Peripheral lymphocyte subsets in chronic hepatitis C: Effects of 12 weeks of antiviral treatment with interferon-alpha plus ribavirin

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A B S T R A C T

Chronic infection with hepatitis C virus (HCV) causes a quantitative and functional alteration in innate and adaptative immunity. In the present work, we determined by flow-cytometry the profile of blood lymphocyte of untreated HCV patients and in subjects of this group that achieved or not an early virologic response at 12-weeks of treatment with interferon-α plus ribavirin. Twenty-six untreated HCV patients and 20 control healthy individuals were enrolled in the study. Untreated HCV patients had a higher proportion of B cell and a lower proportion of CD8+ T cell and NK cells than healthy individuals did, but the proportions of CD4+ T cells and Treg cells (CD4+CD25+Foxp3+) were similar in these patients and controls. Untreated HCV patients presenting cryoglobulinemia had a lower proportion of Treg cells and a lower Treg/NK cell ratio when compared with those without cryoglobulins. Nineteen out of 26 untreated HCV patients remained in the study and were treated with Interferon-α plus ribavirin. At 12-weeks of treatment, 10 of them achieved early virologic response (EVR), whereas 9 were non-responders (NR). EVR patients differed from NR patients in the increase of their proportion of NK cells at 12 weeks of treatment. In conclusion, untreated HCV patients exhibit an altered profile of blood lymphocyte subsets, including a reduction in the proportion of CD4+CD25+FoxP3+T regulatory cells in patients that present cryoglobulinemia. An early virological response at 12-weeks of treatment with IFN-α plus ribavirin seems to be associated a significant improvement in the proportion of NK cells of HCV treated patients.

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1. Introduction

Chronic hepatitis C (CHC) is an important public health problem. According to the World Health Organization (WHO), more than 170 million people are infected worldwide, with limited therapeutic options and without a preventive vaccine [1—3].

The hepatitis C virus causes persistent infection in most infected individuals; however, the mechanisms involved in this chronic infection still need better knowledge. Factors as HCV mutation, inefficient antigen presentation, impaired NK cell function, T cell depletion and immune suppression mediated by regulatory T cells could contribute to the chronic HCV infection [4—7].

Chronic hepatitis C has been associated with autoimmune manifestations, mainly the production of cryoglobulins (cryoprecipitable immune complexes formed by HCV core antigen, core antibodies, and complement C1q) and the production of HCV antibodies that cross-react with self-antigens as non-organ-specific autoantibodies (NOSA) [8,9]. The involvement of regulatory T cells in chronic HCV infection has been reported in some studies, where a decrease in the number of blood Treg cells has been associated with the autoimmune findings found in HCV patients. Other studies have reported an increased proportion of peripheral Treg cell phenotypes represented by CD4+CD25+high and CD25+FoxP3+ in HCV patients, which could down-regulate the anti-HCV immune response of T lymphocytes [10—14].

In previous studies, we have showed a high prevalence of autoimmune findings in Brazilian HCV patients, mainly represented by cryoglobulinemia and significant production of NOSA [15—18]. However, the profile of peripheral lymphocyte subsets

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involved with either the innate or adaptive immune response of these individuals against HCV and self-antigens still need to be better investigated, justifying the present study. In addition, we also investigated how antiviral treatment with IFN-α plus ribavirin could influence the profile of these immune cells in treated patients that achieved an early virologic response or were non-responders at 12-weeks of this treatment.

2. Material and methods

2.1. Patients and controls

In this study were consecutively enrolled 26 HCV patients from both genders (12 men and 14 women), with age ranging from 33 to 65 years, without previous antiviral therapy. The clinical evaluation determined the presence of chronic hepatitis C in these individuals, supported by laboratory tests for aminotransferases and HCV-antibodies, the demonstration of blood HCV-RNA, HCV-genotyping, and liver histology (necroinflammatory activity and fibrosis) [19]. Two patients were evaluated with the Fibroscan® to avoid the risk of bleeding due to liver biopsy (Table 1).

