

## Immunomodulatory Role of Treg Lymphocytes in Chronic Hepatitis C Patients

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### Abstract

**Background:** Regulatory T cells (Tregs) have a fundamental job in keeping up a harmony between forestalling immunopathology and enabling the insusceptible reaction to clear infections, in HCV infection, elevation of Tregs may cause insistent HCV infection.

**Objective:** The current work aimed to clarify the immunomodulatory role of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs in chronic hepatitis C (CHC) patients.

**Subjects and methods:** This study incorporated two groups: 50 patients with chronic HCV infection of different classes of Child-Pugh classification (Child A, B and C) and control group; 25 healthy subjects. All patients were exposed to full history taking and finish clinical examination, as well as routine lab analysis including CBC, AST, ALT, ALP, GGT, PT, INR, blood urea, serum creatinine, ANA. HCV-Abs was measured for both patients and controls groups. Viral load was determined for patients group by HCV-RNA PCR. Immunophenotyping of CD4<sup>+</sup> CD25<sup>+</sup> FoxP3<sup>+</sup> regulatory Treg cells were performed by flow Cytometry for patients and controls groups.

**Results:** There was expansion of CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup> Treg lymphocytes in CHC patients compared with controls with significant difference. Furthermore, there was a highly significant decline in the average of CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup>Treg lymphocytes amongst the 3 different classes of Child-Pugh classification of CHC patients on relating to the control. There were significant correlations between CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup>Treg lymphocytes and liver fibrosis.

**Conclusion:** There is marked increased level of CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup>Treg lymphocytes among CHC patients, in addition to, the 3 different classes of Child-Pugh classification. This might confirm the immunomodulatory role by CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup>Treg during chronic HCV infection that it might contribute to the immune response failure.

**Keywords:** Chronic hepatitis C; Treg lymphocytes; Dendritic cells; Liver cirrhosis

### Introduction

HCV may elude the insusceptible reaction or pass on an explicit resilience to itself to guarantee its survival in tainted patients through systems as viral departure, T-cell energy and acceptance of administrative Lymphocytes (Treg). There are 2 fundamental subsets of Tregs; thymically chosen natural Tregs (nTreg), which are phenotypically divided as CD4<sup>+</sup>CD25<sup>+</sup> FOXP3<sup>+</sup> and "inducible" Treg cells, stimulated in the periphery, named either Tr1 or Th3 characterized as freeing IL-10, TGF-β and presumably IL-4 [1,2].

The FOXP3 is capable of control and inciting the multiplication of CD4<sup>+</sup>CD25<sup>+</sup> Tregs in the fringe course accomplished from HCV infected patients. Besides, FOXP3 raises the inhibitory impact of Tregs on the insusceptible framework and supports a deficient invulnerable reaction to the infection, in the long run prompting an incessant viral disease [3].

Initiated CD4<sup>+</sup> CD25<sup>+</sup> Tregs can stifle the multiplication, separation and emission of CD4<sup>+</sup> and CD8<sup>+</sup> Immune system microorganisms cytokines in a cell contact-subordinate way [4].

Studies on HCV have discovered an elevation of Treg markers in incessantly infected patients when contrasted and settled and non-tainted people, likely causing viral industriousness [3,5].

The current work was planned to clarify the immunomodulatory role of CD4<sup>+</sup> CD25<sup>+</sup> FOXP3<sup>+</sup> T regulatory lymphocytes in CHC patients regarding fibrosis.

### Subjects and Methods

#### Study design

This study was performed in Clinical Pathology and Internal Medicine Departments, Faculty of Medicine, Al-Azhar University in the period from June, 2015 to January, 2018.

## Ethical considerations

Endorsement for achievement this examination was found from Clinical Pathology and Interior Prescription Divisions, in the wake of getting Institutional Audit Board (IRB) and moral advisory group endorsement. Furthermore, composed educated assent was accomplished from every patient

## Subjects

This study included 2 groups:

**Case group:** It included 50 patients. They were 37 (74%) males and 13 (26%) females. Mean  $\pm$  SD of their ages was  $52.3 \pm 3.5$  years. They were present in the outpatient clinic of Internal Medicine Department, Al-Azhar University Hospitals.

