



Original Research Article

The Role of T Regulatory Cells and Pro-and Anti-inflammatory Cytokines in Viral Persistence and Clinical Outcome in HCV-Infected Patients

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ABSTRACT

The immuno pathogenesis of chronic HCV infection is a matter of great controversy. We aimed to investigate the distributional profiles of T-regulatory cells (Tregs) and the balance between pro-inflammatory (interferon [IFN] γ and interleukin-[IL] 2) and anti-inflammatory (transforming growth factor [TGF] β 1 and IL-10) cytokines among chronic HCV- infected patients in comparison to asymptomatic HCV patients and normal healthy controls. Ninety individuals (50 viremic HCV with elevated liver enzymes, 20 asymptomatic HCV patients with normal liver enzymes and 20 healthy blood donors as control) were investigated. Levels of Tregs subpopulation (CD4 + CD25 +, CD4 + FoxP3 + and CD4 + CD25 + FoxP3 +) were analyzed using 4-colour flow cytometry. In addition, IL-10, TGF- β 1, IFN- γ , and IL-2 were measured in serum using ELISA. A significantly higher proportion of CD4 + CD25 + cells were found in those with chronic HCV infection compared to asymptomatic and normal controls). Similar results were obtained when comparing CD4 + FoxP3 + Treg subset among the chronic HCV group and the other two groups with p values equaling 0.009 and 0.04 respectively, similarly, with CD4 +CD25 +FoxP3 + significant differences were obtained between chronic and asymptomatic (p <0.004) and between chronic and control group (p <0.008). A positive correlation was found between CD4 +CD25 +FoxP3 +and HCV RNA titer (R=0.601, P>0.005), meanwhile, no relation between them and degree of fibrosis. Comparison of the cytokines profile of chronic HCV group with the asymptomatic one revealed significantly higher serum levels of IL-2, IL-10, TGF- β and IFN- γ . Patients with chronic viral hepatitis display increased numbers of Tregs compared to other groups which highlight the importance of Tregs in immunopathogenesis of chronic HCV infection.

Keywords

T regulatory cells,
Hepatitis C virus,
CD4 +
CD25 +
FoxP3 +

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver disease, with around 130

million people infected worldwide (1). Egypt has a very high prevalence of HCV

and a high morbidity and mortality from chronic liver disease, cirrhosis, and hepatocellular carcinoma (1-3). Approximately 20% of Egyptian blood donors are anti-HCV positive. The strong homogeneity of HCV subtypes found in Egypt (mostly 4a) suggests an epidemic spread of HCV (2, 3).

HCV establishes a persistent liver infection in the majority (~80%) of cases, resulting in a fibrotic liver disease which progresses over several decades. Liver cirrhosis and hepatocellular carcinoma occur in ~25% of these chronic HCV cases, making HCV infection the main cause of chronic liver disease and the single largest indication for liver transplantation (4).

The mechanisms of HCV persistence are not well known. However, it has been postulated that HCV may evade the immune response or impart a specific tolerance to it to ensure its survival through mechanisms such as, but not exclusive to, viral escape (5), T-cell anergy, and induction of regulatory T cells (Tregs) (6).

In the past 10 years, there has been a steadily increasing interest in Tregs. Several subtypes of Tregs have been defined, each with a distinct phenotype, cytokine-production profile and mechanism of action for suppressing immune responses. Some of these Tregs are CD8⁺ (7) others are CD4⁺. In the CD4⁺ Treg-cell compartment, detailed analysis led to identification of the interleukin (IL)-10-producing Treg- cell type 1 (Tr1)(8), transforming growth factor (TGF) - β secreting T-helper cell type 3 (Th3) (9) and a subpopulation of 'naturally occurring' Tregs that exert their suppressive function in vitro in a contact-dependent manner and preferentially express high levels of CD25 and the forkhead and winged-helix family transcription forkhead box P3 (FOXP3) Tregs.

Accumulating evidence indicates that patients with chronic viral hepatitis display increased numbers of Tregs (both natural and inducible) in peripheral blood (10, 11) or liver (12-14), which in turn exert a suppressive function against specific HCV-effector clones in vitro (10-13). Thus, it has been suggested that the expansion of Tregs during viral hepatitis may contribute to an inadequate immune response, causing persistent viral infection. However, the precise role of Tregs in the pathogenesis of chronic hepatitis is the subject of intense debate, since it has been demonstrated that Tregs suppress the function and the expansion of virus-specific T effector cells ex vivo, irrespective of the patients having chronic or resolved virus infection (14, 15).

Recently Langhans et al. have suggested that T- cell immunoregulatory cytokines play a key role in both HCV viral persistence and liver inflammation (16). While some cytokines may exert a pro-inflammatory activity, such as interleukin (IL)-1 (α and β), interferon (IFN)- α , IL-8, tumor necrosis factor (TNF)- α and IL-2, which can prime T-cells towards a Th-1 type immunity (16), others have a predominantly anti-inflammatory activity, as is the case for IL-4, IL-6 and IL-10, which are involved in Th-2 immunity, and very recent research elucidates the importance of FoxP3⁺ Tregs in disease progression in the inflamed liver (17).

In this study, we aimed to investigate the distributional profiles of Tregs and the balance between pro-inflammatory (IFN- γ and IL -2) and anti-inflammatory (TGF β 1 and IL -10) cytokines in different stages of chronic HCV infection compared to normal control individuals.