Blood samples of these individuals were collected before and at 12 weeks of antiviral treatment with IFN-α plus ribavirin, when the virological response was evaluated, using the Brazilian Clinical Protocol and Therapeutic Guidelines for Viral Hepatitis C and Coinfections [20]. In this way, early virological response (EVR) was defined as a drop of 2 Log in the blood HCV load or a blood HCV load of less than 1/100 of the previous HCV-RNA load. A total early virological response corresponded to the absence of blood HCV-RNA at 12-week treatment. The control group was formed by 20 healthy individuals (10 men and 10 women, with a mean age of 48.7 ± 6.7 years). All tested seronegative for viral hepatitis (A, B and C), HIV, and HTLV infection. The Ethical Committee of the Professor Edgard Santos Hospital (Bahia Federal University) approved the study.

2.2. Cryoglobulin and NOSA

The presence of cryoglobulinemia was tested by both cryoprecipitate and gel-diffusion [21]. Serum rheumatoid factor (RF) was probed by nephelometry, using the IMMAGE System from Beckman Coulter (USA). In this immunoassay, a positive RF titer was >20 IU/ml. Antinuclear autoantibodies (ANA) were detected by the indirect fluorescent antibody test (IFAT) using HEp-2 cell antigen substrate (VIRO-IMMUN Labor-Diagnostika GmbH, Germany). Smooth muscle and LKM-1 autoantibodies were tested using a commercial IFAT with combined tissue sections of a stomach, kidney, and rat liver from the same source. The cut-off in these immune reactions was a positive test with serum diluted at 1:40.

2.3. Blood lymphocytes

Lymphocyte subsets were identified and quantified in the patients' peripheral blood by flow cytometry. The following immune reactants were used in these assays: anti-CD3 phycoerythrin (PE), anti-CD4-fluorescein isothiocyanate (FITC), anti-CD8 peridinin chlorophyll protein (PerCP)-Cy5, anti-CD19-PE, CD56-allophycocyanin (APC) (eBioscience San Diego, CA, USA). The cells were acquired using an FACSCanto II cytometer (BD Biosciences, USA). The phenotypic identification of blood Treg cells was determined using the following immune reactants: anti-CD4-FITC, anti-CD25-APC, and anti-FoxP3-PE (Human/Non-Human Primate Regulatory T Cell Staining Kit, eBioscences). Flow cytometry determined the absolute counts of this T cell subset with the cytometer above. Around 20,000 $CD4^+T$ cells were acquired to determine the frequency of regulatory T cells. The FlowJo software (Tree Star) was used in the cytometric analyzes of the lymphocyte subsets (Fig. 1).

2.4. Statistical analysis

Statistical analysis was performed using the Prism software version 6.0 (GraphPad Software Inc., USA). The continuous variables were expressed as mean ± standard deviation (SD) or median and interquartile range (IQR), in accordance with their distribution in the normality test of D'Agostino and Pearson. The correlation test of Pearson or Spearman was used as required in each situation. The unpaired t-test of Student compared the means of two independent groups, whereas the U test of Mann–Whitney compared the medians. Paired groups were analyzed with the paired t-test of Student or the test of Wilcoxon in accordance with their variable distributions. A p value < 0.05 was considered significant.

3. Results

3.1. Clinical, virological, and demographic findings in untreated HCV patients

Most untreated patients enrolled in this study were infected with HCV-genotype 1, followed by the infection with the HCV-genotype 3, being less frequent the infection with the HCV-genotype 2. A high blood HCV load (>600,000 IU/mL) was detected in the majority of the patients before treatment. Twenty-two subjects had ALT levels above the reference value of 41 U/L, varying from 43 to 459 U/L. Mild to moderate necroinflammatory activity, as well as mild to moderate fibrosis, was prevalent in this group.

