

ROLE OF T REGULATORY CELLS IN CHRONIC HCV INFECTED EGYPTIAN PATIENTS AND THEIR IMPACT ON THE RESPONSE TO PEGYLATED INTERFERON THERAPY

By

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Abstract

Treatment of patients with chronic hepatitis C with the current standard pegylated interferon (PEG-IFN) and ribavirin achieves overall response (SVR) rates of ~55%. A role of CD4⁺ CD25⁺ regulatory T cells (Treg cells) has been proposed as they can suppress HCV-specific T cells in HCV-infected patients. Patients with chronic HCV legible for PEG-IFN plus ribavirin treatment, were classified according to their response to treatment into two groups (responders and non-responders, 32 and 27 patients respectively). Blood and plasma samples were collected at the start of treatment and at 12 and 24 weeks during treatment. Immunophenotyping by flow cytometry for Treg cells, the FOXP-3 expression using real-time PCR and measurement of IL-10, TGF- β CXCL-9 and CXCL-10 were performed. Increased expression of Treg cells was detected in patients who didn't respond to treatment before and during treatment. Also, the levels of IL-10, TGF- β , CXCL-9 and CXCL-10 revealed significant increase in non-responders all through compared to responders group. Evaluation of Treg cells, cytokines (IL-10 & TGF- β) and chemokines (CXCL-9 & CXCL-10) before starting the treatment could be a predictor of response to treatment with PEG-IFN plus ribavirin. The optimum levels which would differentiate between responders and non-responders are needed to be defined before-hand.

Key words: T Regulatory cells, Chronic HCV, Pegylated interferon, Ribavirin, Chemokines- Cytokines.

Introduction

Chronic infection with hepatitis C virus (HCV) is estimated to affect 170 to 200 million people globally (Di Bisceglie, 2000). Egypt has the highest prevalence of chronic hepatitis C virus (HCV) infection in the world, and more than 90% of patients are infected with genotype 4 virus and HCV led to chronic liver disease and HCC (Doss *et al*, 2015).

Combination therapy with pegylated interferon alpha and ribavirin is currently the standard treatment for patients with chronic hepatitis C (Tam *et al*, 1999; Caetano *et al*, 2008). The goal of such a treatment is to obtain sustained virological response (SVR) defined as undetectable HCV RNA in the serum after 24 weeks of post treatment fol-

low up (Asselah *et al*, 2009; Pearlman and Traub, 2011; Romero-Gómez *et al*, 2015).

Treatment of HCV using combination of pegylated interferon alpha with ribavirin yields a sustained response in approximately 55% of the cases for genotype 4. Because of the important side effects and high cost of pegylated interferon alpha plus ribavirin therapy, it is highly important to identify serum markers as predictors of response to treatment (Bolacchi *et al*, 2006; Asselah *et al*, 2010).

Responder's patients defined as undetectable HCV RNA in the serum after 24 weeks of post treatment follow up (Asselah *et al*, 2010). Treg cells are heterogeneous population of cells including the classical CD4⁺ CD25⁺ subset expressing the transcription

factor FoxP3, which is important in the development and suppressive function of CD4⁺ CD25⁺ Treg that can suppress HCV-specific T cells in HCV-infected patients (Sprengers *et al*, 2007; Alatrakchi and Koziel, 2009).

Regulatory T cells (Treg) were identified as a specialized subset of T cells that suppress auto-reactive cells to maintain immunological tolerance and inhibit autoimmunity. Treg have proved to control excessive or chronic immune activation after infections with various pathogens (Belkaid, 2007; Billerbeck *et al*, 2007)

These induced CD4⁺ CD25⁺ cells have been shown to suppress T cell responses to tumors and acute and chronic bacterial and viral infections (Suvas *et al*, 2003; Aandahl *et al.*, 2004). In particular, it has been reported recently that CD4⁺ CD25⁺ T cells suppress HCV specific T cell responses and it has been suggested that they may play a role in viral persistence (Cabrera *et al*, 2004).

