Association of T_{reg} and TH_{17} Cytokines with HCV Pathogenesis and Liver Pathology

Amal A Elkhawaga[1], Amal Hosni[2], Doaa Z. Zaky[3], Amira A. kamel[4], Nahed A Mohamed[4], Mohamed A. A. Abozaid[5], Muhammad A. El-Masry[5]

Departments of [1]Medical Microbiology & Immunology, [2]Clinical Pathology, [4]Medical Biochemistry, [5]Internal Medicine, Faculty of Medicine, Assiut University, Assiut, Egypt and [3]Department of Tropical Medicine, Faculty of Medicine, Ain Shams University, Cairo, Egypt.

Chronic hepatitis C (CHC) infection is considered a high risk for development of end-stage liver diseases, particularly server hepatitis, decompensated liver cirrhosis, and hepatocellular carcinoma. Regulatory T cells (Treg) and T-helper 17 (TH17) associated cytokines presumed to play a pivotal role in the immune pathogenesis of HCV infection and stimulate autoimmune diseases. Herein, we tried to assess the association of Treg and TH 17 cytokines with HCV pathogenesis and liver pathology. Fifty CHC infected patients and twenty HCV free controls were included in this study, IL17, IL21, IL10, IL4, TGF-β and IL35 serum levels were assessed in both groups using enzyme linked immunosorbent assay (ELISA). CHC infected patients had statistically significant higher values of all serum cytokine levels when compared to the control group (P<0.0001) for each. Additionally, serum levels of IL17, IL10 and IL35 were positively correlated with viral load. Also, the serum level of IL17 IL21, IL10 and IL35 was positively correlated with ALT serum levels. Only IL21 and IL10 were positively correlated with AST levels. Serum IL17, IL10, TGF-β and IL35 levels were significantly elevated in CHC patients with advanced fibrosis stages. We concluded that CHC infected patients displayed high serum levels of Treg and TH17 associated cytokines. Collectively, these results support the hypothesis that liver damage in CHC infection might be due to an immune-mediated destructive mechanism rather than to the direct cytopathic effect of the virus itself.

Chronic hepatitis C (CHC) virus remains a very strong topic for studying throughout the world. It is one of the major causes of chronic liver disease globally including liver inflammation, liver cirrhosis and hepatocellular carcinoma. Around 170 million individuals worldwide become infected with hepatitis C virus (HCV) infection [1-6]. Egypt reported the highest prevalence of HCV infection in the world with an estimated prevalence of 14. 7% in 2015 [7]. In addition, HCV genotype 4 is the most predominant isolated genotype from 90% of the HCV-infected patients in Egypt [8-12].

The adaptive effector CD4+ T helper 1 and TH 17-mediated immune response is highly heterogeneous, based on the development of special subsets that present different profiles of cytokine output [13]. Cytokines are regulatory molecules that play a major role in physiological and pathological processes [14].

IL-17A (IL-17) and IL21 are T cell–derived cytokines produced by Th17 CD4 T cells specifically memory CD4+ T cells. Moreover, IL-17 is produced by a vast variety of cell types, including neutrophils, CD8+T cells, γδ T cells, NKT cells and Tregs [15].

TH17 cytokines is a powerful chemoattractant for neutrophils and has been

involved in many immune pathways, they are most prominent in inducing and mediating proinflammatory immune responses e.g. several autoimmune diseases, asthma, allergic diseases and pulmonary infection [13]. Also, IL-17 and Th17 seem to have a remarkable role in viral infections and stronger Th17 responses are associated with higher viral plasma load, elevated levels of serum transaminases, and enhanced activation of liver macrophages as well as blood monocytes[16-19].

Regulatory T (Treg) cells play an important role in the homeostasis of immune system [20-23], via preventing the development of autoimmune diseases. However, in the case of HCV infection, Treg levels are increased and favor persistence of the virus[24, 25].There is growing evidence that Treg cells secrete pro-inflammatory cytokines in many inflammatory conditions [26, 27].

Herein, we aimed to investigate the association of the serum levels of Treg and TH17 cytokines with HCV pathogenesis and liver pathology among chronic HCV infected patients.

Patients and Methods

Patients enrolled

This study includes 50 patients with CHC infection and hospitalized at the Department of Internal Medicine, Division of Gastroenterology, Faculty of Medicine-Assiut University. The study protocol was reviewed and approved by Assiut University Faculty of Medicine Medical ethics Committee (IRB no: 17300297) and an informed written consent was taken from all the participants in the study.

