

The Efficacy of Peg-IFN α Anti-viral Treatment Were Evaluated by Variation of Peripheral Th17 Cells in Chronic Hepatitis C Patients

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Abstract—IL-17-producing *T* helper (Th17) cells have been shown to play an important role in many liver diseases. The aim of this study is to investigate changes in the frequency of Th17 cells in peripheral blood of chronic hepatitis C (CHC) patients. The Th17 frequencies of 36 chronic hepatitis C patients were compared with those of 20 normal controls. All samples were quantitatively analyzed by flow cytometer. Serum IL-17 levels were evaluated using the ELISA assay. There was a higher frequency of circulating Th17 cells and IL-17 levels in CHC patients than controls ($3.46 \pm 1.53\%$ and $2.05 \pm 0.88\%$ for Th17 cells, 86.21 ± 29.28 pg/ml and 58.05 ± 14.17 pg/ml for IL-17 levels) ($P < 0.01$). There were no significant differences in Th17 frequency and IL-17 levels between the groups of CHC patients with HCV RNA genotype 1b and 2a. The percentage of circulating Th17 cells increased significantly, correlating positively with ALT and negatively with HCV RNA. After 4 weeks of peg-IFN α -2a treatment, the patients who acquired rapid virological response (RVR) had a higher pretreatment Th17 frequency compared with that of patients without RVR. During the 24 weeks of treatment with peg-IFN α -2a, Th17 cell frequency increased during the initial 4 weeks, then subsequently declined. In conclusion, Th17 cells and IL-17 were significantly increased in CHC patients and they were a positive correlation with ALT but a negative correlation with HCV RNA. Results suggest that an increase in Th17 cells is associated with inflammatory liver damage and persistent infection of HCV. The characteristics of Th17 variation during peg-IFN α -2a treatment imply that Th17 cells may serve as potent immunological markers for evaluating the efficacy of peg-IFN α anti-viral treatment.

Index Terms—chronic hepatitis C, IL-17, Th17 cells, pathogenesis

I. INTRODUCTION

The Hepatitis C virus (HCV) infection is a leading cause of chronic hepatitis. Chronic hepatitis C (CHC) affects approximately 170 million people worldwide [1]. There are over 37 million people infected with HCV in China [2], and 60-85% of those infected will develop chronic hepatitis C during their lifetime. Of that number, approximately 10-20% of patients will progress to end-stage liver disease or hepatocellular carcinoma (HCC)

[3]-[5]. The most common treatment is with pegylated IFN α -2a and ribavirin, but efficacy is limited, especially in genotype 1b where it is only approximately 30% efficient. [6], [7]. Newly developed direct antiviral drugs show a substantial increase in sustained virological response rates, but due to their expense and limited availability, they are still not widely used [8]. Chronic morbidity and mechanisms of CHC are still unclear, though it is now accepted that the host immune response plays an important role in chronic HCV infection and viral clearance [9], [10]. Th17 cells are a recently discovered group of secreted interleukin -17 (interleukin-17, IL-17) CD4 T cells and it has been confirmed that they play an important role in the pathogenesis of chronic hepatitis B, hepatitis C and autoimmune hepatitis [9]-[11]. However, there is little current research investigating whether or not there is a significant relationship between persistent HCV infection and Th17 cells, especially during antiviral treatment with peg-IFN α . The purpose of this study was to observe the correlation of Th17 cell frequency and IL-17 levels with antiviral efficacy in CHC patients.

II. METHODS

A. Patients

A total of 36 clinically diagnosed CHC patients (21 males, 15 females, mean age 48.3 ± 14.2 years) and 20 healthy controls (12 males and 8 females, mean age 44.3 ± 15.0 years) from PLA Hospital No. 454 were enrolled from October 2013 to August 2014. Non-CHC patient had received any pharmacological treatment for at least 8 months before they were given peg-IFN α -2a (180 μ g/w, Roche Pharmaceutical Co., Ltd., Shanghai, China) plus ribavirin (1000 ~ 1200 mg/d, Genentech, Inc.) anti-viral therapy. Peripheral blood was collected at various times pre- and post- treatment. All cases tested positive for anti-HCV were detected by the ELISA antibody assay, and for serum HCV RNA load by real-time PCR detection of and the exclusion of other hepatitis virus infection and merge autoimmune, alcoholic liver disease, primary liver cancer.

