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*Original Article*

# IFN-γ, IL-17, IL-22$^+$ CD4$^+$ subset in patients with hepatitis C virus and correlation with clinical factor

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**Abstract:** Background: CD4$^+$ T cell responses in HCV infection have a crucial role in the immunopathology of hepatitis C virus (HCV) infection. Our aim was to investigate the frequency of Th1, Th17, and Th22 cells in HCV-infected patients and elucidate their role in the progression of the disease. Methods: Twenty-six HCV-infected patients and 26 healthy individuals were recruited. Peripheral blood mononuclear cells (PBMCs) were stained to separate CD4, IFN-γ, IL-17, and IL-22 producing cells using flow cytometry. Results: Results showed that the mean expression of IL-22 in CD4$^+$ T cells was significantly lower in HCV-infected patients compared to healthy controls. About correlation with clinical factor and T subsets, a negative correlation between the frequency of CD4$^+$ IFN-γ$^+$ cells and Thyroxine level (T4) was observed in the patients. The data showed a positive link between thyroid-stimulating hormone (TSH), cholesterol levels, and the frequency of Th17 cells. In addition, a positive correlation was seen between serum creatinine level with both Th1 and Th17. Ultimately, it was found that there was a positive link between viral burden and IL-17$^+$ IL-22$^+$ cells and a negative correlation between viral load and pure Th22. Conclusions: Our findings indicate that Th22 cells may play a part in the immunopathology of HCV and show the associations between Thelper subsets and the clinical signs of the disease.

**Keywords:** Hepatitis C, Th1, Th17, Th22, inflammation

## Introduction

Hepatitis C is a liver infection that arises from the hepatitis C virus (HCV) categorized as a hepatotropic and non-cytopathic virus that is transmitted through contaminated blood products, sexual activity, and intravenous drug use [1, 2]. HCV infection remains a major global health problem, with around 58 million people of the world population being infected with it, while there is no vaccine for clinical use yet [3]. HCV infection is the prime cause of acute hepatitis, consequently resulting in chronic liver disease, which is characterized by fibrosis and cirrhosis and may ultimately lead to hepatocellular carcinoma (HCC) [4, 5]. In the chronic course of HCV infection, persistent virus replication in the hepatocytes may promote the stimulation of inflammatory responses leading to progressive fibrosis and damage to the liver [6].

It has been established that during HCV infection, a wide variety of cytokines contribute to viral clearance but also tissue injury [7]. The pro-inflammatory cytokines are fundamental prerequisites for initiating the inflammatory cascades and sustaining the chronic infection, which eventually results in hepatocellular liver injury [8]. Besides, chronic and inflammatory courses of HCV infection might cause an imbalance between the expression of pro-inflammatory and anti-inflammatory cytokines and che-

mokines [9]. Furthermore, a large and growing body of research has emphasized the importance of $CD8^+$ cytotoxic T lymphocytes (CTLs) and $CD4^+$ T helper (Th) cells that have influential roles in cell-mediated immunity against HCV infection [10, 11]. Naïve $CD4^+$ T cells can differentiate into various distinct subtypes, including Th1, Th2, Th17, Th22, and regulatory T cells with definite immunoregulatory functions in both steady and inflammatory states [12]. Among these, Th1 cells are characterized by IFN-γ production and robust protection against intracellular pathogens [13]. IFN-γ is a pleiotropic molecule related to mediate. Immune responses including pro-apoptotic antiproliferative and antitumor mechanisms which promote the antiviral activity and facilitate macrophage activation [14-16]. Th17 cells produce pro-inflammatory cytokines such as IL-17 (A, F) and IL-22, which promote the recruitment of other immune cells to the inflamed and injured sites [13, 17]. IL-17, also known as IL-17A, activates the neutrophil mobilization and activation. It can mediate protective innate immunity against pathogens and has been shown to be a key cytokine in protecting the host from mucosal infections [18, 19]. Th22 cells, as another helper subset, are specifically involved in producing IL-22 and can modulate the tissue responses to inflammation. IL-22 is recognized as a unique cytokine that contributes critically to the crosstalk between the immune cells and epithelial cells [20].