All patients were subjected to history taking and clinical examination and abdominal ultrasonography. HCV infection was serologically examined in all participants by Monolisa HCV Ag/Ab ULTRA (Bio-Rad, Marnes la Coquette, France). Quantitative determination of HCV RNA was analyzed by a reference method using Qiagen Gmbh, (Germany) and analyzed by 7500 fast real-time PCR, Applied Biosystems.

Liver function tests were evaluated using Cobas Integra 400 Chemistry Analyzer (Roche Diagnostics GmbH, Mannheim, Germany). The liver stiffness measurements were done by Fibroscan® (EchoSens). All these blood tests were carried out according to relevant manufacturer's instructions.

Exclusion criteria included patients with hepatitis B virus infection, patient with HIV infection, patients who had received antiviral drugs, steroids, or immune suppressive drugs, patients who had other causes of chronic liver injury such as alcohol intake and pregnancy.

In addition, the study included 20 apparently healthy blood donors as a control group. They were negative for known serological markers of HIV, HCV and HBV infections including hepatitis B surface antigen (HBsAg) and anti-HCV antibody.

Methods

Ten ml blood was obtained from each subject into sterile tubes without any anticoagulant. Blood samples were spun down at 500 $_{X}$g for 10 minutes. The Serum was divided into aliquots, labeled appropriately and stored at -20 ^{0}C until used.

- Cytokines assay

IL4, IL10, TGF-β, IL17 and IL21 serum levels were measured using ELISA kits (RayBiotech, USA) while IL35 serum level was measured using ELISA kit (USCN Life Science, US) according to the manufacturer' instructions. Concentrations were calculated from standard curves using recombinant proteins, provided in the kits and expressed in pg/ml.

Statistical Analysis

Data are presented as range (minimum, maximum); and mean±SD. Continuous variables ALT, AST, were expressed as the arithmetic mean±SD, and were compared between the enrolled patients using Student's t test, or Wilcoxon Rank Sum Test, as appropriate with a significance value at $P \leq 0.05$. Correlations between parameters measured were calculated using Spearman's correlation coefficient. All statistical analyses were completed with the help of Graph Pad Prism 7 Software (San Diego, California, USA).

Results

Demographic and laboratory data

This study includes 50 CHC infected patients. 32(64%) males and 18 (36%) females with mean age of 52.93±12.77, ALT mean level of 34.90±8.76 and AST mean level of 56.33±15.32. Control group was formed of 7 (35%) females and 13(65%) males with a mean age of 40.45±10.66, ALT mean of 20.65±5.65 and AST level mean 22.55±7.80. Demographic and clinical characteristics of the subject groups were summarized in table (1).

Table 1. Demographic and clinical characteristics of the studied groups

	Chronic HCV patients N=50	Healthy Controls N=20	P-value
Age (mean±SD)	52.93 ±12.77	40.45 ±10.66	*P* < 0.0001
Gender			
Male: Female	32(64%):18 (36%)	13(65%):7 (35%)	*P* >0.9
ALT(mean±SD)	36.9±8.7	19.35±5.65	*P*< 0.0001
AST (mean±SD)	56.33±15.2	22.55±7.80.	*P* < 0.0001
HCV viral load [10^6 IU/ml, mean±SD)	1.3±1.2	na	na
Distribution of liver fibrosis stage			
Stage 1-2	18	na	na
Stage 3-4	32		
Distribution of liver inflammatory grade			
Grade 1-2	20	na	Na
Grade 3-4	30		

ALT; alanine transferase. AST; aspartate transferase Alb; albumin, na=not applicable. *P*≤0.05 is significant.

Serum levels of Treg and TH17 associated cytokines in the study groups

Table (2), shows a comparison between the serum levels of different cytokines in patients and the control group. CHC infected patients had statistically significant higher values of all serum cytokine levels when compared to the control group (*P*<0.0001) for each.

Table 2. Serum cytokine levels in chronic HCV infected group and the control group.

	Chronic HCV patients N=50	Controls N=20	*P* value
IL17a (mean±SD) Pg/ml	40.41±39.50	1.98±6.09	*P* < 0.0001
IL21 (mean±SD) Pg/ml	144.65±60.66	132.93±12	*P* < 0.0001
IL4 (mean±SD) Pg/ml	120± 18.69	40.64±29.55	*P* < 0.0001
TGF-β (mean±SD) Pg/ml	109.75±45.33	80.43±40.97	*P* < 0.0001
IL10 (mean±SD) Pg/ml	26.45±9.75	17.10±5.44	*P* < 0.0001
IL35 (mean±SD) Pg/ml	40.64±12.54	23.44±3.39	*P* < 0.0001

Mann-Whitney Test was used to compare serum levels of cytokines between the two study groups.

