RESEARCH ARTICLE

Increased Th1, Th17 and pro-fibrotic responses in hepatitis C-infected patients are down-regulated after 12 weeks of treatment with pegylated interferon plus ribavirin

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ABSTRACT. Hepatitis C virus causes significant morbidity and mortality worldwide. The infection induces up-regulation of cytokine and chemokines commonly linked to the development of cellular and pro-inflammatory antiviral responses. The current standard in hepatitis C treatment consists of combination regimens of pegylated interferon-alpha plus ribavirin. The impact of combined treatment in the host immune response is still poorly understood. In the present study, we profiled 27 cytokines, chemokines and growth factors involved in the innate and adaptive responses to the virus in the serum of 27 hepatitis C virus-infected patients, before and after 12 weeks of combined treatment, and compared them to 10 healthy controls. Hepatitis C virus infection induced not only the secretion of chemokines and cytokines participating in Th1 responses (MIP-1α, IP-10, TNF-α, IL-12p70, IL-2), but also cytokines involved in the development of Th17 responses (IL-6, IL-8, IL-9 and IL-17) and two pro-fibrotic factors (FGF-b, VEGF). The most important increases included MIP-1α (4.7-fold increase compared to the control group), TNF-α (3.0-fold), FGF-b (3.4-fold), VEGF (3.5-fold), IP-10 (3.6-fold), IL-17 (107.0-fold), IL-9 (7.5-fold), IL-12p70 (7.0-fold), IL-2 (5.6-fold) and IL-7 (5.6-fold). Combined treatment with pegylated interferon-alpha plus ribavirin down-modulated the secretion of key Th1 and Th17 pro-inflammatory mediators, and pro-fibrotic growth factors as early as 12 weeks after treatment initiation. MIP-1α, FGF-b, IL-17 decreased in a more dramatic manner in the group of responder patients than in the group of non-responders (fold-change in cEVR; fold-change in NcEVR): MIP-1α (4.72;1.71), FGF-b (4.54;1.21), IL-17 (107.1;1.8). Correlation studies demonstrated that the decreases in the levels of these mediators were significantly associated with each other, pointing to a coordinated effect of the treatment on their secretion (r coefficient; p value): [Δ FGF-b versus Δ IL-17 (0.90; 0.00), Δ IL-17 versus Δ VEGF (0.88; 0.00), Δ MIP-1α versus Δ IL-17 (0.84;0.00), Δ FGF-b versus Δ MIP-1α (0.96;0.00), Δ FGF-b versus Δ IL-12p70 (0.90; 0.00), Δ VEGF versus Δ IL-12p70 (0.89; 0.00)]. Th17 immunity has been previously associated with autoimmune diseases and asthma, but this is the first work reporting a role for this profile in viral hepatitis. These results provide an opportunity to evaluate the impact of the treatment with Peg-INF-alpha and RBV on the prevention of immune-driven tissue damage in infected patients.

Keywords: cytokines, HCV, Th1, Th17, treatment

Hepatitis C virus (HCV) causes significant morbidity and mortality worldwide, with nearly 3% of the World population infected by this virus [1]. HCV is a leading cause of end-stage liver disease, and is the most common indication for liver transplantation. HCV nearly always recurs in liver-transplanted patients, and 10 to 25% of them develop cirrhosis within five to 10 years [2]. The current standard in hepatitis C treatment consists of combination regimens of pegylated interferon-alpha (Peg-INF-alpha) with ribavirin (RBV). Such treatment regimens are quite successful in patients with HCV genotypes 2 and 3 infections, but they are much less effective in patients with
genotypes 1 and 4 infections [3]. The combination of Peg-INF-alpha with RBV therapy substantially improves the efficacy of HCV treatment by targeting several steps of viral replication and/or cellular pathways [4, 5]. However, the exact mechanism of action of these drugs is not yet well understood, neither is their impact on the host’s immune response. The objective of this study was to evaluate innate and adaptive host immune responses paralleling treatment with Peg-INF-alpha and RBV, by profiling 27 cytokines and chemokines before and after 12 weeks of treatment. Results demonstrated a down-modulatory effect of the treatment on the Th1 and Th17 responses induced by the virus.

