

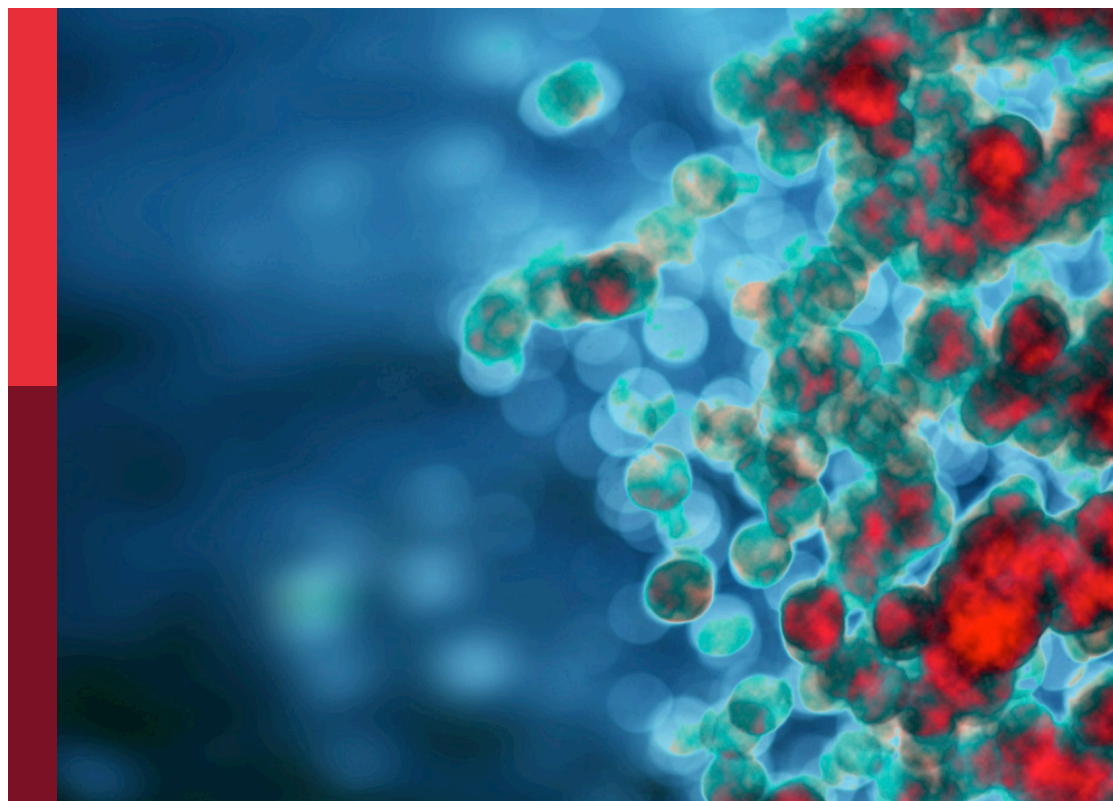
Focus on HBV: Antiviral, pregnancy, and immunization

Edited by

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Focus on HBV: Antiviral, pregnancy, and immunization

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Dynamic changes of cytokine profiles and virological markers during 48 weeks of entecavir treatment for HBeAg-positive chronic hepatitis B

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Objective: The aims of this study were to investigate the kinetic changes of serum, virological, and immunological markers during entecavir (ETV) antiviral therapy and to explore whether these indicators can predict the antiviral efficacy of ETV in hepatitis B e antigen (HBeAg)-positive chronic hepatitis B (CHB) patients.

Methods: HBeAg-positive CHB patients were enrolled and treated with ETV 0.5 mg/day. Clinical biochemical, virological, and serological tests were performed at baseline and every 12 weeks during the 48-week treatment. Plasma levels of cytokines (FIt-3L, IFN- α 2, IFN- γ , IL-10, IL-17A, IL-6, TGF- β 1, TGF- β 2, TGF- β 3, and TNF- α) were measured at baseline and at 12 and 24 weeks after treatment. Analysis of the trends of these clinical indicators in ETV antiviral therapy was performed.

Results: A total of 105 HBeAg-positive CHB patients were enrolled, and 100 of them completed 48 weeks of ETV treatment and follow-up. After 48 weeks of treatment, hepatitis B s antigen (HBsAg) decline $\geq 1 \log_{10}$ was found in seven patients, but no patient achieved HBsAg disappearance. serological HBeAg disappeared in 13 patients, and serological HBeAg transformed in 3 patients. The baseline HBsAg and HBeAg levels, HBV DNA load, IL-10, and TGF- β 1 levels in the complete virological response group were lower than those in the incomplete virological response group, while the ALT level in the complete virological response group was higher than that in the incomplete virological response group. Both univariate analysis and multivariate analysis showed that baseline biochemical indexes, virological indexes, and cytokine levels had no correlation

with the complete virological response at 48 weeks. In multivariate analysis, low baseline HBV DNA load, and HBeAg and IL-10 levels were significantly associated with ALT normalization after 48 weeks of ETV treatment (HBeAg OR = 1.003, 95% CI 1.001–1.006, $p = 0.007$; HBV DNA OR = 0.184, 95% CI 0.046–0.739, $p = 0.017$; IL-10 OR = 0.040, 95% CI 0.972–0.999, $p = 0.040$).

Conclusion: Cytokine levels changed dynamically during ETV antiviral therapy. Low baseline HBV DNA load, and HBeAg and IL-10 levels were significantly associated with ALT normalization after 48 weeks of ETV treatment.

KEYWORDS

chronic hepatitis B, cytokines, entecavir, hepatitis B e antigen, deoxyribose nucleic acid

Introduction

Chronic hepatitis B (CHB) is one of the most serious infectious diseases in China (1, 2). CHB is considered to be an immune-mediated disease, in which the immune response initiated by HBV causes hepatocyte necrosis and inflammation of liver tissue. It causes liver tissue damage while clearing the virus. If there is no immune clearance, long-term HBV infection will not cause liver histological damage (3–5).

Antiviral therapy can significantly reduce the incidence of liver cirrhosis and liver cancer in patients with CHB, and is an important means to delay the progression of liver disease. Interferon and nucleoside (acid) analogs are two types of effective antiviral drugs for CHB as recommended by most guidelines (2, 6, 7). We previously found that the pathogenesis and antiviral efficacy of CHB were related to immune cells and cytokines. The pathogenesis of CHB was positively correlated with interferon-alpha (IFN- α) and negatively correlated with transforming growth factor beta (TGF- β) and interleukin-10 (IL-10) (8, 9), while TGF- β and interferon-gamma (IFN- γ) were related to the efficacy of interferon (10). Our study also found that, compared with entecavir (ETV) therapy, peginterferon-alpha (PEG-IFN- α) treatment could significantly increase natural killer (NK) cell frequency and function (11). Recently, we have reported that patients with lower baseline hepatitis B virus deoxyribose nucleic acid (HBV DNA) load and higher baseline cluster of differentiation86+ (CD86+) plasmacytoid dendritic cell (pDC) % were more likely to obtain functional cure (12), and the dynamic changes of early cytokines and virological markers were associated with clinical cure of hepatitis B e antigen (HBeAg)-positive CHB (13). These results indicate that pathogenesis of CHB and antiviral treatment especially the interferon treatment is related to immunity.

It remains to be determined whether the effect of nucleoside (acid) analogues, another class of first-line drugs for antiviral treatment of CHB, is related to immunity. The purpose of this study is to explore the changes in serology, virology, and cytokine indexes in patients with HBeAg-positive CHB during the 48-week ETV treatment. We detected cytokines that cause liver inflammation and immunosuppression (IL-6, IL-10, TNF- α , and TGF- β), factors that stimulate immune cell function (IFN- α), and factors that have viral clearance (IL-17A and IFN- γ) or can stimulate proliferation of DC cells and NK cells (Flt-3L) (14–17). We hope to find out the serology, virology, and cytokine indexes related to ETV antiviral response, and to provide a basis for the selection of a more reasonable antiviral treatment scheme.

Materials and methods

Subjects and inclusion and exclusion criteria

Subjects of this prospective cohort study were HBeAg-positive patients with CHB. From November 2017 to November 2018, HBeAg-positive CHB patients who were willing to be treated with ETV in the Department of Hepatology Division 2 of Beijing Ditan Hospital Affiliated to Capital Medical University entered the study after signing informed consent.

The inclusion criteria of HBeAg-positive CHB patients were as follows (15): (1) continuous hepatitis B s antigen (HBsAg) positive (HBsAg ≥ 0.05 IU/ml) > 6 M; (2) HBeAg positive (HBeAg ≥ 1.0 S/CO); (3) HBV DNA positive ($>10^4$ IU/ml); (4) ALT abnormality (≥ 80 IU/L) lasting for more than 3 months or significant inflammation (above G2) in liver histological

examination; (5) 18–65 years old; (6) gender is not limited; and (7) no use of hormones and/or immunosuppressants and other liver protection drugs.

Exclusion criteria were as follows: (1) complicated with other virus (EBV, CMV, HIV, HCV, HDV, etc.) infection; (2) combined with autoimmune liver disease; (3) chronic alcoholism; (4) taking other liver damaging drugs; (5) have a history of mental illness; (6) have evidence of liver tumor (clinical diagnosis of liver cancer or AFP > 100 ng/ml); (7) liver fibrosis and cirrhosis (excluded by Fibroscan) (18); (8) those who have serious heart, brain, lung, kidney, and other serious diseases and cannot participate in long-term follow-up; (9) use of hormones and/or immunosuppressants; and (10) there are other liver diseases (fatty liver, metabolic liver disease, and hereditary liver disease).

This study was approved by the Ethics Committee of Beijing Ditan Hospital affiliated to Capital University of Medical Sciences (JDL-2017-034-01), and was registered with Clinical Trials (NCT03210506).

Research design

The patients were treated with ETV 0.5 mg/day and followed up for 48 weeks after enrollment. The virological and serological indexes, biochemistry, and AFP were tested at baseline and every 3 months during ETV treatment. Liver imaging examination was performed every 6 months. Cytokines (Flt-3L, IFN- α 2, IFN- γ , IL-10, IL-17A, IL-6, TGF- β 1, TGF- β 2, TGF- β 3, and TNF- α) were measured at baseline and at the 3rd and 6th months of treatment to analyze the change trend of cytokines in ETV antiviral therapy.

Grouping: According to HBV DNA level at 48 weeks of ETV treatment, the patients were divided into complete virological response group with HBV DNA < 20 IU/ml and incomplete virological response group with HBV DNA $\geq$ 20 IU/ml. According to the ALT level at 48 weeks of ETV treatment, the subgroups were divided into normal ALT group with ALT < 40 U/L and abnormal ALT group with ALT $\geq$ 40 U/L.

This study mainly observed the response of patients during 48 weeks of treatment. After 48 weeks of treatment, the ETV treatment would continue if the response was good. If the response was poor, ETV would be switched to other nucleosides (acids) or interferon alone/in combination according to the patient's wishes.

This is a prospective exploratory study. We previously found that, approximately 80% of CHB patients achieved virological response (clinical outcome event) after 48 weeks of ETV treatment. Six factors (age, gender, HBsAg, HBeAg, HBV DNA, and immune cells) affecting the effect of ETV on CHB treatment were considered, and 15 patients were enrolled for each factor ($15 \times 6 = 90$ patients). Considering a loss rate of 10%–20%, the sample size was determined to be 99–118 cases. In

the end, we enrolled 105 patients with CHB in this prospective study, which met the statistical requirements.

Detection of HBV DNA, HBV serology, and clinical indexes

Blood routine (Sysmex Corporation, Japan), liver function (Wako Pure Chemical Industries, Ltd., Japan), renal function (Sekisui Medical Co, Ltd, Japan), tumor series index (Abbott Ireland Diagnostics Division, Finisklin Business Park, Sligo, Ireland), and serum HBV DNA load (Cobas ampliprep/Cobas TaqMan 96, polymerase chain reaction, PCR, detection limit < 20 IU/ml) were performed.

HBsAg/anti HBs level and HBeAg/anti-HBe level were detected by Abbott Architect i2000 microparticle chemiluminescence reagent. The detection range of HBsAg level is 0.05–250 IU/ml; those with HBsAg level greater than 250 IU/ml will be automatically diluted 500 times. The actual HBsAg level is calculated by multiplying the detection value by 500. HBsAg < 0.05 IU/ml was defined as the disappearance of HBsAg.

Quantitative detection of plasma cytokines

The following cytokines were detected by Luminex Technology: Fms-like tyrosine kinase 3 ligand (Flt-3L), interferon-alpha 2 (IFN- α 2), interferon-gamma (IFN- γ), interleukin-10 (IL-10), interleukin-17A (IL-17A), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF- α), transforming growth factor beta1 (TGF- β 1), transforming growth factor beta2 (TGF- β 2), and transforming growth factor beta 3 (TGF- β 3). All the data were analyzed by Flexmap 3D analyzer.

Our study aimed to investigate whether cytokines can early predict the efficacy of ETV therapy for HBeAg-positive chronic hepatitis B; thus, we tested cytokines primarily at baseline, week 12, and week 24.

Statistical analysis

The counting data were expressed by the number of cases and the percentage, and the Chi-square test was used for comparison between groups. The statistical description of normal distribution measurement data was expressed by means $\pm$ standard deviation (mean $\pm$ SD), and the *t*-test of two independent samples was used for comparison between the two groups. Non-normal distribution data were expressed by median and quartile (median, Q1Q3), and Mann–Whitney *U* test was used for comparison between groups. Spearman or

Pearson correlation test was used for the correlation of variables according to whether they conformed to normal distribution.

All data were statistically analyzed with SPSS (Statistical Package for the Social Sciences) 21.0 software (SPSS, Chicago, IL) and GraphPad Prism 5 software. In the statistical analysis of the data, all tests were performed by two-sided test.

Bonferroni is used to correct the rank-sum test results at each different time point, and the correction level is α . Among them, biochemical, viral, serological, and other indicators were observed at five time points. α was set to 0.01, and $p < 0.01$ was statistically significant. There were three observation time points for cytokines. α was set as 0.013, and $p < 0.013$ was statistically significant.

Results

Basic clinical characteristics of patients

From November 2017 to November 2018, 221 of the 250 eligible HBeAg-positive CHB patients signed the informed consent to enter our study, and the remaining 29 were lost to follow-up. Among the 221 CHB patients, 105 received ETV antiviral therapy, while 116 chose to be treated with PEG-IFN α -2a. In the ETV group, one case withdrew due to the increase in lactate, and four cases did not complete the 48-week follow-up. Finally, a total of 100 CHB patients treated with ETV completed the 48-week follow-up

(Figure 1). The median age was 32 years (28–38), with 61 men and 39 women. After 48 weeks of treatment, HBsAg decreased by ≥ 1 log10 in seven cases, but no patient achieved HBsAg disappearance. serological HBeAg disappeared in 13 cases, and serological HBeAg transformed in 3 cases.

Group analysis according to virological response at week 48 of ETV treatment

According to the HBV DNA level at 48 weeks of ETV treatment, the complete virological response group was defined as HBV DNA < 20 IU/ml, and the incomplete virological response group was defined as HBV DNA ≥ 20 IU/ml. There were 87 cases (63 men) in the complete virological response group and 13 patients (8 men) in the incomplete virological response group. There were no statistical differences in age, biochemical indexes (ALT, AST, TBIL, and ALB), virological indexes (HBV DNA, HBsAg, and HBeAg), or cytokines between the two groups at baseline and 3 months (Figure 2).

At the 6th month, there was significant difference in the median HBeAg between the two groups (57.67 vs. 291.05 S/CO, $Z = -2.260$, $p = 0.024$), but not in HBsAg level, biochemical indexes, or cytokines (Figure 2). After 9 months, the median HBeAg and HBsAg levels in the complete virological response group were significantly lower than those in the incomplete

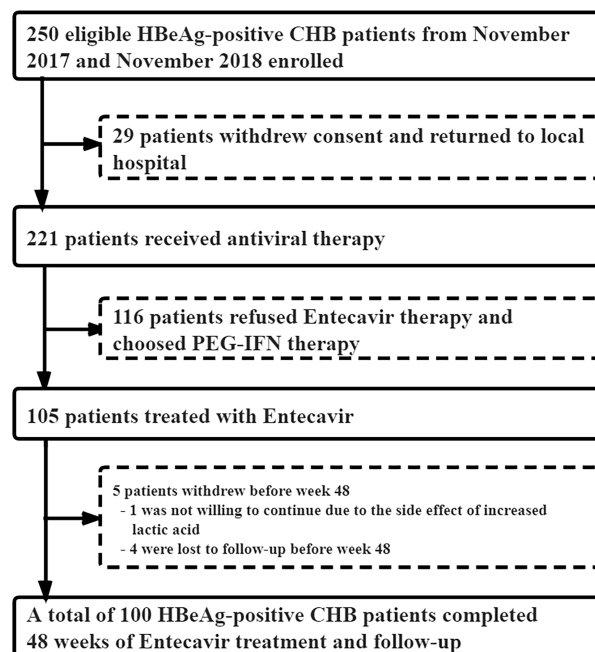


FIGURE 1
Patient enrollment and deposition.

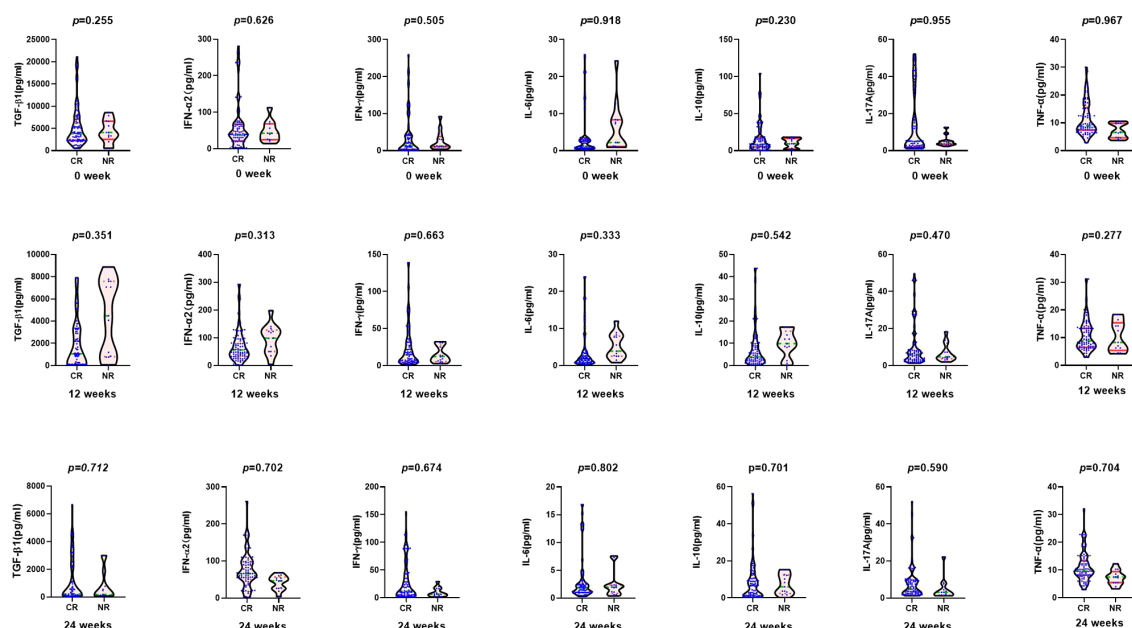


FIGURE 2

Comparison of cytokines between complete virological response group (CR: $n = 87$) and non-complete virological response group (NR: $n = 13$) at baseline, 12, and 24 weeks after entecavir therapy.

virological response group (HBeAg 22.67 vs. 886.21 S/CO, $Z = -4.895$, $p = 0.000$; HBsAg 3.66 log₁₀ vs. 4.06 log₁₀ IU/ml, $Z = -4.034$, $p = 0.017$).

After 12 months of treatment, the median HBeAg and HBsAg levels were significantly lower in the complete virological response group as compared with the incomplete virological response group (HBeAg 11.25 vs. 906.46 S/CO, $Z = -5.007$, $p = 0.000$; HBsAg 3.64 log₁₀ vs. 4.09 log₁₀ IU/ml, $Z = -3.645$, $p = 0.000$), and the median AST was significantly increased (21 vs. 17.40 U/L, $Z = -2.266$, $p = 0.023$). However, there was no difference in ALT, TBil, and ALB between the two groups (Table 1).

Baseline predictors of complete virological response at week 48 of ETV treatment

Both univariate analysis and multivariate analysis showed that baseline biochemical indexes (ALT, AST, TBIL, and ALB), virological indexes (HBV DNA, HBsAg, and HBeAg), and cytokine levels had no correlation with the complete virological response after 48 weeks (Table 2).

Subgroup analysis of ALT level at week 48 of ETV treatment

The normal ALT values for men and women in different regional guidelines are inconsistent. The 2016 AASLD guidelines suggest that the upper limit of normal ALT values for men and women with HBV infection is 30 U/L and 19 U/L, respectively (19). The 2018 AASLD guidelines suggest that the normal ALT values for infected men and women are 35 U/L and 25 U/L (7), respectively. However, the Asia-Pacific guidelines and EASL guidelines have always used 40 U/L as the normal upper limit of ALT levels for men and women (6, 20). Since ALT levels are the most sensitive indicator of hepatocyte necrosis and liver inflammation, patients with abnormal ALT and more pronounced ALT elevations often have more significant liver inflammation than patients with normal ALT level and mildly elevated ALT. Therefore, as proposed in the 2015 APASL and 2017 EASL guidelines, 40 U/L was still used as the normal upper limit of ALT level in this study.

According to the ALT level at 48 weeks of ETV treatment, the subgroups were divided into the normal ALT group with ALT < 40 U/L and the abnormal ALT group with ALT ≥ 40 U/L. There were 90 patients (64 men) in the normal ALT group and

TABLE 1 Comparison of clinical indicators between the complete virological response group ($n = 87$) and incomplete virological response group ($n = 13$) at baseline and 12, 24, 36, and 48 weeks after entecavir therapy.

	Baseline			12 weeks			24 weeks			36 weeks			48 weeks		
	Complete virological response	Incomplete virological response	Z/p	Complete virological response	Incomplete virological response	Z/p	Complete virological response	Incomplete virological response	Z/p	Complete virological response	Incomplete virological response	Z/p	Complete virological response	Incomplete virological response	Z/p
ALT (U/L)	229.30 (121.20–360.94)	217 (153.55–324)	– 0.164/ 0.870	32 (21–49.8)	43.2 (27.35–80.75)	–1.604/ 0.109	32 (21–49.8)	43.2 (27.35–80.75)	–1.082/ 0.279	22.40 (15.80–28.90)	24.90 (22.40–30.80)	–1.528/ 0.127	20.50 (15.30–29.10)	23.10 (16.90–25.60)	0.000/ 1.000
AST (U/L)	101.75 (61.18–195.63)	112.70 (50.35–137.50)	– 0.815/ 0.415	27.7 (19.90–35.50)	30 (21.95–38.75)	–0.856/ 0.392	27.7 (19.90–35.50)	30 (21.95–38.75)	–1.374/ 0.169	21 (16.90–25.40)	22.40 (15.15–25.20)	–0.220/ 0.826	21.40 (17.60–25.00)	17.40 (16.45–20.70)	–2.266/ 0.023
TBil ($\mu\text{mol/L}$)	15.70 (11.55–21.43)	13.70 (11.60–22.85)	– 0.164/ 0.870	12.6 (9.3–18.20)	12.8 (8.40–23.15)	–0.385/ 0.701	12.6 (9.3–18.20)	12.8 (8.40–23.15)	–1.374/ 0.169	13 (10.00–16.20)	12.5 (8.95–13.50)	–1.133/ 0.257	12.20 (9.90–14.90)	13.50 (9.95–17.50)	–0.723/ 0.470
ALB (g/L)	45.80 (40.98–48.40)	45.00 (43.05–46.95)	0.218/ 0.828	47.8 (44.50–49.20)	45.6 (43.65–48.25)	–1.539/ 0.124	47.8 (44.50–49.20)	45.6 (43.65–48.25)	–1.539/ 0.124	48.50 (46–50.20)	48.50 (46.50–49.85)	–0.062/ 0.951	48.20 (46.40–49.80)	49.70 (47.40–49.90)	–1.661/ 0.097
HBsAg (log ₁₀ IU/ml)	3.86 (3.59–4.10)	3.94 (3.70–4.10)	– 0.677/ 0.499	3.77 (3.30–4.04)	3.65 (3.52–3.93)	–0.113/ 0.910	3.77 (3.30–4.04)	3.65 (3.52–3.93)	–0.379/ 0.704	3.66 (3.22–3.92)	4.06 (3.99–4.37)	–4.034/ 0.000	3.64 (3.18–3.92)	4.09 (3.80–4.27)	–3.645/ 0.000
HBeAg (S/CO)	811.69 (437.33–1,173.23)	805.87 (683.66–1,148.33)	– 0.502/ 0.615	57.67 (13.74–665.09)	291.05 (50.02–931.78)	–1.596/ 0.111	57.67 (13.74–665.09)	291.05 (50.02–931.78)	–2.260/ 0.024	22.67 (3.40–96.65)	886.21 (321.53–1,190.18)	–4.895/ 0.000	11.25 (2.30–50.24)	906.46 (164.32–1,194.36)	–5.007/ 0.000
HBV DNA (log ₁₀ IU/ml)	6.63 (6.34–6.96)	7.99 (7.12–8.36)	– 3.536/ <0.001	2.75 (0–3.34)	4.29 (3.94–4.59)	–4.344/ <0.001	0.00 (0.00,2.36)	3.72 (2.77–3.79)	–4.639/ <0.001	0.00 (0.00, 0.00)	3.32 (2.77–3.79)	–7.192/ <0.001	0.00 (0.00, 0.00)	3.07 (2.49–3.44)	–9.918/ <0.001
Flt-3L (pg/ml)	0.11 (0.02–43.59)	NA	NA	1.25 (0.02–28.46)	NA	–1.225/ 0.333	2.24 (0.02–78.56)	NA	–1.225/ 0.221	/	/	/	/	/	/
IFN- α 2 (pg/ml)	41.60 (26.46–74.19)	62.21 (26.21–74.21)	– 0.487/ 0.626	56.01 (34.02–98.29)	72.34 (40.88–112.53)	–1.010/ 0.313	67.89 (52.61–98.74)	46.38 (26.46–56.88)	–0.382/ 0.702	/	/	/	/	/	/
IFN- γ (pg/ml)	19.72 (3.92–45.38)	30.05 (5.89–161.61)	– 0.666/ 0.505	14.43 (5.79–31.55)	10.01 (4.59–24.97)	–0.436/ 0.663	12.94 (5.22–45.28)	6.47 (3.25–16.70)	–0.420/ 0.674	/	/	/	/	/	/
IL-10 (pg/ml)	8.58 (4.94–17.69)	15.60 (8.53–21.89)	– 1.199/ 0.230	4.70 (2.08–10.28)	6.12 (2.54–13.31)	–0.610/ 0.542	6.56 (1.23–12.56)	5.98 (2.40–12.37)	–0.384/ 0.701	/	/	/	/	/	/
IL-17A (pg/ml)	5.20 (2.73–37.25)	5.20 (2.60–25.94)	– 0.056/ 0.955	6.10 (3.12–10.12)	4.09 (2.86–10.47)	–0.723/ 0.470	6.28 (2.86–10.13)	2.29 (1.54–4.05)	–0.538/ 0.590	/	/	/	/	/	/
IL-6 (pg/ml)	2.44 (0.84–7.25)	2.55 (0.71–5.59)	– 0.103/ 0.918	1.89 (0.80–3.76)	2.20 (1.40–7.08)	–0.969/ 0.333	1.92 (1.02–3.20)	1.85 (0.84–2.32)	–0.251/ 0.802	/	/	/	/	/	/

(Continued)

TABLE 1 Continued

	Baseline				12 weeks				24 weeks				36 weeks				48 weeks			
	Complete virological response	Incomplete virological response	Z/p		Complete virological response	Incomplete virological response	Z/p		Complete virological response	Incomplete virological response	Z/p		Complete virological response	Incomplete virological response	Z/p		Complete virological response	Incomplete virological response	Z/p	
TNF- α (pg/ml)	8.79 (6.59–13.30)	8.64 (6.86–15.81)	– 0.041/ 0.967		8.29 (6.29–13.67)	12.51 (6.93–15.95)	–1.087/ 0.277		9.72 (7.69–14.24)	7.53 (5.48–10.30)	–0.379/ 0.704		/	/	/		/	/	/	
TGF- β 1 (pg/ml)	3.874 (2.314–7.019)	5.025 (2.786–10.500)	– 1.138/ 0.255		1.455 (203.13–3.767)	889 (105.47–3,401.50)	–0.933/ 0.351		468 (103.86–2,548)	170.12 (104.78–1,328.32)	–0.369/ 0.712		/	/	/		/	/	/	
TGF- β 2 (pg/ml)	412.21 (217.39–575.38)	NA	NA		1.25 (0.02–28.46)	NA	–1.225/ 0.333		/	/	/		/	/	/		/	/	/	
TGF- β 3 (pg/ml)	187.12 (151.96–209.12)	NA	NA		56.01 (34.02–98.29)	72.34 (40.88–112.53)	–1.010/ 0.313		/	/	/		/	/	/		/	/	/	

Biochemistry and virus indicators: $p < 0.010$ was statistically significant. Cytokines: $p < 0.013$ was statistically significant.

10 patients (7 men) in the abnormal ALT group. They had similar age, biochemical indexes, and cytokines at baseline. The baseline HBsAg and HBeAg levels in the ALT normalization group were significantly lower than that in the non-normalization group. After 3 months, the median HBsAg and HBeAg levels in the normal ALT group were significantly higher as compared with the abnormal ALT group (HBsAg 3.78 log₁₀ vs. 3.29 log₁₀ IU/ml, $Z = -2.632$, $p = 0.008$; HBeAg 86.66 vs. 12.85 S/CO, $Z = -2.654$, $p = 0.008$), and the median TBIL level was significantly lower (TBIL 12.20 vs. 25.50 μ mol/L, $Z = -2.477$, $p = 0.013$).

After 6 months, the median HBsAg and HBeAg levels in the normal ALT group were significantly higher than those in the abnormal ALT group (HBsAg 3.84 log₁₀ vs. 3.23 log₁₀ IU/ml, $Z = -2.356$, $p = 0.018$; HBeAg 86.74 vs. 12.56 S/CO, $Z = -2.287$, $p = 0.022$).

After 9 months, the median HBeAg level in the normal ALT group was significantly higher compared with the abnormal ALT group (57.13 vs. 8.76 S/CO, $Z = -1.976$, $p = 0.048$).

At 12 months, the median HBsAg level in the normal ALT group was significantly higher as compared with the abnormal ALT group (3.68 log₁₀ vs. 3.04 log₁₀ IU/ml, $Z = -2.195$, $p = 0.028$), while the median ALT and AST levels were lower (ALT 20.10 vs. 48.20 U/L, $Z = -5.172$, $p = 0.000$; AST 20.70 vs. 41.90 U/L, $Z = -4.862$, $p = 0.000$) (Table 3).

Baseline predictors of ALT normalization at week 48 of ETV treatment

Univariate analysis showed that baseline HBsAg, HBeAg, and IL-10 were significantly correlated with ALT normalization at 48 weeks after ETV treatment (HBsAg OR = 4.058, 95% CI 1.080–15.245, $p = 0.038$; HBeAg OR = 1.002, 95% CI 1.000–1.004, $p = 0.011$; IL-10 OR = 0.985, 95% CI 0.973–0.997, $p = 0.015$). Multivariate analysis showed that baseline HBV DNA, HBeAg, and IL-10 levels were independent factors significantly related to whether ALT was normal or not after 48 weeks of ETV treatment (HBeAg OR = 1.003, 95% CI 1.001–1.006, $p = 0.007$; HBV DNA OR = 0.184, 95% CI 0.046–0.739, $p = 0.017$; IL-10 OR = 0.040, 95% CI 0.972–0.999, $p = 0.040$) (Table 4).

Discussion

Controlling HBV replication and improving body immunity are important means to reduce liver inflammation and prevent disease progression. Nucleoside (acid) analogues and interferon have become the first-line antiviral drugs for CHB (2, 6, 7). The target site of nucleoside (acid) analogues is the reverse transcriptase required in the process of HBV DNA replication (21). Nucleoside (acid) analogue therapy is fast-acting, safe, and well tolerated, but they are ineffective for covalently closed

TABLE 2 Logistic regression analysis of the baseline predictors of complete virological response after 48 weeks of ETV treatment.

Univariate analysis	OR	95% CI	p-value
HBsAg (log10 IU/ml)	1.993	0.437–9.092	0.373
HBeAg (S/CO)	1.001	0.999–1.002	0.417
HBV DNA (log10 IU/ml)	1.550	0.766–3.136	0.223
ALT (U/L)	0.999	0.995–1.002	0.385
AST (U/L)	0.996	0.990–1.003	0.251
TBil (μmol/L)	0.994	0.924–1.068	0.862
ALB (g/L)	1.014	0.894–1.150	0.829
Flt-3L (pg/ml)	1.013	0.948–1.083	0.704
IFN-α2 (pg/ml)	0.997	0.991–1.003	0.374
IFN-γ (pg/ml)	1.000	0.997–1.004	0.849
IL-10 (pg/ml)	1.000	0.985–1.015	0.993
IL-17A (pg/ml)	0.995	0.981–1.009	0.499
IL-6 (pg/ml)	0.996	0.980–1.011	0.586
TNF-α (pg/ml)	0.988	0.919–1.062	0.742
TGF-β1 (pg/ml)	1.000	1.000–1.000	0.297
TGF-β2 (pg/ml)	1.004	0.991–1.018	0.539
TGF-β3 (pg/ml)	0.991	0.919–1.068	0.805
Multiple factor analysis	OR	95% CI	p-value
HBV DNA (log10 IU/ml)	1.342	0.592–3.042	0.481

ALT, glutamic-pyruvic transaminase; AST, glutamic oxalacetic transaminase; TBil, total bilirubin. ALB, albumin.

circular DNA (cccDNA) in the nucleus, and the viral load will rebound after drug withdrawal. Therefore, patients need to receive long-term drug treatment in most cases.

In this study, we chose high gene barrier resistance and potent ETV treatment, and the overall virological response was good. In our study, 87 of the 100 patients obtained a complete virological response. Although there was no significant difference between the groups, the baseline HBsAg, HBeAg, HBV DNA load, IL-10, and TGF-β1 levels in the complete viral response group were lower, and the level of ALT was higher than those in the incomplete viral response group.

High HBV DNA load, and high HBeAg and HBsAg levels may inhibit the immune cell function of patients, leading to the reduction of Flt-3L, IFN-γ, IL-17A, and other cytokines with virus clearance effect (8, 13, 22–24), and to the increase in the level of the most important cytokine for immunosuppression (IL-10) and other cytokines with immunosuppressive effect such as TGF-β1 (8, 13, 25–27). In this study, compared with the incomplete virus response group, the complete virus response group had lower HBV DNA load, lower HBeAg and HBsAg levels, lower IL-10 level, and lower TGF-β level, which indicate that patients with complete virus response may have better immune clearance ability against virus. HBeAg and IL-10 levels in the complete virological response group were lower than those in the incomplete response group at 3 months of treatment, while IFN-γ and IL-17A were higher. At 6 months of treatment, HBeAg in the complete virological response group

was significantly lower than that in the incomplete virological response group, while IFN-α2, IFN-γ, and IL-17A were higher.

HBsAg and HBeAg levels at 9 and 12 months in the complete virological response group were significantly lower than those in the incomplete response group. The result suggests that the acquisition of complete virological response may be related to the decrease of antigen, the improvement of immune capacity (the increase of the cytokines with immunostimulatory effect), and the decrease of the cytokines with immunosuppressive effect (8, 13, 14, 25, 28, 29). However, it is difficult to clarify the causal relationship between the complete viral response and the increase in the level of the cytokines with immunostimulatory effect and the decrease in the level of the cytokines with immunosuppressive effect. Through univariate and multivariate analysis, no factors related to the virological response at 48 weeks were found, which may be related to the fact that ETV is a potent drug that strongly inhibits HBV replication, thus masking the impact of other factors on the antiviral effect.

The aim of antiviral therapy is to prevent or reduce the inflammation of liver tissue, and to block or delay the progress of liver disease by inhibiting the replication of virus and improving the immune control ability of virus (2, 6, 7). In our study, 90 of 100 patients (90%) achieved ALT normalization after 48 weeks of ETV treatment. The baseline HBsAg and HBeAg levels in the ALT normalization group were significantly lower than that in the non-normalization group. The baseline IL-10 and TGF-β1 in the ALT normalization group were lower than that in the ALT non-normalization group, while the baseline ALT level and IFN-

TABLE 3 Comparison of clinical indicators between the normal ALT group ($n = 90$) and abnormal ALT group ($n = 10$) at baseline and weeks 12, 24, 36, and 48 during entecavir therapy.

	Baseline			12 weeks			24 weeks			36 weeks			48 weeks		
	Normal ALT	Abnormal ALT	Z/p	Normal ALT	Abnormal ALT	Z/p	Normal ALT	Abnormal ALT	Z/p	Normal ALT	Abnormal ALT	Z/p	Normal ALT	Abnormal ALT	Z/p
ALT (U/L)	300.55 (201–743.29)	225.60 (117.65–345.20)	–1.827/0.068	31.90 (22.70–49.90)	41.90 (15.50–86.15)	–0.644/0.520	23.05 (17.85–34.05)	22.40 (11.00–45.50)	–0.483/0.629	22.75 (16.90–28.90)	24.30 (13.10–35.40)	–0.172/0.863	20.10 (15.10–26.45)	48.20 (42.45–75.40)	–5.172/0.000
AST (U/L)	126.60 (88.20–625.80)	100.50 (54.20–179.20)	–1.623/0.103	27.70 (19.93–35.38)	29.70 (19.10–43.10)	–0.689/0.491	22.45 (17.90–27.30)	23.65 (16.30–34.10)	–0.517/0.605	20.90 (16.38–25.60)	21.00 (16.90–25.40)	–0.477/0.633	20.70 (16.50–22.88)	41.90 (29.13–46.80)	–4.862/0.000
TBil (μ mol/L)	21.20 (18.90–28.28)	14.30 (11.40–21.05)	–2.534/0.011	12.20 (9.10–17.85)	25.50 (9.30–32.78)	–2.477/0.013	12.20 (9.50–15.50)	20.90 (8.80–26.68)	–1.644/0.100	12.80 (10.30–15.50)	15.50 (7.30–26.98)	0.425/0.671	12.20 (9.90–14.80)	19.45 (7.90–22.05)	–1.604/0.109
ALB (g/L)	41.25 (39.38–48.93)	45.90 (42.85–47.85)	–1.446/0.148	47.80 (44.30–49.10)	44.70 (44.50–49.93)	–0.290/0.772	47.75 (45.60–49.03)	47.50 (45.30–50.53)	–0.397/0.692	48.50 (46.40–50.20)	48.45 (43.60–49.40)	–0.948/0.343	48.50 (46.50–49.83)	47.85 (45.90–49.80)	–0.523/0.601
HBsAg (log10 IU/ml)	3.76 (2.87–3.91)	3.89 (3.65–4.10)	–2.000/0.045	3.78 (3.28–4.04)	3.29 (3.08–3.68)	–2.632/0.008	3.84 (3.39–4.06)	3.23 (3.02–3.85)	–2.356/0.018	3.77 (3.45–4.01)	3.16 (3.11–3.92)	–1.937/0.053	3.68 (3.37–3.96)	3.04 (3.02–3.86)	–2.195/0.028
HBeAg (S/CO)	358.26 (322.81–651.35)	908.07 (589.15–1,222.08)	–2.896/0.004	86.66 (17.01–719.60)	12.85 (0.78–133.77)	–2.654/0.008	86.74 (7.09–462.31)	12.56 (1.00–98.95)	–2.287/0.022	57.13 (4.36–233.87)	8.76 (1.27–78.81)	–1.976/0.048	22.25 (4.49–136.72)	9.09 (1.11–30.01)	–1.839/0.066
HBV DNA (log10 IU/ml)	6.65 (6.33–7.32)	6.70 (6.45–7.51)	–0.460/0.646	3.01 (1.12–3.77)	2.76 (2.38–3.06)	–0.468/0.640	1.54 (0.00–2.69)	2.36 (0.00–2.68)	–0.290/0.772	0.00 (0.00–1.65)	0.00 (0.00–0.58)	–0.647/0.518	0	0	–1.278/0.201
Flt-3L (pg/ml)	0.07 (0.02–41.09)	NA	NA	0.64 (0.02–1.25)	NA	NA	1.13 (0.02–2.24)	NA	NA	/	/	/	/	/	/
IFN- α 2 (pg/ml)	45.79 (29.69–76.08)	30.72 (4.95–62.21)	–2.029/0.043	57.85 (33.60–98.39)	47.90 (33.37–86.51)	–0.230/0.818	58.34 (36.00–94.46)	71.69 (60.13–119.50)	–1.708/0.088	/	/	/	/	/	/
IFN- γ (pg/ml)	20.16 (3.92–51.00)	20.23 (2.39–107.04)	–0.402/0.688	14.43 (6.12–31.55)	4.71 (3.23–47.09)	–1.735/0.083	14.43 (4.79–43.00)	10.01 (5.34–50.19)	–0.494/0.621	/	/	/	/	/	/
IL-10 (pg/ml)	9.07 (4.94–17.69)	13.25 (1.01–121.22)	–0.322/0.748	5.47 (2.16–10.28)	4.93 (0.92–81.82)	–0.149/0.881	5.30 (1.60–12.30)	6.22 (1.12–49.62)	–0.891/0.373	/	/	/	/	/	/
IL-17A (pg/ml)	5.18 (2.75–35.74)	14.68 (1.25–39.77)	–0.144/0.886	6.47 (3.22–10.12)	4.09 (1.31–15.51)	–1.385/0.166	6.15 (2.56–9.71)	5.09 (2.12–11.70)	–0.408/0.683	/	/	/	/	/	/
IL-6 (pg/ml)	2.49 (0.90–7.83)	1.46 (0.62–2.68)	–1.149/0.250	1.90 (0.88–3.79)	1.40 (0.58–11.75)	–0.345/0.730	2.01 (1.02–2.69)	2.29 (1.15–7.32)	–1.322/0.186	/	/	/	/	/	/
TNF- α (pg/ml)	8.19 (6.23–8.64)	NA	NA	9.42 (6.55–14.05)	6.86 (4.35–16.39)	–1.178/0.239	8.29 (7.60–13.29)	12.51 (6.02–12.20)	–0.138/0.890	/	/	/	/	/	/
TGF- β 1 (pg/ml)	5,114 (2,496.50–5,751.50)	10,817.50 (10,445–10,917)	–0.712/0.476	1,319.69 (268.60–3,611)	152.73 (99.22–3,790.50)	1.517/0.129	2,032 (104.97–2,548)	1,514 (112.50–5,959)	–0.276/0.783	/	/	/	/	/	/

(Continued)

TABLE 3 Continued

	Baseline			12 weeks			24 weeks			36 weeks			48 weeks		
	Normal ALT	Abnormal ALT	Z/p	Normal ALT	Abnormal ALT	Z/p	Normal ALT	Abnormal ALT	Z/p	Normal ALT	Abnormal ALT	Z/p	Normal ALT	Abnormal ALT	Z/p
TGF-β2 (pg/ml)	412.21 (210.48–412.21)	NA	NA	/	/	/	/	/	/	/	/	/	/	/	/
TGF-β3 (pg/ml)	200.31 (147.58–209.12)	NA	NA	/	/	/	/	/	/	/	/	/	/	/	/

biochemistry and virus indicators: $p < 0.010$ was statistically significant; cytokines: $p < 0.013$ was statistically significant.

$\alpha 2$ level were significantly higher. Compared with the ALT non-normalization group, the ALT normalization group had lower baseline HBsAg, HBeAg, IL-10, and TGF- β levels and higher baseline ALT level. This indicates that the ALT normalization group may have better baseline immune control ability (14, 15, 28), and the efficacy of NA treatment is related to the immune and viral factors of patients (2, 6, 7). At 3 and 6 months after treatment, the IFN- γ level of the ALT normalization group was significantly higher than that in the non-normalization group. Given that IFN- γ is an important cytokine for cellular immunity (14, 25, 28, 29), the increase in IFN- γ suggests that ETV can restore part of cellular immune function by reducing HBV DNA load (29).

Logistic regression analyzed risk factors related to whether the ALT is normal after 48 weeks of ETV treatment. Univariate analysis showed that low baseline levels of HBsAg, HBeAg, and IL-10 were significantly related to normal ALT at 48 weeks. Multivariate analysis showed that baseline HBV DNA, HBeAg, and IL-10 were significantly correlated with normal ALT at 48 weeks. After 48 weeks of treatment with ETV, normal ALT means good biochemical response and better control of hepatocyte necrosis and liver tissue inflammation. The results show that the effect of ETV treatment can be affected by baseline viral factors, the degree of liver tissue inflammation, and immune cytokines.

Although univariate analysis showed that baseline HBsAg, HBeAg, and IL-10 were heavily correlated with ALT normalization at 48 weeks after ETV treatment, the levels of HBeAg and HBsAg in the ALT normalization group were higher than those in ALT elevated group after 48-week ETV treatment, and the reason was still considered to be related to the immune function of patients. In patients with chronic HBV infection, the existence of liver diseases and progression are influenced by multiple factors. In terms of HBV, HBV replication can activate the response of host immune cells and cause liver cell necrosis and inflammation. On the other hand, HBV and its antigens can inhibit the immune cell function through multiple ways, making it difficult to clear the virus (14–17). Therefore, the decrease of HBV DNA level in most patients during NA treatment can reduce the stimulation of immune cells and the immune damage of hepatocytes, and thus the clinical manifestations are ALT normalization, and no decrease or even increase in HBsAg and HBeAg. However, in some patients, due to the decrease or even negative transformation of HBV level during NA treatment, the inhibition of immune cell function by HBV is alleviated, and the function of immune cells to inhibit viral replication and clear virus-infected cells is restored. The clinical manifestations are increased ALT, accompanied by decreased HBeAg and HBsAg levels. Thus, the results of this study showed that patients with elevated ALT during ETV treatment had lower HBeAg and HBsAg levels than those with normal ALT.

In conclusion, this prospective study shows that cytokine levels change dynamically in ETV antiviral therapy. Low

TABLE 4 Logistic regression analysis of the baseline predictors of ALT normalization at week 48 of ETV treatment.

Univariate analysis	OR	95% CI	p-value
HBsAg (log10 IU/ml)	4.058	1.080–15.245	0.038
HBeAg (S/CO)	1.002	1.000–1.004	0.011
HBV DNA (log10 IU/ml)	0.846	0.390–1.834	0.672
ALT (U/L)	0.999	0.997–1.001	0.238
AST (U/L)	0.997	0.995–1.000	0.076
TBil (μmol/L)	0.943	0.883–1.008	0.084
ALB (g/L)	1.080	0.953–1.224	0.229
Flt-3L (pg/ml)	0.984	0.939–1.040	0.559
IFN-α2 (pg/ml)	1.006	0.994–1.018	0.340
IFN-γ (pg/ml)	1.000	0.996–1.005	0.871
IL-10 (pg/ml)	0.985	0.973–0.997	0.015
IL-17A (pg/ml)	1.005	0.998–1.022	0.548
IL-6 (pg/ml)	1.061	0.930–1.211	0.380
TNF-α (pg/ml)	1.011	0.934–1.094	0.791
TGF-β1 (pg/ml)	1.000	1.000	0.443
TGF-β2 (pg/ml)	0.995	0.985–1.006	0.364
TGF-β3 (pg/ml)	1.011	0.955–1.069	0.713
Multiple factor analysis	OR	95% CI	p-value
HBV DNA (log10 IU/ml)	0.184	0.046–0.739	0.017
HBeAg (S/CO)	1.003	1.001–1.006	0.007
IL-10 (pg/ml)	0.040	0.972–0.999	0.040

cytokines: $p < 0.013$ was statistically significant.

baseline HBV DNA, HBeAg, and IL-10 levels were significantly associated with ALT normalization after 48 weeks of ETV treatment. The findings warrant further investigation with a large sample size in the future. In this study, we only investigated the kinetic changes of serum, virological, and immunological markers during ETV antiviral therapy in HBeAg-positive CHB patients. In the future, we expect similar studies to be conducted in HBeAg-negative CHB patients.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study was reviewed and approved by The Ethics Committee of Beijing Ditan Hospital affiliated to Capital University of Medical Sciences. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ML, RS, YJ, WY and YX contributed to study concept and design. YG, LY, YanL, WD, TJ, XB, YaoL, LZ,GS, RL, SW, MC, MX and LH performed the data collection. ML, RS, YJ, WY and YX conducted data analysis. ML, YG, LY and YanL wrote the first draft. ML edited the English version. RS, YJ, WY and YX approved the submitted version after modification. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Serum hepatitis B virus large and medium surface proteins as novel tools for predicting HBsAg clearance

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Background: There is still lack of reliable predictors for hepatitis B surface antigen (HBsAg) clearance. Recent studies have shown that the levels of large (LHBs) and medium hepatitis B surface proteins (MHBs) are closely related to antiviral efficacy. This study aimed to investigate the possibility of LHB and MHB levels to predict HBsAg clearance.

Methods: An inactive HBsAg carriers (IHCs) cohort that had received pegylated interferon (Peg-IFN) treatment was divided into the HBsAg-cleared group (R group) and the HBsAg non-cleared group (NR group) based on whether HBsAg was cleared at 96 weeks. We detected the levels of LHBs and MHBs to evaluate the possibility of predicting HBsAg clearance.