Treg cells also operate via an IL-10 and TGF- β dependent mechanism, as they are considered as consecutive inflammatory mediators (Gressner *et al.*, 2007). The presence of TGF- β induces the generation of regulatory T cells (Treg cells) population by the surface expression of CD4⁺ and CD25⁺ and transcription factor FoxP3 (Alatrakchi and Koziel, 2009; Evans *et al*, 2007; Zheng *et al*, 2007).

Chemokines are important in lymphocyte recruitment to the liver in HCV infection. They play an essential part in orchestrating the antiviral immune responses during acute HCV infection. CXCR3 associated chemokines (such as CXCL 9 and CXCL 10) seem to be required for effective antiviral T cell responses during the acute phase of HCV infection. In Chronic HCV infection, on contrary, HCV replicates persistently within the hepatocytes, thus sustaining chemokine production. As a result continuous recruitment of inflammatory cells to the liver is provoked and liver injury continues

without viral clearance. For instance, one study examined the levels of chemokines that bind to CXC chemokine receptor 3 (CXCR3) to determine whether they play a role in the failure of the immune system to clear HCV infection (Butera *et al*, 2005; As-saleh *et al*, 2009). In addition to their roles in liver inflammation, chemokines may also be used as biomarkers of antiviral treatment outcomes of HCV (Sturm *et al*, 2010; Kang and Shin, 2011).

A critical role for CXCR3 in the recruitment of regulatory T cells in response to CXCL9 and CXCL10 by parenchymal cells was reported which posted on the luminal surface of hepatic sinusoidal endothelial cell (HSEC) via interactions with proteoglycans in the glycocalyx (Curbishley *et al*, 2005; Schrage *et al*, 2008). CXCL10 expressed by stromal cells subsequently supported migration of CXCR3 T cells into the hepatic parenchyma or a long myofibroblast conduits in the space of Disse to the portal tracts and biliary epithelium (Holt *et al*, 2009; Ye Htun *et al*, 2012). So, CXCL9 and CXCL10 are considered to be positively associated with the severity of liver fibrosis and significantly correlated with serum levels of the hepatoreactive cytokines interleukins IL-6 and IL-10, suggesting their involvement in a counter-regulatory response during the progression of liver disease, shedding new light on their involvement in the pathophysiology of liver disease (Tacke *et al*, 2011).

In this study cells, a longitudinal analysis of value of CD4⁺ CD25⁺ cells was performed in responder and non-responder patients chronically infected with HCV before and during treatment with peg-IFN α plus ribavirin, to evaluate these cells as predictors for response to treatment.

Patients, Materials and Methods

The present study was conducted on patients with hepatitis C virus infection coming to out patients clinic of Hepato-Gastroenterology at Theodor Bilharz Research In-

stitute (TBRI). The local institutional review board at the institute has approved this study and all individuals gave written informed consent according to the ethical guidelines of the 1975 Declaration of Helsinki. None of them were co-infected with HIV or hepatitis B virus (HBV). All patients received 24 weeks of antiviral therapy consisting of orally administered ribavirin and subcutaneous infusion with pegylated IFN- α , both weight based. All responders complete the 48 weeks of treatment. However non-responders stopped the treatment at 24 weeks. So, for comparative reasons, analysis for both groups had been done at 24 weeks of treatment.

The diagnosis of HCV infection was based on clinical features, laboratory tests and liver biopsy. Full medical history, clinical examination, laboratory investigations and abdominal ultrasonography were performed for all patients at the beginning of treatment. Blood and plasma samples were collected at the start of treatment (0 day), and at 12 & 24 weeks of treatment. According to response of HCV patients to peg IFN α + ribavirin therapy, the patients were classified into 2 groups, 32 patients were responders to treatment (sustained virological response) with PCR for HCV RNA was negative 3 months of treatment and negative 6 months post-treatment. Another 27 patients were non-responders to treatment (non-sustained virological response) with persistence of positivity of HCV-RNA-PCR after 3 months of treatment.