B. Flow Cytometry

Assays of peripheral Th17 cells frequency were similar to a previously published study [12], [13]. Briefly;

peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by Ficoll-Hypaque Solution (Sigma-Alorich, China) density-gradient centrifugation. Mononuclear cells were resuspended in RPMI 1640 (Invitrogen, Shanghai, China) completed with 10% heat-inactivated fetal calf serum (FCS, HyClone, China) and 1% glutamine, and left stimulated with 50 ng/ml Phorbol Myristate Acetate (Sigma-Aldrich, China), 1 µg/ml Ionomycin (Sigma-Aldrich). The stimulation was carried out at 37 °C for 5 h, in the presence of the intracellular trafficking inhibitor Brefeldin A (10 µg/ml BFA; Sigma-Aldrich), before intracellular staining and cytofluorimetric analysis. After stimulation, cells were washed twice in phosphate-buffered saline (PBS) and subsequently treated using the Fix and Perm cell permeabilization kit (Caltag Laboratories, Burlingame, CA), according to the manufacturer's instructions. The end of stimulation was used anti-CD3-PerCP, CD4-APC (BD Biosciences, Shanghai China). The surface each 10 µl fluorescently labeled anti-IL-17-PE (BD Biosciences, Shanghai China) was added 20 µl after each washing rupture with washing machine. The washing cells were performed on a FACS AricII flow cytometer (BD, USA) and analyzed using CELL Quest software and testing. ELISA Commercially available ELISA kits (Labs Biotech, Inc., USA) were used for quantitative analysis of interleukin-17 according to the manufacturer's instructions. The A450 of the plates was determined using a micro plate reader (Thermo Scientific Multiskan GO, USA). The resultant absorbance values were plotted on a graph and fitted to a linear equation for the calibration curve. All the samples were assessed in triplicate. Qualitative detection of serum anti-HCV antibodies was performed using a commercially available ELISA kit supplied by Beijing Science and Health reagents (Limited FQ). The absorbance was measured at 450 nm in a Multiskan Ascent ELISA reader (Thermo Scientific Multiskan GO, USA). RT-PCR The RT-PCR method to detect serum HCV RNA was performed according to the commercial kit manufacturer's protocol (Da An Gene Co., Ltd., Zhongshan Medical University). Statistical analysis Results are expressed as mean ± standard deviation (SD). Data was analyzed using SPSS version 13.0 for Windows (SPSS, Chicago, IL). The t-test was employed to compare the percentage of Th17 or IL-17 levels of CHC patients versus healthy controls and RVR group versus non-RVR group with peg-IFNα-2a treatment. Pearson's correlation test was used to perform the correlation analysis between ALT levels or HCV RNA load with the frequency of Th17 cells or serum IL-17 levels in CHC

patients. All tests were two-tailed and values of $P < 0.05$ were considered to indicate statistical significance.

III. RESULTS

Th17 cells frequency and plasma IL-17 levels analysis in CHC peripheral blood The expression of CD4⁺ T cells to IL-17 Th17 cells showed that the percentage of Th17 cells in CHC patients ($3.46 \pm 1.53\%$) was significantly higher than in the healthy control subjects ($2.05 \pm 0.88\%$), ($t = -4.368$, $P < 0.001$) (Fig. 1A,1B). We detected a statistically significant increase in serum IL-17 levels (86.21 ± 29.28 pg / ml) of CHC patients compared to healthy controls (58.05 ± 14.17 pg / ml), ($t = -4.842$, $P < 0.001$) (Fig. 1C). The relationship of Th17 cells and plasma IL-17 levels and clinical parameters In CHC patients, there was a positive correlation between the frequency of Th17 cells and ALT levels, ($r = 0.632$, $P < 0.001$) (Fig. 2A), while there was a negative correlation with HCV RNA load ($r = -0.401$, $P = 0.015$) (Fig. 2B). CHC patients' serum IL-17 levels and ALT were positively correlated ($r = 0.347$, $P = 0.038$) (Fig. 2C), while there was no statistically significant correlation with HCV RNA ($r = -0.018$, $P = 0.918$) (Fig. 2D). The CHC patients were divided according to type: 1b HCV RNA (26 cases) and 2a (10 cases) groups. Respectively, the percentage of Th17 cells in each group was $3.37 \pm 1.67\%$ and $3.68 \pm 1.15\%$, and the plasma IL-17 levels were 87.72 ± 30.83 pg / ml and 82.29 ± 25.86 pg / ml. These differences were not found to be statistically significant ($P > 0.05$).

