ORIGINAL ARTICLE

A prospective study on Foxp3$^{+}$CD25$^{\text{high}}$CD4$^{+}$ regulatory T cells in chronic hepatitis C infected patients undergoing direct-acting antiviral combination therapy

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Key words:
Chronic HCV, Direct-acting antivirals, flow cytometry, Regulatory T cells

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ABSTRACT

Background: Immunopathology is responsible for clinical sequelae of chronic HCV infection, FoxP3$^{+}$CD25$^{\text{high}}$CD4$^{+}$ regulatory T cells (Tregs) are the master regulators of several immune functions and monitoring their dynamics during HCV infection and after viral clearance is of great importance. Objective: to investigate alterations in FoxP3$^{+}$CD25$^{\text{high}}$CD4$^{+}$Treg cells frequency and their FoxP3 expression level in chronic HCV infected Egyptian patients undergoing interferon-free direct acting antiviral (DAA) therapy in comparison to healthy controls. Methodology: Twenty chronic HCV patients undergoing sofosbuvir/daclatasvir combination therapy were included, circulating FoxP3$^{+}$CD25$^{\text{high}}$CD4$^{+}$ Treg cells frequency and their FoxP3 expression were assessed by flow cytometry before and at 2 time points after completing the treatment course, correlated with clinical and radiological data at enrollment and compared to those of 20 healthy anti-HCV negative blood donors. Results: Circulating FoxP3$^{+}$CD25$^{\text{high}}$CD4$^{+}$ Treg cells frequency and their FoxP3 expression were significantly elevated in chronic HCV patients before treatment with no correlation to virus load, ALT level or fibrosis grade. Treg parameters falls significantly after sofosbuvir/daclatasvir therapy. Treg frequency remained significantly higher than controls 6 months after treatment. Conclusion: DAA therapy was effective at reducing Treg cells frequency and FoxP3 expression but the persistent higher level than normal after 6 months of viral eradication should be further investigated to find out when they will normalize and their influence on the future course of the disease.

INTRODUCTION

Regulatory T cells (Treg cells) are a subpopulation of T cells first described by Sakaguchi and coworkers two decades ago and gained the reputation as “The Master Switch” in immune modulation, homeostasis and maintenance of self-tolerance. This is due to their pronounced ability to suppress many cell types; effector T cells, B cells, natural killer and dendritic cells.

Forkhead box P3 (FoxP3) $^{+}$ Treg cells is the major subset of CD4$^{+}$ Treg family that constitutively express the interleukin-2 (IL-2) receptor α chain (CD25), the transcription factor Foxp3 and the inhibitory receptor cytotoxic T Lymphocyte Antigen 4 (CTLA4). They also express low levels of the IL-7 receptor (CD127) $^{4}$. They have been linked to multiple disorders especially autoimmunity, allergy and some cancers $^{5,6}$. In addition, their role in immunopathogenesis of some infectious diseases has been noted $^{7,8}$. According to the site of their differentiation Foxp3$^{+}$ Treg cells are either thymus-derived Tregs (tTregs) develop in the thymus during the selection process and peripherally derived Tregs (pTregs) that develop in the periphery from naive T cells as a result of antigenic stimulation under the influence of permissive environmental cues.

FOXP3 is the key regulator for the development and suppressive function of Tregs as it is a potent repressor of IL-2 production, but enhances the expression of IL-2 receptor (CD25) and of the Treg marker CTLA4 $^{10}$. It is also an inducer of the anti-inflammatory cytokine IL-10 in a process involving the contribution of the transcription factor STAT3 $^{11}$. Mutations impairing Foxp3 expression lead to the Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome $^{12,13,14}$. A cardinal feature of HCV infection is high tendency of progression to chronicity with low rate of spontaneous viral clearance that doesn’t exceed 20% which always occurs in the acute phase. Different fates of HCV acute infection are highly dependent on the type of CD4$^{+}$ T cells response; a potent CD4$^{+}$ T cell response is important for viral elimination, while a weak or absent CD4$^{+}$ T cells response was detected in cases who developed chronic HCV infection. Factors proposed to result in this inadequate CD4$^{+}$ T cell response and progression to chronicity include;
dendritic cells dysfunction leading to inadequate antigen presentation and co-stimulation, viral mutational changes escaping T cell recognition, high viral replication rate overwhelming host T cell response and Treg suppression which is mediated either by a contact dependent mechanism via cell surface markers or by secretion of cytokines as IL-10, transforming growth factor β, IL-8 and IL-35. Chronic HCV infection, however associated with a moderate inflammatory response, still progresses to hepatic fibrosis, cirrhosis and hepatocellular carcinoma over time and the role of Treg cells in activation of fibrogenesis and their association with hepatocellular carcinoma had been reported.

