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Dual role of IL-12 in the therapeutic efficacy or failure during combined PEG-Interferon-α2A and ribavirin therapy in patients with chronic hepatitis C

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ABSTRACT

Several efforts have been made to establish novel biomarkers with relevant predictive values to monitor HCV-infected patients under pegilated Interferon-α2A-(PEG-IFN-α2A)/ribavirin therapy. The aim of this study was to monitor the kinetics of HCV viral load, serum levels of pro-inflammatory/regulatory cytokines and leukocyte activation status before and after PEG-IFN-α2A/ribavirin therapy in 52 volunteers, including 12 chronic HCV patients and 40 controls. The HCV viral load, serum levels of cytokines (IL-8/IL-6/TNF-α/IL-12/IFN-γ/IL-4/IL-10) and the phenotype of peripheral blood leukocytes were evaluated before and after 4, 12 and 24 weeks following the PEG-IFN-α2A/ribavirin therapy. Our results demonstrated that sustained virological response-(SVR) is associated with early decrease in the viral load after 4 weeks of treatment. The presence of a modulated pro-inflammatory profile at baseline favors SVR, whereas a strong inflammatory response at baseline predisposes to therapeutic failure. Furthermore, a time-dependent increase on serum IL-12 levels in patients under treatment is critical to support the SVR, while the early predominance of IL-10 correlates to late virological relapse. On the other hand, a broad but unguided "cytokine storm" is observed in the non-responder HCV patients after 12 weeks of treatment. Corroborating these findings, monocyte/lymphocyte activation at baseline is associated with the non-responders to therapy whereas high $CD8^+$ T-cell numbers associate with SVR. All in all, these data suggest that the baseline pattern of serum pro-inflammatory/regulatory cytokines and the immunological activation status of chronic HCV patients undergoing PEG-IFN-α2A/ribavirin therapy are closely related with the therapeutic response.

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1. Introduction

Hepatitis C virus (HCV) infects approximately 3% of the world, and causes chronic hepatitis in most cases [1,2], with progressive fibrosis, cirrhosis, liver failure and risk of hepatocellular carcinoma [3,4].

Currently, the therapy based on the combination of PEG-IFN-α2A and ribavirin is a major intervention to reducing the impact

Abbreviations: SVR, sustained virological load; HCV-NR, HCV non-responder; PEG-IFN-α2A, pegilated Interferon-α2A.

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of this worldwide health problem. Host and viral factors have been associated with the effectiveness of PEG-IFN-α2A/ribavirin therapy [5,6]. The treatment outcome may also depend upon the ability of the host's cellular immune response. It has been described that an effective HCV-specific T-cell response along with a pro-inflammatory cytokine microenvironment is necessary for therapeutic success [7–10]. The combination therapy with PEG-IFN-α2A/ribavirin has a potent modulatory effect in the context of HCV infection, especially in regards to the development of a polarized Th1 response. The HCV-specific T-cell response during interferon and ribavirin treatment plays a role in the development of a polarized Th1 response followed by a vigorous inflammatory response [8,9]. Peg-interferon alone or in combination with ribavirin is able to enhance HCV-specific CD4 T-helper 1 responses in patients chronically infected with HCV [9]. While an early and strong pro-inflammatory response seems to play an important role in disease resolution [11], a polarized pro-inflammatory profile may as well contribute to liver injury. Therefore, a balance between pro-inflammatory/regulatory cytokines appears to be important in the success of PEG-IFN-α2A/ribavirin therapy.

A better understanding of the immunological events and molecules involved in the success of treatment would contribute for the establishment of new biological correlates of cure that could be used as biomarkers of treatment success. In this regard, a single-nucleotide polymorphism (SNP) upstream of interleukin (IL)28B was recently identified as an important predictor of the outcome of HCV patients treated with PEG-IFN-α2A/ribavirin [12]. The IL28B genotype appears to be a strong predictor of SVR following PEG-IFN-α2A/ribavirin therapy, however, controversial results were already reported concerning the viral strain and different genetic background of some cohorts [13–15]. In addition, adequate protocols for the follow up of HCV-infected patients under treatment still remain to be established, especially for those with a poor prognosis [12–16]. Considering the widespread infection of HCV and the probability of non-responders to the PEG-IFN-α2A/ribavirin therapy, it is imperative to develop and study putative predictors of therapy success/failure.

In the present investigation we observed that enhanced pro-inflammatory immune activation at treatment baseline has association with the therapeutic failure by triggering an early counter-regulatory immunomodulation. On the other hand, the development of a time-dependent pro-inflammatory response, mediated by IL-12 is relevant to support the sustained virological response following PEG-Interferon-α2A/ribavirin therapy in chronic hepatitis C infection.

2. Materials and methods

2.1. Study population

This is an observational, descriptive, clinical study that was carried out during August 2008 to August 2009 at the Fundação de Medicina Tropical do Amazonas (FMTAM) and Fundação de Hematologia e Hemoterapia do Amazonas (HEMOAM) after approval by the Ethical Committee from the FMTAM (Protocol# 093/2007). All patients signed an informed written consent form that is in accordance with the guidelines established by the 196/1996 resolution of the Brazilian National Health Council.

The study population consisted of a non-probability sample of convenience including fifty-two volunteers segregated into two study groups.