Results: There were 39 patients in the R group and 21 in the NR group. The total HBsAg, LHB, and MHB levels at baseline and at 12 weeks were significantly lower in the R group than in the NR group (all $p < 0.05$). Multivariate logistic regression indicated that LHB and MHB levels at baseline and 12 weeks were independent predictors of HBsAg clearance (OR = 0.435, $p = 0.016$; OR = 0.136, $p = 0.003$; OR = 0.137, $p = 0.033$; OR = 0.049, $p = 0.043$). The area under the curve (AUC) for the baseline and 12-week LHB and MHB levels was 0.827–0.896, which were greater than that of the total HBsAg level at baseline and 12-week (AUC: 0.654–0.755). Compared with the prediction results of a single indicator, the combination of LHB and MHB levels had better value in predicting HBsAg clearance. The AUCs of combination factor 1, constructed from baseline LHB and MHB, and combination factor 2, constructed from 12-week LHB and MHB, were 0.922 and 0.939, respectively, and the sensitivity (82.05%–100.00%) and specificity (85.71%–100.00%) were both high. The combined indicators based on baseline LHBs ≤ 13.99 ng/mL and MHBs ≤ 7.95 ng/mL predicted HBsAg clearance rate of more than 90%.

Conclusion: Baseline and 12-week LHB and MHB levels can predict HBsAg clearance obtained by Peg-IFN therapy in IHCs, and the predictive value is higher than that of the total HBsAg levels.

KEYWORDS

inactive HBsAg carriers, pegylated interferon, HBsAg clearance, LHBs, MHBs, predictor

Introduction

Chronic hepatitis B virus (HBV) infection is an important global public health problem, especially in the Asia-Pacific region. In recent years, domestic and international chronic Hepatitis B (CHB) management guidelines have made hepatitis B surface antigen (HBsAg) clearance as the highest clinical treatment goal (1–3). Because of limited therapeutic drugs, improving prediction efficacy and thus providing precise treatment are key issues for improving the clinical cure rate. But the prediction of clinical cure with antiviral therapy for CHB has not been well established. HBsAg consists of large (LHBs), medium (MHBs) and small surface proteins, and their relative proportions are closely related to disease stage. Recent studies have reported that LHBs and MHBs are associated with HBsAg clearance and that the value of baseline MHBs in predicting clinical cure is better than that of total HBsAg (4). However, that was a small-sample study based on most patients received nucleos(t)ide analogue (NAs) therapy. We accumulated a large number of patients who achieved HBsAg clearance during treatment with pegylated interferon (Peg-IFN). To this end, we investigated the value of LHB and MHB levels in predicting HBsAg clearance in inactive HBsAg carriers (IHCs) treated with Peg-IFN.

Materials and methods

Study population

IHCs who had been treated with Peg-IFN in our hospital were included in this study, and blood samples were collected

Abbreviations: HBV, hepatitis B virus; CHB, chronic Hepatitis B; HBsAg, hepatitis B surface antigen; LHBs, large hepatitis B surface proteins; MHBs, middle hepatitis B surface proteins; NAs, nucleos(t)ide analogue; Peg-IFN, pegylated interferon; IHCs, inactive HBsAg carriers; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; ULN, upper limit of normal; ROC, receiver operating characteristic; OR, odds ratio; AUC, area under the curve; CI, confidence interval.

at baseline and at 12 weeks of treatment. All IHCs met the criteria defined in the prevention and treatment guidelines for chronic hepatitis B (2019 edition) (3). HBsAg positive > 6 months and HBsAg<1000 IU/mL, HBeAg negative, anti-HBe positive; HBV DNA<2000 IU/mL, and ALT and AST remained normal; no signs of liver cirrhosis on imageology examination. Enrolled IHCs were treated with Peg-IFN 135 mg weekly by subcutaneous injection in our hospital, and the total duration of treatment was 96 weeks. Based on the results at 96 weeks of treatment, the patients were divided into the HBsAg-cleared group (responders group, R group) and the HBsAg non-cleared group (non-responders, NR group). Patients in the R group satisfied the following criteria: a sustained virological response (HBV DNA undetectable), hepatitis B e antigen (HBeAg) negative status, HBsAg clearance (HBsAg< 0.05 IU/ml) or seroconversion (hepatitis B surface antibody≥ 10 IU/ml). Those who did not meet any of the above indicators were included in the NR group. All enrolled patients signed an informed consent form, and the study was approved by the Ethics Committee of Beijing Youan Hospital affiliated with Capital Medical University ([2017]24).

Detection indicators and methods

The levels of LHBs and MHBs were detected by enzyme-linked immunosorbent assay (Shanghai Jianglai Biotechnology Co., Ltd., China), the lower limit of detection was 0.5 ng/ml. HBV markers were detected using Elecsys MODULAR ANALYTICS E170 (Roche Diagnostics GmbH, Germany), the lower limit for the quantitative detection of HBsAg was <0.05 IU/ml, anti-HBs >10 IU/ml, and HBeAg >1 COI were considered positive. HBV DNA was detected using the cobas® AmpliPrep/cobas® TaqMan automatic nucleic acid isolation and purification system and PCR analysis system (Roche Diagnostics GmbH, Germany) with a lower limit of detection of 20 IU/ml. Alanine aminotransferase (ALT) was detected using reagents from Shanghai Kehua Dongling Company (China), and a normal value was considered < 40 U/L.

Statistical analysis

SPSS 21.0 (SPSS, USA) and Medcalc Statistical Software version 14.8.1 (MedCalc Software Ltd, Ostend, Belgium) were used for statistical processing. The continuous variables were expressed as the mean $\pm$ standard deviation or median (interquartile range), and categorical variable data were expressed as the number of cases and the percentage [cases (%)]. Continuous parameters were analyzed by Student's t-test or Mann-Whitney U test, categorical parameters were analyzed by the Pearson Chi-square test or Fisher's exact test. Logistic regression analysis was conducted to identify factors associated with HBsAg clearance. Receiver Operating Characteristic (ROC) analysis was performed to analyze the predictive value of the factors in predicting HBsAg clearance. The cut-off value corresponding to the maximum Youden index is regarded as the best cut-off (Youden index = sensitivity + specificity - 1). P-value < 0.05 was considered significant.

Results

Patient characteristics

Sixty patients met the above criteria (39 patients in the R group and 21 patients in the NR group), all of whom were Asian. The average age (years) of patients in the R group was $38.05 \pm$

11.25 years and that of patients in the NR group was 40.62 ± 11.26 years. The proportions of males in the R group and the NR group were 69.23% (27/39) and 76.19% (16/21), respectively. There was no significant difference in age or sex between the two groups ($p = 0.368$, $p = 0.568$, Table 1).

Total HBsAg, LHB and MHB levels in the R group and the NR group

We measured the total HBsAg levels in the R group and the NR group at baseline and at 12 weeks. The results showed that the total HBsAg level in the R group was significantly lower than that in the NR group (all $p < 0.05$, Figure 1A, Table 1). At 12 weeks of Peg-IFN treatment, the total HBsAg level in the two groups decreased compared with the baseline, and the reduction in the R group was greater than that in the NR group ($p = 0.021$; Figure 1B).

Further analysis of LHB and MHB levels at baseline and 12 weeks showed that LHB and MHB levels in the R group were significantly lower than those in the NR group (all $p < 0.001$, Figures 1C, E, Table 1). At 12 weeks of treatment, the levels of LHBs and MHBs decreased compared to those at baseline. The reduction in LHBs was greater in the R group than in the NR group ($p = 0.015$, Figure 1D), while the reduction in MHBs was similar in the two groups ($p = 0.821$; Figure 1F).

TABLE 1 Characteristics of R and NR groups at baseline and at 12 weeks of treatment.

CParameter	All patients (n = 60)	R group (n = 39)	NR group (n = 21)	P-value
Gender (M/F)	43/17	27/12	16/5	0.568
Age (years)	38.95 ± 11.23	38.05 ± 11.25	40.62 ± 11.26	0.368
Baseline				
Total HBsAg, IU/ml	198.78 ± 236.54	155.63 ± 194.33	287.35 ± 291.79	0.046
LHBs, ng/ml	13.28 ± 1.89	12.49 ± 1.53	14.76 ± 1.59	<0.001
MHBs, ng/ml	7.19 ± 1.19	6.62 ± 0.88	8.23 ± 0.99	<0.001
ALT, U/L	31.31 ± 14.51	30.88 ± 14.62	33.17 ± 14.36	0.570
12 weeks				
Total HBsAg, IU/ml	91.93 ± 170.75	37.77 ± 57.15	189.92 ± 250.70	0.001
LHBs, ng/ml	11.23 ± 2.10	10.22 ± 1.64	13.09 ± 1.52	<0.001
MHBs, ng/ml	6.20 ± 1.14	5.70 ± 0.90	7.12 ± 0.94	<0.001
ALT, U/L	76.78 ± 77.74	82.56 ± 89.52	66.05 ± 49.14	0.681
ALT > 1.0 $\times$ ULN	53.33% (32/60)	56.41% (22/39)	47.62% (10/21)	0.515
ALT > 1.5 $\times$ ULN	41.67% (25/60)	51.28% (20/39)	23.81% (5/21)	0.040
ALT > 2.0 $\times$ ULN	23.33% (14/60)	28.20% (11/39)	14.29% (3/21)	0.224
Change from baseline to 12 weeks				
Total HBsAg, log IU/ml	-0.73 ± 0.87	-0.91 ± 0.96	-0.38 ± 0.50	0.021
LHBs, log ng/ml	-0.08 ± 0.06	-0.09 ± 0.07	-0.05 ± 0.04	0.015
MHBs, log ng/ml	-0.07 ± 0.06	-0.07 ± 0.07	-0.06 ± 0.06	0.821

HBsAg, Hepatitis B surface antigen; LHBs, large hepatitis B surface proteins; MHBs, middle hepatitis B surface proteins; ALT, alanine aminotransferase; ULN, upper limit of normal.

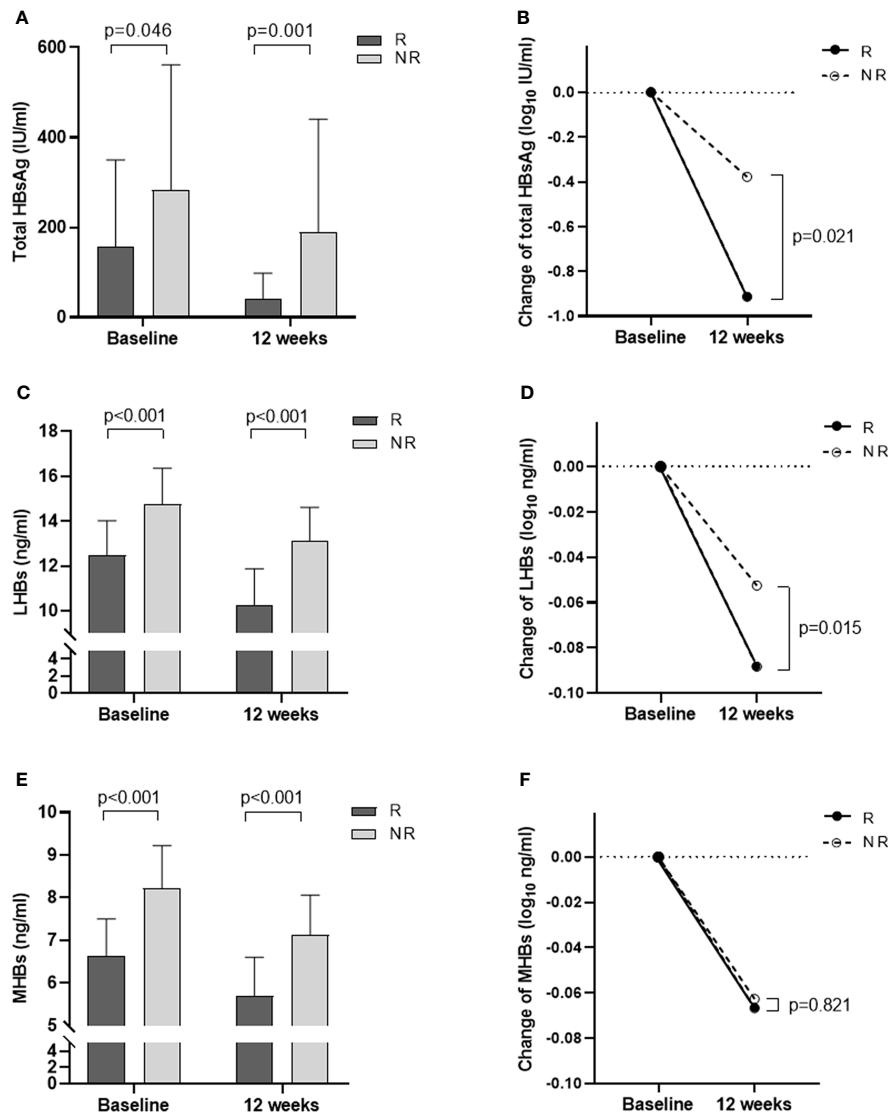


FIGURE 1

Changes in total HBsAg, LHBs, and MHBs at baseline and 12 weeks of treatment in the R and NR groups. (A) Total HBsAg levels at baseline and 12 weeks. (B) The degree of decline in total HBsAg from baseline to 12 weeks (log). (C) LHBs levels at baseline and 12 weeks. (D) The degree of decline in LHBs from baseline to 12 weeks (log). (E) MHBs levels at baseline and 12 weeks. (F) The degree of decline in MHBs from baseline to 12 weeks (log).

Analysis of related influencing factors of HBsAg clearance

Logistic regression

Logistic regression analysis was performed on the factors that may affect HBsAg clearance. Univariate logistic regression analysis showed that baseline LHB and MHB levels, 12-week total HBsAg, LHB and MHB levels, 12-week total HBsAg and LHB reduction (log), and 12-week ALT greater than 1.5 times the upper limit of normal (ULN) were all influencing factors of HBsAg clearance (Table 2). Multivariate logistic regression

analysis showed that only baseline and 12-week LHB and MHB levels were influencing factors of HBsAg clearance [odds ratio (OR) = 0.435, $p = 0.016$; OR = 0.136, $p = 0.003$; OR = 0.137, $p = 0.033$; OR = 0.049, $p = 0.043$; Table 2].

The predictive value of relevant influencing factors for HBsAg clearance by ROC evaluation

Based on the logistic regression results, the ROC curve and area under the curve (AUC) for total HBsAg, LHB and MHB levels at baseline and 12 weeks, reductions (log), and ALT > 1.5 × ULN were calculated (Table 3). The AUCs of LHBs and MHBs at

TABLE 2 Logistic regression.

Parameter	Univariate logistic regression			Multivariate logistic regression		
	OR	95% CI	P-value	OR	95% CI	P-value
Sex	0.703	0.209 - 2.364	0.569	0.316	0.026 - 3.783	0.363
Age	0.980	0.934 - 1.027	0.397	0.933	0.840 - 1.035	0.189
LHBs at baseline	0.364	0.211 - 0.627	<0.001	0.435	0.221 - 0.854	0.016
MHBs at baseline	0.157	0.063 - 0.392	<0.001	0.136	0.037 - 0.502	0.003
Total HBsAg at baseline	0.998	0.995 - 1.000	0.051	0.994	0.998 - 1.000	0.070
LHBs at 12 weeks	0.375	0.231 - 0.610	<0.001	0.137	0.022 - 0.853	0.033
MHBs at 12 weeks	0.185	0.075 - 0.458	<0.001	0.049	0.003 - 0.907	0.043
Total HBsAg at 12 weeks	0.991	0.985 - 0.998	0.007	0.989	0.975 - 1.003	0.128
LHBs decline at 12 weeks (log)	1.010	1.000 - 1.019	0.044	0.968	0.932 - 1.005	0.251
MHBs decline at 12 weeks (log)	1.001	0.993 - 1.009	0.817	0.980	0.948 - 1.014	0.241
Total HBsAg decline at 12 weeks (log)	1.001	1.000 - 1.002	0.030	1.002	0.999 - 1.006	0.431
ALT>1.5×ULN at 12 weeks	0.297	0.091 - 0.970	0.044	0.283	0.012 - 6.580	0.033

1,R group; 0,NR group; 1,male; 0,female; 1,ALT>1.5×ULN at 12 weeks; 0,ALT ≤ 1.5×ULN at 12 weeks); HBsAg, Hepatitis B surface antigen; LHBs, large hepatitis B surface proteins; MHBs, middle hepatitis B surface proteins; ALT, alanine aminotransferase; ULN, upper limit of normal; OR, odds ratio; CI, confidence interval.

baseline were 0.827 and 0.887, respectively, which were greater than the AUC of the total HBsAg level (0.654, **Figure 2A**). The AUC for baseline MHBs was significantly higher than that for total HBsAg ($p = 0.014$, 95% CI: 0.048-0.417; **Table 3**), suggesting that the value of baseline MHBs in predicting HBsAg clearance was significantly better than that of total HBsAg. At 12 weeks of treatment, the AUCs for LHBs and MHBs were 0.896 and 0.863, respectively, which were higher than the AUC for the total HBsAg level (0.755, **Figure 2B**), but the difference was not statistically significant ($p=0.082$, $p=0.179$,

Table 3). In addition, the AUC for the reduction in LHBs at 12 weeks was 0.711, which was higher than the AUCs for the reduction in the total HBsAg and MHBs (**Figure 2C**), though the difference was not significant (**Table 3**). The AUC for ALT > 1.5 × ULN at 12 weeks was 0.637, and its predictive value for HBsAg clearance was lower than that of LHBs and MHBs at 12 weeks.

To further improve the prediction accuracy, we used baseline LHBs (X1) and MHBs (X2) and 12-week LHBs (X3) and MHBs (X4) to construct joint prediction indicators. The

TABLE 3 ROC curves for the prediction of HBsAg loss in patients.

Parameter	ROC curves					Comparison of AUC for indicators			
	AUC	95% CI	cut-off	sensitivity	specificity	indicators	difference between AUC	p value	95% CI
Baseline									
Total HBsAg (a)	0.654	0.521 - 0.773	114.7	61.54	66.67	–	–	–	–
LHBs (b)	0.827	0.707 - 0.912	13.99	87.18	61.90	a vs.b	0.172	0.072	-0.016 - 0.360
MHBs (c)	0.887	0.779 - 0.954	7.95	100.00	71.43	a vs.c	0.233	0.014	0.048 - 0.417
Combination factor 1(d)	0.922	0.823 - 0.975	-0.83	100.00	85.71	a vs.d	0.267	0.004	0.087 - 0.448
After 12 weeks of treatment									
Total HBsAg (e)	0.755	0.626 - 0.857	29.57	69.23	71.43	–	–	–	–
LHBs (f)	0.896	0.790 - 0.960	11.44	84.62	85.71	e vs.f	0.142	0.082	-0.018 - 0.301
MHBs (g)	0.863	0.750 - 0.938	5.84	64.10	100.00	e vs.g	0.109	0.179	-0.050 - 0.267
Combination factor 2(h)	0.939	0.846 - 0.984	1.00	82.05	100.00	e vs.h	0.184	0.015	0.037 - 0.332
Change from baseline at 12 weeks									
The reduction in total HBsAg (i)	0.691	0.559 - 0.804	0.29	74.36	61.90	–	–	–	–
The reduction in LHBs (j)	0.711	0.580 - 0.821	0.07	69.23	76.19	i vs.j	0.020	0.837	-0.172 - 0.212
The reduction in MHBs (k)	0.507	0.374 - 0.638	0.17	84.62	4.76	i vs.k	0.184	0.091	-0.029 - 0.398
ALT>1.5×ULN(l)	0.637	0.503 - 0.758	0.00	51.28	76.19	i vs.l	0.054	0.572	-0.133 - 0.240

HBsAg, Hepatitis B surface antigen; LHBs, large hepatitis B surface proteins; MHBs, middle hepatitis B surface proteins; ALT, alanine aminotransferase; ULN, upper limit of normal; AUC, area under the curve; CI, confidence interval.

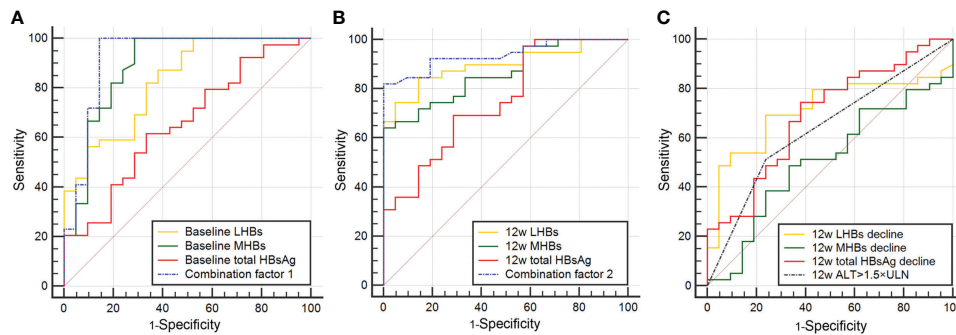


FIGURE 2

ROC curves of HBsAg clearance predictors. (A) ROC curves of total HBsAg, LHBs, MHBs, and combination factor 1 at baseline. (B) ROC curves of total HBsAg, LHBs, MHBs, and combination factor 2 at 12 weeks. (C) ROC curves of the degree of decline in total HBsAg, LHBs, and MHBs from baseline to 12 weeks, and ALT elevations $>1.5 \times \text{ULN}$ at 12 weeks.

logistic regression models and predictive probability equation (PRE) were as follows:

combination factor 1

$$= -23.5594 + 0.84516 \times X1 + 1.55683 \times X2$$

combination factor 1 PRE

$$= \frac{1}{1 + e^{-(-23.5594 + 0.84516 \times X1 + 1.55683 \times X2)}}$$

combination factor 2

$$= -19.2726 + 0.81440 \times X3 + 1.44604 \times X4$$

combination factor 2 PRE

$$= \frac{1}{1 + e^{-(-19.2726 + 0.81440 \times X3 + 1.44604 \times X4)}}$$

The results indicated that the AUCs for combination factor 1 and combination factor 2 constructed from LHBs and MHBs were significantly increased; the AUCs were 0.922 and 0.939, respectively (Figures 2A, B; Table 3). The predictive values of combination factor 1 and combination factor 2 for HBsAg clearance were comparable. The values of combination factor 1 and combination factor 2 in predicting HBsAg clearance were both higher than that of the total HBsAg of the corresponding time point, and the differences were statistically significant ($p < 0.05$, Table 3).

The predictive value of single and combined indicators for HBsAg clearance

To more intuitively and conveniently apply the predictive indicators in clinical practice, we compared the difference between single indicator and combined indicator prediction. The analysis was performed based on the cut-off value for each indicator in Table 3. As shown in Figure 3, when a single indicator was used, the predictive values of baseline LHBs $\leq$

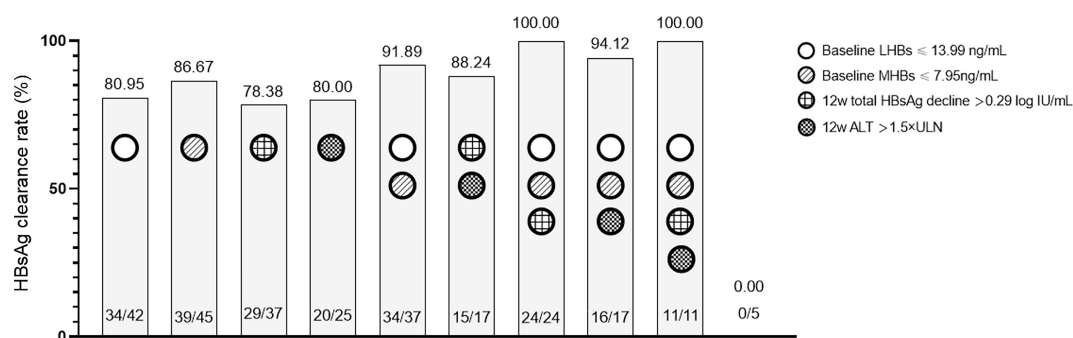


FIGURE 3

The predictive value of single and combined indicators for HBsAg clearance.

13.99 ng/mL and MHBs ≤ 7.95 ng/mL for HBsAg clearance were good (>80.00%). When the combined indicators were used for prediction, the accuracy of the combined prediction of HBsAg clearance based on baseline LHBs ≤ 13.99 ng/mL and MHBs ≤ 7.95 ng/mL was higher than 90.00%. The combination of baseline LHBs ≤ 13.99 ng/mL, MHBs ≤ 7.95 ng/mL, and 12-week total HBsAg reduction > 0.29 log₁₀ IU/mL predicted that the HBsAg clearance rate could reach 100%.

Discussion

HBsAg clearance is currently the highest goal of clinical treatment for chronic hepatitis B, but it is still difficult to achieve using existing treatment regimens (2, 5). Screening the dominant population to administer precise treatment has important guiding significance in clinical practice. In the past, the total HBsAg level was often used to predict clinical cure. Some recent retrospective studies have found that LHB and MHB levels are correlated with HBV DNA response and HBeAg conversion (6–8). However, few studies have reported their correlation with HBsAg clearance. Therefore, this study analyzed the correlation between LHB and MHB levels and HBsAg clearance in an IHCs population treated with Peg-IFN.

The outer membrane protein of hepatitis B virus, namely, total HBsAg, is composed of three components: LHBs, MHBs, and small surface proteins. The 226 amino acid-long S domain comprises the SHBs and forms the carboxy-terminus in LHBs and MHBs. The preS2 domain is present in LHBs and MHBs, while the preS1 domain is present only in LHBs (6). The composition ratio of LHB and MHB levels at different stages of HBV infection is different. Pfefferkorn et al. reported that the baseline LHB and MHB levels in HBeAg-negative CHB were 2.5 ± 0.6 log₁₀ ng/mL and 2.1 ± 0.8 log₁₀ ng/mL, respectively; in HBeAg-positive CHB, the levels were 3.1 ± 0.6 log₁₀ ng/mL and 2.6 ± 0.8 log₁₀ ng/mL, respectively (9). In this study, the levels of LHBs and MHBs at baseline in the 60 IHCs were significantly lower than those reported above, i.e., 13.28 ± 1.89 ng/mL (1.12 ± 0.06 log₁₀ ng/mL) and 7.19 ± 1.19 ng/mL (0.85 ± 0.07 log₁₀ ng/mL), respectively. This is similar to the reported by Peiffer KH and Liu C, who suggested that the proportion of LHBs and MHBs in IHCs was lower than that in HBeAg-negative or HBeAg-positive CHB patients (8, 10).

In this study, there were 39 patients in the R group and 21 patients in the NR group. The total HBsAg, LHB, and MHB levels at baseline were compared between the two groups. The results showed that the levels of total HBsAg, LHB, and MHB in the R group were significantly lower than those in the NR group (all $p < 0.05$, Table 1). The total HBsAg, LHB, and MHB levels in the two groups significantly decreased after Peg-IFN treatment, but the total HBsAg and LHB levels in the R group decreased more at 12 weeks of treatment ($p = 0.021$, Figure 1B; $p = 0.015$, Figure 1D). Recently, researchers studied HBeAg-positive CHB patients treated with Peg-

IFN and reported a similar situation. Rinker et al. found that patients with low levels of LHBs and MHBs were more likely to have high HBeAg conversion after 24 weeks of treatment (6). Zhu et al. reported that patients with low LHB levels at 4 weeks were more likely to obtain HBeAg conversion (7). However, it is not clear whether LHBs and MHBs are associated with HBsAg clearance after Peg-IFN treatment.

It is generally believed that patients with good responsiveness to Peg-IFN are often accompanied by elevated ALT. Similar phenomena were also observed in this study. In patients with HBsAg clearance, the degree of ALT elevation was greater after 12 weeks of Peg-IFN treatment, and when ALT $> 1.5 \times$ ULN, the difference between the two groups was statistically significant ($p = 0.04$, Table 1). This reflects the double-edged sword effect of Peg-IFN treatment in both immune clearance and injury.

Regarding the predictive value of total HBsAg, LHB, and MHB levels for HBsAg clearance, the multivariate logistic regression analysis results indicated that baseline and 12-week LHB and MHB levels were independent predictors. Further ROC curve analyses were performed (Figure 2). The prediction results for a single indicator showed that the value of the baseline or 12-week LHB and MHB levels in predicting HBsAg clearance was greater than that of total HBsAg level at the corresponding time point, the 12-week reduction in total HBsAg or ALT $> 1.5 \times$ ULN. To improve the prediction accuracy, a combined predictive factor was constructed through a logistic regression model (11, 12). Baseline LHBs and MHBs were combined to construct combination factor 1, and the 12-week LHBs and MHBs were combined to construct combination factor 2; the AUCs were 0.922 and 0.939, respectively, indicating that the value of predicting HBsAg clearance was significantly higher than the above single indicators. In view of the complex calculation of the combination factors, for the convenience of clinical use, we performed combined indicators analysis using the cut-off value for each predictive indicator (Table 3, Figure 3). The results showed that the combined indicators based on LHBs and MHBs predicted HBsAg clearance rates of more than 90.0%, among which, the combination of baseline LHBs ≤ 13.99 ng/mL, baseline MHBs ≤ 7.95 ng/mL, and 12-week total HBsAg reduction > 0.29 log₁₀ IU/mL predicted HBsAg clearance rates can reach 100%. In this study, the commonly used clinical efficacy predictors of 12-week total HBsAg reduction (> 0.29 log₁₀ IU/mL) and ALT elevation ($> 1.5 \times$ ULN) combined predicted an HBsAg clearance rate of 88.24%. Similar to our previous study, in IHCs treated with Peg-IFN, when HBsAg decreased > 0.30 log₁₀ IU/mL and ALT elevation (greater than ULN) at 12 weeks were combined, the predicted HBsAg clearance rate was 80.80% (13). The traditional indicators can predict HBsAg clearance, but their predictive value is lower than that of LHBs and MHBs. As seen in Table 3, the combined indicators have higher sensitivity and specificity in predicting HBsAg clearance. But the prediction values (sensitivity and specificity) were without validation from external data, the models could be over fitted based on the sample size. Further

studies are need in the future to confirm the prediction role of Large and Medium Surface Proteins.

LHBs are necessary for the entry of HBV into hepatocytes and the secretion of viral particles from hepatocytes (14, 15). MHBs play a regulatory role in HBV replication, and MHB deletion can affect viral particle secretion (16). In 1987, Gerken et al. (17) demonstrated that a rapid decline in LHBs and MHBs occurred during the early stage of spontaneous HBsAg clearance in patients with acute hepatitis B. At 8 weeks after onset, 90% (18/20) of the patients were negative for serum MHBs, and at 16 weeks, 90% (18/20) of the patients had undetectable levels of LHBs. A similar phenomenon was also observed in the treatment of patients with chronic hepatitis B. Pfefferkorn et al. provided antiviral treatment to 83 patients with CHB, of whom 20 achieved HBsAg clearance (3 patients were in the Peg-IFN group and 17 patients were in the NA group). The results of that study suggested that the proportion of LHBs and MHBs in total HBsAg at baseline was significantly lower in HBsAg-cleared patients than in non-cleared patients and that the decrease in LHBs and MHBs was faster during treatment in HBsAg-cleared patients. The value of the proportion of baseline MHBs in predicting HBsAg clearance (AUC: 0.726) was higher than that of total HBsAg (AUC: 0.390) (4). The results were similar to those of our study. Not only did baseline and 12-week LHBs and MHBs have higher prediction value than total HBsAg in single indicator prediction, but the combination of LHBs and MHBs had better prediction value for HBsAg clearance. Compared with Pfefferkorn et al.'s study, in this study, LHBs and MHBs were better for predicting HBsAg clearance. The possible reason is that the patients included in this study were all IHCs patients, while Pfefferkorn et al. enrolled HBeAg-positive patients. In addition, the 39 patients with HBsAg clearance in our study were all underwent Peg-IFN treatment, while most of the patients with HBsAg clearance in the study by Pfefferkorn et al. underwent NA treatment. In addition, Rinker et al. studied 127 HBeAg-positive patients who received Peg-IFN treatment and found that LHBs and MHBs were similar to total HBsAg in predicting HBeAg conversion and did not have greater advantages (6).

In summary, the predictive value of LHB and MHB levels for HBsAg clearance was good and was better than total HBsAg level at both baseline and 12 weeks. In addition, the combination of LHBs and MHBs had a higher predictive value than did single factors, and the accuracy of predicting HBsAg clearance based on the combined baseline LHBs and MHBs was over 90.00%. Therefore, based on the current treatment strategy, the monitoring of LHBs and MHBs in the process of pursuing HBsAg clearance can provide a reference for clinical treatment. In addition, the detection of LHBs and MHBs is convenient and reproducible, which is important for clinical application. However, this study enrolled an IHCs population treated with Peg-IFN; therefore, the significance of the results needs to be further explored in a wider range of CHB patients.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study was approved by the ethics committee of Beijing Youan Hospital affiliated with Capital Medical University ([2017]24). The patients/participants provided their written informed consent to participate in this study.

Author contributions

XL, YZ, HL, ZC, and XC designed the study. JL, SR, YL, and XW collected the data. XL, YZ, and HL analyzed the data. SZ and LM guided statistical analysis. XL, YZ, and HL drafted the manuscript. ZC and XC contributed to the interpretation of the results and critical revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor WY declared a shared parent affiliation with the authors during the review.

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Clinical characteristics of hepatitis flares during pregnancy and postpartum in Chinese chronic hepatitis B virus carriers—a prospective cohort study of 417 cases

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Background: In China, it is common for pregnant women with a high load of hepatitis B virus (HBV) to take nucleos(t)ide analogue (NA) to prevent maternal-to-child transmission of HBV. However, the impact of NA intervention on virological and biochemical parameters in pregnant and postpartum women and the safety of drug cessation remain unclear. A prospective observational cohort was established in this study to analyze the clinical characteristics of hepatitis flares in pregnant and postpartum chronic HBV carriers, with or without NA intervention.

Methods: Pregnant women who were chronic HBV carriers were enrolled in this study and divided into an NA intervention group and a non-intervention group according to their preferences. Liver function, HBV DNA level, and HBV serological markers were regularly measured during pregnancy and at approximately 6 weeks, 12 weeks, 24 weeks, 36 weeks, and 48 weeks postpartum.

Results: A total of 417 patients were enrolled, including 303 in the NA intervention group and 114 in the non-intervention group. The incidence rates of postpartum hepatitis flares in both groups were higher than that of during pregnancy (45.7% vs 10.9%, $p < 0.001$; 41.2% vs 17.7%, $p < 0.001$). The second trimester was the peak of the incidence of flares during pregnancy and the incidence peak of postpartum flares was about 6 weeks postpartum. A total of 98% (145/148) of postpartum flares occurred within 24 weeks postpartum. After drug cessation, the incidence rate of flares was 34.1% (44/129).

Conclusion: In pregnant chronic HBV carriers, a certain proportion of hepatitis flares occurred during pregnancy and postpartum regardless of whether NA intervention was used, and the incidence of postpartum flares (44.6%) was

significantly higher than that (12.8%) of during pregnancy. The flare incidence peaked at approximately 6 weeks postpartum, which may be the time period suitable for treatment. Since 98% of postpartum flares occurred within 24 weeks postpartum, the follow-up after drug cessation should be at least 24 weeks postpartum.

KEYWORDS

chronic HBV carriers, pregnant women, hepatitis flares, drug cessation, postpartum

1 Introduction

In China, most patients with chronic hepatitis B virus (HBV) infection acquired the infection at a young age, mother-to-child transmission as the main transmission route, accounting for approximately 40% of all HBV infections (1). Among pregnant women with high HBV load, 8–15% of them still can transmit HBV to their infants even after their infants receive conventional immunization (2–4). Growing evidence (4–6) in recent years has confirmed that the administration of nucleos(t)ide analogue (NA) in women with high HBV load during second or third trimester can inhibit viral replication and thereby significantly increase the success rate of prevention of mother-to-child transmission. Therefore, the Chinese and international guidelines for hepatitis B (7–10) have recommended that pregnant women with high HBV load receive NA intervention during second or third trimester. NA intervention does benefit newborns significantly, but the impact of NA intervention on virological and biochemical parameters in pregnant and postpartum women, and the safety of drug cessation are still unclear. This prospective observational cohort study was to investigate the clinical characteristics of hepatitis flares in pregnant and postpartum women.

2 Patients and methods

2.1 Study population

The subjects of the study were chronic HBV carriers visited in Beijing You'an Hospital Affiliated to Capital Medical University (from November 2015 to July 2018), who were planning for pregnancy or pregnant (less than 8 weeks of gestation). The inclusion criteria were as follows: age 18–40 years, hepatitis B surface antigen (HBsAg) positivity for more than 6 months, HBeAg hepatitis B e antigen (HBeAg) positivity,

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; NA, nucleos(t)ide analogue; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; ULN, upper limit of normal; TDF tenofovir disoproxil, LDT telbivudine.

HBV DNA $\geq 2 \times 10^6$ IU/ml, no history of antiviral therapy, and normal alanine aminotransferase (ALT) levels in at least two tests within 2 years before pregnancy. The exclusion criteria were as follows: hepatitis A, C, D, or E infection; other chronic liver diseases; human immunodeficiency virus infection; cirrhosis or hepatocellular carcinoma; and intrahepatic cholestasis of pregnancy and a history of miscarriage.

2.2 Research methods

A prospective, open-cohort design was adopted for this clinical study. The patients were given the choice between joining either the non-intervention group (group A) or the NA intervention group (group B) during pregnancy. Medications used for intervention included telbivudine (LDT) and tenofovir disoproxil (TDF), which were started at 24 weeks to 28 weeks of gestation at a dose of 600 mg/day for LDT and 300 mg/day for TDF. To select LDT or TDF was based on patients' willingness. All patients were informed about this study and signed informed consent forms before enrollment (approval number: JingYou KeLun Zi[2015]21).

The team members of the joint research group of the Department of Obstetrics and the Department of Hepatology followed up the patients regularly from pregnancy to 48 weeks postpartum. Patients were followed up once every 4–6 weeks during pregnancy. The first postpartum follow-up was approximately 6 weeks postpartum (6 ± 2 weeks), and afterwards the patients were followed up approximately every 12 weeks until the completion of the 48-week follow-up. HBV DNA, HBV serological markers, liver function and renal function should be tested during each follow-up. Abnormal liver function was defined as ALT > upper limit of normal (ULN, ULN was 40 U/L), including mildly abnormal liver function ($ULN < ALT < 2 \times ULN$), hepatitis flare ($ALT \geq 2 \times ULN$), and hepatitis exacerbation ($ALT \geq 10 \times ULN$ or Total bilirubin $\geq 5 \times ULN$, ULN was 17.1 μ mol/L). Patients with abnormal liver function test results were re-examined within 2 weeks, and any liver injury-inducing factors, such as alcohol, fat and drugs, had to be excluded by a clinicians' assessment.

2.3 Laboratory test methods

HBV DNA was detected using the Roche Cobas/Taqman Real-Time fluorescence quantitative polymerase chain reaction (PCR) system (Roche Diagnostics GmbH, Germany), and the low limit of detection was 20 IU/mL. The HBV serological markers were detected using the Roche E601 automatic chemiluminescence analyzer, with lower detection limit of 0.05 IU/ml for HBsAg and 1 COI for HBeAg. An OLMPUS-AU5400 biochemistry analyzer was used to detect biochemical parameters such as liver function parameters. HBV was genotyped with the HBV genotyping reagent kit (fluorescence PCR) (Fosun Pharma, Shanghai, China).

2.4 Statistical analysis

SPSS 25.0 (SPSS, USA) software was used for the statistical analysis. Continuous variables were presented as the mean $\pm$ standard deviation or median (interquartile range). Categorical variables were expressed as frequencies and percentages. Continuous variables were assessed using Student's *t* test or Ranksum test. Categorical variables were compared with the Chi-squared test or Fisher's exact test. A two-sided *P* value of < 0.05 indicated statistical significance.

3 Results

3.1 General information of enrolled patients and follow-up

A total of 471 HBsAg-positive women who were pregnant or were planning for pregnancy were screened in this study (Figure 1), and 417 patients with chronic HBV infection met the inclusion criteria, 114 patients joined the non-intervention group during pregnancy (group A) and 303 patients joined the NA intervention group during pregnancy (group B). Two patients, one in group A and one in group B, were excluded during pregnancy due to unexpected causes, and the follow-up and lost follow-up of 415 pregnant women included in the analysis of pregnancy were shown in Figure 1.

There were no significant differences in age, the baseline HBV DNA, HBeAg, and ALT levels, and the genotypes of patients between group A and group B (Table 1). The median time of the initiation of the NA intervention in group B was 28 weeks of gestation.

3.2 Hepatitis flares during pregnancy

The rate of abnormal liver function during pregnancy was 20.9% (87/415), including 34 (8.2%) with mildly abnormal liver

function, 53 (12.8%) with hepatitis flares, and 8 (1.9%) with hepatitis exacerbation. Among the 53 cases of hepatitis flares during pregnancy, 46 happened in non-intervention state, an incidence rate of 11.1% (46/415). Seven patients in group B had hepatitis flares during NA intervention, an incidence rate of 2.3% (7/302). The incidence rate of hepatitis flares in non-intervention state during pregnancy was significantly higher than that in intervention state, the difference was statistically significant (11.1% vs 2.3%, $P < 0.001$). There was no significant difference in the incidence of hepatitis flares with or without NA intervention during pregnancy, with 17.7% (20/113) and 10.9% (33/302) in group A and B, respectively (Table 2). However, all the 8 cases with hepatitis exacerbation occurred in non-intervention state, and all of them received NA for antiviral therapy.

In terms of the time of hepatitis flares, the second trimester was more common, with 65.0% (13/20) of patients with hepatitis flares during pregnancy in group A experienced hepatitis flares and 54.5% (18/33) of patients with hepatitis flares during pregnancy in group B experienced hepatitis flares in the second trimester.

3.3 Hepatitis flares during postpartum

3.3.1 Incidence of postpartum hepatitis flares

After 48 weeks of postpartum follow-up, 30 patients were lost to follow-up. The 53 patients with hepatitis flares during pregnancy were not included in the analysis of postpartum hepatitis flares. Therefore, 332 patients (85 in group A and 247 in group B) were included for the overall postpartum analysis, including 132 (39.7%) with mild abnormal liver function, 148 (44.6%) with hepatitis flares. The incidence rate of hepatitis flares during the postpartum period was significantly higher than that during pregnancy (44.6% vs 12.8%, $P < 0.001$, Table 3). Further comparison of the incidence rates of hepatitis flares in each group during pregnancy and the postpartum period also showed that the incidence rate of hepatitis flares during the postpartum period was significantly higher than that during pregnancy (group A: 41.2% vs 17.7%, $P < 0.001$; group B: 45.7% vs 10.9%, $P < 0.001$; Table 3). The incidence rate of hepatitis exacerbation in the postpartum period was 4.8% (16/332), which was also higher than the 1.9% (8/415) during pregnancy ($P = 0.026$, Table 3). Comparing the incidence rates of hepatitis exacerbation between the two groups, group A was slightly higher than group B, which were 9.4% (8/85) and 3.2% (8/280), respectively, $P = 0.046$.

In group B, about 6 weeks postpartum, 129 patients stopped NA, and the incidence of hepatitis flares in these patients was 34.1% (44/129). The other 138 patients did not stop NA and the incidence of hepatitis flares in these patients was 33.3% (46/138). The difference was not significant ($P = 0.893$).

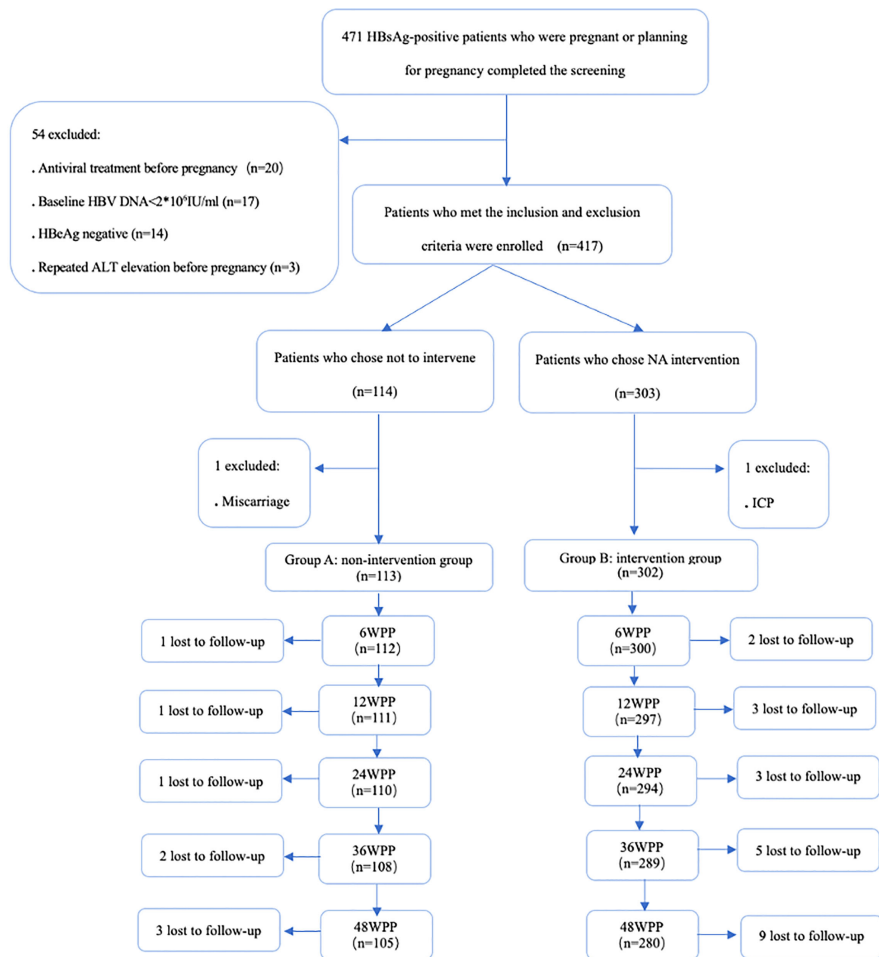


FIGURE 1

Flow chart of enrollment and follow-up of women who were chronic HBV carriers. HBsAg hepatitis B surface antigen, HBV hepatitis B virus, HBeAg hepatitis B e antigen, ALT alanine aminotransferase, NA nucleos(t)ide analogue, ICP intrahepatic cholestasis of pregnancy, WPP weeks postpartum.

3.3.2 Time of postpartum hepatitis flares

The median time of onset of postpartum hepatitis flares in group A and group B was both about 6 weeks postpartum, and the onset of hepatitis flares peaked at approximately 6 weeks postpartum (Table 4). A total of 148 patients had postpartum hepatitis flares, 81.8% (121/148) of them occurring approximately 6 weeks postpartum. The incidence rate of hepatitis flares at this time point was significantly higher than that at other follow-up time points (Table 4). Within 24 weeks postpartum, the cumulative number of hepatitis flare cases was 145, accounting for 98.0% of the total number of cases with hepatitis flares during the entire postpartum follow-up period. Only three cases of hepatitis flares were between the 24th week and the 48th week of the 48-week follow-up period, accounting for 2.0% of all postpartum hepatitis flare cases (Figure 2).

3.3.3 Virological parameters during postpartum hepatitis flares

Within the 48 weeks of the postpartum follow-up, the cumulative number of patients with postpartum hepatitis flares in non-intervention group (group A) was 35, and the median value of HBV DNA during hepatitis flares was 7.6 log₁₀ IU/ml; the cumulative number of cases with postpartum hepatitis flares in intervention group (group B) was 113, including patients with drug cessation and patients without drug cessation, and the median value of HBV DNA during hepatitis flares was 4.4 log₁₀ IU/ml, significantly lower than that in group A ($P < 0.001$). In group B, the HBV DNA levels at the onset of hepatitis flares were compared between patients with drug cessation and patients without drug cessation. The former had 67 cases of hepatitis flares and the median value of HBV DNA was higher, 5.6 log₁₀

TABLE 1 General information of the two groups.

General information ^a	Group A non-intervention group (n=113)	Group B intervention group (n=302)	P-value
Age (years)	27.1 ± 4.3	28.9 ± 4.2	0.400
Baseline HBV DNA (log ₁₀ IU/ml)	7.6 ± 0.5	7.8 ± 0.6	0.327
Baseline HBeAg (log ₁₀ COI)	3.0 ± 0.2	3.1 ± 0.4	0.141
Baseline ALT (U/L)	20.1 (15.9-29.9)	26.3 (19.0-39.2)	0.050
Genotype			0.401
B	33 (29.2%)	72 (23.9%)	
C	80 (70.8%)	229 (75.8%)	
A	0 (0.0%)	1 (0.3%)	—
Weeks of intervention	NA	28 (24-28)	
Types of intervention medications (TDF/LDT)	NA	TDF 203 (67.2%) LDT 99 (32.8%)	—

HBV hepatitis B virus, HBeAg hepatitis B e antigen, ALT alanine aminotransferase, TDF tenofovir disoproxil, LDT telbivudine, NA not available.

^aGeneral information were presented as mean ± standard deviation, median (interquartile range) or n (%).

IU/ml; the latter had 46 cases of hepatitis flares and the median value of HBV DNA was lower, 3.3 log₁₀ IU/ml, the difference was significant ($P < 0.001$).

