Original Article

Dynamic Changes of Multiple Immune Cells in Chronic Hepatitis B Patients Undergoing Antiviral Treatment

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Objective Various immune cells in patients with CHB have been demonstrated to play critical roles in HBV infection. The goal of this study is to observe changes in Th17, Treg, Th1 and B lymphocytes from peripheral blood and to evaluate immune status of CHB patients undergoing antiviral treatment.

Methods Total of 49 CHB patients, 19 asymptomatic carriers and 29 healthy donors were included in our present study. The frequencies of peripheral Th17 cells (CD3⁺CD4⁺IL-17⁺T cells), Treg cells (CD3⁺CD4⁺CD25⁺CD127⁻ T cells), Th1 cells (CD3⁺CD4⁺IFN- γ T cells) and B lymphocytes in chronic hepatitis B (CHB) were analyzed by flow cytometry.

Results The frequency of Th17 cells increased after treatment for 6 months, but there was no statistically significant difference of IL-17 expression between baseline and 6 months after treatment. The frequencies of Treg cells, memory B cells and total CD19⁺ B cells decreased after antiviral treatment. The frequencies of Th1 cells and plasma cells increased after antiviral treatment.

Conclusions This study highlights that the reestablishment of immune function during antiviral treatment in CHB patients, which caused by the antiviral drugs or the patients themselves. CHB patients may exhibit varied responses to these antiviral drugs. It is essential to supplement immune therapy during the antiviral treatment, but Th17 may play a limited role in inflammation during antiviral treatment, targeting Th17 therapy may not be useful for CHB treatment. More time and more experiments are critical to explain it.

Key words: Hepatitis B, chronic; Th17/Treg/Th1/B cells

Patients with chronic Hepatitis B (CHB) often results in dichotomous clinical manifestations, viral resolution and viral persistence. Mounting evidences have well demonstrated that the role of patients' immune status was an important determinant factor to affect the clinical results of CHB. Various immune cells in patients with CHB were shown to play different roles in the pathogenesis of hepatitis B virus (HBV) infection. Two main subsets of T cells, Th1/Th2, act vitally in the pathogenesis of HBV infection in human beings.^{1,2} Recently, the clinical significance of another T cells subsets including Th17 and Tregs cell aroused wide interests in the pathogenesis of HBV infection. Th17 cell, involved in mobilizing, recruiting and activating neutrophils,^{3,4} is recently regarded as a pro-inflammatory CD4⁺ helper cell subset, which is characterized by the production of IL-17. Treg cell was differentiated from naïve CD4⁺ T cells under separate

TGF- β induction and characterized by FOXP3. It has been found that the frequency of CD4⁺ CD25^{high} Treg was significantly increased in CHB patients compared with normal controls, which was closely correlated with serum HBV DNA load in CHB patients.⁵ Th17 cells were closely associated with liver inflammation and Treg cells which participated in the chronicity of hepatitis. Furthermore, B-lymphocytes including early B-cell precursors, mature B-lymphocytes, germinal center B-cells and mature B-lymphocytes, also participated in the resolution or development of CHB and played different roles in the clinical process of HBV infection. Mature B-lymphocytes were generated by the differentiation of early B-cell precursors in bone marrow (BM) and antigen-triggered maturation of germinal center. B-cells often developed to memory B-lymphocytes and plasma blasts (PB) in lymphoid tissues. According to the maturation stage, B-cells resident in periphery blood can be classified into i) immature/transitional, ii) naïve, iii) memory B-lymphocytes, and iv) (PB)/plasma cells.⁶ The role of Th17, Treg cells and B cells subsets in CHB should be further elucidated.¹

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Antiviral treatment of nucleos(t)ides or interferon have been important choices for chronic hepatitis B. Results of clinical trials showed that most patients who suffered from liver transplant recipients and liver cancer, can benefit from antiviral treatment with HBV DNA suppression, normalization of alanine aminotransferase (ALT), and improvement in liver histology, compared with patients who received placebo. However, some patients receive antiviral treatment showed non-response, including HBV DNA and abnormal ALT persistence. However, it's still unknown about the dynamic changes of multiple immune cells in CHB during the therapy. In this study, we aimed to confirm the expression characteristics of different immune cells in CHB and explore their clinical significance during the antiviral therapy in CHB patients.

