Assessment of CD4+CD25+ Regulatory T Lymphocytes Cells in Chronic Hepatitis C, Chronic Hepatitis C– Associated Cirrhosis and Hepatocellular Carcinoma Patients

Mansour J 1, Amal F. Abdel-Hai2, Mohamed M. EL din3, Ahmed E2, and Samira R. Mansour*4

1Clinical Laboratory Department, El-Ghad Faculty for Health Science, Jeddah, KSA.
2Department of Clinical Pathology, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.
3Department of Internal Medicine, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.
4Botany Department, Faculty of Science, Suez Canal University, Ismailia, Egypt.

ABSTRACT

CD4+CD25+ regulatory T lymphocytes are a subset of circulating CD4+ T cells with suppressive properties. CD4+CD25+ regulatory T cells suppress HCV-specific T cell responses and it has been suggested that they may play a role in viral persistence. Our aim was to assess the frequency of regulatory T cells in the different clinical presentations of hepatitis C virus infections in a chosen Egyptian population. Peripheral blood CD4+CD25+ regulatory T cells from patients with different progression of HCV, was conducted and analyzed using flow cytometry. For each stage of HCV progression, twenty patients (n=20) were subjected for analysis in comparison with normal healthy control subjects (n=20). Results showed a significantly higher frequency of CD4+CD25+ regulatory T cells in chronic HCV (3.0 ± 0.9%), HCV related liver cirrhosis (5.7±0.8%), HCV related-HCC (3.9 ±1.6%), when compared to normal healthy controls (2.3 ±0.8%). No statistical significant differences were found when comparing HCV related cirrhotic patients with chronic HCV infected patients (P=0.95). Meanwhile, statistical significant differences were detected when comparing HCV related HCC patients with chronic HCV patients (P=0.047). In conclusion, our results indicated the presence of significant higher than normal frequency of peripheral blood CD4+CD25+ regulatory T cell among Egyptian patients that may help to early detect the viral disease and open new field to find a way to disease recovery or protection. Conversely, within different clinical presentation of hepatitis C virus infection no significant differences were detected. Severity of liver affection was significantly correlated with viral load.

Key words: Chronic Hepatitis, Flow cytometry, Hepatitis C infection, Hepatitis associated cirrhosis, Hepatocellular Carcinoma, Liver disease, Regulatory T cells.

INTRODUCTION

CD4+CD25+ regulatory T lymphocytes (Treg) are a subset of circulating CD4+ T cells with suppressive properties (Jonuleit et al., 2001; Stephens et al., 2001). There are two general categories of CD4+CD25+ T cells. One Treg cell subset develops during the process of T cell maturation in the thymus, resulting in the generation of a naturally occurring population of Treg cells poised to prevent autoimmune responses by suppressing auto-reactive T cells (Sakaguchi, 2000; Shevach, 2000). The second subset of Treg cells develops as a consequence of ex vivo peripheral activation of naïve CD4+CD25- T cells (Bluestone and Abbas 2003). These induced CD4+CD25+ which have been shown to suppress T cell responses to tumors, acute and chronic bacterial and viral infections (Woo et al., 2002; Belkaid et al., 2002). Treg cells suppress the proliferation, cytokine-production (IFN-γ, IL-2) and cytolytic activity of naïve and antigen specific CD4+ and CD8+ cells. In addition, Treg cells are able to suppress the functions of antigen presenting cells and B cells (von Boehmer, 2005).

Treg cells may mediate their suppressive activity either through the secretion of anti-inflammatory cytokines like IL-10 or TGF-β, direct killing of the target cells or distinct cell-cell contact dependent mechanisms (von Boehmer, 2005). However, the mechanisms and the antigen-specificity of Treg cell mediated immune-suppression are still largely unknown. Indeed, the virus specific induction of Treg cells may have two very different consequences: first, it may help the virus to establish viral persistence and second, it may be an important process that occurs to prevent excessive immune pathological damage (Mills, 2004; Belkaid and Rouse 2005).