4 Discussion

According to the recommendations of the recent guidelines for the management of hepatitis B (7–10), NAs are widely used in pregnant chronic HBV carriers in China to prevent maternal-to-child transmission. We conducted a prospective cohort study to investigate whether drug cessation after NA intervention was

safe for mothers and the characteristics of the hepatitis flares in pregnant and postpartum women. The results of our study suggested that the overall incidence rates of hepatitis flares and hepatitis exacerbation were 12.8% and 1.9% in 415 chronic HBV carriers during pregnancy. These results were consistent with previous studies. The retrospective study by Kushner (11) suggested that the incidence of hepatitis flares during pregnancy in women with chronic HBV infection was 14% and that the incidence rate of ALT $\geq 10 \times$ ULN was 2%. Our study also observed that the incidence rate of hepatitis flares during pregnancy was 11.1% (46/415) in non-intervention state, which was much higher than that (2.3%, 7/302) in intervention

TABLE 2 Incidence of hepatitis flare during pregnancy in two groups.

	Group A non-intervention group (n=113)	Group B intervention group (n=302)	P-value
Proportion of mildly abnormal liver function [✱] n (%)	5 (4.4%)	29 (9.6%)	0.087
Hepatitis flare [*] incidence n (%)	20 (17.7%)	33 (10.9%)	0.066
Proportion of hepatitis exacerbation [★] n (%)	1 (0.9%)	7 (2.3%)	0.345
Time of hepatitis flare onset(week) [§]	14 (12-32)	17 (13-28)	0.730
Hepatitis flare incidence at each follow-up time point [§]			
First trimester	6/113 (5.3%)	8/302 (2.6%)	0.181
Second trimester	13/107 (12.1%)	18/294 (6.1%)	0.046
Third trimester	1/94 (1.1%)	7/276 (2.5%)	0.662
The level of HBV DNA at the onset of hepatitis flare (log ₁₀ IU/ml) ^b	7.7 ± 0.7	7.3 ± 1.1	0.570

HBV hepatitis B virus.

^amedian (interquartile range).

^bmean ± standard deviation.

✱Mildly abnormal liver function, upper limit of normal (ULN) < alanine aminotransferase(ALT) < 2 × ULN (ULN was 40 U/L).

*Hepatitis flare, ALT $\geq 2 \times$ ULN.

★Hepatitis exacerbation, ALT $\geq 10 \times$ ULN or Total bilirubin $\geq 5 \times$ ULN, ULN was 17.1 μmol/L.

Patients with abnormal liver function test results were re-examined within 2 weeks, and any liver injury-inducing factors, such as alcohol, fat, immunity, and drugs, had to be excluded by a clinicians' assessment.

§ When calculating the incidence rate of hepatitis flare at each follow-up time point during pregnancy, the total number of patients at each follow-up time point did not include patients who have had hepatitis flares before and those who were lost to follow-up.

P<0.05 is shown in bold.

TABLE 3 Comparison of incidence of hepatitis flare during pregnancy and postpartum.

	Total			Group A non-intervention group			Group B intervention group		
	Pregnancy (n=415)	Postpartum (n=332)	P- value	Pregnancy (n=113)	Postpartum (n=85)	P- value	Pregnancy (n=302)	Postpartum (n=247)	P- value
Hepatitis flare* incidence ^a	53 (12.8%)	148 (44.6%)	<0.001	20 (17.7%)	35 (41.2%)	<0.001	33 (10.9%)	113 (45.7%)	<0.001
Hepatitis exacerbation★ incidence ^a	8 (1.9%)	16 (4.8%)	0.026	1 (0.9%)	8 (9.4%)	0.012	7 (2.3%)	8 (3.2%)	0.510

^an (%).*Hepatitis flare, ALT $\geq 2 \times$ ULN.★Hepatitis exacerbation, ALT $\geq 10 \times$ ULN or Total bilirubin $\geq 5 \times$ ULN, ULN was 17.1 μ mol/L.

Patients with abnormal liver function test results were re-examined within 2 weeks, and any liver injury-inducing factors, such as alcohol, fat, immunity, and drugs, had to be excluded by a clinicians' assessment.

P<0.05 is shown in bold.

state. This suggests that hepatitis flares during pregnancy might not be associated with NA intervention because hepatitis flares occurred regardless of whether the intervention was applied, and the incidence of flares in non-intervention state was higher. The incidence of hepatitis flares might be mainly due to changes in immune function caused by physiological changes during pregnancy. However, there were inconsistent reports. Bzowej et al. (12) reported that the incidence rate of hepatitis flares in pregnant women with chronic HBV infection without intervention was 3.4% (5/149), which was comparable to the incidence rate of flares in pregnant women with intervention (2.0%).

In this study, among the 332 patients who were followed up to 48 weeks postpartum and included in the postpartum analysis, the incidence rate was 39.7% for mildly abnormal liver function, 44.6% for hepatitis flares, and 4.8% for hepatitis exacerbation. In the non-intervention group and the intervention group, mildly abnormal liver function and hepatitis flares were 36.5% and 40.9%, 41.2% and 45.7%, respectively, and the differences were not significant. But the proportion of hepatitis exacerbation in the non-intervention group (9.4%) was higher than that in the intervention group (3.2%), the difference was significant. Pan et al. (5) reported that in pregnant women with a high viral load of HBV, the incidence rates of abnormal ALT (ALT > 40 U/L) and severe hepatitis (ALT > 400 U/L) between 5 and 28 weeks postpartum were 45% and 1% in the TDF intervention group and 30% and 3% in the non-intervention group respectively. The incidence rates of mildly abnormal liver function in the study of Pan et al. were consistent with the results of our study, but there was no difference in the proportion of severe hepatitis in the TDF intervention group and the non-intervention group, which was inconsistent with the results of our study. In this study, we specifically subgrouped the proportion of hepatitis flares (ALT $\geq 2 \times$ ULN), which can reach 41.2% in the non-intervention group and 45.7% in the intervention group respectively during postpartum period. According to the domestic and foreign

hepatitis B guidelines (7–9) recommended standards for treatment, people with hepatitis flares are the focus of the recommended population in need of treatment, especially worthy of clinical attention.

Besides, our study also suggested that regardless of whether NA intervention was applied, the incidence rates of hepatitis flares and hepatitis exacerbation was significantly higher in the postpartum period than during pregnancy (44.6% vs 12.8%, $P < 0.001$; 4.8% vs 1.9%, $P = 0.026$). Hepatitis flares could not be avoided even during NA intervention, and the incidence rates of hepatitis flares were 2.3% in pregnant women during NA intervention and 33.3% in postpartum women. Bzowej (12) et al. also had similar report that the incidence of hepatitis flares (ALT > 100U/L) during NA intervention in pregnant women with chronic HBV infection was 2%. The incidence rate of abnormal ALT (ALT > 40 U/L) was 34% during NA intervention within 12 weeks postpartum reported by Liu (13). The results of these studies all suggested that whether the pregnant or postpartum women were in the state of intervention, drug cessation or non-intervention, the changes in ALT level were needed to be observed closely, especially the postpartum women required closer follow-up.

Regarding the time of onset of hepatitis flares during pregnancy, it had been reported (13) that about half of hepatitis flares occurred in the first and second trimester. The findings of our study showed the median time of onset of hepatitis flares was 14 weeks and 17 weeks of gestation in group A and group B, respectively and the hepatitis flares occurring in the second trimester in group A and group B accounted for 65.0% and 54.5% of all flare cases in the respective groups during pregnancy. In addition, our study also observed that the incidence of postpartum flares peaked at approximately 6 weeks postpartum, and the incidence rates of flares at this time point in group A and group B (33.7% and 33.7%) were significantly higher than those at other follow-up time points. However, some studies reported that the peak of postpartum hepatitis flares onset was about 12 weeks postpartum (14, 15).

TABLE 4 Incidence of postpartum hepatitis flare in the two groups.

	Group A non-intervention group	Group B intervention group	P-value
Proportion of mildly abnormal liver function [‡]	31/85 (36.5%)	101/247 (40.9%)	0.473
Hepatitis flare* incidence	35/85 (41.2%)	113/247 (45.7%)	0.464
Proportion of hepatitis exacerbation [★]	8/85 (9.4%)	8/247 (3.2%)	0.046
Time of hepatitis flare onset (week) [§]	6 (4-7)	6 (5-8)	0.257
Hepatitis flare incidence at each follow-up time point [§]			
6 ± 2WPP	31/92 (33.7%)	90/267 (33.7%)	0.998
12 ± 2WPP	2/60 (3.3%)	14/174 (8.0%)	0.342
24 ± 2WPP	1/57 (1.8%)	7/157 (4.5%)	0.607
36 ± 2WPP	0/54 (0.0%)	0/145 (0.0%)	—
48WPP	1/51 (2.0%)	2/136 (1.5%)	1.000
The level of HBV DNA at the onset of hepatitis flare (log ₁₀ IU/ml) [§]	7.6 (7.1-8.2)	4.4 (3.3-6.6)	<0.001

HBV hepatitis B virus, WPP weeks postpartum.

[‡]median (interquartile range).

[‡]Mildly abnormal liver function, upper limit of normal (ULN) < alanine aminotransferase (ALT) < 2 × ULN (ULN was 40 U/L).

*Hepatitis flare, ALT ≥ 2 × ULN.

★Hepatitis exacerbation, ALT ≥ 10 × ULN or Total bilirubin ≥ 5 × ULN, ULN was 17.1 μmol/L.

Patients with abnormal liver function test results were re-examined within 2 weeks, and any liver injury-inducing factors, such as alcohol, fat, immunity, and drugs, had to be excluded by a clinicians' assessment.

§ When calculating the incidence rate of hepatitis flare at each postpartum follow-up time point, the total number of patients at each follow-up time point did not include patients who have had hepatitis flares before and those who were lost to follow-up.

P < 0.05 is shown in bold.

The differences may be associated with the differences in the time of the first postpartum visit and the interval between follow-up visits.

Regarding the safe time for drug cessation, 92.3% (385/417) of patients completed the 48-week follow-up, and 98% of postpartum hepatitis flares (145/148) occurred within 24 weeks of postpartum, only 2% after 24 weeks. A retrospective study (16) showed that 96% of abnormal liver function occurred within 6 weeks postpartum in postpartum women who were chronic HBV carriers followed up to 6 months postpartum. Another article (17) reported that 93.3% (42/45) of abnormal liver function occurred within 3 months postpartum in a sample of 114 pregnant women with chronic HBV infection that was followed up to 6 months postpartum. The findings of these two studies above were different from the results of our study. According to our data, although the incidence rate of postpartum hepatitis flares was already high within 12 weeks postpartum, eight new cases of hepatitis flares and one case of hepatitis exacerbation occurred from 12 weeks to 24 weeks postpartum, and only 3 new hepatitis flares occurred from 24 weeks to 48 weeks. Therefore, we believe that postpartum follow-up should be conducted for at least 24 weeks, which can cover 98% of postpartum hepatitis flare cases.

In addition, the HBV DNA load varied significantly among the patients with hepatitis flares in the intervention group in this study. Virological rebound occurred first in patients who stopped taking NA postpartum, and the HBV DNA level increased (5.6 log₁₀ IU/ml) and then could cause hepatitis flares, which was in line with the general pattern of hepatitis

flares, namely, high viral load and high ALT level. However, the hepatitis flare in patients who did not stop NA was different from the above. Even when the HBV DNA load was lower (3.3 log₁₀ IU/ml), the ALT level was still elevated. The pattern of hepatitis flares in patients who did not stop NA was characterized by high ALT level but low viral load. The difference between the pattern of hepatitis flare before drug cessation and that after drug cessation was related to the different status of drug administration. In general, when the HBV DNA load is low, it usually does not trigger hepatitis flares. If the ALT level increases when the viral load declines, it is more likely to indicate the enhancement of immune function and the transition from immune tolerance to immune clearance period. In addition, other liver injury-inducing factors, such as fatty liver and drug-induced hepatitis, were excluded in this study. Pregnancy was generally considered to be in a relatively immunosuppressive state (18) to avoid rejection, which is conducive to the growth and development of the offspring, but the immune function is relatively enhanced during the postpartum period (2, 19). In addition, with high viral load during pregnancy, the body tended to be immune tolerant, and the decline of viral load after delivery is equivalent to weakened immune tolerance. Some scholars also have reported that many autoimmune diseases can be relieved during pregnancy but often recur after delivery (20). These mechanisms might partly explain the reason why the incidence of postpartum flares (44.6%) was significantly higher than that (12.8%) of during pregnancy. However, it has not been reported whether the high ALT level but low viral load hepatitis flares observed in this study has a

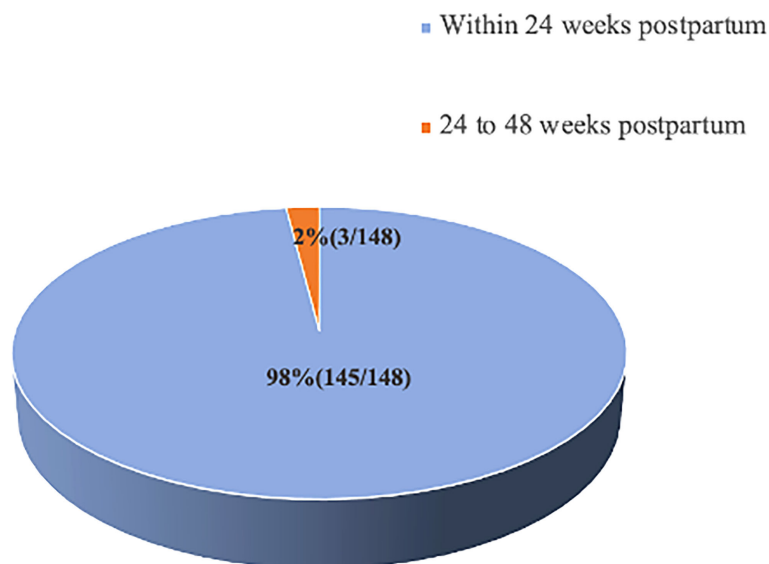


FIGURE 2
Distribution of postpartum hepatitis flare.

similar immune mechanism. To explore the therapeutic effects under this condition, our team (21) treated patients with pegylated interferon and NA for 96 weeks and achieved satisfactory treatment outcomes: The HBeAg clearance rate can reach 56.7% (17/30), and the HBsAg clearance rate was 26.7% (8/30), suggesting that women who did not stop treatment after delivery may achieve better therapeutic effects if they had antiviral therapy timely for postpartum hepatitis flares.

In summary, the status of immune tolerance in pregnant women who were chronic HBV carriers was not always unchanged. Regardless of whether NA intervention was applied, a certain proportion of hepatitis flares occurred during pregnancy and postpartum period, and the incidence rate of hepatitis flares during postpartum (44.6%) was significantly higher than that during pregnancy (12.8%). The peak of postpartum hepatitis flare was in 6 weeks. Since 98% of the postpartum hepatitis flares occurred within 24 weeks postpartum, patients should be followed up for at least 24 weeks postpartum to determine whether they can safely stop the treatment.

Capital Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

XC, XW, and AS conceived and designed the protocol and study. AS, XL and SR collected the data. XW analyzed the data and drafted the manuscript. YZ performed the follow up of patients. JL, SZ, and LM guided statistical analysis. XC contributed to the interpretation of the results and critical revision of the manuscript for important intellectual content and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by The ethics committee of Beijing Youan Hospital,

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a shared parent affiliation with the authors at the time of review.

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Cytokine profiles and CD8+ T cells in the occurrence of acute and chronic hepatitis B

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Objective: We explore the expression of functional molecules on CD8+ T lymphocytes, cytokines concentration, and their correlation to occurrence of hepatitis B and hepatitis B virus (HBV) deoxyribose nucleic acid (DNA), hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), and alanine aminotransferase (ALT) in patients infected with HBV.

Methods: This is a single center study. 32 patients with acute hepatitis B (AHB), 30 patients with immune tolerant (IT) phase chronic HBV infected, and 50 patients with chronic hepatitis B (CHB) were enrolled. The activation molecules (CD69) and the apoptosis-inducing molecules (CD178) on surface of CD8+ T lymphocytes were tested by the flow cytometry. Fms-like tyrosine kinase 3 ligand (Flt-3L), interleukin 17A (IL-17A), interferon γ (IFN- γ), and Interferon α 2 (IFN- α 2) were quantitated by Luminex assay. We use linear regression analysis to analyze their correlations to ALT, HBV DNA, HBsAg, and HBeAg.

Results: The frequency of CD69+CD8+ T lymphocytes in CHB and AHB groups were increased significantly compared with IT group (4.19[3.01, 6.18]% and 4.45[2.93, 6.71]% vs. 3.02[2.17, 3.44]%; $H=26.207$, $P=0.001$; $H=28.585$, $P=0.002$), and the mean fluorescence intensity (MFI) of CD69 in AHB group was significantly higher than IT and CHB groups (27.35[24.88, 32.25] vs. 20.45[19.05, 27.75] and 23.40[16.78, 28.13]; $H=25.832$, $P=0.005$ and $H=22.056$, $P=0.008$). In IT group, HBsAg levels and HBV DNA loads were negatively correlated with CD69MFI ($\beta=-0.025$, $t=-2.613$, $P=0.014$; $\beta=-0.021$, $t=-2.286$, $P=0.030$), meanwhile, HBeAg was negatively related to the frequency of CD69+CD8+ T lymphocytes ($\beta=-61.306$, $t=-2.116$, $P=0.043$). In AHB group, IFN- α 2 was positively related to the frequency of CD8+ T lymphocytes ($\beta=6.798$, $t=2.629$, $P=0.016$); however, in CHB group, IFN- α 2 was negatively associated with frequency of CD8+ T lymphocytes ($\beta=-14.534$, $t=-2.085$, $P=0.043$). In CHB group, HBeAg was positively associated with

frequency of CD69+CD8+ T lymphocytes ($\beta=43.912$, $t=2.027$, $P=0.048$). In AHB group, ALT was positively related to CD69MFI ($\beta=35.042$, $t=2.896$, $P=0.007$), but HBsAg was negatively related to CD178MFI ($\beta=-0.137$, $t=-3.273$, $P=0.003$).

Conclusions: The activation of CD8+ T lymphocytes was associated with the occurrence of AHB and CHB. However, due to the insufficient expression of functional molecules of CD8+ T lymphocytes and the depletion of CD8+ T lymphocytes, CHB patients were difficult to recover from HBV infection.

KEYWORDS

chronic hepatitis B, acute hepatitis B, cytokines, CD8+ T cells, immune tolerance

Introduction

Long-term progression of hepatitis B virus (HBV) infection will lead to liver failure, cirrhosis, and liver cancer, which will bring great threat to patients' lives and economic situation. The occurrence and development of hepatitis are closely associated with immune environment of hosts and virus after HBV infection (1). HBV infection always persists in majority of chronic hepatitis B (CHB), but can be cleared spontaneously in acute hepatitis B (AHB). We know that there are four phases in the natural outcome of CHB patients depending on different immune states (2, 3). These differences mainly depend on the differential immune responses of patients.

Various immune cells played a role in the immune response of patients with hepatitis B. Innate immunity mediated by natural killer (NK) cells, NKT cells, dendritic cells (DCs), and mononuclear macrophages can inhibit virus spread, participate in antigen presentation and activate specific immunity, which is the first line of antiviral defense. Adaptive immunity, mainly involving B cells, CD8+ T lymphocytes and CD4+ T lymphocytes, is considered to not only inhibit virus replication, but also play an important role in virus clearance. CD8+ T lymphocytes are of crucial importance in HBV clearance. In addition, it is difficult to achieve persistent viral clearance and hepatitis B surface antigen (HBsAg) serological conversion in patients infected with HBV, which may be associated with the deficiency of HBV-specific adaptive immunity (4). This study tried to investigate the function of CD8+ T lymphocytes and related cytokines to illustrate different immune state in HBV infected patients. In HBV infection, CD8+ T lymphocytes can inhibit viral replication by secreting interferon γ (IFN- γ), and induce apoptosis of infected hepatocytes through the secretion of perforin, which causes the rupture of HBV infected hepatocytes and the expression of apoptotic molecules. Thus, CD8+ T lymphocytes are of crucial

importance in HBV clearance. The major of adults infected with HBV can recover from infection. The immune response caused by HBV infection leads to severe hepatic inflammation in the liver. However, this immune response in CHB patients is not effective in clearing HBV. Many kinds of immune cells, like NK cells, DCs, mononuclear macrophages, CD4+ T lymphocytes, CD8+ T lymphocytes and their secreted cytokines, are involved in the occurrence of liver inflammation and viral clearance. The outcome of HBV infection depends on the function of HBV-specific cellular immunity. In our research, we investigate the relationship between the immune environment and clinical indicators in patients infected with HBV.

Materials and methods

Patients

Eligible patients attending Beijing Ditan Hospital from February 2014 to December 2016 were consecutively enrolled. The enrolled objects in the study were assigned to one of the three subgroups: immune tolerant (IT) phase chronic HBV infection group, hepatitis B envelope antigen (HBeAg)-positive CHB group, and AHB group. IT phase chronic HBV infection was defined as HBsAg positive for > 24 weeks, HBV desoxyribose nucleic acid (DNA) $>10^7$ IU/ml, HBeAg positive, consistently normal alanine aminotransferase (ALT) (<30 U/L in male, <19 U/L in female), and/or no obvious liver fibrosis and inflammation in histology. CHB was defined as HBsAg positive for > 24 weeks, ALT in abnormal level (>120 U/L) for more than 12 weeks, and/or obvious liver fibrosis and inflammation in histology, with positive HBeAg. AHB was defined as no HBV infection 24 weeks ago, HBsAg positive, abnormal ALT, and anti-HBc-IgM for $> 1:1000$ or confirmed by histopathological examination (2, 3). Exclusion criteria: co-infected with other

hepatitis viruses, co-infected with other viruses, with other liver diseases (such as alcoholic, immune, metabolic), and liver cancer; with cirrhosis or fibrosis diagnosed by transient elastography (5). Patients with autoimmune diseases, and subjects using immunosuppressive or regulatory drugs were also excluded. The liver cancer was excluded by CT examination (Computed Tomography System, 103 LightSpeed VCT, LightSpeed Pro32, Tokyo, Japan) or ultrasound (Acuson Sequoia, Siemens, Erlangen, Germany). The Liver cirrhosis or fibrosis test was taken by FibroScan (FibroScan 502, EchoscansTM, Paris, 102 France).

This is a single center study. Our research was approved by Beijing Ditan Hospital Institutional Review Board. All objects have signed informed consent before enrollment.

Clinical indicators, serology and HBV DNA detection

The biochemical indicators were tested by Hitachi 7600 automatic biochemical analyzer (Hitachi 7600-11; Hitachi, Tokyo, Japan). Virological indicator was measured by Roche Cobas AmpliPrep/Cobas TaqMan 96 automatic real-time fluorescence quantitative polymerase chain reaction detection (PCR) detection reagent (Roche, Pleasanton, CA, USA; with a lower detection limit of 20IU/ml). Serological indexes were quantitated by Abbott Architect i2000 detection reagent (Abbott Diagnostics, USA).

Functional molecular expression and frequency of CD8+ T lymphocytes

The frequency of CD8+ T lymphocytes of all patients in leukomonocytes, CD69+CD8+ T lymphocytes, CD178+CD8+ T lymphocytes, and the counts of CD69 and CD178 expressed on CD8+ T lymphocytes were detected by flow cytometry (FACS Caliburflow Cytometer, USA). It was performed as follow steps: 1) using monoclonal Antibodies (mAbs) of CD3-peridinin chlorophyll protein (PerCP), CD8-human antigen presenting cells (APC), CD69-phycoerythrin (PE), and CD178-PE (BD Biosciences, Cowley, UK) incubated 100µl of whole peripheral blood samples in the dark for 20 minutes; 2) incubated with 2ml fluorescently activated cell classifier (FACS) lysate for 5 mins. 3) after centrifuged at 300×g 5 minutes and aspirated the supernatant, 2ml phosphate buffered saline (PBS) was added and vortex lightly; 4) after centrifuged at 300×g 5 minutes and aspirated the supernatant, the sample was re-suspended with 200µl of PBS and finally analyzed by FACS flow cytometer. CD8+T lymphocytes were identified as monocytes which were in the subsequent showing CD3+/CD8+. CD3+CD8+CD69+ was considered to be the

activated CD8+T lymphocytes, CD3+CD8+CD178+ was considered to be the apoptosis-inducing CD8+T lymphocytes.

Plasma cytokines quantitation

The serum cytokines were quantitated by Luminex assay widespread using for serum cytokine measure. The plasma was separated from EDTA anticoagulant peripheral venous blood, and at -80°C in refrigerator for the cytokine test. Interferon α 2 (IFN- α 2), IFN- γ , fms-like tyrosine kinase 3 ligand (Flt-3L), and interleukin 17A (IL-17A) were measured. Each sample was measured duplicate, and the standard cytokine supplied by the manufacturer were run on each plate. The operation process was taken according to manufacturer's instructions. The cytokine levels were obtained by the data acquired by Luminex assay and analyzed using FlexMap 3D analyzer (Austin, TX, USA).

Statistical methods

In our study, SPSS 22.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Categorical variables were expressed as absolute values, and were compared using Fisher's exact test or Chi-square test between different groups. In this study, we express continuous variables in terms of mean $\pm$ standard deviation or median (Q1, Q3). Continuous variables were compared using analysis of variance and independent samples *t*-test or Kruskal-Wallis test between groups, according to whether they are normal distribution. We use linear regression analysis to analyze correlation of CD8+ T lymphocytes with clinical indexes (ALT level, HBsAg level, HBeAg content, and HBV DNA load). It was considered to be statistically significant if $P < 0.05$.

Results

Clinical features of the patients

112 patients (63 males, 49 females) participated in this research, including 30 patients in IT phase, 32 patients with AHB, and 50 patients with CHB, with median age of 30 (26, 39) years. Significant differences were showed in age (27[25.75, 31.25], 30[27.00, 36.25], 33[27.00, 49.75]y; $H=7.904$, $P=0.019$), ALT levels (29.85[21.83, 39.85], 252.70[129.45, 361.50], 930.10[447.25, 1902.48] U/L; $H=74.981$, $P=0.000$), total bilirubin (TBIL) levels (10.25[7.33, 14.43], 14.10[11.90, 18.15], 52.55[25.48, 111.02] µmol/L; $H=55.928$, $P=0.000$), albumin (ALB) (47.45[45.68, 49.30], 44.90[42.30, 47.00], 42.95[38.05, 44.35] g/L; $H=28.965$, $P=0.000$), HBsAg levels (4.79[4.59, 4.93], 3.89[3.46,

4.19], 2.80[2.18, 3.59] $\log_{10}$ IU/ml; $H=60.732$, $P=0.000$), HBeAg content (1606.36[1556.53, 1679.44], 933.34[467.38, 1316.67], 5.93[0.92, 59.31] S/CO; $H=77.956$, $P=0.000$), and HBV DNA (8.11[8.11 $\pm$ 0.48], 7.05[7.05 $\pm$ 1.23], 4.61[4.61 $\pm$ 1.40] $\log_{10}$ IU/ml; $H=61.988$, $P=0.000$) among the three groups (Table 1).

Frequency and functional molecular expression of CD8+ T lymphocytes

Frequency of CD8+ T lymphocytes in leukomonocytes was similar between the three groups. Frequency of CD69+CD8+ T lymphocytes in CHB and AHB groups were increased significantly compared with IT group (4.19[3.01, 6.18] % and 4.45[2.93, 6.71] % vs. 3.02[2.17, 3.44] %; $H=26.207$, $P=0.001$; $H=28.585$, $P=0.002$). The CD69 mean fluorescence intensity (MFI) in AHB group was significantly higher than that in IT and CHB groups (27.35[24.88, 32.25] vs. 20.45[19.05, 27.75] and 23.40[16.78, 28.13]; $H=25.832$, $P=0.005$ and $H=22.056$, $P=0.008$). Frequency of CD178+CD8+ T lymphocytes and the CD178MFI in CHB and AHB groups were

slightly higher than those in IT group (1.16[0.85, 1.50] % and 1.16 [0.90, 1.93] % vs. 1.03[0.83, 1.67] %; 22.10 $\pm$ 6.05 and 23.9 $\pm$ 25.27 vs. 21.00 $\pm$ 4.73), but no significant differences ($P>0.05$) were found (Figure 1 and Table 1).

Correlation of CD8+ T lymphocytes with clinical indexes and cytokines

In IT group, HBsAg and HBV DNA load had negative correlation with CD69MFI ($\beta=-0.025$, $t=-2.613$, $P=0.014$; $\beta=-0.021$, $t=-2.286$, $P=0.030$), and HBeAg was negatively correlated with frequency of CD69+CD8+ T lymphocytes ($\beta=-61.306$, $t=-2.116$, $P=0.043$). In CHB group, HBeAg was positively related to frequency of CD69+CD8+ T lymphocytes ($\beta=43.912$, $t=2.027$, $P=0.048$), and IFN- $\alpha 2$ was negatively related to frequency of CD8+ T lymphocytes ($\beta=-14.534$, $t=-2.085$, $P=0.043$). In AHB group, ALT was positively related to CD69MFI ($\beta=35.042$, $t=2.896$, $P=0.007$), HBsAg was negatively related to CD178MFI ($\beta=-0.137$, $t=-3.273$, $P=0.003$), and IFN- $\alpha 2$ was positively related to the frequency of CD8+ T lymphocytes ($\beta=6.798$, $t=2.629$, $P=0.016$) (Table 2).

TABLE 1 Comparison of clinical characteristics, frequency and functional molecular expression of CD8+ T cells between the three groups.

Values	IT group (n=30)	CHB group (n=50)	AHB group (n=32)	P	P1 (IT vs CHB)	P2 (IT vs AHB)	P3 (CHB vs AHB)
Sex (male/female)	14/16	28/22	21/11	$\chi^2=2.264$, $P=0.322$	$\chi^2=0.655$, $P=0.418$	$\chi^2=2.264$, $P=0.132$	$\chi^2=0.752$, $P=0.386$
Age (years) (median [Q1, Q3])	27.00 (25.75, 31.25)	30.00 (27.00, 36.25)	33.00 (27.00, 49.75)	$H=7.904$, $P=0.019$	$H=13.960$, $P=0.187$	$H=22.988$, $P=0.016$	$H=9.028$, $P=0.656$
ALT (U/L) (median [Q1, Q3])	29.85 (21.83, 39.85)	252.70 (129.45, 361.50)	930.10 (447.25, 1902.48)	$H=74.981$, $P=0.000$	$H=42.435$, $P=0.000$	$H=70.360$, $P=0.000$	$H=27.925$, $P=0.000$
TBIL (μ mol/L) (median [Q1, Q3])	10.25 (7.33, 14.43)	14.10 (11.90, 18.15)	52.55 (25.48, 111.02)	$H=55.928$, $P=0.000$	$H=19.529$, $P=0.027$	$H=59.358$, $P=0.000$	$H=39.829$, $P=0.000$
ALB (g/L) (median [Q1, Q3])	47.45 (45.68, 49.30)	44.90 (42.30, 47.00)	42.95 (38.05, 44.35)	$H=28.965$, $P=0.000$	$H=-17.692$, $P=0.047$	$H=-43.774$, $P=0.000$	$H=-26.082$, $P=0.001$
HBsAg ($\log_{10}$ IU/ml) (median [Q1, Q3])	4.79 (4.59, 4.93)	3.89 (3.46, 4.19)	2.80 (2.18, 3.59)	$H=60.732$, $P=0.000$	$H=-37.003$, $P=0.000$	$H=64.099$, $P=0.000$	$H=-27.096$, $P=0.001$
HBeAg (S/CO) (median [Q1, Q3])	1606.36 (1556.53, 1679.44)	933.34 (467.38, 1316.67)	5.93 (0.92, 59.31)	$H=77.956$, $P=0.000$	$H=-35.893$, $P=0.000$	$H=-72.833$, $P=0.000$	$H=-36.940$, $P=0.000$
HBV DNA ($\log_{10}$ IU/ml) (mean $\pm$ SD)	8.11 $\pm$ 0.48	7.05 $\pm$ 1.23	4.61 $\pm$ 1.40	$H=61.988$, $P=0.000$	$H=-25.993$, $P=0.001$	$H=-63.741$, $P=0.000$	$H=-37.748$, $P=0.000$
CD8+/LC (%) (mean $\pm$ SD)	25.05 $\pm$ 6.31	24.60 $\pm$ 7.24	27.11 $\pm$ 7.14	$F=1.333$, $P=0.268$	$t=0.287$, $P=0.775$	$t=-1.201$, $P=0.235$	$t=-1.544$, $P=0.127$
CD69+/CD8+ (%) (median [Q1, Q3])	3.02 (2.17, 3.44)	4.19 (3.01, 6.18)	4.45 (2.93, 6.71)	$H=15.439$, $P=0.000$	$H=26.207$, $P=0.001$	$H=28.585$, $P=0.002$	$H=2.379$, $P=1.000$
CD8+CD69MFI (median [Q1, Q3])	20.45 (19.05, 27.75)	23.40 (16.78, 28.13)	27.35 (24.88, 32.25)	$H=12.195$, $P=0.002$	$H=3.777$, $P=1.000$	$H=25.832$, $P=0.005$	$H=22.056$, $P=0.008$
CD178+/CD8+ (%) (median [Q1, Q3])	1.03 (0.83, 1.67)	1.16 (0.85, 1.50)	1.16 (0.90, 1.93)	$H=1.622$, $P=0.445$	$H=0.714$, $P=0.398$	$H=1.485$, $P=0.223$	$H=0.388$, $P=0.533$
CD8+CD178MFI (mean $\pm$ SD)	21.00 $\pm$ 4.73	22.10 $\pm$ 6.05	23.92 $\pm$ 5.27	$F=0.642$, $P=0.528$	$t=-0.846$, $P=0.400$	$t=-2.284$, $P=0.026$	$t=-1.395$, $P=0.167$

ALT, Alanine aminotransferase; TBIL, total bilirubin; ALB, albumin; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus desoxyribose nucleic acid; MFI, mean fluorescence intensity; IT, immune tolerance; CHB, chronic hepatitis B; AHB, acute hepatitis B.

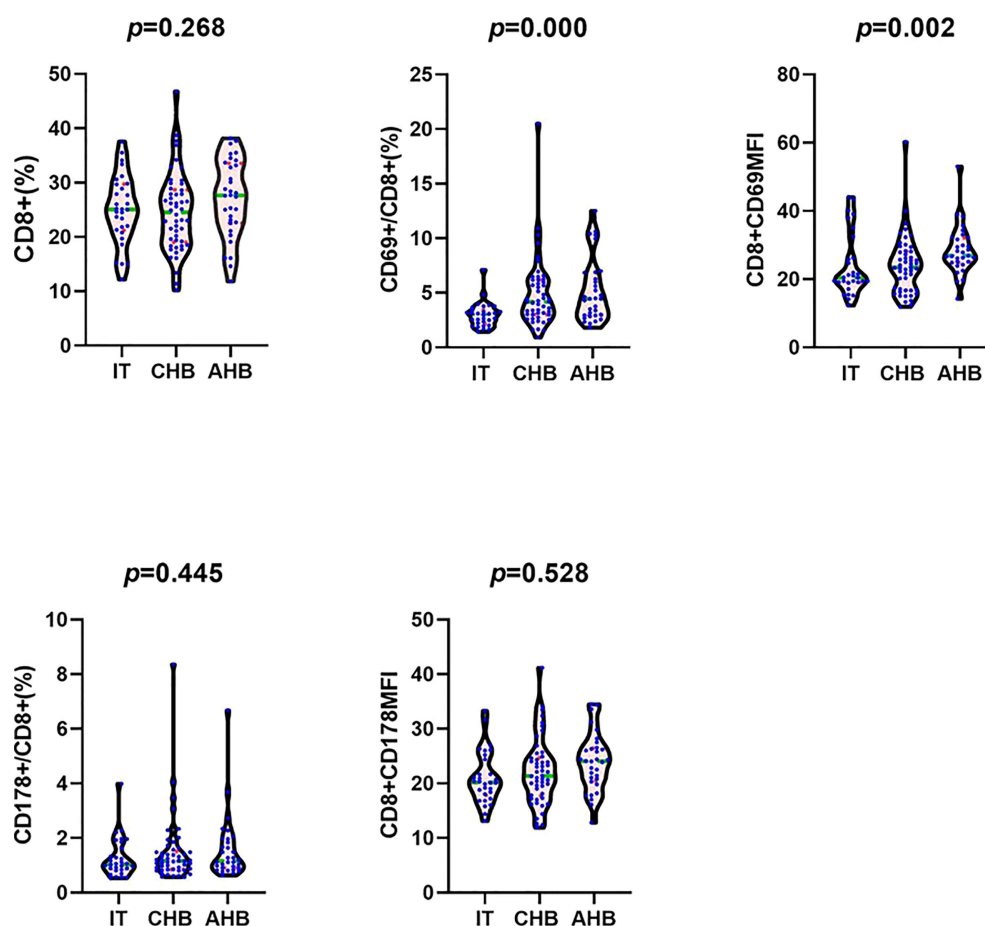


FIGURE 1

Comparison of the frequency of CD8+ T cells in leukomonocytes, the frequency of CD69+CD8+ T cells in CD8+ T cells, the CD8+CD69MFI, the frequency of CD178+CD8+ T cells in CD8+ T cells, and the CD8+CD178MFI between IT, CHB, and AHB groups. Note: MFI, mean fluorescence intensity; IT, immune tolerance; CHB, chronic hepatitis B; AHB, acute hepatitis B.

Correlation of cytokines with clinical parameters

HBV DNA was positively related to Flt-3L, IL-17A and IFN- γ ($\beta=0.001$, $t=2.254$, $P=0.034$; $\beta=0.002$, $t=2.269$, $P=0.033$; $\beta=0.001$, $t=2.205$, $P=0.038$) in IT group. There was no significant correlation between cytokines and HBV DNA, ALT, HBsAg, and HBeAg in CHB patients. In AHB patients, HBeAg was positively related to IL-17A, Flt-3L and IFN- γ ($\beta=7.057$, $t=7.763$, $P=0.000$; $\beta=1.695$, $t=7.123$, $P=0.000$; $\beta=2.949$, $t=11.940$, $P=0.000$), and HBV DNA was also positively related to Flt-3L, IL-17A and IFN- γ ($\beta=0.004$, $t=2.343$, $P=0.029$; $\beta=0.015$, $t=2.320$, $P=0.030$; $\beta=0.006$, $t=2.671$, $P=0.014$) (Table 3).

Discussion

As a non-cytotoxic hepatotropic virus, the clinical manifestation and outcome of HBV infection largely depend on host immunity. In general, a well-developed immune response is likely to induce severe liver injury and viral clearance, so that over 95% patients infecting HBV in adults can recover spontaneously. Conversely, an immature immune response leads to the persistence of HBV, just as the 90% patients infecting HBV in infants become chronic infection (6). HBV-induced immunity is coordinated by innate immunity, specific immunity and cytokines, in which the specific immunity plays an important role in virus clearance (7, 8). Cytotoxic lymphocytes (CTLs) are of crucial importance in HBV-specific immunity. There are rich of HBV-specific CD8+ T

TABLE 2 Correlation of CD8+ T cells with clinical indexes and cytokines.

	IT group					CHB group					AHB group				
	CD8+ (%)	CD69+ (%)	CD69MFI	CD178+ (%)	CD178MFI	CD8+ (%)	CD69+ (%)	CD69MFI	CD178+ (%)	CD178MFI	CD8+ (%)	CD69+ (%)	CD69MFI	CD178+ (%)	CD178MFI
ALT (U/L)	$t=1.227$, $P=0.230$	$t=-0.777$, $P=0.444$	$t=-0.620$, $P=0.540$	$t=-1.358$, $P=0.185$	$t=1.474$, $P=0.152$	$t=-0.668$, $P=0.508$	$t=-0.638$, $P=0.527$	$t=-0.161$, $P=0.873$	$t=0.094$, $P=0.925$	$t=-0.348$, $P=0.729$	$t=0.752$, $P=0.458$	$t=0.128$, $P=0.899$	$t=2.896$, $P=0.007$	$t=-0.471$, $P=0.641$	$t=1.178$, $P=0.248$
HBsAg (log ₁₀ IU/ ml)	$t=0.118$, $P=0.907$	$t=-1.378$, $P=0.179$	$t=-2.613$, $P=0.014$	$t=1.119$, $P=0.273$	$t=0.972$, $P=0.339$	$t=0.713$, $P=0.480$	$t=0.967$, $P=0.338$	$t=-0.345$, $P=0.731$	$t=0.459$, $P=0.648$	$t=-0.969$, $P=0.337$	$t=0.825$, $P=0.416$	$t=1.426$, $P=0.164$	$t=-0.209$, $P=0.836$	$t=-0.233$, $P=0.817$	$t=-3.273$, $P=0.003$
HBeAg (S/CO)	$t=1.659$, $P=0.108$	$t=-2.116$, $P=0.043$	$t=-1.494$, $P=0.146$	$t=-0.112$, $P=0.911$	$t=0.914$, $P=0.369$	$t=1.313$, $P=0.196$	$t=2.027$, $P=0.048$	$t=0.053$, $P=0.958$	$t=-0.021$, $P=0.983$	$t=-0.164$, $P=0.870$	$t=0.672$, $P=0.507$	$t=-0.261$, $P=0.796$	$t=-0.770$, $P=0.447$	$t=0.010$, $P=0.992$	$t=-1.618$, $P=0.116$
HBV DNA (log ₁₀ IU/ ml)	$t=-0.712$, $P=0.482$	$t=-0.770$, $P=0.448$	$t=-2.286$, $P=0.030$	$t=1.427$, $P=0.165$	$t=0.567$, $P=0.575$	$t=0.585$, $P=0.561$	$t=1.870$, $P=0.068$	$t=1.210$, $P=0.232$	$t=0.599$, $P=0.552$	$t=-0.561$, $P=0.578$	$t=0.190$, $P=0.851$	$t=-0.191$, $P=0.850$	$t=0.958$, $P=0.346$	$t=-0.519$, $P=0.607$	$t=-0.793$, $P=0.434$
Flt-3L (pg/ml)	$t=-0.202$, $P=0.842$	$t=-0.374$, $P=0.712$	$t=-1.029$, $P=0.315$	$t=1.891$, $P=0.072$	$t=0.050$, $P=0.961$	$t=-1.915$, $P=0.062$	$t=-1.028$, $P=0.310$	$t=-0.409$, $P=0.685$	$t=-1.108$, $P=0.274$	$t=-0.594$, $P=0.556$	$t=0.760$, $P=0.456$	$t=-1.500$, $P=0.149$	$t=-0.473$, $P=0.641$	$t=0.089$, $P=0.930$	$t=-0.960$, $P=0.348$
IFN- α 2 (pg/ml)	$t=-0.272$, $P=0.788$	$t=-0.900$, $P=0.378$	$t=-1.080$, $P=0.292$	$t=-0.513$, $P=0.613$	$t=-0.474$, $P=0.640$	$t=-2.085$, $P=0.043$	$t=-0.497$, $P=0.622$	$t=-0.870$, $P=0.389$	$t=-0.776$, $P=0.442$	$t=-0.844$, $P=0.403$	$t=2.629$, $P=0.016$	$t=0.485$, $P=0.633$	$t=1.517$, $P=0.144$	$t=2.025$, $P=0.056$	$t=0.439$, $P=0.665$
IFN- γ (pg/ml)	$t=-0.116$, $P=0.909$	$t=-0.165$, $P=0.870$	$t=-1.471$, $P=0.156$	$t=0.867$, $P=0.395$	$t=-0.994$, $P=0.331$	$t=-1.231$, $P=0.225$	$t=-0.533$, $P=0.597$	$t=-0.511$, $P=0.612$	$t=-0.819$, $P=0.417$	$t=-1.220$, $P=0.229$	$t=0.632$, $P=0.534$	$t=-0.945$, $P=0.355$	$t=-1.004$, $P=0.327$	$t=0.069$, $P=0.946$	$t=-1.574$, $P=0.131$
IL-17A (pg/ml)	$t=0.122$, $P=0.904$	$t=-0.359$, $P=0.723$	$t=-1.424$, $P=0.168$	$t=-0.904$, $P=0.376$	$t=-0.857$, $P=0.400$	$t=-1.230$, $P=0.226$	$t=-0.483$, $P=0.632$	$t=-0.486$, $P=0.630$	$t=-0.699$, $P=0.489$	$t=-1.463$, $P=0.151$	$t=1.440$, $P=0.165$	$t=-0.616$, $P=0.544$	$t=-0.923$, $P=0.367$	$t=-0.032$, $P=0.975$	$t=-1.521$, $P=0.143$

ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribose nucleic acid; Flt-3L, fms-like tyrosine kinase 3 ligand; IFN- α 2, interferon α 2; IFN- γ , interferon γ ; IL-17A, interleukin 17A; IT, immune tolerance; CHB, chronic hepatitis B; AHB, acute hepatitis B; MFI, mean fluorescence intensity.

TABLE 3 Correlation of cytokines with clinical indexes.

	IT group				CHB group				AHB group			
	Flt-3L (pg/ml)	IFN- α 2 (pg/ml)	IFN- γ (pg/ml)	IL-17A (pg/ml)	Flt-3L (pg/ml)	IFN- α 2 (pg/ml)	IFN- γ (pg/ml)	IL-17A (pg/ml)	Flt-3L (pg/ml)	IFN- α 2 (pg/ml)	IFN- γ (pg/ml)	IL-17A (pg/ml)
ALT (U/L)	$t=-0.487$, $P=0.631$	$t=-0.622$, $P=0.540$	$t=0.594$, $P=0.559$	$t=0.347$, $P=0.732$	$t=-0.648$, $P=0.521$	$t=0.255$, $P=0.800$	$t=-0.777$, $P=0.442$	$t=-1.004$, $P=0.321$	$t=0.561$, $P=0.581$	$t=-0.785$, $P=0.441$	$t=-0.010$, $P=0.992$	$t=0.738$, $P=0.469$
HBsAg (log ₁₀ IU/ml)	$t=1.561$, $P=0.133$	$t=0.716$, $P=0.482$	$t=1.088$, $P=0.288$	$t=1.301$, $P=0.207$	$t=-0.347$, $P=0.731$	$t=-1.022$, $P=0.312$	$t=-0.035$, $P=0.972$	$t=0.240$, $P=0.812$	$t=0.942$, $P=0.357$	$t=-0.063$, $P=0.950$	$t=1.855$, $P=0.078$	$t=1.522$, $P=0.143$
HBeAg (S/CO)	$t=0.907$, $P=0.374$	$t=1.200$, $P=0.243$	$t=0.969$, $P=0.343$	$t=1.294$, $P=0.209$	$t=1.328$, $P=0.191$	$t=1.068$, $P=0.292$	$t=1.723$, $P=0.092$	$t=1.726$, $P=0.092$	$t=7.123$, $P=0.000$	$t=0.718$, $P=0.480$	$t=11.940$, $P=0.000$	$t=7.763$, $P=0.000$
HBV DNA (log ₁₀ IU/ml)	$t=2.254$, $P=0.034$	$t=1.403$, $P=0.174$	$t=2.205$, $P=0.038$	$t=2.269$, $P=0.033$	$t=-0.206$, $P=0.838$	$t=-0.783$, $P=0.438$	$t=-0.064$, $P=0.950$	$t=0.109$, $P=0.914$	$t=2.343$, $P=0.029$	$t=-0.188$, $P=0.852$	$t=2.671$, $P=0.014$	$t=2.320$, $P=0.030$

ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus desoxyribose nucleic acid; IT, immune tolerance; CHB, chronic hepatitis B; AHB, acute hepatitis B. Flt-3L, fms-like tyrosine kinase 3 ligand; IFN- α 2, interferon α 2; IFN- γ , interferon γ ; IL-17A, interleukin 17A.

lymphocytes in our liver, which can clear HBV by cytosolic or non-cytolytic pathway (9–11). We investigated the function of CD8+ T lymphocytes and related cytokines in HBV infected patients of different states, to explore their role in clinical outcome and to better understand HBV-induced immune pathogenesis.

Specific CD8+ T lymphocytes need to be stimulated by cytokines such as IFN- α to clear HBV. When receiving the stimulation of viral antigen, they secrete cytokines and perforin to inhibit viral replication. And HBV-specific CD8+ T lymphocytes can lead apoptosis of HBV-infected hepatocytes by inducing expression of apoptotic molecules. In our study, we examined major cytokines with virus-clearing effects, expression of CD8+ T lymphocytes activating molecules and apoptosis-inducing molecules in patients with IT, CHB, and AHB patients.

CD69 is the marker of early activated lymphocytes, and it can be up-regulated when lymphocytes are stimulated by antigens. In our study, Patients with AHB and CHB had a significant higher frequency of CD69+CD8+ T lymphocytes compared with IT patients, which may suggest that the occurrence of hepatitis is linked to increased proportion of activated CD8+ T lymphocytes. In HBV infected patients, CD8+ T lymphocytes activated by IFN- α play a role in killing infected hepatocytes (12), and it has been widely used for immune control and clinical cure of CHB (13, 14). Our research also indicated why interferon therapy is more effective in CHB patients than IT patients, especially in HBV infected patients with severe hepatitis (15, 16). However, although being activated, CD69 molecular expressed on CD8+ T lymphocytes surface just increased slightly in CHB group compared with IT group, but increased significantly in AHB patients compared with IT and CHB patients as shown in our study. This may partly explain why most CHB patients can not eliminate the virus and recover from HBV infection like AHB patients. CD178 (Fas-L) is an apoptosis-inducing molecule on surface of CD8+ T lymphocytes, and can mediate apoptosis of infected liver cells by Fas/Fas-L pathway. In our study, frequency of CD178+CD8+ T lymphocytes and the CD178MFI in AHB

and CHB group were slightly higher than those in IT patients. These results indicated that, in addition to mediating the apoptosis of target cells, killing the infected cells directly by secreting cytotoxic molecules like granzyme and perforin, and secreting cytokines involving IFN- γ and TNF- α to inhibit spread and replication of virus are also important ways of CTLs to eliminate HBV (9–11).