In this study, we aimed to examine the frequency of peripheral Th1, Th17, and Th22 subsets and the level of their corresponding cytokines, including IFN-γ, IL-17, and IL-22, in patients with chronic HCV infection. Furthermore, we investigated whether the alterations in the frequency of Th subsets were associated with the clinical features of the patients, including certain hematological and biochemical findings.

## Materials and methods

### *Study population*

This study was carried out on 26 patients (24 males and 2 females) who were infected with hepatitis C infection. Our study was conducted before admitting any antiviral or immunomodulatory treatments. The subjects (42.26±9.8 years old) were recruited from Motahari Clinic affiliated with Shiraz University of Medical Sciences over a one-year period. Twenty-six age- and gender-matched healthy individuals (42.20±10 years old) were also included in the study as the control group. All enrolled subjects provided written informed consent according to the Declaration of Helsinki and the research was reviewed and confirmed by the Medical Ethics Committee of Shiraz University of Medical Sciences (IR.SUMS.REC. 1396. S731).

### *Peripheral blood mononuclear cell (PBMC) isolation*

Five milliliters (ml) of heparinized whole peripheral blood were collected from all healthy controls and patients before receiving any treatment. PBMCs were isolated by Ficoll density gradient centrifugation. The viability of the cells was determined using the trypan blue exclusion test (Sigma-Aldrich, Germany). PBMCs were counted and suspended in 2 ml of complete culture medium (RPMI 1640) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 10% heat-inactivated fetal calf serum, at a density of $2\times10^6$ cells/ml. All were purchased from Gibco, USA.

### *Cell activating and staining*

The PBMCs were stimulated with 50 ng/mL phorbol myristate acetate (PMA) plus ionomycin (1 µg/ml) (both from Sigma-Aldrich) in the presence of 0.7 µl Brefeldin A, 0.7 µl Monensin, and 2 µl DNase (BD Biosciences, USA) for 5 hours in a $CO_2$ incubator [21, 22]. After the incubation period, the cells were harvested and centrifuged at 350 g and 4°C for 10 min. The supernatant was removed, and the pellet was washed with 2 ml of 1× phosphate-buffered saline (PBS); then, the cells were fixed with 300 µl 1% paraformaldehyde (Sigma-Aldrich) at 4°C for 15 minutes. Following washing with 1× PBS, the pellet was diluted in Perm/Wash buffer (Biolegend, USA) and incubated for 15 min in the dark at room temperature for permeabilization. Then, the tube was centrifuged for 10 min at 500 g, and the supernatant was discarded. The cells were transferred into flow cytometry tubes, stained with PerCP/Cyanine5.5 CD4 (RPA-T4) FITC anti-human IFN-γ (B27), APC anti-human IL-17A (BL168), and PE anti-human IL-22 antibodies (2G12A41) (all from Biolegend); subsequently, they were incubated for 30 min at 4°C in the dark. After we washed the tubes to remove the extra antibodies, the supernatant was dis-