Detection of Treg cells by flow cytometry: Venous peripheral blood samples were drawn into sterile tubes containing EDTA anticoagulant and processed within 2 hours of sample collection. For CD4⁺CD25⁺ co-expression, 100 μ l of blood samples were mixed with each of 10 of anti-CD4⁺-fluorescein isothiocyanate (FITC) and 10 μ l of anti-CD25⁺-phycoerythrin (PE) monoclonal antibodies (Immunotech, A Beckman Coulter Company, France) and allowed to

incubate for 10 minutes at room temperature. Red cells were lysed using 500 μ l of OptiLyse C solution (Immunotech, A Beckman Coulter Co., France) for 10 min., followed by the addition of 500 μ l of phosphate buffered saline (PBS), pH 7.4. The cells were washed by adding 1 ml of PBS to each tube and the tubes were centrifuged at 300 x g for 10 min. The supernatant was removed by aspiration and the cell pellet was resuspended in PBS. Samples were analyzed using COULTER EPICS XL flow cytometer (Beckman Coulter Inc., Fullerton, California, USA). Using unstained cells, the negative threshold for CD4⁺CD25⁺ was set at 0.5% and gated at main cell population.

Measurement of FOXP-3 by real-time PCR: RNA was extracted from blood samples using Trizol (Invitrogen). Isolated pure RNA for reverse transcriptase-polymerase chain reaction (RT-PCR) was prepared by treating RNA samples with RNase free DNase I (Promega Corporation, Madison, Wisconsin, USA). Samples were extracted with buffered phenol and then chloroform: isoamylalcohol (24:1). The recovered RNA was ethanol precipitated and stored in RNase-free distilled water. The purity of RNA samples was assessed spectrophotometrically. The presence of relative mRNA species was evaluated by the SYBR Green method using the ABI Prism 7700 Sequence Detector System (Applied Biosystems, Foster City, California, USA). Primers were designed for PCR by using Gene Runner Software (Hasting Software, Inc., Hasting, NY, USA). The RNA sequences used for primer design were obtained from the Gene Bank (forward, 5'-GGC CCT TCT CCA GGA CAG A-3' and reverse 5'-GCT GAT CAT GGC TGG GTT GT-3'). All primer sets had a calculated annealing at 60°C. RT-PCR was performed in duplicate in a 25 μ l reaction volume containing 2X SYBR Green PCR Master Mix (Applied Bio-systems), 900nm of each primer, 2-3 μ l of cDNA and the volume was adjusted with pure distilled water.

RT-PCR amplifications were 2min. at 50° followed by 40 cycles of denaturation for 15 sec and annealing/extension at 60° for 1 min. Real-Time PCR data were analyzed using v.1.7 Sequence Detection Software (PE Biosystems, Foster City, California, USA). Relative expression of the genes was calculated by using comparative CT method. Relative transcript levels were normalized to the 18s rRNA genes. For comparison, transcript levels were set at 1 and data from different studied groups are reported as fold increase over 1 baseline. Dissociation curves were used to verify specificity of the PCR products.

Measurement of IL-10 & TGF- β by ELISA
IL-10: Quantitative determination of human IL-10 concentrations in serum using Quantikine immunoassay ELISA kit (Catalog no D1000B). Mouse monoclonal antibody against IL-10 conjugated to horseradish peroxidase used as conjugate. Color reagent A (stabilized hydrogen peroxide) and reagent B (stabilized chromogen) were mixed in equal volumes and used as substrate solution. Serial standard dilutions were prepared using calibrator diluents (animal serum; 500, 250, 125, 62.5, 31.2, 15.6, 7.8pg/ml).