Data are represented as means ± SEM. *P*≤0.05 is significant.

Association of serum cytokine profile with different laboratory parameters in CHC infected patients

Serum levels of IL17, IL10 and IL35 was positively correlated with viral load (r =0.4, *P* = 0.001), (r = 0.49, *P*= 0.002) and (r = 0.49, *P*= 0.002). Also, the serum level of IL17, IL21, IL10 and IL35 was positively correlated with ALT (r =0.32, *P* = 0.04), (r = 0.44, *P*= 0.01), (r= 0.35, *P* = 0.03) and (r = 0.4, *P* = 0.02). Only IL21 and IL10 was positively correlated with AST levels (r = 0.35, *P*= 0.02) and (r = 0.49, *P* = 0.001).

However, no significant correlation was found between serum level of IL4 and TGF-β and any of the liver enzymes (ALT, AST) and HCV viral load (Table 3).

Table 3. Correlation between cytokine levels and ALT, AST and HCV viral load.

Parameter	ALT		AST		Viral Load	
	r	*P* value	r	*P* value	r	*P* value
IL17	0.32	0.04	0.12	NS	0.4	0.001
IL21	0.44	0.01	0.35	0.02	0.21	NS
IL10	0.35	0.03	0.49	0.001	0.49	0.002
IL35	0.4	0.02	0.24	NS	0.39	0.03

r = spearman coefficient *P*>0.05 is not significant NS).

Association between serum cytokines profile and liver fibrosis among CHC infected patients

The serum levels of IL17a, TGF-β, IL10 and IL35 was significantly higher in chronic HCV patients at stage 3-4 when compared to CHC at stage 1-2 (P = 0.01, P = 0.03, P = 0.008 and P = 0.01) respectively Figure (1) a, b, c, d.

However, no significant difference was found in the serum levels of IL4 and IL21 among HCV patients with different fibrosis stages.

