Serum levels of interleukin 17 and its activators in chronic hepatitis C patients

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Abstract

Immune dysregulations in patients with chronic hepatitis C are still poorly understood, particularly their inflammatory pathways. Recent studies have shown that transforming growth factor β (TGF-β) alone promotes the generation of anti-inflammatory regulatory T lymphocytes (T-reg) from naïve T CD4+ cells, while TGF-β in concert with interleukin 6 (IL-6) promotes Th17 cells that produce IL-17. We studied serum levels of IL-17 and its known activators (IL-6, TGF-β) in 55 hepatitis C patients and 33 age- and gender-matched healthy subjects. Cytokine levels were quantified by sandwich enzyme-linked immunosorbent assays. Surprisingly, IL-17 serum levels were significantly higher in healthy subjects than in hepatitis C patients (16.5 ±6.6 pg/ml vs. 8.1 ±5.5 pg/ml; p < 0.0000). By contrast, serum TGF-β levels were significantly lower in healthy subjects (30.9 ±7.4 ng/ml vs. 40.7 ±20.6 ng/ml; p = 0.0327). Serum levels of IL-6 did not differ between groups but correlated positively with the stage of fibrosis in hepatitis C patients (r = 0.36; p = 0.005). Our findings suggest that hepatitis C virus down-regulates IL-17 production and up-regulates TGF-β production. The increased level of TGF-β in HCV patients may be responsible for suppressing IL-17 production by Th17 cells.

Key words: hepatitis C, interleukin 17, TGF-β.

Introduction

The hepatitis C virus (HCV) is an RNA virus that primarily infects hepatocytes and induces an immune-related necroinflammatory hepatic reaction, frequently leading to significant fibrosis or cirrhosis. While HCV-specific Th-1 response is associated with at least transient control of viral replication and Th-2 response is linked with viral persistence, many aspects of immune dysregulations in hepatitis C are still unclear, especially the inflammatory pathways [1].

Recently described novel lineage of interleukin 17 (IL-17) secreting CD4+ T cells seems to be a fascinating link between HCV and mechanisms of immune regulations. Th-17 cells are the primary pathogenic T cells in organ-specific autoimmune diseases [2, 3]. They also serve a protective role against certain bacterial and fungal infections by promoting chemokines and proinflammatory cytokines with concurrent recruitment and activation of macrophages and neutrophiles. Thus, the inflammatory functions of Th-17 cells are important for microorganism's clearance, and in specific conditions these cells may also damage host tissues.

Several groups of investigators recognized a dichotomy in the generation of T cells, showing that transforming growth factor β (TGF-β) alone promote the generation of anti-inflammatory regulatory T cells from naive CD4+ T cells, whereas TGF-β in the presence of IL-6 promote 17 Th17 cells (Fig. 1) [4-6].

Until this time, there has been no investigation of the role of IL-17 in HCV infected patients, especially in relation to hepatic inflammation and fibrosis. To address this issue we studied serum levels of IL-17 and its activators (IL-6, TGF-β) in chronic hepatitis C patients and age and gender-matched healthy subjects.

Material and methods

The study group comprised 55 chronically HCV infected patients aged 20-69 years, including 30 women and

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25 men. All individuals were tested for antibodies to HCV using a third-generation enzyme immunoassay, which was confirmed with a positive result for HCV RNA by a qualitative RT-PCR. Liver biopsies were performed employing the Menghini technique under local anesthesia of 2% lidocaine solution with the use of HEPAFIX® G16 (1.6 mm) needles, produced by B. Braun Melsungen AG, Melsungen 34209, Germany. Microscopic evaluation of liver tissue specimens was performed by an experienced pathologist using an 18 point Knodell scale modified by Ishak to evaluate the necro-inflammatory activity (*Histological activity index* – HAI, *grading*) and 6 grade Ishak scale for the degree of fibrosis evaluation (*staging*).

The control group consisted of 33 healthy volunteers (21 women, 12 men, aged 20-62 years). In all of them acute and chronic diseases were excluded based on anamnesis, results of blood morphology, concentration of C-reactive protein and serum level of alanine and aspartate aminotransferase which were within reference ranges.

## Sample collection and storage

IL-6 and IL-17

From each patient we took 6 ml of peripheral blood sample into a serum separator tube and allowed it to clot for 30 minutes at room temperature before centrifugation for 15 minutes at $1000 \times$ g. Then, we removed serum immediately, separated into Eppendorf tubes and stored samples at –70˚C.