Around 17 (65%) of the 26 untreated HCV patients were positive for at least one autoimmune marker; 13 of them had cryoglobulinemia, whereas 11 exhibited NOSA. Rheumatoid factor was found in 5 out of 26 subjects, antinuclear antibodies were detected in 5, and 9 (35%) untreated patients were positive for smooth muscle antibodies.
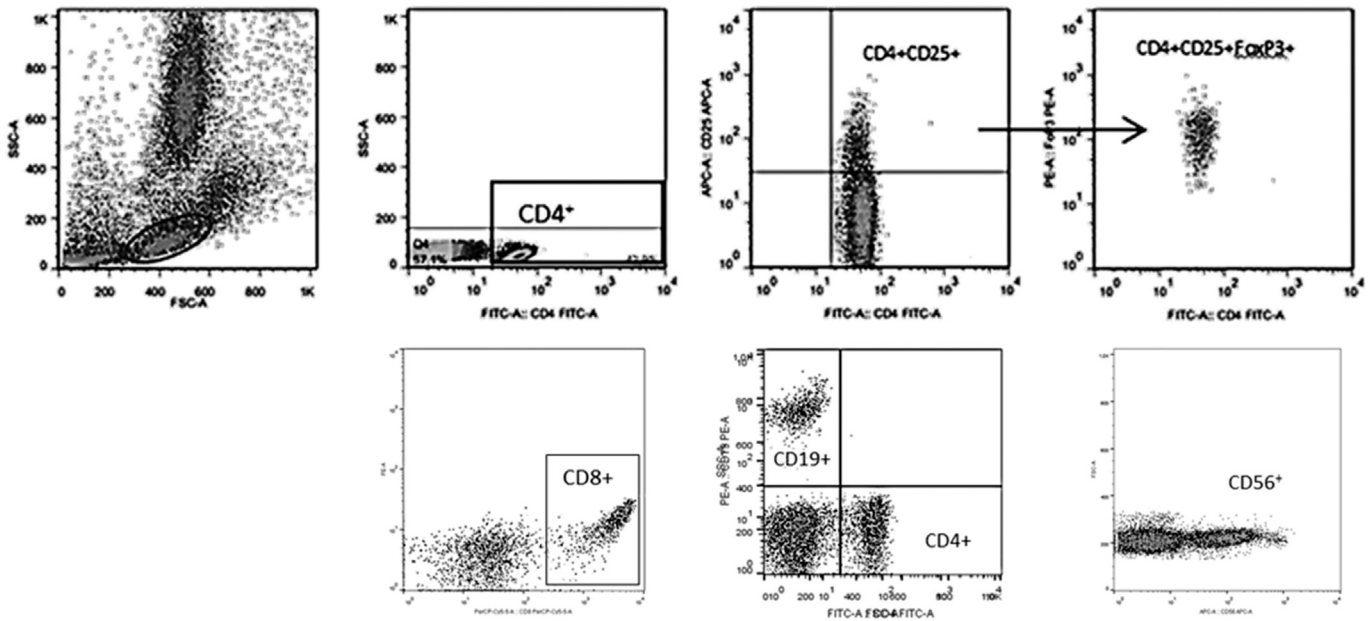
Table 1
Demographic and clinical findings in 26 patients with chronic hepatitis C (CHC) before antiviral treatment with Interferon-α plus ribavirin (T0).

Characteristic	CHC − T0 n 26	(%)
Gender		
Man (age 50.2 ± 9.2 years)	12	46
Woman (Age 50.5 ± 7.3 years)	14	54
Genotype		
1	17	65
2	2	7
3	7	27
Necroinflammatory activity		
0	7	27
1	8	31
2	7	27
3	1	3
Not evaluated	3	12
Fibrosis		
1	4	16
2	15	58
3	2	8
4	2	8
Not evaluated	3	12
Viral load		
Low (<600.000 IU/mL)	9	35
High (≥600.000 IU/mL)	17	65

Fig. 1. Flow cytometry analysis of peripheral blood lymphocyte subsets performed in chronic hepatitis C patients and healthy individuals.