The group of twelve untreated HCV patients (HCV-NT), included 58.3% of males and 41.7% females, age ranging from 31 to 63 years. All HCV patients were infected with genotype 1 and were submitted to treatment with weekly subcutaneous dose of PEG-Interferon-α2A(40 kD) at 180 μcg in combination with daily oral dose of ribavirin at 1.0 g. Patients were monitored by clinical and laboratorial records (viral load and serum cytokine analysis) at four distinct times, including before treatment (T0), at week 4 (T4), week 12 (T12) and week 24 (T24) of treatment. Following PEG-IFN-α2A/ribavirin therapy the patients were categorized into two subgroups referred as Sustained Virological Response (SVR = 9) and non-responders (NR = 3) according to the recommendations of the Brazilian Ministry of Health. Briefly, SVR was classified as serum HCV RNA that was undetectable 24 weeks after completing therapy. A therapeutic failure (nonresponse) was defined as detectable HCV RNA during the treatment course and after 24 weeks of treatment completion.

The control group included forty healthy blood donors, consisting of 71% of males and 29% females, age ranging from 19-51 years, contacted at HEMOAM, Manaus, AM, Brazil. All subjects in the CT group presented negative results for relevant blood-borne pathogens including the human immunodeficiency virus (HIV), Human T-cell Lymphotropic Virus type 1/2 (HTLV-1/2), the hepatitis C virus (HCV), the hepatitis B virus (HBV), the *Treponema pallidum*, and the *Trypanosoma cruzi*.

Exclusion criteria included: pregnant women (screening by beta-HCG), patients co-infected with HIV (anti-HIV-1 and 2), HTLV (anti-HTLV-1/2), hepatitis B (anti-HBc, HBsAg), syphilis (ELISA), Chagas disease (ELISA), patients with decompensated cirrhosis, diabetes, history of illicit drug use, daily alcohol consumption; psychiatric disorders, chronic renal failure and metabolic syndromes.

2.2. Serological diagnosis of HCV infection

The diagnosis of HCV infection was performed by a third generation enzyme immunoassay ELISA (Murex anti-HCV, Chicago, IL, USA) and confirmed by Immunoblot (RIBA HCV 3.0 SIA, Chiron Health Care Ireland Ltd, Dublin, Ireland) at the Fundação de Medicina Tropical do Amazonas.

2.3. Molecular diagnosis of HCV infection, genotyping and HCV viral load analysis

HCV genomic RNA test (HCV-RNA) was performed in the Amplicor RT-PCR (Roche, NJ, USA), that presents a sensitivity of 50 IU/mL. Samples with detectable HCV-RNA were further genotyped by an in-house nested RT-PCR and RFLP analysis. An in-house standardized nested-PCR was used to detect HCV RNA [17]. Genotype assignment was based on type-specific motifs on the sequenced amplicons delimited by primers HC11/HC18 from the 5′ untranslated region [18]. Viral load was determined by RT-PCR (Amplicor HCV Monitor, Roche, NJ, USA) with data expressed as IU/mL.

2.4. Analysis of serum cytokine by enzyme immunoassay (ELISA)

The serum levels of cytokines (IL-8, IL-6, TNF-α, IL-12p70, IFN-γ, IL-4 and IL-10) were quantified using ELISA immunoassay kit, as recommended by the manufacturer (OpteiaTM Set Human – Becton Dickinson Biosciences Pharmigen, San Diego, CA). The limits of detection for each cytokine were 4.7 pg/mL for IL-6 and IFN-g, 3.1 pg/mL for IL-8, 7.8 pg/mL for TNF, IL-4 and IL-10, and IL-12p70.

2.5. Immunophenotyping of peripheral blood leukocytes by flow cytometry

The immunophenotypic profile of peripheral blood leukocytes (T-cells and monocytes) was performed by flow cytometry as described by Maia et al. [19].

2.6. Data analysis

The data sets of viral load, cytokine serum levels and peripheral blood leukocytes immunophenotypic profile displayed normal distribution and, therefore, analyses amongst groups were performed by parametric test such as T-student test (Fig. 1) or by One-way ANOVA analysis of variance followed by Tukey post-test (Figs. 2 and 5). The statistical analyses were performed using the software GraphPad Prism 5.0 (San Diego, CA, USA) and significant differences considered in all cases when $p < 0.05$.

Additionally, the comparative analysis of paired cytokine profiles (Fig. 3) was performed using the mean value for each cytokine observed in the control group of uninfected individuals (CT) as the cut-off. The analysis of the mean proportional change in the cytokine profiles was carried out normalizing the data relative to the baseline (T0) of serum cytokine levels. Cytokines showing peak of baseline fold changes at each time ratio (T4/T0, T12/T0 and T24/T0) above the cut off points established were highlighted and considered as the most relevant finding.

Multivariate analyses and Receiver Operating Characteristic (ROC) curve analyses were not fitted due to the very small sample size.

3. Results

3.1. Untreated chronic hepatitis C display a mixed pattern of pro-inflammatory/regulatory cytokines

The profile of pro-inflammatory/regulatory cytokines in the HCV-NT group demonstrated decreased levels of IL-8 ($p = 0.001$), higher levels of IL-6 ($p < 0.0001$), TNF-α ($p < 0.0001$), IL-4 ($p = 0.002$) and IL-10 ($p < 0.0001$), which were the major features of HCV-NT, suggesting that chronic hepatitis C evolve with a general mixed pro-inflammatory/regulatory cytokines pattern (Fig. 1).