It is known that HBV can impair the functions and maturation of DCs (17, 18), and DCs will show tolerance subtypes under long-term stimulation of high levels of HBV and its antigens in CHB (19, 20). In this research, HBV DNA and HBsAg were negatively correlated with CD69MFI, and there was negative correlation between HBeAg level and frequency of CD69+CD8+ T lymphocytes in IT group. These results showed that activation of CD8+ T lymphocytes was also suppressed by long-term high levels of HBV and its antigens. It is consistent with previous researches that the activation of HBV-specific immunity will be affected when innate immune is weakened (21, 22). As mentioned above, IFN- α can stimulate CD8+ T lymphocytes to activate immune response (12). In our study, IFN- α 2 was positively related to frequency of CD8+ T lymphocytes in AHB group, which could confirm this statement. Interestingly, we found that IFN- α 2 was negatively related to frequency of CD8+ T lymphocytes in CHB group. It may indicate why the HBV-specific CD8+ T lymphocytes exhaust during interferon therapy and lead to a decline in efficacy in CHB group (23, 24).

The outcome of HBV infection is determined by the status of HBV infection and the function of HBV-specific CD8+ T lymphocytes. 95% of adults with acute HBV infection can recover spontaneously, which is correlated with the function of CD8+ T lymphocytes (25). However, it is difficult to recover from chronic HBV infection due to impaired CD8+ T lymphocytes function (26, 27). The response of CD8+ T lymphocytes is also associated with liver inflammation in chronic HBV infection (28). The aim of this study was to investigate the function of CD8+ T lymphocytes in HBV

infection, including the expression of activation molecules (CD69) and apoptosis-inducing molecules (CD178) in the pathogenesis of chronic HBV infection (IT and HBeAg-positive CHB), and their role in hepatitis B (CHB and AHB). In our study, patients with CHB had a significant higher frequency of CD69+CD8+ T lymphocytes compared with IT patients, but frequency of CD8+ T lymphocytes, frequency of CD178+CD8+ T lymphocytes and CD178 molecular expressed on CD8+ T lymphocytes surface were all similar between the two groups. Meanwhile, in CHB patients, frequency of CD8+ T lymphocytes, CD69+CD8+ T lymphocytes, CD178+CD8+ T lymphocytes and expression of CD69, CD178 molecules on CD8+ T lymphocytes surface had no significant correlation with HBV DNA load. The results suggested that the activation of CD8+ T lymphocytes might be associated with the onset of hepatitis in chronic HBV infection, but it couldn't inhibit replication of virus or eliminate infected hepatocytes effectively. This may be related to the presence of high levels of viral antigens in chronic HBV infection, which can suppress the function of HBV-specific CD8+ T lymphocytes (29). And the detection of CD8+ T lymphocytes in peripheral blood may not fully reflect its distribution in liver histology too. We found that frequency of CD8+ T lymphocytes, CD69+CD8+ T lymphocytes and CD178+CD8+ T lymphocytes in AHB patients were also similar to CHB patients. In addition, in AHB patients, frequency of CD8+ T lymphocytes, CD69+CD8+ T lymphocytes, CD178+CD8+ T lymphocytes and expression of CD69, CD178 molecules on CD8+ T lymphocytes surface also had no significant correlation with HBV DNA load. Although the ALT in CHB and AHB groups significantly increased in our study, it might not mainly caused by CD8+ T lymphocytes. Previous studies have found that the hepatitis in chronic HBV infection is associated with the activation and phenotype of NK cells (30). And meta-analysis showed that there was no significant correlation between the function of CD8+ T lymphocytes and ALT level in chronic HBV infection (31). Some studies have found that the inflammatory phase of AHB is also associated with the activation of NK cells (32), and the activation of NK cells may negatively regulate HBV-specific CD8+ T cells (33). Therefore, the occurrence of hepatitis in HBV infection is not only related to the function of CD8+ T lymphocytes, but also related to the function of various immune cells such as NK cells.

Cytokines play a vital role in virus clearance. Cytokines can directly inhibit virus replication, leading necrosis of hepatocytes infected with virus, and stimulate or inhibit other immune cells to conduct immune regulation and form a certain immune environment. We also explored the relativity of cytokines and clinical indexes in this study. Flt-3L also plays an important role as an essential growth factor for NK and DCs (34). IFN- γ is an essential cytokine for CTLs to inhibit HBV replication and eliminate HBV in a non-cytolytic way (35). IL-17A is related to degree of inflammation as a pro-inflammatory cytokine (36). In our

study, HBeAg and HBV DNA were positively related to Flt-3L, IL-17A and IFN- γ in AHB patients, which showed the activation of inflammation by HBV. We found that HBV DNA was also positively related to Flt-3L, IL-17A and IFN- γ in IT patients. Although the immune response is deeply downregulated in IT patient, the HBV DNA may still activate mild inflammation.

Our study still had some limitations. First, we only enrolled HBeAg-positive patients in this study for the sake of investigate the correlation of CD8+ T lymphocytes and cytokines with HBV antigens. Second, we didn't pair baseline parameters among the three groups because it was difficult when patients were consecutively enrolled, which might lead to a bias. Third, the sample was small. Since patients were consecutively enrolled in this study and the sample size was relatively small, it was difficult to handle baseline parameters by propensity score matching. We will conduct large-sample studies with matched baseline parameters in the future to further avoid selection bias. Since occurrence and clearance of hepatitis caused by HBV involve various immune cells and cytokines, the outcome of CHB patients depends on the immune environment. Only some of the cytokines that contribute to CD8+ T lymphocytes function were investigated in this study.

Conclusion

In conclusion, the activation of CD8+ T lymphocytes was related to occurrence of AHB and CHB. Meanwhile, it is difficult to recover from HBV infection because of its insufficient expression of functional molecules of CD8+ T lymphocytes and depletion of CD8+ T lymphocytes.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board of Beijing Ditan Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

ML, SX, YaoL, RS, and YX contributed to design and concept. XB, LY, WD, TJ, YanL, SW, LZ, RL, MC, SW, YG, HH, GS, MX, XC, and LH performed the data collection. YX, SX, WD, TJ, YaoL, RS, and ML guided statistical analysis. SX wrote

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Effect of the change in antiviral therapy indication on identifying significant liver injury among chronic hepatitis B virus infections in the grey zone

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Objective: In clinical practice, a substantial proportion of chronic hepatitis B virus (HBV) infections that do not fit into any of the usual immune states are considered to be in the “grey zone (GZ)”. This study aimed to investigate the effect of the change in antiviral therapy indication on identifying significant hepatic injury among GZ patients.

Methods: Patients with chronic HBV infections and a persistent normal alanine aminotransferase (ALT) level (PNALT) who underwent ultrasonography-guided percutaneous liver biopsy were examined retrospectively. Evidenced hepatic injury (EHI) was defined as an inflammation grade ≥ 2 ($\geq G2$) and/or fibrosis stage ≥ 2 ($\geq F2$). Complete clinical data, liver inflammation, and fibrosis grades were collected, and the levels of cytokines were detected by the Luminex technique, all of which were analysed to investigate the immune and histopathology states of the liver.

Results: A total of 347 patients with chronic HBV infections and PNALT were categorized into immune tolerant (IT, $n = 108$), inactive HBV surface antigen (HBsAg) carrier (IHC, $n = 61$), GZ-1 (HBsAg positive in GZ, $n = 92$), and GZ-2 (HBsAg negative in GZ, $n = 68$) phases. Among them, 51.3% were in the GZ phase, and 50.1% presented with EHI. The IL-6 levels were higher in the EHI group than in the non-EHI group (2.77 vs. 1.53 pg/ml, $Z = -13.32$, $p = 0.028$). The monocyte chemoattractant protein 1 (MCP-1) level was positively correlated with HBV DNA ($R = 0.64$, $p < 0.001$) and HBsAg ($R = 0.5$, $p < 0.001$) but negatively correlated with fibrosis grade ($R = -0.26$, $p = 0.048$). The ratio of EHI in the GZ phase was 60.55%, which was significantly higher than that in patients in the IT (39.8%) and IHC phases (37.7%) ($\chi^2 = 10.4$, $p = 0.006$). A total of 46.69% of all patients exceeded the new ALT antiviral treatment threshold (30 U/L for men and 19 U/L for women). The EHI values in the IT and IHC phases below the new ALT threshold were 32.6% and 37.8%, respectively, whereas higher EHI values of 67.4% and 68.4% were seen in GZ-1 and GZ-2 patients, respectively, exceeding the new ALT threshold, and

the difference was statistically significant ($\chi^2 = 11.13$, $p < 0.001$; $\chi^2 = 14.22$, $p = 0.002$). The median age in our cohort was 38.91 years, and only 21.03% were less than 30 years old. The EHI values in the IT and IHC patients <30 years old were 32.4% and 35.8%, respectively, while the ratio of EHI increased to 43.2% once patients were older than 30 years but still in the IT and IHC stages.

Conclusion: Setting 30 years old as a cut-off and lowering the ALT threshold could facilitate screening for the presence of significant liver injury, especially for GZ patients. IL-6 was a good indicator of EHI, and MCP-1 was significantly positively correlated with HBV DNA but negatively correlated with liver fibrosis.

Clinical Trial Registration: <https://www.chictr.org.cn/com/25/edit.aspx?pid=655567&htm=4>, identifier (ChiCTR2000040971).

KEYWORDS

chronic hepatitis B (CHB), grey zone (GZ), liver biopsy, significant liver injury, antiviral therapy, alanine aminotransferase (ALT), indication

Introduction

Chronic hepatitis B virus (HBV) infection is a public health problem, with approximately 257 million cases worldwide (1) and 70 million chronic HBV infections in China (2). Chronic HBV infection is a dynamic process that reflects the interaction between HBV replication and the host immune response. Investigators from Taiwan and Hong Kong, China, have introduced the concept of host immunity, and after several revisions of the paradigm, the natural history of chronic HBV infection is currently stratified into four phases: immune tolerance (IT, HBeAg positive with a high HBV DNA load), immune clearance (IC), inactive HBsAg carrier (IHC; HBeAg negative with HBV DNA <2,000 IU/ml), and reactivation (3–5); this has been widely adopted by guidelines (6–9). Antiviral therapy is recommended for patients within the IC and reactive phases due to elevated alanine aminotransferase (ALT), while IT and IHC patients with normal ALT levels could skip antivirals for the time being, as their inflammation/fibrosis in the liver tissue is not significant.

However, in clinical practice, some patients who are not categorized into any of the above four phases are considered to be in the “indeterminate phase” (10) or “grey zone (GZ)”. Especially in cases of normal ALT, HBeAg-positive patients may have a mild HBV DNA load, and HBeAg-negative patients can have HBV DNA $\geq 2,000$ IU/ml. Therefore, the threshold of ALT and the level of HBV DNA are critical for distinguishing chronic infection from chronic hepatitis. Recently, “The Chronic Hepatitis B Treatment Algorithm” (11) in the United States recommended that regardless of HBeAg positivity or negativity, any case with elevated ALT (>30 U/L for men and >19 U/L for women) and HBV DNA $\geq 2,000$ IU/ml should receive antiviral therapy.

Subsequently, “The Expert Opinion on Expanding Antiviral Therapy for Chronic Hepatitis B” (12) in China adopted the same ALT threshold but lowered the indication further: once positive for HBV DNA [lower limit of detection (LLV) = 10–20 IU/ml] with elevated ALT, antiviral therapy was recommended.

Increasing evidence (13) of a benefit from antiviral therapy for patients outside the current criteria, as well as increasing accessibility and affordability of highly effective, low-drug-resistant drugs, makes it feasible to expand the indications for antiviral therapy. However, the effect of the change in antiviral therapy indication on identifying significant liver injury in the GZ phase remains uncertain. Therefore, we aimed to establish a retrospective cohort of chronic HBV infections with normal ALT levels who underwent ultrasonography-guided percutaneous liver biopsy. Complete clinical data, liver inflammation, and fibrosis grades were collected to investigate the effect of an expansion in indications of antiviral therapy on identifying significant liver injury among GZ patients and to select appropriate antiviral therapy (ART) strategies to maximize the benefits of treatment for the patients.

Methods

Study population

A total of 780 patients with chronic HBV infections who underwent liver biopsy at Beijing You'an Hospital, Capital Medical University (Beijing, China), from January 2018 to September 2021 were enrolled in this retrospective study. Chronic HBV infection was defined as the persistent presence

of serum HBsAg for >6 months (6). Persistent normal ALT level (PNALT) was defined as normal ALT (upper limit of normal (ULN): ≤ 40 U/L) measured on at least two to three occasions at intervals of 3 months over 12 months. Patients with the following conditions were excluded: alcohol consumption >20 g/day (47 patients); accompanied by non-alcoholic fatty liver disease (99 patients); accompanied by autoimmune liver disease (27 patients); coinfection with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus (48 patients); prior or current antiviral treatment (52 patients); ALT > 1 $\times$ ULN (148 patients); and the interpretations of a biopsy sample by two pathologists were not consistent (12 patients). Finally, 347 patients with chronic HBV infection and PNALT levels were included in this study (Figure 1).

The study protocol was approved by the Beijing You'an Hospital, Capital Medical ethics committee and was conducted in accordance with the 1975 Declaration of Helsinki and revised in 1983 (The Code of Ethics Approval (2020):089). The study had registered at clinicaltrials.gov (ID: ChiCTR2000040971, <http://www.chictr.org.cn/com/25/edit.aspx?pid=65556&htm=4>)

Liver histological examination

Ultrasonography-guided percutaneous liver biopsy was performed using a 16-G disposable needle (Hepafix, B. Braun, Melsungen, Germany) under local anaesthesia. Liver tissue

samples of minimum length (15 mm) or larger were immediately fixed with 10% formalin and embedded in paraffin. Liver tissue with at least six portal tracts was sufficient for histological scoring. The METAVIR scoring system was adopted as the diagnostic standard for liver inflammation and fibrosis according to the recommended Chinese guidelines on HBV prevention and treatment (2019 version) (6). The histological grading of liver inflammation was classified into G0–G4, and fibrosis was staged at F0–F4. Evidence of hepatic injury (EHI) was defined as an inflammation grade ≥ 2 ($\geq G2$) and/or fibrosis stage ≥ 2 ($\geq F2$). All of the biopsy samples were interpreted independently by at least two liver pathologists. If the interpretations of a biopsy sample by two pathologists were not consistent, we discarded the data (Figure 1).

Laboratory tests

Fasting blood samples were obtained, and routine laboratory tests were performed at the same time as the liver biopsies. The serological markers of HBV were detected using commercially available enzyme-linked immunosorbent assay (ELISA) kits (ARCHITECT i2000 SR; Abbott, Wiesbaden, Germany). The serum biochemical parameters, including ALT, aspartate transaminase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), total bilirubin, albumin, and globulin, were measured on an automated biochemistry analyser

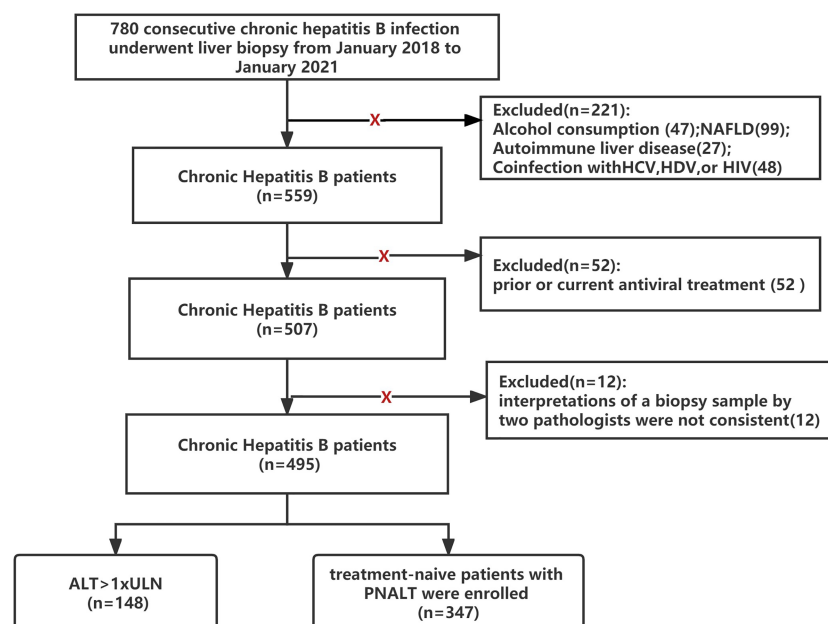


FIGURE 1

Flowchart of the study design (NAFLD, nonalcoholic fatty liver disease; HCV, hepatitis C virus; HDV, hepatitis D virus; HIV, human immunodeficiency virus; PNALT, persistent normal ALT; ULN, <40 U/L).

(7600 Series; Hitachi, Tokyo, Japan). Routine blood test results, including white blood cell (WBC) count and platelet count, were evaluated on an automated haematology analyser (XT-2000i; Sysmex, Kobe, Japan). HBV DNA was quantified on a real-time polymerase chain reaction (PCR) system (ABI 7500; Applied Biosystems, Foster City, CA, USA) with the lowest detection limit at 20 IU/ml.

Cytokines detected with Luminex kits

Each serum sample was analysed for cytokines, including monocyte chemoattractant protein 1 (MCP-1), soluble CD40 L (sCD40 L), fibroblast growth factor 2 (FGF2), interferon α (IFN- α), interferon gamma (IFN- γ), interleukin-1 (IL-1), IL-2, IL-6, IL-8, IL-10, IL-17, IL-21, tumour necrosis factor- α (TNF- α), interferon- γ -inducible protein 10 (IP-10), and vascular endothelial growth factor- α (VEGF- α) by Luminex bead-based MILLIPLEX[®] assays using the Human Cytokine Panel A Magnetic Bead Panel kit (Millipore, Billerica, MA, USA) with the FlexMAP3D (Luminex) platform, and the cytokine level data were analysed using xPONENT[®] software following the manufacturer's instructions (14).

Statistical analysis

The normality of all data was assessed using the Kolmogorov–Smirnov test. The baseline characteristics of the enrolled patients are as follows: normally distributed data are presented as the mean $\pm$ standard deviation, non-normally distributed continuous data are given as the median [interquartile range (IQR)], and categorical variables are given as the number (percentage). Chi-square tests (for categorical variables), Mann–Whitney tests (for non-normally distributed continuous variables), and t-tests (for normally distributed variables) were conducted to identify the significant differences between groups. Univariate and multiple logistic regression analyses were performed to identify the independent predictors of EHI. All significance tests were two-tailed, and $p < 0.05$ was considered statistically significant. All statistical analyses were carried out using SPSS statistical software version 23.0 (SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics of the enrolled patients

The baseline characteristics of 347 chronic HBV infections with PNAIT are summarized in Table 1. The majority of the

enrolled patients were men (210, 60.5%), HBeAg-positive (200, 57.64%), and middle-aged [38.91 (16–66) years], and 51.3% were in the GZ phase. The distribution of their different immune statuses was as follows based on the HBV Guidelines in China (2019 version) (8): IT (HBeAg-positive, HBV DNA $> 2 \times 10^7$ IU/ml), 108 (31.12%) patients; GZ-1 (HBeAg-positive with HBV DNA $\leq 2 \times 10^7$ IU/ml), 92 (26.52%) patients; GZ-2 (HBeAg-negative, HBV DNA $> 2,000$ IU/ml), 86 (24.78%) patients; and IHC (HBeAg-negative, HBV DNA $\leq 2,000$ IU/ml), 61 (17.58%) patients. The patients with HBeAg-negative HBV infections were older Figure 3C, especially the GZ-2 patients (median, 43.5 vs. 34.5–40 years, $p < 0.001$). A significantly higher ALT level was observed in HBeAg-positive patients than in HBeAg-negative patients (28.36 ± 9.49 vs. 23.59 ± 9.62 U/L, $p < 0.001$).

The histopathology data of the liver biopsy samples are shown in Table 1 and Figure 2. A total of 32.9% of patients had G2–4 disease. Among these patients, GZ-1 patients had a significantly higher rate of G2–4 than IT patients (46.7% vs. 30.6%, $\chi^2 = 5.52$, $p = 0.019$), while GZ-2 patients had a significantly higher rate of G2–4 than IHC patients (31.4% vs. 18%, $\chi^2 = 3.32$, $p = 0.049$). In the case of liver fibrosis, GZ-1 patients had a significantly higher rate of F2–4 than IT patients (50% vs. 23.1%, $\chi^2 = 15.64$, $p < 0.001$), while GZ-2 patients had a significantly higher rate of F2–4 than IHC patients (46.5% vs. 27.9%, $\chi^2 = 5.23$, $p = 0.049$). Among all patients, 50.1% had an EHI $\geq$ G2 and/or $\geq$ F2. Typically, GZ patients had a higher rate of EHI, including GZ-1 (GZ-1: 63% vs. IT: 39.8%, $\chi^2 = 10.72$, $p = 0.001$) and GZ-2 (GZ-2: 58.1% vs. IHC: 37.7%, $\chi^2 = 5.96$, $p = 0.015$). Univariate analysis indicated that ALT, AST, HBV DNA, HBeAg, HBsAg, and FibroScan were associated with EHI. However, multiple logistic regression analysis indicated that HBsAg and FibroScan were associated with EHI in HBeAg-positive patients and that AST, HBsAg, and FibroScan were associated with EHI in HBeAg-negative patients (Supplementary Table 1).

In the HBeAg-positive cohort ($n = 200$), the overall proportion with EHI was 50.5% (101/200). Based on their different levels of HBV DNA load, the subjects were stratified into four groups, namely, > 7 log (54%, 108/200), 6–7 log (15%, 30/200), 4–6 log (21%, 42/200), and < 4 log (10%, 20/200), and liver biopsies with EHI in the different groups are depicted in Figure 2D: 39.8% of patients with > 7 log DNA load had EHI (43/108), and in order of their HBV DNA load decreasing, 60% (18/30), 61.9% (26/42) and 70% (14/20) of the subjects had EHI, respectively ($\chi^2 = 11.2$, $p = 0.01$). For the HBeAg-negative cohort ($n = 147$), the overall ratio of EHI was 49.7% (73/147). Based on the different levels of HBV DNA load, the subjects were stratified into three groups, namely, $< 2,000$ (41.5%, 61/147), 2,000–20,000 (28.6%, 42/147), and $> 20,000$ (29.9%, 44/147). The EHI rate in IHC (HBV DNA $< 2,000$ IU/ml) was significantly lower (39.8%) than that in 2,000–20,000 (50%) and $> 20,000$ (65.91%) patients ($\chi^2 = 10.4$, $p = 0.02$).

TABLE 1 Baseline characteristics of enrolled patients.

Clinical characteristics	Total (n = 347)	HBeAg-positive patients (n = 200, 57.64%)		HBeAg-negative patients (n = 147, 42.36%)		Value
		IT (n = 108)	GZ-1 (n = 92)	GZ-2 (n = 86)	IHC (n = 61)	
Age (years)	38.91 (16–66)	34.5 (19–54)	37.63 (16–61)	43.5 (23–66)	40 (24–61)	Z = 39.78, p < 0.001
Male, n (%)	210 (60.5%)	63 (58.3%)	65 (70.65%)	43 (50%)	39 (63.9%)	$\chi^2 = 8.45$, p = 0.038
BMI (kg/m ²)	22.82 ± 4.23	21.42 ± 4.23	23.1 ± 3.92	22.32 ± 5.12	20.11 ± 4.12	F = 0.28, p = 0.34
ALT (U/L)	26.34 ± 9.82	28.64 ± 9.38	28.03 ± 9.66	24.68 ± 9.90	22.07 ± 9.07	F = 8.02, p < 0.001
AST (U/L)	23.21 ± 11.24	22.10 ± 10.12	26.82 ± 15.46	23.14 ± 8.81	22.07 ± 9.07	F = 8.02, p < 0.001
PLT (10 ⁹ /L)	206 ± 78.82	248 ± 96.00	218 ± 86.00	185 ± 62.00	209 ± 89.00	F = 0.83, p = 0.09
ALB (g/L)	43.0 ± 4.20	45.0 ± 5.17	43.0 ± 3.65	44.0 ± 3.78	41.32 ± 5.16	F = 0.78, p = 0.64
ALP (U/L)	70.0 ± 29.0	72.0 ± 28.0	73.2 ± 23.4	76.0 ± 21.8	71.0 ± 28.0	F = 0.66, p = 0.61
GGT (U/L)	22.0 ± 27.0	20.0 ± 13.0	24.0 ± 18.0	20.9 ± 14.8	23.4 ± 17.0	F = 0.59, p = 0.56
Genotype C (n, %)	266 (76.1%)	82 (75.92%)	69 (75%)	34 (77.27%)	84 (81.53%)	$\chi^2 = 3.46$, p = 0.09
HBV DNA (log ₁₀ IU/ml)	5.40 ± 2.25	8.14 ± 0.60	4.99 ± 1.47	4.40 ± 1.03	2.57 ± 0.56	F = 655.4, p < 0.001
HBeAg (log ₁₀ IU/ml)	3.55 ± 0.94	4.07 ± 0.77	3.58 ± 0.74	3.23 ± 0.65	2.86 ± 1.22	F = 22.78, p < 0.001
HBeAg (COI/ml)	565.49 ± 826	1,386.68 ± 703.65	506.97 ± 834.9	0.13 ± 0.15	0.11 ± 0.04	F = 92.51, p < 0.001
FibroScan:LSM (kPa)	6.14 ± 4.02	6.10 ± 6.35	6.42 ± 2.72	6.46 ± 2.38	5.31 ± 1.04	F = 1.21, p = 0.31
Liver histopathology						
Inflammation (G0–1 vs. G2–4)	233 vs. 114 (67.1 vs. 32.9)	75 vs. 33 (69.4 vs. 30.6)	49 vs. 43 (53.3 vs. 46.7)	59 vs. 27 (68.6 vs. 31.4)	50 vs. 11 (82 vs. 18)	$\chi^2 = 14.45$, p = 0.002
Fibrosis (F0–1 vs. F2–4)	219 vs. 128 (63.1 vs. 36.9)	83 vs. 25 (76.9 vs. 23.1)	46 vs. 46 (50 vs. 50)	46 vs. 40 (53.5 vs. 46.5)	44 vs. 17 (72.1 vs. 27.9)	$\chi^2 = 21$, p < 0.001
EHI vs. non-EHI	174 vs. 173 (50.1 vs. 49.9)	43 vs. 65 (39.8 vs. 60.2)	58 vs. 34 (63 vs. 37)	50 vs. 36 (58.1 vs. 41.9)	23 vs. 38 (37.7 vs. 62.3)	$\chi^2 = 16.7$, p = 0.001

BMI, body mass index; PLT, platelet count; ALB, albumin; ALP, alkaline phosphatase; GGT, g-glutamyl transferase; evidenced hepatic injury (EHI) was defined as inflammation grade ≥ 2 ($\geq G2$) and/or fibrosis stage ≥ 2 ($\geq F2$).

Effect of the change in antiviral therapy indication on identifying evidenced hepatic injury

New alanine aminotransferase treatment thresholds for antiviral therapy

Based on the new recommendations for the ALT antiviral treatment threshold (30 U/L for men and 19 U/L for women) (7), we further evaluated the ratio of EHI in different groups. Among the 347 chronic HBV infections, nearly half of them (162/347, 46.69%) were above this new threshold **Figure 3A**. Among these 162 patients, 56.2% (91/162) had EHI, which was significantly higher than that among the other patients (44.9%, 83/185), and was less than the new threshold ($\chi^2 = 4.42$, p = 0.036). In total, the proportion of patients with ALT ≤ 40 U/L who required antiviral therapy was 70.61% [(162 + 83)/347] according to the “Expert Opinion on Expanding Anti-HBV treatment” (7) in China.

In the HBeAg-positive cohort, 57.4% (62/108) of IT and 50% (46/92) of GZ-1 patients were above this new ALT threshold, and the EHI ratios were 45.2% and 67.4%, respectively **Figure 3B**. Among the patients below the ALT threshold, the ratio of EHI in GZ-1 was significantly higher than that in the IT group (58.7% vs. 32.6%, $\chi^2 = 6.72$, p = 0.01). However, there was no significant difference in the proportion of EHI between GZ-1 patients above the new ALT threshold and below it (67.4% vs. 58.7%, $\chi^2 = 1.12$, p = 0.34), and both were relatively high. It is worth noting that the ratio of EHI in IT patients with $\leq$ “normal” ALT levels was significantly lower than that among GZ-1 patients >ALT threshold (32.6% vs. 67.4%, $\chi^2 = 11.13$, p < 0.001). The former was in the “truly” IT phase because of a high HBV DNA load ($>2 \times 10^7$ IU/ml) and quite low ALT levels (≤ 30 U/L for men and 19 U/L for women).

More significant discrepancies were seen between IHC and GZ-2 in the HBeAg-negative cohort. The vast majority (73.8%, 45/61) of IHC patients were within the new ALT threshold, and

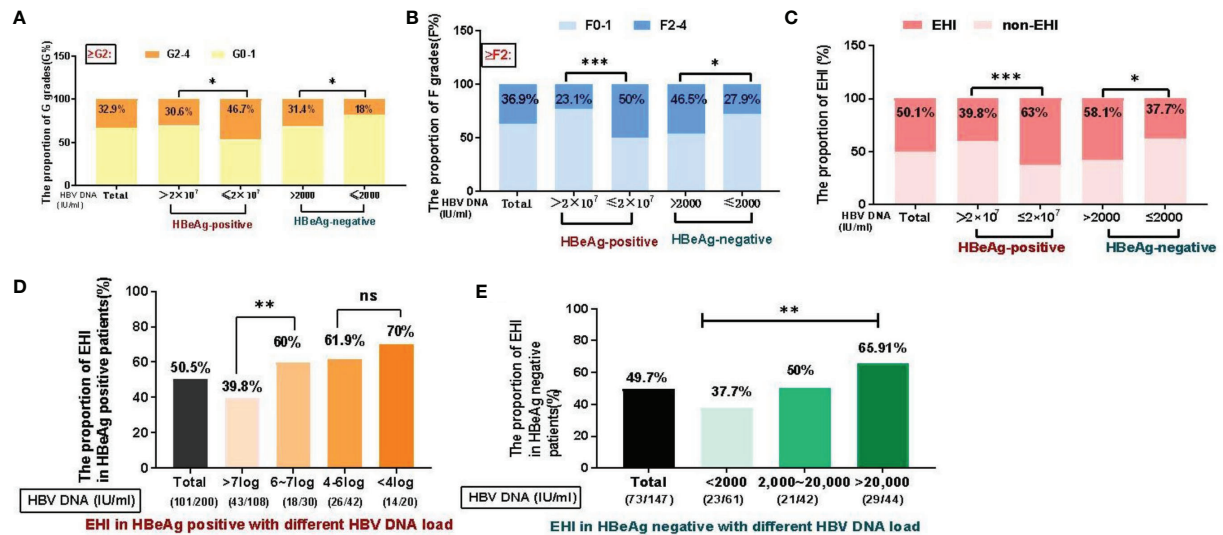


FIGURE 2

Distribution of liver inflammation and fibrosis in different immune status groups (%). (A) Significant differences in liver inflammation were observed among the groups. (B) Significant differences in liver fibrosis were observed among the groups. (C) EHI in different immune phases. (D) In the HBeAg-positive patient cohort, the overall proportion of EHI was 50.5% (101/200), 39.8% of patients with >7 log DNA load had EHI (43/108), followed by a decreased HBV DNA load, and 60–70% of subjects (18/30, 26/42, 14/20) had EHI ($X^2=11.2$, $P=0.01$). (E) For HBeAg-negative patients, the overall proportion of EHI was 49.7% (73/147). The proportion of EHI in IHC (HBV DNA<2000 IU/ml) was significantly lower (37.7%) than that in patients with a higher HBV DNA load (50%–77.3%, $X^2=10.4$, $P=0.02$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

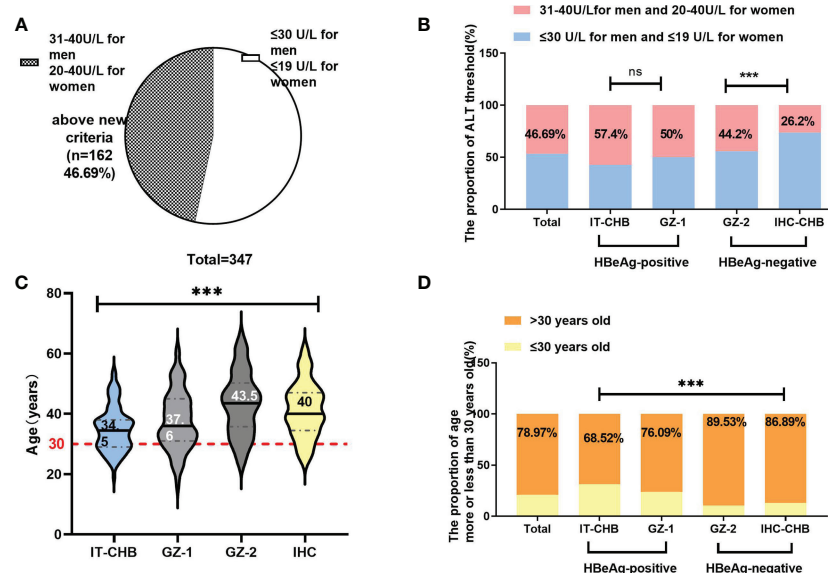


FIGURE 3

The change in antiviral therapy indication by ALT and age. (A) Among patients with ALT<40 U/L, only 53.3% were under the new threshold of ALT (30 U/L for men and 19 U/L for women). (B) Comparison of the proportion of patients exceeding the ALT threshold in different groups. (C) Comparison of the age in different groups (median); (D) Overall, 78.97% (274/347) of patients were over 30 years old. Comparison of the proportion of patients older than 30 years in different groups. *** $p < 0.001$; ns, not significant.

the ratio of EHI was comparable regardless of whether they exceeded or were under the new ALT threshold (37.5% vs. 37.8%, $\chi^2 = 0.61$, $p = 0.87$). In contrast, GZ-2 patients had a significantly higher EHI of 68.7% because of HBV DNA >2,000 IU/ml and ALT >new threshold ($\chi^2 = 14.22$, $p = 0.002$).

Age is a critical factor in identifying evidenced hepatic injury

The median age in our cohort was 38.91 years, and 78.97% (274/347) of them were >30 years old. Among them, almost 90% of HBeAg-negative patients, including 89.53% of GZ-2 and 86.89% of IHC, were >30 years old (Figure 3D), and the ratio was significantly higher than that in HBeAg-positive patients (IT: 68.52%, GZ-1: 76.09%, $\chi^2 = 15.64$, $p = 0.001$). In total, the ratio of EHI in patients >30 years old was 51.5% (Figure 4B), and there was no significant difference in the ratio of EHI among GZ patients regardless of whether they were older or younger than 30 (55.6%–64.3%, $\chi^2 = 1.34$, $p = 0.29$). The ratio of EHI in the IT group was 32.4% for those <30 years old, while it increased to 43.2% for those >30 years old even though they were still in the IT stage ($\chi^2 = 4.65$, $p = 0.04$). A similar difference was observed in the IHC group, and the ratio of EHI was 35.8% in IHC patients <30 years old and increased to 50% when they were >30 years old ($\chi^2 = 5.72$, $p = 0.03$).

Correlation of cytokines with clinical characteristics and evidenced hepatic injury

Plasma cytokines, including sCD40 L, FGF-2, IFN- α , IL-1, IL-2, IL-6, IL-8, IL-10, IL-18, IL-21, IP-10, MCP-1, VEGF- α , TNF- α , and IFN- γ , were measured in 168 out of HBeAg-positive patients with HBV DNA >10⁴ IU/ml and 20 healthy controls to evaluate their clinical characteristics and EHI upon liver biopsy (Figure 4A). In immune-tolerant patients, when cytokine levels were included as independent variables, HBV DNA (log10) levels were positively correlated with MCP-1 and IL-8 ($R =$

0.64, $p < 0.001$; $R = 0.48$, $p < 0.001$), HBeAg levels were positively correlated with MCP-1 and IL-8 ($R = 0.5$, $p < 0.001$; $R = 0.45$, $p < 0.001$), and MCP-1 levels were negatively correlated with the histological grading of fibrosis ($R = -0.26$, $p = 0.048$) (Figure 5A).

The median levels of sCD40 L, FGF-2, IFN- α 2, IL-1 β , IL-8, IL-21, TNF- α , and VEGF- α in healthy donors were significantly higher than those in patients with chronic HBV infections and normal ALT levels. However, there was no significant difference between EHI and non-EHI (Supplementary Table 2). Only the IL-6 level was significantly higher in the EHI group than in the non-EHI group (2.77 vs. 1.53, $Z = -13.32$, $p = 0.028$) (Figure 5B). As all IT phase patients had a normal ALT level, the potential correlation between histological grading of fibrosis, which was staged from F0 to F4, and immune cytokines was analysed. We found that the MCP-1 level was significantly higher in patients with fibrosis stage ≥ 2 ($\geq F2$) than in patients with F0–1 (170.89 ± 64.78 vs. 135.86 ± 36.05 , $F = 11.38$, $p = 0.04$), and both were significantly higher than those in healthy donors (Supplementary Table 2 and Figure 5C). Except for MCP-1, the IFN- γ and IL-2 levels in the EHI group increased markedly (8.65 vs. 3.64 pg/ml, $Z = 7.89$, $p = 0.011$; 2.33 vs. 0.55 pg/ml, $Z = 27.25$, $p = 0.001$) (Supplementary Table 3, Figures 5D, E).

Discussion

This study was based on a retrospective cohort of patients with chronic HBV infections and normal ALT levels (≤ 40 U/L) who underwent ultrasonography-guided percutaneous liver biopsy at Beijing You'an Hospital, Capital Medical University. Among these patients, 78.97% were over 30 years of age, 60.5% were men, and the overall proportion with EHI ($\geq G2$ and/or $\geq F2$) was 50.1%. The proportion of chronic HBV infections in the GZ phase was 51.3%, and the EHI ratio in the GZ phase was

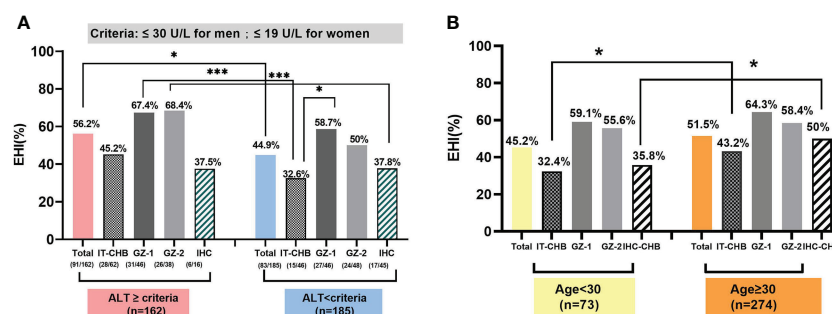


FIGURE 4

The effect of the change in antiviral therapy indication for age and ALT threshold in identifying EHI among those with chronic HBV infections. (A) Based on the new ALT threshold, IT and IHC patients presented a low EHI. (B) The proportion of EHI in patients more than 30 years old was 51.5%. Comparison of the proportion of EHI in each group, based on 30 years as the cut-off value. * $p < 0.05$, *** $p < 0.001$.

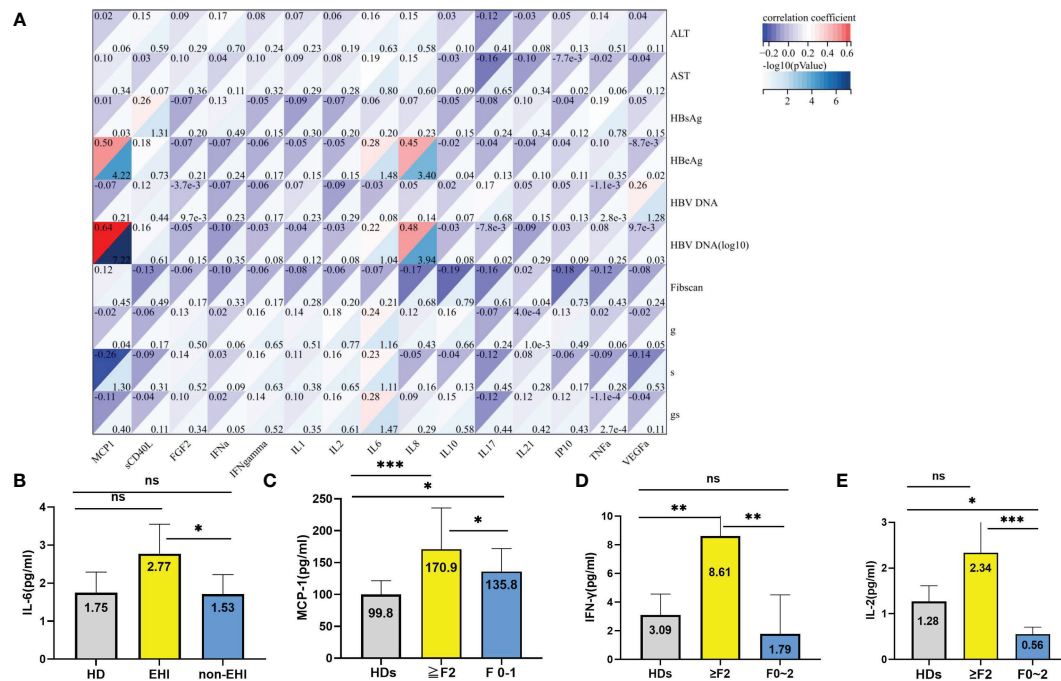


FIGURE 5

Relationship of the plasma cytokines with EHI. (A) Correlation of the cytokines with clinical characteristics and significant liver injury. (B) Comparison of the IL-6 levels in different groups. (C) Comparison of the MCP-1 levels in different groups. (D) Comparison of the IFN-γ levels in different groups; (E) Comparison of the IL-2 levels in different groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

60.55% (63% in GZ-1 and 58.1% in GZ-2), which was significantly higher than that in the IT (39.8%) and IHC (37.7%) stages. This finding was consistent with the results of previous studies that pointed out the high proportion of patients in the GZ phase among 179 patients in the IT phase and 327 patients in the IHC phase (15). The EHI ratio of GZ accounted for 57.5%–81.8% and 51.6%–

chronic HBV infections have already been established during the middle youth stage and have been included in the considerations for antiviral therapy.

In our study, IT phase patients were the youngest at 34.5 years, followed by GZ-1 patients at 37.63 years, while HBeAg-negative patients were significantly older at 40 and 43.5 years for IHC and GZ-2, respectively. Setting 30 years old as a threshold is critical for IT and IHC patients, but not for the GZ phase: EHI in GZ patients was the highest (55%–65%), irrespective of age, while EHI in IT and IHC increased significantly from 32.4% to 43.2% and 35.8% to 50% if the age threshold was >30 years, respectively. This is also consistent with the current guidelines that >30 years old is an independent risk factor for disease progression and can be an indication for initiating antiviral therapy. In a large US cohort study of 8,539 patients with chronic HBV infection followed up for 12 years, 317 developed HCC, age and family history were independent risk factors for HCC in patients without cirrhosis, and the OR was 32.9 (21). A study based on the Chinese Health Statistics Yearbook on the relative risk of HBV-related death suggested that the risk of HCC was significantly higher in patients >30 years old (22).

In particular, our findings suggest the potential for using the IL-6 level as a prognostic biomarker for EHI among patients with chronic HBV infections and normal ALT levels. IL-6 has also been implicated in HBV-induced hepatic necroinflammation (23). Furthermore, patients with chronic HBV infection and cirrhosis had significantly higher plasma IL-6 levels and more severe liver inflammation (24). Consistent with these reports, we observed that high IL-6 levels could indicate the severity of the inflammatory response and the risk of hepatic necrosis. MCP-1 attracts monocytes, T cells, and dendritic cells during infection, providing a sustained inflammatory bridge between innate and acquired immunity (25). In previous studies, MCP-1 levels were positively correlated with the viral load in HIV infection (26). One study (27) compared patients with chronic HBV infection and healthy individuals. The MCP-1 level was higher in chronic HBV infection, and a positive correlation of MCP-1 with HBV viral load and HBsAg level was discovered. In our study, the levels of HBeAg and HBV DNA were positively correlated with the levels of MCP-1, which was negatively correlated with the histological grading of fibrosis, implying the possible role of this chemokine in host immunity against viral replication. As mentioned previously, a higher level of MCP-1 is associated with breaking the balance and facilitating HBV immune tolerance. Further investigations are needed to clarify the underlying mechanism.

In addition, higher levels of serum IFN- γ and IL-2 were observed in patients with $F \geq 2$. IFN- γ can be secreted by CD8⁺ T cells and NK cells (28), and IL-2 production by HBV-specific CD4⁺ T cells (29) plays an important role in the efficient development of cytotoxic effector CD8⁺ T cells that contribute

to the elimination of infection. These results may partly explain the pathogenesis of fibrosis progression as part of the immune response to chronic HBV infection, even within the immune-tolerant state with normal ALT levels.

The cytokine IL-8, also known as C-X-C motif ligand 8 (CXCL8), is a proinflammatory chemokine that acts as a chemoattractant of leukocytes (30). The correlation of viral load and HBeAg level with IL-8 showed a weak relationship in the HBV group, perhaps suggesting that high IL-8 favours the replication of the virus in chronic hepatitis B (31). Increased IL-8 was related to severe liver damage, and its levels decreased when these conditions improved in HBV (32). Additionally, this cytokine is considered to be one of those responsible for maintaining the inflammatory environment in HBV infection and was associated with HCC interfering with antitumour immunity (33).

Nevertheless, the present study has several limitations that affect the interpretation of our findings. This was a retrospective cross-sectional study that could not exclude selection bias. Moreover, the number of patients aged over 30 years was not large, and the follow-up of patients after the liver biopsy was insufficient.

The expansion of antiviral therapy indications may significantly increase the proportion of patients with chronic HBV infection receiving antiviral treatment. In our study, patients in the GZ phase, as well as IT and IHC patients older than 30 years, would indicate EHI. Lowering the antiviral therapy threshold for ALT was effective in screening out patients who already had EHI. Guided by the expansion of antiviral therapy indications, these patients could accept antiviral therapy in a timely manner, which might improve their long-term prognosis. In addition, our study showed that IL-6 levels were a good indicator of EHI even in the presence of an immune tolerance phase with normal ALT levels, and MCP-1 levels could reflect significant liver fibrosis at high levels of HBV DNA loads.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding authors.

Ethics statement

The study was approved by the ethics committee of Beijing Youan Hospital affiliated with Capital Medical University ([2020]089). The patients/participants provided their written informed consent to participate in this study.

Author contributions

SR and XC contributed to the conception of the study. WW contributed to cytokine detection with Luminex. KW and YZ contributed to the collection of the clinical data. SR and JL contributed significantly to the analysis and manuscript preparation. SR and LM performed the data analyses and wrote the manuscript. XC and SZ helped perform the analysis with constructive discussions. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.1035923/full#supplementary-material>

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The relation of the frequency and functional molecules expression on plasmacytoid dendritic cells to postpartum hepatitis in women with HBeAg-positive chronic hepatitis B virus infection

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Objective: To explore the correlation between postpartum hepatitis and changes of plasmacytoid dendritic cells' (pDC) function and frequency in hepatitis B e antigen (HBeAg)-positive pregnant women with chronic hepatitis B virus (HBV) infection.

Methods: Pregnant women with chronic HBV infection receiving antiviral treatment (treated group) or not receiving antiviral treatment (untreated group) were enrolled and demographic information was collected before delivery. Clinical biochemical, virological serology, pDC frequency and functional molecular expression were tested before delivery and at 6, 12, 24 weeks after delivery.

Results: 90 eligible pregnant women were enrolled, 36 in the untreated group and 54 in the treated group. 36 patients developed postpartum hepatitis, including 17 (17/36, 47.2%) in the untreated group and 19 (19/54, 35.2%) in the treated group ($\chi^2 = 1.304$ $p=0.253$), and 22 cases of hepatitis occurred at 6 weeks postpartum, 12 at 12 weeks postpartum, and 2 at 24 weeks postpartum. The alanine transaminase (ALT) levels at any time postpartum were significantly

higher than that of the antepartum, especially at 6 weeks and 12 weeks postpartum. However, the frequencies of pDCs, CD83⁺ pDCs and CD86⁺ pDCs antepartum had no significant difference from any time postpartum. The frequencies of CD83⁺ pDCs, CD86⁺ pDCs in the treated group antepartum were significantly higher than those in the untreated group [12.70 (9.46, 15.08) vs. 10.20 (7.96, 11.85), $p=0.007$; 22.05 (19.28, 33.03) vs. 18.05 (14.33, 22.95), $p=0.011$], and the same at 12 weeks postpartum [12.80 (10.50, 15.50) vs. 9.38 (7.73, 12.60), $p=0.017$; 22.50 (16.80, 31.20) vs. 16.50 (12.65, 20.80), $p=0.001$]. The frequency of CD86⁺ pDCs in the treated group was significantly higher than that in the untreated group at 24 weeks postpartum [22.10 (16.70, 30.00) vs. 17.10 (13.70, 20.05), $p=0.006$].