MATERIALS AND METHODS

Patients and controls

Forty-nine treatment-naïve patients with CHB who visited the Shenzhen Third People's hospital were enrolled in this study. All of them were diagnosed according to the described criteria.⁷⁻⁹ No patients have received antiviral treatment [such as interferon or nucleos(t)ides analogues] for at least six months. Individuals with concurrent hepatitis C virus, hepatitis D virus, hepatitis G virus, or human immunodeficiency virus (HIV) infection, individuals with autoimmune liver diseases, and individuals who met clinical or biological criteria of bacterial or fungal infection were excluded. Twenty-nine age- and sex-matched healthy controls (HC) were enrolled as controls. The study protocol was approved by the ethics committee of Shenzhen Third People's Hospital and written informed consent was obtained from each subject.

Detection of serum ALT and HBV DNA levels

Serum creatinine and ALT were measured enzymatically (Olympus AU640, Japan). Serum HBV DNA load was measured by quantitative PCR assay (PG Biotech, Shenzhen, China), with a sensitivity of 5×10^2 copies/ml. Serum HBsAg, anti-HBe, hepatitis B e antigen (HBeAg), antibody to hepatitis B e antigen (anti-HBe) and antibody to hepatitis B surface antigen (anti-HBs) were detected by commercially available microparticle immunoassays (AXSYM, Abbott Laboratories, Chicago, USA).

Flow cytometric analysis

The frequencies of Th1, Th17, Treg and B cells were measured by flow cytometry. All antibodies were purchased from BD Biosciences (San Jose, USA). For Treg cell staining, fresh heparinized peripheral blood (100 μ l) was stained with anti-CD3, anti-CD4, anti-CD25, anti-127 monoclonal antibodies (BD

Biosciences, San Jose, USA) at room temperature for 20 minutes. The blood was then lysed with lysing solution (BD Pharmingen USA). For Th17 and Th1 cells staining, fresh heparinized peripheral blood (250 μ l) was incubated with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml; Sigma-Aldrich, St. Louis, MO), ionomycin (1 μ g/ml, Sigma-Aldrich) and BFA (0.4 μ mol/L, BD Pharmingen) in 750 μ l RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) for 6 hours at 37°C. Cells were stained with anti-CD3, anti-CD4 monoclonal antibodies at first, and then the blood was lysed with fluorescence-activated cell sorting (FACS) lysing solution (BD Pharmingen) and further permeabilities, and stained with anti-IL-17, anti-IFN- γ antibodies. For B cells staining, peripheral blood mononuclear cell (PBMC) was isolated from fresh whole blood by density gradient centrifugation using Lymphoprep (Axis-Shield, Oslo, Norway). PBMC (2×10^5 /100 μ l) were stained with anti-CD10, anti-CD19, anti-CD20, anti-IgM, anti-CD27, anti-CD38 monoclonal antibodies. All stained cells were acquired by FACS Calibur (BD Biosciences) and data were analyzed by FACS Diva software (BD Biosciences).

Statistical analysis

All statistical tests were performed with Prism 3.0 (GraphPad, La Jolla, CA). The one-way analysis of variance was applied for statistical analysis to compare the differences among multiple groups. For the frequencies of all cells at different time points, statistical comparison was analyzed by Wilcoxon matched pair's test. The unpaired t-test was applied to analyze the difference with other two groups. For all tests, two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Clinical markers of all subjects and the dynamic changes in CHB patients

Total of 49 CHB patients, 19 asymptomatic HBV carrier (AsC) and 29 age- and sex-matched healthy donors (HD) were included in this study. The basic characteristics of the subjects are listed in Table 1. Thirty five CHB patients receiving antiviral treatment [include nucleos(t)ides and interferon] were followed with protocol visits for 6 months, 28 of whom were men and 7 were women (mean age as 34 years).

