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Original Article

Increased values of peripheral blood γδT cells, Th17 cells, IL-17, ALT, AST, TB, and DB are closely related to the severity of chronic hepatitis B

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Abstract: Objective: To investigate the correlation between the increase of peripheral blood γδT cells, Th17 cells, IL-17, ALT, AST, TB, DB and the severity of chronic hepatitis B. Methods: The number of γδT cells and Th17 cells in the peripheral blood of 20 healthy individuals, 20 asymptomatic carrier CHB patients, 20 mild CHB patients, and 20 patients with moderate and severe CHB were assessed using flow cytometry. The levels of IL-17 cytokines in the serum of each group were measured by ELISA. The concentrations of ALT, AST, TB, and DB in the serum of each group were quantified using an automatic biochemical analyzer (AU-640). The concentration of HBV-DNA in the serum of each group was measured using the PCR-fluorescence probe method. Results: The expression levels of the γδT cells in healthy individuals, AsC patients, mild CHB patients, and moderate and severe CHB patients were 1.258±0.1348%, 2.178±0.1946%, 4.160±0.0693%, and 7.058±0.9699%, respectively. The differences were statistically significant ($P < 0.05$. $F=498.35$, $P=0.000$); The expression levels of the Th17 cells were 1.252±0.1545%, 1.714±0.1031%, 2.338±0.2337%, and 3.826±0.4884%, respectively, and the differences were statistically significant ($F=310.65$, $P=0.000$). The concentrations of IL-17 (pg/ml) were 16.307±19.25, 92.706±16.85, 147.635±6.32, and 391.787±28.52, respectively. The differences were statistically significant ($F=349.41$, $P=0.000$). There was a significant positive correlation between γδT cells, Th17, cells IL-17 and the clinical parameters ALT, AST, TB, DB, $P < 0.05$, but no correlation with HBV-DNA was found, $P > 0.05$. Conclusion: With the deepening of the severity of CHB, the expression of peripheral blood γδT cells, Th17 cells, and IL-17 also increased, indicating that γδT cells and Th17 cells may be involved in the immune response and tissue damage caused by body infection. Increased values of peripheral blood γδT cells, Th17 cells, IL-17, ALT, AST, TB, and DB are closely related to the severity of chronic hepatitis B.

Keywords: Chronic hepatitis B, γδT cells, Th17 cells, IL-17, cytokines

Introduction

Hepatitis B virus (HBV) is a hepatotropic DNA virus that does not directly cause cell damage after it enters the body but has a strong ability to infect [1], and persistent HBV infection can develop into chronic hepatitis, eventually causing liver cirrhosis and liver cancer in patients [2, 3], seriously endangering their health. Currently, various drugs and treatment methods cannot effectively remove the hepatitis B virus that has been integrated into hepatocytes. γδT cells are innate immune T cells that are widely distributed in mucosal epithelial tissues. Their effects are similar to those of αβ T cells. γδT cells can recognize some non-polypeptide antigens in an unlimited manner using MHC molecules and can participate in antigen presentation, instead of functioning as dendritic cells [4]. γδT cells account for only 0.5 to 5% of adult human peripheral blood and are mainly distributed in the mucosa and subcutaneous tissue, such as in human-intestinal epithelial lymphocytes (intra-epithelial lymphocyte, IEL) and they account for 10~18%, 25~37% of human large intestine IEL, and 50% of mouse IEL [5, 6]. Activated γδT cells have anti-infective and anti-tumor effects [7-12]. Th17 cells (T helper type

17 cells) are a subset of αβ T cells and a subset of CD4+ T cells secreting pro-inflammatory cytokines. They are widely involved in the occurrence and development of chronic inflammation, autoimmune diseases, tumors, and infectious diseases [13-16], mainly secreting the cytokine IL-17. IL-17 is an important effector of γδT cells and Th17 cells and has a strong pro-inflammatory effect [17, 18]. In this study, flow cytometry (FCM) and ELISA were used to detect the number of γδT cells, Th17 cells, and the expression of cytokine IL-17 in the peripheral blood of patients with chronic hepatitis B to investigate the correlation between γδT cells, Th17 cells, IL-17, and serum ALT, AST, TB, DB, HBV-DNA in patients with chronic hepatitis B in order to provide a theoretical basis for the treatment of chronic hepatitis B to delay the progression of liver cirrhosis or liver cancer.