Subjects and Methods

Study population

A descriptive study was conducted on individuals attending the Gastroenterology and Hepatology Unit at the Suez Canal University Hospital. The study population included a total of 90 individuals that were divided into 3 groups. The first group included 50 chronic HCV patients with the following inclusion criteria: (i) an age between 18-60 years, (ii) a confirmed HCV infection (by a third-generation ELISA), (iii) a positive qualitative and quantitative PCR for HCV-RNA, (iv) a 1.5-4.00 fold increase in serum alanine aminotransferase (ALT) and (v) a liver biopsy confirming chronic hepatitis, graded and staged according to Metavir with fibrosis or cirrhosis.

The second group included 20 HCV patients with the following inclusion criteria: (i) an age between 18-60 years, (ii) a confirmed HCV infection (by a third-generation ELISA), (iii) a negative qualitative and quantitative PCR for HCV-RNA, (iv) normal serum ALT. The third (control) group included 20 healthy blood donors with normal liver enzymes and negative for HCV antibodies. The last group was recruited from Suez Canal University Hospital Blood Bank. Ethical approval to perform the study was obtained from the ethics committee in the Faculty of Medicine, Suez Canal University and the management board of the hospital. All the included patients consented to the collection of specimens before the study was initiated.

Patients with any other hepatic diseases, previous immunosuppressive or interferon treatment, or concurrent malignant disease were excluded. Table 1 summarizes the main characteristics of the study population.

Sample and tissue collection: After obtaining a detailed history and clinical examination, five ml of peripheral blood (PB) were drawn from the patients and control subjects. Each blood sample was divided into two tubes, 2 ml in EDTA tube for immunophenotyping, and 3 ml in plain tube for enzymatic assay, cytokines assay and HCV load estimation. Flow cytometry was performed on the same day of the arrival of the specimens, while the serum from the other tube was separated and kept at -20 0C until the time for the test. Needle liver biopsies were obtained percutaneously from the patients with approved chronic HCV on a later session.

Clinical laboratory analysis: Serum transaminases levels were measured by using a Modular Analytics instrument (Roche, Diagnostics, Indianapolis, IN, USA). Quantification of HCV-RNA was measured with an Amplicor HCV Monitor (Roche Diagnostic Systems, Mannheim, Germany) according to the manufacturer's instructions at a lower detection limit of 50 IU/mL.

Histological evaluation: Biopsy specimens were taken from the chronic HCV group and were fixed in 10% formalin and sent to pathology laboratory. The specimens were graded and staged according to Metavir scoring system (22). The grade gives an indication of the activity or amount of inflammation and the stage represents the amount of fibrosis or scarring. The grade is assigned a number based on the degree of inflammation, which is usually scored from 0-4 with 0 being no activity and 3 or 4 considered severe activity. The fibrosis score is also assigned a number from 0-4: 0 = no scarring, 1=minimal scarring, 2 = scarring has occurred and extends outside the areas in the liver that contains blood vessels, 3=bridging fibrosis is spreading and

connecting to other areas that contain fibrosis, 4=cirrhosis or advanced scarring of the liver.

Flow cytometry: Two ml of whole blood were collected in EDTA vacutainer tubes. T-reg subsets were identified from CD4+ T-cells according to their expression of CD25 and Foxp3. The following markers were used: anti-CD3 FITC conjugated monoclonal antibodies, anti-CD4 Percp conjugated monoclonal antibodies, anti-FOXP3 PE conjugated monoclonal antibodies, and anti CD25 APC conjugated monoclonal antibodies (Becton Dickinson (BD), Franklin Lakes, NJ, USA). Surface staining was done first, for CD3, CD4, and CD25 following the manufacturer's instructions. Intracellular staining for FOXP3 using the FOXP3 BD kit, followed the surface staining.

Appropriate isotype controls were used. After staining, data acquisition by Accuri C6 (BD Bio-sciences, San José, CA USA) and analysis by FlowJo software v.10.0.7 (TreeStar CA, USA) was performed. Lymphocytes were gated according to their light scatter properties (SSC vs FSC) followed by gating of CD3+CD4+ cells. T-reg subtypes were then identified as follows: CD4+CD25+Foxp3-, CD4+CD25+FOXP3+ and CD4+CD25-FOXP3+. Percentages of CD4+ Tregs subpopulation are given as the frequency (%) of CD4+ Tregs.

Cytokine assay: A quantitative measurement of serum cytokines was performed for the cases and blood donor controls using a commercially available enzyme linked immunosorbent assay, according to the manufacturer's instructions. The following cytokines were assessed: IL-2 IL-10, IFN- γ (R&D Systems Minneapolis, USA) and TGF- β (DRG Instruments GmbH, Germany).

Statistical analysis

For descriptive analyses, the means and standard deviations, or the medians med and interquartile ranges, are reported. Comparisons of proportions between the cases and controls were assessed by Chi-square test or Fisher's exact test, when appropriate. Continuous and ordinal data were compared by MannWhitney rank sum test.

The Spearman's rank correlation coefficient was used to assess correlations between the continuous variables. A significance level of 0.05 was used and all of the p values were two-sided. All of the analyses were conducted in SPSS version 12 (2002).

Result and Discussion

Characteristics of the cases and controls

A total of 90 individuals divided into three groups were included in this study. The first group included 50 HCV patients with chronic hepatitis, the second group included 20 HCV patients assigned as asymptomatic HCV-infected patients and the third one included 20 healthy blood donors serving as control. The demographic, biochemical, and histological characteristics of the the three study groups were shown in Table I. Thirty two (64%) chronic HCV patients were males and 18 (36%) were females, with a median age of 44.5 years (range 22-58). The cases, asymptomatic and control groups did not differ significantly with regard to gender or age.

