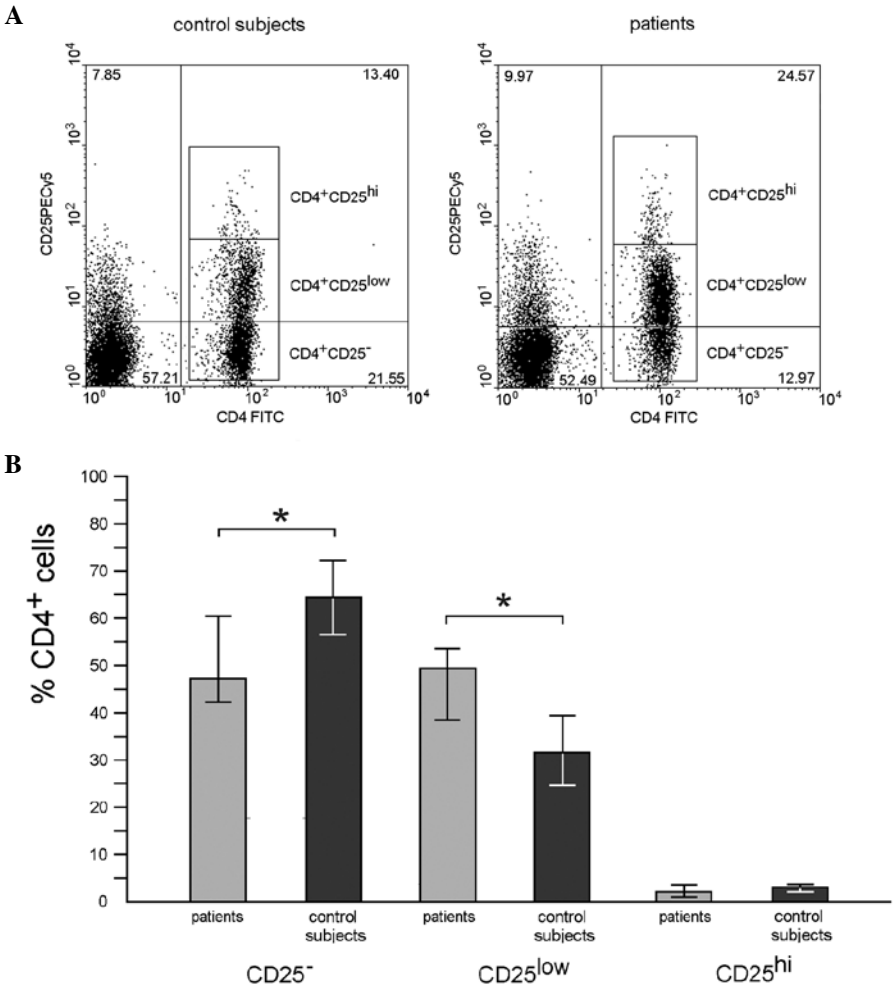

Figure 2. (A) Representative dot plots from peripheral blood. The cytograms show the expression of CD4 and CD25 in the peripheral blood lymphocyte population of healthy controls and patients. (B) Bar histogram representing the mean percentages of $CD25^-$, $CD25^{low}$ and $CD25^{hi}$ cell populations 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 the controls. *p<0.0001.

Statistical analysis. The percentages of T cell subsets in the 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 patients with HCV-associated liver disease displayed a grading ranging from 1/0, with 1 point for fibrosis, to 3/2 with 3 points for fibrosis. The immunohistochemical analysis for Foxp3 detected positive cells in 11 out of 30 patients (Table I), and showed that $Foxp3^+$ cells were found among the lymphocytes infiltrating the portal area (Fig. 1).

Transaminase levels were elevated in most patients: 29 and 19 patients for alanine transaminase (ALT) and aspartate aminotransferase (AST), respectively. The mean ALT serum level was 97.27±32.09 U/l in $Foxp3^+$ patients vs. 127±33.22 in $Foxp3^-$ patients; the mean AST serum level was 56.73±28.09 U/l in $Foxp3^+$ patients vs. 76.21±32.21 in $Foxp3^-$ patients. Such a difference between the mean values of $Foxp3^+$ and $Foxp3^-$ patients resulted in a statistical significance for ALT (p=0.03) (Table II).