Virological response to peg-IFNα-2a anti-retroviral therapy In accordance with the peg-IFNα-2a treatment whether patients undergoing RVR, that is, when 4 weeks of treatment if the patient HCV RNA into negative ($\leq 1 \times 10^3$ IU/mL), we were divided into two groups (15 cases RVR group, 21 cases of non-RVR group). In the RVR group, we found that the number of Th17 cells prior to treatment ($4.18 \pm 1.81\%$) was significantly higher than that of the non-RVR group ($2.95 \pm 1.07\%$), and the difference was statistically significant ($t = 2.349$, $P = 0.029$) (Figure 3A). IL-17 levels in the RVR group before treatment (92.97 ± 36.95 pg / ml) were higher than in the non-RVR group (81.38 ± 22.03 pg / ml), but the difference was not statistically significant ($t = 1.177$, $P = 0.247$) (Fig. 3B). We also investigated the percentage of Th17 cells of 12 CHC patients during the 24 weeks of their anti-viral therapy. All patients reached HCV RNA negative by the end of the treatment period. We found that the percentage of Th17 cells appeared significantly increased after treatment for 2 weeks and 4 weeks, but then decreased gradually.

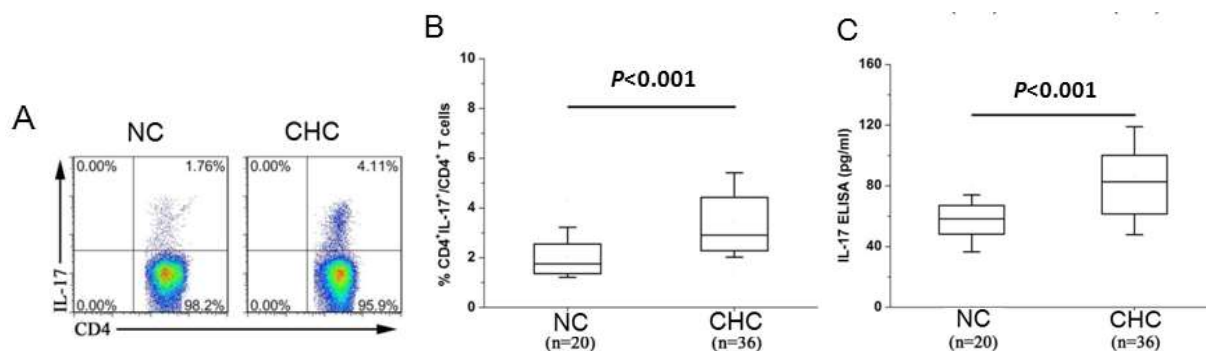


Figure 1. Frequency of peripheral Th17 cells and level of IL-17 in patients of CHC. The frequency of Th17 cells were observed between the group CHC and normal by flow cytometry. A statistically significant increase of frequency of Th17 cells (A, B) and level of IL-17 (C). In B, C horizontal bars represent the median values of indicated index.