The median aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were 54 IU/L (range 18-87) and 58.5 IU/L (range 22-122), respectively with significant difference between chronic HCV group and asymptomatic one ($p < 0.05$) and

chronic HCV group and control one ($p < 0.05$) (Table 1). No significant difference was obtained between the asymptomatic HCV patients and the control groups. The frequency of different Treg cell subsets defined by CD4+ CD25+, CD4+ FoxP3+, and CD4+ CD25+ FoxP3+ respectively.

The frequency of CD4 + CD25 + cells in HCV infection was measured in chronic infection, asymptomatic infection, and normal controls by flow cytometry. A significantly higher proportion of CD4 + CD25 +, cells were found in those with chronic infection (median, 8.5% range 1.06-13; mean, 8.08%; \pm 3.18) when compared with asymptomatic (median 3.2%, range 1.9-5.3, mean 3.49 \pm 1.08), $P = 0.001$) and normal controls (median 4%, range 2.4-9, mean 4.62% \pm 1.56% $P = 0.02$), suggesting a relationship with viral persistence (Table 2; Fig. 1a). Additionally, no significant difference in the frequency of CD4 + CD25 + cells was found in the asymptomatic group when compared with normal controls (Table 2).

When comparing CD4 + FoxP3 + Treg subsets among the 3 study groups, similar results were obtained with significance difference between chronic HCV group compared to the asymptomatic and control groups with p values equaling 0.009 and 0.04 respectively (Fig. 1b).

Similarly, when comparing CD4 + CD25 + FoxP3 + which were considered by many authors as true Treg cells, significant differences were obtained between chronic and asymptomatic ($p < 0.004$) and between chronic and control group ($p < 0.008$). Meanwhile no significant difference was observed when comparing asymptomatic group with the control one (Fig. 1c and Fig. 2).

Analysis of chronic infected patients ($n = 50$) showed a positive correlation between CD4 + CD25 + FoxP3 + and HCV RNA titer ($R = 0.601$, $P > 0.005$) (Table 3). Meanwhile, there was no significant difference between CD4 + CD25 + FoxP3 + frequency and degree of fibrosis ($P = 0.476$) (table 4).

Cytokine patterns in the three study groups

When comparing the cytokine pattern of the chronic HCV group (group 1) with the asymptomatic one (group 2), the chronic HCV group had significantly higher serum levels of IL-2 (30.28 ± 3.26 vs. 8.55 ± 2.82 pg/mL; $p < 0.01$), IL-10 (41.28 ± 8.19 vs. 8.6 ± 2.54 pg/mL; $p < 0.01$), TGF- β (780.8 ± 247.77 vs. 168 ± 136.67 pg/mL; $p < 0.01$) and IFN- γ (40.9 ± 8.78 vs. 7.1 ± 2.36 pg/mL; $p < 0.01$) (table 5). Similar significant results were obtained when comparing the chronic HCV group with the normal control group (table 5). On the other hand IL-2 and IL-10 showed no difference between the asymptomatic and the control groups while TGF- β and IFN- γ showed significant one ($p = 0.03$ for TGF- β and $p = 0.0001$ for IFN- γ).

Among the chronic HCV patients a positive correlation was found between IL-2, IL-10 and TGF- β and viral load. On the contrary, IFN- γ showed a negative correlation ($R = -0.581$) with viral load (Table 3). No difference could be demonstrated between the tested cytokines and the degree of fibrosis (Table 4).

This study was designed to find association between Treg subsets as well as pro-inflammatory and anti-inflammatory cytokines profile and development of chronic HCV infection. For this purpose, we measured CD4 + CD25 +, CD4 + FoxP3 +, CD4 + CD25 + FoxP3 + in blood from two groups of patients (chronic HCV and

asymptomatic HCV patients) as well as from a group of healthy uninfected blood donor individuals. Also, we measured the serum level of IL-2, IFN- γ (pro-inflammatory cytokines), IL-10 and TGF- β (anti-inflammatory cytokines) in the serum of the three groups.

In this study, a significantly higher proportion of CD4 + CD25 +, CD4 + FoxP3 + and CD4 + CD25 + FoxP3 + cells were found in those with chronic HCV infection group compared with the asymptomatic or the control group. Different groups have analyzed levels of Tregs in HCV infection (16- 23). Not in all, but in the majority of reports, an increased frequency of CD4+CD25+ Tregs in chronically HCV-infected patients has been found (21, 24, 25), supporting the contribution of Tregs to the impaired virus-specific T-cell responses in chronic HCV infection.

In some controversial results, no increase in the level of Tregs in chronically HCV infected patients was obtained (18,23), however, this result was not supported by Yoshizawa et al. (21). Several parameters may contribute to the discrepancy between the present data and those with no increase in the level of Tregs. These include differences in patient profiles, stage and the identification method of circulating Tregs.

CD4+CD25+ T cells seem to play a major role in the control of Th1-mediated immune responses, which are the main mediators for HCV disease resolution (26). Our results seem to be in agreement with this point of view as we demonstrated a positive correlation between Treg subsets and viral load (Table 4) and significant differences in ALT and AST levels between chronic HCV group and asymptomatic or control groups (Table 1).

Although we demonstrated significant difference in Treg subsets between chronic HCV group and the other two groups, this difference was insignificant between the asymptomatic group and the control one (data not shown). This finding could support the possibility stated by Cabrera et al. (27), that the steady-state level of regulatory CD4+CD25+ cells before infection may predetermine and/or predict whether individuals will clear or develop chronic infection.