DONORS AND METHODS

Study design and patients
A prospective study was carried out in the Hepatology Services of the “Hospital Clínico Universitario” and of the “Hospital Universitario Río Hortega” in Valladolid, Spain. Twenty seven patients were recruited between May 2008 and July 2009 in these two hospitals.
– Inclusion criteria: patients with HCV RNA present in blood, diagnosed by molecular biology-based methods, and programmed for treatment with Peg-INF-alpha (1.5 μg/kg/week) plus RBV (1,000–1,200 mg/day according to weight).
– Exclusion criteria: those patients who abandoned the treatment and those who discontinued their participation for personal reasons were excluded from the study. Patients not giving informed consent were also excluded from the study.
Healthy controls (n = 10) were voluntary health workers of similar age, with no relevant clinical antecedents. Informed consent was obtained directly from each patient and also from the healthy controls before enrolment. Approval of the study protocol, for both the scientific and the ethical aspects, was obtained from the Scientific Committee for Clinical Research of the two participating hospitals.

Abbreviations

cEVR: Complete early virological response
FGF-b: Fibroblast growth factor - basic
G-CSF: Granulocyte colony-stimulating factor
GM-CSF: Granulocyte macrophage colony-stimulating factor
HCV: Hepatitis C virus
IFN-γ: Interferon γ
IP-10: Interferon-gamma inducible protein-10
MCP-1: Monocyte chemoattractant protein-1
MIP-1α: Macrophage inflammatory protein-1α
MIP-1β: Macrophage inflammatory protein-1β
NC-EVR: Non-complete early virological response
PEG: Pegylated interferon-alpha
INF-alpha: Interferon alpha
PDGF: Platelet-derived growth factor
RANTES: Regulated upon activation, normal T-cell, expressed, and secreted
RBV: Ribavirin
TNF-α: Tumor necrosis factor α
VEGF: Vascular endothelial growth factor

Samples
A blood sample was collected into an EDTA tube before the beginning of the treatment. A second blood sample was obtained 12 weeks after treatment initiation. Plasma was obtained after appropriate centrifugation and was immediately frozen at -70°C until quantification of the immune mediators. A second blood sample into an EDTA tube was obtained at the same time points for blood cell count, along with a third blood sample for quantification of biochemical mediators in serum. Healthy controls were asked to donate one single EDTA tube-blood sample for cytokine comparison purposes in plasma.

Cytokine and chemokine quantification
Plasma chemokine and cytokine levels were evaluated using the multiplex Bioread® 27 plex assay following manufacturer’s instructions. This system allows for quantitative measurement of 27 different chemokines, cytokines, growth-factors and immune mediators, while consuming a small amount of biological material. Furthermore, this system has good representation of analytes for inflammatory cytokines, anti-inflammatory cytokines, Th1 cytokines, Th2 cytokines, Th17 cytokines and chemokines, allowing for the testing of differential levels of regulatory cytokines in patients serum.

Viral load and viral genotype
HCV viral load was determined from serum using the COBAS® TaqMan HCV Test for use with the COBAS® AmpliPrep instrument (Roche®), and the genotype was identified using the VERSANT HCV Amplification 2.0 kit (LI-PA) and VERSANT® HCV Genotype 2.0 assay (LI-PA) Siemens® using the Auto-LIPA 48 instrument (INNOGENETICS®). The viral RNA load was measured before beginning of the treatment and again 12 weeks after treatment initiation. A complete early virological response (cEVR) was defined as undetectable viral load 12 weeks after treatment initiation.

Duration of HCV infection
The duration of HCV infection in patients with a history of drug abuse was estimated, taking as the initial point the moment they started administering drugs intravenously. For blood recipients, the initial point for estimating the duration of the infection was the moment the first transfusion was received. For those patients with unidentified transmission origin, the initial point was the moment of diagnosis.