The last few years had witnessed a substantial shift in chronic HCV treatment by the discovery and approval of 4 HCV DAA (Direct-acting Antiviral Agents) classes that replaced the conventional pegylated interferon (Peg-IFN) based therapy with high success rates in terms of virus elimination and achievement of sustained virological response (SVR) with negligible side effects. They are known to act directly without affecting the immune system. However, resetting of the immune response to the pre-infection state and halting the progression of HCV-induced immunopathology after virus elimination is an important determinant of the future course of the disease and long term outcomes of therapy. Since multiple immune functions are modified by Treg cells, it is important to know their dynamics during HCV infection and after viral clearance. Data from previous studies on IFN-based therapy show controversy regarding long persistence of Tregs in patients’ livers and blood even after successful clearance. Data from previous studies on IFN-based therapy show controversy regarding long persistence of Tregs in patients’ livers and blood even after successful clearance. Data from previous studies on IFN-based therapy show controversy regarding long persistence of Tregs in patients’ livers and blood even after successful clearance.

This study was conducted to investigate alterations in FoxP3+CD25highCD4+Treg cells frequency and their FoxP3 expression level in chronic HCV Egyptian patients undergoing IFN-free DAA therapy in comparison to healthy controls.

**METHODOLOGY**

**Patients and setting:**

This study was conducted in Medical Microbiology and Immunology and Tropical Medicine Departments, and the Flow Cytometry Unit, Faculty of Medicine, Zagazig University, during period from January till October 2018. The study was approved by the Ethical Committee of Faculty of Medicine, Zagazig University. All subjects had given a written informed consent before participation in the study.

Chronic HCV patients that were eligible for an IFN-free all-oral DAA therapy in the form of a 12 weeks course of sofosbuvir/daclatasvir combination (400/60 mg/d respectively) received according to the 'National Committee for Control of Viral Hepatitis' (NCCVH) HCV treatment program were recruited for this study. The included patients had been anti-hepatitis C seropositive for at least 6 months and had detectable serum HCV RNA levels at the time of enrollment. Patients with previous IFN treatment failure, incomplete treatment course, malignant neoplasm, decompensated liver disease, autoimmune disease, HBV or HIV coinfection were excluded. Matched healthy, anti-HCV negative, blood donors were included as controls.

The severity of liver disease was assessed at the time of enrollment both biochemically - liver function tests (ALT, AST, total and direct bilirubin, serum albumin and INR) – and radiologically using Fibroscan (Echosens, France) for assessment of liver stiffness in kipolascales (KPa) and fibrosis grading as follows: F0=0-2.9 KPa; F1= 3-5.9 KPa; F2=6-8.9KPa; F3=9-16.9 KPa; and F4=17-75 KPa.

HCV RNA detection and quantification was determined before starting DAA therapy, at end of treatment course and 12 weeks after (for SVR), using Artus® HCV RG RT-PCR Kit on Rotor-Gene Q real time cycler (Qiagen, Hilden, Germany) as per manufacturer’s instructions.