We observed that the frequency of Tregs in chronic hepatitis was not related to the grade of inflammation (table 4). This could be attributed to the fact that most of the chronic HCV patients were with mild fibrosis (90%) while the rest (10%) were with severe one.

Cytokines are key mediators of inflammation, apoptosis, necrosis and fibrosis and they are actively involved in the regeneration process of liver tissue after injury. It has been hypothesised that successful treatment of hepatitis C depends on a complex balance between pro and anti-inflammatory responses (28-30). To observe the imbalance between T helper cell Th1 and Th2 cytokines in chronic HCV infection, we measured the cytokine levels of Th1 (IL-2 and IFN- γ) and Th2 (IL-10 and TGF- β) by the ELISA technique in the sera of 50 patients with chronic liver disease. In addition, 20 asymptomatic hepatitis C virus carriers and 20 healthy blood donors' subjects negative for hepatitis C virus markers served as controls.

In our study, a significantly elevated level of Th1 profile (IL-2 and IFN- γ) was demonstrated in the chronic HCV patients when compared to either the asymptomatic or the control (Table 5) group. This result is in agreement with the results of Missale et al. (31) and Cacciarelli et al. (32).

Table.1 The demographic, biochemical and histological characteristics of study population

	Chronic HCV N= 50	Asymptomatic HCV N= 20	Controls N= 20
	Mean \pm SD Median (range)	Mean \pm SD Median (range)	Mean \pm SD Median (range)
Age (years)	44.46 \pm 8.3 44.5(22-58)	45.95 \pm 6.35 45(35-58)	44.8 \pm 7.75 46(32-56)
Sex			
Male n (%)	32(64%)	13(65%)	10(50%)
Female n (%)	18(36%)	7(35%)	10(50%)
WBC (x10 ³)	5.5 \pm 1.31 5.3(3.6-9.1)	6.1 \pm 1.03 6.25(4.2-7.5)	5.52 \pm 1.04 5.4(3.9-7.7)
Albumin(mg/l)	4.25 \pm 0.49 4.2(3.4-5.3)	4.22 \pm 0.39 4.2(3.5-5)	4.22 \pm 0.36 4.1(3.6-5)
ALT (u/L) *†	57.18 \pm 21.89 58.5(22-122)	13.1 \pm 4.27 12(7-26)	13.35 \pm 3.83 13(8-19)
AST (u/L) *†	58.65 \pm 63.6 54(19-103)	16.85 \pm 6.55 15.5(8-30)	13.35 \pm 3.83 13(8-19)
Quantitative HCV - RNA(IU/mL x10 ³)	502.02 \pm 768.38 225(3.03-4000)	-ve	-ve
Histology			
Grading	2(0-4)	ND‡	ND
Staging	1(0-4)	ND	ND

*P value is significant comparing chronic HCV to asymptomatic group.

† P value is significant comparing chronic HCV to control group.

‡ Not done .

Table.2 Treg cell subsets in the three study groups

	Chronic HCV N=50	Asymptomatic N=20	Control N=20
	Mean \pm SD Median(Range)	Mean \pm SD Median(Range)	Mean \pm SD Median(Range)
Total CD4 T cells†‡	28.26 \pm 5.21 29 (18-42)	26.55 \pm 5.61 27(19-38)	20.15 \pm 4 19(15-29)
Fox P3*†‡	4.55 \pm 2.03 4(1.36-8)	3.09 \pm 1.26 2.75(1.4-6)	1.06 \pm 0.47 0.9(0.5-2.4)
CD25*†‡	8.08 \pm 3.18 8.5(1.06-13)	3.49 \pm 1.08 3.2(1.9-5.3)	4.62 \pm 1.56 4(2.4-9)
Fox P3CD 25+*†	0.631 \pm 0.309 0.58(0.07-1.3)	0.4 \pm 0.17 0.41(0.1-0.7)	0.38 \pm 0.2 0.32(0.1-0.8)

*P value is significant comparing chronic HCV to asymptomatic group.

† P value is significant comparing chronic HCV to control group.

‡ P value is significant comparing asymptomatic to control groups.

Table.3 Spearman correlation of HCV viral load with T-regulatory cells and cytokines profile

	HCV RNA	
	Spearman Rho	P value
CD4+CD25-Fox P3+	0.546	0*
CD4+CD25+foxP3-	0.662	0*
CD4+CD25+Fox P3+	0.601	0*
Total CD4+cells	-0.156	0.279
IL-2 (pg/mL)	0.437	0.002*
IL-10 (pg/mL)	0.857	0*
TGF-β (pg/mL)	0.611	0*
IFN-γ (pg/mL)	-0.581	0*

*P value is significant.