Alcohol consumption
Patients were questioned in relation to alcohol consumption. We considered the consumption of more than 50 grams of alcohol per day for ≥12 months as a high alcohol intake. An APRI (aspartate aminotransferase-to-platelet count ratio index) calculation was performed as follows: [AST level (ULN)/platelet counts x 103/μL] x 100
(39 IU/L being the upper limit of normality (ULN) in our laboratory). An APRI index ≤ 0.5 was considered as absence of hepatic fibrosis; an APRI index > 1.5 was considered as indicative of fibrosis, and APRI scores between 0.5 and 1.5 are related to progressive stages of fibrosis [6].

**HOMA (homeostasis model assessment) calculation**

Insulin resistance (IR) was estimated using the HOMA, a validated model derived from normal volunteers. A HOMA calculation was performed as follows: insulin (μU/ml) × glucose (mg/dL) / 405.

**Statistics**

Data analysis was performed using SPSS 15.0. Comparisons of cytokine levels between patients and controls were performed using the non-parametric U-Mann Whitney test, since the Sapiro-Wilk test revealed an absence of a normal distribution of immune mediator levels in the cohorts compared. Differences in cytokine levels before and after treatment were assessed using the non-parametric Wilcoxon test. Associations between cytokine level increments were studied by calculating the Spearman correlation coefficient (r) and data were shown as (r, p-value). Significance was fixed at p value < 0.05.

**RESULTS**

**Clinical, virological and biochemical parameters**

Twenty-seven patients were included in the study: 20 of them showed a complete, early virological response (cEVR); seven patients were classified as non-responders (NcEVR), as they showed detectable viral load after 12 weeks of treatment. While 60% of cEVR patients showed genotype 1 of HCV, 100% of NcEVR load after 12 weeks of treatment. While 60% of cEVR responders (NcEVR), as they showed detectable viral levels in the cohorts compared. Differences in cytokine levels before and after treatment were assessed using the non-parametric Wilcoxon test. Associations between cytokine level increments were studied by calculating the Spearman correlation coefficient (r) and data were shown as (r, p-value). Significance was fixed at p value < 0.05.

**Effect of the virus on the host immune mediator profiles**

The infection by HCV induced the systemic increase, compared to control levels, of a group of chemokines involved in innate immune responses (MCP-1, MIP-1α, MIP-1β and IP-10), in cytokines participating in T-helper 1 responses (IFN-γ, TNF-α, IL-12p70 and IL-2), in T-helper 2 responses (IL-9 and IL-13), in T-helper 17 responses (IL-8, IL-17 and IL-6), and finally of other mediators participating in regulatory responses (IL-10 and IL-1RA), in the induction of fibrogenesis (FGF-b, VEGF), and in the mobilization of T lymphocytes such as IL-7 (table 1, figure 1). The most important increases corresponded to MIP-1α (4.7-fold increase compared to control group), TNF-α (3.0-fold), FGF-b (3.4-fold), VEGF (3.5-fold), IP-10 (3.6-fold), IL-17 (107.0-fold), IL-9 (7.5-fold), IL-12p70 (7.0-fold), IL-2 (5.6-fold), IL-7 (5.6-fold) (table 2).