3.2. Viral response at 12-weeks of treatment with IFN-α plus ribavirin

Twenty-three out of 26 untreated patients started antiviral treatment of IFN-α plus ribavirin, but 4 of them abandoned the study. Thus, only 19 patients completed 12-weeks of treatment and were monitored for their infection status at this time. Ten out of these 19 treated HCV patients presented an early virologic response: 2 were blood HCV-RNA negative, whereas 8 exhibited a significant drop in their previous HCV load. In contrast, 9 patients of 19 treated patients did not respond to the antiviral treatment and were classified as non-responders (NR). The viral load of HCV before treatment was higher in NR patients. The ALT levels were similar in NR and EVR patients, and no prevalence of HCV genotype 1 was observed in non-responders patients (Table 2).

3.3. Lymphocyte subsets in untreated HCV patients

The proportions of B cells (CD19$^+$), CD8$^+$ T cells, and NK cells were different, comparing healthy controls and untreated HCV patients. There was an increase in the proportion of B cells in the patients ($p = 0.042$). However, they had a lower proportion of both CD8$^+$T cells and NK cells than controls ($p = 0.048$ and $p = 0.028$, respectively). Nonetheless, their proportions of CD4$^+$ T cells and CD4$^+$CD25$^+$FoxP3$^+$Treg cells were similar to those from the controls ($p = 0.513$ and $p = 0.309$, respectively) (Fig. 2).

The proportion of CD4$^+$CD25$^+$FoxP3$^+$Treg cells in HCV patients
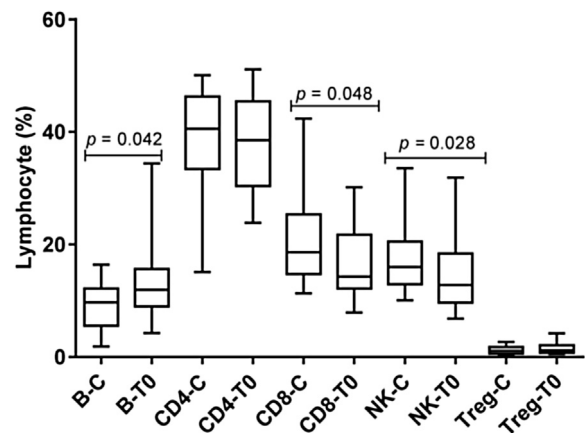

Fig. 2. Proportion of blood lymphocytes subtypes in 20 control healthy individuals (B-C, CD4-C, CD8-C, NK-C, Treg-C) and in 26 untreated HCV patients (B-T0, CD4-T0, CD8-T0, NK-T0 and Treg-T0). Box and whiskers represent the median, interquartile range (25%—75%) and maximum and minimum. (B = B cell, CD4 = CD4$^+$T cell, CD8 = CD8$^+$T cell, NK = NK cell and Treg = CD4$^+$CD25$^+$FoxP3$^+$Treg cell).

was not affected by their viral load of HCV, but it was lower in patients with cryoglobulinemia in comparison to that exhibited by patients without cryoglobulins (mean $1.13 \pm 0.64\%$ against $1.90 \pm 1.06\%$, $p = 0.038$) (Fig. 3). However, the proportion of these cells was similar in patients with or without NOSA ($p = 0.181$). The

Table 2
Laboratory findings in 19 patients with chronic hepatitis C (CHC) before (T0) and after 12-weeks treatment (T12).