TGF- β : Quantitative determination of human TGF- β concentration in serum began with activation of latent TGF- β to immune reactive TGF- β detectable by Quantikine TGF- β immunoassay (Cat. no. DB100B). Activation was performed by acid activation using 1 N HCl and neutralization to pH 7.2-7.6 by 1.2 N NaOH/ 0.5M HEPES. Polyclonal antibody against TGF- β conjugated to horseradish peroxidase was used as conjugate. Color reagent A (stabilized hydrogen peroxide) and reagent B (stabilized chromogen) were mixed in equal volumes and used as substrate solution. Serial standard dilutions were prepared using calibrator diluents (concentrated buffered protein) (1000, 500, 250, 125, 62.5, 31.2 pg/m).

Measurement of chemokines by ELISA
CXCL9 and CXCL10: Quantitative deter-

mination of human chemokine CXCL9 and CXCL10 induced by gamma interferon concentrations in plasma was performed using Quantikine immunoassay ELISA kit (Cat. no. DCX 900). Polyclonal antibody against CXCL9 or CXCL10 conjugated to horse radish peroxidase was used as conjugate. Color reagent A (stabilized hydrogen peroxide) and reagent B (stabilized chromogen) mixed in equal volumes was used as substrate solution. Serial dilutions for CXCL9 were prepared using calibrator diluents (concentrated buffered protein base) (1000, 500, 250, 125, 62.5, 31.2 pg/ml). Serial dilutions for CXCL10 were prepared using calibrator diluents (buffered animal serum; 500, 250, 125, 62.5, 31.2, 15.6, 7.8pg/ml).

Calculations: Average of duplicate readings for each standard, control, and sample were linearized. Optical density for standards was plotted versus the concentration of the standards and the curve was drawn. Data were linearized by using log/log paper and regression analysis was applied to the log transformation. The resultant concentration of each sample was determined by applying optical density (OD) on standard curve.

Statistical analysis: Data are expressed as mean \pm Standard Error or Percentage (%). Comparison between the mean values of different parameters in both groups was performed using unpaired (t) test. Comparison between categorical data was performed using (Chi Square test). Correlation between variables was performed using (Pearson Correlation), SPSS program version (12) was used in data analysis, (p) Value less than or equal to 0.05 was considered significant & < 0.01 was considered highly significant.

Results

The biochemical findings measured before treatment for all patients showed highly significant increase in value of ALT and AST in non-responder group compared to responder group ($p < 0.01$) without significant

change in value in the other biochemical parameters between both groups (Tab. 1).

Table 1: The Biochemical findings measured before and during treatment for all patients.

variants	NR (n= 25)	R (n= 25)	P value
ALT (U/L)	47.64 ± 28.71	23.16 ± 11.60	0.001**
AST (U/L)	47.76 ± 30.21	23.04 ± 10.59	0.001**
Total Protein (g/dL)	7.79 ± 0.48	7.73 ± 0.57	0.689 ^{NS}
Albumin (g/dL)	4.06 ± 0.46	3.94 ± 0.33	0.290 ^{NS}
Total bilirubin(mg/dL)	0.95 ± 0.85	0.84 ± 0.38	0.550 ^{NS}
Direct bilirubin(mg/dL)	0.32 ± 0.30	0.41 ± 0.22	0.220 ^{NS}
AFP(U/ml)	7.80 ± 11.69	6.78 ± 4.89	0.687 ^{NS}
Createnin (mg/dL)	0.91 ± 0.13	1.01 ± 0.26	0.105 ^{NS}
Urea (mg/dL)	25.44 ± 7.74	25.96 ± 9.07	0.828 ^{NS}

p> 0.05= not significant, ** p< 0.01 significant versus non-responders, ALT: Alanine amino transferase, AST: Aspartate amino transferase, AFP: Alpha feto protein, Normal range for albumin: 3.5-5 g/dL, Normal range for ALT and AST: up to 40 U/mL, Normal range for AFP:< or = 20 U/ml.