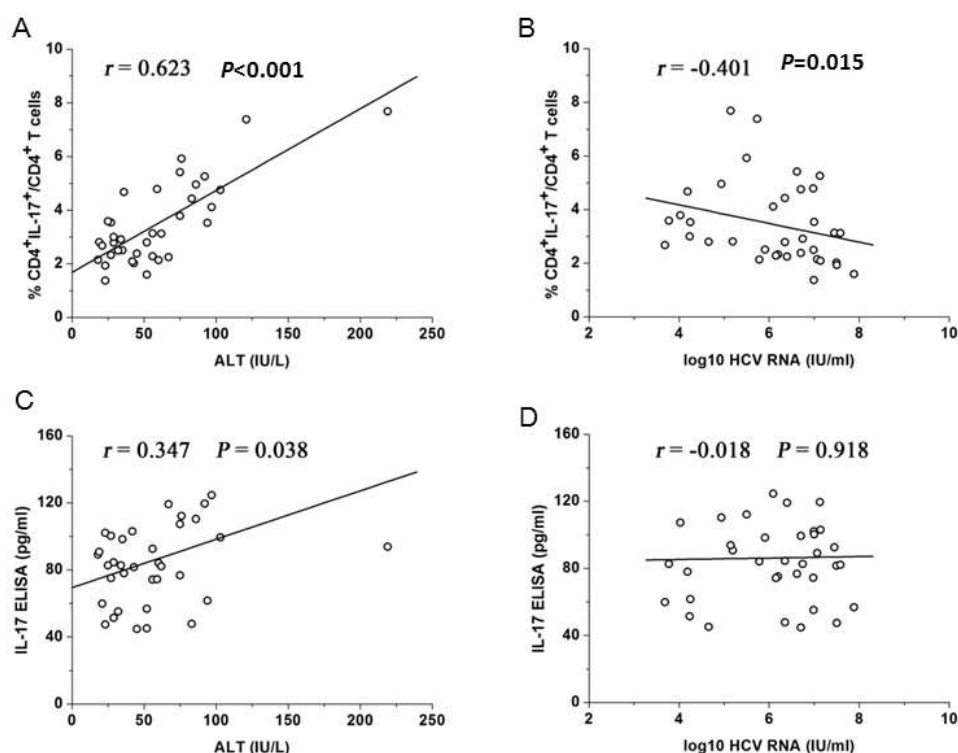


Figure 2. Th17 cells or IL-17 relationship with HCV RNA or ALT in peripheral blood of CHC patients. Th17 cells in patients of CHC correlated with level of peripheral blood HCV RNA was positively, and with ALT was negatively (A, B). Level of IL-17 was positively correlated with ALT (C) but showed no correlation with HCV RNA (D).

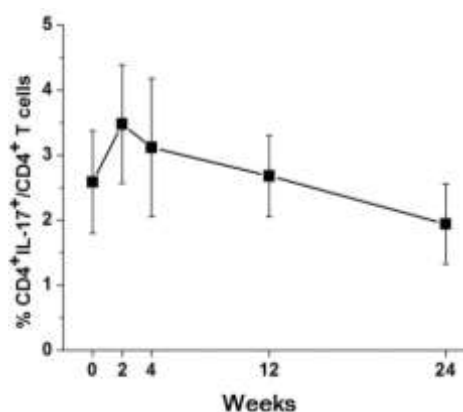


Figure 3. Th17 and IL-17 potential influencing RVR to Peg-IFN α -2a antiviral therapy in patients of CHC. There was a statistically significant difference in the higher frequency of Th17 cells in those patients who achieved RVR than the patients who did not achieve RVR (A). The difference was not statistically significant compared to the peripheral blood of IL 17 levels in the patients who achieved RVR when compared with the patients who did not achieve RVR (B).

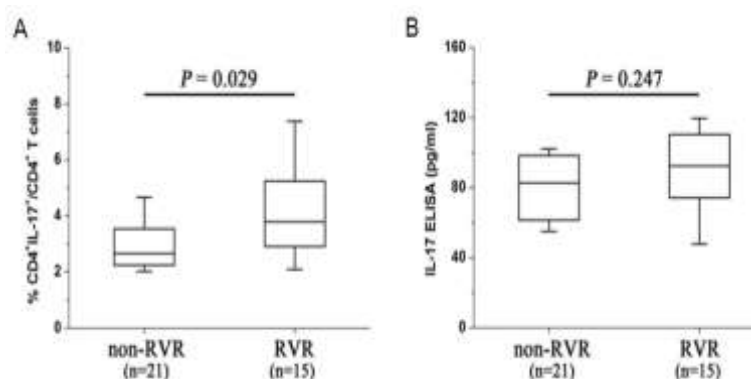


Figure 4. The changes of the frequency of Th17 cells with peg-IFN α -2a antiviral therapy in patients of CHC. During peg-IFN α -2a antiviral therapy, the frequency of Th17 cells increased after the first decline in CHC patients.