Materials and methods

General information

We randomly selected 60 cases who were carriers of or inpatients with chronic asymptomatic HBV or CHB during the period November 2015 to April 2016 in our hospital The cohort included 28 males and 32 females, aged 19-70 years with an average age of 35.5±6.7. According to *The Guidelines for the Prevention and Treatment of Chronic Hepatitis B* and The Viral Hepatitis Prevention and Control Program [19, 20], the groups were divided as follows: a. 20 cases of chronic asymptomatic HBV carriers (AsC, serum transaminases and bilirubin were in the normal range); b. 20 cases of patients with mild CHB (serum transaminases were 0 to 3 times the normal reference value, serum bilirubin was 0 to 2 times the normal reference value); c. 20 cases of patients with moderate or severe CHB (with normal serum transaminase ≥ 3 times the normal reference value, serum gallbladder Red pigment ≥ 2 times the normal reference value). The inclusion criteria were: HBsAg positive patients with no less than 6 months with a positive HBV-DNA test; no other symptoms; no anti-HBV treatment; no systemic or topical glucocorticoid treatment within 1 month; no administration of antihistamines or immunotherapy. The healthy control group: 20 healthy volunteers were selected from the physical examination center of our hospital during the same period, including 12 males and 8 females, aged 30-56 with an average age of 38.2±5.8; five serological tests for hepatitis B were negative; HBV-DNA was negative; serum transaminases and serum bilirubin were in the normal range; liver tissue examination had no obvious abnormalities; there were no other symptoms, and no current history of infection existed. All subjects received the tests after providing an informed consent.

Main instruments and reagents

Flow cytometry (FACS101 Handbook, Becton Dickinson, USA); microplate reader (RT-6000, Rayto, USA); PCR amplification (MJ Research); AU-5831 automatic biochemical analysis Instrument (U.S. Beckman Coulter Co., Ltd.); CD3 mAb (Becton Dickinson, USA, product number: 561806); phorbol ester (PMA) (Sigma, USA, product number: P1585); ionomycin (IM) (Sigma Corporation, USA, product number: I0643); γδTCR (Becton Dickinson, USA, product number: 561995); IL-17 ELISA kit (US Elabscience, product number: E-EL-H0105c).

Methods

Collection of specimens

In the early morning, 6 ml of peripheral venous blood was collected from the subjects, 2 ml of heparin was used for anticoagulation, and the γδT cells and Th17 cells were detected by flow cytometry within 2 hours. Another 4 ml was centrifuged at 3000 r/min for 15 minutes to collect serum to determine the concentrations of IL-17, ALT, AST, TB, DB, and HBV-DNA in the serum.

Expression of γδT cells by flow cytometry

20 μl of CD3 and 20 μl of γδTCR antibodies and 50 μl of anticoagulant blood were added to a flow test tube and mixed gently by shaking and sheltered from light for 15 minutes at room temperature. 1 ml of 1 × FACS Lysing Solution was added to each tub, which were mixed gently by shaking and sheltered from light for 10 minutes at room temperature; they were then centrifuged at 1,000 r/min for 5 minutes; 500 μl PBS was added to resuspend; the up-flow cytometry was examined, and the CellQuest data was analyzed.

Flow cytometry detection of Th17 cell expression

250 μl of peripheral blood was taken with the addition of 50 μg/L of PMA, 750 μmol/L of ion-

Table 1. The expressions of γδT and Th17 cells in each group of peripheral blood by flow cytometry [($\bar{x}$ ± s)%], n=20 in each group

Groups	Healthy person	AsC	CHB light	CHB medium and heavy
γδT cells	1.258±0.1348	2.178±0.1946	4.160±0.0693	7.058±0.9699
Th17 cells	1.252±0.1545	1.714±0.1031	2.338±0.2337	3.826±0.4884

Note: γδT cells, F=498.35, P=0.000; Th17 cells, F=310.65, P=0.000.