**Inclusion criteria:** Chronic hepatitis C patients in various clinical states (Child A, B and C) who had positive HCV antibody with persistently elevated ALT activity and measurable serum level of HCV RNA by PCR for at least 6 months. They were collected from patients' files.

**Exclusion criteria:** Patients with medication or liquor misuse, HBV, HDV, or immune system hepatitis (positive ANA), metabolic liver infection, introduction to hepatotoxin or immunosuppressive treatment. In addition, those with any chronic illness, thyroid and psychiatric diseases, diabetes mellitus, renal or heart failure. Pregnant or lactating females or those were taking contraceptive pills or obese were excluded.

**Control group (II):** (25 Healthy subjects): They matched patients as regards age and sex. They were 18 (72%) males and 7 (28%) females. Mean  $\pm$  SD of their ages is  $51.9 \pm 3.8$  years. They were evidenced to be negative for HCV Abs by ELISA and did not have history of liver diseases.

## Data collection

Clinical evaluation that includes age and sex, general, abdominal, chest and heart examinations.

## Sampling

An 8 mL Blood samples were collected from each subject and separated for routine lab investigations, detection of HCV Ab. Furthermore, 6 mL blood samples were collected in a sterile tube containing EDTA and divided into three parts 2 mL for flow Cytometry, 2 mL for CBC and 2 mL for real-time PCR (CHC patient only). EDTA was used for flow cytometry as the reagent's performance may be affected by the use of other anticoagulant (Figure 1) [6].

The sample was stained freshly for flow cytometry within 6 hours for optimal results, during this period; the blood was kept at room temperature (20-25°C). Tests with hemolysis, clots or suspended cell aggregates were rejected (Figure 1).

## Methods

### A. Laboratory investigations include:

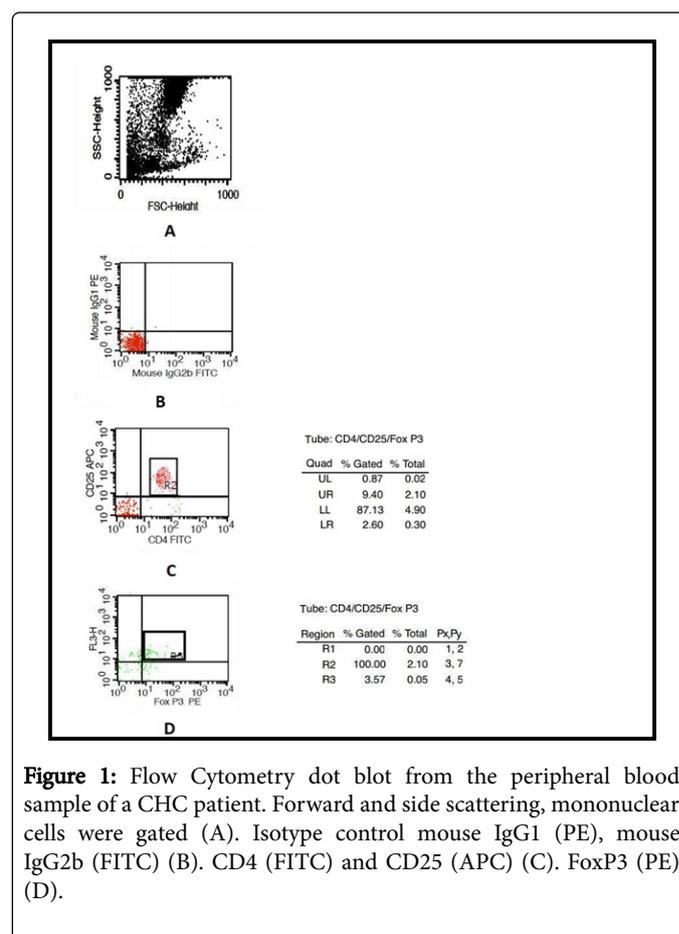
- Complete blood picture (CBC) (Sysmex KX-21, Japan).