Hepatitis C virus (HCV) is a disease with a significant global impact. According to the World Health Organization (WHO) there are 200 million people infected with HCV (WHO, 2012). In Egypt, HCV is considered a serious problem due to its propensity to chronicity, which often resulting in liver cirrhosis and hepatocellular carcinoma, and the high prevalence of antibodies to HCV among apparently healthy Egyptian population (Perz and Alter, 2006; Miller and Laith, 2010). It also has extremely high HCV seroprevalence among Egyptian children (Nahla et al., 2011). Acute infection usually is not spontaneously cleared in part due to immune escape by emerging quasispecies and virus-induced immune dysfunction.

In particular, it has been reported that Treg cells suppress HCV-specific T cell responses and suggested that they may play a role in viral persistence (Cabrera et al., 2004). Many investigators reported a higher frequency of Treg cells in the blood of chronically HCV infected persons versus recovered or healthy persons (Sugimoto et al., 2003; Boettler et al., 2005). It has also been reported an increment of Treg cells in peripheral blood mononuclear cells (PBMCs) of Hepatocellular...
carcinoma patients which was positively correlation with tumour burden (Mengde et al., 2007).

Therefore, the aim of this study was to assess the frequency of T_{reg} cells in different clinical presentation of chronic hepatitis C virus infection, in a chosen Egyptian population, which may be linked to choriocity and severity or progression of HCV disease. Furthermore, may these findings may facilitate the understanding of the mechanisms underlying the pathogenesis of liver damage and of hepatic immune cells during inflammation.

**MATERIALS AND METHODS**

**Study Subjects**

Sixty patients, receiving care at Suez Canal University Hospital, were categorized into 3 groups, depending on progression of HCV disease. First group was patients with chronic hepatitis C virus (CHCV), second group included patients with HCV related liver cirrhosis and third group included patients with HCV related-HCC. However, reference group was selected from healthy blood donor without a history of HCV infection. Each group was represented by twenty individuals.

CHCV was diagnosed if the following criteria were present after a 6-month follow-up: a) significant and persistent symptoms, b) fluctuating or persistently elevated ALT and AST (> 1.5 fold of normal levels), normal serum albumin and prothrombin time, c) ultrasonography revealed an enlarged bright texture ± portal tract thickening ± normal spleen. Cases were clinically diagnosed as cirrhosis if they had coarse texture, attenuated hepatic veins; irregular surface, shrunken liver, enlarged spleen ± dilated portal and splenic veins ± hypoalbuminemia ± prolonged prothrombin time and ascites. HCC was diagnosed according to the diagnostic guidelines of the AASLD (Bruix et al., 2005).

Patients and control subjects were subjected to a detailed history taking and clinical examination. None of the patients had fever, evidence of other infectious diseases, hepatitis B virus co-infection (excluded serologically), or any other autoimmune inflammatory disorders, at the time of the blood samples were obtained. No patients had received previous anti-viral, anti-cancer or immuno-modulatory treatment. Informed consent was obtained from each patient included in the study. CHCV patients were 4 females and 16 males, their ages ranged between 35 and 50 (45.5±9.3) years. Cirrhotic patients were 5 females and 15 males, their ages ranged between 40 and 60 (51±7.7) years. HCC patients were 3 females and 17 males, their ages ranged between 45 and 60 (52±8.4) years. Control subjects were 6 females and 14 males and their ages ranged between 35 and 48 (43±8.5) years.

Venous blood samples (7ml in EDTA and plain tubes) were withdrawn from each subject. Serum samples were obtained after clotting of the blood. For PCR testing, serum samples were freeze-stored until the time of assay. All samples were subjected to the following laboratory investigations:

1-Complete blood count using automated cell counter Cell-DYN170 (Abbott, USA).
2-Liver function tests including, alanine aminotransferas (ALT), aspartate aminotransferase (AST) total bilirubin,direct bilirubin, and serum albumin (Cobas integra autoanalyzer, Roche). Alpha-fetoprotein was assessed by enzyme linked fluorescent assay (ELFA) using Vidas autoanalyzer, according to the manufacture's instructions (Biomerieux, France).
3-Viral hepatitis markers: HCV antibodies were assessed by microparticle enzyme immunoassay (MEIA) and HBsAg by chemiluminescent immunoassay (AXSYM (Abbott laboratories, USA) according to the manufacture’s instructions.
4-Quantitative real-time PCR: Quantitative PCR was performed for chronic HCV patients by TaqMan amplification system with an internal positive control for HCV RNA quantitation according to manufacture instructions (Applied Biosystem).
5-Flow cytometry analysis: T_{reg} cells were assessed by flow cytometry using FACS Calibur (BD Bio-sciences, San Jose’s, CA, USA).