The other 14 CHB patients were lost to follow-up. At baseline, their alanine aminotransferase (ALT) levels were twice as normal upper limits (range: 96-1776 U/L). Their HBV DNA levels were $> 5 \times 10^2$ copies/ml. (range: 3.80×10^4 - 1.06×10^{10} copies/ml). Among the 35 CHB patients, there were 8 patients (23%) with undetectable serum HBeAg and 27 patients (77%) with detectable serum HBeAg upon admission. For the 35 CHB patients, the

Table 1. The basic characteristics of all subjects

Group	Cases	Sex (male/female)	Age (year)	ALT (U/L)	AST (U/L)	HBV DNA (log ₁₀ copies/ml)	HBsAg (+/-)	HBeAg (+/-)
HC	29	15/14	30.4 (21 - 60)	< 40	< 40	ND	0/29	0/29
AsC	19	14/5	30.5 (18 - 58)	20 (9- 38)	21 (11 - 32)	5.6 (2.7 - 8.3)	19/0	12/7
CHB	49	37/12	33.0 (18 - 59)	324 (86 - 1776)	171 (38 - 1049)	6.9 (3.8 - 9.3)	49/0	37/12

levels of serum HBV DNA, alanine aminotransferase (ALT) and glutamic-oxalacetic (AST) were determined at baseline, as well as 1, 3 and 6 months after antiviral treatment. Our results showed that ALT, AST and HBV DNA in CHB patients were at peak levels upon admission. ALT, AST and HBV DNA after treatment for 1 month were considerably lower than those at baseline and they gradually decreased to normal range. At the end of the following-up, HBV DNA levels were undetectable in 27 patients (77%) and ALT levels were normal in 29 (83%) patients. Loss of serum HBeAg occurred in 2 patients, while loss of serum HBsAg did not occur in any patient during the treatment. Changes in the levels of clinical markers in these subjects are shown in Figure 1.

Figure 1. Clinical characteristics of CHB patients undergoing antiviral treatment.

Notes: Serum ALT levels, AST levels and HBV DNA levels of the 35 patients at baseline and during 6 months of antiviral therapy. The levels of HBV DNA and ALT 1 month after treatment were significantly lower than those at baseline ($P < 0.05$). Each symbol represents an individual and each line represents changes in an individual patients' DNA or ALT levels from the baseline to the endpoint.

ALT: alanine aminotransferase; AST: glutamic-oxalacetic transaminase. HBV: hepatitis B virus.

Th17, Treg and Th1 cells responses and their dynamic changes in peripheral blood of CHB patients

As shown in Figure 2A and 2B, all subjects clearly displayed the CD4 T-cell subsets ($n = 49$). Frequencies of Th17 and Treg cells in CHB patients ($4.04\% \pm 0.29\%$, $8.2\% \pm 0.26\%$) were significantly higher compared with healthy subjects ($2.9\% \pm 0.27\%$, $6.2\% \pm 0.26\%$) (Figure 2C, 2D). Despite Th1 can generate from the same naive T cell pool that generate Th17 and Treg cells, no statistical differences were observed on the frequencies of Th1 cells between CHB patients and healthy controls (HC) (Figure 2E). To better understand the effect of antiviral therapy on T-helper subsets, we longitudinally determined the proportion

of CD4⁺T-cell subsets in the blood samples of 35 CHB patients by flow cytometric assay. All patients showed distinct compositions of CD4⁺T-cell subsets at different treatment time points. The frequencies of Th17 cells increased after treatment for 6 months, but there was no statistically significant difference of IL-17 expression between baseline and 6 months after treatment (Figure 2F). Frequencies of Th1 reached the maximal peak 6 months after treatment, which also significantly increased 1 month after treatment than those at baseline (Figure 2G). While frequency of Treg cells after antiviral treatment was lower than baseline (Figure 2H).