To determine the contribution of CD4⁺ CD25⁺ Treg to the regulation of immune responses to HCV, peripheral blood samples were collected and evaluated by flow cytometry at the beginning of treatment (0 day) and during therapy at 12 & 24 weeks of treatment.

Expression% of cell of CD4⁺CD25⁺ Treg cells subset before and during treatment in responders and non-responders: Before treatment (0day), expression percent of Treg cells showed highly significant increase (p<0.001) in non-responders (48.58±0.3) compared to responders (20.8±1.2). Highly significant increase in percent expression of Treg cells (p<0.001) was also detected after 12 weeks of treatment in non-responders (72.23±1.3) as compared to responders (23.59±1.08) and after 24 weeks of treatment in non-responders (94.82±1.8) compared to responders (23.76±0.8). FOXP-3 expression in blood samples of different groups using quantitative real time PCR: Before treatment (0 day), the FOXP-3 expression increased significantly from 5.3±1.3 fold in responders group to 10.6±1.5 fold in non-responders group comparing to normalization baseline. Highly significant increase in FOXP-3 expression (p<0.001) was also detected 12 weeks post treatment in non-responders (12.23±1.2) compared to responders (6.59±1.08) and 24 weeks post

treatment in non-responders (15.12±1.1) compared to responders (6.76±0.8). Cytokines levels in responders & non-responders: Levels of IL-10 & TGF-β (pg/ml) were measured before treatment and showed highly significant increase (p<0.01) in non-responders (IL-10=220.98±1.07 & TGF-β=91.72±1.65) compared to responders (IL-10=170.04±1.39 & TGF-β=56.50±1.66). Highly significant increase (p<0.001) was detected after 12 weeks in non-responders (IL-10=519.67±3.69 & TG 90.01±7.46) compared to responders (IL-10= 180.22±1.1 & TGF-β=49.58±1.6) and after 24 weeks in non-responders(IL-10=520.03±1.15 & TGF-β=109.85±1.71) compared to responders (IL-10=180.22±1.6 & TGF-β=50.12 ±1.1).

Levels of chemokines in responders and non-responders: Level of CXCL 9 & CXCL 10 were measured before treatment showed highly significant increase (p< 0.001) in non-responders (CXCL 9 = 862.87 ±3.15 & CXCL10=1360.58±3.1) compared to responders ones (CXCL9=134.38±2.6 & CXCL10=167.64±2.9). Highly significant increase (p<0.001) was detected after 12 weeks in non-responders (CXCL9= 803.07 ±2.3 & CXCL10=1352.58±4.02) compared to responders (CXCL9=161.19±2.54 and CXCL10=163.62±3.3) and after 24 weeks in non-responders (CXCL9=856.09±1.48 and CXCL 10=1507.46±1.5) compared to res-

ponders (CXCL9=158.10±1.7 & CXCL10 = 166.16±1.6). Details are given in figures (1, 2, 3, 4, 5, 6 & 7).

Discussion

Large numbers of highly activated and differentiated CD4⁺ CD25⁺ Treg cells localize to the infiltrated chronic HCV-infected liver. They play a pivotal role in limiting collateral damage by suppressing excessive HCV-infected immune activation (Claassen *et al*, 2010). It is suggested that Treg cells secreting IL-10 and TGF-β may lead to attenuation of T-cell responses and amelioration of liver inflammation (Alatrakchi and Koziel, 2009). At the same time, chemokines like CXCR3 and its ligands CXCL9 and CXCL10 promote recruitment of Treg cells from blood into the liver indicated their involvement in a counter-regulatory response during the progression of liver disease (Tacke *et al*, 2011; Ye Htun *et al*, 2012).

In response to treatment with peg IFN α and ribavirin, CD4⁺ CD25⁺ Treg frequencies caused increased in the liver of chronically HCV infected patients and accumulated in peripheral blood (Cabrera *et al*, 2004; Claassen *et al*, 2011).