**Treg cells workup:**

EDTA anti-coagulated blood was collected from each patient at times of assessment. [at baseline, i.e. before starting DAA course (BL), at the end of treatment (ET) and 3 months or more after achieving SVR i.e. after 6 months of follow up after treatment i.e., end of follow up (EF)] and kept at 4°C. Treg cells frequency (as % of CD4+Tcells) was determined by flow cytometric immunophenotyping and the level of Foxp3 expression per cell was determined by calculating the relative fluorescent intensity (RFI) as follows:

Blood samples were processed within 24 hours of collection. Briefly, 100 µL blood were mixed with 10 µL of each of fluorescein isothiocyanate (FITC) conjugated anti-Human CD4 and phycoerythrin (PE) conjugated anti-Human CD25 (Affymetrix, eBioscience, Inc., USA) in the test tube, and with PE and FITC isotype controls (Affymetrix) in the control tube. Both tubes were incubated for 20 minutes in the dark at the room temperature. Two mL of fluorescence activated cell sorting (FACS) lysing solution (Becton Dickinson (BD), USA) were added and incubated for 10 minutes in the dark at the room temperature. The cell pellet was washed twice with 2 mL phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich Chemie Gmbh). For intracellular staining, 1 mL of fixation/permeabilization solution (Affymetrix) was added to the cell pellet and incubated for 30 minutes at 4°C. The cell pellet was washed twice in 2 mL of permeabilization buffer. Ten µL of allophycocyanin (APC) conjugated anti-Human FoxP3 and APC isotype control (Affymetrix) were added to the cell pellet in the test and control tubes, respectively and incubated in the
dark at the room temperature for 30 minutes. After washing twice in 2 mL of permeabilization buffer, the cells were suspended in 200 μL PBS for analysis by the flowcytometer (FACSCalibur, BD) using CELL Quest TM software.

Gating was done on lymphocytes using side scatter (SSC) and forward scatter (FSC) strategy. CD4+ T cells expressing high levels of CD25 were further gated and analyzed for FoxP3. FoxP3 Tregs were defined by co-expression of CD4, CD25<sup>high</sup> and FoxP3 (Figure 1). Treg cells frequency is the percentage of FoxP3<sup>+</sup>CD25<sup>high</sup>CD4<sup>+</sup>T cells among total CD4<sup>+</sup>T cells.

FoxP3 expression level was determined by calculating the Relative Fluorescence Intensity (RFI) of FoxP3 using the following formula: mean fluorescence intensity (MFI)/MFI with isotype control antibody.<sup>24</sup>

**RESULTS**

Twenty chronic HCV patients that completed a 12 weeks coarse of Sofosbuvir/daclatasvir combination, showed an end of treatment response and completed a follow up period of 6 more months were included in the final analysis. Their characters at enrollment (baseline, BL) in comparison to 20 anti-HCV negative healthy blood donors are presented in table 1. They showed a highly significant increase in Treg cells frequency in comparison to healthy controls.

**Table 1: Baseline characteristics of studied subjects**

<table>
<thead>
<tr>
<th>Character</th>
<th>Cases (n=20)</th>
<th>Control (n=20)</th>
<th>P(t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Mean ±SD</td>
<td></td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>(min-max)</td>
<td>50±11</td>
<td>48.6±11.4</td>
</tr>
<tr>
<td>Gender No (%)</td>
<td>Female</td>
<td>7(35)</td>
<td>5(25)</td>
</tr>
<tr>
<td></td>
<td>male</td>
<td>13(65)</td>
<td>15(75)</td>
</tr>
<tr>
<td>Liver fibrosis</td>
<td>F0</td>
<td>5(25)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>grade</td>
<td>F1</td>
<td>6(30)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>5(25)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>4(20)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>0(0)</td>
<td></td>
</tr>
<tr>
<td>Viral Load</td>
<td>Mean ±SD</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(min-max)</td>
<td>(1.34±1.68) x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Not applicable</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24, 827 - 5.7x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td>Mean ±SD</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(min-max)</td>
<td>157±136</td>
<td>Not measured</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32-548</td>
<td></td>
</tr>
<tr>
<td>Treg (BL)</td>
<td>Mean ±SD</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>(min-max)</td>
<td>5.05±1.14</td>
<td>(HS)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.2-7</td>
<td>2.6±0.96</td>
</tr>
<tr>
<td>FOXP3 RFI (BL)</td>
<td>Mean ±SD</td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>(min-max)</td>
<td>19±6.2</td>
<td>18±4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11-31</td>
<td>10-25</td>
</tr>
</tbody>
</table>

HS= highly significant <sup>1</sup> Chi-square test

Correlation between each of Treg cells % and FoxP3 RFI before treatment (BL) and, HCV viral load (an indicator of HCV replication), ALT level (an indicator of liver damage and hepatocyte death) and fibrosis grade didn’t show statistically significant results. (Table 2)
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Table 2: Correlation between Treg% and Foxp3 expression at baseline (BL) and each of (viral load, ALT, fibrosis grade).