- Total and direct bilirubin, serum albumin, ALT, AST, ALP, GGT) (COBAS INTEGRA® 400 plus, Roche Diagnostics Ltd., Switzerland). PT was measured by (Coatron M1, Germany).
- Blood urea and serum creatinine (COBAS INTEGRA® 400 plus, Roche Diagnostics Ltd., Switzerland)
- ANA and HCV-Abs using ELISA technique (Axiom, Worm (WO), Germany).

B. HCV RNA detection by real-time PCR was performed using "DNA-Technology, Research and Production LLC, Russia"

C. Flow Cytometric analysis that includes immunophenotyping of CD4+ CD25+ FoxP3+ regulatory T-reg cells using (Flow XTM Human Regulatory T Cell Multi-Color Flow Kit) (Cat. no. FMC021) (R and D Systems Inc., Minneapolis, USA). Investigation was finished utilizing stream Cytometry (FACS caliber, Becton Dickinson, San Jose, California, USA) and CELLQuest™ Software (BD Biosciences, San Diego, USA) to patients and controls groups (Table 1-4).

**Statistical analysis:** The gathered information were displayed, organized, and broke down utilizing the Statistical Package for Social Sciences (SPSS version 19.0).



**Figure 1:** Flow Cytometry dot blot from the peripheral blood sample of a CHC patient. Forward and side scattering, mononuclear cells were gated (A). Isotype control mouse IgG1 (PE), mouse IgG2b (FITC) (B). CD4 (FITC) and CD25 (APC) (C). FoxP3 (PE) (D).

## Results

Parameter	Child A (No=20)	Child B (No=15)	Child C (No=15)	Control	F	P
Hb (g/dL)	13.6 ± 1.75	9.4 ± 1.85	10.2 ± 2.4	14.3 ± 1.5	31.3	<0.001**
	9.9 - 16.6	5 - 12	6.1 - 15.5	12 - 17		
TLC (× 10 <sup>3</sup> /mm <sup>3</sup> )	6.0 ± 1.76	4.1 ± 3.2	3.6 ± 1.4	7.1 ± 1.7	17.3	<0.001**
	2.5 - 8.5	1.4 - 14.5	1.9 - 7	4 - 10		
PLT (× 10 <sup>3</sup> /mm <sup>3</sup> )	149.6 ± 70.2	93.5 ± 25	80 ± 42.2	280.4 ± 68	53.1	<0.001**
	37 - 314	43 - 140	37 - 188	180 - 401		
PT (seconds)	13.5 ± 1.5	20.6 ± 3.0	25.85 ± 6.4	13.5 ± 1.5	85.0	<0.001**
	12.2 - 17.8	17.3 - 27.5	18.3 - 42.9	12.2 - 17.4		
INR	1.17 ± 0.14	1.9 ± 0.3	2.34 ± 0.7	1.1 ± 0.08	65.1	<0.001**
	1.02 - 1.51	1.49 - 2.37	1.06 - 3.64	1 - 1.2		
Total Bilirubin (mg/dL)	1.1 ± 0.42	3.9 ± 1.4	6.3 ± 2.2	0.8 ± 0.2	82.4	<0.001**
	0.6 - 2.1	2.5 - 6.5	2.5 - 10.4	0.5 - 1.18		
Direct Bilirubin (mg/dL)	0.32 ± 0.24	2.7 ± 1.0	3.6 ± 1.2	0.18 ± 0.06	97.2	<0.001**
	0.1 - 0.98	1.4 - 4.7	1.6 - 6.3	0.1 - 0.3		
Albumin (g/L)	3.9 ± 0.4	2.76 ± 0.4	2.37 ± 0.2	4.4 ± 0.4	121.1	<0.001**
	2.9 - 4.8	2 - 3	1.9 - 2.8	3.8 - 5.1		

There is significant decrease of Hb, TLC, platelets count and albumin among Child-Pugh classes of patients.  
There is significant increase of PT and INR, total and direct bilirubin among Child-Pugh classes of patients.