In this study, the biochemical findings measured before treatment for all patients showed highly significant increase in value of ALT & AST in non-responder group compared to responder ones. But, there was no significant change in value of other biochemical parameters between both groups. This is due to the high percentage of Treg cells in non-responders group. Treg cells suppress the T lymphocytes that fight the virus replication (Billerbeck *et al*, 2007).

The current debate was to determine whether Treg cells play a role in the immune dysfunction and if they can be used as predictor for response to treatment.

The duration of the treatment varied depending on the genotype. Forty eight weeks are recommended for patients infected with genotypes 1 & 4; responder patients are de-

finied to have undetectable HCV RNA in the serum after 24 weeks of post treatment follow up. Non responder patients were defined to have persistence of HCV-RNA-PCR in spite of peg IFN therapy for at least 6 months (Alatrakchi and Koziel, 2009; As-selah *et al*, 2009).

In the present study, before the start of treatment and during the treatment, when both groups were compared, chronically infected patients who did not respond to therapy showed significantly higher levels of percent expression of CD4⁺ CD25⁺ cells than responder patients at all-time points studied. The non-responder patients showed gradually increasing percentage of CD4⁺ CD25⁺ cells from the beginning of treatment till the end of treatment. In non-responders an increment of CD4⁺ CD25⁺ Treg frequency during peg IFN α plus ribavirin therapy was suggested to negatively influence T cell reactivity to the virus, as illustrated by the reduced Th-cell proliferation, contributing to treatment failure (Sprengers *et al*, 2007).

On the other hand, the delicate balance between intrahepatic CD4⁺ CD25⁺ Treg cells and effector T cells explained the response to IFN-α based treatment in a subset of chronic the HCV patients (Claassen *et al*, 2011; Prati *et al*, 2012).

Shuo *et al*. (2009) hypothesis, supported by their data, stated that a proportion of the regulatory T cells are specifically stimulated by the virus and that these cells are a stable cell population. They found evidence that these suppressive cells have a distinct set of genes activated and importantly might have a survival advantage over effector T cells, which helps to explain why regulatory T cells may influence the outcome of HCV infection.

Several studies indicated that patients chronically infected with HCV generally show a weak peripheral blood T cell response against HCV, which is insufficient to eradicate the virus. The peripheral blood regulatory T cells (Treg) from HCV infected

patients suppressed both HCV specific T cell proliferation and IFN-gamma production. Also, HCV proteins encode some activities to interfere and limit the interferon signaling pathway. In non-responders, some interferon-stimulated genes were up-regulated before treatment (Jacobson *et al*, 2007; Kau *et al*, 2008; Patri *et al*, 2012). On the other hand, RT-PCR analysis indicated significant correlations between FoxP3 and CD8 transcription of IL 10, TGF-beta and IFN-gamma (Sturm *et al*, 2010).

IL-10 is a T helper type 2 cytokine, which plays a major role in T & B cell regulation. Peripheral blood mononuclear cells (PBMCs) from patients with chronic hepatitis C had increased IL-10 mRNA and protein expression, The IL-10 inhibited the production of IFN- α by stimulation of viral infections. The strong relationship was found between IL-10 polymorphism and response to IFN- α treatment (Estrabaud *et al*, 2012).

Sprengers *et al*. (2007) found that an increase of the frequencies of IL -10 producing cells and CD4⁺ CD25⁺ Treg during treatment was inversely correlated to treatment response. Sturm *et al*. (2010) also reported that TGF-beta may have an important role in chronic hepatitis and cirrhosis, as successful IFN treatment in patients with chronic hepatitis C led to a decrease in the expression of TGF-beta messenger (m) RNA and pro collagen type 1 mRNA in the liver. Moreover, TGF-beta is known to suppress the antiviral effects of IFN therapy perhaps leading to viral replication and persistence in host cells, whereas inhibition of TGF-beta *in vitro* has been shown to enhance anti HCV cytotoxic T lymphocyte activity and as reported before that secretion of TGF- β is considered as an important factor for the local survival and function of natural Treg cells.