omycin, 2.0 μmol/L of the Golgi blocker monensin, all mixed in an incubator of 50 ml/L CO_2 for 4 hours at 37°C. The cell suspension was transferred to a 1.5 ml EP tube and centrifuged at 1,000 r/min for 6 minutes. The supernatant was discarded and washed twice in PBS before being analyzed by flow cytometry. 10 μl of PECy5-anti-CD3 and 10 μl of FITC-anti-CD8 were added to the incubator, and kept in the dark at room temperature for 30 minutes, washed in PBS twice with the addition of 300 μl of fixative, and then incubated in the dark at 4°C for 15 minutes. Then we centrifuged and discarded the supernatant; a membrane-breaking solution was added with centrifugation at 2,000 r/min, and then the supernatant was discarded and washed twice in PBS and divided into 2 tubes. 20 μl of PE-anti-IL-17 and 10 μl of isotype control PE-IgG1 were added respectively. The incubation was sheltered from light at room temperature for 30 minutes and washed twice in PBS. The cells were resuspended in 0.3 ml PBS. The flow cytometry was checked and the data were analyzed using the CellQuest software.

ELISA detection of serum IL-17 concentration

① Preparation of the standard curve: the concentration volumes of the IL-17 standard samples were 0, 31.3, 62.5, 125, 250, 500, 1000, and 2000 pg/ml. The test was done three times, and we calculated the OD value of the average and used an Excel table to create scatter plots to draw a standard curve; ② According to the ELISA kit's instructions, specimens of IL-17 were detected, their OD values were calculated, and the standard curve of the IL-17 concentration was checked.

Detection of ALT, AST, TB, DB, HBV-DNA

The concentrations of ALT, AST, TB, and DB were measured using an automatic biochemical analyzer (AU-640). The HBV-DNA was detected using the PCR-fluorescence probe method. The above were all tested according to the operating instructions.

Statistical analysis

SPSS 16.0 software was used for the statistical analysis. The normal distribution of measurement data used the $\bar{x}$ ± s description. The F test was applied in the comparisons between groups; the correlation of the parameters was analyzed using a Pearson correlation analysis, and $P < 0.05$ was considered statistically significant.

Results

Flow cytometry detection of the γδT and Th17 cell expressions

The expressions of the γδT cells and Th17 cells in the peripheral blood of each group were determined using flow cytometry, as shown in **Table 1**; **Figures 1** and **2**. From **Table 1**, it can be seen that the γδT and Th17 cells can be detected in the peripheral blood of the CHB patients and healthy volunteers, and the number of γδT and Th17 cells gradually increases with the degree of CHB disease progression. That is, for CHB medium-heavy > CHB. Mild > AsC > healthy people, the difference was statistically significant.

ELISA detection of IL-17 concentration in the serum

① Preparation of the standard curve: the concentrations of the IL-17 standard samples were 0, 31.3, 62.5, 125, 250, 500, 1000, and 2000 pg/ml. The examination was determined by machines three times, and the average OD value was obtained. An excel chart was used to draw the standard curves of the scatter plots. The linear relationship between the OD value and the concentration of IL-17 in the serum as determined by ELISA is shown in **Figure 3**. ② The IL-17 concentrations in the serum in each group examined by ELISA are shown in **Table 2**. As can be seen from **Table 2**, IL-17 cytokines can be detected in the serum of patients with chronic hepatitis B and in the healthy subjects. The concentrations of the IL-17 cytokines gradually increased with worsening CHB disease. That is, CHB medium > CHB mild > AsC > healthy people. The difference was statistically significant.