Table II. Mean ALT and AST serum concentrations in $Foxp3^+$ and $Foxp3^-$ patient livers at the immunohistochemical level.

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

Numbers in bold indicate a statistically significant difference in ALT levels between $Foxp3^+$ and $Foxp3^-$ patients (p=0.03).

Flow cytometry analysis of the PB samples showed a relative increase in the total amount of lymphocytes in the patients compared to the controls; the median percentage of circulating

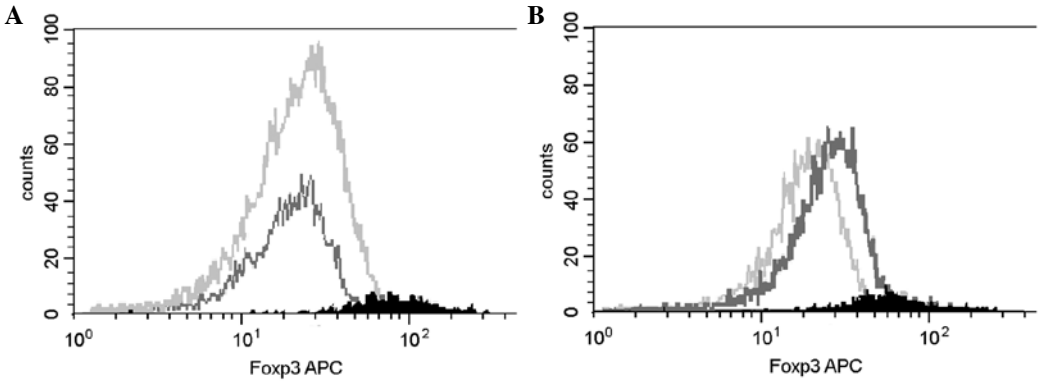

Figure 3. Histograms representing Foxp3 expression intensity among the $CD25^-$, $CD25^{low}$ (empty histograms) and $CD25^{hi}$ (filled histograms) cell populations in controls (A) and patients (B). The $CD4^+CD25^{hi}$ cells are $Foxp3^+$ at low intensity, while the $CD4^+CD25^-$ and the $CD4^+CD25^{low}$ cells are $Foxp3^-$. The fluorescence intensity of the isotype control was similar to the intensity of the anti-Foxp3 antibody in the $CD4^+CD25^-$ and $CD4^+CD25^{low}$ cells (data not shown).

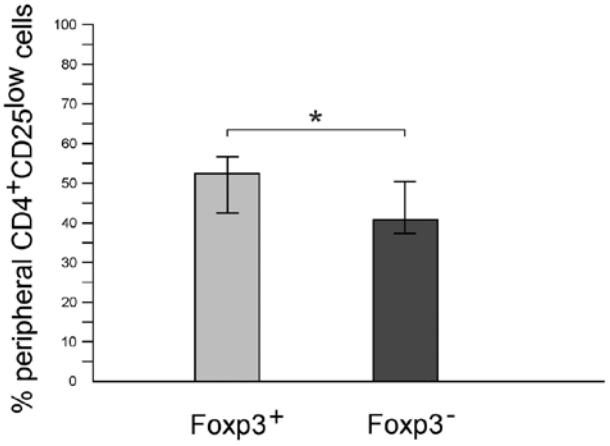

Figure 4. Median percentages of $CD25^{low}$ cells in peripheral $CD4^+$ T cell population in patients subgrouped for liver Foxp3 expression. $^*p<0.05$.

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 percentage of T helper $CD4^+$ cells was 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 was 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, the expression of CD25 distinguished 3 subsets: $CD4^+CD25^-$, $CD4^+CD25^{low}$ and $CD4^+CD25^{hi}$. The marker separating the $CD25^{low}$ and $CD25^{hi}$ subset was set at 1 logarithmic decade from the $CD25^-/CD25^{low}$ cut-off value (Fig. 2A). Within the $CD4^+$ cells, the 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. The proportions of $CD25^{low}$ cells were significantly higher and those of $CD25^-$ cells significantly lower in the patients in comparison to the controls (p<0.0001), whereas those of $CD25^{hi}$ cells were not different between the 2 groups (p=0.08) (Fig. 2B).