In this study, differences between sustained responder and non-responder patients were further confirmed by measuring the levels of IL 10 and TGF- β before and during

the course of peg IFN- α plus ribavirin therapy showed in contrast to the responders, that the non-responders had highly significant increase in levels of IL 10 and TGF- β .

The chemokines are considered to be useful biomarkers of treatment outcomes of HCV. In this study, the highly increased levels of CXCL-9 and CXCL-10 were detected in non-responder patients compared to responder patients before and during treatment (Casrouge *et al*, 2011). The elevated plasma levels of pretreatment CXCL-10 are correlated with therapeutic non-response in chronic hepatitis C patients treated with a combination of peg IFN and ribavirin (Romero *et al*, 2007). The CXCL-10 and CXCL-9 decreased during successful anti HCV treatment Askarieh *et al*, 2010; Kang *et al*, 2011).

Casrouge, *et al*. (2011) proposed an interesting explanation to the paradoxical phenomenon that increased CXCL-10 level in non-responder patients is in fact due to the N-terminal-cleaved-form of CXCL=10 that acts as a CXCR3 antagonist. Thus the N-terminal-cleaved form of CXCL-10 inhibits migration of effector T cells from the blood into the liver.

Another study observed that elevated CXCL-10 measured in the plasma may represent overproduction in the liver, or it may be produced by cells distant from the liver as part of a broader antiviral response. These results suggested that plasma concentrations of immune-reactive CXCL-10 may be a predictor of response to peg IFN- α with ribavirin (Assaleh *et al*, 2009; Sturm *et al*, 2010).

Ethical concerns have limited the study of intrahepatic immune response during antiviral therapy, because such studies would entail repeated biopsies. In addition, studies of peripheral blood T cell responses to HCV antigens during antiviral therapy have yielded conflicting results (Classan *et al*, 2011).

So, detection of responder patients by measuring Treg cells, IL-10, TGF- β and chemokines levels in blood or plasma of pa-

tients before starting any treatment may be a very important issue because the standard treatment is physically and economically demanding.

Therefore, in non-responder patients the immune-modulatory effects of peg IFN- α plus ribavirin therapy may result in an increment in Treg frequency and an up-regulation of IL 10, TGF- β producing cells which together could negatively influence T cell reactivity to the virus and contribute to treatment failure.

Conclusion

Treg cells can be considered to be predictor of response of HCV patients to treatment with Peg-IFN. A wild scale screening of patients is needed to define the optimal level of Treg cells which differentiates between responder and non-responder patients before application in medical practice.