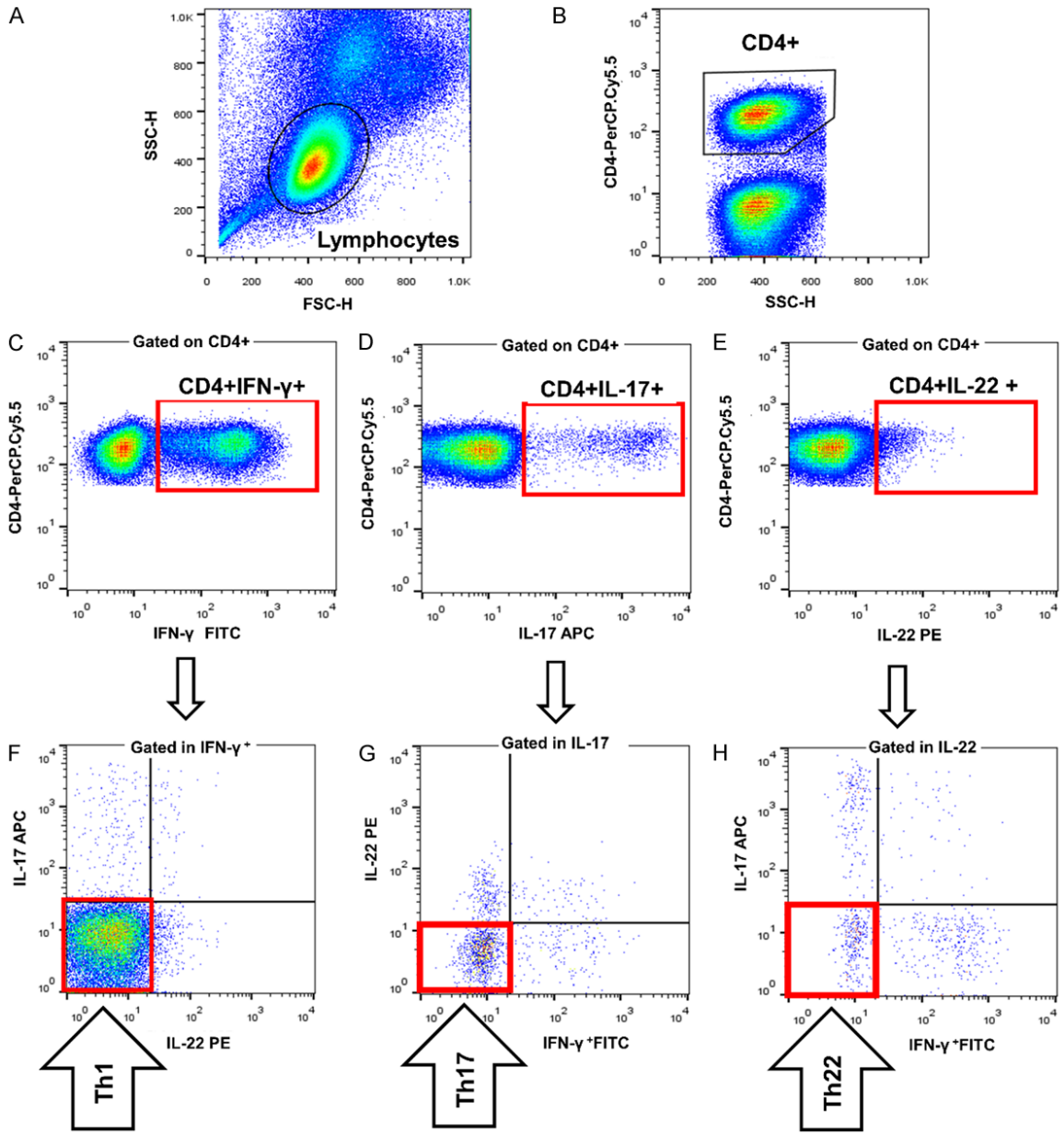


**Figure 1.** Flow cytometry analysis of different CD4⁺ T helper cell subsets. A. Lymphocytes were first gated based on their relative forward and side scatters. B. Cells with high CD4 expression were then gated among the lymphocytes. C-E. The frequency of IFN-γ, IL-17, and IL-22-producing CD4⁺ T cells was determined in CD4⁺ lymphocytes. F-H. The up arrows under the figures show the pure Th1, Th17, and Th22 populations.

carded and 300 µl 1% paraformaldehyde was added to the tubes for 15 min at 4°C. Subsequently, the cells were washed and resuspended in 300 µl 1× PBS. To control the background staining, we used appropriate matched isotype control antibodies, including FITC mouse IgG1, PE mouse IgG2a, PerCP/Cy5.5 mouse IgG1, and APC mouse IgG2b (all from Biolegend). Flow cytometric analysis was carried out using a FACS-Calibur flow cytometer (BD Bioscience), and the obtained data were further analyzed using FlowJo software 7.6.1 (Tree Star Inc., USA).