Foxp3 staining was positive but at a low intensity in the $CD4^+CD25^{hi}$ cells only (Fig. 3), and the Foxp3 mean fluorescence in this population was not significantly different between the patients and the controls (21.61±9.39 vs. 20.34±10.21, p=0.65). Of note, the proportion of PB $CD4^+CD25^{low}$ cells was significantly higher in the patients displaying a positive immunolocalization of $Foxp3^+$ compared to those displaying a negative one (52.87%, IR 41.80-57.73% vs. 41.26, IR 37.54-50.24%; p=0.04) (Fig. 4). The liver biopsies from the 2 patients receiving therapy were positive for Foxp3. No difference was found in the $CD4^+CD25^{hi}$ and $CD4^+CD25^-$ cell proportions between the patients who were $Foxp3^+$ and $Foxp3^-$ at the immunohistochemical level.

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%) in comparison to the controls (17.13%, IR 13.91-20.40%) (p<0.0001); this difference was significant in both $CD4^+$ (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 *in vitro* stimulation, the proportion of $Foxp3^+$ cells was still not significantly different between the patients and controls: 1.21% (IR 0.15-2.87%) in the 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).

Discussion

In HCV-associated chronic liver disease, the immune system is unable to effectively clear the viral infection, despite the activation of the humoral and cellular immune response (10). A number of viral factors are involved in this process, such as the ability of HCV to modify its surface antigens and to exploit different entry mechanisms to infect the target cells (11). However, the liver microenvironment may also inhibit an effective immune response. In fact, 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 (9,12,13).

In the present study, we showed that the proportions of circulating $CD4^+CD25^{low}$ T cells, comprising the activated/effector compartments of T helper (Th) cells, were increased