<table>
<thead>
<tr>
<th>Character</th>
<th>Treg% (BL)</th>
<th>Foxp3 RFI (BL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(r)</td>
<td>(r)</td>
</tr>
<tr>
<td>Foxp3 RFI (BL)</td>
<td>-0.18</td>
<td>0.46</td>
</tr>
<tr>
<td>Viral Load</td>
<td>0.16</td>
<td>0.51</td>
</tr>
<tr>
<td>ALT</td>
<td>0.06</td>
<td>-0.097</td>
</tr>
<tr>
<td>Fibrosis Grade</td>
<td>-0.22</td>
<td>0.36</td>
</tr>
</tbody>
</table>

Mean Treg cells frequency and Foxp3 RFI were further assessed at the end of treatment coarse (ET) and 6 or more months later (EF) and a statistically significant decrease in both parameters at end of treatment and end of follow up relative to pretreatment measurements was observed. Also, LSD between pairs found that mean Treg % and Foxp3 RFI at baseline were statistically higher than those at end of treatment (p=0.001 and 0.026 respectively) and at end of follow up (p=0.001 and 0.002), also measurements at end of treatment was statistically higher than those at follow up (p=0.004 and 0.001). (Table 3 & Figure 2).

Table 3: comparison between Treg % (Mean ±SD) and Foxp3 expression (Mean ±SD) at different time points throughout follow up

<table>
<thead>
<tr>
<th>Character</th>
<th>Cases</th>
<th>Repeated measure ANOVA</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BL</td>
<td>ET</td>
<td>EF</td>
</tr>
<tr>
<td>Treg %</td>
<td>Mean ±SD</td>
<td>5.05±1.14</td>
<td>3.93±1</td>
</tr>
<tr>
<td></td>
<td>(min-max)</td>
<td>3.2-7</td>
<td>2.4-5.8</td>
</tr>
<tr>
<td>Foxp3 RFI</td>
<td>Mean ±SD</td>
<td>19.1±6.2</td>
<td>17.25±4.3</td>
</tr>
<tr>
<td></td>
<td>(min-max)</td>
<td>11-31</td>
<td>11-28</td>
</tr>
</tbody>
</table>

BL=baseline  ET=end of treatment  EF= end of follow-up

Despite the significant drop in Treg frequency at end of follow up, they remained significantly higher than normal subjects (p=0.01). On the contrary, Foxp3 RFI (EF) was lower than normal but the difference was not statistically significant (p=0.054), (Figure 2).

Fig. 2: Treg% and foxP3 expression in patients (at BL, ET, EF) and control

DISCUSSION

The progression of acute HCV infection into a chronic one entails a failure of the host’s HCV-specific immunity. Cellular immunity is the principle player in virus elimination while the role of antibodies is not clear. Several mechanisms of CD4+ T cell failure have been proposed and the contribution of Treg cells was established.³

Treg cells thus limit the extent of immune-mediated liver damage associated with an excessive inflammatory process supposed to be maintained by persistent HCV infection and they have long been thought to limit hepatic fibrosis and subsequent cirrhosis resulting from such injury ²⁵. Nevertheless, the majority of chronic HCV carriers still possess the tendency to develop liver fibrosis and cirrhosis and ultimately hepatocellular carcinoma ²⁶. There is an increasing body of evidence on the role of Treg cells in chronic HCV-induced liver fibrosis either via local production of IL-8 that stimulate hepatic stellate cells promoting fibrogenesis ¹⁶, or by inhibiting fibrosis resolution by disturbing the balance between matrix metalloproteinases (MMPs) – collagenases and gelatinases responsible for fibrous tissue degradation-and tissue inhibitors of matrix metalloproteinase (TIMPs) by suppressing (MMPs) secretion from Kupffer cells.²⁷

In addition, the role of Treg cells in suppressing effector cells of immune surveillance and thus the development of hepatocellular carcinoma has been extensively elucidated.²⁶,²⁸

Treg cells are thus important determinants of the natural course of liver disease accompanying chronic
HCV infection and studying Treg cells dynamics throughout the infectious process and in the post-treatment period is of paramount importance.