Conclusions: Postpartum hepatitis in HBV infected women mainly occurs at 6–12 weeks postpartum. Antiviral treatment during pregnancy can significantly increase the frequencies of CD83⁺ pDCs and CD86⁺ pDCs in pregnant women with chronic HBV infection.

KEYWORDS

plasmacytoid dendritic cells, chronic hepatitis B, antiviral treatment, postpartum, immunity

Introduction

Mother-to-child transmission (MTCT) of HBV is the main cause of chronic HBV infection in China. The rate of HBV MTCT maintained 7%–11% in newborns of HBeAg-positive mothers despite combined active-passive immunization with a hepatitis B vaccine and hepatitis B immunoglobulin (HBIG) shortly after birth. HBeAg positive and high HBV DNA level (HBV DNA > 10⁵ IU/ml) in pregnant women are the main cause of HBV MTCT (1, 2). Antiviral treatment during the third trimester can significantly reduce the rate of MTCT in pregnant women with high HBV DNA level (3, 4).

pDCs can secrete a large amount of cytokine interferon (IFN) with pleiotropic effect during viral infection, and they have a strong ability to activate the initial immune response and activate natural killer (NK) cells and T lymphocytes to further activate and regulate the antiviral immune response (5). They play a vital role in the control and prognosis of HBV infection. Studies have shown that hepatitis B surface antigen (HBsAg) and HBeAg can inhibit the frequency and function of pDCs in chronic HBV infected patients, leading to the impairment of antigen presenting function of pDCs (6, 7).

The elevation of postpartum ALT is a common phenomenon. It is believed that due to the decrease of steroid hormone levels after delivery, the immunosuppressive state during pregnancy is rapidly relieved, triggering the immune response to HBV and mediating liver tissue injury and hepatocyte necrosis in patients

with chronic hepatitis B (CHB). Hepatocyte necrosis leads to elevation of ALT and indicates that these women have entered the immune clearance period or the reactivation period (8), which is a good time to treat hepatitis (7, 9).

However, there are few studies on the changes of postpartum immune cell function of HBeAg-positive pregnant women with chronic HBV infection and its association with postpartum hepatitis, and few reports about the effects of antiviral treatment during pregnancy on postpartum immune cell function and postpartum hepatitis. Therefore, we designed a prospective case-cohort study and recruited HBeAg-positive chronic HBV infected pregnant women after informed consent. We measured the frequency of pDCs and the expression of functional molecules in peripheral blood antepartum and at 6, 12, 24 weeks postpartum to explore the frequency and functional changes of pDCs after delivery.

Materials and methods

Research objects

Pregnant women with chronic HBV infection were enrolled in the study from January 2016 to January 2018. Inclusion criteria: 1. Between the ages of 20 and 40; 2. HBsAg and HBeAg positive over 6 months, and serum HBV DNA > 2.0 × 10⁵ IU/ml; 3. Liver function remained normal; 4. With or

without antiviral treatment at 24–28 weeks of gestation as they wish. Exclusion criteria: 1. History of amniocentesis during pregnancy; 2. History of hereditary diseases in one or both families of the couple; 3. Fetal abnormalities detected before enrollment by imaging or other prenatal tests, including genetic testing; 4. Coinfection with hepatitis C virus, hepatitis D virus, human immunodeficiency virus, syphilis, toxoplasmosis, rubella, or cytomegalovirus; 5. History of two or more spontaneous abortions; 6. Previous delivery of a child with deformity; 7. Twin or multiple pregnancies; 8. The mother had a history of cancer or autoimmune diseases.

Pregnant women were classified into treated group (with antiviral treatment at 24–28 weeks of gestation) and untreated group (without antiviral treatment during pregnancy). Antiviral therapy was stopped immediately after delivery and all women were followed up at 6, 12 and 24 weeks postpartum.

All subjects signed an informed consent. The study was approved by the ethics committee of Beijing Ditan Hospital, Capital Medical University (JDL-2017-004-01), and registered at clinicaltrials.gov (Clinical registration No: NCT03214302).

Blood specimen collection

2 ml of peripheral venous blood of all pregnant women was collected by EDTA anticoagulant violet tube at the time of antepartum (baseline) and 6, 12, 24 weeks postpartum, cells were collected by BD FACS Calibur within 4 hours of blood collection.

Virological indexes and clinical biochemical indicators

Virological indexes (HBeAg, HBsAg and HBV DNA) and ALT, glutamic oxaloacetic transaminase (AST), total bilirubin (TBIL), albumin (ALB) were tested by the laboratory of Beijing Ditan Hospital, Capital Medical University.

pDC functional detection

pDCs were detected with Lineage1-FITC, HLA-DR-PerCP, Anti-CD123-APC, Anti-CD83-PE, Anti-CD86-PE antibodies, and CD83MFI and CD86MFI data were analyzed by FlowJo7.6 software.

Statistical analysis

Data were analyzed by SPSS 19.0 software. All data were presented as mean $\pm$ standard deviation (mean $\pm$ SD) or median, interquartile interval [median (Q1, Q3)], box-and-whisker plots

or percentage. Student t-test was used for normal distribution data and Mann-Whitney test was used for skewness distribution data between groups. $P < 0.05$ was considered statistically significant.

Results

Baseline characteristics

90 pregnant women were enrolled, including 54 cases with antiviral treatment (treated group) and 36 cases without antiviral treatment (untreated group). There was no difference between the two groups in age, gestational weeks, pregnancy complications, neonatal weight, length, Apgar score at 5 minutes and neonatal malformations ($P > 0.05$). The number of gravidity and parity in the untreated group were significantly higher than those in the treated group ($p = 0.015$ and 0.005 , respectively). The level of TBIL was $7.05 \pm 2.18 \mu\text{mol/l}$ in the treated group and $8.18 \pm 2.73 \mu\text{mol/l}$ in the untreated group ($P = 0.033$), but TBIL in both groups was within the normal range. The levels of HBsAg, HBeAg and HBV DNA antepartum in the treated group were significantly lower than those in the untreated group ($P < 0.05$), which was related to antiviral therapy. The basic information is shown in [Table 1](#).

Changes of pDC and ALT after delivery

In this study we use $\text{ALT} \geq 2$ times the upper limit of normal (40 U/L) as the diagnostic criterion for the occurrence of hepatitis according to the Asian-Pacific clinical practice guidelines on the management of hepatitis B (10). The ALT levels of all pregnant women enrolled were significantly higher at 6, 12 and 24 weeks postpartum than those of antepartum ($P < 0.05$), postpartum hepatitis occurred in 36 cases, 22 cases occurred at 6 weeks postpartum, 12 at 12 weeks postpartum, 2 at 24 weeks postpartum. 17 cases (17/36) of abnormal ALT occurred in the untreated group and 19 (19/54) occurred in the treated group, with no significant difference ($\chi^2 = 1.304$, $p = 0.253$). The frequencies of antepartum pDCs, CD83^+ pDCs and CD86^+ pDCs in all pregnant women enrolled were not significantly different from those at 6, 12 and 24 weeks postpartum ($P > 0.05$) ([Table 2](#)).

Frequency and expression of functional molecules of pDC cells and ALT in HBV infected pregnant women postpartum

The ALT levels were significantly higher at 6, 12, and 24 weeks postpartum than those of the antepartum in both treated and untreated group ($p < 0.001$). In the treated group, the

TABLE 1 Basic characteristics of the subjects.

Values	All patients (n=90)	Treated group (n=54)	Untreated group (n=36)	t/χ ²	P value
Mothers					
Ages (year)	29.79±3.86	30.20±4.20	29.17±3.24	-1.254	0.213
No. of gravidity (times)	2.17±1.07	1.94±1.02	2.50±1.08	2.474	0.015*
No. of parity (times)	1.50±0.55	1.37±0.49	1.69±0.58	2.870	0.005*
Gestational weeks (weeks)	39.24±1.03	39.41±1.00	39.00±1.04	-1.862	0.066
Vaginal delivery(%)	51(56.67%)	34(62.96%)	17(47.22%)	2.179	0.140
precipitate labour (%)	4(4.44%)	4(7.41%)	0(0%)	Fisher	0.147
Postpartum hemorrhage (%)	5(5.56%)	3(5.56%)	2(5.56%)	Fisher	1.000
Hypertension disorder of pregnancy (%)	1(1.11%)	1(1.85%)	0(0%)	Fisher	1.000
Diabetes (%)	24(26.67%)	15(27.78%)	9(25%)	0.085	0.770
Hypothyroidism (%)	3(3.33%)	2(3.7%)	1(2.78%)	Fisher	1.000
Intrahepatic cholestasis of pregnancy (%)	1(1.11%)	1(1.85%)	0(0%)	Fisher	1.000
Anemia (%)	3(3.33%)	2(3.7%)	1(2.78%)	Fisher	1.000
ALT level (U/L)	22.51±29.05	20.46±8.84	25.60±44.84	0.679	0.502
AST level (U/L)	23.58±18.12	22.95±5.91	24.53±27.95	0.333	0.741
TBIL level (umol/L)	7.50±2.46	7.05±2.18	8.18±2.73	2.161	0.033*
ALB level (g/L)	36.32±2.17	36.26±2.26	36.40±2.07	0.293	0.770
HBsAg level (log ₁₀ IU/ml)	4.40±0.41	4.29±0.42	4.56±0.35	3.127	0.002*
HBsAg level (S/CO)	1360.43±442.35	1207.54±465.36	1589.77±281.92	4.847	0.000*
HBV DNA level (log ₁₀ IU/ml)	5.79±2.02	4.28±0.96	8.06±0.33	26.577	0.000*
Infants					
Male (%)	52(50.00%)	27(50.00%)	19(52.77%)	0.067	0.796
Length (cm, mean ± SD)	50.09±0.83	50.11±1.02	50.06±0.41	-0.310	0.758
Weight (kg, mean ± SD)	3338.33±395.93	3297.04±435.00	3400.28±324.79	1.215	0.228
Apgar score at 5 min (mean ± SD)	9.97±0.32	10.00±0.00	9.92±0.50	-1.000	0.324
Neonatal malformations (%)	3(3.33%)	1(1.85%)	2(5.55%)	Fisher	0.561

*P < 0.05, was considered statistically significant.

frequency of CD83⁺ pDCs antepartum was significantly higher than that of the 24 weeks postpartum [12.70(9.46, 15.08) vs. 10.40(9.39, 12.10), p=0.026]. In the untreated group, the frequency of CD83⁺ pDCs antepartum was significantly lower than that of the 6 weeks postpartum [10.20 (7.96, 11.85) vs. 11.70 (9.12, 14.23), p=0.040] (Table 3).

Effect of antiviral therapy on the frequency and the expression of functional molecules of pDCs and ALT

The frequencies of CD83⁺ pDCs and CD86⁺ pDCs in the treated group were significantly higher than those in the untreated group before delivery [12.70 (9.46, 15.08) vs. 10.20 (7.96, 11.85), p=0.007; 22.05 (19.28, 33.03) vs. 18.05 (14.33, 22.95), p=0.011]. The frequencies of CD83⁺ pDCs and CD86⁺ pDCs in the treated group at 12 weeks postpartum were significantly higher than those in the untreated group [12.80 (10.50, 15.50) vs. 9.38 (7.73, 12.60), p=0.017; 22.50 (16.80, 31.20) vs. 16.50 (12.65, 20.80), p=0.001]. The frequency of CD86⁺ pDCs in the treated group at 24 weeks

postpartum was significantly higher than that in the untreated group [22.10 (16.70, 30.00) vs. 17.10 (13.70, 20.05), p=0.006]. It is suggested that antiviral treatment in the third trimester of pregnancy can significantly increase the frequencies of CD83⁺ pDCs and CD86⁺ pDCs in peripheral blood antepartum and postpartum (Table 4).

Discussion

Dendritic cells (DCs) can be divided into pDCs and myeloid dendritic cells (mDCs) according to the source of precursor cells. According to specific functions, DCs can be divided into immature DCs and mature DCs, most of which are immature DCs under normal circumstances and have a strong ability to capture antigens (11, 12). However, the surface of immature DCs lacks some costimulatory molecules, which cannot provide necessary costimulatory signals for T cell activation, making T cells unable to be fully activated and in a state of loss of function, meanwhile, immature DCs also induce antigen specific regulatory T cells, block the functional response of effector T cells, and also

TABLE 2 ALT and pDC levels antepartum and postpartum of all patients.

Values	0 week	6 week	12 week	24 week	Z or T value/P value
ALT (U/L)	17.15 (13.58, 22.63)	45.35 (28.63, 85.00)	55.30 (32.60, 96.60)	29.00 (23.18, 42.93)	a1=-9.362/0.000* a2=-9.590/0.000* a3=-6.968/0.000*
pDC/PBMC (%)	0.20 (0.14, 0.26)	0.20 (0.14, 0.25)	0.18 (0.13, 0.25)	0.21 (0.16, 0.29)	a1=-0.086/0.932 a2=-0.781/0.435 a3=-1.510/0.131
CD83+pDC (%)	11.20 (8.48, 14.35)	11.70 (9.18, 14.25)	12.25 (9.34, 14.45)	10.25 (8.50, 12.18)	a1=-0.452/0.651 a2=-1.033/0.302 a3=-1.753/0.080
CD83MFI (%)	18.30 (14.7, 21.6)	17.90 (15.35, 22.40)	18.00 (15.88, 23.25)	18.35 (15.50, 21.40)	a1=-0.708/0.479 a2=-0.753/0.451 a3=-0.354/0.723
CD86+pDC (%)	20.50 (16.78, 28.5)	19.80 (15.45, 26.95)	19.50 (15.48, 27.73)	19.55 (15.78, 26.98)	a1=-0.504/0.614 a2=-0.796/0.426 a3=-0.908/0.364
CD86MFI (%)	87.00 (72.15, 104.25)	88.20 (75.35, 106.00)	89.00 (71.70, 115.00)	99.10 (78.78, 109.75)	a1=-0.322/0.747 a2=-0.798/0.425 a3=-1.773/0.076

1. 0 week=antepartum 6 week=6 weeks postpartum 12 week=12 weeks postpartum 24 week=24 weeks postpartum.

2. a1: 0 week vs. 6 week a2: 0 week vs. 12 week a3: 0 week vs. 24 week.

3. * P < 0.05, was considered statistically significant.

4. pDCs frequency and function include pDC%, CD83⁺pDC%, CD83MFI and CD86⁺ pDC%, CD86MFI.

5. The expression of CD83 and CD86 molecules on the surface of pDC cells is also the mean fluorescence intensity (MFI).

can induce immune tolerance (12–14). Mature DCs stimulate T lymphocytes by expressing major histocompatibility complexes and costimulatory molecules, and secreting cytokines such as IFN, thus initiating the cellular immune response (14, 15). At

present, research suggests that CD86 is a marker of DC activation, and CD83 is a specific marker of DC maturation. DC activation and maturation increase the expression of cell surface molecules CD86 and CD83, otherwise inhibit the differentiation and

TABLE 3 The changes of postpartum ALT and pDC levels in Treated and Untreated group.

Values	Treated group (n=54)					Untreated group (n=36)				
	0 week	6 week	12 week	24 week	Z or T/P value	0 week	6 week	12 week	24 week	Z or T/P value
ALT (U/L)	17.95 (14.85, 24.03)	40.25 (28.18, 62.68)	49.35 (31.28, 78.80)	27.85 (23.63, 35.90)	a1=-6.925/0.000* a2=-7.549/0.000* a3=-4.889/0.000*	15.10 (12.00, 21.68)	53.40 (30.73, 157.45)	68.30 (40.55, 146.80)	33.75 (23.00, 58.00)	a1=-6.163/0.000* a2=-5.810/0.000* a3=-4.848/0.000*
pDC/PBMC (%)	0.20 (0.15, 0.26)	0.20 (0.16, 0.25)	0.19 (0.13, 0.26)	0.21 (0.17, 0.30)	a1=-0.347/0.728 a2=-0.164/0.870 a3=-1.328/0.184	0.19 (0.13, 0.26)	0.18 (0.12, 0.25)	0.18 (0.11, 0.23)	0.22 (0.12, 0.28)	a1=-0.628/0.530 a2=-1.250/0.211 a3=-0.654/0.513
CD83 ⁺ pDC (%)	12.70 (9.46, 15.08)	11.70 (9.27, 14.30)	12.80 (10.50, 15.50)	10.40 (9.39, 12.10)	a1=-1.047/0.295 a2=-0.689/0.491 a3=-2.230/0.026*	10.20 (7.96, 11.85)	11.70 (9.12, 14.23)	9.38 (7.73, 12.60)	10.10 (5.59, 12.30)	a1=-2.053/0.040* a2=-0.128/0.898 a3=-0.422/0.673
CD83MFI (%)	18.70 (15.23, 22.6)	19.30 (16.45, 23.70)	19.80 (16.70, 24.80)	19.80 (15.80, 24.80)	a1=-0.773/0.440 a2=-1.223/0.221 a3=-0.735/0.462	16.75 (14.40, 19.05)	16.65 (14.20, 20.50)	16.50 (13.55, 18.25)	16.70 (14.10, 19.15)	a1=-0.081/0.936 a2=-0.599/0.549 a3=-0.314/0.753
CD86 ⁺ pDC (%)	22.05 (19.28, 33.03)	20.80 (16.65, 29.05)	22.50 (16.80, 31.20)	22.10 (16.70, 30.00)	a1=-1.218/0.223 a2=-0.592/0.554 a3=-0.760/0.447	18.05 (14.33, 22.95)	18.25 (14.48, 25.05)	16.50 (12.65, 20.80)	17.10 (13.70, 20.05)	a1=-0.644/0.519 a2=-0.932/0.351 a3=-0.596/0.551
CD86MFI (%)	90.60 (72.83, 114.25)	89.00 (80.25, 106.00)	94.70 (77.00, 118.00)	104.00 (82.00, 113.00)	a1=-0.088/0.932 a2=-1.047/0.295 a3=-1.273/0.203	83.95 (69.13, 93.70)	85.85 (68.68, 107.25)	78.40 (67.30, 103.50)	89.80 (78.05, 104.50)	a1=-0.299/0.765 a2=-0.539/0.590 a3=-1.332/0.183

1. 0 week=antepartum 6 week=6 weeks postpartum 12 week=12 weeks postpartum 24 week=24 weeks postpartum.

2. a1: 0 week vs. 6 week a2: 0 week vs. 12 week a3: 0 week vs. 24 week, they were comparison within groups.

3. * P < 0.05, was considered statistically significant.

4. pDCs frequency and function include pDC%, CD83⁺ pDC%, CD83MFI and CD86⁺ pDC%, CD86MFI.

TABLE 4 Comparison of ALT and pDC levels at different time points between two groups.

Values	Treated group (n=54)				Untreated group (n=36)				T value/P value
	0 week	6 week	12 week	24 week	0 week	6 week	12 week	24 week	
ALT (U/L)	17.95 (14.85, 24.03)	40.25 (28.18, 62.68)	49.35 (31.28, 78.80)	27.85 (23.63, 35.90)	15.10 (12.00, 21.68)	53.40 (30.73, 157.45)	68.30 (40.55, 146.80)	33.75 (23.00, 58.00)	a1 = 0.679/0.502 a2 = 1.717/ 0.095 a3 = 1.724/0.097 a4 = 1.547/ 0.133
pDC/PBMC (%)	0.20 (0.15, 0.26)	0.20 (0.16, 0.25)	0.19 (0.13, 0.26)	0.21 (0.17, 0.30)	0.19 (0.13, 0.26)	0.18 (0.12, 0.25)	0.18 (0.11, 0.23)	0.22 (0.12, 0.28)	a1=-0.138/0.891 a2=-0.405/ 0.686 a3=-1.425/0.159 a4=-0.686/ 0.496
CD83 ⁺ pDC (%)	12.70 (9.46, 15.08)	11.70 (9.27, 14.30)	12.80 (10.50, 15.50)	10.40 (9.39, 12.10)	10.20 (7.96, 11.85)	11.70 (9.12, 14.23)	9.38 (7.73, 12.60)	10.10 (5.59, 12.30)	a1=-2.786/0.007* a2 = 0.731/ 0.467 a3=-2.446/0.017* a4=-1.075/ 0.291
CD83MFI (%)	18.70 (15.23, 22.6)	19.30 (16.45, 23.70)	19.80 (16.70, 24.80)	19.80 (15.80, 24.80)	16.75 (14.40, 19.05)	16.65 (14.20, 20.50)	16.50 (13.55, 18.25)	16.70 (14.10, 19.15)	a1=-1.260/0.211 a2=-0.189/ 0.851 a3=-2.811/0.007* a4=-1.966/ 0.054
CD86 ⁺ pDC (%)	22.05 (19.28, 33.03)	20.80 (16.65, 29.05)	22.50 (16.80, 31.20)	22.10 (16.70, 30.00)	18.05 (14.33, 22.95)	18.25 (14.48, 25.05)	16.50 (12.65, 20.80)	17.10 (13.70, 20.05)	a1=-2.594/0.011* a2=-1.410/ 0.162 a3=-3.393/0.001* a4=-2.867/ 0.006*
CD86MFI (%)	90.60 (72.83, 114.25)	89.00 (80.25, 106.00)	94.70 (77.00, 118.00)	104.00 (82.00, 113.00)	83.95 (69.13, 93.70)	85.85 (68.68, 107.25)	78.40 (67.30, 103.50)	89.80 (78.05, 104.50)	a1=-1.378/0.172 a2=-0.565/ 0.574 a3=-2.062/0.044* a4=-1.527/ 0.133

1. 0 week=antepartum 6 week=6 weeks postpartum 12 week=12 weeks postpartum 24 week=24 weeks postpartum.

2. a1: 0 week vs. 0week a2: 6 week vs. 6 week a3: 12 week vs. 12 week a4: 24 week vs. 24 week. they were comparison between groups.

3. * P < 0.05, was considered statistically significant.

4. pDCs frequency and function include pDC%, CD83⁺ pDC%, CD83MFI and CD86⁺ pDC%, CD86MFI.

maturation of DCs, and reduce the expression of cell surface molecules CD86 and CD83 (16–19).

Recently, the function of pDCs has attracted extensive attention in the field of chronic HBV infection. As the only innate immune cells with antigen presenting ability, which can activate non-sensitized initial T cells, pDC is the main effector cell of human antiviral immunity and plays a double-acting role in antiviral treatment, which can mediate both immune response and immune tolerance (12, 14, 16). Mature pDCs can secrete a large amount of cytokines with pleiotropic effects to directly inhibit viral replication during viral infection, and can also activate mDCs, T cells, NK cells and B lymphocytes to form protective immune response, becoming a bridge connecting natural immunity and acquired immunity, with strong ability to activate initial immune response. Immature pDCs have a strong ability to capture antigen, but the ability to present antigen is weak, making T cells cannot be fully activated, and have a strong ability to induce regulatory T cells, which mediates the induction and maintenance of immune tolerance (14–18).

Studies have suggested that HBsAg and HBeAg in patients with chronic HBV infection can inhibit the function of DCs, which leads to a decrease in the frequency and function of pDCs in patients with chronic HBV infection (20, 21). Our previous study on the immune function of HBeAg positive chronic

hepatitis B patients found that the frequency of pDCs was related to the incidence of CHB, the lower the frequency of pDCs in CHB patients, the more likely they were to develop hepatitis. However, we did not distinguish immature pDCs and mature pDCs frequency changes on the impact of hepatitis in this study (7, 22). Our previous retrospective study and this prospective study both suggest that postpartum liver dysfunction is common in women with chronic HBV infection, we also found that the abnormal liver function of HBV infected women mainly occurred in 6–12 weeks postpartum (23, 24), which indicated the importance of preventing postpartum hepatitis. We further studied the effect of antiviral therapy in the third trimester on postpartum hepatitis, and found that with the reduction of HBV DNA level due to the effective antiviral therapy during pregnancy, the inhibition of the virus on the pDCs function was alleviated. Based on this result, we also studied the effect of antiviral treatment in the third trimester of HBeAg-positive chronic HBV infected women on the frequency and function of postpartum peripheral blood pDCs and ALT level. Our study found that the ALT level of the pregnant women receiving antiviral treatment in the third trimester has a downward trend at 6–12 weeks postpartum, but there is no statistical significance between the two groups, which may be related to the limited number of patients in our study. Our study

also found that there was no significant change in the frequency of pDCs after antiviral treatment during pregnancy, but it could significantly increase the frequencies of CD83⁺ pDCs and CD86⁺ pDCs in pregnant women with chronic HBV infection before delivery and 12 weeks postpartum. This might indicate that antiviral therapy in late pregnancy increases the proportion of mature pDCs, the immune activity and antiviral function of pDCs, which may improve the success rate of postpartum hepatitis treatment. However, this study had a small sample, and only involved the changes of pDCs in peripheral blood, without discussing the changes of the immune function in the liver, therefore, if we want to elucidate the above causal relationship, we need to further enlarge the sample and investigate the changes of the immune function in liver, as well as in peripheral blood, which would shed new light on the prevention and treatment of postpartum hepatitis in HBeAg positive chronic HBV infected pregnant women.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by The ethics committee of Beijing Ditan Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

FW, GW, LZ, ML, and YX contributed to study concept and design. MS, YH, YLi, XB, YLu, TJ, WD, SyW, FS, ZZ, GS, RL, MC, SIW, YG, HH, MX, XC, and LH performed the data collection. FW, GW, LZ, ML, and YX conducted data analysis.

FW, MS, YH, LY, and XB wrote the first draft. FW edited the English version. GW, LZ, ML, and YX approved the submitted version after modification. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Hepatitis B functional cure and immune response

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Hepatitis B virus (HBV) is a hepatotropic virus, which damage to hepatocytes is not direct, but through the immune system. HBV specific CD4⁺ T cells can induce HBV specific B cells and CD8⁺ T cells. HBV specific B cells produce antibodies to control HBV infection, while HBV specific CD8⁺ T cells destroy infected hepatocytes. One of the reasons for the chronicity of HBV infection is that it cannot effectively activate adoptive immunity and the function of virus specific immune cells is exhausted. Among them, virus antigens (including HBV surface antigen, e antigen, core antigen, etc.) can inhibit the function of immune cells and induce immune tolerance. Long term nucleos(t)ide analogues (NAs) treatment and inactive HBsAg carriers with low HBsAg level may “wake up” immune cells with abnormal function due to the decrease of viral antigen level in blood and liver, and the specific immune function of HBV will recover to a certain extent, thus becoming the “dominant population” for functional cure. In turn, the functional cure will further promote the recovery of HBV specific immune function, which is also the theoretical basis for complete cure of hepatitis B. In the future, the complete cure of chronic HBV infection must be the combination of three drugs: inhibiting virus replication, reducing surface antigen levels and specific immune regulation, among which specific immunotherapy is indispensable. Here we review the relationship, mechanism and clinical significance between the cure of hepatitis B and immune system.

KEYWORDS

chronic hepatitis B, functional cure, innate immunity, adaptive immunity, antiviral

Introduction

The infection of chronic hepatitis B virus (HBV) is still a worldwide problem that seriously threatens human health. It is the principal reason of end-stage liver diseases, including cirrhosis and hepatocellular carcinoma (HCC). As WHO estimated, there were 290 million people infected with chronic HBV all over the world in 2019, and about 1.5 million people were newly infected each year (1). The prevalence rate of HBsAg among the general people in China is 6.1%, and there are about 86 million people with positive HBsAg (2). More than 90% of HCC in China is related to chronic HBV infection.

At present, there are two kinds of widely used anti-HBV drugs, namely nucleos(t)ide analogues (NAs) as well as peginterferon- α (PegIFN α). The treatment goal determined by domestic and foreign guidelines is to improve the long-term outcomes by maximizing the persistent inhibition of HBV (3–5). The goal of CHB treatment is to pursue cure. According to HBV related biomarkers and possible outcomes, hepatitis B cure can be divided into partial cure, functional cure (clinical cure) and complete cure (6). Because of covalently closed circularDNA (cccDNA) existence and integrated HBV DNA in hepatocytes, HBV cannot be completely eliminated, but functional cure, that is, the clearance of HBsAg, becomes possible. The so-called functional cure refers to achieving HBsAg seroclearance, with or without positive anti-HBs, based on continuous undetectable HBV DNA and HBeAg after a finite course of treatment. It can be originated from the induction of PegIFN based regimens or NAs, and the spontaneous clearance of HBsAg (7).

Chronic hepatitis B (CHB) is an immune related disease. HBV does not directly damage the liver but through abnormal immune response. The interaction between the replication of virus and the antiviral immunity of host determines the final result of HBV infection. The responses of host's innate and specific immunity to the virus promote the clearance of HBV in patients with acute hepatitis B. HBV continues to replicate and the host immune response is insufficient in patients with CHB. Integration of HBV DNA in hepatocytes, persistent presence of HBV cccDNA, dysfunction of T cells and insufficient response of B cells are the major obstacles to eliminate HBV. This determines that CHB cannot be completely cured through direct antiviral agents as chronic hepatitis C. Therefore, in order to cure hepatitis B, it is more important to restore host immune function in addition to inhibiting virus replication, reducing or even eliminating virus antigens (8). Here, we focus on the chronicity of HBV infection, the relationship between CHB functional cure and host innate immunity, specific cellular and humoral immunity, and the possible role of new immunomodulators in functional cure.

Persistent HBV infection and host immune response

Persistent HBV infection and host innate immune response

As a crucial immune organ of the human body, the liver is rich in natural killer cells (NK cells), natural killer T cell (NKT cells), as well as macrophages (eg. Kupffer cells), of which NK cells and NKT cells making up half in terms of numbers of lymphocytes in the liver. As the first line of defense against HBV, the natural immune system is greatly important in the initial and chronic process of HBV infection. In the process of early virus clearance and specific immune response, the innate immune

response is greatly important, whose deficiency is an important reason for persistent HBV infection. HBV has evolved an active strategy to actively evade the natural immune recognition or actively interfere with the natural immune signaling pathway to induce immune suppression, which is conducive to self-replication (9).

Age at exposure contributed greatly to the chronicity of the human HBV infection, which is associated with the immature development of the innate immunity in perinatal and infant periods and the unstable intestinal microbiota (10). Lebossé et al. conducted a study involved 105 untreated CHB patients to explore the expression profiles of 67 genes, which were involved in the innate immune pathways in the hepatic tissues of 105 untreated CHB patients. The results proved that the expression profile of genes in the liver of CHB patients showed significant down-regulation of antiviral effectors, IFN stimulating genes (ISGs), Toll-like and pathogen recognition receptors (PPR) pathway genes compared with that of the healthy control group. In CHB patients with negative HBeAg, some genes expression was negatively correlated with the level of qHBsAg, but not directly related to HBV replication. It is suggested that the innate immunity in the liver of CHB patients is seriously impaired, especially at high levels of HBsAg (11). More and more studies have found that viral antigens, including HBsAg, is crucial to suppress natural immunity and promoting viral immune escape. HBsAg can inhibit stimulator of interferon genes (STING) expression in NK cells and inhibit NK's response to cGAMP (12). Interaction of HBsAg as well as TGF β - Activated kinase 1 (TAK1) - TAB2 complex s inhibits NF- κ B signal pathway by reducing post-translational modification and autophagic degradation (13). HBsAg promotes the production of GP73 and HBV replication by inhibiting NF- κ B signaling pathway (14). HBsAg inhibits interferon regulatory factor (IRF)- α and Toll-like receptor 9 (TLR9) nuclear translocation, thereby specifically inhibiting TLR9 mediated interferon production. HBsAg can directly cause dysfunction of bone marrow dendritic cells in CHB patients, which is regarded as a potential mechanism for HBV escaping from the immune system (15). These outcomes reveal new insights into the mechanism of the persistence of HBV in the liver. Methods aimed at reducing the level of HBsAg may restore the immune response upon the virus, which are considered as one of the strategies to cure CHB.

Persistent HBV infection and cellular immunity

In the liver puncture samples of CHB patients, inflammatory necrosis and fibrosis of different degrees were found in and around the portal area of the liver and the infiltrating inflammatory cells were mainly lymphocytes, indicating the great importance of immune cells in its pathogenesis (5). T

cell exhaustion (Tex) is one of the characteristics of CHB patients. It means that when the virus is chronically infected, owing to being exposed to persistent antigens and an inflammatory environment for a long time, T cells have a constantly stimulation and gradually lose their effective function, and memory T cell characteristics begin to be lost. Tex cells have independent differentiation pathways, phenotypes, functions, and subpopulation dynamics. In addition to the decrease in number, its functions such as rapid growth and cytokine secretion are lost in a gradual and graded manner (16). Currently, it is recognized that it may be the enhanced expression of inhibitory molecules, such as programmed cell death protein-1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) (17). Studies have found that by blocking the interaction between PD-1 and programmed death ligand-1 (PD-L1), Tex can be partially reversed (18). In addition, the transcriptional programs and metabolic patterns of Tex cells have also changed. Based on the latest results of single-cell transcriptome analysis, study has found that the exhausted CD8⁺T cells of hepatitis B patients at different stages come from different sources, in which patients at the immune activation stage mainly come from autologous clonal expansion, while patients at the recovery stage of acute infection are more from the transformation and infiltration of peripheral effector T cells (19).

CD8⁺T cells play an important role in cellular immunity, which are considered to be effectors of virus clearance, killing HBV-infected hepatocytes with antigenic specificity restricted by MHC-I molecules (20). A few studies have revealed that in patients with acute hepatitis, activated CD8⁺T cells targeting HBsAg are abundant, but in patients with chronic hepatitis B, the number of CD8⁺T cells is reduced and their functions are impaired, leading to persistent viral replication (21, 22). In the chimpanzee model infected with HBV, a considerable delay in viral clearance was observed by using CD8⁺T cells blocking antibodies (23). The immune response of HBV-specific CD8⁺T cell relies on the development of the disease course and is greatly susceptible to the influence of the level of HBV replication. For patients with viral load >107 copies/ml, it is difficult to detect CD8⁺T cells, whose deletion mechanism is still unclear, but it has been reported that it may be related in part to increased apoptosis of CD8⁺T cells (24). A recent research has explored the causes and found that HBV can eliminate immature HBsAg-specific T cells in the thymus through monocytic myeloid derived suppressor cells (mMDSCs), thus forming an HBV-specific T-cell tolerance environment (25).

CD4⁺T cells have significant heterogeneity, in terms of their cytokine expression, CD4⁺T helper cells (Th) can be classified into Th1, Th2, Th17, and regulatory T cells (Treg) subsets (26). Maintaining a balance in these T cells is crucial to liver immune homeostasis. The depletion of HBV-specific CD4⁺T cells and the imbalance of regulatory T cells may be significant features of CHB (27). Although the pathogenesis of T cells in different

stages of HBV is still poorly understood at present, the rise of single-cell sequencing technology may provide new insights for delineating the profile of liver immune cells in different stages of HBV and exploring the mechanism (19).

Some studies have shown that the expression of Treg and Th17 cells greatly increased in CHB patients (28, 29). Compared with convalescent patients and healthy people, the proportion of peripheral blood Treg cells had a significant increase in CHB patients, and the proportion as well as the number of Treg cells positively correlated with serum HBV DNA content (30). Treg cells contributed a lot to maintain the immune tolerance and inhibit the activation and proliferation of T lymphocytes and the maturation of dendritic cells (DC) (31). In addition, Treg could also inhibit the ability of T follicular helper cells (Tfh) through its expression of CTLA-4, resulting in its inability to assist the functional maturation of B cells to produce neutralizing antibodies, leading to persistent HBV infection (32).

Persistent HBV infection and humoral immune response

Over the past 20 years, most researches on the pathogenesis of HBV have paid attention to the T cell responses and the chronic phase clearance deficiency caused by virus specific T cell failure, while B cells have been neglected for a long time (33). Actually, humoral immune responses mediated by B cells are important for controlling and clearing HBV, whose infection has a great influence on the secretion of HBV-specific antibody and compartment of global B cells (34). The reactivation of HBV most often occurs in patients receiving cancer chemotherapy, especially those receiving B cell depleting agents, for example, rituximab and orfatumumab for blood or solid organ malignancies and patients receiving hematopoietic stem cell transplantation without antiviral prevention (35). One major way in which B cells are involved in anti-HBV infection is the production of specific antibodies against diverse HBV protein components, such as antibody to hepatitis B s antigen (HBsAb), antibody to hepatitis B e antigen (HBeAb) and antibody to hepatitis B core antigen (HBcAb). HBcAb and HBeAb are considered as the markers for the diagnosis of HBV infection, while only HBsAb can recognize and bind to HBsAg (36), thus playing an essential part in the clearance of HBsAg (37). HBsAb can not only prevent HBV from entering the body through acting as a protective neutralizing antibody to combine with free HBV virus particles, thus reducing the viral load *in vivo* (38), but also eliminate infected cells by mediating antigen dependent cytotoxicity and phagocytosis (39). In chronic HBV infection, global peripheral B cells are activated and hypofunctioning, while B cells which secrete anti-HBs are rarely detected (40). A recent study enrolled 13 healthy individuals without vaccination (HBsAg, HBsAb and HBcAb are negative) and 38 treatment naïve CHB patients using RNA sequencing of B cells showed that, compared with peripheral B cells, a upregulation of B cell receptor pathway was seen in liver B

cells (41). Zhou et al. found that in CHB patients, the expression of skewed CD39 and CD73 on B cells was correlated with a high viral burden, liver inflammation and antiviral efficacy, and the skewed CD39/CD73/adenosine pathway played an essential part in the hyperactivation of B cells (42). Additionally, although in CHB patients could detect a significantly higher total immunoglobulin G (IgG) in the serum than that in healthy individuals, HBV-specific antibodies were lacking (34, 43). The immune dysfunction in CHB patients might be related to B cells hyperactivation, impaired differentiation, inhibitory signal as well as regulatory B cells activation (44). A recent study isolating CD19⁺B cells from peripheral blood of 4 healthy controls and 4 CHB patients, and from the B cell transcriptome of all participants, totally 1401 genes with different expression were identified. The results revealed that B cells function of CHB patients was impaired, with the increasing expression of TLR4, the activation of NF- κ B pathway, as well as the alteration of mitochondrial function (45). During the persistent infection of HBV, the enrichment of IL-35⁺B cell could be seen in CD19⁺CD24^{hi}CD38^{hi} B cell. Meanwhile, Breg cells caused dysfunction of T cells through IL-35 dependent mechanism, as well as the intercellular contact exerted dysregulation (46).

Additionally, studies suggested that Tfh, which regulates B cells-mediated humoral immune response, is phenotypically different, and leads to deficiency in humoral immunity of CHB patients (39). Tfh cells are located in secondary lymphoid organs and promote the differentiation of B cells by secreting IL-21 (47). The reaction of Tfh cells to HBsAg to produce IL-21 is defective during CHB. However, despite of the low levels of IL-21, Tfh cells can effectively do a favor to B cell responses by producing IL-27 despite low levels of IL-21, which directs naive and memory B cells to form plasmablasts as well as plasma cells by enhancing B lymphocyte-induced maturation protein-1 (48). Ayithan et al. found that treatment of cytokine IL-21 induced by TLR8 agonists on peripheral blood mononuclear cells (PBMCs) in CHB patients was achieved by enhancing Tfh cells co-expressing IL-21⁺BCL-6⁺ and ICOS⁺BCL-6⁺ (49). Tfh cells contribute to differentiate B cells into antibody producing plasma cells and provide lifelong protection (50). Evidence have proved that depletion of Tfh cells is a significant cytokine for B cells maturation and clearance of chronic viral infections (16, 51).

Anti-viral therapy and host immune response

Anti-viral therapy and host innate immune response

Conventional IFN α has been used to treat CHB for more than 30 years, and was replaced by PegIFN α in 2005. It has anti-HBV and immunomodulatory effects, and plays a variety of key roles in innate and adaptive immune responses (52). Many studies have proved that the therapeutic effect of PegIFN α for

its induction of the repression of specific interferon-stimulated genes (ISGs) and secretion of antiviral proteins (53, 54).

IFN α can drive the proliferation, activation and antiviral ability of NK cells *in vivo*, mainly showing that the activated CD56^{bright} NK cells significantly expand, thereby indirectly inhibiting HBV replication, but this effect will be reduced after the treatment cessation (55). In HBeAg positive CHB patients who experienced 48 weeks of PegIFN α treatment, CD56^{bright} NK cells of sequenced NAs treatment were still higher than the baseline level, while PegIFN α could promote its expansion and increased activating receptors NKp30 and NKp46 expression in NK cells. NK cells activation and proliferation in CHB patients under PegIFN α treatment followed by NAs were significantly stronger than those with NAs or PegIFN α alone (56).

A prospective cohort study analyzed the changes of NK cells at week 12 and 24 in CHB patients received PegIFN α 2 α and entecavir (ETV) treatment. In patients treated with PegIFN α , a increase was seen in the frequency of CD56^{bright} NK cells, while there was a decrease in that of CD56^{dim} NK cells, and the expression of NKp46 and IFNAR2 receptors increased. In patients treated with ETV, although the frequency of NK cells increased, no difference was found in that of CD56^{bright} and CD56^{dim} NK cells and the expression of IFNAR2 at baseline and after treatment. Based on the 60% decrease of HBsAg level from baseline, patients were classified into responders and non-responders. In PegIFN α responders, the frequency of CD56^{bright} NK cells and the expression of IFNAR2 increased significantly after treatment compared with that of the baseline, while there were no changes in PegIFN α non-responders and ETV treatment responders. In patients with ETV treatment, NK cell frequency increased significantly, while the frequency of NKp46^{bright} and IFNAR2⁺NK and IFNAR2 MFI decreased greatly at 12 and 24 weeks. It is suggested that in CHB patients, PegIFN α could significantly enhance the frequency and function of NK cells compared with ETV treatment, and the efficacy of IFN α or ETV is associated with the improvement of NK cell function (57). A meta-analysis found that there was no significant difference in NK frequency between CHB patients before and after NAs antiviral therapy, and the activated receptor was up-regulated, while the inhibitory receptor was similar to the healthy control (58).

Anti-viral therapy and cellular immunity

PegIFN α plays a dual role of immune regulation and antiviral as mentioned above and NAs can strongly interfere with HBV replication as a reverse transcriptase inhibitor, which is easy to use and well tolerated (59). However, it is a pity that the clearance effect of both drugs on HBsAg is not ideal. Therefore, exploring the immune changes of the host after drug treatment and find an immunotherapy scheme may provide new insights

into improving the therapeutic effect of patients and achieving clinical cures as much as possible.

Targeted recovery of exhausted T cells may be the focus of HBV treatment in the future, and several studies have been explored accordingly. Among them, blocking agents of immune checkpoints like PD-1/PD-L1 can alleviate the down-regulation of specific T cells and resuscitate the exhaustion of T cells (18). Blockers of some other checkpoint molecules, like T-cell immunoglobulin domain and mucin domain-containing molecule-3 (TIM-3) and CTLA-4, can restore virus-specific CD8⁺ T-cell responses in CHB patients. Inhibitors of metabolic checkpoints may also be an effective means to restore T cells exhaustion. Acyl coenzyme A-cholesterol acyltransferase (ACAT), an enzyme that regulates cellular cholesterol, its inhibitors can improve the ability of antiviral T cells to clear the virus and rescue exhausted T cells (60). In addition, research has proved that the use of T cell immunotherapy can achieve effective control of HBV (61).

The ideal antiviral therapy for CHB patients may eventually lead to the effective recovery of HBV-specific T cells frequency and function (62). Among them, virus-specific CD8⁺T's number and function recovery might be a research hotspot of current therapy (63). Several studies have shown that the cytotoxic and non-cytotoxic abilities of virus-specific CD8⁺T cells are significantly enhanced with a decreased viral load after antiviral treatment, which is closely associated with HBeAg negative conversion. Some researches have also focused on the exploration of the effect of new therapeutic drugs on CD8⁺T cells (64, 65). These results indicate an interaction between antiviral therapy and virus-specific cellular immune function, and the regulation of immune cells can improve the immune microenvironment and further affect antiviral therapy.

The negative regulation of Treg cells can contribute to the deficiency and absence of HBV-specific T cell response, which is an important mechanism of CHB persistence. Several studies have shown that the proportion of Treg cells had a close association with the clinical efficacy of PegIFN α (66). In the course of antiviral treatment, it has been found that NAs could lead to a decrease in Treg cell levels, which may be related to the targeting effect of NK cells (67). In addition, it can also inhibit the level of virus-specific Th17 cells in the peripheral blood (67), and the serum levels of IL-22 and IL-23 also decreased (68), which may be related to direct viral inhibition and inflammation regulation. The restoration of Th17/Treg balance may also be important for reducing HBsAg levels in patients (69).

An important feature of CHB patients is the decreased number and changed ratio of Th1 and Th2 cells. Several previous research have proved that the number of Th1 and Th2 cells and the expression of related cytokines increased after antiviral treatment (70), which is related to the decrease of HBV DNA viral loads (71). However, compared with Th1 and Th2 cells of chronic HBV patients, Treg cells and Th17 cells are more sensitive subtypes of HBV replication inhibition induced by ETV (70).

Anti-viral therapy and humoral immune response

Except for the proven effect of PegIFN α on natural killer cells and T cells (55), B cells are greatly important in this process as well (72). PegIFN α treatment may play an immunomodulatory role through remodeling the B cell compartments, which is related to continuously increased sCD30 levels and decreased plasma HBsAg (73). HBcAb is an important indicator of host humoral immune response. A retrospective cohort study lasted for 2 years showed that CHB patients whose HBV DNA <9 log₁₀ copies/mL and anti-HBc ≥ 4.4 log₁₀ IU/mL at baseline level had 37.1% and 65.8% HBeAg seroconversion rates under NAs and PegIFN treatment, respectively (74). Another study involved 139 Chinese patients receiving entecavir or entecavir maleate treatment for 240 weeks also showed that 25.2% of the patients reached the goal of serological response (HBeAg seroconversion), at the same time HBcAb level of these patients increased significantly at 240 weeks (75). A recent research showed that a *de novo* higher level of HBcAb was related to the sustention of response, the decline of HBsAg, the clearance of HBsAg and the loss of HBeAg after *de novo* PegIFN α ($p < 0.050$), and for patients received add-on PegIFN α , higher HBcAb was related to the loss of HBeAg ($p = 0.012$) (76). Thus, baseline HBcAb titre can be concerned as an effective predictor of the efficacy of PegIFN α and NAs therapy in CHB patients with positive HBeAg.

CHB functional cure and host immune response

CHB functional cure and host innate immune response

PegIFN α combined with NAs treatment significantly affects the function and phenotype of NK cells, thereby playing an important role in clearing HBsAg. Stelma et al. followed up CHB patients under PegIFN α treatment combined with adefovir for 48 weeks, and analyzed the function and phenotype of NK cells in 7 patients with functional cure, 7 matched patients without functional cure, and 7 healthy controls. They found that the absolute number and the proportion of CD56^{bright} NK cells had a significant increase, while those of CD56^{dim} NK cells had a decrease in CHB patients received combination therapy. Compared with those without functional cure, the expression of chemokine receptor CX3CR1 on CD56^{bright} NK cells and the suppressor receptor NKG2A on CD56^{dim} NK lymphocytes decreased significantly at baseline, and higher CD56^{bright} TNF-related apoptosis-inducing ligand (TRAIL) expression and IFN γ were obtained at the end of treatment in patients with functional cure, compared with those without it (72). Inactive HBsAg carriers (IHC), which were previously believed to require no treatment, can achieve higher HBsAg seroconversion rate after

PegIFN α . Cao et al. compared the changes of NK cells in IHCs with and without the seroconversion of HBsAg after 48 weeks of PegIFN α therapy. They found that the proportion, the IFN γ secretion and CD107a expression of NK cells were greatly higher in HBsAg seroconversion group compared with those without HBsAg seroconversion both at baseline and during PegIFN α treatment. In addition, NK cell activity had an increase in patients with HBsAg seroconversion group, especially before the seroconversion of HBsAg, which were not seen in patients without HBsAg seroconversion. In summary, PegIFN α induced NK cells to increase with enhanced activity, which is conducive to the HBsAg seroconversion of IHCs (77).

NAs play a direct antiviral role mainly by inhibiting HBV DNA polymerase. Does it affect the immune function of the body? Liu et al. conducted immunological studies on CHB patients with decreased HBsAg levels, HBV DNA <1000 IU/mL and negative HBeAg levels after NAs treatment. Compared with untreated CHB patients, the proportion of total NK cells, CD56^{dim}NK cells increased, and that of CD56^{bright}NK cells decreased in these patients, but there was no significant difference in the healthy controls. It suggested that peripheral blood CD56^{dim} NK cells recovered after inhibition of virus replication, which could be conducive to reduction of the HBsAg levels in CHB patients (69). The potency of TDF antiviral therapy upon the frequency of NK cells in peripheral blood in pregnant women whose HBV DNA was positive was analyzed. The frequency of NK cells, NKp46^{dim} NK cells and CD56^{bright} NK cells after delivery was markedly higher than that before delivery both in the treatment and untreated groups. Compared with untreated group, a significant increase of the frequency of NK cells and CD56^{bright} NK cells after delivery could be seen in the treatment group, while that of CD56^{dim} NK cells was significantly lower. The frequency of postpartum NK cells increased significantly in pregnant women under antiviral treatment. Unfortunately, the impact of TDF on HBsAg levels was not analyzed (78). After NAs treatment, the body immunity can be partially recovered. Then, for CHB patients treated with NAs and low HBsAg levels, whether adding Peg IFN has stronger immune effect than continuing NAs treatment? A recent study proved that for CHB patients with low HBsAg levels who received NAs treatment, the addition of PegIFN α was more likely to lead to the decrease or even elimination of HBsAg by increasing the activity of CD56^{bright}NK cells (79).