Table.4 Comparison of T-regulatory cells and cytokines profile between mild and severe fibrosis in the study cases group (viremic HCV+)

	Mild Fibrosis N=45	Severe Fibrosis N=5	
	Mean±SD Median(Range)	Mean±SD Median(Range)	P value
CD4+CD25-Fox P3+	4.69±2.03 (1.9-8)	2.2±1.46 1.9(0.5-6)	0.131
CD4+CD25+foxP3-	8.29±3.09 9(3-13)	4.29±1.91 3.9(1.06-11)	0.211
CD4+CD25+Fox P3+	0.65±0.31 0.58(0.07-1.3)	0.4±0.19 0.(0.1-0.8)	0.476
Total CD4+cells	28.29±5.34 29(18-42)	23.87±5.8 23(15-38)	0.897
IL-2 (pg/mL)	30.33±3.37 30(21-36)	10.31±7.2 8(3-33)	0.568
IL-10 (pg/mL)	41.51±8.26 40(28-57)	12.87±10.18 11(4-49)	0.604
TGF-β (pg/mL)	782.22±260.24 760(330-1250)	194.27±231.7 110(12-880)	0.884
IFN-γ (pg/mL)	40.58±8.77 39(28-57)	9.62±12.84 6(1-56)	0.4

Table.5 Cytokine levels in the three study groups

	Chronic HCV N=50	Asymptomatic N=20	Control N=20
Interleukin Level (Pg/mL)	Mean±SD Median(Range)	Mean±SD Median(Range)	Mean±SD Median(Range)
IL-2*†	30.28±3.26 30(21-36)	8.55±2.82 8.5(3-13)	7.2±2.21 7(4-11)
IL-10*†	41.28±8.19 40(28-57)	8.6±2.54 8.5(4-12)	10.55±3.52 11(4-16)
TGF-β*†‡	780.8±247.77 765(330-1250)	168±136.67 167.5(18-510)	77.1±49.07 65(12-190)
IFN-γ*†‡	40.9±8.78 39(28-57)	7.1±2.36 7.5(3-11)	3.6±2.35 3(1-8)

*P value is significant comparing chronic HCV to asymptomatic HCV groups.

† P value is significant comparing viremic to control groups.

‡ P value is significant comparing aviremic to control groups.

Figure.1 Boxplot showing comparison of the frequency of different Treg cell subsets defined by CD4+ CD25+, CD4+ FoxP3+, and CD4+ CD25+ FoxP3+ respectively