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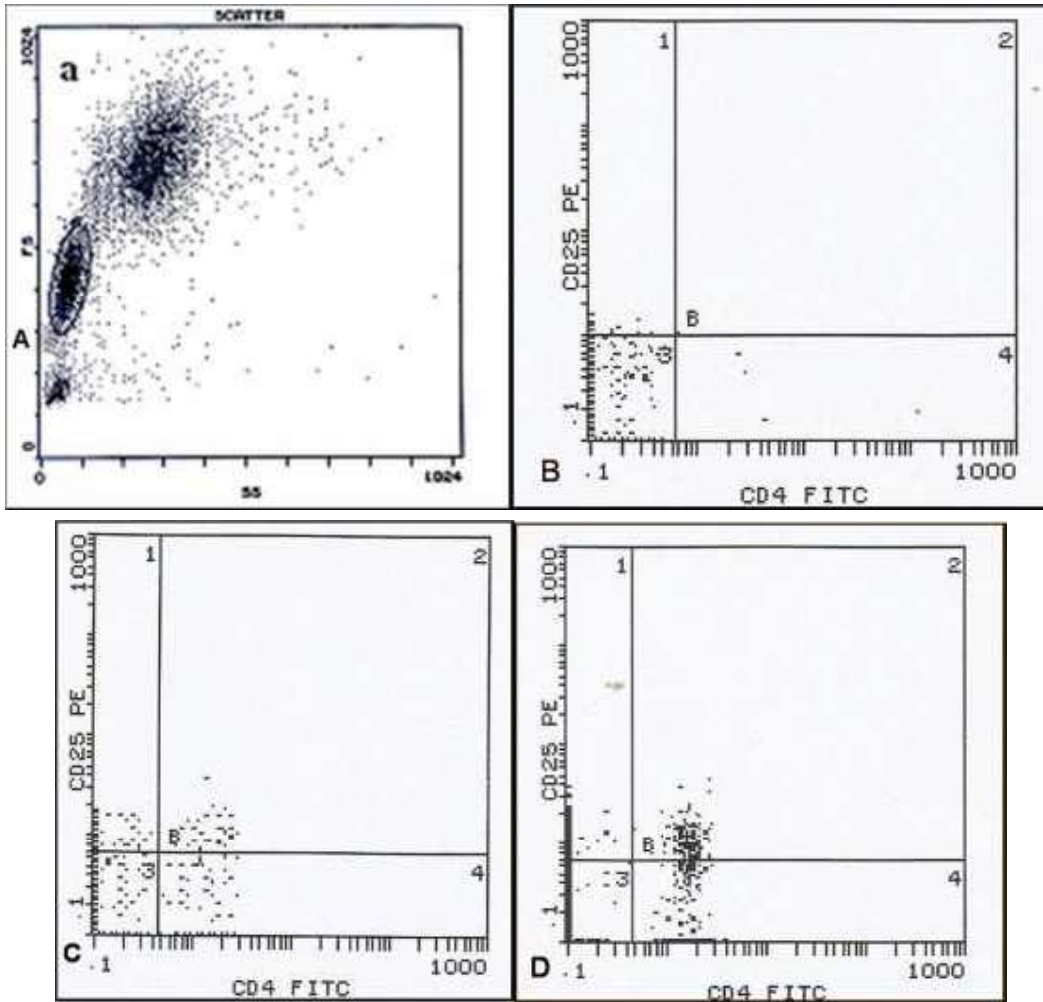


Fig 2: Flow cytometric analysis of circulating Treg cells subset stained for expression of CD4+CD25+. (A) A histogram demonstrating the lymphoblast region gated using forward and wide scatter. (B) A histogram of horizontal and vertical lines showing negative threshold (auto-fluorescence background) for CD4⁺ and CD25⁺ set at 0.5% using unstained cells. (C) Horizontal and vertical lines mark fluorescence intensity higher than background observed with FITC- and PE-conjugated anti CD4⁺ and anti-CD25⁺; respectively, denoting the dual expression of both markers of circulating lymphocytes in responders group. (D) Horizontal and vertical lines mark intense fluorescence observed with FITC- and PE-conjugated anti-CD4⁺ and anti-CD25⁺; respectively, denoting up-regulation of both markers expression on circulating lymphocytes in non-responders

Explanations of figures

Fig 1: Expression percentage of Treg cells subset (%) before and during antiviral treatment in both groups. * $p < 0.001$ significant versus non-responders.

Fig. 3: Expression of FOXP-3 (*Fold Change*) before and during antiviral treatment in both groups. * $p < 0.001$ significant versus responders group.

Fig. 4: Levels of IL-10 (*pg/ml*) before and during antiviral treatment in both groups. * $p < 0.001$ significant versus non-responders.

Fig. 5: Levels of TGF- β (*pg/ml*) before and during antiviral treatment in both groups. * $p < 0.001$ significant versus non-responders.

Fig. 6: Levels of CXCL-9 (*pg/ml*) before and during antiviral treatment in both groups. * $p < 0.001$ significant versus non-responders.

Fig. 7: Levels of CXCL-10 (*pg/ml*) before and during antiviral treatment in both groups. * $p < 0.01$ significant versus non-responders.