Several studies have shown that CHB patients who comply with the guideline recommendation to discontinue NAs therapy may have HBsAg seroclearance (80, 81). The best target is CHB patients with no liver cirrhosis, undetectable HBV DNA and HBeAg, as well as low HBsAg levels, especially Asians with HBsAg <100 IU/mL and white people with HBsAg <1000 IU/mL (82). Stopping NAs treatment had no significant effects on NK cells phenotype. On the contrary, the cytotoxic reaction of NK cells increased after NAs cessation, especially in patients with HBsAg clearance. The enhancement of this cytotoxic reaction

was timely related to the increase of ALT in patients, which may indicate the role played by NK cells in the clearance of HBV (83). For patients with functional cure after withdrawal of NAs, increased frequency of functional HBV specific CD8⁺T cells at baseline was related to the continued viral control after withdrawal of NAs. After the end of treatment, these HBV specific T cell responses persisted, but did not continue to increase. HBV specific CD4⁺T cell reaction has a similar trend, but there is no statistical significance (84).

CHB functional cure and cellular immunity

HBV infection would cause damage to innate and adaptive immunity in patients. Previous studies have shown that NAs and PegIFN α offered effective complementary mechanisms of T cells immune reconstitution and NK cells immunity to better achieve the goal of clinical cure (85). In a study comparing NAs and PegIFN α combination therapy with NAs monotherapy, it was found that with the extended treatment time, the number of CD4⁺ and CD8⁺ cells decreased, but no significant difference was seen between the two groups (79). This was also in line with the previous studies that long-term treatment with PegIFN α might lead to depletion of CD8⁺T cells, showing a decreased percentage and absolute values. Additionally, the recovery of HBV-specific CD8⁺T cell function was limited (55). Therefore, suspending PegIFN α while maintaining NAs sequential and combined therapy may better assist the reconstruction of specific immunity, which may be an effective way to achieve clinical cure (86).

Further research of CD4⁺ cell subsets found that a transient increase was seen in Treg cells in the beginning, while decreased in the late stage of treatment. No significant difference was seen in the number of CD4⁺CD25⁺ and CD4⁺CD25^{low} cells between the combination therapy group and the monotherapy group, while only CD4⁺CD25⁺CD127^{low} cells increased significantly in the combination therapy group after 24 weeks of treatment (55). Several studies have shown that NK cells could secrete IFN- γ to inhibit the proliferation and differentiation of Treg cells, moreover, PegIFN α could improve the inhibitory effect of NK cells on Treg cells in CHB patients (67).

As for Th cells, with the increase of treatment time, Th1 cells gradually increased, while Th17 cells gradually decreased. There was no significant difference in the number of Th1, Th2, and Th17 cells between the two groups (79). The interaction between Tfh cells and B cells was critical to humoral immunity, and in a study of patients treated with PegIFN α , the proportion of Tfh cells significantly increased after drug withdrawal in those who achieved complete response (87). Using PegIFN α as a monotherapy in another study found a increasing number of CD40L-expressing Tfh cells in patients with complete response. Tfh cells contributed to the increase of the proportion of specific

B cell subsets and the induction of antibody, thereby improving HBsAg serologic conversion rates in CHB patients (88).

Several studies have confirmed that PegIFN α treatment in children with CHB could achieve a higher HBsAg seroconversion rate and better prognosis than adults. A study of pediatric patients with CHB receiving PegIFN α treatment showed that there were significantly more activated CD8 $^{+}$ cells (CD69 $^{+}$ CD8 $^{+}$, TRAIL $^{+}$ CD8 $^{+}$) and activated CD4 $^{+}$ cells (TRAIL $^{+}$ CD4 $^{+}$) in those who achieved full response compared with those who did not. The HBV-specific T cell responses of CD4 $^{+}$ and CD8 $^{+}$ continued to increase with the advancement of treatment (89). All of the above elucidated the importance of PegIFN α in promoting the activation of immune response and its close association with HBV-specific T cells, revealing part of the mechanism of inducing HBsAg serological transformation.

CHB functional cure and humoral immune response

Targeting HBV-specific B cells as well as inducing anti-HBs antibody responses are considered as important strategies for the rational and effective CHB cure (90). Several research groups have tried different methods targeting vaccinated and CHB patients to detect the responses of HBsAg-specific memory B cell. Through the cultivation of enriched CD19 $^{+}$ cells and the stimulation of CD40-CD40L for 5 days, an HBs-ELISPOT assay was used for the identification anti-HBs secreting B cells (91). The other group used HBsAg-binding microspheres in individuals with enriched CD19 $^{+}$ cells to directly detect HBsAg-specific memory B cells through FACS *in vitro* and showed a significant low HBsAg-specific memory B cells frequency (92). Both of these methods could effectively detect HBsAg-specific B cells in individuals inoculated with HBsAg, however, it was not true in CHB patients. Two recent studies analyzed HBsAg specific B cells in patients with the infection of HBV and used fluorochrome-labeled recombinant HBsAg as “bait” (43, 93). These two groups obtained similar results that a similarity of the frequencies of HBsAg-specific B cells can be found in acute, chronic as well as recovery phase of HBV infection, which had no linkage with HBsAg, HBV DNA and ALT levels. A longitudinal analysis showed that Tfh cells, particularly which expressed CD40L could stimulate the differentiation of B cell and improve the rate of HBsAg seroconversion in CHB patients who receiving PegIFN α monotherapy (88).

Clinical experience indicated that patients with low HBsAg levels who discontinued long-term NAs therapy (94), or shifting to PegIFN α therapy (95) can easily reach HBsAg loss. Therefore, therapeutic effect of B cells targeted vaccination could be optimized through selecting reasonably upon patients with low HBsAg levels who have been treated with NAs for a long time. Actually, a recent study enrolled 20 patients with negative

HBeAg as well as HBsAg < 1000 IU/ml and found that HBsAg decreased significantly in 14 patients, and 2 patients had a HBsAg loss after inoculated conventional HBsAg-based vaccination (96). Another research showed that for HBeAg-negative patients, switching from long-term ETV therapy to adding HBsAg-based vaccination, IFN α -2b and IL-2 as a combination, there was a higher rate of HBsAg loss (9.38%) compared with patients using IFN α -2b alone (3.03%) or continuing entecavir (3.70%) treatment. Moreover, the loss rate of HBsAg was 27.3% in the combination treatment group for patients whose HBsAg titers at baseline level was in the range of 100 to 1500 IU/mL (97). These studies showed that routine HBsAg therapeutic vaccination may increase the loss of HBsAg in CHB patients treated with NAs for a long time, especially whose baseline HBsAg level were low. A recent preliminary immunization study of Sci-BVAcTM selecting NAs-treated CHB patients with low HBsAg levels showed that after vaccination, three patients cleared HBsAg and developed anti-HBs. Although the study involved only a small number of patients, the result was inspiring and suggested the latent utility of this method (98).

CHB functional cure and novel immunomodulatory agents

CHB functional cure and host innate immune activators

PRRs agonists to activate the host's natural immunity lead to the production/expression of proinflammatory cytokines and ISGs, which constitute an antiviral state (99). The effects of TLR-8 agonist GS-9688 on immune cell subsets were studied *in vitro* using PBMC from CHB patients and healthy participants *in vitro* and the results showed that GS-9688 could increase the frequency of CD4 $^{+}$ Tfh cells, activated NK cells, mucosal associated variant T cells and HBV specific CD8 $^{+}$ T cells, as well as reduce that of CD4 $^{+}$ regulatory T cells and MDSCs (65). SB9200 is an activator of NOD2 and RIG-I, which can induce the activation of IFN- α/β and ISG in the blood or liver of WHV infected marmosets and reduce the serum WHV DNA and WHBsAg levels. The study *in vitro* showed that activating the Recombinant Retinoic Acid Inducible Gene I Protein (RIG-I) pathway and the interaction regulatory factor (IRF) 3 induced the innate immune response which could inhibit or even eliminate cccDNA (100). Interferon gene stimulator (STING) is a major regulator of DNA mediated activation of innate immunity and a latent therapeutic target for the infection of virus. In the chronic HBV mouse model, activation of STING signal pathway can inhibit HBV replication by inhibiting the epigenetics of cccDNA, as well as attenuate HBV induced liver fibrosis by inhibiting macrophage inflammatory bodies (101). APOBEC-3 enzyme may mediate lymphotoxin and type I

interferon antiviral activity through cytidine deamination, resulting in the degradation of cccDNA and participating in the host's innate immunity to HBV (102). The α -kinase 1 (ALPK1) can activate NF- κ B pathway and stimulate PRRs of innate immunity. Using mouse and primary human HBV hepatocyte models, a preclinical study investigated the anti-HBV effect of ALPK1 agonist DF-006. The results indicated that DF-006, mainly located in the liver, could activate the innate immunity in the liver and play an anti-HBV role (103).

Unless the subsequent adaptive immunity plays a good role, HBV infection may not be controlled only by activating the innate immunity. Therefore, understanding the characteristics of these innate immune activators not only helps to activate innate immunity, but also helps to discover their role in connecting with adaptive immunity. The role played by the immunomodulatory drugs in development of CHB infection is summarized in Figure 1 and Table 1 (104–129).

CHB functional cure and cellular immune activators

Based on the immune mechanism of the infection of chronic HCV, a variety of immunomodulatory drugs can strengthen the

specific cellular immune response of HBV patients by addressing immunologic targets. Immune checkpoint (PD-1/PD-L1) inhibition can attenuate negative regulation of specific or resuscitate depleted T cells (130). PD-1 blockers have been proved to restore virus-specific CD8⁺T cells, but when they are depleted, blocking PD-1/PD-L1 has limited effect in CHB patients (131, 132). Other checkpoint blockers like CTLA-4 and TIM-3 also restored virus-specific CD8⁺T cells and multiple cellular immunity in CHB patients (132, 133). Lymphocyte-activation gene 3 (LAG-3), as a crucial immunomodulatory molecule, combine with MHC-class II molecules to transmit negative regulatory signals and inhibit T cell activation. Researches have found that impaired CD8⁺T cells recover better when using anti-PD-1 and anti-LAG-3 antibodies at the same time (134).

With the development of gene editing technology, edited T cells can target virus-specific immune dominant epitopes to play a long-term role in controlling HBV infection. For example, in preclinical models, CAR T cells targeting HBsAg have shown anti-HBV ability, but further research and optimization are needed for clinical application (135).

A variety of vaccines are of significant importance in enhancing cellular immune responses in patients with HBV (136). When IL-12 was used as an adjuvant, HBsAg

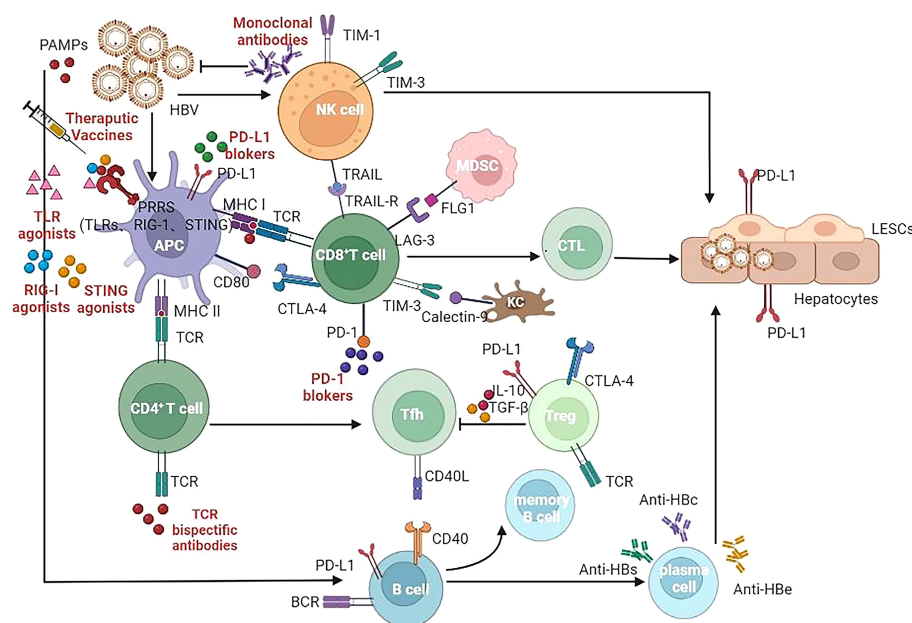


FIGURE 1

Target of immunomodulatory drugs in development for CHB infection. APC, Antigen presenting cells; NK, Natural killer (cells); MDSC, myeloid derived suppressor cells; CTL, cytotoxic lymphocyte; Tfh, T follicular helper (cells); Treg, regulatory T cells; LSEC, liver sinusoidal endothelial cells; TLR, Toll-like receptor; RIG-I, Retinoic Acid Inducible Gene I Protein; STING, stimulator of interferon genes; TCR, T cell receptor; BCR, B cell receptor; MHC, major histocompatibility complex; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; PD-1, programmed death -1; PD-L1, programmed death ligand-1; TIM-3, T-cell immunoglobulin domain and mucin domain-containing molecule-3; TRAIL, TNF-related apoptosis-inducing ligand; LAG-3, Lymphocyte-activation gene 3; FLG1, Fibrinogen Like Protein 1.

TABLE 1 Summary of immunomodulatory drugs in development for CHB infection.

Drug name	Developer, country	Development phase	Main research results	Reference
Therapeutic vaccine: Induce HBV-specific B- and/or T-cell responses or generate new B/T cells				
CVI-HBV-002	CHA Vaccine Institute, Korea	Phase IIb	—	—
GS-4774	Gobellmune with Gilead, USA	Phase II	(Phase II) Safe and well tolerated, no significant HBsAg decreases but increased production of IFN, TNF and IL2 by CD8 ⁺ T cells	(104, 105)
HepTcell	Altimune, USA	Phase II	(Phase Ib) Safe and well tolerated	(106)
VVX001	Viravaxx, Austria	Phase II	(Phase II) Safe and well tolerated, induced preS-specific IgG responses in all patients but declined during follow-up, 87% of vaccinated patients did not repeat NAs after discontinuation until end of 8-month follow-up	(107)
VBI-2601 (BR11-179)	VBI Vaccines, USA with Brii Biosciences, China	Phase IIa/IIb	(Phase Ib/IIa) Safe and well tolerated, anti-HBs responses were induced in >30% patients, moderate anti-Pre-S1 or anti-Pre-S2 antibody responses were only observed in combination with IFN- α therapy, no notable HBsAg reduction was observed	(108)
AIC649	AiCuris, Germany	Phase II	(Phase I) Safe and well tolerated, increased IL-1 β , IL-6, IL-8 and IFN- γ levels, decreased IL-10 levels, the HBV DNA and HBsAg levels were essentially unchanged in most patients	(109)
GSK3528869A	GSK Biologicals, UK	Phase II	—	—
GSK4388067A	GSK Biologicals, UK	Phase II	—	—
HB-110	Ichor Medical Systems with Genexine, USA	Phase II	(Preclinical and Phase I) Safe and tolerable, induced HBV-specific T-cell responses in a portion of patients, exhibited positive effects on ALT normalization and maintenance of HBeAg seroconversion	(110)
VTP-300	Vaccitech, USA	Phase Ib/IIa	(Phase Ib/IIa) Monotherapy: induced HBV specific T cell responses and declined HBsAg only in three patients with low initial HBsAg (-0.7, -0.7 and -1.4 log ₁₀ respectively); Combined with low-dose nivolumab at the boosting time point: induced HBV specific T cell responses and declined HBsAg in most patients (>1 log ₁₀ at month 6)	(111)
VRON-0200	Viron Therapeutics, USA	Phase Ib	(Preclinical) Mouse model: elicited potent and broad CD8 ⁺ T cell responses, decreased HBV DNA by 2-3 log ₁₀ , decreased HBsAg slightly (<1 log ₁₀)	(112)
JNJ 64300545	Janssen, Ireland with Ichor Medical Systems, USA	Phase I	—	—
JNJ 64300535	Janssen, Ireland	Phase I	(Phase I) A ≥ 3 -fold increase in anti-HBc and/or anti-pol responses was observed in 50% of CHB patients and 91.7% of healthy individuals; HBV-specific T-cell responses were stronger in healthy individuals than CHB patients	(113)
TVAX-008	CGE HEALTHCARE, China	Phase I	—	—
CARG-201	CaroGen Crop, USA	Phase I	—	—
Chimigen HBV	Akshaya Bio Inc., Canada	Preclinical	(Preclinical) Cell model: induced vigorous T cell proliferation and enhanced expression of IFN- γ , TNF- α , perforin and granzyme B in both CD4 ⁺ and CD8 ⁺ T cell subsets; Animal model: induced a dose-dependent S1/S2-Core specific antibody response	(114, 115)
HBV	HOOKIPA Pharma, Austria, with Gilead, USA	Preclinical	—	—
TherVacB	Helmholtz Zentrum Muenchen, Germany	Preclinical	(Preclinical) Mouse model: silencing PD-L1 using siRNA enhanced the HBV-specific CD8 ⁺ T cell responses of TherVacB	(116)
PRGN-2013	Precigen, USA	Preclinical	—	—
ISA104	ISA Pharma, Netherlands	Preclinical	—	—

(Continued)

TABLE 1 Continued

Drug name	Developer, country	Development phase	Main research results	Reference
CLB-3000	Clear B Therapeutics, USA and Australia	Preclinical	(Preclinical) Mouse model: reduced HBsAg by more than 2.5 log IU/mL, 80% achieved functional cure and induced anti-HBs responses	(117)
FNX008	FANXI Biopharma, China	Preclinical	—	—
Decoy20	Indaptus Therapeutics, USA	Preclinical	—	—
Innate immune activator: Stimulation of innate immunity effector cells and cytokines, such as T cells, IFNα, etc.				
GS9688 (TLR8 agonist)	Gilead Sciences, USA	Phase II	(Preclinical and Phase II) Good absorption and high first pass clearance in preclinical species; reduced viral markers in HBV-infected primary human hepatocytes; combined with tenofovir alafenamide resulted in a decrease of HBsAg $\geq 0.5 \log_{10}$ IU/mL in 7.4% of patients	(118, 119)
RG7854 (TLR7 agonist)	Roche, Switzerland	Phase II	(Phase I) Safe and acceptably tolerated	(120)
TQA3334 (TLR7 agonist)	ChiaTai TianQing, China	Phase II	(Phase I) Safe and tolerated	(121)
CB06 (TLR8 agonist)	ZhiMeng Biopharma, PR China	Phase I	—	—
HRS9950 (TLR8 agonist)	Hengrui Pharmaceuticals, China	Phase I	—	—
SBT8230 (TLR8 agonist)	Silverback Therapeutics, USA	Preclinical	(Preclinical) NHP: well-tolerated and efficiently targeted to the liver; Mouse model: reduced HBsAg about 2 log and driven seroconversion, lowered HBV DNA titers 2.5 logs, and induced anti-viral antigen T and B cell responses	(122)
YS-HBV-002 (TLR3, RIG1, MDA5 agonist)	YiSheng Biopharma, China	Preclinical	—	—
Apoptosis inducer: Induce and accelerate the process of apoptosis				
APG-1387	Ascentage Pharma, China	Phase II	(Preclinical) Mouse model: completely cleared of HBsAg, HBeAg and HBV DNA in serum, as well as HBcAg and HBV replicative intermediates in infected livers, no relapse after stop therapy; upregulated HBV-specific CD4 ⁺ and CD8 ⁺ T cells frequency and function; upregulated immune-related genes	(123)
Monoclonal antibody: Neutralize HBV-related antigens and inhibit HBV cell entry, thereby stimulate the immune responses				
GC1102	GC Pharma, South Korea	Phase II	(Phase I) Safe and tolerable, multiple doses of 80,000 IU and 240,000 IU resulted in HBsAg loss in 12.5% and 22.2% of patients, and all patients had baseline HBsAg ≤ 1 000 IU/mL	(124)
VIR-3434	Vir Biotech, USA with Bria Biosciences, China	Phase I	(Phase I) A single dose of 6 mg, 18 mg, or 75 mg induced mean HBsAg decreases of 1.30, 1.27 and 1.96 $\log_{10}$ IU/mL, but apparent rebound in HBsAg levels at week 8 was observed in all groups	(125)
162	Yangshengtang Co., Ltd, China	Phase I	—	—
BJT-778	Blue Jay Therapeutics, USA	Preclinical	—	—
KW-027	Kawin Technology, China	Preclinical	—	—
Immune checkpoint inhibitor: Reversal of T cell exhaustion by inhibiting PD-1 or PD-L1				
ASC22 (Anti-PD-L1)	Ascleptis Pharma, PR China	Phase IIb	(Phase IIb) Reduced HBsAg by more than 0.5 $\log_{10}$ IU/mL in 21% patients and among those patients 28.6% and 14.3% achieved HBsAg seroclearance and HBsAg seroconversion, respectively	(126)

(Continued)

TABLE 1 Continued

Drug name	Developer, country	Development phase	Main research results	Reference
RG6084 (Anti-PD-L1)	Roche, Switzerland	Phase II	—	—
GS4224 (Anti-PD-L1)	Gilead Sciences, USA	Phase I	—	—
JNJ 63723283 (Anti-PD-L1)	Janssen, Ireland	Phase I	—	—
AB-101 (Anti-PD-L1)	Arbutus Biopharma, USA	Preclinical	(Preclinical) MC38 tumor model: reduced tumor by activating T cells with considerable potency as anti-PD-L1 antibody; PBMCs from CHB patients: reinvigorated HBV-specific T cells	(127)
ARB-272572 (Anti-PD-L1)	Arbutus Biopharma, USA	Preclinical	—	—
ALG-093453 (Anti-PD-1/PD-L1)	Aligos Therapeutics, USA	Preclinical	(Preclinical) PBMCs from CHB patients: activated HBV-specific T cells to similar or higher extend as nivolumab and durvalumab	(128)
Other immunological candidates: Redirect nonHBV-specific T cells or stimulate innate immunity to eliminate virus-infected cells				
IMC-I109V	Immunocore, USA	Phase I/II	(Phase I/II) Safe, decreased HBsAg levels transiently by 11–15% and then returned to baseline within 3weeks after a single low dose	(129)
LT-V11	Lion TCR, Singapore	Preclinical	—	—
TCR bispecific antibodies	Lion TCR, Singapore	Preclinical	—	—
ALVR107	AlloVir, USA	Preclinical	—	—

CHB, chronic hepatitis B; HBV, hepatitis B virus; TLR, Toll-like receptor; RIG-I, Retinoic Acid Inducible Gene I Protein; PD-L1, programmed death ligand-1.

immunization effectively reversed the systemic tolerance to HBV protein in HBV infected mice, correlated with the enhancement of HBV-specific CD4⁺/CD8⁺ T cell responses as well as the decrease of CD4⁺Foxp3⁺Treg cells (137). HBcAg-specific cytotoxic T cells are essential in controlling HBV replication, but unfortunately, vaccines containing HBcAg core 18-27 and Th epitopes are not very effective in eliminating HBV (138). On the other hand, the HBsAg/HBcAg composite vaccine developed based on the above can induce Th1/Th2 response of HBsAg, as well as maintain a balance towards Th1 type response (139). Safe and effective treatment was demonstrated in a two-year follow-up clinical study (140).

At present, there are still a variety of new immunomodulatory drugs under research, has achieved positive results. IMC-I190V is a novel bispecific protein immunotherapy that specifically clears HBsAg expressing hepatocytes infected with HBV by T cell reorientation. LT-V11 is a T-cell receptor that enhances host immunity by recruiting T cells to clear hepatocytes containing cccDNA or integrating HBV DNA. Undisclosed (CBE) can produce nonsense mutations in HBsAg and HBeAg/HBcAg ORFs, and can also cause cccDNA enrichment regions to affect HBV replication. ALVR107 is a kind of allograft under research, which aims to target HBV infected cells, and its own or allogeneic reactivity is relatively small, and has achieved very impressive early results.

CHB functional cure and humoral immune activators

Studies have shown that adoptive transfer of anti-HBs-positive bone marrow to CHB patients can eliminate HBV in recipients, and that transfer of anti-HBs-positive donor immune cells toward HBV-infected recipients through liver transplantation can partially control HBV infection in the transplanted liver, suggesting that immune modulation is one of the essential strategies to achieve functional cure of CHB (141). Research had indicated that the acquisition and maintenance of HBsAb could be achieved through the co-activation of B cells and lineages after vaccination (142).

The likelihood of antibody mediated immunotherapy against CHB infection increased thanks to the success of polyclonal hepatitis B immunoglobulins (HBIG) (143). A pilot study involved eight amivudine-treated CHB patients treated with monthly HBIG injections and then received hepatitis B vaccination showed that after 1 year treatment, serum HBsAg decreased significantly in half of the patients. Notably, three patients were positive for anti-HBs, thus achieving a functional cure. This suggested that the combination of antiviral therapy and antibody mediated immunotherapy can trigger the B cell response after vaccination and achieved continuous HBsAg loss and functional cure (144). Zhong et al. conducted a study including 36 healthy individuals without previous history of

HBV infection and HBsAb negative were given a series of three HB vaccine doses for some time of 0, 1, and 6 months with longitudinal follow-up. The result revealed that with the progress of vaccination, the overall trend of the small S protein of hepatitis B virus (SHBs) peptide coverage increased and the individual subregion recognition rate was closely related to the anti-HBs titers (145).

Problems and prospects

IFN-based regimens have many disadvantages: inconvenient administration, long treatment duration, many and severe adverse reactions, and low functional cure rate. The host immune system is an organic whole, and the synergistic effect of natural immunity and adoptive immunity can eliminate HBV. Unlike the immunomodulatory drugs being developed that act on one or several link points of the host immune system, IFN is greatly important in both natural and adoptive immunity. Therefore, in the foreseeable future, IFN cannot be separated from the pursuit of CHB cure (146). The improvement of IFN itself (longer half-life, higher targeting of hepatocytes, fewer and lighter adverse reactions) and the optimization of IFN-based treatment scheme (higher functional cure rate) are one of the future development directions (147).

Although the research and development of new drugs are in full swing, it is far from the clinical practice. Many drugs targeting the human immune system, including therapeutic vaccines, TLR agonists, apoptosis inducers, immune checkpoint inhibitors, are in Phase 2 of clinical trials, and some are still in the preclinical exploration stage (148). The effectiveness and safety need to be further verified.

Even if novel agents go on the market, there is still a lot of work to optimize the treatment regimens. It is generally believed that the cure of CHB must be the combination of targeted HBV and host drugs, including inhibition of HBV replication, reduction of viral antigen level, immune regulation, etc. There are many drugs that in clinical trials or have been on the market in each aspect. Immune regulation involves innate immunity, specific cellular immunity, specific humoral immunity, cytokines, etc. The successful treatment of chronic HBV infection depends on not only activation of the innate immunity, but also restoration of the specific adoptive response.

Powerful and low drug resistant drugs that inhibit HBV replication have been used for many years, and one of the first-line NAs can be selected. There are many kinds of drugs to reduce the level of viral antigen, involving all aspects of viral replication cycle. The drugs regulating immune function are more. In the future, the combination of 3-4 or more drugs is likely to be required to pursue the cure of CHB. It is hoped that in the future, as in the treatment of CHC, there will be drug combinations or compound preparations with fewer times of

medication, fewer tablets, fewer adverse reactions, less treatment time and higher functional cure rate.

The indications for antiviral therapy of CHB are gradually expanding. Not limited to those patients in immune clearance period (HBeAg positive) and replication period (HBeAg negative), inactive HBsAg carriers in low replication period and chronic HBV infected persons in “gray area” are gradually included in the scope of antiviral treatment. Patients in immune tolerance period begin to enter the agenda (149). Even more and more hepatologists have put forward the idea of treatment for all CHB patients. These have brought great challenges to the research and development of antiviral drugs and the exploration of treatment schemes.

Functional cure is not equal to complete cure. There is still about 10% recurrence rate and 1-2% incidence of HCC. The detection of cccDNA and integrated HBV DNA was not standardized, and the sensitivity was low. As a “hard indicator” of functional cure, HBsAg is not an ideal one for early evaluation of clinical efficacy. The search for the biomarkers closest to complete cure including immunological markers is also needed in clinical practice (150).

Author contributions

Study concept, design and supervision (BF), acquisition, analysis and interpretation of data, drafting of the manuscript (J-RZ), critical revision of the manuscript for important intellectual content (Z-LW). All authors have made a significant contribution to this study and have approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Functional molecular expression of nature killer cells correlated to HBsAg clearance in HBeAg-positive chronic hepatitis B patients during PEG-IFN α -2a therapy

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Objective: To explore whether the frequencies and functional molecules expression of Natural Killer cells (NK cells) are related to hepatitis B surface antigen (HBsAg) disappearance in hepatitis B e envelope antigen (HBeAg)-positive patients with chronic hepatitis B (CHB) throughout peginterferon alpha-2a (PEG-IFN α -2a) treatment.

Methods: In this prospective research, HBeAg-positive patients with CHB received PEG-IFN α -2a treatment, completing 4-year follow-up. After PEG-IFN α -2a treatment, undetectable HBV DNA, HBsAg loss, and HBeAg disappearance were defined as functional cure. Proportions of NK, CD56^{dim}, CD56^{bright}, NKp46⁺, NKp46^{dim}, NKp46^{high}, and interferon alpha receptor 2 (IFNAR2)⁺ NK cells, and the mean fluorescence intensity (MFI) of NK cell surface receptors IFNAR2 and NKp46 were detected.

Results: 66 patients were enrolled into the study in which 17 patients obtained functional cure. At baseline, hepatitis B virus deoxyribose nucleic acid (HBV DNA) titer in patients with functional cure was remarkably lower than that in Non-functional cure group. Compared with baseline, HBV DNA levels, HBsAg levels, and HBeAg levels significantly declined at week 12 and 24 of therapy in patients with functional cure. At baseline, the negative correlation between CD56^{bright} NK% and HBV DNA and the negative correlation between CD56^{dim}

NK% and HBV DNA was showed; CD56^{bright} NK% and IFNAR2 MFI in patients with functional cure were remarkably higher than those in patients without functional cure. After therapy, CD56^{bright} NK% and NKp46^{high} NK% in patients with functional cure were higher than those in patients without functional cure. In Functional cure group, after 24 weeks of treatment NK%, CD56^{bright} NK%, IFNAR2 MFI weakly increased, and NKp46^{high} NK% and NKp46 MFI significantly increased, meanwhile, CD56^{dim} NK% and NKp46^{dim} NK% decreased. Only NKp46 MFI increased after therapy in patients without functional cure.

Conclusion: The lower HBV DNA load and the higher CD56^{bright} NK% before therapy, and the higher the post-treatment CD56^{bright} NK%, IFNAR2 MFI, NKp46^{high} NK%, the easier to achieve functional cure.

KEYWORDS

nature killer cells, chronic hepatitis B, PEG-IFN α -2a, hepatitis B virus, HBsAg loss

Introduction

Hepatitis B virus (HBV) infection is still a public health problem worldwide; especially chronic HBV infection which is very harmful to human health (1, 2). Therefore, eliminating hepatitis B has become the topical issue over recent years. Annually, the rate of spontaneous HBsAg clearance is approximately 1% in natural history of chronic HBV infection (3, 4). It is urgent to explore the optimal treatment strategies for CHB. At present, the most effective treatment to delay the progress of CHB is antiviral therapy. However, if we want to enhance living quality and lengthen the survival period to the most extent, “functional cure”, identified as undetectable HBV DNA, persistent HBsAg loss, whether or not accompanied by HBsAg serological conversion (5, 6), is pursued as the treatment end point (7, 8). Currently, by NAs therapy, CHB patients achieve HBsAg loss at a lower rate <1% per year, while HBsAg clearance rate is the highest in CHB patients with HBeAg positivity, who receive IFN therapy (9–12). Therefore, improving the hosts’ immune response through PEG-IFN optimal treatment can more effectively eliminate the virus and even achieve clinical cure (13–15).

As a crucial component in innate immunity, NK cells have a key part in eliminating viruses as well as immunologic defence (16). NK cells consist of CD56^{bright} NK cells and CD56^{dim} NK cells. Generally, CD56^{bright} NK cells have a key role mainly in triggering the specific immune response by secreting immunomodulation cytokines and CD56^{dim} NK cells have a key role in directly eliminating target cells (17, 18). The function of NK cells is induced by an array of inhibiting or active surface receptors (19). NKp46, as the activated receptor on the surface of NK cells, is a key receptor to activate the killing activity of NK

cells. IFNAR2 expressed on the surface of NK cells plays an antiviral role when the expression was upregulated and can make IFN- α more easily to activate NK cells, helping to eliminate viruses. IFNAR2 deficiency results in dysregulation of NK cell functions (20). In humans, some studies showed that NK cells were activated in patients with acute HBV infection (21), with the increased expression of activation markers including NKp46, the increased CD56^{bright} NK cells subpopulation, the cytotoxicity and IFN- γ production of NK cells (22–24). However, the activities of NK cells are significantly damaged in CHB patients. HBsAg and HBeAg directly inhibit function of NK cells by multi-signal pathways correlated to the activation of NK cells (25–27). The up-regulation of inhibition receptors and the down-regulation of activation receptors, as the phenotypic characteristics of NK cells, are commonly found in chronic infection, leading to the impairment of effector functions, which may lead to the persistence of viruses (28).

PEG-IFN α increases the inhibiting effect of NK cells on regulatory T cells by IFN- γ in CHB (29). After IFN treatment, remarkable increase in expansion and activation of CD56^{bright} NK cells, IFN- γ production, and augmentation of NK cell activating receptors (NKp46 and NKp30) expression are observed (30, 31). After PEG-IFN treatment, the recovery of CD56^{bright} NK cells function and expansion of percentages of NKp46⁺ and CD56^{bright} NK cells were revealed in inactive HBsAg carriers and CHB patients with NAs therapy (32, 33). The percentage and functional molecular expression of NK cells correlated to HBsAg clearance in treatment-naïve HBeAg-positive CHB patients during PEG-IFN α -2a therapy are relatively rare. Thereby, the aim of the research is to probe in treatment-naïve CHB patients with HBeAg positivity, whether proportion of NK cells and functional molecules expression on

the surface of NK cells are correlated to functional cure throughout PEG-IFN α -2a treatment.

Materials and methods

Subjects

In the nonrandomized, single center, prospective cohort study, 66 HBeAg (+) CHB patients with naïve therapy were enrolled from October 2014 to November 2017 visiting the Liver Disease Center of Beijing Ditan Hospital, Capital Medical University. The inclusion criteria were detectable HBV DNA, HBsAg positive for > 24 weeks, HBeAg positive, and continuously abnormal alanine aminotransferase (ALT) for > 12 weeks, or liver puncture indicating apparent inflammation. Exclusion criteria were the followings (1): co-infected with other viruses (HDV, HIV, or HCV et al) (2); HCV, HIV or syphilis antibodies positive (3); complicated with other liver diseases consisting of fatty liver, hepatic cirrhosis, hepatocellular carcinoma, autoimmune hepatitis, metabolic liver disease, alcoholic liver disease et al (4); use of hormone or immunomodulatory medication. All patients signed written informed consent. Our study was approved by Beijing Ditan Hospital Institutional Review Board and registered at clinicaltrials.gov (ID: NCT03208998).

All patients received individualized treatment and followed up for 4 years. As follows, patients were treated with PEG-IFN α -2a alone, 180 μ g subcutaneous injection weekly for 24 weeks. After 24 weeks of treatment, HBV DNA negative patients would continue PEG-IFN α -2a single-drug treatment. However, patients who didn't achieve HBV DNA negative needed PEG-IFN α -2a combined with tenofovir or entecavir treatment, and specific need for treatment with entecavir or tenofovir depended on previous medical history and health status of those patients. In the follow-up therapy, on the basis of the decrease of HBsAg and HBeAg levels, if HBsAg and HBeAg levels continued to decrease, or even HBsAg clearance, patients were continually treated with PEG-IFN α -2a consolidation therapy for 12 weeks-24 weeks, then drug withdrawal. Nevertheless, if HBsAg and HBeAg levels didn't decrease continuously and remained at the platform period, PEG-IFN α -2a needed to be stopped, then the maintenance monotherapy of ETV or TDF would be continued. HBsAg/HBsAb levels, HBeAg/HBeAb levels, HBV DNA titers and ALT were tested before therapy and per 12 weeks after treatment till HBsAg disappeared. Measurements of the frequency and functional molecule expression of NK cells were performed before therapy and at 12 weeks and 24 weeks of therapy.

Functional cure was as follow: persistent HBsAg disappearance, whether or not accompanied by HBsAg serological conversion, HBeAg loss with or without seroconversion, and HBV DNA negative. Conversely, it was defined as non functional cure.

Assessment of clinical parameters

Serum HBsAg/HBsAb and HBeAg/HBeAb levels were quantitated by Abbott Architect i2000 Detection Reagent (Abbott Diagnostics, Abbott Park, IL); HBV DNA titers were detected by Roche Cobas AmpliPrep/Cobas TaqMan 96 full-automatic real-time fluorescent quantitative PCR detection reagent (Roche, Pleasanton, CA); and biochemical indexes were measured by Hitachi 7600 full-automatic biochemical analyzer (Hitachi, Japan). The detection range of HBsAg was 0.05-250 IU/ml, HBeAg ≥ 1 S/CO was positive, and HBeAb < 1 S/CO was positive. The lower limit of serum HBV DNA detection value was 20 IU/ml. The upper limit of normal ALT value was 40 U/L.

Detection of NK cells

Peripheral venous blood of all patients was collected by EDTA anticoagulant violet tube before therapy (baseline), at the 12 and 24 weeks after PEG-IFN therapy. Cell phenotyping stained with four monoclonal Antibodies (mAbs) including Mouse anti-Human-CD3-PerCP (Becton-Dickinson, USA; clone: SP34-2), Mouse anti-Human CD56-APC (Becton-Dickinson, USA clone: B159), anti-Human CD335 (NKp46)-PE (Biolegend, USA; clone: 9E2), and Anti-Human IFNAR2-FITC (Sino Biological, Beijing, China; clone: 07), were analyzed by flow cytometry (FACS Caliburflow Cytometer, USA) within 4 hours after collection, as shown in [Supplementary Figure 1](#). The proportions of NK, CD56^{dim} and CD56^{bright}, NKp46⁺, and IFNAR2⁺ NK cells, and MFI of NKp46 and IFNAR2 were analyzed by FlowJo 7.6.1 software. Expression of surface receptors NKp46 and IFNAR2 on NK cells represented NK cell function.

Statistical methods

In the research, statistical data were processed by SPSS 26 (SPSS Inc., Chicago, USA). continuous variables were represented by mean $\pm$ standard deviation or median (Q1, Q3). Repeated-measures analysis of variance was used for changes of the indexes at three observation time points, and p values for multiplicity were adjusted by Bonferroni, and p < 0.016 was considered as statistical significance. Independent sample t-test was used to analyze normal distribution data and Mann-Whitney nonparametric U test was used to analyze skewness distribution data between two groups. Spearman's correlation was applied for the association of variables. We used binary logistic regression analysis to analyze independent factors of functional cure. P < 0.05 was considered to be statistically significant.

Results

Baseline features of various indicators in patients with CHB

In our research, 66 HBeAg (+) patients with CHB received PEG-IFN- α -2a individually, following up for 4 years, including 40 males and 26 females. Patients with undetectable HBV DNA and HBsAg disappearance were considered as functional cure, on the contrary, as non functional cure, with 17 cases obtaining functional cure. Among them, 3 patients with HBsAg clearance lost the detection of the frequency and functional molecules expression of NK cells at 12 weeks or 24 weeks of therapy, and 9 patients without HBsAg clearance lost the detection of the frequency and functional molecules expression of NK cells at 12 weeks or 24 weeks of therapy.

The baseline serological virological indicators of the two groups were compared. We found that the serum HBsAg, HBeAg and ALT had no remarkable statistical differences, respectively, between two groups (Table 1), but the HBV DNA titers were lower in functional cure group than those in non functional cure group ($P = 0.001$) in Table 1. As shown in Table 1, at 12 weeks and 24 weeks of therapy, HBV DNA ($P = 0.034(12w)$, $P = 0.005(24w)$), HBsAg ($P = 0.000(12w)$, $P = 0.000(24w)$), as well as HBeAg ($P = 0.030(12w)$, $P = 0.011(24w)$) in patients without functional cure were remarkably higher than those in patients with functional cure. Before

therapy, CD56^{bright} NK/NK% ($P=0.000$), CD56^{dim} NK/NK% ($P=0.000$) and IFNAR2 MFI ($P=0.043$) had significant statistical differences between functional cure group and non functional cure group (Table 2).

We used Binary logistic regression analysis to analyze baseline clinical indicators, proportions and functional molecules expression of NK cells in two groups, but no independent influencing factors of functional cure were observed.

Correlation of the parameters of NK cells with virological indicators and ALT at baseline

At baseline, we performed the correlation analysis on the frequency of NK cells, the expression of surface functional molecules NKp46, INFAR2 and HBV virological characteristics. We found that there were no correlations between NK cell frequency or function (NK%, IFNAR2+ NK/NK%, and IFNAR2 MFI) and serological and virological indicators (HBsAg, HBeAg, and HBV DNA, ALT). (Figure 1A-F, H-J, L-T) And there were no correlations between the expression of surface functional molecules NKp46 and virological indicators. The negative correlation between CD56^{bright} NK% and HBV DNA ($r=-0.243$, $P = 0.049$) (Figure 1G) and the negative correlation between CD56^{dim} NK% and HBV DNA ($r=0.243$, $P = 0.049$) (Figure 1K) was observed.

TABLE 1 Comparison of clinical characteristics.

TItem	All patients N = 66	Baseline		P value	12w		P value	24w		P value
		Non-functional cure group N = 49	Functional cure group N = 17		Non-functional cure group N = 40	Functional cure group N = 14		Non-functional cure group N = 40	Functional cure group N = 14	
Male/ Female	40/26	32/17	8/9		25/15	7/7		25/15	7/7	
Age(yrs)	30 (20–51)	30 (20–51)	32 (24–49)	0.476	30 (20–51)	31 (24–49)	0.976	30 (20–51)	31 (24–49)	0.976
HBsAg (log10 IU/ mL)	3.864 $\pm$ 0.678	3.911 $\pm$ 0.667	3.727 $\pm$ 0.710	0.833	3.377 $\pm$ 0.702	1.720 $\pm$ 1.470	0.000	3.196 $\pm$ 0.802	1.509 $\pm$ 1.683	0.000
HBV DNA (log10 IU/ mL)	7.070 $\pm$ 1.136	7.329 $\pm$ 0.908	6.324 $\pm$ 1.405	0.001	4.095 $\pm$ 1.555	3.146 $\pm$ 1.560	0.034	2.084 $\pm$ 0.760	1.233 $\pm$ 0.879	0.005
HBeAg (PEIU/mL)	879.460 (372.835, 1396.338)	878.350 (369.29, 1350.645)	880.570 (441.715, 1409.685)	0.809	59.895 (16.438, 406.53)	21.895 (0.353, 88.013)	0.030	18.920 (5.728, 82.250)	1.855 (0.325, 17.475)	0.011
ALT level (U/L)	253.100 (129.525, 355.550)	234.700 (127.400, 357.400)	273.000 (135.250, 371.150)	0.337	67.150 (49.450, 82.050)	64.250 (20.50, 101.825)	0.608	44.250 (28.150, 56.425)	28.700 (22.350, 67.975)	0.424

ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen. P value: Non-functional cure group vs Functional cure group. P value <0.05, statistical significance.

TABLE 2 and surface receptors NKp46 and INFAR2 expression of NK cells at baseline between functional cure group and non-functional cure group.

Item	All subjects (n = 66)	Non-functional cure group (n = 49)	Functional cure group (n = 17)	P value*
Gender(M/F)	40/26	32/17	8/9	
NK(%)	11.024 ± 6.011	11.361 ± 6.201	10.054 ± 5.486	0.491
CD56 ^{bright} NK/NK(%)	14.367 ± 7.661	12.466 ± 6.739	19.848 ± 7.695	0.000
CD56 ^{dim} NK/NK(%)	85.633 ± 7.661	87.534 ± 6.739	80.152 ± 7.695	0.000
IFNAR2 ⁺ NK/NK(%)	4.998 ± 2.788	5.115 ± 2.809	6.664 ± 2.782	0.450
IFNAR2 MFI	21.750 (15.175, 27.200)	19.900 (14.450, 25.900)	26.300 (18.375, 39.800)	0.043
NKp46 ⁺ NK/NK(%)	90.765 ± 6.621	90.363 ± 7.509	91.924 ± 2.646	0.687
NKp46 ^{bright} NK/NK(%)	17.886 ± 8.506	17.342 ± 8.911	19.456 ± 7.224	0.194
NKp46 ^{dim} NK/NK(%)	82.114 ± 8.506	82.658 ± 8.911	80.544 ± 7.224	0.194
NKp46 MFI	91.600 (63.775, 138.750)	88.400 (57.200, 134.500)	107.000 (87.050, 159.500)	0.086

*Non-functional-cure group vs. Functional cure group. In all analyses, P value < 0.05, statistical significance.

NK cells function and CHB function cure

The baseline P values in Table 2 and Figure 2 were different, because Table 2 showed the total number of patients in the functional cure group and the non functional cure group, and at 12 and 24 weeks of treatment, 3 patients in the functional cure group and 9 patients in the non functional cure group were lost to follow-up. Figure 2 showed the statistical analysis after excluding the lost patients. At baseline and at 12 weeks and 24 weeks of therapy, CD56^{bright} NK/NK% ($P = 0.001$ (0w); $P = 0.012$ (12w); $P = 0.001$ (24w)), NKp46^{high} NK/NK% ($P = 0.048$ (0w); $P = 0.004$ (12w); $P = 0.004$ (24w)) in functional cure group were markedly higher than those in non functional cure group. CD56^{dim} NK/NK% ($P = 0.001$ (0w); $P = 0.012$ (12w); $P = 0.0001$ (24w)), NKp46^{dim} NK/NK% ($P = 0.048$ (0w); $P = 0.004$ (12w); $P = 0.004$ (24w)) in functional cure group were markedly lower than those in non functional cure group in Figure 2.

In Figure 3, in functional cure group, our results showed NK %, CD56^{bright} NK/NK% and IFNAR2 MFI weakly increased after 24 weeks of treatment, CD56^{dim} NK/NK% decreased after treatment. However, p values for multiplicity were adjusted by Bonferroni, so $p > 0.016$ has no significant statistical significance.

As shown in Figure 4, in functional cure group, NKp46^{high} NK/NK% after 12 weeks of treatment was dramatically higher than that at baseline ($P = 0.005$), NKp46^{dim} NK/NK% after 12 weeks of treatment was markedly lower than that before therapy ($P = 0.005$), and NKp46 MFI after 24 weeks of treatment ($P = 0.005$) was dramatically higher than that before treatment. In non functional cure group, NKp46 MFI was significantly higher after treatment ($P = 0.000$) than that before treatment.

Discussion

NK cells, as the main components of innate immunity, play a crucial part in viral elimination, meanwhile, they participate in the

process of liver tissue damage (34). CD56^{bright} and CD56^{dim} NK cells are the subsets of NK cells. The main function of CD56^{bright} NK cells is to secrete cytokines, including TNF- α , IFN- γ and others, and play an antiviral role through non cytotoxic effects. CD56^{dim} NK cells kill viral infected liver cells through cytotoxic effects mediated by granzyme and perforin (35). NKp46 is an activated receptor on NK cell surface. In acute hepatitis B infection, expression of activating receptor NKp46 is up-regulated, which resulted in enhancement of NK cell activity (23); The surface of NK cells also expresses the receptor IFNAR2, and the up regulation of IFNAR2 expression can make IFN- α more easier to activate NK cells, play an antiviral role, and help to eliminate viruses. However, several studies have reported that in chronic HBV infection, dysfunction of NK cells is revealed, which is not conducive to the elimination of HBV (36). Therefore, we need to choose the optimal treatment to restore and even enhance the immune cell function, so as to enhance the hosts' immune response to the viruses, which may lead to HBsAg loss and achieve functional cure. In a variety of treatment methods for CHB, PEG-IFN has antiviral role and immunomodulatory effect. Previous studies have shown that PEG-IFN therapy can enhance function of NK cells and facilitate virus clearance (13, 31–33). NK cells were the important part of innate immunity. Interferon triggered innate immunity, which took effect at 12 weeks of treatment and peaks at 24 weeks. The early to mid-term efficacy of interferon on natural killer cell induction determined the subsequent efficacy, so the changes in the characteristics of NK cells at 12 and 24 weeks of treatment were tested (25). In our research, we explored in CHB patients with HBeAg positivity, whether proportions and function of NK cells were related to functional cure throughout IFN treatment.