**Table 1:** Results of different classes of Child-Pugh classification in CHC patients.

Parameter	Mean ± SD	Range	T	P
CHC Patients	2.33 ± 2.2	0.1 - 6.32	2.04	0.04
Controls	0.32 ± 0.2	0.98 - 1.7		

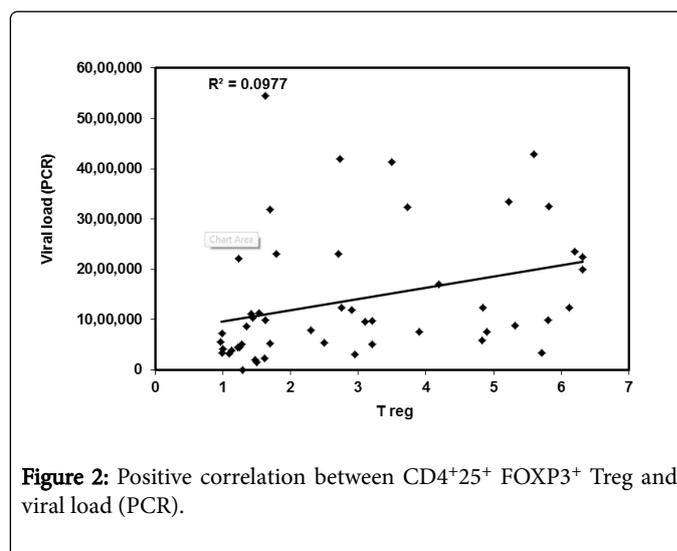
**Table 2:** CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup>Treg lymphocytes in CHC patients and controls.

Parameter	Child A	Child B	Child C	Controls	F	P
Treg lymphocytes	1.26 ± 0.2	2.82 ± 0.6	5.15 ± 1.2	0.26 ± 0.02	67.5	<0.001
	0.98 - 1.7	1.7 - 3.9	1.64 - 6.32	0.1 - 0.4		

**Table 3:** CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup>Treg lymphocytes in different classes of Child-Pugh classification in CHC patients.

Parameter	R <sup>2</sup>	P
Viral load	0.0977	<0.05

**Table 4:** Correlations between CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup> Treg lymphocytes and the Viral load in CHC patients.



**Figure 2:** Positive correlation between CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup> Treg and viral load (PCR).

There is huge increment of Treg lymphocytes in CHC patients contrasted with the control gathering and furthermore, they have critical increment among the 3 unique classes of Child-Pugh order of CHC patients on contrasting and the controls. Moreover, there were

significant positive correlation between them and positive viral load (Figure 2).

## Discussion

Treg are a key CD4<sup>+</sup> White blood cell subpopulation that are principal in keeping up parity of the insusceptible framework and assume a fundamental job responsible for insusceptibility to remote antigens including infections [2,7].

The presence of the Forkhead Box Protein 3 (FOXP3) and low levels of alpha chain IL-7 receptor (CD127) on CD4<sup>+</sup>CD25<sup>+</sup> T cells are currently utilized as the gold standard phenotypic markers of circulating Treg [8].

Starting here of view, our examination was intended to illuminate the immuno-modulatory job of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T administrative cells in CHC patients.

In this research, there was expansion in CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup> Treg lymphocytes with statistically significant difference (P<0.05).

Perrella, et al. were the chief to show that Treg (CD4<sup>+</sup> CD25<sup>+</sup>) were actually elevated during the acute phase of HCV infection in the peripheral blood of patients. Furthermore, patients who later developed chronic infection had higher blood levels of CD4<sup>+</sup> CD25<sup>+</sup> T-cells at the onset of the infection than those who got rid of the infection. Be that as it may, this was not the situation in two subsequently distributed autonomous investigations with respect to both the CD4<sup>+</sup> CD25<sup>+</sup> White blood cell populace and the HCV-explicit CD4<sup>+</sup> FoxP3<sup>+</sup> populace [9].