3.2. The PEG-IFN-α2A/ribavirin therapy leads to a significant decrease in the HCV viral load concomitant with a time-dependent increase of IL-12 and declining levels of IL-10 at week 24 after treatment

Our data demonstrate that PEG-IFN-α2A/ribavirin therapy induced a substantial decrease in the HCV viral load starting at T4 after treatment and reaching lower mean levels at T24 (Fig. 2).

Despite no changes in the serum levels of IL-8, IL-6, TNF-α, IFN-γ and IL-4, the treatment led to continuous increase of IL-12, which were above the mean average observed for CT group early at T4.

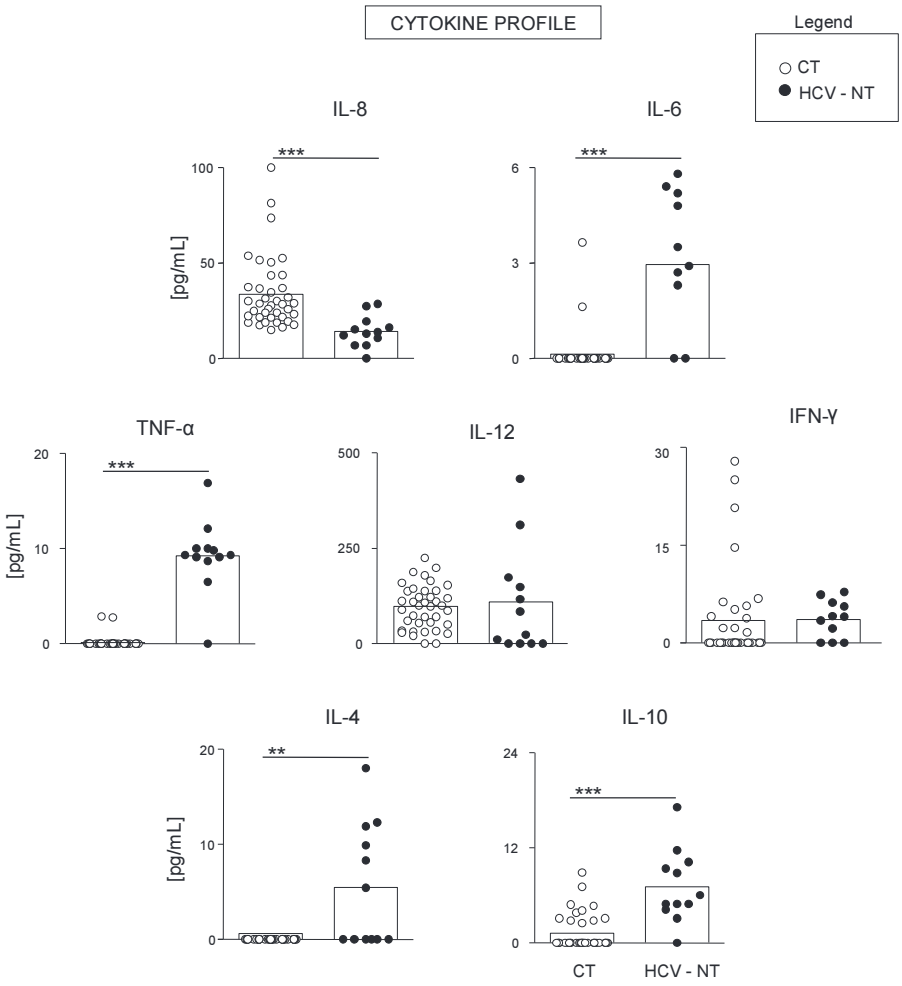

Fig. 1. Serum cytokine profile of non-treated *hepatitis C patients (HCV-NT = ●)* and *healthy controls (CT = ○)*. Data are expressed as scattering of individual levels and mean of IL-8, IL-6, TNF-α, IL-12, IFN-γ, IL-4 and IL-10, all reported in pg/mL. Significant differences are highlighted by connecting lines and ** or *** for significance at $p < 0.001$ and $p < 0.0001$, respectively.