*Gating strategy for evaluating the frequency of Th1, Th17, and Th22 cells*

PBMCs were first divided into two distinct populations of lymphocytes and monocytes based on their relative forward and side scatter (**Figure 1A**). Next, the lymphocytes with a high expression of CD4 were gated (**Figure 1B**), and

**Table 1.** Frequencies of various $CD4^+$ T helper subsets in untreated HCV-infected patients and controls

| Lymphocyte subset | Markers | Untreated HCV-infected patients (Mean ± SD) | Controls (Mean ± SD) | *P* value |
|---|---|---|---|---|
| $CD4^+$ | $CD4^+$ | 31.53±14.99 | 39.61±8.74 | 0.058 |
| IFN-γ-producing | $CD4^+$ IFN-$\gamma^+$ | 17.43±10.64 | 16.45±5.96 | 0.952 |
| IL-17-producing | $CD4^+$ IL-$17^+$ | 1.67±0.85 | 1.65±0.65 | 0.680 |
| IL-22-producing | $CD4^+$ IL-$22^+$ | 0.96±0.65 | 0.83±0.37 | 0.656 |
| Pure $CD4^+$ T helper subsets | | | | |
| Pure Th1 | $CD4^+$ IFN-$\gamma^+$ IL-$17^-$ IL-$22^-$ | 16.14±10.31 | 16.66±6.20 | 0.477 |
| Pure Th17 | $CD4^+$ IL-$17^+$ IFN-$\gamma^-$ IL-$22^-$ | 1.26±0.63 | 1.22±0.49 | 0.805 |
| Pure Th22 | $CD4^+$ IL-$22^+$ IFN-$\gamma^-$ IL-$17^-$ | 0.36±0.26 | 0.31±0.17 | 0.878 |

the frequency of different Th subsets was determined based on the expression of IFN-γ (**Figure 1C**), IL-17 (**Figure 1D**), and IL-22 (**Figure 1E**). The IFN-γ, IL-17, and IL-22 single-producing T cells (negative for the two other cytokines) were defined as pure Th1, Th17, and Th22, respectively (**Figure 1F-H**). The percentages of double-positive IL-17/IFN-γ, IL-22/IFN-γ, and IL17/IL-22 cells were also determined. The frequency of the defined populations was calculated as a percentage of $CD4^+$ lymphocytes. The geometric mean fluorescent intensity (MFI) of each cytokine was normalized by dividing the MFI of positive cells by the MFI of negative cells and considered as a criterion for its expression level per target cell.

*Statistical analysis*

All data were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The distribution of the data was checked using Koromosmirnow test and accordingly non-parametric tests were used to analyze the data. Mann-Whitney U test was carried out to compare the difference between the two groups, along with the Kruskal-Wallis test for multiple comparisons. The Spearman correlation test was run to detect the potential correlations between the variables. The quantitative data were presented as the mean ± standard deviation (SD), and *p*-values less than 0.05 were regarded statistically significant.

## Results

Distribution of $CD4^+$ T helper subsets expressed IFN-γ, IL-17, and IL-22 in HCV-infected patients and controls. Flow cytometry data indicated that the average frequency of various $CD4^+$ Th subsets in HCV-infected patients compared to healthy controls was not significantly different. However, analyzing the frequency of total $CD4^+$ lymphocytes indicated a trend towards a lower frequency of $CD4^+$ T cells in HCV-infected patients compared to the healthy controls (P = 0.058).