Monoclonal antibodies against CD4 and CD25 were purchased from IQ Product (Groningen, Netherlands), 100 µl of whole blood was incubated with 20 µl FITC-conjugated anti-CD25 monoclonal antibodies (mAb) and PE-conjugated anti-CD4 (mAb) for 20 min at room temperature, in the dark. This was followed by red cell lysis and wash. Cells were then re-suspended in 300 µl of PBS. Data were analyzed using Paint-A-Gate software, the typical forwards and side scatters were used for lymphocytes gating. CD4CD25^{high} T cells were assessed as percentage of gated CD4^{+} lymphocytes. High CD25 expression (CD25^{high}) was defined at a threshold where most CD4-negative cells lose CD25 expression according to Baecher-Allan et al., (2000).

**Statistical analysis**

Data were analyzed using computer program SPSS, ver.11. Values were given as mean ± SD. Statistical analysis was performed using t-test. Correlation analysis was carried out using Spearman’s test and P-values less than 0.05 were considered to be significant.

**RESULTS**

**Studied HCV-population characteristics**

Clinical characteristics and laboratory parameters of the three studied groups enrolled in this study are listed in (Table 1). Data analysis demonstrated a statistical significant difference in the liver function of the three studied groups when compared with controls. The hematological features of the three studied groups, including white blood cells, haemoglobin and platelets showed a statistical significant difference when compared with controls.

**Frequency of CD4CD25^{high} T cells in the studied groups**

In control subjects, the percentage of CD4CD25^{high} T cells ranging between 1-3.5% (2.3 ±0.8), while for
CHCV patients, HCV-related cirrhosis patients and HCC patients the percentages were 2.5-5% (3 ±0.9), 2.3-4.7% (3 ±0.8) and 2-8% (3.9 ±1.6), respectively as shown in (Table 2). A significantly higher proportion of CD4CD25\textsuperscript{High} T cells were found in those with chronic infection when compared with normal controls (P ≤0.034). Also, comparing HCV related cirrhosis or HCV related HCC patients with controls showed a statistical significant differences (P ≤0.023) and (P ≤0.001) respectively. No statistical significant differences were detected when comparing HCV related cirrhotic patients with chronic HCV infected patients (P ≤0.95).

Meanwhile, high frequency of CD4CD25\textsuperscript{High} T cells of HCV related HCC patients (Table 3) showed a statistical significant differences when compared with chronic HCV patients (P ≤0.047) or HCV-related cirrhosis patients (P ≤0.036). The flow cytometric analysis of CD4CD25\textsuperscript{High} T cells for a control subject and a patient of each group are shown in Figure 1. A significant positive correlation between the frequency of CD4CD25\textsuperscript{High} T-cells and HCV viral load of chronic hepatitis C group was also detected (r = 0.47, p<0.05) (Figure 2).

Table (1): Clinical characteristics and laboratory parameters of study population.