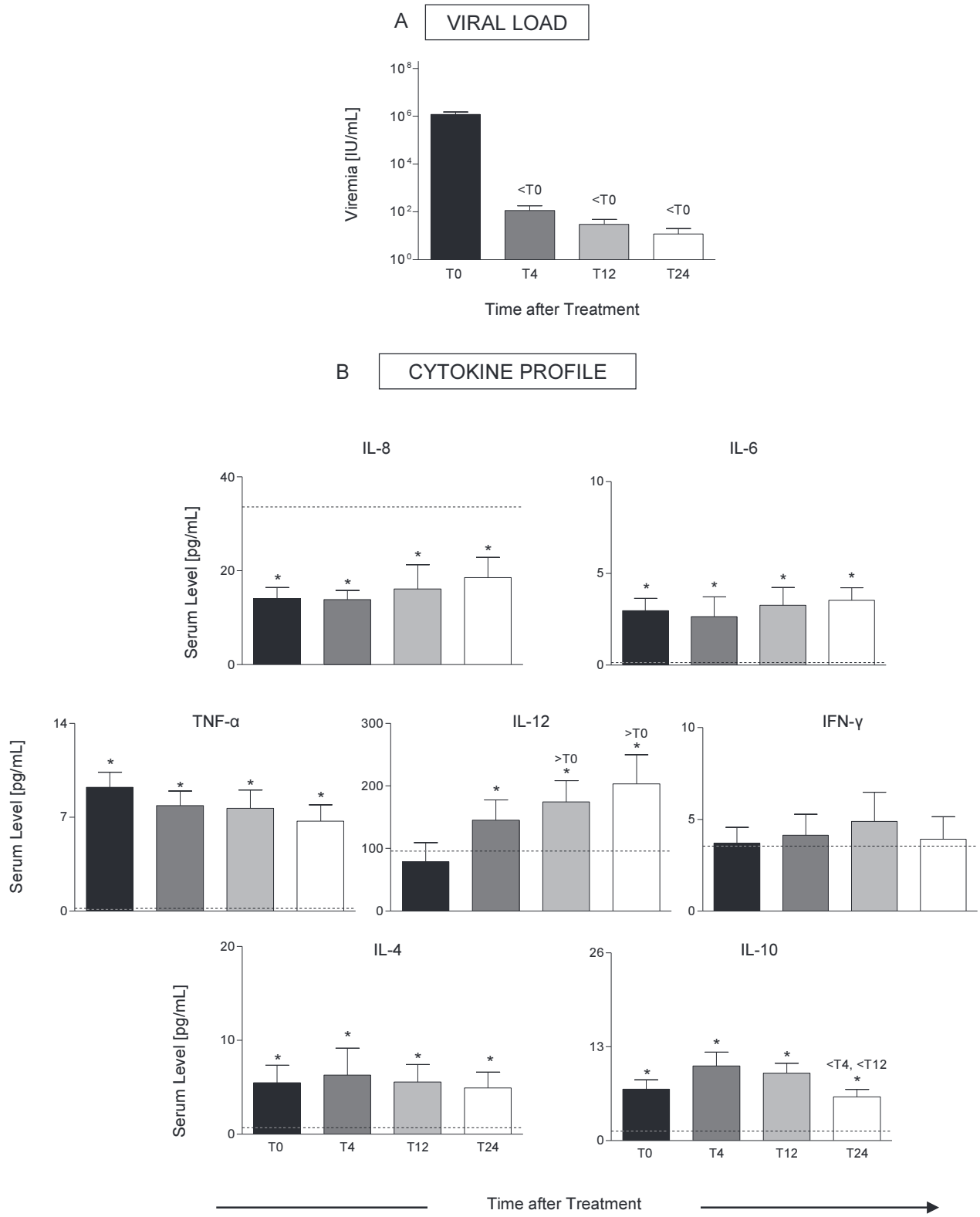

Fig. 2. *Kinetics of virological and immunological features following PEG-IFN-α2A/ribavirin therapy of chronic hepatitis C patients.* The viral load (A), expressed as mean ± SE IU/mL and the serum cytokine profile for IL-8, IL-6, TNF-α, IL-12, IFN-γ, IL-4 and IL-10 (B), reported as mean ± SE pg/mL, were determined at treatment baseline (T0 = ■) and at 4-weeks (■), 12-weeks (■) and 24-weeks (T24 = □) of treatment. Significant differences at $p < 0.05$ are highlighted by * for comparisons with the mean values observed for the health control group (dashed lines). Additionally, significant increase (>) or decrease (<) as compared to T0, T4 and T12 are indicated in the figure.