Our results demonstrated that HBV DNA titers in patients without HBsAg clearance were significantly higher than those in patients with HBsAg clearance, meanwhile, HBV DNA, HBsAg, and HBeAg dramatically declined during IFN therapy in patients with functional cure, which correspond to previous studies demonstrating the lower HBV DNA, the lower transcription and

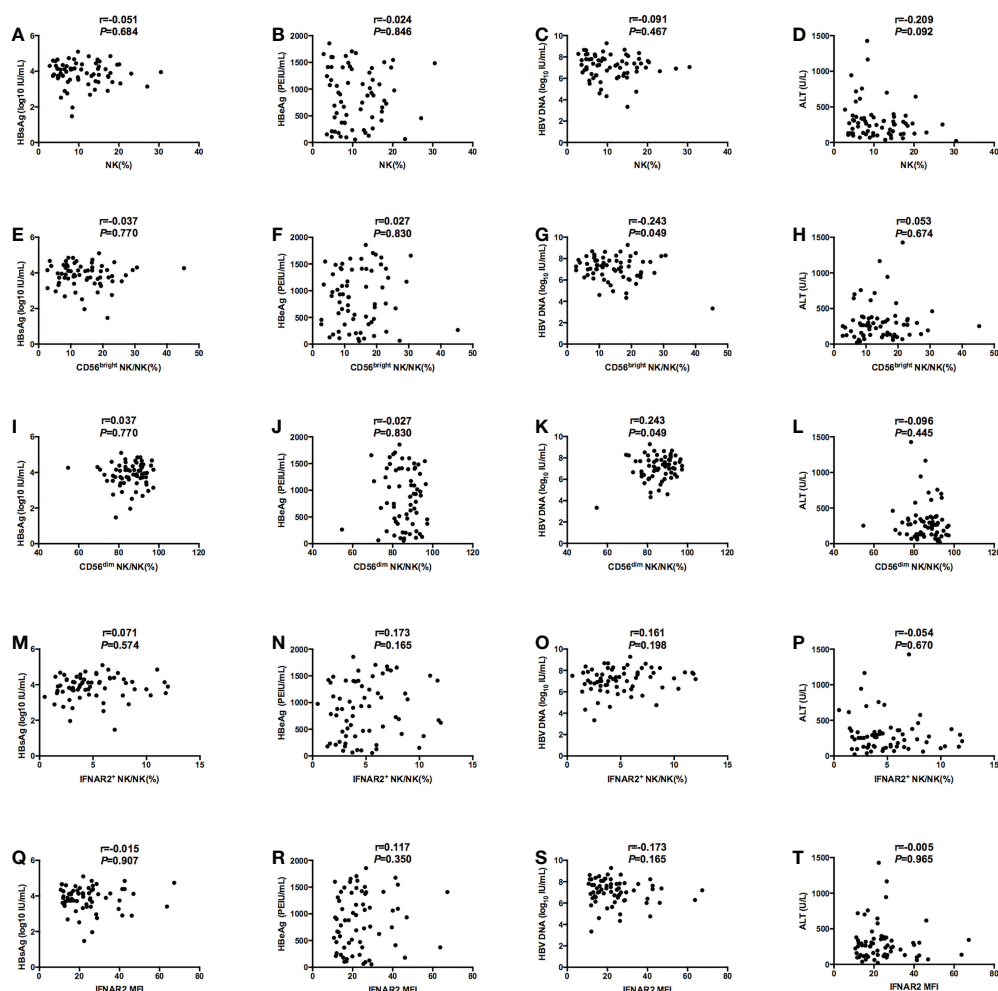


FIGURE 1

Correlations between NK cell frequency or function (NK%, CD56^{bright} NK/NK%, CD56^{dim} NK/NK%, IFNAR2⁺ NK/NK%, and IFNAR2 MFI) and serological and virological indicators (HBsAg, HBeAg, and HBV DNA, ALT). (A–D) Correlations between NK% and serological and virological indicators (HBsAg (A), HBeAg (B), and HBV DNA (C), ALT (D)). (E–H) Correlations between CD56^{bright} NK/NK% and serological and virological indicators (HBsAg (E), HBeAg (F), and HBV DNA (G), ALT (H)). (I–L) Correlations between CD56^{dim} NK/NK% and serological and virological indicators (HBsAg (I), HBeAg (J), and HBV DNA (K), ALT (L)). (M–P) Correlations between IFNAR2⁺ NK/NK% and serological and virological indicators (HBsAg (M), HBeAg (N), and HBV DNA (O), ALT (P)). (Q–T) Correlations between IFNAR2 MFI and serological and virological indicators (HBsAg (Q), HBeAg (R), and HBV DNA (S), ALT (T)). P < 0.05 was considered to be statistically significant.

replication activities of HBV DNA, which leads to the decrease of viral protein production and even the reduction of cccDNA (37), the easier to achieve HBsAg loss and even functional cure.

Previous studies suggested that HBsAg and HBeAg could inhibit the function of NK cells resulting in insufficient immune response without the virus clearance (25–27). Negative correlation between HBsAg level and percentage of CD56^{bright} NK cells was found in previous study (31). Even though we didn't observe the correlation between CD56^{bright} NK cells and HBsAg, our result demonstrated that the baseline CD56^{bright} NK was negatively correlated with HBV DNA. Tjwa ET et al. found that the reduction of viral load improved the function of NK cells in chronic HBV infected patients (38). Some studies suggested that

the decrease of viral load achieved through antiviral treatment partially reshaped phenotype and function of NK cells (13, 17), which indirectly indicated that HBV DNA had an inhibiting role on NK cells. In our study, we found that HBV DNA before therapy in patients with functional cure was dramatically lower than that in patients without non functional cure, as well as CD56^{bright} NK increased after PEG-IFN therapy. Therefore, our results were consistent with the above conclusions. Our study suggested that the baseline CD56^{bright} NK% at baseline and post-treatment in the functional cure group were higher than those in the non-functional cure group, meanwhile, CD56^{bright} NK% increased, therefore, the higher baseline CD56^{bright} NK%, the more likely to obtain functional cure. After PEG-IFN treatment, the immunity was

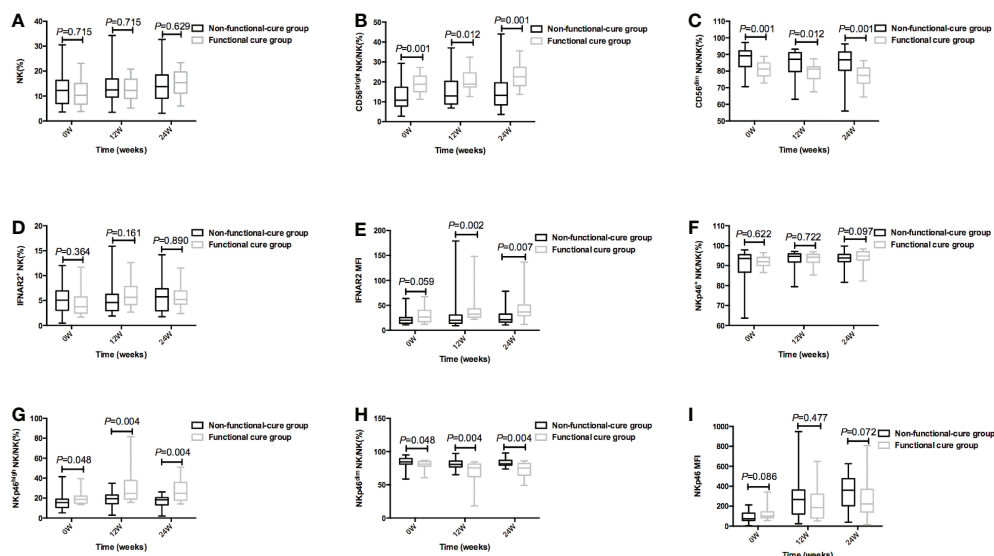


FIGURE 2

During PEG-IFN α -2a therapy, the frequency of NK cells and surface functional molecules [NK% (A), CD56^{bright} NK/NK% (B), CD56^{dim} NK/NK% (C), IFNAR2+ NK/NK% (D), IFNAR2 MFI (E), Nkp46 NK/NK% (F), Nkp46^{high} NK/NK% (G), Nkp46^{dim} NK/NK% (H), and Nkp46MFI (I)] between Non-functional-cure group and Functional cure group were compared before therapy and at 12 weeks and 24 weeks of therapy, respectively. $P < 0.05$ was statistically significant.

up-regulated, and the immune response in the functional cure group was better, which made CD56^{bright} NK increase. Thus, patients with high baseline level and good immune response after treatment were more likely to achieve functional cure. Our data also showed that CD56^{bright} NK was negatively correlated with HBV DNA at baseline and significantly higher in patients with functional cure than those without functional cure, meanwhile it also increased in the former group after PEG-IFN therapy, which

were in line with previous studies indicating that the ability of CD56^{bright} NK cells to secrete IFN- γ was significantly enhanced during HBeAg seroconversion, which proved the importance of IFN- γ secreted by CD56^{bright} NK cells in controlling virus replication (39). Not only did IFN- α have direct antiviral role, but also made the up-regulation of TRAIL expression on the surface of NK cells, activated CD56^{bright} NK cells, and increased IFN- γ secretion (40). Activated CD56^{bright} NK cells may be

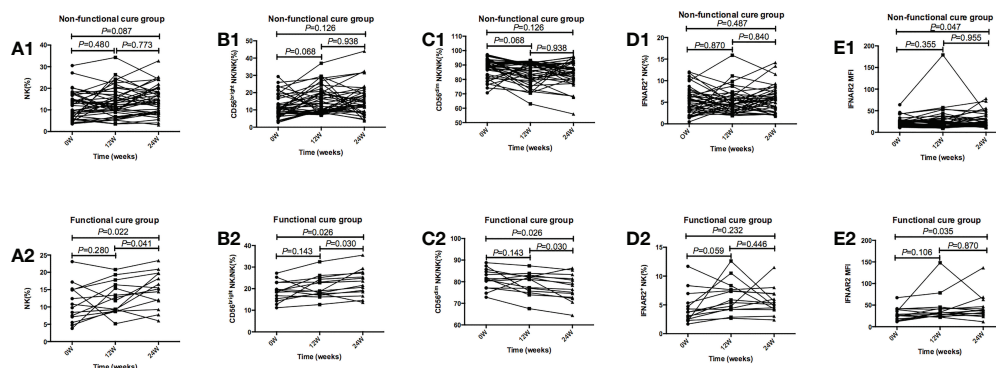


FIGURE 3

Tendencies of NK cells frequency and function, including NK%, CD56^{bright} NK/NK%, CD56^{dim} NK/NK%, IFNAR2+ NK/NK%, and IFNAR2 MFI at baseline and after 12 and 24 weeks of treatment in the Functional cure group and Non-functional-cure group. (A1–E1) Tendencies of NK% (A1), CD56^{bright} NK/NK% (B1), CD56^{dim} NK/NK% (C1), IFNAR2+ NK/NK% (D1), and IFNAR2 MFI (E1) at baseline and after 12 and 24 weeks of treatment in the Non-functional-cure group. (A2–E2) Tendencies of NK% (A2), CD56^{bright} NK/NK% (B2), CD56^{dim} NK/NK% (C2), IFNAR2+ NK/NK% (D2), and IFNAR2 MFI (E2) at baseline and after 12 and 24 weeks of treatment in the Functional cure group. p values for multiplicity were adjusted by Bonferroni, and $P < 0.016$ was identified as significant statistical difference.

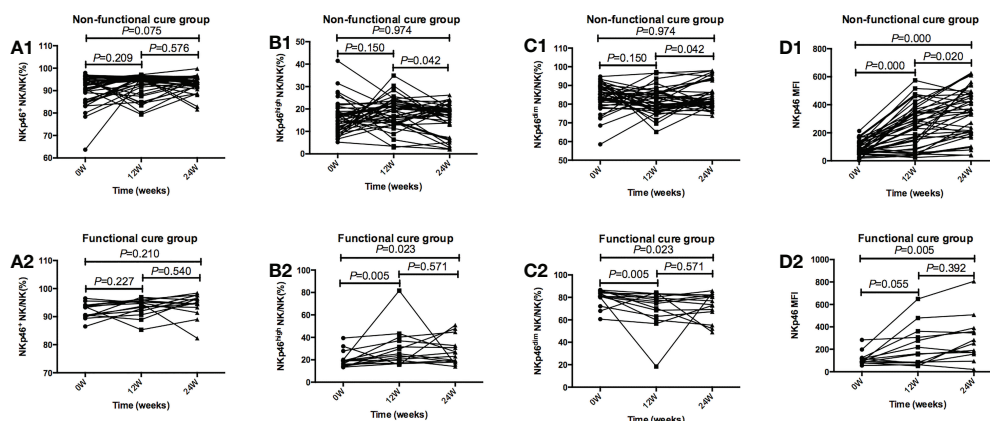


FIGURE 4

Tendencies of NK cells frequency and function, including NKp46 NK/NK%, NKp46high NK/NK%, NKp46dim NK/NK%, and NKp46MFI at baseline and after 12 and 24 weeks of treatment in the Functional cure group and Non-functional-cure group. (A1–D1) Tendencies of NKp46 NK/NK% (A1), NKp46high NK/NK% (B1), NKp46dim NK/NK% (C1), and NKp46MFI (D1) at baseline and after 12 and 24 weeks of treatment in the Non-functional-cure group. (A2–D2) Tendencies of NKp46 NK/NK% (A2), NKp46high NK/NK% (B2), NKp46dim NK/NK% (C2), and NKp46MFI (D2) at baseline and after 12 and 24 weeks of treatment in the Functional cure group. p values for multiplicity were adjusted by Bonferroni, and $P < 0.016$ was identified as significant statistical difference.

conductive to lead to HBsAg decrease or HBsAg disappearance and even obtain functional cure.

Receptors NKp46 and IFNAR2 on NK cell surface play crucial effects in chronic HBV infection. Type I interferon receptor (IFNAR) has been involved in the progression of CHB. IFNAR2 plays a vital part in initiation of type I interferon-induced JAK-STAT signaling and activation of STATs (41). IFNAR2 deficiency results in dysregulation of the function of NK cells (20). Our data showed that after PEG-IFN treatment, the expression of reporters IFNAR2 on the surface of NK cells in patients with functional cure significantly increased, which suggested that the up-regulation of IFNAR2 made NK cells more easily activated by IFN- α and contributed to clearing HBV. NKp46, as the activating receptor of NK cells, may regulate the activity of NK cells in the suppression of HBV replication and HBV-related liver injury, meanwhile, it is crucial for the activation of NK cells in chronic HBV infection (42). Our results showed that after therapy, the frequency of NKp46^{high} NK cells and surface reporters IFNAR2 and NKp46 expression on NK cells in patients with HBsAg loss significantly increased, which was consistent with some studies suggesting that in patient with CHB, the expression of IFNAR2 and NKp46 on the surface of NK cells elevated after PEG-IFN therapy (32, 33), NKp46^{high} NK cells may be closely correlated to HBV clearance, and up-regulated expression of NKp46^{high} is beneficial to the clearance of HBV.

In conclusion, this study adds to our knowledge of the relevance of functional cure and frequency and function of NK cells in CHB patients with PEG-IFN α -2a treatment. Our results demonstrate the lower HBV DNA before therapy, the higher CD56^{bright} NK% at baseline, and the higher CD56^{bright} NK%, IFNAR2 MFI, NKp46^{high} NK% after therapy, the easier to achieve HBsAg clearance. Functional cure is dependent on low HBV DNA

titer, the recovery and enhancement of NK cells function at the early phase of PEG-IFN α -2a therapy. These conclusions may be conducive to identify those easier to obtain functional cure and optimizing therapeutic strategies for CHB to pursue functional cure.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Review Committee of Beijing Ditan Hospital of Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

WC, YX, and ML made contribution to conception and design of the study. HL, LuxZ, SWang, WD, TJ, YLin, LY, GS, XB, YLu, LuZ, RL, MC, SWu, YG, MX, HH, LH, and XC organized the data. WC, YX, and ML guided statistical analysis. WC wrote the first manuscript. HL, LuxZ, SWang, WD, YX, and ML edited and modified the English manuscript. ML submitted the modified version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.1067362/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Using FlowJo software for CD3-CD56+ NK cell image analysis to illustrative dot plots related to the FACS data.

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Study on liver histopathology of chronic HBV infected patients with different normal ALT values

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Aims: Comparison of liver histopathological findings to explore the occurrence of liver inflammation in patients with chronic hepatitis B (CHB) under different alanine aminotransferase (ALT) normal values.

Methods: The patients who were diagnosed as chronic hepatitis B virus (HBV) infection by liver histopathology at the Department of Pathology, Beijing Ditan Hospital due to clinical difficulty in defining the degree of liver inflammation or fibrosis were retrospectively enrolled from May 2008 to November 2020. Study of the incidence of significant hepatic histopathology in enrolled patients according to different ALT normal values. Using logistic regression to investigate the relevant factors of significant hepatic histopathology.

Results: A total of 1474 patients were enrolled, 56.20% of the patients were male, and the overall patients' age was 36.80 ± 10.60 years. 39.00% of patients had liver inflammation grade $G > 1$, 34.70% liver fibrosis stage $S > 1$, and 48.17% patients had significant hepatic histopathology ($G > 1$ and/or $S > 1$). Among patients with normal ALT values, 36.40% and 40.40% had significant hepatic histopathology by American Association for the Study of Liver Diseases (AASLD) criteria and Chinese guideline criteria, respectively, but the difference was not statistically significant ($\chi^2 = 3.38$, $P = 0.066$). In contrast, among patients with abnormal ALT values, 58.90% and 62.20% of patients had significant hepatic histopathology by AASLD criteria and Chinese guideline criteria, respectively, with no significant difference ($\chi^2 = 2.28$, $P = 0.131$). ALT ($P < 0.001$, OR=1.019), hepatitis B surface antigen (HBsAg) ($P < 0.001$, OR=0.665) and hepatitis B e antigen (HBeAg) status ($P < 0.001$, OR=2.238) were relevant factors in the occurrence of significant hepatic histopathology. ALT was positively correlated with grade of inflammation G ($r = 0.194$, $P < 0.001$) and negatively correlated with liver fibrosis stage S ($r = -0.066$, $P = 0.021$).

Conclusions: Our study found no statistically significant differences in the presence of significant hepatic histopathology under the two ALT criteria. ALT, HBsAg and HBeAg status were related to the occurrence of significant hepatic histopathology.

KEYWORDS

chronic HBV-infection, ALT value, HBV therapy, liver histopathology, clinical indicators

Introduction

Since the widespread application of the recombinant hepatitis B vaccine in China in 1992, the prevalence of HBV among the Chinese population has declined year by year. The prevalence of HBsAg in the serological survey of HBV in China in 2006 and 2014 decreased by 46% and 52%, respectively, compared with 1992 (1), and the prevalence of children under the age of 5 decreased by 97% (1). With the further application of vaccines, the chronic infection rate of HBV in the Chinese population will further decline. Nevertheless, there are still about 70 million chronic HBV-infected people in China, 20 to 30 million of which are CHB patients requiring treatment, and about 1 million people are newly diagnosed as hepatitis patients every year (2).

The typical natural history of chronic HBV infection includes HBeAg positive chronic infection, HBeAg positive chronic hepatitis, HBeAg negative chronic infection, HBeAg negative chronic hepatitis, and HBsAg negative stage (2, 3). The key to whether patients with chronic HBV infection need to start antiviral treatment is whether hepatitis occurs. If there is inflammation, it needs to be treated. At present, in the latest prevention and treatment guidelines for CHB in China, indicators such as ALT, hepatitis B virus deoxyribonucleic acid (HBV DNA), HBsAg, HBeAg, and liver pathology are used to assess the infection stage of the infected people. Based on the diagnosis and treatment guidelines for CHB updated in 2019, Chinese scholars put forward the expert opinion on expanding the antiviral treatment of CHB (4). The opinion pointed out that the screening of HBsAg in the population should be expanded and highly sensitive HBV DNA detection methods should be used. Lowering the ALT threshold for CHB patients to start treatment, and actively treating patients who are at risk of disease progression or in the gray area (4). The purpose of our study is to retrospectively collect the biochemical indicators and liver pathology results of patients who were chronic infected by HBV, compare the accuracy of ALT normal value under different definitions of CHB guidelines (2, 3, 5, 6) in judging whether there are significant hepatic histopathology in patients with chronic HBV infection, and discuss the necessity of lowering the ALT threshold of patients with CHB for antiviral treatment.

Methods and materials

Patients

Patients with chronic HBV infection who had undergone liver biopsy in Beijing Ditan Hospital from May 2008 to November 2020 were enrolled. Demographic data, antiviral treatment information, HBV DNA load, and serological test results, as well as the examination results of liver function, renal function, blood routine and coagulation function indicators were collected. According to the inclusion and exclusion conditions, patients were enrolled and statistically analyzed.

Inclusion criteria: 1) 18-65 years old; 2) The duration of HBsAg positive was more than 6 months; 3) Having not received HBV antiviral treatment; 4) The diagnosis result of liver puncture pathology was clear; 5) Having the complete results of liver function, coagulation function, blood routine test, viral load, and serological index within 2 weeks before or after the biopsy.

Exclusion criteria: 1) Exclude other types of viral hepatitis (including hepatitis A, hepatitis C, and hepatitis E), alcoholic liver disease, non-alcoholic fatty liver disease, drug-related liver injury, autoimmune liver injury, cirrhosis, liver cancer, and other liver diseases; 2) Co-infection with other viruses (such as human immunodeficiency virus, cytomegalovirus, etc.); 3) Patients' biopsy results didn't suggest a definite inflammatory grade or fibrosis stage; 4) Those with heart and kidney dysfunction.

The patients are chronic HBV-infected people who are difficult to judge whether there is significant hepatic histopathological change through clinical indicators, and carry out liver puncture examinations.

Study parameters

Aspartate aminotransferase (AST), ALT, albumin (ALB), total bilirubin (TBIL), cholinesterase (CHE), platelet (PLT), prothrombin time activity (PTA), international normalized ratio (INR), HBsAg, HBeAg, HBV DNA.

The results of liver pathological examination included grade of inflammation G0-G4, and liver fibrosis stage S0-S4. Using Scheuer scoring system.

In this study, $G > 1$ means that the liver has significant inflammation, and $G \leq 1$ means that the liver has no significant inflammation. $S > 1$ is defined as significant fibrosis of the liver, $S0$ as no fibrosis, and $0 < S \leq 1$ as slight fibrosis. $G \leq 1$ and $S \leq 1$ is defined as no significant hepatic histopathology, $G > 1$ and/or $S > 1$ as significant hepatic histopathology.

Study content

Patients were divided into normal ALT group and abnormal ALT group according to the standard of normal ALT (≤ 40 U/L) in 2019 Chinese Hepatitis B Treatment Guidelines, 2019 European Association for the Study of the Liver (EASL) Guidelines, and 2016 Asian Pacific Association for the Study of the Liver (APASL) Guidelines.

According to the guidelines of the American Association for the Study of Liver Diseases (AASLD) for chronic hepatitis B, male patients with ALT ≤ 35 U/L and female patients with ALT ≤ 25 U/L were assigned to normal ALT group, and the rest were classified as abnormal ALT group.

Considering the liver pathology, the incidence of significant hepatic histopathology in normal and abnormal patients was evaluated to compare the difference under the two ALT standards, and the impact of different ALT thresholds on the accuracy of diagnosing significant hepatic histopathology in patients with chronic hepatitis B was studied.

ALT, HBsAg, HBeAg status and HBV DNA load were used as independent variables for univariate regression analysis for the occurrence of significant hepatic histopathology, respectively. Those with statistically significant differences were then selected for multivariate regression analysis. The cut-off values of the factors associated with the occurrence of significant hepatic histopathology were calculated separately.

Finally, patients with significant hepatic histopathology were selected and the correlation between ALT and G, S in these patients were analyzed, respectively.

Statistical analysis

Describing counting data with mean $\pm$ standard deviation (normal data) or quartile (non-normal data), describing classified data with percentage. Comparing the classified data with chi-square test, and analyzing the relevant factors in the occurrence of significant hepatic histopathology with univariate binary logistic regression and receiver operating characteristic curve (ROC). Kendall's tau-b correlation analysis was used in categorical data. All data were analyzed with IBM SPSS 25.0, and graphed with Graphpad Prism 8.0 and Microsoft Excel 2019. The significance of all tests shall be subject to bilateral tests. If $P < 0.05$, it means there is statistical significance.

Results

Patients

There were 4421 patients with definite liver histopathological diagnoses. Demographic data, HBV virus content, serological indicators, clinical biochemical, and blood routine indicators were collected. 1870 patients with fatty liver, 8 patients with chronic hepatitis C, 219 patients with no clear indication of disease grade by the pathological diagnosis of liver, 226 patients undergoing HBV therapy at the time of enrollment, 428 patients missing HBV DNA detection results at the time of pathological examination of liver tissue, 165 patients missing HBeAg results, and 31 patients missing ALT results were excluded. Finally, 1474 patients with definite liver histopathological diagnosis, completing virus content and serological, liver function indicators and peripheral blood indicators, were enrolled (Figure 1).

Among all patients, 56.20% were male, 36.8 ± 10.6 years old, and 48.17% had significant hepatic histopathology. Patients with significant hepatic histopathology had higher percentage of HBeAg positive patients (59.00% vs 46.70%, $\chi^2 = 22.30$, $P < 0.001$), higher age (38.00 ± 10.80 vs 35.70 ± 10.20 years old, $t = -4.13$, $P < 0.001$), higher ALT levels [38.2 (25.7, 58.1) vs 26.8 (18.9, 40.7) U/L, $z = -10.28$, $P < 0.001$], and higher AST levels [32.8 (24.4, 48.9) vs 24 (19.3, 33.5) U/L, $z = -11.69$, $P < 0.001$]. Patients with significant hepatic histopathology had lower HBsAg levels (3.20 ± 1.18 vs 3.38 ± 1.25 log10 IU/ml, $t = 2.25$, $P = 0.025$), lower ALB levels (44.10 ± 4.49 vs 46.20 ± 3.58 g/L, $t = 9.73$, $P < 0.001$), lower PLT counts (166.00 ± 57.90 vs 199.00 ± 51.20 10⁹/L, $t = 11.52$, $P < 0.001$), and lower CHE levels (7449.00 ± 2286.00 vs 8687.00 ± 2270.00 U/L, $t = 10.30$, $P < 0.001$) (Table 1).

Liver histopathology

Of the pathological diagnosis of liver tissue, the grade of liver inflammation: no G0, the proportion of $G \leq 1$ was 61.0% (899 cases), the proportion of $1 < G \leq 2$ was 24.2% (356 cases), the proportion of $2 < G \leq 3$ was 13.7% (202 cases), and the proportion of $3 < G \leq 4$ was 1.2% (16 cases). Grading of liver fibrosis: S0 accounts for 0.8% (11 cases), $S \leq 1$ accounts for 64.5% (951 cases), $1 < S \leq 2$ accounts for 20.6% (304 cases), $2 < S \leq 3$ accounts for 10.7% (157 cases), and $3 < S \leq 4$ accounts for 3.4% (50 cases) in Table 2.

Comparison of hepatitis incidence under ALT threshold recommended by different guidelines

In the diagnostic criteria of 2018 AASLD chronic hepatitis B guidelines, the definition of normal ALT for men is ALT $\leq$

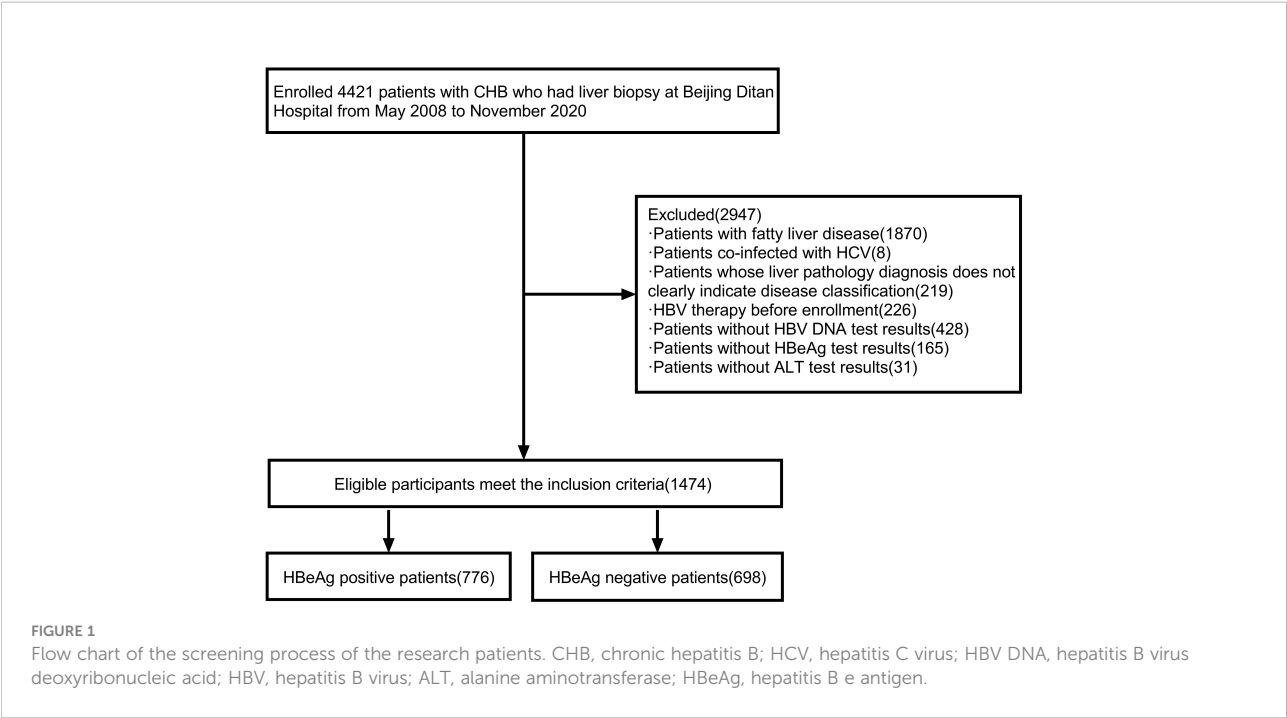


TABLE 1 Overall patients' clinical characteristics and their comparisons between patients with and without significant hepatic histopathology.

Characteristics	Total (N = 1474)	No significant hepatic histopathology group (N = 764)	Significant hepatic histopathology group (N = 710)	$\chi^2/t/z$	P
Male (%)	56.20%	52.90%	59.90%	7.28	0.007
Age (years)	36.80 ± 10.60	35.70 ± 10.20	38.00 ± 10.80	-4.13	<0.001
HBsAg (log ₁₀ IU/ml)	3.30 ± 1.22	3.38 ± 1.25	3.20 ± 1.18	2.25	0.025
Positive HBeAg (%)	52.60%	46.70%	59.00%	22.3	<0.001
HBV DNA (log ₁₀ IU/ml)	5.30 ± 2.13	5.27 ± 2.27	5.32 ± 1.96	-0.44	0.663
ALT (U/L)	31.80 (21.20, 49.20)	26.80 (18.90, 40.70)	38.20 (25.70, 58.10)	-10.28	<0.001
AST (U/L)	27.80 (21.10, 40.20)	24.00 (19.30, 33.50)	32.80 (24.40, 48.90)	-11.69	<0.001
TBIL (μmol/L)	12.90 (10.00, 17.20)	12.40 (9.97, 16.40)	13.20 (10.10, 17.90)	-2.63	0.008
ALB (g/L)	45.20 ± 4.17	46.20 ± 3.58	44.10 ± 4.49	9.73	<0.001
CHE (U/L)	8087.00 ± 2359.00	8687.00 ± 2270.00	7449.00 ± 2286.00	10.36	<0.001
PLT (10 ⁹ /L)	183.00 ± 56.90	199.00 ± 51.20	166.00 ± 57.90	11.52	<0.001
PTA (%)	89.20 ± 12.60	91.80 ± 11.80	86.90 ± 12.90	5.83	<0.001
INR	1.03 ± 0.09	1.03 ± 0.08	1.04 ± 0.10	-2.08	0.038

HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALB, albumin; TBIL, total bilirubin; CHE, cholinesterase; PTA, prothrombin time activity; PLT, platelet; INR, international normalized ratio.

35U/L, and that for women is ALT ≤ 25U/L. According to AASLD, 47.70% of patients in our study were normal and 52.30% were abnormal. Taking the normal range of ALT ≤ 40 U/L defined in 2019 Chinese Guidelines, 2019 EASL Guidelines, and 2016 APASL Guidelines as the normal range,

64.30% of patients in this study were normal patients and 35.70% were abnormal patients. Among the patients with normal ALT values, 36.40% and 40.40% of the patients under the two standards had significant hepatic histopathology, but without statistical difference ($\chi^2 = 3.38$, $P = 0.066$). In patients

TABLE 2 Histopathological grade distribution of the liver biopsy.

Classification	Degree of lesion	Grade	Number of people (%)
G	Insignificant	G0	0
		$G \leq 1$	61.0%
	Significant	$1 < G \leq 2$	24.2%
		$2 < G \leq 3$	13.7%
		$3 < G \leq 4$	1.2%
S	Insignificant	S0	0.8%
		$S \leq 1$	64.5%
	Significant	$1 < S \leq 2$	20.6%
		$2 < S \leq 3$	10.7%
		$3 < S \leq 4$	3.4%

G, inflammation grade; S, liver fibrosis stage.

with abnormal ALT values, 58.90% and 62.20% of the patients with the two criteria had significant hepatic histopathology, with no significant difference ($\chi^2 = 2.28$, $P = 0.131$) (Table 3).

Analysis of factors associated with significant hepatic histopathology

In the analysis of factors associated with significant hepatic histopathology, by univariate logistic regression analysis, the results showed that significant hepatic histopathology were significantly associated with patients' HBsAg ($P = 0.025$, OR = 0.883, 95% CI = 0.791 ~ 0.985), HBeAg positivity ($P < 0.001$, OR = 1.642, 95% CI = 1.335 ~ 2.018), and ALT levels ($P < 0.001$, OR=1.019, 95% CI=1.015~1.024) were significantly associated. By multivariate logistic regression analysis, the results showed that, HBsAg level ($P < 0.001$, OR=0.665, 95% CI=0.577~0.767), HBeAg positivity ($P < 0.001$, OR=2.131 95% CI=1.288~3.525), and ALT level ($P < 0.001$, OR=1.014, 95% CI=1.002~1.025) were independent correlates of significant hepatic histopathology in chronic HBV infection (Table 4).

The cutoff values were calculated from the receiver operating characteristic curve (ROC) and the Jorden index, and the results showed that HBsAg (3.85 log10 IU/ml, sensitivity 42.60%, specificity 72.80%, AUC=0.56) and ALT (33.45 U/L, sensitivity 59.40%, specificity 65.40%, AUC=0.66) (Table 4; Figure 2).

Correlation analysis of ALT with G and S in patients with significant hepatic histopathology

In patients with significant hepatic histopathology, grade of inflammation G gradually increased with increasing ALT levels ($r = 0.194$, $P < 0.001$), while liver fibrosis stage S gradually decreased ($r = -0.066$, $P = 0.021$).

Discussion

As we know, $G \geq 2$ means the presence of significant inflammation and $S \geq 2$ means the presence of significant fibrosis. In our study, we found that 5.3% (78) patients had

TABLE 3 Comparison of the occurrence of significant hepatic histopathology between normal and abnormal ALT groups under different guidelines.

	Guideline of AASLD	Guideline of China, EASL, APASL	χ^2 and P
Normal value of ALT	Male ≤ 35 U/L, Female ≤ 25 U/L	≤ 40 U/L	NA
Percentage of ALT normal	47.70% (703/1474)	64.30% (948/1474)	NA
Percentage of ALT abnormalities	52.30% (771/1474)	35.70% (526/1474)	NA
Percentage of patients with normal ALT values but with significant hepatic histopathology	36.40% (256/703)	40.40% (383/948)	$\chi^2 = 3.38$, $P = 0.066$
Percentage of patients with abnormal ALT values but with significant hepatic histopathology	58.90% (454/771)	62.20% (327/526)	$\chi^2 = 2.28$, $P = 0.131$

ALT, alanine aminotransferase; EASL, European Association for the Study of the Liver; APASL, Asian-Pacific Association for the Study of the Liver; AASLD, American Association for the Study of Liver Diseases.

TABLE 4 Table of binary logistic regression analysis of overall patients regarding the occurrence of significant hepatic histopathology and cut off value for the variables.

Univariate binary logistic regression analysis

	B	P	OR	95% CI
HBsAg(log ₁₀ IU/ml)	-0.13	0.025	0.883	0.791 ~ 0.985
HBeAg-negative	Ref	N/A	N/A	N/A
HBeAg-positive	0.50	<0.001	1.642	1.335 ~ 2.018
HBV DNA(log ₁₀ IU/ml)	0.01	0.663	1.011	0.962 ~ 1.060
ALT(U/L)	0.02	<0.001	1.019	1.015 ~ 1.024
Multivariate binary logistic regression analysis				
	B	P	OR	95% CI
HBsAg(log ₁₀ IU/ml)	-0.41	<0.001	0.665	0.577 ~ 0.767
HBeAg-negative	Ref	N/A	N/A	N/A
HBeAg-positive	0.81	<0.001	2.238	1.596 ~ 3.139
ALT(U/L)	0.02	<0.001	1.019	1.013 ~ 1.025
Cut off value and AUC				
	Cut off	sensitivity	specificity	AUC
HBsAg(log ₁₀ IU/ml)	3.85	42.60%	72.80%	0.56
HBeAg	0.50	59.00%	53.30%	0.56
ALT(U/L)	33.45	59.40%	65.40%	0.66

HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; ALT, alanine aminotransferase; Ref, reference; N/A, not available; AUC, area under curve.

pathological findings suggestive of G or S between 1-2 by analyzing the liver biopsy results of all patients. We defined G>1 and/or S>1 as the presence of significant hepatic histopathology in order to cover those patients between 1-2, because significant hepatic histopathology were truly absent in patients with G ≤ 1 and S ≤ 1. We believed that patients between 1-2 were in the early stages of inflammation or fibrosis and that disease progression would occur without intervention.

HBV is a non-cytopathogenic hepatophilic virus, and it is currently believed that it does not cause damage to liver cells per se. Liver inflammation occurs when HBV induces an immune response in the host, causing hepatocyte necrosis *via* immune damage, which may lead to cirrhosis or even liver cancer if left untreated. The key to whether chronic HBV-infected patients need to initiate antiviral therapy is the presence of liver inflammation, with inflammation requiring treatment. HBV stimulates the body's immune response, thereby causing hepatocyte necrosis, liver tissue inflammation, and fibroplasia

through immune cells and secreted cytokines, which results in the occurrence of chronic hepatitis B. However, viral antigens inhibit the effective clearance of viruses and infected liver cells by virus-specific immune cells, making the disease persistent and preventing spontaneous recovery from infection. Therefore, antiviral therapy is the most important way to stop the progression of liver disease.

The key to the need for antiviral therapy is the presence of liver inflammation, then it is very important to accurately identify the presence of liver inflammation. Clinical indicators in patients with HBeAg positive chronic infection stage are characterized by high HBV DNA load, HBeAg positivity and normal ALT (2). Truly immune-tolerant patients usually meet these criteria, and most scholars now believe that patients in the immune-tolerant phase have no or only mild liver inflammation and therefore do not require antiviral therapy (7–9). ALT has the most sensitive reaction of hepatocyte inflammation and necrosis, and a small amount of hepatocyte necrosis can cause an increase

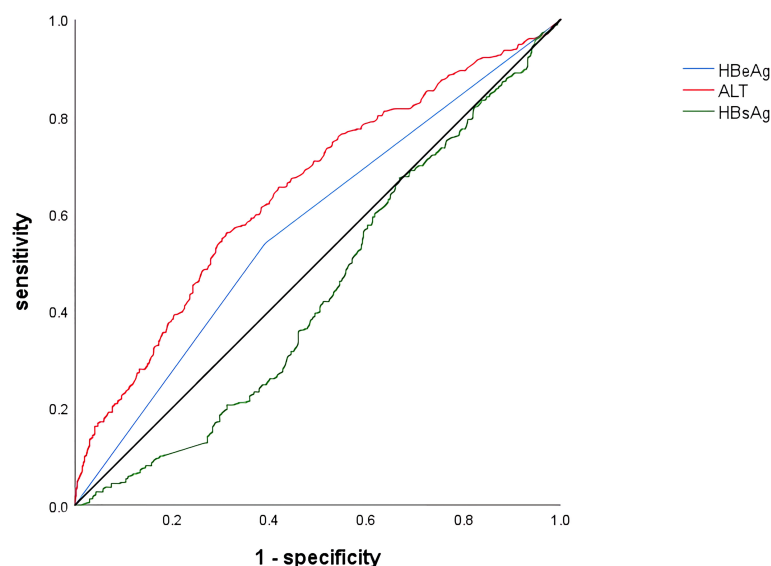


FIGURE 2

Receiver operating characteristic curve of variables associated with significant hepatic histopathology. HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen.

in ALT, but not all patients with hepatic histological lesions exhibit elevated ALT, meaning that even if the criteria for the immune tolerance period are met, significant hepatic histopathology may still be present.

The latest version of the Hepatitis B guidelines, including China, EASL and APASL, all define a normal ALT value as less than 40 U/L (2, 3, 5). However, many scholars have studied the liver pathology findings of chronic HBV-infected patients with normal ALT and found that different proportions of this group of patients already had hepatitis (10–12), that is, the criteria for antiviral treatment were met. In our previous study, we found that 20.2% of patients with chronic HBV infection who met the conditions of HBV DNA $>2 \times 10^7$ IU/ml, HBeAg positivity, and ALT ≤ 40 U/L had liver puncture biopsies result suggesting liver inflammation (13).

In addition, with the growth of age, more and more patients in the immune tolerance phase will break tolerance to hepatitis and enter the immune clearance phase, and a previous study found that the median age of immune tolerance breakage was 30.7 years (14). In view of the above characteristics, the need to lower the ALT threshold for initiating antiviral therapy in patients who were chronic infected with HBV has been widely discussed by scholars in recent years. Most scholars believe that lowering the ALT threshold for antiviral therapy can effectively improve the diagnosis rate as well as the treatment rate of hepatitis B, so that more patients with hepatitis B can benefit from it (4, 15).

In this study, the histopathological findings of the liver of patients with ALT ≤ 40 U/L were analyzed separately without

considering other clinical indicators, and the results showed that 40.40% had significant hepatic histopathology. We compared the diagnostic criteria of ALT ≤ 35 U/L in men and ALT ≤ 25 U/L in women with the ALT ≤ 40 U/L recommended by the 2019 edition of the Chinese guidelines for chronic hepatitis B, the 2019 edition of the EASL guidelines and the 2016 edition of the APASL guidelines by referring to the ALT diagnostic criteria of the 2018 edition of the AASLD guidelines for chronic hepatitis B. The results showed that 36.40% and 40.40% of patients with normal ALT under the AASLD criteria and the remaining guideline criteria had significant hepatic histopathology, respectively, but with no statistically significant differences. In contrast, among patients with abnormal ALT values, 58.90% and 62.20% of patients with significant hepatic histopathology by AASLD criteria and Chinese guideline criteria, respectively, and the difference was also not significant.

After studying the role of ALT to predict liver fibrosis, Korean scholars found that there was a positive correlation between ALT levels derived from the liver and liver fibrosis stage, but no correlation was found between overall serum ALT levels and fibrosis stage (16). In our study, we found that ALT levels were positively correlated with G, but inversely correlated with S. Combined with the studies of previous scholars, we believed that the reason for the difference might be due to variances in scoring criteria and ALT measurement methods. The correlation coefficient between ALT and S was only -0.066, and the clinical significance of this result was too small to be used for clinical guidance. However, we can also learn from this that the relationship between ALT and G is stronger than the

relationship between ALT and S, which is in accordance with our knowledge.

It is not enough to rely on ALT alone as an indicator to assess the necessity of HBV therapy, as hepatitis attacks may be accompanied by changes in several clinical indicators, such as HBsAg, HBV DNA, alpha fetoprotein (AFP), etc (17, 18). Also, when evaluating a patient, some demographic characteristics such as age and gender are factors that we must consider (19). The best strategy is to evaluate the need to initiate antiviral therapy in patients with CHB by combining multiple indicators, as our current treatment goals for CHB have changed, and clinical cure based on delaying disease progression is the best (20, 21). Similar conclusions were found in our study. After analyzing the risk factors associated with significant hepatic histopathology, we found that in addition to ALT, HBsAg levels and HBeAg status were also associated with significant hepatic histopathology. Although the diagnostic ability of each indicator alone is unsatisfactory, the combination of several indicators to determine the disease progression status in clinical work can make the diagnosis more reliable.

We found that 48.17% of untreated patients had significant hepatic histopathology by enrolling a large sample size of HBV-infected patients, that means a significant proportion of patients requiring treatment existed under the normal ALT criteria of different guidelines, which suggested that lowering the ALT threshold for initiating treatment in CHB patients was important and necessary to improve the prognosis and reduce the burden of disease. At the same time, there were some shortcomings in this study, as we were not able to calculate the optimal threshold for initiation of treatment, and the enrolled patients did not cover multiple ethnic groups. However, these problems need to be solved by designing a more elaborate study protocol.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

ML and YX contributed to the study design. ZZ, HH, XB, YLi, LY, SW contributed to the data analysis. GS, MC, YLu, YG,

RL, MX, XC, LH, LZ contributed to the recruitment, enrolment, and assessment of participants, as well as following up with the patients. TJ, WD, FS, HL contributed to data collection. ZZ and ML wrote the first draft of the manuscript. YX revised the manuscript and is the guarantor of the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

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Sustained viral response and relapse after discontinuation of oral antiviral drugs in HBeAg-positive patients with chronic hepatitis B infection

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Objective: To investigate the sustained virological response and relapse in chronic hepatitis B (CHB) patients with hepatitis B e antigen (HBeAg) positive after stopping oral antiviral drugs, and to monitor the disease progression and the incidence of adverse events such as liver cirrhosis and hepatocellular carcinoma.

Methods: This is a prospective observational study. Patients who continued nucleos(t)ide analogue (NA) treatment after achieving HBeAg seroconversion for more than 3 years were enrolled. After signing the informed consent form, patients stopped NA treatment and received follow-up. During the follow-up, the antiviral treatment information of the patients was collected, and the follow-up observation was carried out every 3 months since the enrollment. We monitored the virological indexes, liver and kidney function, serology and liver imaging during follow-up. The purpose of this study was to explore the sustained virological response rate, HBV DNA recurrence rate, clinical relapse rate and the related factors after drug withdrawal.

Results: A total of 82 patients were enrolled, including 42 males (51.22%) and 40 females (48.78%), with a median age of 34.00 (31.00, 37.25) years. All enrolled patients were followed up for 1 year. At the end of the follow-up, 36.59% (30/82) of patients had sustained virological response, 63.41% (52/82) of patients had HBV DNA reactivation, 17.07% (14/82) of patients had clinical relapse, and 10.98% (9/82) of patients had HBeAg reversion. During the follow-up, there were no adverse events such as liver cirrhosis and

hepatocellular carcinoma. The median level of hepatitis B surface antigen (HBsAg) in patients with sustained virological response was lower than that in patients with HBV DNA reactivation (2.92 vs. 3.18 $\log_{10}$ IU/mL, $Z = -1.492/P = 0.136$), and the median level of baseline HBsAg in patients with HBV DNA reactivation was lower than that in patients with clinical relapse (3.01 vs. 3.45 $\log_{10}$ IU/mL, $Z = -1.795/P = 0.073$), but the difference was not significant. There was no significant statistical difference between patients with sustained virological response and HBV DNA reactivation of the median total treatment time [69.50 (56.25, 86.00) vs. 62.50 (44.00, 88.50) months, $Z = -0.689/P = 0.491$], and the consolidation treatment time [41.50 (36.75, 54.75) vs. 40.50 (36.00, 53.75) months, $Z = -0.419/P = 0.675$].

Conclusion: The sustained virological response rate of HBeAg positive CHB patients after stopping oral antiviral treatment is lower, and it is more common in patients with lower HBsAg levels. Patients still need to be closely monitored after stopping NA therapy.

KEYWORDS

chronic hepatitis B, HBeAg, HBV DNA, nucleos(t)ide analogue, clinical relapse, sustained virological response

Introduction

CHB is a world public health problem. According to the statistics of the World Health Organization, 296 million people were living with chronic hepatitis B virus (HBV) infection in 2019. CHB resulted in an about 820,000 deaths, mainly due to cirrhosis and hepatocellular carcinoma (1, 2). CHB is an immune-mediated liver inflammatory injury. In the pathogenesis of CHB, the virus stimulates the host to cause immune response, and then immune factors induce the inflammation of liver tissue and liver cell damage (3–6). It is generally believed that patients can inhibit virus replication through antiviral treatment, thereby reducing the stimulation of virus on immunity, alleviating liver inflammation, reducing the occurrence of cirrhosis and hepatocellular carcinoma, prolonging the life span of patients and improving the quality of life (3, 4, 7, 8).

NA is a commonly used oral antiviral drug, which is widely used because of its strong antiviral effect, convenient administration and a few side effects. However, patients often need long-term medication, for NA cannot improve the host's immune control of the virus. Long term drug use will also bring many problems: although there are few adverse reactions of NA,

its long-term safety remains to be confirmed (9). Long term treatment has significantly increased the economic burden of the national health care system. In many regions, the cost of NA treatment cannot be reimbursed or can only be partially reimbursed, bringing great economic burden to individuals and families. It is difficult to define the time of drug withdrawal which brings out great psychological pressure to patients. What needs more attention is that even if the drug is given for a long time, it is difficult to induce a lasting response after drug withdrawal. It is unclear how long NA should be treated, which drug withdrawal criteria is most effective and conforms to the principles of health economics, and which indicators can effectively predict the relapse after drug withdrawal. Therefore, it is particularly important to formulate a reasonable and feasible study to stop oral antiviral treatment.

In recent years, some scholars have proposed that CHB patients receiving long-term oral antiviral treatment may benefit from drug withdrawal. Liver inflammatory mediated by immune response after drug withdrawal is more likely to induce virus clearance and achieve negative transformation of HBsAg (10–13). In 2015, China's prevention and treatment guidelines for CHB proposed that drug withdrawal can be considered when patients with HBeAg positive achieved HBeAg serological

conversion, HBV DNA turns negative and continued to consolidate treatment for 3 years (14). However, whether patients can maintain virological response after stopping treatment and the safety after stopping treatment still need our further research to verify.