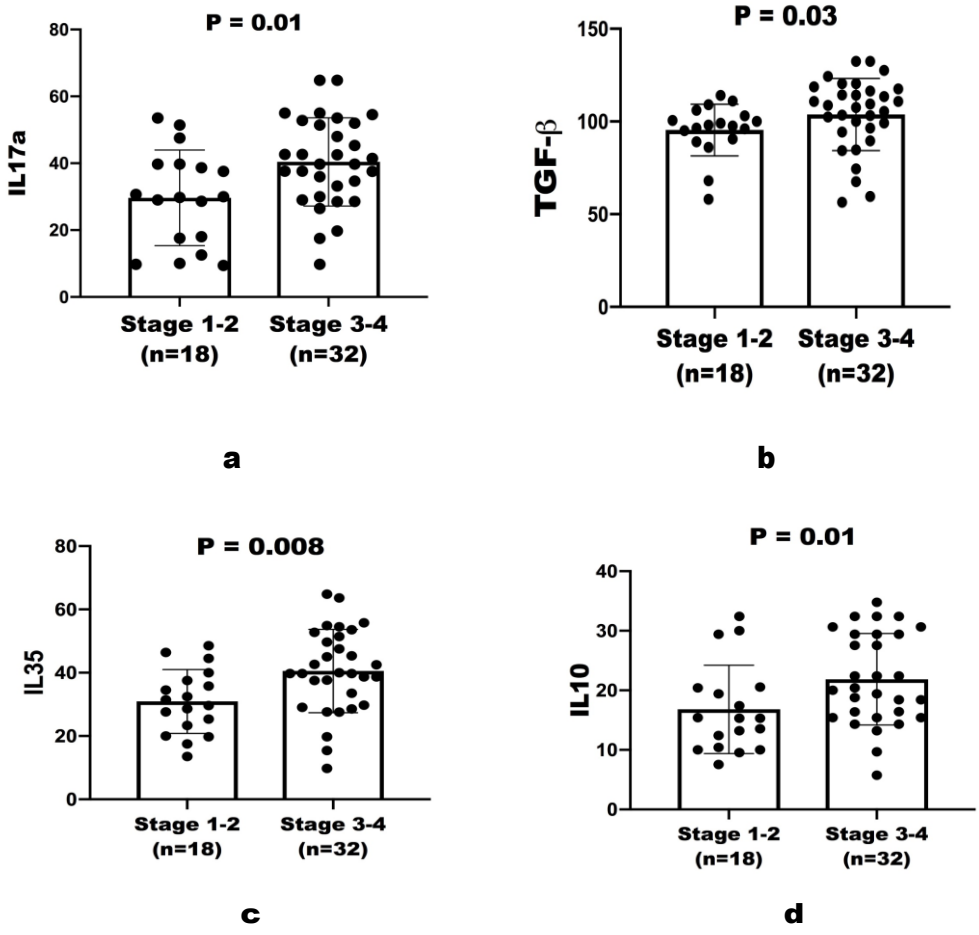

Figure 1. Serum levels of different cytokines among chronic HCV infected patients with different liver fibrosis stages.

Discussion

HCV is one of the inflammatory liver disease and a leading cause of chronic hepatitis, cirrhosis, liver cancer, and is a primary indication for liver transplantation [28-30]. Changes in various cytokine activities that occur during the inflammatory response against HCV and other hepatitis

viruses is responsible for variable degree of liver damage[31-34]. The present study was performed to investigate the involvement of the Th17 and Treg cells associated cytokines in immune response by determining serum levels of the Treg and TH17 associated cytokine, in patients with chronic hepatitis C and compare such data to these of a control group.

Previous studies showed that plasma cytokines levels are altered during HCV infection compared to uninfected control group [35, 36]. The chronic HCV infected patients in this study displayed elevated levels of IL-17a and IL21 in comparison to controls with a significant association between their levels and ALT, AST levels and viral load. These results were compatible with a previous study[19]. Moreover, in a recent study it was demonstrated that untreated CHC patients have higher serum IL-21 levels than uninfected adults [37], confirming that IL-21 may compromise host immune responses to the virus. IL-21 is an important component of TH17cells in specifically sustaining C8+ T cell effector activity during chronic viral infection.

Also, our results are in agreement with finding of other studies, indicating that increasing circulating Th17, intrahepatic IL-17 positive cells, and HCV- specific Th17 cells were correlated with severity of liver inflammation in chronic HCV patients [38],[39]. A previous study by Jimenez-Sousa (2010) showed that IL-17 and Th17 have an important role in viral infections and stronger Th17 responses are associated with higher viral plasma load, increased levels of ALT and AST, and enhanced activation of blood monocytes as well as liver macrophages [39].

We observed a significant correlation between the serum concentration of IL-17 and IL21 and that of some indicators of liver function e.g. ALT, AST, viral load and the degree of liver fibrosis. Such findings, suggest that TH17 associated cytokines may be associated with the extent of liver damage and liver fibrosis. In addition, the significant correlation between serum IL-17 and ALT is in accordance with several studies which showed that IL-17 level correlated directly with severity of liver inflammation. Our results are in agreement with previous studies which reported a significant positive correlation with ALT serum level [38, 40]. Previous studies have reported pivotal role of the Th17/IL-17 axis in liver fibro genesis However, the stimuli that attract Th17 cells to the liver are not completely elucidated.

IL10 is predominately secreted by immune cells including T lymphocytes, macrophages and natural killer (NK) cells and regulatory cells including T and B cells[41]. In the current study CHC patients presented higher serum levels of IL10 with association with viral load. Our results came in accordance with several previous studies [42-44]. These results suggest that elevation of serum IL-10 might be involved in downregulation of the inflammatory response in chronic liver disease [42]. The association between IL10 serum levels and ALT, AST and degree of liver fibrosis is an index to reflect the degree of inflammation in the liver[45].

In the present study, we observed that IL-35 was elevated in the serum of patients with chronic hepatitis C when compared to the control group. Moreover, the elevation of IL35 serum levels was significantly associated with ALT, AST and viral load. Such findings may indicate the involvement of IL-35 in the pathogenesis of HCV related liver diseases [46].

Our results revealed that, serum levels of TGF-β1 were significantly higher in HCV

patients compared with control subjects. In addition, TGFβ1serum levels were positively correlated with stage of liver fibrosis. Our results came in accordance with previous studies that reported increased serum levels of TGF-β1 in HCV-related chronic liver disease and positively correlated with the stage of liver fibrosis [47]. These data show that serum TGF-β1 is a promising marker of hepatic fibrosis in chronic HCV patients.

In conclusion, these results support the hypothesis that liver damage in CHC infection may be due to an immune-mediated destructive mechanism rather than to the direct cytopathic effect of the virus itself.

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