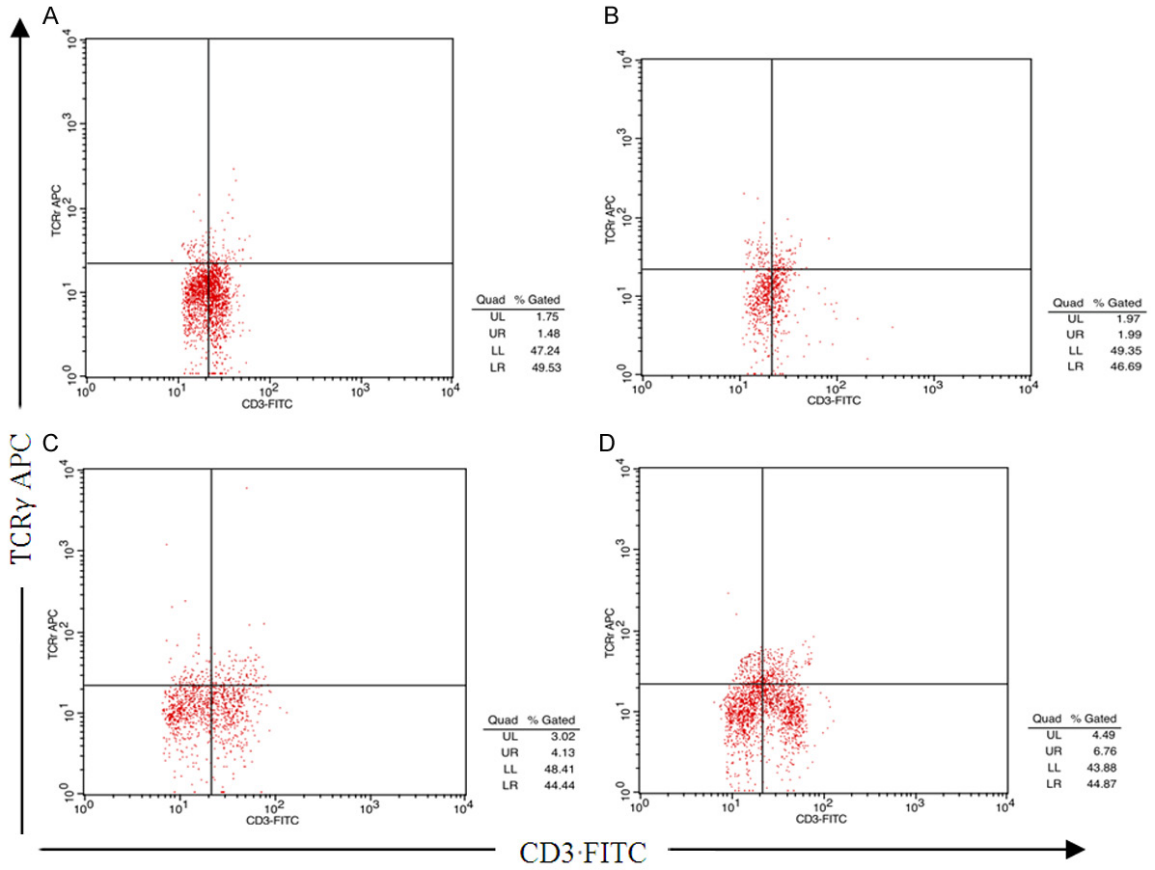

Figure 1. The quantification of γδT cell expressions in the peripheral blood of various groups determined using flow cytometry. A: The expression of γδT cells in healthy people; B: The expression of γδT cells in AsC; C: Expression of γδT cells in mild CHB; D: Expression of γδT cells in heavy CHB.

Quantification results of ALT, AST, TB, DB, and HBV-DNA in each group

The concentrations of ALT, AST, TB and DB in serum of each group were quantified by an automatic biochemical analyzer. The values in the HBV-groups were determined using the PCR-fluorescence probe method. The content of the DNA is shown in **Table 3**. The results showed that there were significant differences between ALT, AST, TB, DB, and the HBV-DNA groups, $P < 0.05$.

The correlation of γδT cells, Th17 cells, and IL-17 with ALT, AST, TB, DB, HBV-DNA

γδT cells, Th17 cells and IL-17, and clinical indicators of ALT, AST, TB, DB, HBV-DNA. The correlation is shown in **Table 4**. The results showed that there was a significant positive correlation between γδT cells, Th17 cells, and IL-17 and the clinical parameters ALT, AST, TB, and DB, $P < 0.05$, but no correlation with HBV-DNA, $P > 0.05$.