Chronic HCV patients in our study showed higher levels of circulating FoxP3+CD25high CD4+Treg cells frequency and FoxP3 expression compared to control group. Unexpectedly, this elevation was not correlated to viral load, ALT levels or fibrosis grade. Tsang and colleagues, in accordance with our results, reported an elevated Treg frequency in blood of chronic HCV patients with no correlation to liver function. Similarily, Fouad et al in a recent Egyptian study showed that chronic HCV patients exhibited higher levels of circulating Tregs and FoxP3 expression in comparison to healthy control group which decreased significantly after peg-IFN ribavirin therapy. Another Egyptian study found no correlation between circulating Tregs and any laboratory parameters including liver functions and viral load. The same finding was reported by other studies.

Classin et al. reported similar frequencies of CD4+FoxP3+ Tregs in blood of HCV patients and controls but patients showed higher intrahepatic CD4+FoxP3+ Treg frequency compared to controls that was inversely correlated to the extent of fibrosis but was not correlated to virus load or ALT level; one drawback of this study is that it didn’t consider CD25 expression in Treg cells identification. During the course of chronic HCV infection, Treg cells number and function as well as Foxp3 expression are fine-tuned relative to effector conventional T cells in infected livers so as to minimize immune-mediated injury without complete loss of immune responses that limit viral dissemination. Galectin (Gal)-9/T cell immunoglobulin and mucin domain 3 (Tim-3) and programmed cell death receptor-1 (PD-1)/its ligand (PD-L1) interactions were proposed as possible mechanisms.

All patients in our study responded to sofosbuvir/daclatasvir combination therapy and showed undetectable serum viral RNA at end of treatment and 3 months later (SVR12). Treg cells frequency showed significant drop when assessed at end of treatment and 6 months or more later where Treg frequency, despite such significant reduction, remained significantly higher than that of the control group. (3.6±1.1 vs 2.6±0.96, p=0.01). Childs et al reported a significant decrease in Treg cells frequency in responders to DAA therapy (sofosbuvir/daclatasvir or ledipasvir).

Langhans et al. recently studied the levels of Tregs and the expression of Treg cells activation markers at different time points during and after successful treatment with sofosbuvir/IFN combination and with IFN-free regimens. They reported an increased frequency of Tregs in chronic HCV infection before treatment that did not normalize in either treatment protocols. Moreover, Tregs activation status remained high even 1 year after successful treatment. Accordingly, they postulated that all DAA including regimens, with or without IFN, failed to reset Treg frequency and activity to normal after viral elimination which may impair restoration of other immune functions, including cancer immune-surveillance.

Nevertheless, The Treg frequency does not necessarily follow Treg function. We tried to predict Treg cell suppressor function alteration by examining FoxP3 expression at different time points after successful DAA therapy and our results unveiled a significant drop of the mean FoxP3 expression on sequential assessment which fall below the mean FoxP3 expression in normal subjects 6 months after treatment but the difference was not statistically significant (15.4±3.8 vs 18±4.3, p= 0.054). This was in line with other reports suggesting immune reconstitution after DAA therapy including NK cell activity and HCV-specific CD8+ T-cell function.

CONCLUSION

In light of the above mentioned results, we recommend testing for Treg cells function alterations after DAA therapy by co-culture assays i.e. culturing separated Treg cells with autologous effector CD4+ conventional T cells and determining the percentage inhibition of CD4+ conventional T cell proliferation and/or IFN gamma production after in-vitro stimulation. In addition, other Treg cells markers, contact-acting molecules and cytokines are better tested, either by flow cytometry or RT-PCR targeting their mRNAs, to monitor dynamic changes in their expression in response to treatment. Finally, Intrahepatic Treg cells remain the best representatives of Treg cell dynamics in response to HCV infection and DAA-induced clearance but ethical and medical considerations were constraints on obtaining liver biopsies for this research.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.

This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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