B cells response and their dynamic changes in peripheral blood in CHB patients

Previous studies on CHB patients suggested that B cells play an important role in HBV induced inflammatory responses. To better understand the role of B cells in HBV infection, we extended these findings by comparing B cells responses in patients with asymptomatic HBV carrier (AsC) ($n = 19$), as well as patients with CHB ($n = 49$). As shown in Figure 3A, all subjects clearly displayed the B lymphocyte subsets. Meanwhile, we investigated the immature B cells ($p3$ and $p5$, IgM⁺CD10⁺dCD19⁺CD20⁺CD27⁻CD38⁺⁺), naive B cells ($p3$, $p6$ and $p7$, IgM⁺CD10⁻CD19⁺CD20⁺CD27⁻CD38^{+/+}), memory B lymphocytes ($p3$, $p4$ and $p8$, IgM^{+/+}CD10⁻CD20⁺CD27⁺CD38^{+/+}) and plasmablasts ($p9$ and $p10$, IgM⁻CD10⁻CD19⁺CD20⁺CD27^{+/+}CD38⁺⁺). The results showed that B cells response was significantly increased in asymptomatic carriers compared with healthy donors. The frequency of B cells in CHB patients was higher than HC, but there was no significant difference ($P = 0.0573$) (Figure 3B). The subsets of B cells were also analysed. As shown in Figure 3C, there were no obvious difference of immature B cells frequencies among CHB, AsC and HC ($P = 0.4778$). The frequencies of memory B cells showed the similar results ($P = 0.2432$) (Figure 3D). While the frequencies of plasmablasts were higher in AsC than that in HC ($P = 0.012$). However, we find no significant difference between CHB patients and HC ($P = 0.0817$), there were no obvious difference of plasmablasts between CHB patients and AsC ($P = 0.1172$) (Figure 3E). As shown in Figure 3F, frequencies of naïve B cells in CHB and HC were higher than that in AsC ($P = 0.0004$, $P = 0.0052$), but the difference between CHB and HC were not obvious ($P = 0.4703$).

To assess the effect of antiviral treatment on B

Figure 2. Treg, Th17, Th1 cell frequency from CHB patients and their dynamic changes during antiviral treatment.

Notes: A: Dot plots show CD25⁺CD127⁻ CD4 T cells (Treg) in the peripheral blood of HD and CHB; B: Dot plots show IL-17-producing CD4 T cells (Th17) and IFN-γ-producing CD4 T cells (Th1) in the peripheral blood of HC and CHB. Pooled data indicates the percentage of C: Th17, D:Treg and E: Th1 within CD4 T cells. The frequencies of CD4 T cell subsets in antiviral-treated patients during the course of therapy; F: The frequencies of Th17 cells; G: The frequencies of Th1 cells; H: The frequencies of Treg cells were observed from the baseline to the endpoint month 6. The P values between different time points are shown. Each symbol represent some one individual. Horizontal bars represent the median values; ns: no significance.

lymphocyte's subsets, we only got 19 CHB patients in test group at the end of follow-up. The frequencies of CD19 B cells reached the miximal peak after treatment for 6 months, which were not lower than HC (Figure 3G). After treatment for 3 months, the frequency of immature B cells showed a reverse "v" type changes with the maximal peak (Figure 3H). While the frequencies of naïve B cells gradually decreased durning the following-up (Figure 3I). The frequencies of memory B cells after antiviral treatment were lower than baseline (Figure 3J). The frequencies of plasma cells gradually increased and showed the maximal peak 6 months after antiviral treatment (Figure 3K).

Comparison of immune cells levels with different clinical results

Previous studies suggested that immune status was closely related to clinical results.³ In our study, we found the patients with normal liver function had higher level of Th17 cells 6 months after treatment than those with abnormal live function 3 months after treatment ($P = 0.0458$) (Figure 4A). The frequencies of Th1 cells showed the same tendency (Figure 4B). After treatment for 6 months, levels of Th17 cells in patients with HBeAg were lower than those with negative HBeAg (Figure 4C). Levels of Treg cells in patients with HBV DNA at baseline were higher than those with

Figure 3. Frequencies of B cells from CHB patients and their dynamic changes during antiviral treatment.

Notes: A(1-7): We measured the frequency of B cells using flow cytometric analysis. P3 and P5 showed the immature B cell. P3 and P6 and P7 showed the naive B cell. P3 and P4 and P8 showed the memory B cells. P9 and P10 showed the plasmablasts; B: the level of CD19⁺ cells; C: the level of immature B cells; D: the level of memory B cells; E: the level of plasma cells; F: the level of naive B cells were shown among CHB patients, AsC patients and HC. Comparisons between groups were calculated using the unpaired t-test. The frequencies of B-lymphocyte's subsets in antiviral treated patients during the course of therapy; G: the frequency of CD19⁺ B cells; H: the frequency of immature B cells; I: the frequency of naive B cells; J: the frequency of memory B cells; K: the frequencies of plasma cells were observed from the baseline to the endpoint (month 6). Comparisons between groups were calculated using the paired t-test. The *P* values between different time points are shown. ns, not significant. Each symbol represents one individual, and each line represents the changes from the baseline to the endpoint (month 6). Horizontal bars represent the median values of indicated index.

Figure 4. Difference of cells frequencies among different clinical results.