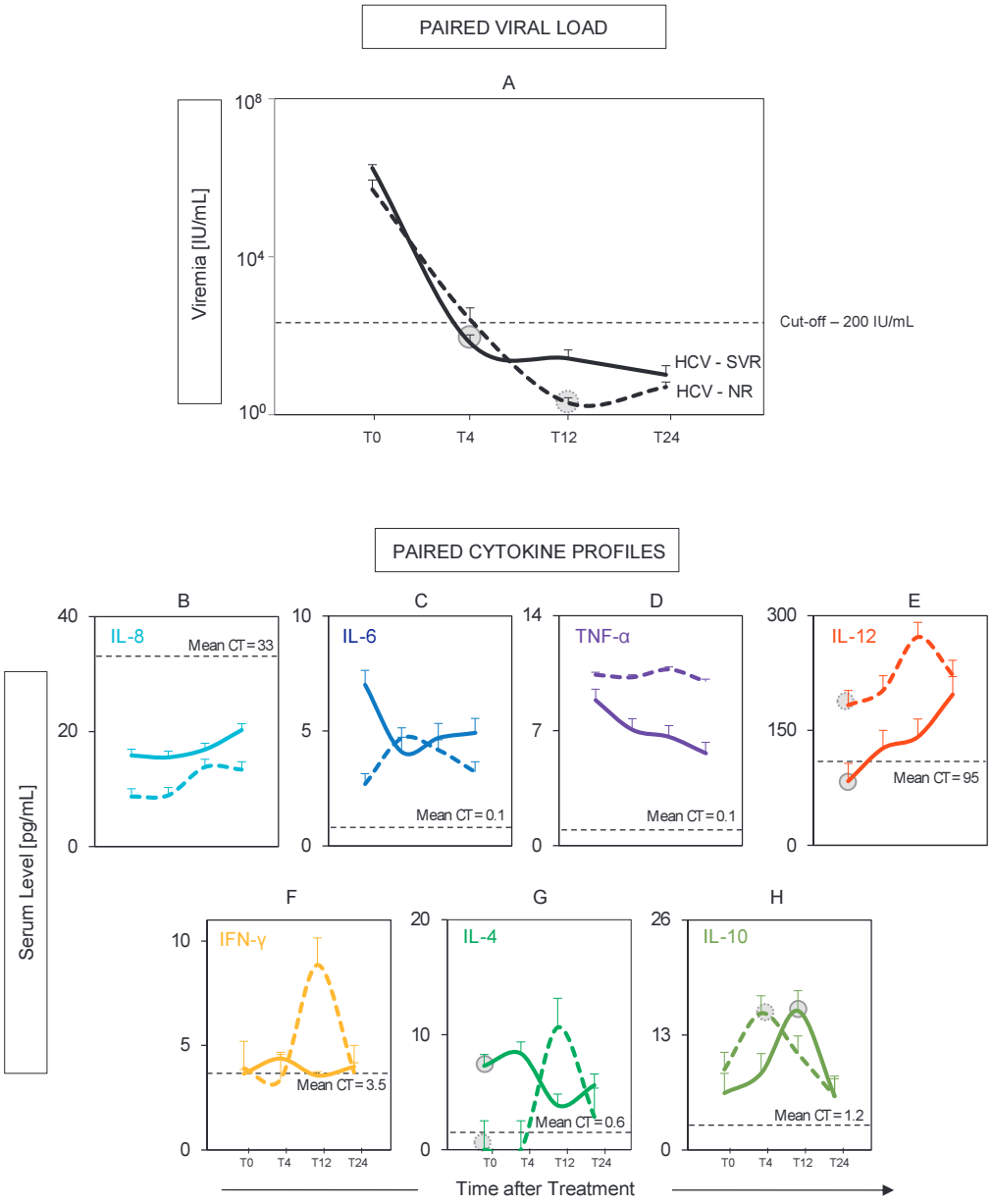

Fig. 3. Virological and immunological features associated with therapeutic effectiveness following PEG-IFN-α2A/ribavirin treatment in chronic hepatitis C patients. Paired analysis were performed to characterize the viral load and cytokine profiles of chronic hepatitis C patients showing distinct profile of therapeutic response, referred as patients with sustained virological response (HCV-SVR = continuous line) and those considered non-responders (HCV-NR = dashed line). The viral load (A), reported as average (IU/mL) and the serum cytokine profile of IL-8 (B), IL-6 (C), TNF-α (D), IL-12 (E), IFN-γ (F), IL-4 (G) and IL-10 (H), expressed as average (pg/mL), were plotted for treatment baseline (T0) and at 4-weeks (T4), 12-weeks (T12) and 24-weeks (T24) after treatment with their respective standard error bars. Comparative analyses were performed using a cut-off edge for viral load (200 IU/mL) and the mean values observed in the healthy control groups (CT) as reference (dashed line across the graphs). Additionally, highlighting circles (,) were used to identify major differences between HCV-SVR and HCV-NR subgroups.

IL-12 levels remained high at T12 and T24 as compared to T0. Although, the levels of IL-10 remained above the mean values of CT throughout the treatment evaluation, it declined significantly at T24 as compared to T4 and T12 (Fig. 2).

3.3. SVR is associated with early decrease in the viral load at week 4 after treatment

Our findings demonstrated that HCV-SVR presented an early decrease in the viral load at week 4 after treatment, whereas HCV-NR showed a decrease in the viral load only at week 12 after treatment (Fig. 3).

3.4. Increased IL-12 response at baseline predisposes patients to become non-responders, whereas an IL-4-modulated pro-inflammatory profile favors SVR after PEG-IFN-α2A/ribavirin therapy

Despite of a mixed pro-inflammatory/regulatory cytokines pattern observed in both HCV subgroups, the HCV-NR presented more prominent IL-12 response at the baseline (T0), whereas patients showing a sustained virological response at week 48, presented a baseline cytokine profile characterized by an IL-4-modulated pro-inflammatory response. These findings suggested that higher levels of IL-12 before treatment (Fig. 3E – highlighting circles) do not favor the establishment of SVR. Our