Numerous investigations of patients with interminable HCV disease demonstrated a hoisted recurrence of CD4<sup>+</sup> CD25<sup>+</sup> Treg cells in the fringe blood contrasted and suddenly settling patients or solid controls, perhaps prompting viral perseverance. Cabrera, et al. performed examination and found that CD4<sup>+</sup>CD25<sup>+</sup>cells discharge TGF-β1 and IL-10. The inhibitory role for TGF-β1 was affirmed by anti-TGF-β1 [10]. Additionally, they found that HCV- explicit IFN-γ movement was upgraded in marginal blood mononuclear cells drained of CD4<sup>+</sup>CD25<sup>+</sup> and stifled in fringe blood mononuclear cells enhanced with CD4<sup>+</sup>CD25<sup>+</sup> [3,10,11].

Consumption of CD4<sup>+</sup>CD25<sup>+</sup> cells additionally improved HCV-explicit CD4<sup>+</sup> and CD8<sup>+</sup> T cell expansion. In addition, they showed that CD4<sup>+</sup>CD25<sup>+</sup> mediated suppression to be dose dependent and requiring cell contact. Furthermore Boettler, et al. and Shin, et al. discovered that Treg cells from constantly infected patients show morally higher inhibitory action against T cell expansion and IFN-γ emission. Besides, Treg cells from HCV- tainted individuals suppress CD8<sup>+</sup> T cells in an antigen-non- explicit way and stifle HCV-specific T cells but as well as flu -, CMV- and EBV-explicit Lymphocytes [12-14].

In a study performed by Manigold, et al. they uncovered that CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Treg cells smother both IFN-γ emission and the development of HCV-explicit T cells and actuation prompted cell passing of HCV-explicit T cells in HCV-uncovered chimpanzees, in both those with suddenly recuperated from HCV disease and with interminable HCV infection [15].

Hence, it was proposed that Treg cells could control HCV-specific memory T cell responses by restricting their activation and inhibiting apoptosis in patients cured from HCV infection [16].

As respects the dispersion of Treg cells inside the HCV-infected liver, found that a high number of penetrating CD4<sup>+</sup> FoxP3<sup>+</sup> T-cells were detected in the livers of chronic HCV patients, while they were absent from healthy livers. These Tregs were completely separated and very actuated phenotype, while about half of them did not show CD25. They clarified that they may result from two primary components specifically influenced by the infection: First, HCV may invigorate the dynamic enlistment of existing Treg by adjusting safe cells like dendritic cells and B cells. They recognized that monocyte-inferred dendritic cells (mo-DC) augmented their secretion of the Treg-attracting chemokines Chemokine (C-C motif) ligand (CCL) 17 and CCL22 when they appended to HCV-tainted Huh7 hepatoma cells [17,18].

Besides, they found that in chronic HCV patients isolated mo-DC have been seen to own a greater ability to induce Treg from simple CD4<sup>+</sup> T-cells than mo-DC from a healthy control. Lastly, B cells to HCV have been shown to display an improved ability to generate Treg. Secondly, HCV-infected cells may straightforwardly associate with T-lymphocytes and support their change into Treg. At the point when actuated CD4<sup>+</sup> T cells were co- developed with HCV-infected Huh7 cells, they communicated more dimensions of the Treg markers CD25 and FoxP3. This impact was started likewise by TGF beta and galectin-9, the declaration of which is increased in infected Huh7 cells and are seen in the serum and liver of HCV-infected patients. Kupffer cells and infected hepatocytes are the major sources of galectin-9 in the liver [18-20].

On the other hand, HCV proteins may attach directly to T-cells and making them immunosuppressive. Jurkat T-cells expressing HCV core protein increase FoxP3 and gain oppressive role and the transduction of HCV core protein in CD4<sup>+</sup> T-cells prompts the expression of FoxP3 and Treg markers [21].