Discussion

HBV is a hepatotropic circular DNA virus with a genome length of 3.2 kb [21] and partially double stranded. Hepatitis B caused by HBV infection is currently prevalent in China. About 130 million people are carriers of HBV, and about 23 million people are progressing to CHB [22]. HBV infection seriously jeopardizes people's health and is a known important cause of the triad of hepatitis B, cirrhosis, and liver cancer [23]. The main reason for the chronicity of HBV infection is the body's immune tolerance to HBV, including the inability to maintain the original equilibrium state among various T cell subpopulations in the body, resulting in the failure of a specific immune function to work, so the body loses its mechanism to eliminate the HBV virus. Therefore, HBV can exist in the body for a

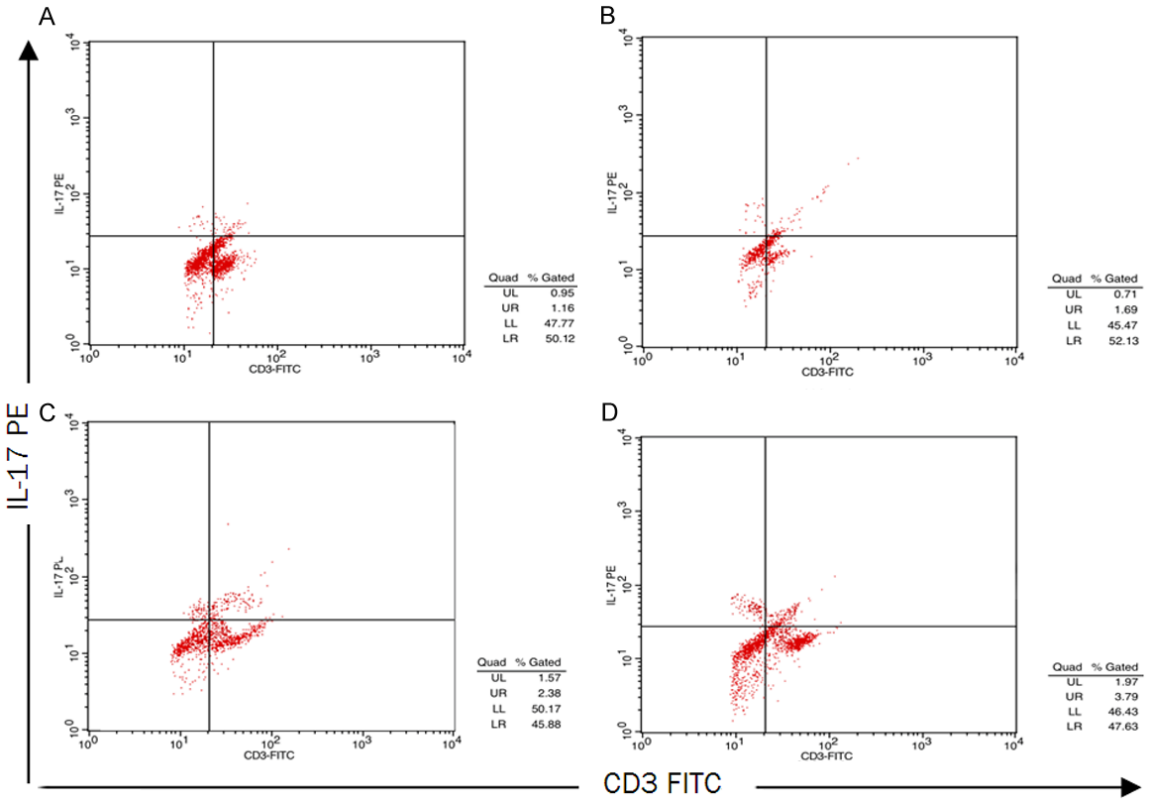

Figure 2. The expressions of Th17 cells in the peripheral blood of each group determined using flow cytometry. A: Th17 cell expression in healthy people; B: Th17 cell expression in AsC; C: The amount of Th17 cells expressed lightly in CHB; D: The amount of Th17 cells that are heavily expressed in CHB.

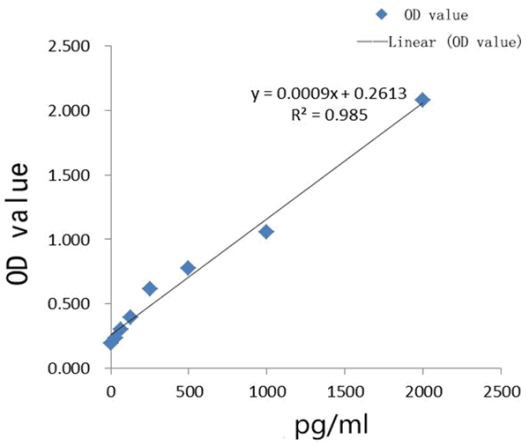

Figure 3. Linearity between the OD value and the concentration of IL-17 in serum as determined by ELISA.

long period of time [24], causing the body to have a persistent HBV infection, leading to the chronicity of hepatitis B [25]. The specific immune response is the key to clearing the virus from the chronic development of hepatitis B. The immune cells of specific cellular immunity are the "sentinels" that resist HBV infection and are at the forefront of anti-HBV, including γδT and Th17 cells derived from the liver. The role of these cells in anti-HBV infection remains unclear and is currently a hot issue [26].