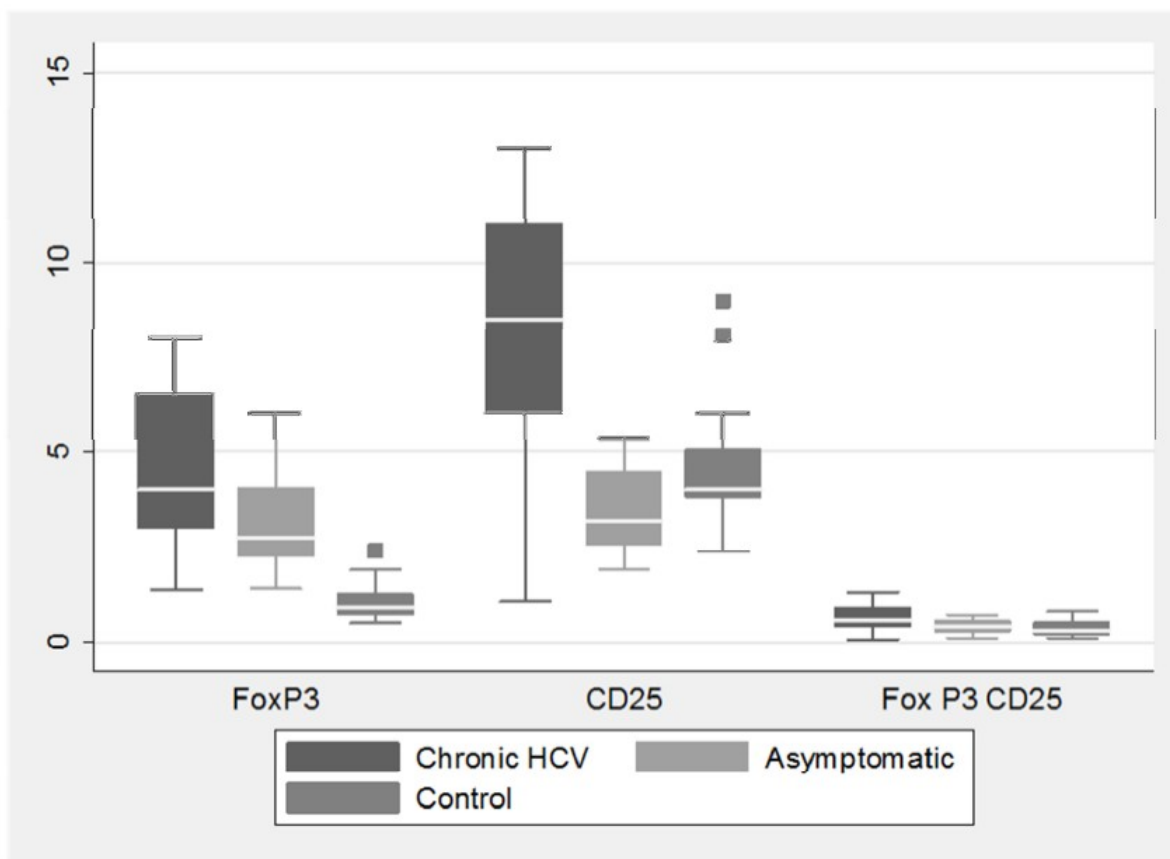


Figure.2 Treg in study groups

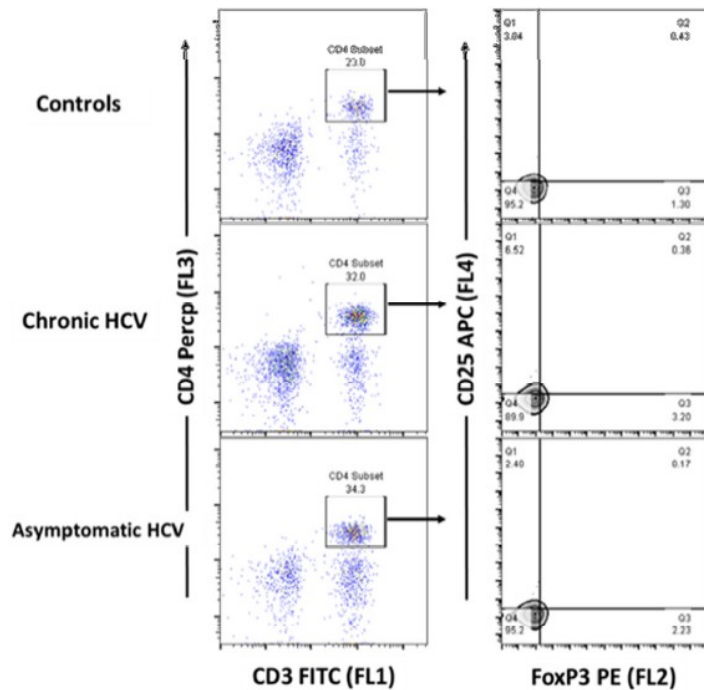


Figure 2. Treg in study groups. PB sample cells were surface stained using CD3 (FITC), CD4 (Percp), and CD25 (APC). Surface staining was followed by intracellular staining with Foxp3 (PE). Pseudocolor plots (left) show the gating and frequency of CD4⁺ T-cells from lymphocytes of the 3 study groups, i.e. Healthy Control (upper), Viremic (middle), and Aviremic (lower). Zebra plots (right) show the frequency of Treg on the Quadrants through the expression of Foxp3 and CD25 on the gated CD4⁺ T-cells.

However, Simsek and Kadayifci (33) found that serum IL-2 had no significant change and they attributed that to the presence of soluble receptor binding to IL-2. On the contrary, Urbani et al. demonstrated a Th1 profile in individuals with a favorable outcome and were associated with the production of elevated amounts of both IFN- γ and IL-2, while patients with a chronically evolving disease displayed an impaired production of Th1 cytokines.

Similarly, the Th2 cytokine response (IL-10 and TGF- β) was significantly higher in chronic HCV group in comparison to the asymptomatic or control groups. Higher serum concentrations of IL-10 were reported by one study (35) and disease progression was associated with high serum level of IL-10 by another (36). The positive correlation found in our study between IL-10 and HCV RNA level ($r=0.8$) (Table 3) supports the idea of the possible role of IL-10 in viral persistence. Also, one study supporting this

idea (19) has demonstrated a minority of Treg subsets displaying HCV specificity to IL-10 secretion which may play a regulatory role in promoting viral persistence. Such finding may provide a novel mechanism used by HCV to evade the immune system and have a significant implication in the arena of vaccine development.

IL-10 has a modulatory effect on hepatic fibrogenesis by downregulating the pro-inflammatory response, including the antigen-presenting cells and in a controlled study, IL-10 has also been shown to reduce liver damage (37, 38). Our results did not resolve any significant difference in IL-10 levels and degree of fibrosis (Table 4) as the majority of our cases (90%) were with mild fibrosis. T-cell secretion of TGF- β in response to HCV has been described for CD4⁺CD25⁺ Tregs in subjects with chronic HCV infection and an anti-inflammatory role for TGF- β during chronic HCV infection has been suggested (39).

In our study, we found significant difference in TGF- β levels between the chronic group and the other two groups separately (table 5). In agreement with our results, one study (40) demonstrated that TGF- β blockade significantly increased IFN- γ response to HCV, confirming the predominant involvement of TGF- β in this suppressive activity. Also, our study revealed a positive correlation between TGF- β and viral load ($r= 0.6$). This finding, supports the idea that TGF- β can diminish the possibility of the infected host eliminating the virus, thereby enhancing the likelihood of HCV persistence.

TGF- β is also a key cytokine driving liver fibrogenesis (41). It has been shown by one study that TGF- β inversely correlated not only with liver inflammation, but also with liver fibrosis progression and fibrogenic hepatic stellate cell (HSC) gene expression (17, 42). In our study, no significant difference between TGF- β levels and degree of fibrosis could be demonstrated, as most of the patients in our chronic HCV group were having mild degree of fibrosis.

The studied cytokines in our work showed that all of them were significantly different when comparing the chronic HCV group with the asymptomatic or the control group (Table 5). This finding could support the idea of Brown & Neuman (43) who stated that it is unclear whether several inflammatory processes are the result of pure Th-1 or Th-2 type responses, or, as in hepatitis C, both arms (Th1 + Th2) are involved.

In conclusion, we simultaneously assessed pro-and anti-inflammatory related cytokines and cell populations of Tregs in chronic HCV patients and compared them to asymptomatic HCV infected patients and healthy blood donor control groups and have

shown that (i) The frequency of different Treg cell subsets defined by CD4+CD25+, CD4+FoxP3+, and CD4+CD25+ FoxP3+ were significantly higher in the chronic HCV group compared to the other two groups, (ii) positive correlation between Treg cell subsets and viral load and no correlation between them and degree of fibrosis, (iii) both pro and anti-inflammatory cytokines were significantly elevated in the chronic group compared to the other two groups, (iv) IL-2, IL-10 and TGF- β showed positive correlation with HCV RNA load, while IFN- γ showed inverse correlation and (v) no correlation between the studied cytokines and degree of fibrosis. Ex-vivo studies are needed to be thoroughly extended using monoclonal antibodies directed against Tregs markers (anti CD25 and/or anti CD FoxP3) or against its related cytokines (IL-10 and/or TGF- β) before tailoring the proper immunotherapy cocktail against chronic HCV infection.