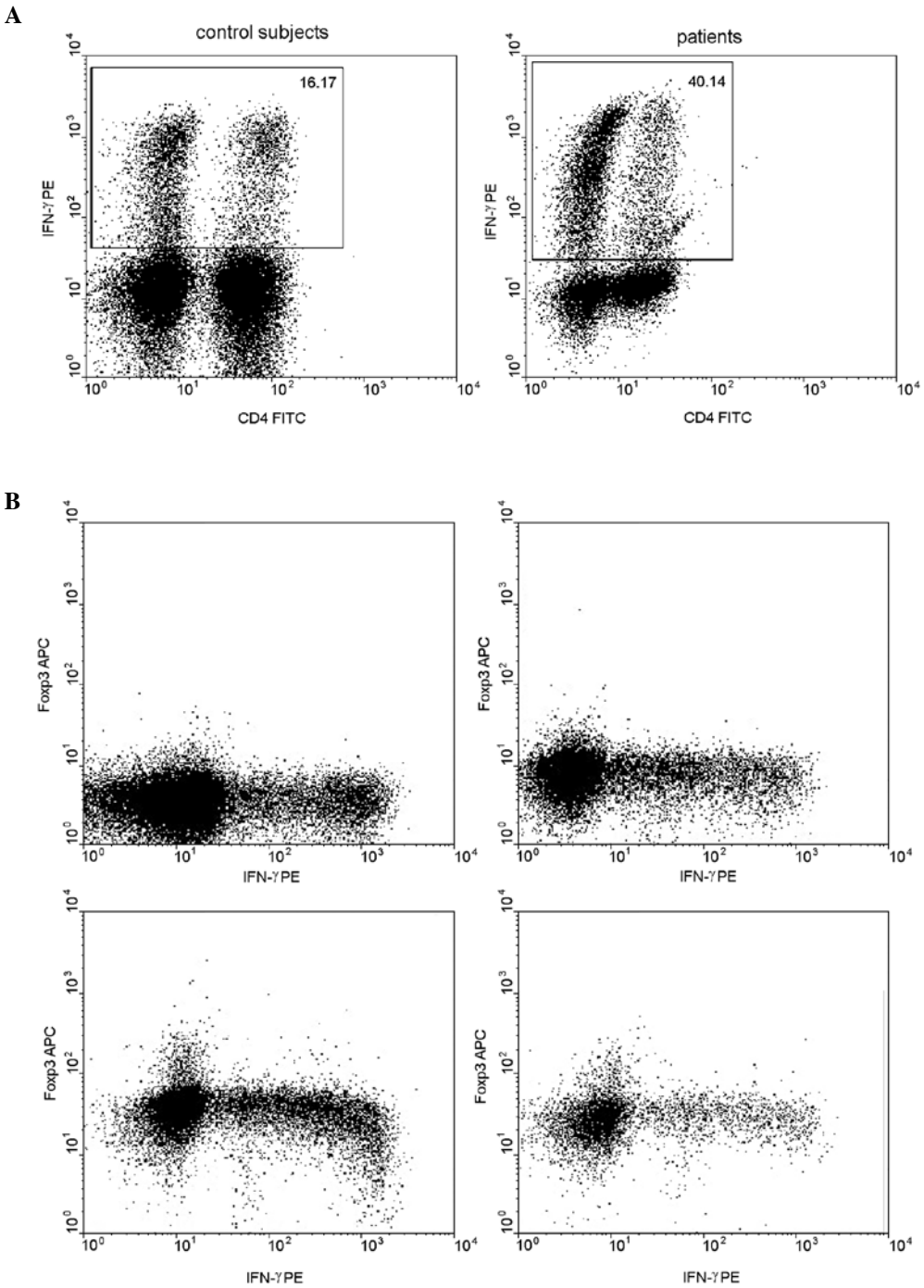

Figure 5. (A) Representative dot plot cytograms showing the expression of CD4 and IFN-γ after stimulation with 25 ng of PMA and 1 μg of ionomycin, in the peripheral blood lymphocyte population, in the healthy controls and in the patients. The percentage of IFN-γ^+ cells is increased in the patients, 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 2 markers.

in the patients compared to the controls. This supports their possible involvement in the anti-viral response. In line with these findings, the proportions of IFN-γ-secreting cells were also increased in both $CD4^+$ and $CD4^-$ lymphocyte cell subsets as expected in a viral infection.

By contrast, in our study, the proportions of circulating $CD4^+CD25^{hi}Foxp3^+$ T cells, comprising cells with Treg activity (referred to as natural Tregs), were not significantly different in the patients and the controls. Similar to the results from other studies (14,15), no double-positive IFN-γ^+Foxp3^+ cells were found in our experiments, making it unlikely that $Foxp3^+$ cells are simply activated effector cells.

Of note, the concentrations of serum transaminases were lower in the patients displaying $Foxp3^+$ cells in the liver inflammatory infiltrates than in those lacking these cells, although these results were only statistically significant as regards ALT. Moreover, the proportion of PB $CD4^+CD25^{low}$ cells was significantly higher in the same patients.

Our findings are consistent with the study of Ward *et al* (16) in the idea that the main effect of the Treg presence and activa-

tion within the liver parenchyma during HCV infection leads to a relative reduction in liver inflammation while the Treg presence may favour fibrogenesis by the production of TGF-β. However, since the correlation with 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 studies.

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 (17). Previous studies have pointed out that Tregs play a key role in allowing the establishment of a long-term immunity to Leishmania infection (18). They suggested that the presence of Tregs within the inflammatory infiltrates has a double purpose: on the one hand, it prevents the exhaustion of effector cells, while on the other hand, by preventing a complete pathogen clearance, it allows the constitution of an antigen reservoir that chronically stimulates the immune system and creates a long-term immunity.

It must be underlined that other subsets of Tregs may be involved in the disease, but they have not been evaluated in this study since their detection is elusive (6); these different subsets (indicated as peripherally-induced Tregs), which can be either $CD4^+$ or $CD8^+$, are often $Foxp3^-$ and may differentiate from inappropriately activated naive 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 (19). 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 a control, however, would function as a general mechanism to limit tissue damage, and to prevent the exhaustion of effector cells. It is also reasonable, however, that because of their great heterogeneity and plasticity, certain liver-infiltrating T cells, conditioned by the local cytokine milieu, may develop phenotypes which may favour fibrogenesis. Moreover, as a result of an aberrant activation mechanism during HCV-associated chronic inflammation, certain HCV-specific T cells displaying a regulatory phenotype, can differentiate in the liver from naive $CD4^+$ T cells, being able to inappropriately inhibit the anti-viral immune response (6,7). Further studies are required to investigate the interplay between thymus-derived natural Tregs and peripherally-induced Tregs.

Acknowledgements

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