**Effect of the treatment on host cytokine and chemokine profiles**

The Mann-Whitney test demonstrated that treatment normalized the levels of the following mediators: MIP-1α, MIP-1β, FGF-b, VEGF, IL-8, IL-17, IL-6, IFN-γ, TNF-α, IL-12p70, IL-2, IL-10, IL-7 and IL-1RA in the cEVR group, as compared to the disappearance of the differences in controls 12 weeks after the beginning of the treatment (table 2). Similarly to that which occurred with cEVR, in the differences in these mediators seen in the NcEVR group compared with the control also disappeared 12 weeks after treatment (table 2). MIP-1α, FGF-b, IL-17 decreased in a more dramatic manner in the group of responder patients than in the group of non-responders (fold-change in cEVR; fold-change in NcEVR): MIP-1α (4.72;1.71), FGF-b (4.54;1.21), IL-17 (107.1;1.8) (figure 1). A number of mediators evolved in a different manner. MCP-1, IP-10, IL-9 and IL-13 still showed higher levels than controls 12 weeks after the beginning of the treatment in both groups (table 2). The Wilcoxon test demonstrated, in the cEVR group, a significant decrease in the vast majority of the mediators studied, 12 weeks after treatment, apart from MCP-1, which actually increased (data not shown), and IL-9 and IL-13, which did not change in a significant manner as a consequence of the treatment. The Wilcoxon test failed to demonstrate significant differences before and after treatment initiation for cytokine and chemokine levels in the NcEVR group (probably due to the low number of patients in this group). When immune mediator levels were compared between NcEVR and cEVR groups 12 weeks after the beginning of the treatment, the Mann-Whitney test revealed no significant differences between any of the mediators studied. The analysis of the correlations between the cytokine increments before and after treatment revealed an association between the variation of innate immunity (MIP-1a), Th1 (IL12p70) and Th17 (IL-17) pro-inflammatory mediators, with relevant pro-fibrotic factors such as VEGF and FGF-b (figure 2). Spearman correlation coefficients and p values for each comparison were as follows: ΔFGF-b versus ΔIL-17 (0.90; 0.00), ΔIL-17 versus ΔVEGF (0.88; 0.00), ΔMIP-1α...
versus ∆IL-17 (0.84; 0.00), ∆FGF-b versus ∆MIP-1α (0.96; 0.00), ∆FGF-b versus ∆IL-12p70 (0.90; 0.00), ∆VEGF versus ∆IL-12p70 (0.89; 0.00).

DISCUSSION

The results presented here show that HCV infection induces the activation of a broad range of immune mediators participating in both innate (CXC and CC chemokines) and adaptive responses (T helper cytokines) to the virus, along with mediators involved in fibrogenesis. The highest levels corresponded to two chemokines (MIP-1α, IP-10), to three Th1 cytokines (TNF-α, IL-12p70, IL-2), to two pro-fibrotic factors (FGF-b, VEGF), to a T cell mobilization-inducer (IL-7), and remarkably, to IL-17, a cytokine that promotes Th17 responses [7]. While participation of Th1 cytokines and chemokines in HCV infection has been extensively documented in the literature [8-11], the induction of Th17 responses in this disease had not been reported until the present moment. Th17 immunity participates in clearing pathogens during...
Table 2