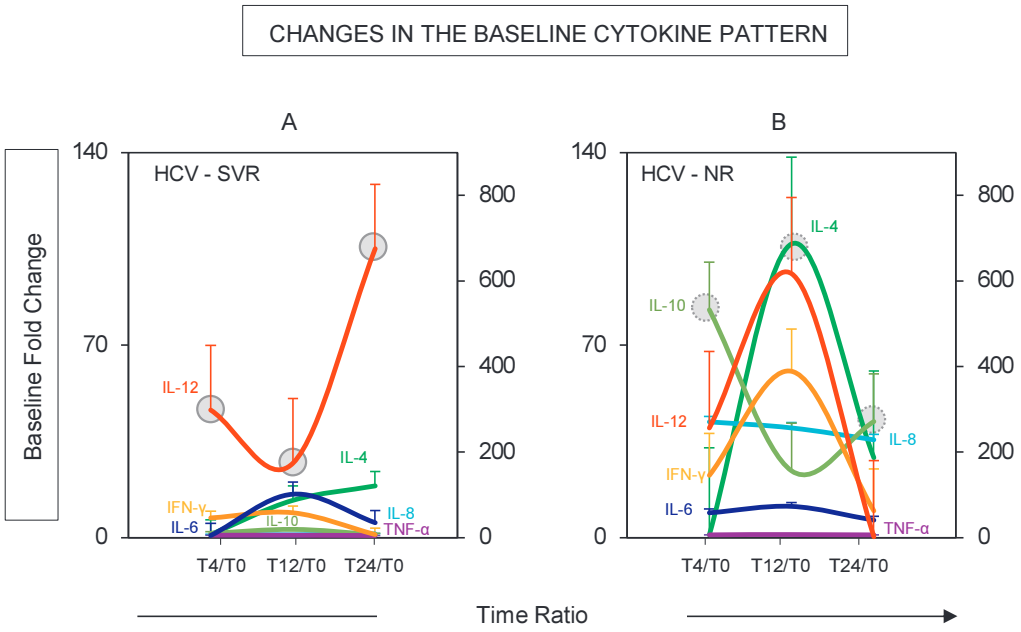

Fig. 4. Changes in the average baseline cytokine patterns associated with therapeutic effectiveness or failure following PEG-IFN-α2A/ribavirin treatment in chronic hepatitis C patients. The cytokine profiles of patients showing sustained virological response – HCV-SVR (A) and those considered non-responders – HCV-NR (B) are expressed as average baseline fold changes considering the time-points of 4-weeks (T4/T0), 12-weeks (T12/T0) and 24-weeks (T24/T0) with their respective standard error bars. Highlighting circles (,) were used to identify the biomarkers associated with therapeutic effectiveness or failure at each time-point.

data supports the hypothesis that these patients are unable to uphold their pro-inflammatory response after the PEG-IFN-α2A therapy onset. Under an environment composed of the "high IL-12 baseline" plus the "pro-inflammatory impact of PEG-IFN-α2A therapy", the immune system undergoes a counter-regulatory response as a compensatory mechanism to control the extreme pro-inflammatory environment. This hypothesis is supported by our findings that HCV-NR presented an early IL-10 response at week 4 after treatment. This immune over-activation associated with chronic HCV infection raises the question of whether the intense pro-inflammatory profile at baseline can lead to an early perturbation in the immune response triggered by the PEG-IFN-α2A/ribavirin therapy that could subsequently affect the treatment outcome. On the other hand, if the PEG-IFN-α2A therapy is started when the HCV patient present an IL-4-modulated pro-inflammatory profile (Fig. 3G – highlighting circles), the immune system is able to properly respond to the IFN-α2A therapy and mount an early pro-inflammatory response at week 4 after treatment. In HCV-SVR patients, the counter-regulatory events are observed only after 12 weeks of treatment characterized by a late-peak increase in the levels of the immunoregulatory cytokine IL-10 (Fig. 3H–highlighting circles).

3.5. *Early changes in the baseline cytokine pattern toward an increased IL-12 response is critical to support SVR while the predominance of IL-10 over the baseline status lead to virological relapse after PEG-IFN-α2A/ribavirin therapy*

Fig. 4 illustrates the kinetics of serum cytokine levels following PEG-IFN-α2A/ribavirin therapy. The average fold changes (relative to baseline) over time of treatment are displayed for each cytokine in the HCV subgroups (HCV-SVR and HCV-NR). Our findings demonstrated that the kinectics of IL-12 is associated with SVR (Fig. 4A – highlighting circles), whereas a perturbation in the cytokine profile is observed on non-responders, with evident peaks of the immunoregulatory cytokine IL-10 (Fig. 4B – highlighting circles).

3.6. *Increased monocytes/lymphocyte activation at baseline predisposes to treatment failure, whereas high CD8⁺ T-cell counts at treatment-baseline favors a SVR after PEG-IFN-α2A/ribavirin therapy*

Our data demonstrate interesting differences in the kinetics of $CD4^+$ and $CD8^+$ T-cells in patients with SVR as compared to non-responders (Fig. 5). More specifically, the HCV-SVR group presented an increased frequency of $CD8^+$ T-cells at treatment-baseline that remains elevated up to week 12 after treatment and returned to normal levels later at week 24 when a late increase in the $CD4^+$ T-cells was observed (Fig. 5B). On the other hand, the HCV-NR group showed an early increase of $CD4^+$ T-cells at weeks 4 and 12 after treatment along with unchangeable percentages of $CD8^+$ T-cells (Fig. 5B).

The analysis of $CD69^+$ expression in monocytes and lymphocytes (Fig. 5) support the hypothesis that high baseline activation status predisposes patients to become non-responders after PEG-IFN-α2A/ribavirin therapy. As demonstrated in the Fig. 5A, although the HCV-SVR presented higher levels of activated monocytes and lymphocytes as compared to the reference group (dashed line in the Fig. 5), this mild activation status still allow the immune system to provide a well-timed activation upon the PEG-IFN-α2A/ribavirin therapy. On the other hand, the HCV-NR presented very high baseline levels of activated monocytes and lymphocytes that remains elevated throughout the PEG-IFN-α2A/ribavirin therapy (Fig. 5B).