TGF-β

From each patient we took 6 ml of peripheral blood sample into a serum separator tube and allow it to clot for 30 minutes at room temperature. For complete release of TGF-β, samples were incubated overnight at 2-8˚C before centrifugation. Than we centrifuged collected samples for 15 minutes at $1000 \times$ g, removed serum and stored at –70˚C.

Quantification of cytokines

All cytokines were quantified in serum by sandwich ELISA (R&D Systems, Minneapolis, MN, USA). The assay employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6, IL-17 or TGF-β has been pre-coated onto a microplate. Standards, controls and samples were pipetted into the wells and any IL-6, IL-17 or TGF-β present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6, IL-17 or TGF-β was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color develops in proportion to the amount of IL-6, IL-17 or TGF-β bound in the initial step. The color development was stopped and the intensity of the color was measured spectrophotometrically (450 nm wave), by microplate reader *μQiand* of Bio-Tek Instruments.
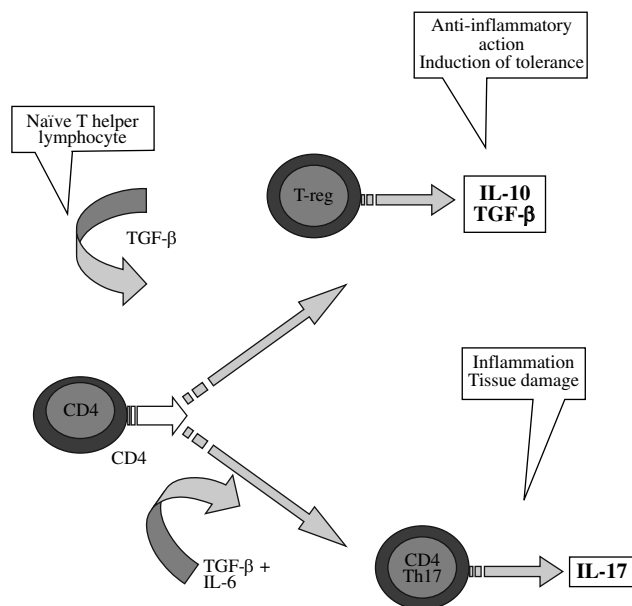
Fig. 1. Activation of naïve T helper lymphocyte

Statistical methods

All data were analyzed using the STATISTICA package (version 8) and were presented as medians, means, and standard deviations. Mann-Whitney test was used for comparison of serum cytokine concentrations between analyzed groups and Spearman rang correlation coefficient *r* for correlations between grading and staging and serum cytokine concentrations. A *p*-value of less than 0.05 was considered statistically significant.

Ethics

Appropriate informed consent was obtained from each participant included to the study. The study protocol was approved by the appropriate local ethics committee and conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008).

Results

We found, that IL-17 serum levels were significantly higher in healthy subjects than in HCV patients (16.5 ±6.6 vs. 8.1 ±5.5; *p* = 0.000) (Fig. 2).

By contrast, in our control group serum TGF-β levels were significantly lower than in HCV patients (30.9 ±7.4 ng/ml vs. 40.7 ±20.6 ng/ml; *p* = 0.0327) (Fig. 3).

We did not find any important correlations between grading and cytokine serum levels in HCV infected patients, but we found positive correlation between serum IL-6 level and the stage of liver fibrosis in HCV cohort (*r* = 0.36; *p* = 0.005) (Table 1).
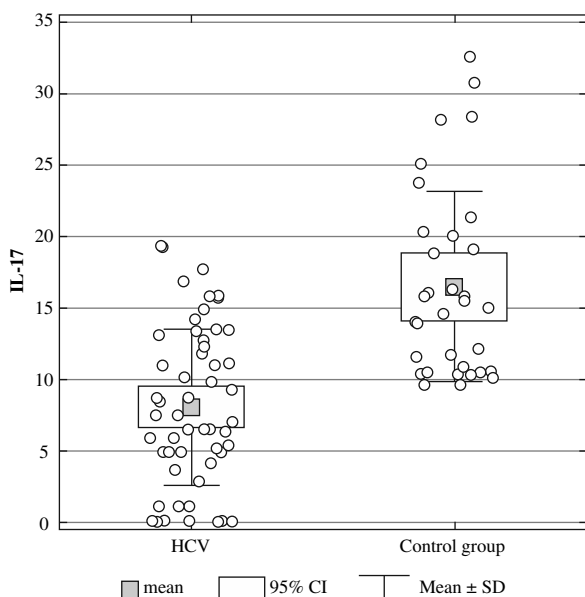
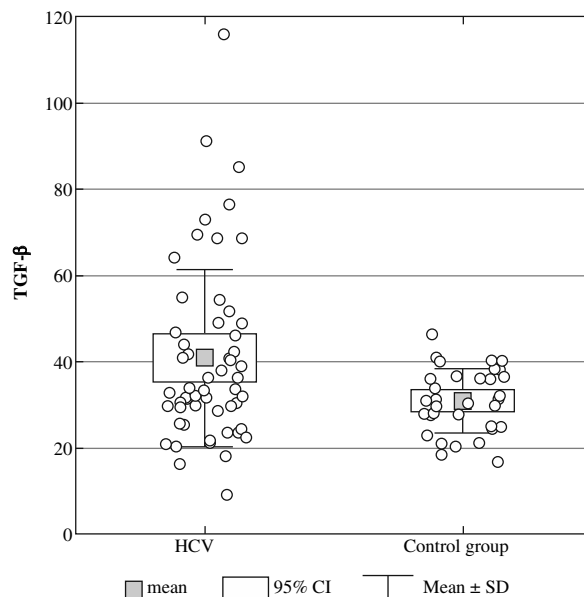**Fig. 2.** Serum concentration of IL-17 in HCV and control group