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References

- 1) Papatheodoridis G, Hatzakis A. Public health issues of hepatitis C virus infection. *Best Pract Res Clin Gastroenterol.* 2012 Aug; 26(4):371-380.
- 2) Alter MJ. Epidemiology of hepatitis C virus infection. *World J Gastroenterol.* 2007; 13: 2436-2441.
- 3) El-Zanaty F, Way A. Egypt demographic and health survey 2008. Cairo, Egypt: Ministry of Health, El-Zanaty and Associates, and Macro

- International; 2009. Available at <http://www.measuredhs.com/pubs/pdf/fr220/fr220.pdf>. Accessed July 18, 2012.
- 4) Wali M, Harrison RF, Gow PJ, Mutimer D. Advancing donor liver age and rapid fibrosis progression following transplantation for hepatitis C. *Gut*. 2002 ; 51(2):248-252.
 - 5) Petrovic D, Dempsey E, Doherty DG, Kelleher D, Long A. Hepatitis C virus--T-cell responses and viral escape mutations. *Eur J Immunol*. 2012 ; 42(1):17-26
 - 6) Spengler U, Nattermann J. Immunopathogenesis in hepatitis C virus cirrhosis. *Clin Sci (Lond)*. 2007 ; 112(3):141-155.
 - 7) Sarantopoulos S, Lu L, Cantor H. Qa-1 restriction of CD8+ suppressor T cells. *J Clin Invest*. 2004; 114:1218–1221.
 - 8) Roncarolo MG, Bacchetta R, Bordignon C, Narula S, Levings MK. Type 1 T regulatory cells. *Immunol Rev*. 2001; 182:68–79. 18
 - 9) Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev*. 2001; 182:207–214.
 - 10) Sugimoto K, Ikeda F, Stadanlick J, Nune F, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology*. 2003; 38(6): 1437–1448.
 - 11) Stoop JN, Van DerMolen RG, Baan CC. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. *Hepatology*. 2005; 41(4): 771–778.
 - 12) Xu D, Fu J, Jin L. Circulating and liver resident CD4+CD25+ regulatory T cells actively influence the antiviral immune response and disease progression in patients with hepatitis B. *J Immunol*. 2006; 177(1): 739–747.
 - 13) Ward SM, Fox BC, Brown PJ. Quantification and localisation of FOXP3+ T lymphocytes and relation to hepatic inflammation during chronic HCV infection. *J Hepatol*. 2007; 47(3): 316–324.
 - 14) Sathaliyawala T, Kubota M, Yudanin N, Turner D, Camp P, Thome JJC, et al. Distribution and compartmentalization of human circulating and tissue resident memory T cell subsets. *Immunity*. 2013;38 : 178–197.
 - 15) Manigold T, Racanelli V. T-cell regulation by CD4 regulatory T cells during hepatitis B and C virus infections: facts and controversies. *Lancet Infectious Diseases* 2007; 7(12): 804–813. 19
 - 16) Langhans B, Kramer B, Louis M, Nischalke HD, Huneburg R, Staratschek-Jox A, et al. Intrahepatic IL-8 producing Foxp3(+)CD4(+) regulatory T cells and fibrogenesis in chronic hepatitis C. *J Hepatol*. 2013;59:229–235
 - 17) Carlo S, and Vincenzo M. Controversy on the role of FoxP3+ regulatory T cells in fibrogenesis in chronic hepatitis C virus infections. *Journal of Hepatol*. 2014 (60) 229–236.
 - 18) Rallón NI, López M, Soriano V, García-Samaniego J, Romero M, Labarga P, et al. Level, phenotype and activation status of CD4+FoxP3+ regulatory T cells in patients chronically infected with human immunodeficiency virus and/or hepatitis C virus. *Clin Exp Immunol*. 2009; 155:35-43.
 - 19) Cabrera R, Tu Z, Xu Y, Firpi RJ, Rosen