We adopted an approach to identify the pure cells. Pure Th1 ($CD4^+$ IFN-$\gamma^+$ IL-$17^-$ IL-$22^-$), pure Th17 ($CD4^+$ IL-$17^+$ IFN-$\gamma^-$ IL-$22^-$), and pure Th22 ($CD4^+$ IL-$22^+$ IFN-$\gamma^-$ IL-$17^-$) cells in patient and control groups, exclusively expressed only one cytokine by gating cells. They were identified as pure cells. However, they were not significant between the patient and control groups (**Table 1**). We also examined the cells that expressed two cytokines simultaneously; there were no significant differences in the frequency of double-positive Th cells, including IFN-$\gamma^+$ IL-$17^+$, IFN-$\gamma^+$ IL-$22^+$, and IL-$17^+$ IL-$22^+$ cells between patients and healthy controls. This date is not shown in the table.

*Mean expression of IFN-γ, IL-17, and IL-22 cytokines in T helper subsets*

MFI is the average amount of fluorescence emitted by specific cells in a cell population, defined as a measure of the number of antigens on the surface and within cells in mean or median fluorescence units [23]. We utilized MFI for IFN-γ, IL-17, and IL-22 expression levels in the corresponding T cell subsets (i.e., $CD4^+$ IFN-$\gamma^+$ cells, $CD4^+$ IL-$17^+$ cells, and $CD4^+$ IL-$22^+$ cells) as well as pure Th subsets (i.e., pure Th1, Th17, and Th22 cells). The comparison of HCV-infected patients and healthy controls demonstrated a lower level of IL-22 in $CD4^+$ IL-$22^+$ T cells of HCV-infected patients compared to the

**Table 2.** MFI of IFN-γ, IL-17, and IL-22 in Th1, Th17, and Th22 cells of patients and controls

| Cytokines | Corresponding lymphocyte subsets | HCV-infected patients (Mean ± SD) | Controls (Mean ± SD) | *P* value |
|---|---|---|---|---|
| IFN-γ | $CD4^+$ $IFN\text{-}\gamma^+$ | 107.76±111.92 | 99.71±46.34 | 0.286 |
| IL-17 | $CD4^+$ $IL\text{-}17^+$ | 215.07±175.58 | 290.68±172.94 | 0.060 |
| IL-22 | $CD4^+$ $IL\text{-}22^+$ | 6.21±1.70 | 7.50±1.70 | 0.0008* |
| MFI of pure CD4+ T helper subsets | | | | |
| Pure Th1 | $CD4^+$ $IFN\text{-}\gamma^+$ $IL\text{-}17^-$ $IL\text{-}22^-$ | 86.74±48.99 | 90.44±28.66 | 0.459 |
| Pure Th17 | $CD4^+$ $IL\text{-}17^+$ $IFN\text{-}\gamma^-$ $IL\text{-}22^-$ | 208.95±167.34 | 276.90±182.16 | 0.105 |
| Pure Th22 | $CD4^+$ $IL\text{-}22^+$ $IFN\text{-}\gamma^-$ $IL\text{-}17^-$ | 16.47±7.08 | 19.26±9.57 | 0.370 |

*P<0.05: statistically significant; based on Mann-Whitney U test. MFI: Mean Fluorescence Intensity.