γδT cells are a subset of T cells first identified in 1986, and are mostly CD4-, CD8- cells, and a few CD4+ and CD8+ cells. CD4+ γδT cells secrete cytokines and participate in immune regulation, and the CD8+ γδT cells mainly participate in immune response effects [27]. In intracellular bacterial infections, γδT cells can produce interleukin 2 (IL-2) and gamma interferon (IFN-γ), showing Th1 (helper lymphocyte type 1 cell)-like effects; while in the extracellular environment, in helminth infections, γδT cells produce IL-4, IL-5, and IL-10, stimulate B cells, and show Th2 (helper lymphocyte type 2 cells)-like effects [28]. Activated γδT cells can inhibit the proliferation of Foxp3+ Tregs (regulatory T cells) [29] and can also produce IL-10, TGF-β (transforming growth factor-β) to exert an

Table 2. The results of IL-17 concentration in each group in serum by ELISA ($\bar{x} \pm s$), n=20 in each group

Groups	Healthy person	AsC	Mild CHB	Moderate and severe CHB
OD value	0.2581±0.0190	0.3322±0.0166	0.3881±0.0064	0.6240±0.0271
Concentration (pg/ml)	16.307±19.25	92.706±16.85	147.635±6.32	391.787±28.52

Note: OD value, F=353.457, P=0.000; concentration, F=349.41, P=0.000.

Table 3. Results of the quantification of ALT, AST, TB, DB, and HBV-DNA in each group ($\bar{x} \pm s$)

Value	Healthy person group	AsC group	CHB light group	Moderate and severe CHB group	*F* Value	*P* Value
ALT (U/L)	21.85±7.26	30.12±9.12	66.52±14.81	462.09±352.01	17.55	0.000
AST (U/L)	22.37±7.65	28.75±10.88	63.42±25.01	350.23±245.01	9.92	0.000
TB (μM/L)	11.36±6.27	19.01±9.71	28.03±13.49	119.03±84.36	5.93	0.001
DB (μM/L)	6.55±3.76	7.61±5.83	15.23±6.56	75.89±19.73	5.89	0.001
HBV-DNA#	3.89±1.27	5.11±1.94	5.22±2.48	6.23±2.30	4.98	0.003

#Lg (copies/mL).

Table 4. The correlation of γδT cells, Th17 cells, and IL17 with the clinical parameters ALT, AST, TB, DB, HBV-DNA

Groups	ALT		AST		TB		DB		HBV-DNA	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
γδT	0.875	0.001	0.882	0.001	0.881	0.001	0.957	0.000	0.473	0.167
Th17	0.825	0.003	0.835	0.003	0.835	0.003	0.929	0.000	0.438	0.206
IL-17	0.844	0.002	0.843	0.002	0.836	0.003	0.979	0.000	0.409	0.240

immunomodulatory effect [30]. The results of this study showed that the number of γδT cells in healthy individuals, AsC, mild CHB, and severe CHB patients increased with the severity of CHB, which was inconsistent with the findings of Chen Min et al. [31] and Tseng et al. [32]. The reported results of hepatitis C peripheral blood were more consistent. To analyze the causes, Chen Min et al. studied γδT, δ2T, and memory or activated γδT and δ2T cells, but we mainly focused on the cell surface expression of CD3 and TCRγ molecules as detection points. In addition, due to the limitation in the number of cases, or exposure to certain unknown antigens, the differences may have a certain influence on the results. However, the percentage of γδT cells in this experiment is consistent with that of Tseng et al. [32]. Therefore, we believe that the results obtained are reliable. The results of this study show that γδT cell values are positively correlated with serum ALT, AST, TB, and DB levels, and have little correlation with the HBV-DNA load, indicating that the severity of CHB patients is closely related to the number of peripheral blood γδT cells. It is suggested that γδT cells may be involved in the immune response and the tissue damage caused by infection with the virus.