Tseng, et al. established that Treg-mediated immunosuppression reduction during and after combination therapy with PEG-IFN and RBV, irrespective of the treatment response [22].

In this study, there is positive correlation between CD4<sup>+</sup> 25<sup>+</sup> FOXP3<sup>+</sup> Treg lymphocytes and age (P<0.05) in CHC patients. There is supporting information originated from human examinations demonstrating a higher level of CD4<sup>+</sup> FOXP3<sup>+</sup> Tregs in biopsies from skin from senior people contrasted with more youthful grown-ups. This assigns CD4<sup>+</sup> Treg compartment increments with age, near to the aggregate CD4<sup>+</sup> T cells. Though, since a number of CD4<sup>+</sup> T cell subsets decrease in the elder individuals, it is not evident whether this near increment is expected to absence of naive CD4<sup>+</sup> T cells or a bona fide expansion in absolute Treg numbers. In contrast, Tseng, et al. found that age did not associate with the CD4<sup>+</sup>FoxP3<sup>+</sup> Treg frequency [22,23].

Interminable HCV infection is identified with a hazard of liver fibrosis that can develop into liver cirrhosis and HCC. The capacity of Treg in this procedure is discussed. Intrahepatic CD4<sup>+</sup> FoxP3<sup>+</sup> T cells are available at more elevated amounts in HCV-infected livers with constrained fibrosis, and the proportion of FoxP3<sup>+</sup>/CD8<sup>+</sup> cells is lessened in livers with serious HCV-related fibrosis. Additionally, TGF-β created by HCV-explicit CD4<sup>+</sup> and CD8<sup>+</sup> T-cells seem to restrain liver fibro-beginning as an option to act as an ace fibrotic factor [24,25].

On contrary, the declaration of Treg markers (FoxP3, CTLA4 and GITR) and Tr1 markers (CD49b, CD18 and IL10) was enlarged in the livers of patients with HCV-related cirrhosis when contrasted with

constantly infected patients without liver cirrhosis. No rise in Th1 and Th2 markers was identified.

Furthermore, initiated CD4<sup>+</sup>FoxP3<sup>+</sup> T cells communicating IL8 were expanded in the liver in closeness to alpha-SMA<sup>+</sup> hepatic stellate cells and territories of fibrosis. The generation of IL8 by Treg is explicitly connected with HCV disease and the way of life supernatant of IL8<sup>+</sup> Treg clones initiates hepatic stellate cells by means of IL8.

Generally, these results recommend that diverse subsets of regulatory T-cells could have a reverse influence on HCV-related fibrosis [26].

In the current work, the average of CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup> Treg lymphocytes was elevated morally in patients with liver cirrhosis measured using Child-Pugh classification, as related to the normal controls [27].

In agreement with the current results, Aleem AA, et al. and Li, et al. stated that the proportion of Treg cells in cirrhotic patients was dramatically more than those in controls. Also, Huang, et al. detected that the percentage of Treg cells elevated significantly in patients with liver cirrhosis when comparing with normal volunteers [28,29].

In concurrence with the acquired outcomes Huang, et al. found that in patients with liver cirrhosis, the expanded dimension of Treg cells was fundamentally connected with viral load (P<0.05) [29].

In this investigation, a positive connection among Treg and viral load, this is coordinated with Barjon, et al. who found that a positive connection was recognized between CD4<sup>+</sup>CD25<sup>+</sup> T cell recurrence and HCV RNA titer, though a reverse connection was found with liver incendiary movement [17].

These showed that CD4<sup>+</sup>CD25<sup>+</sup> T cells can react straightforwardly to HCV antigens and smother the HCV-explicit invulnerable reaction in a portion subordinate cell contact way, which may advance HCV survival inside the host.

## Conclusion

There is marked increased level of CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup>Treg lymphocytes among CHC patients, in addition to, the 3 different classes of Child-Pugh classification. This might confirm the immunomodulatory role by CD4<sup>+</sup>25<sup>+</sup> FOXP3<sup>+</sup>Treg during chronic HCV infection that it might contribute to the immune response failure.

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