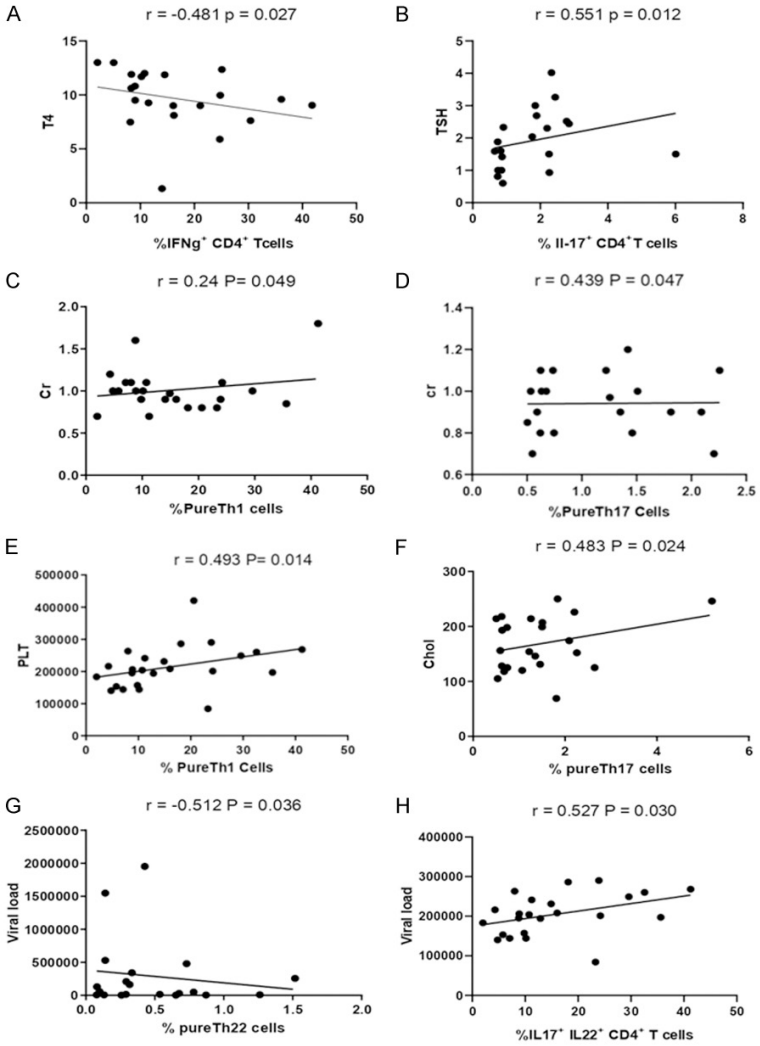


**Figure 2.** Correlations between different T helper subsets and various biochemical characteristics and viral load in untreated HCV-infected patients.

controls (P = 0.0008). We also found a trend towards a lower expression level of IL-17 in $CD4^+$ $IL\text{-}17^+$ lymphocytes of the patients compared to the controls (P = 0.060). The expression level of each cytokine was also analyzed in pure Th subsets, which revealed no significant differences between the patients and controls (**Table 2**).

*Correlation of $CD4^+$ Th subsets with the hematological and biochemical parameters of the patients with hepatitis C*

Examining the potential correlations between different Th subsets and the clinical parameters of the patients, which were collected from the history of the patients, revealed a negative correlation between the frequency of $CD4^+$ $IFN\text{-}\gamma^+$ cells and T4 levels (R = -0.481, P = 0.027) (**Figure 2A**); however, there was a positive correlation between $CD4^+$ $IL\text{-}17^+$ Th cells and serum TSH levels (R = 0.551, P = 0.012) (**Figure 2B**). Regarding the serum creatinine level (**Figure 2C** and **2D**), we noticed a positive correlation with the frequency of both pure Th1 cells (R = 0.24, P = 0.049) and pure Th17 cells (R = 0.439, P = 0.047). Moreover, the frequency of pure Th1 cells was positively correlated with the platelet count of the patients (R = 0.493, P = 0.014) (**Figure 2E**). Furthermore, a higher number of Th17 cells were found in patients with higher cholesterol levels (R = 0.483, P = 0.024) (**Figure**

**2F**). We also observed a negative correlation between the viral load and the frequency of pure Th22 (R = -0.512, P = 0.036) (**Figure 2G**) and a positive correlation between the viral load and IL-17$^+$ IL-22$^+$ cells (R = 0.527, P = 0.030) (**Figure 2H**).