4. Discussion

Sustained virological response (SRV) at week 48 has been the gold standard biomarker to determine the therapeutic effectiveness following PEG-IFN-α2A/ribavirin treatment in chronic hepatitis C patients. However, monitoring non-responders from earlier time points would increase significantly the success of treatment and the quality of life of the patients. Therefore, understanding the mechanisms underlying the success of PEG-IFN-α2A/ribavirin therapy would allow for a better follow up of patients under treatment.

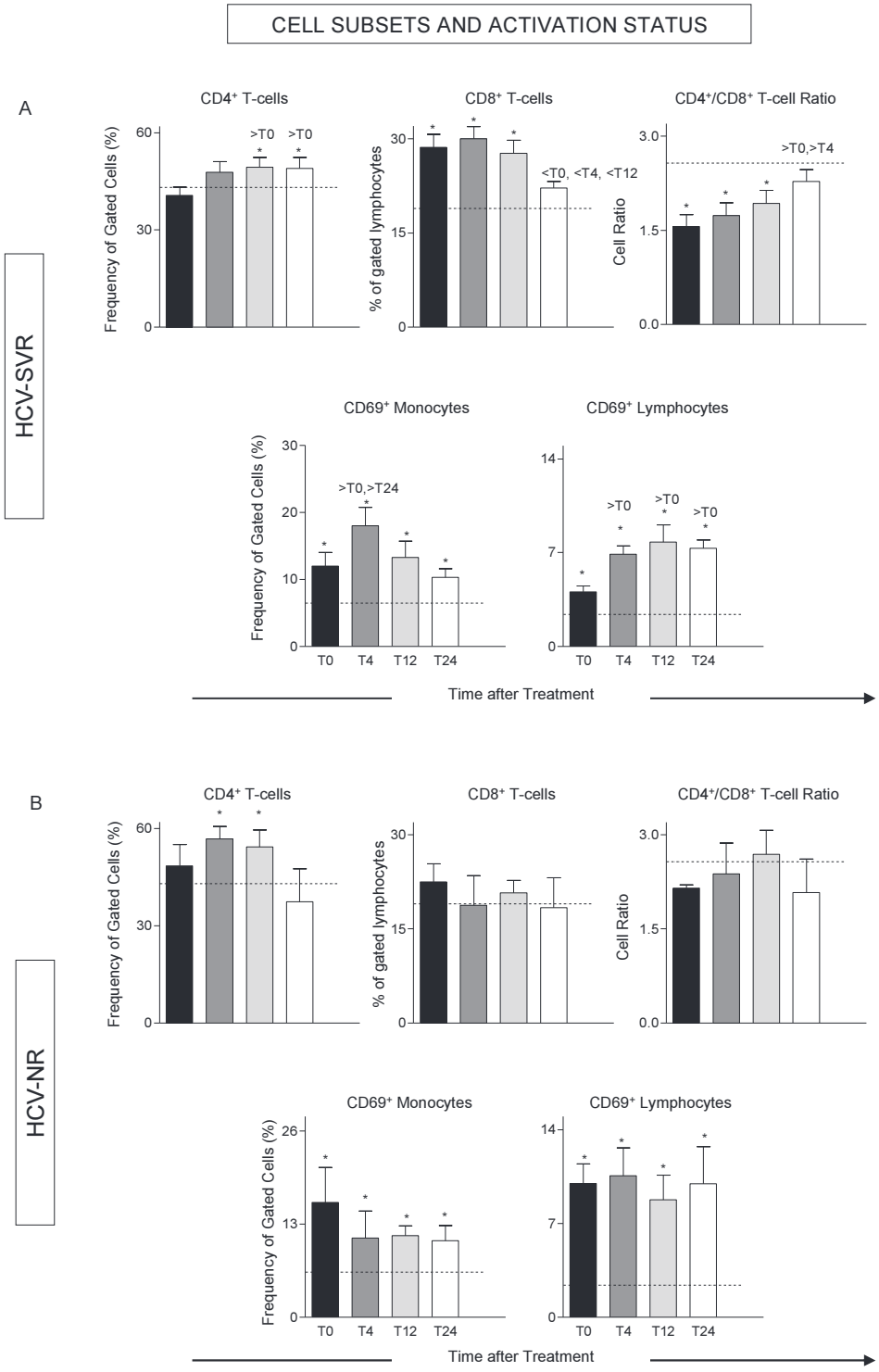

Fig. 5. Cell subsets and activation status of circulating monocytes and lymphocytes associated with therapeutic effectiveness or failure following PEG-IFN-α2A/ribavirin treatment in chronic hepatitis C patients. T-cell subsets ($CD4^+$, $CD8^+$ and $CD4^+/CD8^+$ ratio) and the activation status ($CD69^+$) of circulating monocytes and lymphocytes were determined at treatment baseline (T0 = ■) and at 4-weeks (■), 12-weeks (■) and 24-weeks (T24 = □) of treatment. Paired analysis were performed to characterize the phenotypic profile of circulating monocytes and lymphocytes from chronic hepatitis C patients showing distinct profile of therapeutic response, referred as patients with sustained virological response – HCV-SVR (A) and those considered non-responders – HCV-NR (B). Significant differences at $p < 0.05$ are highlighted by * for comparisons with the mean values observed for the health control group (dashed lines). Additionally, significant increase (>) or decrease (<) as compared to T0, T4, T12 and T24 are indicated in the figure.