- HR, Liu C, et al. An immunomodulatory role for CD4(+)CD25(+) regulatory T lymphocytes in hepatitis C virus infection. *Hepatology*. 2004; 40:1062-1071.
- 20) Sugimoto K, Ikeda F, Stadanlick J, Nunes FA, Alter HJ, Chang KM. Suppression of HCV-specific T cells without differential hierarchy demonstrated ex vivo in persistent HCV infection. *Hepatology*. 2003; 38:1437-1448.
- 21) Yoshizawa K, Abe H, Kubo Y, Kitahara T, Aizawa R, Matsuoka M, et al. Expansion of CD4+CD25+FoxP3+ regulatory T cells in hepatitis C virus-related chronic hepatitis, cirrhosis and hepatocellular carcinoma. *Hepatology Res*. 2010; 40:179-187.
- 22) Heeg MHJ, Ulsenheimer A, Grüner NH, Zachoval R, Jung MC, Gerlach T. FOXP3 Expression in hepatitis C virus-specific CD4+ T cells during acute hepatitis C. *Gastroenterol*. 2009; 137:1280-1288
- 23) Desmet VJ, Gerber M, Hoofnagle JH. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology*. 1994; 19:1513-1520.
- 24) Ward SM, Fox BC, Brown PJ, Worthington J, Fox SB, Chapman RW. Quantification and localisation of FOXP3+ T lymphocytes and relation to hepatic inflammation during chronic HCV infection. *J Hepatology*. 2007; 47:316-24.
- 25) Jones RC, Capen DE, Cohen KS, Munn LL, Jain RK, Duda DG. A protocol for phenotypic detection and characterization of vascular cells of different origins in a lung neovascularization model in rodents. *Nature protocols*. 2008; 3:388-397.
- 26) Claassen MA, de Knecht RJ, Janssen HL, Boonstra A. Retention of CD4+CD25+FoxP3+ regulatory T cells in the liver after therapy-induced hepatitis C virus eradication in humans. *J Virol* 2011;85: 5323–5330.
- 27) Cabrera, R., Tu, Z., Xu, Y., Firpi, R. J., Rosen, H. R., Liu, C., & Nelson, D. R. virus infection. *Hepatology*. 2004; 40(5), 1062-1071.
- 28) Bertolotti A, D'Elia MM, Boni C, De Carli M, Zignego AL, Durazzo M, Missale G, Penna A, Fiaccadori F, Del Prete G, Ferrari C. Different cytokine profiles of intraphepatic T cells in chronic hepatitis B and hepatitis C virus infections. *Gastroenterol*. 1997; 112: 193-199.
- 29) Tsai SL, Sheen IS, Chien RN, Chu CM, Huang HC, Chuang YL, Lee TH, Liao SK, Lin CL, Kuo GC, Liaw YF. Activation of Th1 immunity is a common immune mechanism for the successful treatment of hepatitis B and C: tetramer assay and therapeutic implications. *J Biomed Sci*. 2003;10: 120-135.
- 30) Wan L, Kung YJ, Lin YJ, Liao CC, Sheu JJ, Tsai Y, Lai HC, Peng CY, Tsai FJ 2009. Th1 and Th2 cytokines are elevated in HCV-infected SVR (-) patients treated with interferon-alpha. *Biochem Biophys Res Commun*. 2009; 379: 855-860.
- 31) Missale G, Ferrari C, Fiaccadori F. Cytokine mediators in acute inflammation and chronic course of viral hepatitis. *Ann Ital Med Int*. 1995; 10: 14-18
- 32) Cacciarelli TV, Martinez OM, Gish RG, Villanueva JC, Krams SM. Immunoregulatory cytokines in chronic hepatitis C virus infection: pre- and posttreatment with interferon alfa. *Hepatology*. 1996; 24: 6-9.

- 33) Simsek H, Kadayifci A. Serum interleukin 2 and soluble interleukin 2 receptor in chronic active hepatitis C: effect of interferon therapy. *J Int Med Res.* 1996; 24: 239-245.
- 34) Urbani S, Amadei B, Fiscaro P, Tola D, Orlandini A, Sacchelli L, Mori C, Missale G, and Ferrari C. Outcome of Acute Hepatitis C Is Related to Virus Specific CD4 Function and Maturation of Antiviral Memory CD8 Responses. *Hepatology.* 2006; 44(1): 126-139.
- 35) A T R-Viso, M I S Duarte, C Pagliari, E R Fernandes, R A Brasil, G Benard, C C Romano, S Ogusuku, N P Cavalheiro, C E Melo, A A Barone. Tissue and serum immune response in chronic hepatitis C with mild histological lesions. *Memórias do Instituto Oswaldo Cruz Rio de Janeiro.* 2010; 105(1):25-32.
- 36- Gramenzi A, Andreone P, Loggi E, Foschi FG, Cursaro C, Margotti M, Biselli M, Bernardi M. Cytokine profile of peripheral blood mononuclear cells from patients with different outcomes of hepatitis C virus infection. *J Viral Hepat.* 2005; 12: 525-530.
- 37) Nelson DR, Lauwers GY, Lau JY, Davis GL 2000. Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon non-responders. *Gastroenterol.* 2000; 118: 655-660.
- 38) Nelson DR, Tu Z, Soldevila-Pico C, Abdelmalek M, Zhu H, Xu YL, Cabrera R, Liu C, Davis GL. Long-term interleukin 10 therapy in chronic hepatitis C patients has a proviral and anti-inflammatory effect. *Hepatology.* 2003; 38: 859-868.
- 39) Bolacchi F, Sinistro A, Ciaprini C, Demin F, Capozzi M, Carducci FC, et al. Increased hepatitis C virus (HCV)-specific CD4+CD25+ regulatory T lymphocytes and reduced HCV-specific CD4 β T cell response in HCV-infected patients with normal versus abnormal alanine aminotransferase levels. *Clin Exp Immunol.* 2006; 144: 188-196.
- 40) Naicui Zhai, Xiumei Chi, Tianyang Li, Hongxiao Song, Haijun Li, Xia Jin, Ian Nicholas Crispe, Lishan Su, Junqi Niu, Zhengkun Tu. Hepatitis C virus core protein triggers expansion and activation of CD4+CD25+ regulatory T cells in chronic hepatitis C patients. *Cel and Mol Immunol advance online publication* 22 December 2014; 10.1038/cmi.119
- 41) Schuppan D, Krebs A, Bauer M, Hahn EG. Hepatitis C and liver fibrosis. *Cell Death Differ* 2003; 10(Suppl 1):S59-S67.
- 42) Li S, Vriend LE, Nasser IA, Popov Y, Afdhal NH, Koziel MJ, Schuppan D, Exley MA, Alatrakchi N Hepatitis C virus-specific T-cell-derived transforming growth factor beta is associated with slow hepatic fibrogenesis, *Hepatology.* 2012; 56(6): 2094–2105.
- 43) Brown PMJ, Neuman MG. Immunopathogenesis of hepatitis C viral infection: Th1/Th2 responses and the role of cytokines. *Clin Biochemist.* 2001; 34: 167-171.