IV. DISCUSSION

HCV infection can be a cause of liver inflammation and necrosis, and can further lead to the development of cirrhosis and hepatocellular carcinoma [14]. More recent evidence has also shown that Th17 cells and the secretion of IL-17 play an important role in inflammatory liver injury due to HCV infection [14], [15]. We found that there is a significant increase in the number of Th17 cells and IL-17 levels in patients with CHC peripheral blood, a result that is consistent with other studies [16], [17]. Further analysis revealed that in CHC patients, Th17 cells, IL-17 levels and ALT levels were positively correlated, suggesting that Th17 cells may contribute to aggravating inflammatory liver injury. Our results indicated that in patients with CHC, Th17 cell frequency and HCV replication level were negatively correlated, suggesting that Th17 cells cause liver damage and may also inhibit or remove related viruses at the same time. It has been reported that Th17 cells play an important role [18]-[20] in the control of fungal and parasitic infections, while in vitro cell culture experiments also confirm that IL-17 can inhibit the replication of HCV in cell lines. Seetharam et al. report spontaneous clearance of the virus in liver transplant patients with higher HCV antigen specific Th17 cells in HCV recurrence. It remains controversial whether Th17 cells play a role in viral clearance, although more evidence exists in favor of Th17 cell support in HCV infection control. Studies have found that after acute HCV infection, there is no difference that Th17 cells can spontaneously clear the virus in patients with acute HCV infection and chronic HCV infection [15]. Our data has not shown that plasma IL-17 levels in patients with CHC have any correlation with HCV RNA levels. In fact, unlike Th17 cells, the plasma IL-17 levels compared with ALT levels, correlation was significantly loose, which may be because IL-17 levels are not entirely dependent on secretion by Th17 cells. It has been reported that CD8⁺ T cells, NKT cells, $\gamma\delta$ T cells, neutrophils and even some regulatory T cells under certain circumstances have the ability to secrete IL-17 with some stimulation [18]. However, it may be because HCV infection leads to an increase in the number of Th17 cells in the liver tissue and the secretion IL-17 in the peripheral blood. Another possible reason may be that HCV infection leads to a large number of

Th17 cells in the liver tissue and also secretion of IL-17, but those resulting changes in the peripheral blood were not obvious. Chang et al reported that the degree of inflammation of liver and liver tissue levels of IL-17 is proportional in patients with CHC [14]. Currently, the cellular and molecular mechanisms do not confirm that Th17 cell differentiation and proliferation are caused by HCV infection. The studies have found that HCV stimulates thymus stromal lymphopoietin production in infected hepatocytes and then stimulates dendritic cells to promote Th17 cell differentiation. The HCV core protein and NS3 protein also, through direct activation of dendritic cells, further promote the generation of Th17 cells. Healthy human peripheral blood mononuclear cells co-cultured with HCV infection hepatocytes also appear to increase Th17 cells [16]. These results suggest that HCV is an important factor in the induction of Th17 cell increase. Based on these reports, we hypothesized that the virus is controlled or removed, resulting in a reduction of viral nucleic acid and antigens which may then reduce the number of Th17 cells. Our follow-up study confirmed this hypothesis. After clearing HCV RNA in peripheral blood of all patients who underwent 24 weeks of peg-IFN α -2a antiviral therapy, we observed that the number of Th17 cells appeared to exhibit a more significant decline. Lee et al. reported that in peg-IFN α treatment week 48, CHC patients' serum IL-17 levels were significantly decreased and close to healthy. Our data also indicated that 4 weeks after the treatment, there was a significant increase in Th17 cells and by 12 weeks Th17 cells had returned to pre-treatment levels. This change may be because liver inflammation activity in the initial treatment of interferon itself intensified and the immune response caused the infected liver cell damage, while virus control improved liver inflammation. Foreign study also found that when the peg-IFN α treatment lasted 12 weeks, serum IL-17 levels did not change significantly, but the study lacks data for more therapeutic points in time. Further study confirmed this phenomenon, but to clarify its mechanisms, further expansion of the number of cases and longer follow-up observation periods are still needed.

This study indicated that with peg-IFN α -2a preliminary, Th17 cell levels change relative to the body of virological response in CHC patients. This data

suggests that Th17 cells are one of the potential predictive measures of the antiviral efficacy of immunological parameters in CHC patients. To summarize, in patients with CHC, increased expression of Th17 cells is closely related to viral replication and liver inflammation. Thus, Th17 levels could potentially be an effective immunological parameter for examining peg-IFN α antiviral efficacy. Expanding the number of cases studied, increasing follow-up time, and improving liver tissue in situ study of Th17 cells, in particular the number and functional characteristics observed in HCV-specific Th17 cells, could further clarify the chronicity of HCV infection pathogenesis and assist in the development of a more reasonably effective anti-viral therapy program.

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