## Discussion

Hepatitis C is one of the most significant public health challenges and concerns. The therapeutic effectiveness of various anti-viral regimens for HCV eradication depends on the maintenance of a fine balance among various pro- and anti-inflammatory cells and their mediators [8]. The CD4$^+$ T cell response in HCV infection has a crucial role in viral control, and liver damage can induce HCV infections [7]. To investigate the variations in the frequency of peripheral blood T cell subsets, we examined the frequency of peripheral Th1, Th17, and Th22 cells in untreated HCV-infected patients and healthy controls. In addition, any possible relationships between the Th subsets and clinical laboratory findings of the patients with hepatitis C were evaluated. We aim to elucidate potential associations that may contribute to a better understanding of the complex interplay between the host immune response and the clinical manifestations of HCV infection and also recognition of damage to different body organs caused by viral infection.

Th1 cells are essential for virus clearance in HCV infection and play a crucial role in the immune response to HCV. However, during the clearance of this virus, the Th1 cytokines contribute to necrosis of the hepatocytes. We found no significant differences in the frequency of IFN-γ$^+$ CD4$^+$ lymphocytes as well as the mean expression of IFN-γ in these cells between HCV-infected patients and healthy controls. This observation aligns with the findings of a study that reported no significant alteration in serum IFN-γ levels among individuals with HCV infection compared to the healthy control group [8], but contrary to another findings which was reported a higher level of IFN-γ in chronic HCV infection compared with healthy controls [24]. A higher expression of IFN-γ assessed by enzyme-linked immunosorbent spot assay was also observed in the chronic HCV cases [25].

Regarding IL-17-producing lymphocytes, no significant changes were found in HCV-infected patients compared with the controls, while previous studies reported an increase in both the frequency of Th17 and the serum level of IL-17 in these patients [26, 27]. Many studies have revealed the detrimental and beneficial impacts of IL-17 on virus-induced pathology. IL-17 function is crucial in fostering viral infections by enhancing virus replication and suppressing antiviral Th1 or CTL immunity to maintain the persistence of HCV in the chronic cases. Moreover, it can inhibit the development of regulatory cells and promote Th2 immune responses, which induce hepatic fibrosis [26, 28].

Th22 cells and IL-22 have been observed to have varies functions in the immune system. Several studies have reported an association between increased levels of IL-22 and the severity of hepatic diseases, which can be attributed to the proliferative and anti-apoptotic effects of IL-22 [29, 30]. Detrimental prion flammatory effects have been illustrated to deteriorate certain conditions, such as hepatitis B and Helicobacter pylori infection [31]; also, previous research has highlighted that IL-22 and IL-22BP have a role in liver diseases with a particular focus on the leading causes of advanced liver failure, namely liver infections, liver damage, and liver malignancies [32].

However, some related studies reported the upregulation of IL-22-producing CD4$^+$ T cells in patients with acute hepatitis B compared to the controls [31, 33]. Also, examining the serum level of patients with mild/moderate COVID-19 and healthy controls, researcherobserved the upregulation of IL-22 cytokines levels in COVID-19 patients [34].

IL-22 also has beneficial anti-inflammatory features that help impede the advancement of infections such as influenza and HIV. The protective role of IL-22 against liver fibrosis and its contribution to hepatic tissue regeneration through prompting the expression of metalloproteinase and inducing the antimicrobial activity have been demonstrated [31, 35]. We found a lower MFI of IL-22 in IL-22$^+$ CD4$^+$ T cells in patients with hepatitis C compared to the healthy controls. Our results are in the same line with another study [36]. Given the decreased MFI of IL-22 in the patients with chronic hepatitis C compared with the controls, it can be implied that persistent viral infection leads to the immune exhaustion of certain subsets of the helper cells, Th22 cells in par-

ticular, and reduces the IL-22 production [37]. Another underlying mechanism could be explained by the ability of regulatory T cells to suppress Th22 and Th17 cells as the main sources of IL-22 production, which is mediated by the altered expression of inhibitory receptors including PD-1, CTLA-4, and TIM-3 and regulatory cytokines such as IL-10 [38].

T4 is a hormone that is mainly synthesized in the thyroid follicules and plays an important role in regulating the metabolism. TSH is produced in the brain to stimulate the thyroid gland in order to produce and release more thyroid hormone [39]. Viral diseases like Chronic HBV (Hepatitis B viruses) infections and Covid viruses can be associated with changes in the level of these hormones [40, 41].