Fig. 3. Comparison of serum concentration of TGF-β in HCV and control group

Table 1. Correlations between cytokine concentrations and inflammatory and fibrosis indexes

Cytokine concentration	Index	
	grading	staging
IL-6 [pg/ml]	0.08	0.36
IL-17 [pg/ml]	–0.11	0.06
TGF-β [ng/ml]	0.10	–0.05

Discussion

Our data showed that serum IL-17 levels were substantially higher in healthy subjects than in HCV patients. This finding also demonstrates that serum level of IL-17 in hepatitis C patients behaves differently than in other chronic liver diseases. Patients with autoimmune hepatitis or alcoholic liver disease have elevated levels of this interleukin [7, 8]. Rowan *et al.* [9] provided a possible explanation for this complex phenomenon. These authors reported for the first time, that antigen-specific Th-17 cells are promoted in hepatitis C patients, however, TGF-β and IL-10, which are induced by the viral nonstructural protein 4 (NS4), effectively suppress Th-1 and Th-17 responses. Thus, the NS4 protein may promote differentiation of naïve T cells into Th-17 but would inhibit expansion of Th-17 cells. The Rowan's *in vitro* data on HCV-specific CD4+ T cells also show that the prevailing effect of the TGF-β and IL-10 (induced by NS4 protein) is suppression of cytokine production by memory Th-17 and Th-1 cells [9]. These find-

ings are consistent with the immune-subversive role of the NS4 protein in promoting chronic infection with HCV by suppressing virus-specific Th-1 and Th-17 cells.

In our study, serum TGF-β levels were significantly lower in healthy controls than in hepatitis C patients. It is currently known that the role of TGF-β in induction or suppression of human Th-17 cells depends on its concentration. Zhou *et al.* [10], demonstrated that low concentrations of TGF-β (combined with IL-6 and IL-21) can enhance IL-23R expression and Th-17 differentiation, whereas high concentrations of TGF-β suppress IL-23R and promote Foxp3 expression that inhibits RORγt and differentiation of Th-17 cells. In contrast, the neutralization of TGF-β enhances virus-specific memory Th-17 cells *in vitro* [9]. In view of Rowan's and Zhou's data our results suggest that increased production of TGF-β in hepatitis C patients may be one of important factors responsible for suppression of the IL-17 pathway.

In the analyzed group of HCV infected patients we did not find any correlations between serum levels of IL-17 and liver inflammation activity or fibrosis stage. The lack of such relationships may result from suppression of Th-17 cells. In contrast, naïve autoimmune hepatitis patients exhibit strong negative correlation between serum IL-17 level and activity of hepatic inflammation [7]. Low serum levels of IL-17 in autoimmune hepatitis predict high inflammatory activity within the liver due to hepatic recruitment of Th-17 cells.

Many biological effects of IL-6 depend on naturally occurring soluble IL-6 receptors (sIL-6R and sgp130). In our study the serum levels of IL-6 in hepatitis C patients

did not differ from healthy subjects, but positively correlated with stage of fibrosis. Similar results were published by Migita *et al.* [11], who showed that IL-6 levels in patients with chronic hepatitis C correlated with both the liver function impairment and the degree of fibrosis. They also found a correlation between IL-6 soluble receptor levels and the degree of liver fibrosis. Those observations suggest that the balance of IL-6 and its soluble receptors may correspond to the state of liver damage in HCV infected patients.

In conclusion, our study shows that serum IL-17 levels are substantially decreased in hepatitis C patients. Further studies on the role of HCV in down-regulation of Th-17 response may yield diagnostic and therapeutic benefits.

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