Th17 cells are an independent subpopulation of Th cells discovered in 2005. Their differentiation process is different from that of Th1 and Th2 cells such as IL-2 and IFN-γ, and does not depend on the Cytokines and transcription factors which Th1 and Th2 cells differentiation needs. They have their own unique differentiation pathways [33]. Th17 cells act as a subset of the CD4+ T cells that secrete pro-inflammatory cytokines and secrete IL-17, IL-21, IL-6, TNF-α, GM-CSF, but not IL-4, IFN- γ and are widely involved in the occurrence and development of chronic inflammation, autoimmune diseases, tumors, and infectious diseases [13-16]. In studies related to liver diseases, Zhang et al. [34] and Ge et al. [35] found that Th17 cells in the peripheral blood and liver tissues of patients with chronic liver injury caused by hepatitis B virus infection also increased signifi-

cantly, and were found to be associated with ALT in serum. There is a significant positive correlation between levels. Wu et al. [36] also pointed out that the number of Th17 cells in the peripheral blood of patients with chronic severe hepatitis B is significantly higher than it is in patients with chronic mild hepatitis B and healthy individuals. As the infection progresses, Th17 acts as the center of the pro-inflammatory pathway, and Th17 cells and the cytokines secreted by them gradually increase, causing the destruction of liver tissues and causing liver failure. Therefore, Th17 cells are considered to be closely related to liver damage. This study found that the number of Th17 cells in healthy people, AsC, mild CHB, and severe CHB patients increased with the severity of CHB, and was positively correlated with serum ALT, AST, TB, and DB levels. The correlation with HBV-DNA load is not significant, indicating that the severity of the disease in CHB patients is closely related to the number of Th17 cells in the peripheral blood, which is consistent with previous reports [35, 37], suggesting that Th17 cells are involved in the infection of the body caused by the immune response and the tissue damage process.

IL-17 is an important effector of Th17 cells and has a strong proinflammatory effect. IL-17 expression is increased in many types of inflammation [17, 18]. Yu Xiaohui et al. [38] found that after treatment with entecavir in patients with chronic hepatitis B, the IL-17 cell levels in peripheral blood were significantly reduced; Cui et al. [39] found in vitro that interferon can inhibit PBMCs, or that the initial CD4+ T cells secrete IL-17, while at the same time they promote the secretion of cytokine IL-10; this study found that the number of IL-17 in healthy individuals, AsC, mild CHB, and severe CHB patients increased with the severity of the disease. It correlates positively with the serum levels of ALT, AST, TB, and DB, but has little correlation with the HBV-DNA load. It was confirmed that IL-17 may be involved in the pathological process of liver damage caused by hepatitis B virus infection.

In summary, the expression of γδT cells, Th17 cells, and IL-17 in the peripheral blood of patients with CHB is increased, positively correlates with serum ALT, AST, TB, and DB levels, and has little correlation with HBV-DNA load, confirming that γδT cells, Th17 cells, and IL-17 may be involved in the immune response and tissue damage process caused by viral infection. This mechanism may be due to liver damage after HBV infection, as the liver inflammatory environment after injury promotes the differentiation of γδT cells and Th17 cells, which further directly leads to an increase in the secretion of IL-17 cytokines. As the major pro-inflammatory factor, IL-17 activates a variety of immune cells in the body, resulting in the release of more inflammatory mediators. For example, IL-17 cytokines can exert the cytotoxic effects of cells by inducing a Th1 immune response [40]. As a result of this cyclical reciprocation, the liver tissue of the body is repeatedly irreversibly inflammatory, resulting in decreased liver function. Thus, γδT cells, Th17 cells, and IL-17 may serve as predictors of poor prognosis and provide a reference for the clinical treatment of CHB.

Acknowledgements

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All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and with the national research committee and with the Helsinki declaration and its later amendments or comparable ethical standards. The study was performed according to the Declaration of Helsinki and was approved by the ethics committee of the affiliated hospital of Taishan University. Written informed consents were obtained from all the subjects recruited into our study.

Disclosure of conflict of interest

None.

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