Several efforts have been made to establish novel biomarkers with relevant predictive values that could be used as laboratorial tools for prognostic purposes. The analysis of early virological response at week 4 after treatment has been considered as a relevant indicator for predicting sustained virological response. Despite of the limitation of the present study (the relatively small number of patients that were used in each group), our results corroborate previous findings that show that sustained virological response (SVR) is associated with early decrease in the viral load at week 4 after treatment [20] although later time points were also previously reported

as optimal for confirmation of a fully sustained virological response. Assessment of serum HCV-RNA at week 12 after treatment, using the highly sensitive real-time PCR assay, was demonstrated to be almost as effective as week 24 after treatment to predict SVR in a larger cohort of chronically HCV infected patients from Japan [21].

Although a mixed pattern of pro-inflammatory/regulatory cytokines was observed in most untreated HCV patients, the presence of modulated pro-inflammatory profile at baseline favors SVR whereas a prominent inflammatory response at baseline seems to predispose patients to therapeutic failure. The present data suggests that the analysis of the magnitude of subject-specific fold changes at the baseline level of cytokines is imperative to monitor the dynamic changes occurring for each cytokine during PEG-IFN-α2A/ribavirn therapy. The use of general and inflexible reference levels may not be relevant for the follow-up of a given subject who has lower (or higher) baseline levels when compared to the general population, even if the changes in magnitude are evident.

Previous reports are in agreement with our findings, which show that measurements of pre-treatment cytokine levels could contribute for the understanding of therapy course. Umemura et al. demonstrated that high baseline levels of IL-10 and low levels of IL-12p40 were significantly associated with therapeutic failure [22]. In agreement with these findings, our results demonstrated a time-dependent change in the baseline cytokine pattern toward an increasing IL-12 response. These results indicate that the IL-12 kinectics is critical to support the SVR, while the early predominance of IL-10 surrenders the patients to late virological relapse.

A broad but unguided "cytokine storm" is observed in the non-responder HCV patients at week 12 after treatment, which is consistent with their failure to clear the HCV infection. This unregulated and unbalanced production of cytokines prior to treatment may be associated to exhaustion of T-cell response [23–26]. The expression of exhaustion markers by T-cells has been associated with reversible immune dysfunction [27,28]. It has been shown that patients with SVR present reversing exhaustion T-cell response and, therefore, leading toward a higher and faster control of viral spread and persistence. These exhaustion molecules have been shown to modulate the expression of IL-12 [24,27], which corroborate the putative role of this cytokine in HCV treatment success observed in the present study.

Several efforts have been made to establish novel biomarkers with relevant predictive values to monitor the PEG-IFN-α2a/ribavirin therapy of HCV infection [5,22,29–33]. Seeking associations between cytokine levels and viral protein expression, Yoneda et al., demonstrated that IL-10, IL-12, and IL-18 levels are predictive of the response to HCV treatment with pegylated interferon and ribavirin and are associated with amino acid substitutions in the interferon sensitivity-determining region (ISDR) and core region of HCV in a Japanese cohort [29]. In this study, a high baseline of IL-10 levels were associated with a non-response to treatment, which is in agreement with our data demonstrating that the early predominance of IL-10 correlates to late virological relapse. IL-12p40 levels were decreased after 4 and 72 weeks of treatment and remained low in SVR patients from the Japanese cohort, however, the IL-12p70 kinetics is not clear in the study. Conversely, our study demonstrates that a time-dependent increase on serum IL-12p70 levels in patients under treatment supports SVR. This difference may be due to the genetic background of the population, which are different concerning the HLA haplotypes and other immunological factors. Additionally, the environmental conditions may also account for the disparity of IL-12p70 levels in SVR versus non-SVR patients of these two cohorts. The Brazilian individuals of this study are settled in a tropical area and are exposed to a variety of pathogens such as protozoa and flaviruses at a very high rate. Our population is majorly vaccinated to yellow fever and other infectious pathogens, whereas the Japanese cohort was settled in a non-tropical area with lower exposure to infectious pathogens. These and other previous reports are in agreement with our findings that IL-12 and T-cell activation and frequency are key regulators of success of therapy. The fine tuning orchestrated by IL-12 and T-cells may be the key to find relevant biomarkers to monitor chronically HCV infected patients under PEG-IFN-α2a/ribavirin therapy.

5. Conclusion

The baseline pattern of serum pro-inflammatory/regulatory cytokines and the immunological activation status are closely related with the therapeutic response of chronic HCV patients undergoing PEG-IFN-α2A/ribavirin therapy. Further studies with larger groups of patients are suggested to confirm these findings.

Conflict of interest

We wish to confirm that there are no conflicts of interest associated with this publication and that the Funding agencies did not participate or influence on the design and execution of this study.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship. We further confirm that the order of authors listed in the manuscript has been approved by all of the authors.

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