With respect to the clinical aspects, we observed a negative correlation between T4 level and the frequency of $CD4^+$ IFN-$\gamma^+$ T cells and a positive correlation between TSH level and the percentage of $CD4^+$ IL-$17^+$ T cells. The production of IFN-γ, as a necro-inflammatory cytokine, by $CD4^+$ lymphocytes may result in liver injury which consequently reduces the T4 level through the secretion of thyroid hormone-binding globulin (T4-TBG) in the liver [42, 43]. There have been several reports of elevated levels of TSH in patients with chronic hepatitis C, notably those who had not received any treatment; it can be attributed to hypothyroidism following persistent HCV infection [44, 45]. Furthermore, $CD4^+$ IL-$17^+$ T cells have a key role in stimulating the production of thyroid autoantibodies and certain Th17-related chemokines involved in the pathogenesis of thyroid diseases [46, 47]. Creatinine is a waste product created in the body from the breakdown of creatine, a chemical that supplies the muscles with energy. Depending on the muscle mass, it is released from the body at a constant rate. Creatinine is primarily filtered from the blood by the kidneys and excreted in the urine [48, 49]. Another important finding of this study was a positive correlation between the creatinine level and the frequency of pure Th1 and Th17 cells in untreated HCV patients. This is in the same line with the results of some studies carried out on renal insufficiency and higher creatinine levels in HCV-infected cases, showing that there is very little scientific understanding of the role of HCV in kidney failure [50]. A study has shown Th1 and Th17 cells are involved in the pathogenesis of HCV in patients with glomerulopathy [51]. As to our finding about platelets, we observed a positive correlation between PLT and pure Th1, similar to a study in this examination, they found that the pathogenesis of enterovirus 71 (EV71)-caused hand, foot, and mouth disease (HFMD) had a positive correlation between the platelets with the frequency of Th1 cells. They concluded that PLT were responsible for the regulation of pathogenic Th1 cells differentiation and function in the pathogenesis of the disease [52].

Cholesterol homeostasis is dependent on the activation of LXR nuclear receptors. This receptor can have a significant impact on Th17 cell differentiation. HCV is able to affect these nuclear receptors and cholesterol metabolism through direct and indirect mechanisms [53]. Our result from the present study was a direct correlation between the cholesterol level and frequency of pure Th17 cells, which is consistent with previous studies indicating altered lipid metabolism in HCV infection [54].

Interestingly, there was a negative correlation between the viral load and frequency of pure Th22. Several roles have been considered for this cytokine such as anti-oxidation, anti-bacterial anti-viruses, anti-fibrosis, anti-inflammatory and regenerative stimulation in protecting against liver and gut damage [23, 31]. This cytokine has a protective role in the liver and gut epithelium, when its levels are decreased. We can suggest that by decreasing IL-22 the viral load is increased and probably the liver and gut epithelial have been damaged after infections; as a result, the viral load has increased, eventually causing T cell exhaustion by transferring the virus to the blood [55, 56].

Since Th17 cells are mainly the producers of IL-17 and IL-22; also, the cytokines are involved in inflammation and maintenance of gut epithelial barrier function. In addition, a positive correlation with IL-$17^+$ IL-$22^+$ T cells and viral load was observed. Thus, with the increase in the viral load, Th17 cells also increased, and it can be mentioned that this subset of cells has been trying to improve the mucosal damage [57, 58].

Taken together, the observed inconsistencies between our results and those of others might be attributed to variations in the sample size and methodology, along with dissimilarities in

the viral load, genotypes, the extent of liver fibrosis and damage, and duration of inflammatory responses. Notwithstanding these limitations, our results provide new insights into the role of different helper subsets and their cytokine profiles in the pathology of HCV infection, which would be a fruitful area for further research.

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## Disclosure of conflict of interest

None.

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