	CHC-T0	CHC-T12	p-value
VL (log$_{10}$)[a]	5.76 ± 0.67 (4.56—6.94)	3.85 (0—6.16) (0—6.74)	0.001
ALT (U/L)[a]	53 (43—122) (20—459)	39.3 ± 21.4 (11—91)	0.001
Leukocytes[a] (cells/mm^3)	5.315 ± 1.515 (2.280—8.840)	3.075 (2.350—4.330) (1.930—15.050)	0.005
Hemoglobin[a] (g/dL)	13.6 (12.9—14.3) (4.8—16.9)	11.7 ± 1.3 (9.4—14.9)	0.005
Platelets[b] (cells/mm^3)	189.6 ± 59.2 (67—286)	146.3 ± 52.8 (46—236)	<0.001

VL: viral load; ALT: alanine aminotransferase.
[a] Wilcoxon test, median and IQR (25%—75%).
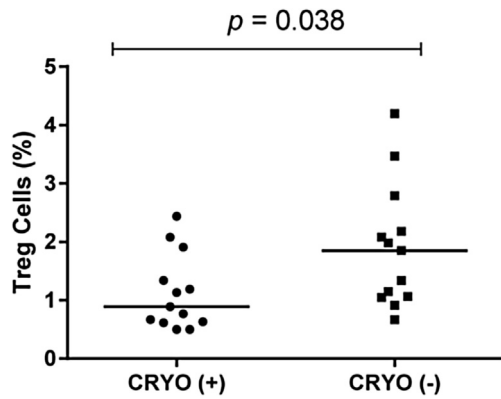[b] Paired t-test, mean ± SD.

Fig. 3. Frequency of Treg cells ($CD4^+CD25^+FoxP3^+$) in the peripheral blood of chronic hepatitis C patients having cryoglobulinemia (Positive, n = 13) and without this cryoprecipitate (Negative, n = 26), before treatment. Horizontal bars represent the means.

ratio of Treg cell/NK cell was higher in untreated HCV patients in comparison with that from healthy individuals. In addition, it was higher in untreated patients without cryoglobulins ($p = 0.039$) (Figs. 4 and 5).

3.4. Lymphocyte subsets in treated HCV patients

At week 12 of antiviral therapy, the proportions of B cells, $CD4^+T$ cells, $CD8^+T$ cells, and Treg cells in patients that had achieved an early virologic response (EVR) or were not responders (NR) were similar to those presented by these individuals before the antiviral treatment (T0). However, there was an increase in the proportion of NK cells in EVR patients when this subset of lymphocytes was compared with that presented by these subjects before antiviral therapy ($p = 0.024$) (Fig. 6).

4. Discussion

The present study evaluated the profile of different subsets of peripheral lymphocytes of patients chronically infected with HCV, before and at week 12 of treatment with interferon-α plus ribavirin. Chronic hepatitis C caused an increase in the proportion of B cells in untreated patients, which could represent the proliferative effect of HCV on these cells [4,22,23]. On the other hand, the increase in B cell proportion in the patients could be associated with the production of NOSA and anti-HCV antibodies. Additionally, there is an increase in BAFF production during chronic HCV infection [16,24]. Nonetheless, the proportion of B cells was similar in 12-week
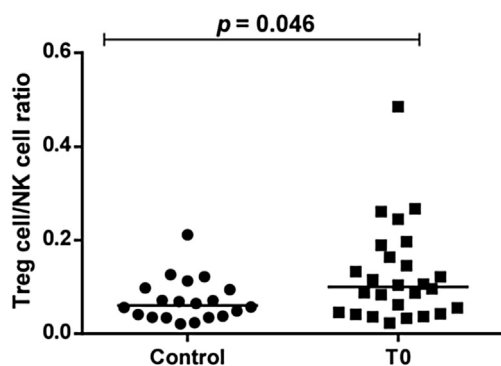