Notes: The patients were divided into different groups according to different clinical results. A: the comparison of Th17 frequency; B: the comparison of Th1 frequency; C: the comparison of Th17 frequency 6 months after treatment between HBeAg(+) patients and HBeAg (-) patients; D: the comparison of Treg frequency. E: the comparison of Treg frequency between male and female. Figure C and E were calculated by unpaired t-test. Figure A, B and D were calculated by paired t-test. *P* values among groups are also shown. Ns: not significant. Each dot represents one individual. Horizontal bars represent the median values of indicated index.

negative HBV DNA patients 1 month after treatment ($P = 0.0272$) (Figure 4D). As shown in Figure E, male had higher levels of Treg cells than female at baseline.

DISCUSSION

Close cooperation of T lymphocyte and B lymphocyte cells are critical for host immune response against HBV. The balance of effectors CD4⁺T cells (Th17 and Th1 responses) and regulatory responses is an important element of immune regulation.¹⁰ Previous study showed that ADV treatment increases the immunity of Th1/Th2 cells in CHB patients, and increases of cytokines partly reflect the efficacy of antiviral treatment.¹¹ Our studies suggested frequencies of Th1 cells increased during the antiviral treatment, which set IFN- γ and promote the cytotoxicity caused by CTLs to effectively eliminate the virus. Antigen non-specific Th17 response increased in patients with CHB, and frequency of peripheral Th17 cells in CHB patients were closely associated with the degree of liver damage, which determined by serum alanine amino-transferase (ALT) levels and liver histological activity index (HAI) scores. With the clearance of HBV and the normalization of liver function, the frequencies of Th17 cells reached the maximal peak 1 month after treatment, which remained higher during treatment for 9 months than those at baseline and the endpoint. In contrast, the frequencies

of Tregs gradually decreased during the 6 months' treatment. They showed a reverse 'V' type change with the maximal peak 9 months after treatment.¹² Adefovir-induced viral load reduction lead to the decline on the number of circulating Tregs together with a partial recovery of the immune response.^{13,14} In our study, Th17 cells frequencies 6 months after treatment were higher than those 1 month and 3 months after treatment. The frequencies of Treg cells after antiviral treatment were lower than that at baseline. The change of Th17 cells and Treg cells are complicated, which may be affected by many factors. More time and samples are needed to get the changing patterns of these cells. Great deal of evidences have suggested humoral immune responses are required for viral clearance in addition to cellular immune responses.^{15,16} Our study showed that more memory B cells differentiate into plasma cells, whose frequencies increased during the antiviral treatment. The memory B cells have a long cycle, the decreased number of which may explain the total B cells reduction. So we get the conclusion that antiviral drugs directly block viral replication and may get the immune function reestablished. Treg cells decreased in the early stage of antiviral treatment and anti-HBV specific proliferation reactions were strengthened. Memory B cells differentiate into plasma cells more quickly, and effectively eliminate the virus in liver and blood. However, the limited time and samples in our study precluded us from certifying the relationship

between the loss of HBeAg and immune cells.

In conclusion, this study highlights that the reestablishment of immune function during antiviral treatment in CHB patients, which caused by the antiviral drugs or the patients themselves. CHB patients may exhibit varied responses to these antiviral drugs.^{17,18} It is essential to supplement immune therapy during the antiviral treatment, but Th17 may play a limited role in inflammation during antiviral treatment, targeting Th17 therapy may not be useful for treatment of CHB. More time and more experiments are critical to explain it.

List of Abbreviations

Th17: Interleukin-17-producing CD4⁺ T cell; HBV: hepatitis B virus; CHB, chronic hepatitis B; ALT: alanine amino-transferase; AST: glutamic-oxalacetic transaminase. HBsAg: Hepatitis B surface antigen; HIV: human immunodeficiency virus; AsC: asymptomatic HBV carrier; HC: health controls; PBMCs: Peripheral blood mononuclear cells;

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Bo-ping Zhou and Xin-chun Chen co-defined the research theme. Xin-chun Chen designed the methods and experiments. Pei-yan Zhang carried out the laboratory experiments, analyzed the data and drafted the manuscript. Si-min Yao, Jing Yuan, and Wei Li co-worked on the associated data collection and their interpretation. Bo-ping Zhou and Xin-chun Chen revised the paper critically for important intellectual content. All authors have seen and approved the manuscript.

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