Comparison of cytokine levels against controls

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pre-treatment</th>
<th>Week 12</th>
<th>Control</th>
<th>Pre-t/ Cont</th>
<th>cEVR/ Cont</th>
<th>NcEVR/ Cont</th>
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<td></td>
<td></td>
<td>cEVR</td>
<td>NcEVR</td>
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<tr>
<td>MCP-1</td>
<td>45.41 [36.42]</td>
<td>61.4 [75.6]</td>
<td>46.1 [32.7]</td>
<td>17.7 [12.1]</td>
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<td>MIP-1a</td>
<td>13.77 [22.87]</td>
<td>2.9 [10.3]</td>
<td>8.0 [18.8]</td>
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<td>4.7 1.0 2.8</td>
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<td>MIP-1β</td>
<td>131.99 [81.29]</td>
<td>44.2 [26.7]</td>
<td>38.5 [56.9]</td>
<td>52.1 [23.9]</td>
<td>2.5 0.8 0.7</td>
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<td>FGF-b</td>
<td>109.52 [186.15]</td>
<td>24.6 [52.5]</td>
<td>90.0 [229.9]</td>
<td>32.1 [82.8]</td>
<td>3.4 0.8 2.8</td>
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<tr>
<td>GM-CSF</td>
<td>99.74 [130.48]</td>
<td>64.4 [72.5]</td>
<td>103.9 [83.4]</td>
<td>65.0 [94.5]</td>
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<td>G-CSF</td>
<td>121.93 [161.45]</td>
<td>156.5 [75.0]</td>
<td>203.9 [108.8]</td>
<td>156.5 [64.5]</td>
<td>1.4 1.0 1.3</td>
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<td>VEGF</td>
<td>132.74 [321.85]</td>
<td>43.5 [29.1]</td>
<td>54.5 [239.6]</td>
<td>38.1 [30.1]</td>
<td>3.5 1.1 1.4</td>
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<tr>
<td>IP-10</td>
<td>8810.89 [6891.89]</td>
<td>6710.8 [2749.4]</td>
<td>5928.8 [3703.8]</td>
<td>2415.2 [438.3]</td>
<td>3.6 2.8 2.5*</td>
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<td>Eotaxin</td>
<td>573.58 [555.74]</td>
<td>431.8 [467.0]</td>
<td>458.8 [705.3]</td>
<td>324.9 [386.9]</td>
<td>1.8 1.3 1.4</td>
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<td>PDGF-bb</td>
<td>6695.62 [12041.36]</td>
<td>3915.4 [4629.9]</td>
<td>4378.5 [112856.2]</td>
<td>11328.6 [10103.8]</td>
<td>0.6 0.3 0.4*</td>
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<td>RANTES</td>
<td>30865 [68304.29]</td>
<td>67759.305 [112856.2]</td>
<td>30865 [93778.2]</td>
<td>54219.705 [411613.27]</td>
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<td>23.42 [18.7]</td>
<td>12.0 [7.9]</td>
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<td>1.8 0.9 1.4</td>
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<td>IL-17</td>
<td>182.36 [439.46]</td>
<td>1.7 [63.5]</td>
<td>101.1 [134.5]</td>
<td>1.7 [136.4]</td>
<td>107.5 1.0 59.5</td>
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<td>IL-6</td>
<td>16.92 [18.53]</td>
<td>8.0 [8.2]</td>
<td>10.2 [9.2]</td>
<td>8.0 [8.1]</td>
<td>2.1 1.0 1.3</td>
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<tr>
<td>IL-9</td>
<td>85.01 [235.68]</td>
<td>40.8 [88.5]</td>
<td>44.3 [55.4]</td>
<td>11.4 [17.4]</td>
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<td>IL-13</td>
<td>41.62 [33.36]</td>
<td>43.2 [23.9]</td>
<td>47.4 [30.1]</td>
<td>16.1 [12.4]</td>
<td>2.6 2.7 2.9</td>
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<tr>
<td>IL-4</td>
<td>8.53 [9.32]</td>
<td>4.2 [3.4]</td>
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<td>5.6 [4.8]</td>
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<td>IL-5</td>
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<td>5.7 [7.4]</td>
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<td>6.7 [5.8]</td>
<td>1.7 0.9 1.5</td>
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<tr>
<td>IL-β</td>
<td>5.57 [18.18]</td>
<td>2.6 [2.3]</td>
<td>4.0 [6.1]</td>
<td>3.7 [1.9]</td>
<td>1.5 0.7 1.1</td>
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<td>IFN-γ</td>
<td>1622.84 [1301.61]</td>
<td>832.5 [1052.9]</td>
<td>631.4 [1211.2]</td>
<td>939.0 [664.5]</td>
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<td>TNF-α</td>
<td>70.86 [102.92]</td>
<td>29.3 [50.2]</td>
<td>52.5 [32.9]</td>
<td>23.7 [56.8]</td>
<td>3.0 1.2 2.2*</td>
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<td>IL-12p70</td>
<td>42.74 [76.74]</td>
<td>10.1 [18.6]</td>
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<td>6.1 [12.9]</td>
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<td>IL-15</td>
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<td>IL-2</td>
<td>15.81 [25.21]</td>
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<tr>
<td>IL-10</td>
<td>5.91 [4.61]</td>
<td>4.4 [1.2]</td>
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<td>3.6 [2.2]</td>
<td>1.6 1.2 1.7</td>
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<tr>
<td>IL-7</td>
<td>37.43 [47.53]</td>
<td>5.7 [7.4]</td>
<td>10.0 [17.8]</td>
<td>6.7 [5.8]</td>
<td>5.6 0.8** 0.8</td>
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<tr>
<td>IL-1RA</td>
<td>454.06 [453.31]</td>
<td>171.1 [234.8]</td>
<td>250.3 [343.3]</td>
<td>221.7 [188.9]</td>
<td>2.0 0.8 1.1</td>
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Results are expressed as pg/mL. Data are displayed as (median, [interquartile rank]). * p < 0.05; ** p < 0.1.