Fig. 4. Treg/NK cell ratio in 20 control healthy individuals and 26 untreated hepatitis C patients before treatment (T0). Horizontal lines represent the medians.
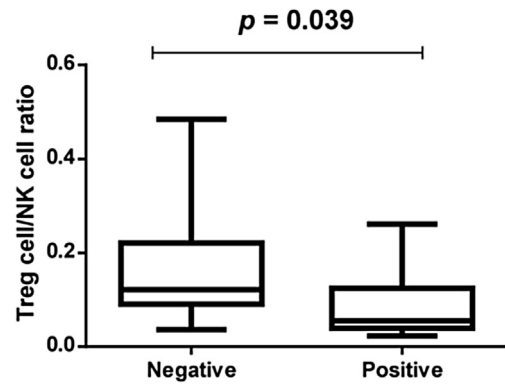

Fig. 5. Treg/NK cell ratio in untreated HCV patients that were negative (n = 13) or positive (n = 13) for the presence of cryoglobulinemia.

treated patients and controls, a finding that could be associated with the decrease in HCV antigen burden.

In our study, NK cell proportion was lower in untreated HCV patients in comparison with those from healthy controls. This finding has been confirmed in some studies, reporting low NK cell proportion and altered NK functions in chronic HCV infection [25–27]. The interaction CD81/HCV glycoprotein E2 on NK cells blocks NK cell activation, cytokine production, cytotoxic granule release, and proliferation [28,29]. Here, NK cell counts were normalized in EVR treated patients, associating this finding with the drop in HCV load caused by the IFN-α treatment. Nevertheless, the increase in NK cell proportion could be also associated with the acquisition of an NK activated cell phenotype following treatment with IFN-α [30]. Interestingly, we verified that the Treg/NK cell ratio was higher in untreated HCV patients, suggesting a negative control of Treg cell on the NK cell activation during HCV chronic infection, which could be down-modulated by the antiviral treatment with IFN-α/ribavirin.

Differently, $CD4^+T$ cell proportion was similar to that from healthy controls in agreement with previous reports. The $CD4^+T$ lymphocyte subset is necessary for the initiation and maintenance of the adaptive immune response against HCV of both B cell and $CD8^+T$ cell to resolve the acute viral infection. Nevertheless, such importance of HCV-specific $CD4^+$ T cell is apparently lost during the chronic infection, which can be demonstrated by decreased IL-2 secretion and a lack of proliferative capacity [31–33].

The proportion of $CD4^+CD25^+FoxP3^+$Treg cells was not affected by the HCV load, as well as not being lower in untreated patients that were seropositive for NOSA or influenced by the antiviral treatment. However, the proportion of these cells was lower in patients with cryoglobulinemia. Such result suggested that these cells could exert an adverse immune regulation in the formation of this immune complex formed by antibodies against HCV core, IgM anti-IgG antibodies, and complement C1q, which is less efficient in cryoglobulinemia patients. Several studies have reported an increased proportion of regulatory T cells in patients chronically infected with HCV, which can be demonstrated in both liver and peripheral blood. However, other groups, confirming our results, have not found such findings [11–14].

In this work, the proportion of $CD8^+T$ cells was lower in untreated HCV patients than in the controls. However, the percentage of these cells in EVR- and NR-treated patients was similar, suggesting a slow restoration of this lymphocyte subset after antiviral treatment. In chronic HCV infection, the $CD8^+T$ cell immune response is weak or absent, presenting reduced cytotoxicity, insufficient secretion of antiviral cytokines including IFN-γ, and low proliferative capacity. Some factors contribute to this $CD8^+T$