<table>
<thead>
<tr>
<th>Variables</th>
<th>CHCV N</th>
<th>CHCV %</th>
<th>Cirrhosis N</th>
<th>Cirrhosis %</th>
<th>HCC N</th>
<th>HCC %</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk factors:</td>
<td></td>
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<tr>
<td>Blood transfusion</td>
<td>3</td>
<td>15</td>
<td>3</td>
<td>15</td>
<td>3</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Anti-Bilharzial</td>
<td>4</td>
<td>20</td>
<td>12</td>
<td>60</td>
<td>11</td>
<td>55</td>
<td>0</td>
</tr>
<tr>
<td>Previous surgery</td>
<td>3</td>
<td>15</td>
<td>3</td>
<td>15</td>
<td>4</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Tattoos</td>
<td>1</td>
<td>5</td>
<td>3</td>
<td>15</td>
<td>3</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Tooth extraction</td>
<td>14</td>
<td>70</td>
<td>5</td>
<td>25</td>
<td>4</td>
<td>20</td>
<td>0</td>
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<td>Liver function:</td>
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<tr>
<td>ALT(U/L)</td>
<td>45.3 ±11\textsuperscript{§}</td>
<td>55.2 ±23\textsuperscript{§}</td>
<td>49 ±130\textsuperscript{§}</td>
<td>16±7</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>AST(U/L)</td>
<td>58.7±14\textsuperscript{§}</td>
<td>64.1±21\textsuperscript{§}</td>
<td>123±263\textsuperscript{§}</td>
<td>18±7</td>
<td></td>
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<tr>
<td>T-Bilirubin(mg/dl)</td>
<td>0.6±0.8</td>
<td>3.2 ±1.9\textsuperscript{§}</td>
<td>2.9 ±1.3\textsuperscript{§}</td>
<td>0.6±0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Bilirubin(mg/dl)</td>
<td>2.0±0.2\textsuperscript{§}</td>
<td>1.7±1.3\textsuperscript{§}</td>
<td>1.1±1.0\textsuperscript{§}</td>
<td>0.08±0.03</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Albumin(g/l)</td>
<td>4.0±0.7</td>
<td>2.4±1.3</td>
<td>3.3±0.4\textsuperscript{§}</td>
<td>4.0±0.3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Haematological parameters:</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Hemoglobin(g/dl)</td>
<td>11.9±3.2</td>
<td>10.3±2.3\textsuperscript{§}</td>
<td>9.7±1.9\textsuperscript{§}</td>
<td>14.9±0.8</td>
<td></td>
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<td></td>
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<tr>
<td>WBCs (cells/ul)</td>
<td>6625±1657</td>
<td>9910±8679</td>
<td>5605±2384</td>
<td>5910±1848</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Platelets countx10\textsuperscript{3}</td>
<td>130±78\textsuperscript{§}</td>
<td>105±41\textsuperscript{§}</td>
<td>80±74\textsuperscript{§}</td>
<td>265±64</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral load:(IU/mlx103)</td>
<td>1313±146</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

\# Number of individuals/HCV groups= 20.
\* Significant p < 0.05 compared with Controls.
\textsuperscript{†} Significant p < 0.01 compared with Controls.
\textsuperscript{§} Significant p < 0.001 compared with Controls.

Table (2): Frequency of CD4CD25\textsuperscript{High} T cells in the studied groups compared with controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control group</th>
<th>CHCV group</th>
<th>Cirrhotic group</th>
<th>HCC group</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Treg cells</td>
<td>1-3.5% (2.3±0.8)</td>
<td>2.5% (3.0±0.9)</td>
<td>0.034</td>
<td>2.3-4.7% (3.0±0.8)</td>
</tr>
</tbody>
</table>
Table (3): Illustrate the 2-tailed significance values of t-test of the frequency of CD4CD25\(^{\text{High}}\) T cells in the studied groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>CHCV group</th>
<th>Cirrhotic group</th>
<th>HCC group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCV group</td>
<td></td>
<td>0.95</td>
<td>0.047</td>
</tr>
<tr>
<td>Cirrhotic group</td>
<td>0.95</td>
<td></td>
<td>0.036</td>
</tr>
<tr>
<td>HCC group</td>
<td>0.047</td>
<td>0.036</td>
<td></td>
</tr>
</tbody>
</table>

Figure (1): Flow cytometry analysis of CD4CD25\(^{\text{High}}\) T cells.

a) Representative dot plots of CD4CD25\(^{\text{High}}\) T cells in chronic hepatitis subject C
b) Representative dot plots of CD4CD25\(^{\text{High}}\) T cells in HCV related cirrhotic subject.
c) Representative dot plots of CD4CD25\(^{\text{High}}\) T cells in HCV related HCC subject
d) Representative dot plots of CD4CD25\(^{\text{High}}\) T cells in normal healthy subject.


**DISCUSSION**

About 3% of the world populations are HCV carriers, a proportion of whom will eventually develop cirrhosis, cancer, or liver failure. Persistent HCV infection is associated with a weak, narrow cell-mediated immune response that is characterized by a low frequency, typically <0.3%, of HCV-specific IFN-γ-producing T cells (Cooper et al., 1999, Thimme et al., 2001, Chisari, 2005) and an even lower frequency of IL-2-producing cells (Semmo et al., 2005).