host defense reactions, but is also involved in tissue inflammation in several autoimmune diseases, allergic diseases, and asthma [12-14]. To this end, we have recently described the induction of Th1 and Th17 cytokine profiles by pandemic influenza virus infection [15]. Patients showed a slight increase in IL-10 and IL-1RA over control values (table 2). Being anti-inflammatory cytokines, the increases in IL-10 and IL-1RA may represent an immune subversion mechanism by the virus to evade Th1 and Th17 host-protective antiviral responses [16], or alternatively they could represent homeostatic mechanisms aimed at avoiding potential tissue damage secondary to inflammation [17]. Patients also showed increased levels of IL-13. This could represent a viral evasion strategy, since this cytokine attenuates Th17 cytokine production [18] or, as in the case of IL-10 and IL-1RA, correspond to a regulatory mechanism aimed at controlling inflammation. Increases in IL-7 could reflect T-lymphocyte mobilization and proliferation, in response to the infection by HCV.

While treatment induced a dramatic decrease in viral load in early responders, leading to undetectable levels of virus in blood in 100% of patients with virus genotype 2 or 3 (table 1), seven patients with genotype 1 virus (37%) showed detectable viremia by week 12 after treatment initiation. These percentages of response correspond to those previously published for the different viral genotypes [3]. Interestingly, in spite of the different behaviour in terms of viral load evolution, the treatment with Peg-INF-alpha and RBV induced in both groups (responders and non-responders), a normalization of Th1 cytokines and chemokines (IFN-γ, TNF-α, IL-12p70, IL-2, MIP-1α, MIP-1β), Th17 cytokines and chemokines (IL-6, IL-17, IL-8), and pro-fibrotic factors (FGF-b and VEGF), and of IL-7, IL-1RA, but conversely it failed to normalize levels of two pro-inflammatory chemokines (IP-10, MCP-1) or IL-9 and IL-13. The most obvious effect of the treatment on levels of MIP-1α, FGF-b, and IL-17 in the responder group compared to the non-responder group, revealed a key role for these mediators in the clinical and biochemical improvement in the cEVR group. The effect of the combined treatment with Peg-INF-alpha and RBV on cytokine and chemokine levels in the HCV-infected patients is probably due to the immunomodulatory properties of these drugs [19-21]. Thus, studies on the correlations between mediator levels before and after treatment revealed that the combined treatment with RBV and Peg-INF-alpha induced the simultaneous modulation of a group of pro-inflammatory molecules participating in the innate and adaptive response, and also of key pro-fibrotic factors, pointing to a coordinated effect of the treatment on the expression of these genes. Since the hepatitis viruses use host intracellular...
signalling pathways to replicate [22], down-modulation of signalling molecules such as that described in this study, can interfere with the virus replication cycle, diminishing viral load and also preventing further development of liver fibrosis processes. In consequence, interfering with virus-induced host responses could represent a major avenue for the development of better treatment strategies in this disease [22, 23]. Additional research is needed to clarify these particular aspects, since down-modulation of Th1 and Th17 cytokines and chemokines do not translate into viral control in all cases, as demonstrated in this work.

In conclusion, infection with HCV induces a predominant activation of both innate and adaptive Th1 and Th17 cytokine and chemokine responses. Co-existence of Th1 and Th17 profiles seems to constitute a pivotal, antiviral response, as recently demonstrated in the context of pandemic influenza. The combined treatment with Peg-INF-alpha and RBV, instead of stimulating cytokine and chemokine antiviral responses, down-modulates the secretion of key pro-inflammatory and pro-fibrotic mediators as early as 12 weeks after treatment in the infected host. However, this immunomodulatory effect is not necessarily accompanied by a control of the viral load. More work is needed to evaluate the influence of other factors (such as host genetics) in the response to the treatment. These results provide the opportunity to evaluate the impact of treatment with Peg-INF-alpha and RBV on prevention of the immune-driven tissue damage, the hepatic inflammation, and progression to liver cirrhosis in infected patients.

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Correlations between increments in immune mediator levels before and 12 weeks after treatment initiation. Data are shown as (r, p-value). Significance was fixed at p-value < 0.05.

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