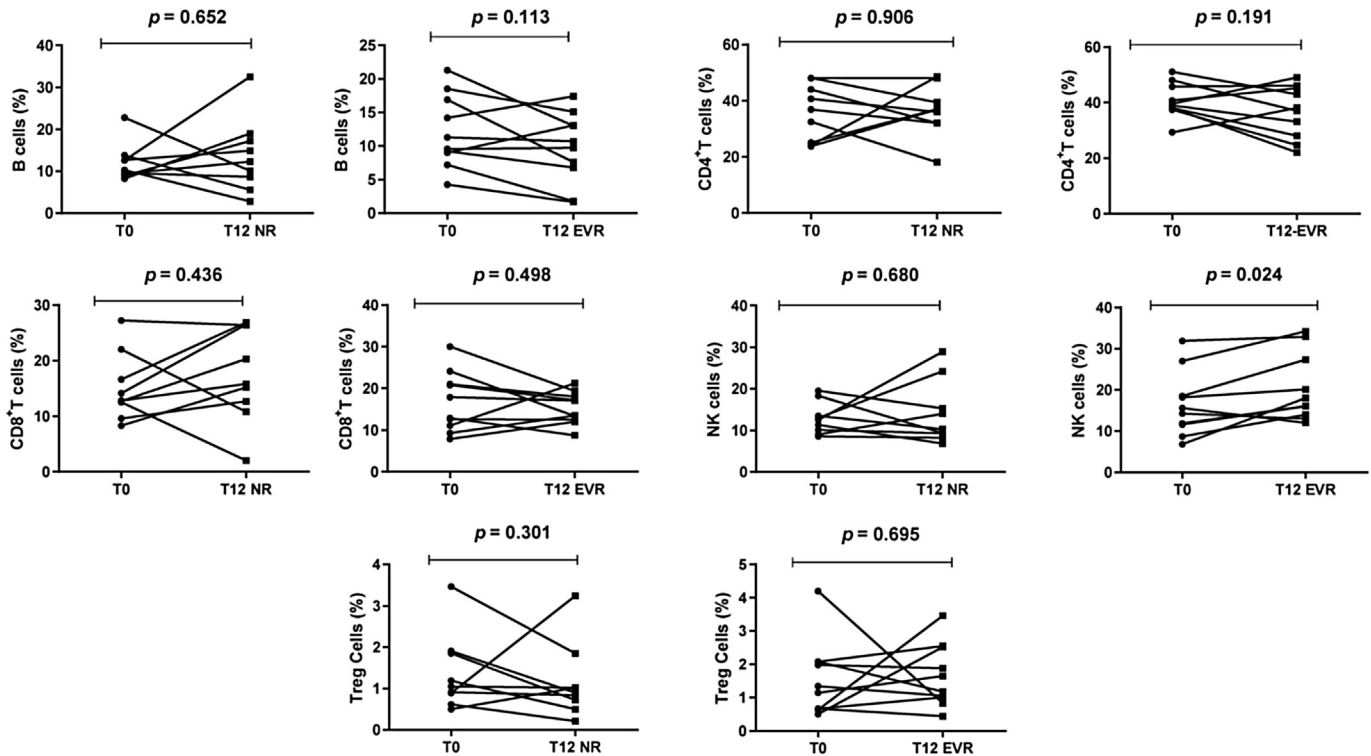**Fig. 6.** Proportion of lymphocyte subsets (B cells, CD4$^+$T cells, CD8$^+$T cells, NK cells and CD4$^+$CD25$^+$Foxp3$^+$T reg cells) before treatment (T0), and at 12-weeks of treatment with interferon-α plus ribavirin (T12). NR are nine treated patients that were non-responders and EVR are ten treated patients that achieved an early virologic response.

cell exhaustion in chronic HCV infection as high viral load, the down-regulation of T cell receptors, and the physical deletion of HCV-specific CD8$^+$T cells [34,35].

In conclusion, we demonstrated an altered profile of blood lymphocyte subsets in untreated HCV patients, including a reduction in the proportion of CD4$^+$CD25$^+$FoxP3$^+$T regulatory cells in untreated patients with cryoglobulinemia. An early virological response at 12-weeks of treatment with IFN-α plus ribavirin is associated with a significant improvement in the proportion of NK cells.

Conflict of interest

The authors have no conflict of interest that is directly relevant to the content of this manuscript.

Acknowledgments

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