In this study, the frequency of CD4CD25High T-cells in peripheral blood of patients with different clinical presentation of chronic hepatitis C virus infection was found to be increased. These results agreed with that reported by Sugimoto et al., (2003) and Cabrera et al., (2004). They reported the presence of a higher proportion of circulating CD4CD25+ cells in the blood of HCV carriers than in convalescent patients and uninfected control individuals. Also, Rushbrook et al., (2005) and Ward et al., (2007) found a much higher percentages of CD4CD25+ cells in the infected liver than in the blood. Dolganiuc et al., (2008) indicated a role for myeloid dendritic cells in expansion of Treg to promote chronic infection of patients with HCV. The CD4CD25+ T-cell fraction isolated from PBMC of infected patients suppressed virus-specific CD8+ T-cell proliferation and IFN-γ production, and depletion of these cells resulted in increased IFN-γ production by the remaining cells in response to HCV proteins (Boettler et al., 2005 and Rushbrook et al., 2005). These studies suggested an important role for Treg in establishing and/or maintaining HCV persistence.

In contrast to the results described above, Treg deficiency was reported in patients with persistent HCV infection. These patients often develop mixed cryoglobulinemia (MC), an autoimmune B-cell proliferative disorder (Boyer et al., 2004). In a comparison of HCV/MC patients to healthy donors, circulating Treg frequency was found to be significantly reduced in HCV/MC patients, and these Treg showed reduced immunosuppressive activity. In addition, Ormandy et al., (2005) reported absences of any significant differences in the number of regulatory T cells between healthy controls and patients with chronic HCV/HBV infection.

Comparing HCV related cirrhotic patients with chronic HCV infected patients, we did not find any statistically significant differences in the two groups, these results agreed with the finding of Mengde et al., (2007) who reported that the presence or absence of cirrhosis did not change the level of CD4CD25+ T cells in patients with chronic HCV infection. However, Delhem et al., (2008) evaluated the existence of Treg cells in liver biopsies of chronically infected patients including patients without liver lesions, with cirrhosis and with Hepatocellular carcinoma of HCV-1b infected patients. They observed installation of Treg cells in the liver of chronically infected patients, with increased frequency in cirrhosis and HCC; that suggested a significant role for these cells in the aggravation of liver pathology. The contradiction between these results may
be attributed to a compartmentalization of T_{reg} cells to liver, the site of infection.

A significant increase of circulating regulatory T cells was seen in HCV related HCC patients compared with chronic HCV patients. These results agreed with the data reported by Mengde et al., (2007). They showed that T_{reg} are increased in peripheral blood mononuclear cells from HCC patients and positively correlated with tumor burden. This also agreed with the data reported by Ormandy et al., (2005) & Yang et al., (2006) that the frequencies of T_{reg} are increased in both peripheral blood and the tumor microenvironment in HCC, and T_{reg} from HCC can suppress proliferation and perforin expression of autologous T cells in vitro.

T_{reg} have also been suggested to impede immune surveillance against cancer. (Linehan and Goedegebuure 2005). Their inactivation improves tumor-specific immunity and leads to more efficient tumor rejection (Yu et al., 2005). In addition, Sasaki et al., (2008) indicated that the high number of hepatocellular carcinoma -infiltrating T_{reg} cells is an independent predictive factor of tumor recurrence after hepatic resection for HCC patients. The increase of the peripheral blood T_{reg} cells may not be related to the aggravation of liver pathology in HCV infection, this may be explained by Mengde et al., (2007) who reported that HCC culture supernatants promoted expansion of T_{reg} and enhanced their suppressive function. This supports the hypothesis that the proliferation of T_{reg} is tumor driven through the secretion of soluble factors and explains absence of their increase in HCV related cirrhosis.

The significant positive correlation between the frequency of T_{reg} and HCV viral load of chronic hepatitis C patients agrees with the results reported by Cabrer et al., (2004). In contrast to previous study, Zarife et al., (2009) observed a parallel increase of CD4CD25^{high} T-cells in the low viral load blood donors; they suggest that CD4CD25^{high} T-cells might play an important role in controlling viremia.

In conclusion, our results indicated that the higher frequency of peripheral blood CD4CD25^{high} regulatory T cell in Egyptian patients with different clinical presentation of chronic hepatitis C virus infection , that was not related to the aggravation of liver pathology and its positive correlation with HCV viral load further.

REFERENCES


Received July 10, 2012
Accepted September 12, 2012