In this study, patients with CHB who met the drug withdrawal criteria were followed up after drug withdrawal, to determine the sustained virological response rate of patients after stopping oral antiviral treatment and explore its related influencing factors, in order to provide help for us to explore the drug withdrawal mode of HBeAg positive CHB patients.

Materials and methods

Subjects and study design

This is a prospective study of CHB patients who were admitted to the Department of Hepatology Division 2 of Beijing Ditan Hospital from January 1, 2017 to December 30, 2020. Eligible patients who were HBeAg positive before treatment and stopped taking drugs in accordance with the Chinese Guidelines for the Prevention and Treatment of Chronic Hepatitis B (2015) were rolled. This study was approved by the Ethics Committee of Beijing Ditan Hospital affiliated to Capital Medical University (Jing Di Lun Ke Zi 2017 No. 003 - 02).

Inclusion criteria: 1) Age above 18 (including 18 when signing the informed consent form); 2) Gender is not limited; 3) HBsAg (i.e. HBsAg ≥ 0.05 IU/mL) is positive for more than 6 months; 4) HBeAg positive (HBeAg ≥ 1 S/CO) before antiviral treatment; 5) Use oral antiviral drugs such as nucleos(t)ide analog (NA) for treatment; 6) Meet the drug withdrawal standard of the Chinese Guidelines for the Prevention and Treatment of Chronic Hepatitis B (2015), and have continued treatment for at least 3 years after HBV DNA negative conversion and HBeAg seroconversion (14); 7) Volunteer to participate in this study, sign the informed consent form, and be able to participate in the follow-up on schedule.

Exclusion criteria: 1) Those who have used interferon antiviral therapy before; 2) The patients were treated with glucocorticoid, thymosin and other immunomodulators before and during the follow-up period; 3) Patients with liver cirrhosis or liver cancer; 4) During screening, alpha fetoprotein (AFP) and liver imaging indicated that patients may have malignancy; 5) Patients with other virus infections (HCV, HDV, HIV, EBV, CMV, etc); 6) Complicated with autoimmune liver disease, alcoholic liver disease, genetic metabolic liver disease, fatty liver disease, drug induced liver disease and other liver diseases; 7) Patients with other malignant tumors or undergoing immunomodulation therapy due to other diseases; 8) The investigator believes that patients who are not suitable to participate in this study.

Follow-up time: Follow up every 3 months from the cessation of antiviral treatment to 1 year after the cessation of antiviral treatment.

Collection of clinical data

- 1) Demographic data: patient's sex, age, family history of liver cancer, transmission route;
- 2) Collection of enrollment data: antiviral drug treatment information, including treatment drugs, time of starting antiviral treatment, total antiviral treatment time, consolidation treatment time, application history of other drugs, biochemical indicators before and during treatment, virological indicators, immunological indicators, imaging data, etc., and complications before and during treatment. However, we failed to collect HBV genotype at the enrollment.
- 3) Monitoring indicators during the follow-up: patients were monitored every 3 months during the follow-up, including: alanine aminotransferase (ALT), aspartate aminotransferase (AST), direct bilirubin (DBIL), total bilirubin (TBIL), albumin (ALB), AFP, blood routine, HBsAg, HBeAg, HBeAb, HBV DNA, abdominal ultrasound, liver elasticity examination, etc.

Biochemical examination

HBV serological markers were measured by chemiluminescent microparticle immunoassay (Architect i2000 analyzer; Abbott Laboratories). The detection range of HBsAg was 0.05–250 IU/mL, HBeAg ≥ 1 S/CO was positive, and HBeAb < 1 was positive. Serum HBV DNA was detected by fluorescence quantitative polymerase chain reaction (Roche, Switzerland, Light Cycle 480 PCR system), and the lower limit of detection value was 20 IU/mL. The liver function index was detected with Hitachi 7600 automatic biochemical analyzer, and the upper limit of normal ALT value was 40U/L (Osaka and TanPUR Chemical Industry Co., Ltd., Japan).

Off-therapy outcome measures

Main evaluation measures: The sustained off-treatment virological response rate after discontinuation of oral antiviral therapy, which was defined as HBV DNA remained below the lower limit of detection after drug withdrawal during the follow-up.

Secondary evaluation indexes: 1) Virological re-positive rate; 2) Clinical relapse rate; 3) HBeAg reversion rate; 4) HBsAg

negative rate; 5) Incidence rate of adverse events such as liver cirrhosis and liver cancer.

Various definitions

- 1) Antiviral treatment time: the time from the start of antiviral treatment to drug withdrawal;
- 2) Consolidation treatment time: The time from HBeAg serological conversion and HBV DNA negative conversion to stop antiviral treatment for HBeAg positive patients during the treatment period;
- 3) Sustained off-treatment virological response: HBV DNA level of CHB patients is still lower than the lower limit of detection after stopping oral antiviral treatment 1 year;
- 4) Viral relapse: HBV DNA were positive for two consecutive times (3 months apart) after stopping oral antiviral treatment;
- 5) Clinical relapse: Patients' HBV DNA were positive and ALT $\geq 2 \times$ ULN at the same visit (the upper limit of ALT detection value in our hospital is 40U/L);
- 6) HBeAg reversion: Patients' HBeAg appear positive again after HBeAg seroconversion;
- 7) HBsAg negative: Patients with chronic HBV infection show sustained off-treatment viral response, and the level of HBsAg is less than 0.05 IU/mL;
- 8) Liver fibrosis score (APRI, FIB-4 score): APRI ≥ 2 indicates liver cirrhosis, APRI < 1 excludes liver cirrhosis (3), FIB-4 ≥ 3.25 indicates progressive fibrosis, FIB-4 < 1.45 excludes progressive liver fibrosis (15, 16).

Statistical analysis

The continuous variables were presented as mean, standard deviation, median and interquartile range. T-tests and Mann-whitney U test were used for comparison between two groups. The categorical variables were presented as frequencies and proportions. The chi-square analysis and Fisher test were used to compare between the two groups. Pearson and Spearman correlation analysis were used for correlation analysis of two continuous variables. Logistic regression was used to analyze the influencing factors of sustained off-treatment virological response. All tests were performed on both sides, and $P < 0.05$ was defined as statistically significant. Biochemical, viral and serological indicators were observed at five time points and $P < 0.01$ was statistically significant. For all analyses, SPSS software (version 19.0) was used.

Result

Baseline demographic and clinical characteristics of enrolled patients

A total of 151 patients were initially HBeAg positive and continued consolidation treatment for 3 years after achieved HBeAg seroconversion. Among them, 56 patients used interferon intermittently, 5 patients refused to follow up, and 8 patients could not come to the hospital regularly because they returned to the local hospital. Finally, 82 patients were included in this study (Figure 1).

Of the 82 patients, 42 were male (51.22%) and 40 were female (48.78%), with a median age of 34.00 (31.00, 37.25) years old. Eight patients (9.76%) had a family history of liver cancer.

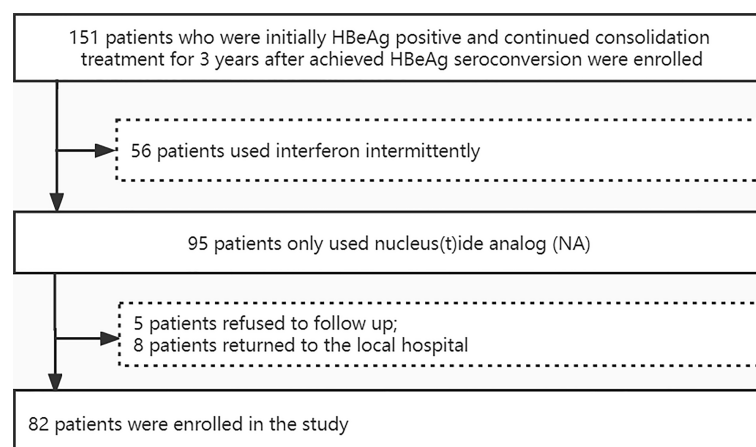


FIGURE 1
Patient enrollment and deposition.

Among the infection routes of all enrolled patients, 31 (37.80%) patients were infected with HBV by mother-to-child transmission, 24 (29.27%) patients had a long-term contact history with hepatitis B patients, 2 (2.44%) patients may be infected through infusion or surgery, 1 (1.22%) patient had a history of blood transfusion and close contact with hepatitis B patients, and the others were unknown.

The median total treatment time was 66.50 (52.75, 86.25) months, and the median consolidation treatment time was 41.00 (36.00, 54.00) months. During the treatment, some patients changed the oral antiviral drug due to drug resistance, drug safety and other side effects. 46 patients (56.10%) used only one oral antiviral drug, 27 patients (32.93%) used two oral antiviral drugs, and 9 patients (10.98%) used three oral antiviral drugs successively. Before stopping antiviral treatment, 62 patients (75.61%) used entecavir (ETV), and 17 patients (20.73%) used tenofovir disoproxil fumarate (TDF), two patients (2.44%) used adefovir dipivoxil (ADV) and one patient (1.22%) used lamivudine (LAM) antiviral therapy.

The median level of HBsAg was 3.06 (2.79, 3.51) $\log_{10}$ IU/mL when patients stopped oral antiviral therapy, of which 12.20% (10/82) patients had HBsAg $\leq$ 100 IU/mL, and 87.80% (72/82) patients had HBsAg > 100 IU/mL. All patients had normal liver function, and the median APRI score at baseline was 0.26 (0.19, 0.44). According to APRI score, no patient had cirrhosis, and 79 patients could completely exclude cirrhosis. The median FIB-4 score at baseline was 0.85 (0.63, 1.22). According to the FIB-4 score, no patients considered advanced liver fibrosis, and 72 patients could exclude advanced liver fibrosis. No patients had liver cirrhosis or malignant tumor according to their imaging examination (Table 1).

Different prognosis after discontinuation of oral antiviral therapy

Within 12 months after discontinuing oral antiviral therapy, 36.59% (30/82) of the patients maintained virological response, 63.41% (52/82) of the patients had virological relapse, of which 28.05% (23/82) relapse at 3month, 20.73% (17/82) relapse at 6month, 8.54% (7/82) relapse at 9 month, and 6.10% (5/82) relapse at 12month. 17.07% (14/82) of patients had clinical relapse during the follow-up, of which 1.22% (1/82) had clinical relapse at 3 month, 6.10% (5/82) had clinical relapse at 6 month, 7.32% (6/82) had clinical relapse at 9 month, and 2.44% (2/82) had clinical relapse at 12 month (Figure 2).

In this study, 10.98% (9/82) of patients had HBeAg reversion during the 12-month follow-up period, which all occurred in patients with clinical relapse, and mainly occurred at 6 months after stopping treatment. During the follow-up, 3.66% (3/82) of the patients had HBsAg decrease > 1 $\log_{10}$ IU/mL, no patients achieved HBsAg negative, and no patients developed to cirrhosis and liver cancer.

Clinical relapse means that HBV replication in patients is active, which induces the immune defense of the body and causes hepatitis. Some patients may progress to liver failure during this process. Therefore, all patients with clinical relapse received antiviral treatment again for safety in our study, of which 7 patients (50.00%) received ETV 0.5 mg daily, 3 patients (21.43%) received TDF 300 mg daily, and 1 patient (7.14%) received pegylated interferon α - 2a 180ug combined with ETV 0.5 mg, 2 patients (14.29%) received pegylated interferon α - 2a 180ug combined with TDF 300mg, one patient (7.14%) received pegylated interferon α - 2a 180ug combined with 25 mg propofol tenofovir fumarate. The patients treated with pegylated interferon α - 2a and ETV achieved clinical cure, and the remaining patients were negative for HBV DNA after treatment, and the patients with HBeAg reversion achieved HBeAg serological conversion again.

TABLE 1 Baseline characteristic of the subjects.

Clinical features	Values
Age [year, median (Q1, Q3)]	34.00 (31.00, 37.25)
Male (n, %)	42 (51.22%)
Family history of liver cancer (n, %)	8 (9.76%)
Antiviral treatment time [month, median (Q1, Q3)]	66.50 (52.75, 86.25)
Consolidation treatment time [month, median (Q1, Q3)]	41.00 (36.00, 54.00)
Kinds of oral antiviral treatment drugs (n, %)	
1	46 (56.10%)
2	27 (32.93%)
3	9 (10.98%)
HBsAg ($\log_{10}$ IU/mL)	3.06 (2.79, 3.51)
HBsAg (IU/mL)	
$\leq$ 100	10 (12.20%)
>100	72 (87.80%)
AFP (ng/mL)	2.20 (1.70, 3.23)
ALT (U/L)	19.65 (12.78, 33.05)
AST (U/L)	20.15 (17.48, 26.25)
TBIL (umol/L)	10.50 (8.38, 13.63)
DBIL (umol/L)	3.80 (3.10, 4.90)
ALB (g/L)	47.50 (45.03, 49.23)
APRI [median (Q1, Q3)]	0.26 (0.19, 0.44)
APRI $\geq$ 2 (n, %)	0
APRI<1 (n, %)	79 (96.34%)
FIB-4 [median (Q1, Q3)]	0.85 (0.63, 1.22)
FIB-4 $\geq$ 3.25 (n, %)	0
FIB-4<1.45 (n, %)	72 (87.80%)

82 patients were enrolled in the study; Values are presented as n (%), or median (Q1, Q3). HBsAg, hepatitis B surface antigen; AFP, alpha fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; DBIL, direct bilirubin; ALB, albumin; APRI = [AST (U/L)/ULN $\times$ 100]/PLT (10^9 /L) note: ULN= the upper limit of AST (40U/L) FIB-4 = [Age $\times$ AST (U/L)]/[PLT (10^9 /L) $\times$ $\sqrt$ ALT (U/L)] APRI $\geq$ 2 represents liver cirrhosis, APRI<1 excludes liver cirrhosis; FIB-4 $\geq$ 3.25 represents progressive liver fibrosis, FIB-4<1.45 excludes progressive liver fibrosis.

Characteristic of patients with sustained virological response and viral relapse at baseline

In this study, 30 (36.59%) patients had sustained virological response and 52 (63.41%) patients had viral relapse after 12-months of follow-up. The comparison of the characteristic between the two groups showed that the median age of patients in the sustained virological response group [34.50 (30.75, 39.25) vs. 33.50 (31.00, 37.00) years, $Z=-0.391/P=0.696$], antiviral treatment time [69.50 (56.25, 86.00) vs. 62.50 (44.00, 88.50) months, $Z=-0.689/P=0.491$], the consolidation treatment time [41.50 (36.75, 54.75) vs. 40.50 (36.00, 53.75) months, $Z=-0.419/P=0.675$], had no significant difference with viral relapse patients. There was no significant difference between the two groups in gender, family history of liver cancer and the kinds of oral antiviral drugs ($P>0.05$). The median level of HBsAg in the sustained virological response group was lower than that in the viral relapse group, but the difference was not statistically significant [2.92 (2.36, 3.52) vs. 3.18 (2.90, 3.54) $\log_{10}$ IU/mL, $Z=-1.492/P=0.136$]. The ALT, AST, TBIL, DBIL, ALB and other indicators of the patients in the sustained virological response group and the viral relapse group were not significantly different. The baseline median APRI of the sustained virological response group was 0.28 (0.18, 0.41), and that of the viral relapse group was 0.25 (0.21, 0.33). According to the APRI score, 29 (96.70%) patients in the sustained virological response group could exclude cirrhosis, and 50 (96.20%) patients in the viral relapse group could exclude cirrhosis. In addition, the median baseline FIB-4 in the sustained virological response group was 0.61 (0.49, 1.05), and that in the viral relapse was 0.74 (0.58, 0.90). According to the FIB-4 score, 26 (86.70%) patients in the sustained virological response group could exclude advanced

liver fibrosis, and 46 (88.50%) patients in the viral relapse group could exclude advanced liver fibrosis (Table 2).

Changes in clinical indicators of patients with sustained virological response and viral relapse during follow-up

After CHB patients stopped oral antiviral treatment, HBsAg, ALT, TBIL, ALB and other indicators did not change significantly at different time. The median of the above clinical indicators between the two groups were compared (Figure 3). The levels of HBsAg in patients with viral relapse at different time were higher than those in sustained virological response patients, but there was no statistically difference ($P>0.01$). The median ALT, TBIL, ALB levels of viral relapse patients at different time were also higher than those in sustained virological response patients, and the differences were not significant ($P>0.01$). In the sustained virological response group, HBsAg level decreased slightly after 6 months of follow-up, while it increased slightly in the viral relapse group. The ALT level of patients with sustained virological response also decreased slightly after 6 months, while that of patients with viral relapse increased slightly.

Characteristic of patients with clinical relapse and viral relapse at baseline

During the follow-up, there were 52 patients (63.41%) had viral relapse, 14 of whom (26.92%) had clinical relapse. Baseline characteristics were compared between the two groups (Table 3). It shows that the median age of viral relapse patients at baseline

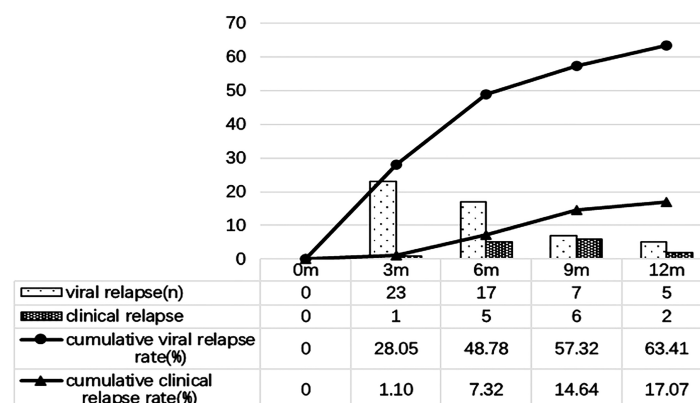


FIGURE 2

Viral relapse and clinical relapse in patients after stopping oral antiviral treatment.

was 32.50 (30.75, 37.00) years, and the median age of clinical relapse patients was 34.00 (32.00, 40.25) years. There was no significant difference between the two groups ($P=0.315$). In the viral relapse group, the median antiviral treatment time was 60.50 (44.00, 89.50) months, the median consolidation treatment time was 40.50 (36.00, 53.25) months, the median antiviral treatment time for patients with clinical relapse was 65.50 (40.75, 88.25) months, and the median consolidation treatment time was 43.00 (36.00, 56.00) months. There was no significant difference between groups ($P>0.05$). There were no significant differences in sex, family history of liver cancer, and types of antiviral drugs between the two groups ($P>0.05$). The baseline median HBsAg level of viral relapse patients was lower than that of patients with clinical relapse [3.01 (2.87, 3.46) vs. 3.45 (3.15, 3.65) $\log_{10}$ IU/mL, $Z=-1.795/P=0.073$]. In this study, 10.53% (4/38) of patients in viral relapse group had HBsAg ≤ 100 IU/mL, and all the patients in clinical relapse group was > 100 IU/mL. However, the difference between the two groups was not significant. The baseline AFP, ALT, TBIL, ALB and other indicators of viral relapse patients and clinical relapse patients were compared, and there was no significant difference between the two groups. According to APRI score, 94.74% (36/38) of viral relapse patients could exclude cirrhosis, and all the patients with clinical relapse could exclude cirrhosis. According to FIB-4 score, 84.21% (32/38) of viral relapse patients could exclude advanced liver fibrosis, and all patients with clinical relapse could exclude advanced liver fibrosis.

Correlation between recurrence time and baseline indicators

In order to determine the relationship between the recurrence time and the antiviral treatment time, consolidation treatment time, HBsAg, ALT in CHB patients after stopping oral antiviral treatment, Spearman correlation analysis was conducted among the above indicators, virological relapse time, and clinical relapse time. The results showed that the Spearman correlation coefficients between the viral relapse and the above indicators were as, antiviral treatment time ($r_s=0.040$, $P=0.780$), consolidation treatment time ($r_s=0.243$, $P=0.082$), HBsAg ($r_s=-0.173$, $P=0.221$), ALT ($r_s=0.081$, $P=0.568$). The Spearman correlation coefficients between the clinical relapse time and the above indicators were as, antiviral treatment time ($r_s=-0.146$, $P=0.620$), consolidation treatment time ($r_s=-0.149$, $P=0.612$), HBsAg ($r_s=0.103$, $P=0.725$), ALT ($r_s=0.437$, $P=0.119$), and there was no significant correlation ($P>0.05$) (Table 4).

Discussion

CHB is liver inflammation caused by HBV (3), and is one of the most important infectious diseases in China. The occurrence of CHB is the result of the interaction between the virus and its stimulated immune system. Antiviral therapy is always used to reduce the incidence of liver cirrhosis and hepatocellular

TABLE 2 Characteristic of patients with sustained virological response and Viral relapse at baseline.

	Sustained virological response (n=30)	Viral relapse (n=52)	Z/χ^2	P
Age [year, median (Q1, Q3)]	34.50 (30.75, 39.25)	33.50 (31.00, 37.00)	-0.391	0.696
Male (n, %)	14 (46.70%)	28 (53.80%)	0.392	0.531
Family history of liver cancer (n, %)	2 (8.70%)	6 (11.50%)	0.109	0.742
Kinds of oral antiviral treatment drugs (n, %)				0.944
1	18 (60.00%)	28 (53.80%)		
2	9 (30.00%)	18 (34.60%)		
3	3 (10.00%)	6 (11.50%)		
Antiviral treatment time [month, median (Q1, Q3)]	69.50 (56.25, 86.00)	62.50 (44.00, 88.50)	-0.689	0.491
Consolidation treatment time [month, median (Q1, Q3)]	41.50 (36.75, 54.75)	40.50 (36.00, 53.75)	-0.419	0.675
HBsAg ($\log_{10}$ IU/mL)	2.92 (2.36, 3.52)	3.18 (2.90, 3.54)	-1.492	0.136
HBsAg ≤ 100 IU/mL	6 (20.00%)	4 (7.70%)	1.665	0.197
AFP (ng/mL)	2.00 (1.78, 3.15)	2.20 (1.70, 3.28)	-0.578	0.563
ALT (U/L)	19.05 (12.70, 35.70)	20.45 (12.90, 29.20)	-0.212	0.832
AST (U/L)	21.35 (17.38, 29.03)	19.40 (17.42, 24.93)	-0.361	0.718
TBIL (μ mol/L)	10.60 (8.28, 13.60)	10.45 (8.40, 14.10)	-0.274	0.784
DBIL (μ mol/L)	3.80 (3.08, 5.53)	3.75 (3.10, 4.68)	-0.024	0.981
ALB (g/L)	46.50 (44.58, 48.32)	47.80 (45.40, 49.78)	-1.931	0.054
APRI [median (Q1, Q3)]	0.28 (0.18, 0.41)	0.25 (0.21, 0.33)	-0.241	0.810
APRI <1 (n, %)	29 (96.670%)	50 (96.15%)	0	1.000
FIB-4 [median (Q1, Q3)]	0.61 (0.49, 1.05)	0.74 (0.58, 0.90)	-0.289	0.773
FIB-4 <1.45 (n, %)	26 (86.67%)	46 (88.46%)	0	1

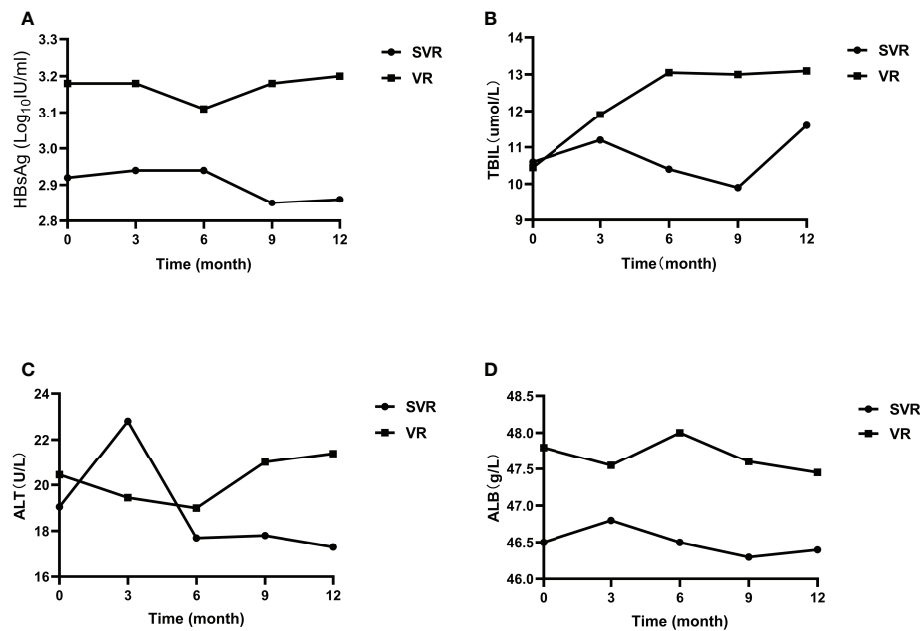


FIGURE 3

Clinical indicators of patients with sustained virological response and viral relapse. (A) the change of HBsAg; (B) the change of TBIL; (C) the change of ALT; (D) the change of ALB. SVR, sustained virological response; VR, viral relapse.

TABLE 3 Characteristic of patients with clinical relapse and viral relapse at baseline.

	Viral relapse (n=38)	clinical relapse (n=14)	Z/ χ^2	P
Age [year, median (Q1, Q3)]	32.50 (30.75, 37.00)	34.00 (32.00, 40.25)	-1.006	0.315
Male (n, %)	22 (57.89%)	6 (42.86%)	0.931	0.335
Family history of liver cancer (n, %)	4 (10.50%)	2 (14.30%)	0	1.000
Kinds of oral antiviral treatment drugs (n, %)				0.490*
1	21 (55.26%)	7 (50.00%)		
2	14 (36.84%)	4 (28.57%)		
3	3 (7.90%)	3 (21.40%)		
Antiviral treatment time [month, median (Q1, Q3)]	60.50 (44.00, 89.50)	65.50 (40.75, 88.25)	-0.186	0.853
Consolidation treatment time [month, median (Q1, Q3)]	40.50 (36.00, 53.25)	43.00 (36.00, 56.00)	-0.052	0.958
HBsAg (log ₁₀ IU/mL)	3.01 (2.87, 3.46)	3.45 (3.15, 3.65)	-1.795	0.073
HBsAg ≤ 100 IU/mL	4 (10.53%)	0	0.458	0.498
AFP (ng/mL)	2.35 (1.68, 3.43)	2.00 (1.85, 2.90)	-0.310	0.757
ALT (U/L)	22.30 (13.10, 34.05)	17.60 (10.67, 25.83)	-0.990	0.322
AST (U/L)	20.40 (17.48, 26.83)	18.65 (16.30, 21.75)	-1.207	0.227
TBIL (umol/L)	10.20 (8.35, 14.35)	10.85 (8.30, 14.40)	-0.165	0.869
DBIL (umol/L)	3.70 (3.08, 4.83)	3.75 (3.18, 4.28)	-0.124	0.901
ALB (g/L)	48.25 (46.15, 50.25)	47.00 (44.18, 48.10)	-1.888	0.059
APRI [median (Q1, Q3)]	0.27 (0.20, 0.50)	0.22 (0.17, 0.33)	-1.485	0.137
APRI<1 (n, %)	36 (94.74%)	14 (100.00%)		1*
FIB-4 [median (Q1, Q3)]	0.85 (0.69, 1.27)	0.85 (0.55, 1.18)	-1.093	0.274
FIB-4<1.45 (n, %)	32 (84.21%)	14 (100.00%)	1.191	0.275

*Fisher's exact test.

carcinoma in CHB patients (17–19). Controlling HBV replication and improving human immunization are important ways to reduce liver inflammation and prevent disease progression. China's 2019 Guidelines for the Prevention and Treatment of Chronic Hepatitis B further expanded the indications for antiviral treatment of chronic HBV infection (3). For patients with chronic HBV infection, the current major antiviral treatment are NA and interferon, both of which have advantages and disadvantages (3). The clinical cure rate of interferon is higher than that of NA, which can significantly reduce the risk of liver cirrhosis and hepatocellular carcinoma (20, 21). However, it is expensive and is inconvenient to use. Some patients stop interferon treatment because they cannot tolerate side effects. NA is easy to use and can effectively suppressed virus replication to delay the progress of liver disease, but they often relapse after drug withdrawal and need long-term treatment. Compared with interferon, NA have no direct immunomodulatory effect (3, 4, 7). Therefore, the limited course of NA cannot induce a lasting response after drug withdrawal theoretically. However, in recent years, some scholars have proposed that if patients with CHB can maintain virological response and consolidate treatment for a certain period of time, they can also consider discontinuing the drug even if HBsAg don't turn negative (22, 23). Some studies have shown that the negative rate of HBsAg after drug withdrawal is higher than that during treatment (22). At present, domestic and foreign guidelines have proposed that it is feasible to stop oral antiviral treatment for patients with long-term oral NA before HBsAg turns negative (3, 4, 7, 24). However, the sustained virological response rate and safety after drug withdrawal still need to be verified. At present, the research on patients with liver cirrhosis after drug withdrawal is limited. According to the existing studies, patients with liver cirrhosis are not recommended to stop oral antiviral treatment. Therefore, we didn't include patients with liver cirrhosis in this study. The purpose of our study is to establish a follow-up cohort of non-liver cirrhosis HBeAg positive initially CHB patients who stopped taking NA, observe the sustained virological response and relapse and explore the relevant factors, which could help explore a clinically feasible NA mode for HBeAg positive CHB patients to stop taking NA, achieve the rationalization and

scientization of drug use, and obtain the maximum health economic benefits.

In this study, all enrolled patients used NA before stopping treatment, and 43.90% (36/82) of patients used two or more NAs due to drug resistance or other reasons. There is no significant statistical difference in the types of oral antiviral drugs between patients with sustained virological response and those with viral relapse. Considering that on the one hand, the sample size may not be large enough, on the other hand, patients all used first-line antiviral drugs such as ETV and TDF before stopping oral antiviral treatment, and long-term ETV or TDF treatment may affect the immune response of the body, narrowing the differences between groups. The third reason is that we didn't acquire test results before starting antiviral treatment. It is difficult to define the changes of patients' indicators during antiviral treatment. This requires us to expand the sample in the future.

NA mainly inhibits viral replication and delay disease progression by competitively inhibiting HBV DNA polymerase, but has no significant impact on the level of covalently closed circular DNA (cccDNA), integrated HBV DNA, HBeAg, and HBsAg in the liver (3, 25–27). During the follow-up period after drug withdrawal, only 36.59% (30/82) of patients had sustained virological response, 63.41% (52/82) had viral relapse, and 17.07% (14/82) had clinical relapse. All patients have consolidated treatment for more than 3 years after achieving HBeAg seroconversion according to the guidelines, however, most patients had viral relapse during the follow-up, indicating that for HBeAg positive patients, the drug should not be stopped easily even if HBeAg seroconversion has been achieved through NA treatment. In previous studies, most of the them set viral relapse (HBV DNA > 2000 IU/mL) or clinical relapse (HBV DNA > 2000 IU/mL, ALT > 2 × ULN) as the main evaluation index. Some studies have shown that the cumulative viral relapse rate of HBeAg positive CHB patients was 50% after stopping antiviral treatment for 2 years (28). HBV DNA is the most direct marker of HBV replication and activity with high sensitivity and specificity. Positive HBV DNA indicates HBV reactivation. For patients who have achieved HBeAg seroconversion and HBV DNA clearance after oral drug treatment, positive HBV DNA indicates that the balance

TABLE 4 Correlation between recurrence time and baseline indicators.

Variable	Viral relapse time (month)		Clinical relapse (month)	
	<i>rs</i>	<i>P</i>	<i>rs</i>	<i>P</i>
Antiviral treatment time (month)	0.040	0.780	-0.146	0.620
Consolidation treatment time (month)	0.243	0.082	-0.149	0.612
HBsAg (log ₁₀ IU/mL)	-0.173	0.221	0.103	0.725
ALT (U/L)	0.081	0.568	0.437	0.119

between human immunity and HBV is broken, and the risk of disease progression is increased. Therefore, it is meaningful and feasible to list positive HBV DNA as the evaluation index of research. In this study, we compared the relevant factors of patients with sustained virological response and patients with viral relapse, and found no reliable factors that can predict the relapse of the virus. At present, we need to further explore other indicators to guide the safe drug withdrawal in CHB patients.

A number of studies have shown that the low concentration of HBsAg at the time of stopping oral antiviral treatment is related to the high clinical cure and the low viral relapse and clinical relapse after stopping antiviral treatment (29–32). Our research supports this view. In this study, patients with sustained virological response had lower HBsAg levels than those with viral relapse. At the same time, the HBsAg level in patients with viral relapse was also lower than that in patients with clinical relapse, suggesting that the HBsAg level may be a reliable marker for evaluating the sustained virological response after drug withdrawal. HBsAg level in serum is in direct proportion to the cccDNA and integrated HBV DNA in liver tissue (27). When the host is infected with HBV, HBV enters the liver cell through sodium taurocholate cotransporting polypeptide (NTCP) on the surface of the liver cell membrane (3, 27), and forms cccDNA in the host liver cell. On the one hand, cccDNA maintains its own level stability through transcription and reverse transcription, and on the other hand, it releases HBsAg into the blood through transcriptional and translation (3, 27). Therefore, HBsAg level in serum can partly reflect the transcription activity of cccDNA *in vivo*. The lower level of HBsAg, the lower content and transcription of cccDNA in patients. At present, there is no drug that can help the host clear the cccDNA in the liver, which is the main reason why CHB is easy to relapse after drug withdrawal. In this study, HBsAg level decreased slightly after 6 months, while the viral relapse group increased slightly. However, there was no significant difference in the HBsAg level between the two groups. On the one hand, the sample size of this study is relatively small, and on the other hand, it is related to the relatively short follow-up time.

Previous research shows that serum HBcAb level can also reflect liver inflammation and predict the efficacy of antiviral therapy (33). A prospective study involving 100 patients showed that 21% of patients with HBcAb ≥ 1000 IU/mL at the end of treatment had clinical relapse within 4 years after discontinuing antiviral therapy, while 85% of patients with HBcAb < 100 IU/mL had clinical relapse. The study showed that HBcAb level was related to the clinical relapse after drug withdrawal, and it might guide CHB patients stop antiviral treatment safely (34). Unfortunately, in this study, we did not conduct quantitative analysis of HBcAb, which needs to be perfected in future studies.

In this study, 10 patients with clinical relapse received NA treatment again, and 4 patients received combination treatment

of NA and peg-interferon α -2a, 1 case achieved clinical cure, and no adverse events such as cirrhosis or hepatocellular carcinoma occurred during the follow-up. Although only one case achieved clinical cure after combination treatment, it also provided a new direction for the clinical treatment of such patients. The outcome of CHB is related to the interaction between HBV and host immunity. Long term low-level antigen stimulation and long-term use of NA will affect the function of HBV specific CD8 cells (35). Studies have shown that the negative change of HBsAg after drug withdrawal was higher than that during treatment, and the negative rate of HBsAg in patients without relapse after drug withdrawal was the highest, followed by patients with relapse and untreated (22). In this study, no patient's HBsAg converted to negative within 1 year after stopping oral antiviral treatment, 3 patients had HBsAg decrease $> 1 \log_{10}$ IU/mL, and 1 patient achieved clinical cure through combined treatment after clinical relapse. It is suggested that for patients with clinical relapse after drug withdrawal, the use of peg-interferon α -2a may be helpful to achieve clinical cure (HBsAg turns negative). The disappearance of HBsAg is considered as "clinical cure", which is an ideal treatment target for the antiviral treatment of chronic hepatitis B infection (36). In order to achieve the immune control goal characterized by HBeAg seroconversion or HBsAg disappearance, interferon has greater advantages than NA. Because interferon treatment has higher HBeAg seroconversion rate and HBsAg disappearance rate than NA, and once interferon treatment achieves HBeAg seroconversion or HBsAg disappearance, it will bring long-term good clinical outcomes after stopping treatment (37).

In conclusion, our study shows that according to the existing drug withdrawal criteria, the rate of sustained off-treatment virological response of CHB patients with HBeAg positive initially is low, and it always occurred in patients with low HBsAg levels. Patients are more likely to have viral relapse or clinical relapse after stopping oral antiviral treatment. The patients with clinical relapse are expected to achieve clinical cure after combining peg-interferon α -2a with NA. Due to the limited number of patients who can reach the withdrawal standard of oral antiviral drugs in clinical practice, our sample size is not large. If we can expand the sample size in future studies, the conclusion may be more reliable. And monitoring for only 12 months may overlook the incidence of viral relapse, cirrhosis and hepatocellular carcinoma, so it's recommended to follow up at least 24 months in future studies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Beijing Ditan Hospital affiliated to Capital Medical University (Jing Di Lun Ke Zi 2017 No. 003 - 02). The patients/participants provided their written informed consent to participate in this study.

Author contributions

ML, JD, and YX contributed to the study design. ML, FS, ZL, and LH contributed to the data analysis. ML, FS, ZL, LH, WD, TJ, SYW, YL, LZ, GS, RL, SLW, MC and ML contributed to the recruitment, enrolment, and assessment of participants, as well as data collection. FS, WD, TJ, XB, HL, YG, HH, MX, and XC contributed to following up with the patients. ZZ, YJL, and LY managed all aspects of laboratory support. FS wrote the first draft of the manuscript. YX revised the manuscript and is the guarantor of the article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A case report of hepatitis B virus reactivation 19 months after cessation of chemotherapy with rituximab

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A 72-year-old woman presented to our hospital with elevation of serum transaminases. Her blood tests showed the hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) negative. Rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) were given for the diffuse large B-cell lymphoma. She didn't receive anti-hepatitis B virus (HBV) drug for the isolated HBcAb positive. HBV reactivation confirmed based on the serum HBV DNA detectable until 19 months after stopping R-CHOP regimen. HBV DNA became undetectable after 4 weeks therapy with Tenofovir alafenamide fumarate (TAF). Serum transaminases went down to normal 3 months later after receiving TAF. HBV reactivation is a substantial risk for patients with isolated HBcAb positive receiving rituximab-containing chemotherapy without anti-HBV drug. Regular monitoring with a frequency of 1-3 months is the basis for timely diagnosis and treatment of HBV reactivation. Serum transaminases abnormalities may be the initial manifestation of HBV reactivation.

KEYWORDS

hepatitis B virus reactivation, lymphoma, rituximab, hepatitis B virus, CD20 monoclonal antibody

Introduction

HBV reactivation is a well-known complication of HBV infected patients undergoing chemotherapy or immunosuppressive therapy. It is well documented (1–7) that prophylactic anti-HBV therapy should be given to HBsAg-negative, HBcAb-positive patients before receiving CD20 monoclonal antibody drugs, but there is no consensus on the duration of prophylactic medication, frequency of monitoring during and after prophylactic treatment, or monitoring indicators. The duration of prophylactic

medication recommended by Chinese Society of Hepatology (5) and European Association for the Study of the Liver (EASL) (3) is at least 18 months since the cessation of immunosuppressive therapy. Here we report a case of HBV reactivation 19 months after cessation of chemotherapy with rituximab for diffuse large B-cell lymphoma in a patient with resolved hepatitis B infection (HBsAg negative, HBcAb positive and HBV DNA negative).

Case report

72-year-old women presented to our hospital with elevation of serum transaminases for one day on August 19, 2021. Liver chemistry showed alanine aminotransferase (ALT) 132 U/L, aspartate aminotransferase (AST) 92 U/L, alkaline phosphatase (ALP) 113 U/L, γ -glutamyl transferase (GGT) 56 U/L. She has no fatigue, anorexia, nausea, abdominal pain, dark urine, pale stool, itchy skin, or other symptoms. Past medical history: She underwent splenectomy and was diagnosed with diffuse large B-cell lymphoma due to splenomegaly in September 2019. She started R-CHOP regimen in November 2019. Her laboratory finding showed isolated HBcAb positive (HBcAb+/HBsAb-/HBsAg-/HBeAb-/HBeAg-), undetected (< 20 IU/ml) HBV DNA, and normal transaminases before and during chemotherapy. Complete response to chemotherapy was achieved and immunosuppressive therapy was withdrawn in June 2020 after completing 4 cycles of it. Monitoring of HBV markers and aminotransferases every 1-3 months after chemotherapy showed no change through 14 months after cessation of chemotherapy. She had no history of receiving anti-HBV drug, liver diseases and blood transfusion. She has no history of taking Chinese traditional medicine, health products or other drugs. She had no history of alcohol consumption. Physical examination didn't show any signs of liver disease (jaundice, liver palm, spider angioma, hepatomegaly, shifting dullness or edema in lower limbs). Blood tests still showed isolated HBcAb positive and

undetected HBV DNA. The patient was tested negative for antibodies of hepatitis A virus (HAV) IgM, anti-hepatitis C virus (HCV) IgG, anti-hepatitis E virus (HEV) IgM and IgG. The serum Cytomegalovirus-DNA and Epstein Barr viruses-RNA were negative (< 500 copies/ml). Anti-nuclear antibody (ANA) was borderline positive (1:100); Anti-soluble liver antigen and anti-liver-pancreas antibodies (Anti-SLA/LP), anti-mitochondrial autoantibodies (AMA), liver kidney microsomal antigen (LKM) and smooth muscle antibody (SMA) were negative. Serum immunoglobulin G (IgG) and immunoglobulin M (IgM) were normal. Ultrasonography showed markedly increased echogenicity of the liver, thickening of the gallbladder wall, and right renal cyst. Fibro Scan showed an increased CAP score (300 dB/m) and a fibrosis score of 7.1 Kpa.

On August 19, 2021, she was prescribed diammonium glycyrrhizinate enteric-coated capsules and bicyclol tablets for liver protection. One month later (September-2021), ALT level decreased to normal (27 U/L). However, AST level kept abnormal. Four months later (January-2022), HBV DNA increased to 7.8×10^1 IU/ml. Although blood test on the same day still showed isolated HBcAb positive, HBV reactivation was considered, and antiviral therapy was recommended to her. Unfortunately, she refused anti-HBV therapy. She was advised to come back to the hospital for monthly follow-up, but she did not until April 2022.

In April 2022, HBV DNA level increased significantly to 1.86×10^4 IU/ml, together with positive of HBsAg, HBeAg and HBcAb. Liver biochemistry showed ALT 20 U/L, AST 47 U/L. She was prescribed TAF immediately. One month later (May-2022), HBV DNA turn back to undetectable. Three months later (July-2022), complete response (undetectable HBV DNA, HBsAg loss and transaminase normalization) was achieved. Re-examined Fibro Scan in July 2022 showed a CAP score of 255 dB/m and a fibrosis score of 4.0 Kpa. The laboratory findings during the monitoring period are shown in Figure 1.

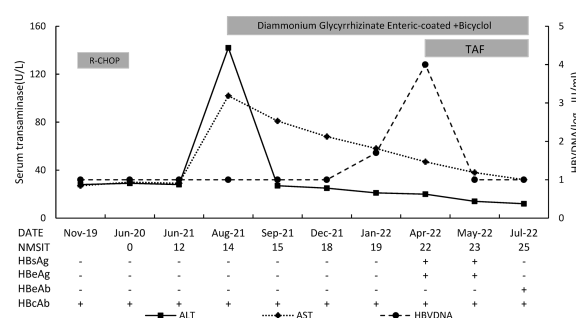


FIGURE 1

The sketch map of this patient. NMSIT: the number of months after stopping immunosuppressive therapy.

Discussion

HBV reactivation is a substantial risk for patients with isolated HBcAb positive receiving rituximab-containing chemotherapy. Studies (8–12) showed that the incidence of HBV reactivation was 3%–68.3% in patients with isolated HBcAb positive who didn't take prophylactic antiviral therapy while receiving rituximab. The incidence of liver failure after HBV reactivation ranges from 0% to 37.5%, resulting in a mortality rate of 0% to 12.5% (11, 13, 14). Prophylaxis anti-HBV therapy is recommended in current guidelines for lymphoma patients with isolated HBcAb positive who received R-CHOP. This patient did not receive anti-HBV drugs for prophylaxis prior to receiving rituximab and subsequently developed HBV reactivation and ALT flare. This case suggested a necessity of prophylactic antiviral therapy for the patients with isolated HBcAb positive in this clinical setting.

In addition, this case also reminds us that the time between stopping of immunosuppressive therapy and HBV reactivation can exceed 18 months, which is the course recommended by EASL and Chinese Society of Hepatology (3, 5) for antiviral prophylaxis in patients receiving B-cell monoclonal antibody therapy. Surprisingly, HBV reactivation happened 19 months after withdrawal of immunosuppressive therapy in this patient. Although HBV reactivation was reported (15) in a patient 55 months after treatment with autologous peripheral blood stem cell transplantation and R-CHOP chemotherapy, late HBV reactivation is rare in patients treated with R-CHOP regimen alone. Researches (16, 17) showed 83%–86% of HBV reactivation in individuals with isolated HBcAb positive receiving rituximab-containing chemotherapy occurred within 12 months after treatment with R-CHOP regimen. Therefore, both physicians and patients may ignore the necessity of subsequent monitoring for HBV reactivation 12 months after chemotherapy. The timely diagnosis of HBV reactivation becomes difficult when it appears 12 months or later after prophylactic therapy, resulting in a delay in intervention and even liver failure or death. So, this case reminds clinicians that long-term, at least 19 months, monitoring of HBV reactivation is required after discontinuation of rituximab therapy in individuals with isolated HBcAb positive.

Although the monitoring of HBV reactivation for patients with isolated HBcAb positive receiving rituximab-containing chemotherapy has become a focus of clinical attention, international consensus and details of monitoring are missing. EASL and Chinese Society of Hepatology (3, 5) recommend prophylaxis should continue for at least 18 months after stopping immunosuppression and monitoring should continue for at least 12 months after prophylaxis withdrawal. Only Chinese Society of Hepatology mentioned testing HBVDNA every 1–3 months after prophylaxis treatment. Other guidelines (1, 3, 4, 6, 7, 18) have no mention on frequency and tests advised for patients who finished monitoring as long as 12 months or 18 months after stopping immunosuppression treatment. However, there is no

answer to the question on which indicators are the best, or the most sensitive to diagnosis of reactivation.

In this patient, ALT abnormality was the first manifestation, serum HBV DNA appeared 5 months later, and HBsAg turned positive 8 months later. It indicates that transaminase is an early, sensitive indicator for the detection of HBV reactivation. Therefore, transaminase testing should be performed to early diagnose of HBV reactivation in resource-limited areas or when HBsAg and HBV DNA cannot be detected promptly. However, in fact, when the patient visits the doctor with elevation of transaminases, we did not identify the cause as HBV reactivation immediately, considering that the cause might be viral hepatitis, drug-induced liver injury, nonalcoholic steatohepatitis (NASH), and autoimmune hepatitis, and after further examination the possibility of NASH was considered. After treatment for NASH (including oral diammonium glycyrrhizinate enteric-coated capsules and bicyclol tablets, adjusting dietary structure, appropriate activity, and weight loss), although the patient's ALT decreased (possibly originating from bicyclol's ALT lowering effect), AST didn't turn to normal, which indicates persistent liver injury, until HBV becomes undetectable 3 months since TAF administration as showed in Figure 1. In short, the patient's transaminases continued to be abnormal and did not return to normal until antiviral therapy took effect. From the characteristics of the response after treatment, we judged that the elevated transaminases at the time of the patient's visit were caused by HBV. This means that ALT abnormalities may be the initial manifestation of HBV reactivation.

When considering HBV reactivation caused the transaminase elevation in this patient, there might be a puzzle: why serum HBV DNA remains undetectable (<20 IU/ml) while ALT is elevated? We suspect that the serum HBV DNA level was too low at that time, possibly below 20 IU/ml (lower limit of detection), so we could not detect it. In fact, when the HBV DNA test result is not 'target not detected', it means there may be HBV DNA at a low level. Candotti, D et al. (19) reported that even when donors were initially screened for highly sensitive HBsAg negative and highly sensitive HBV DNA < 3.4 IU/ml, HBV transmission still occurred by blood transfusion from 3 individuals with occult hepatitis B infection (OBI). In addition, even if this patient's serum HBV DNA is indeed negative, there may still be HBV DNA replication in the liver tissue (20). Indeed, the detection of HBV DNA in liver tissue, instead of the serum HBV DNA, is the gold standard for the diagnosis of OBI in HBsAg negative individuals. Our guess could be further established if we performed liver biopsy at that time and detect HBV DNA in liver tissue. This reflects the diagnostic value of liver biopsy for HBV infection. In conclusion, we suggest that serum HBV DNA may not be a sensitive marker for the diagnosis of HBV reactivation and may lag behind changes in liver biochemistry.

Fortunately, this patient responded well to TAF treatment. After 4 weeks of antiviral treatment with TAF, virological response (HBV DNA <20 IU/ml) was obtained, and complete response was obtained after 12 weeks of treatment. The rapid response is due to

the following factors: Firstly, because of timely surveillance, HBV reactivation was diagnosed early. Secondly, considering the safety, TAF was selected as anti-HBV medications. Thirdly, the patient's lymphoma was in complete remission at the time of HBV reactivation, and she had no history of chronic liver diseases, which was the basis for achieving a complete response. Timely surveillance, diagnosis and intervention is the key to manage HBV reactivation. In terms of medication, current guidelines recommend entecavir (ETV), tenofovir disoproxil fumarate (TDF) and TAF as the first-line antiviral treatment (3,7,18). Seto W K et al. (8) reported that monitoring every 4 weeks after rituximab treatment in HBsAg-negative HBcAb-positive lymphoma patients is feasible and effective, and that HBVDNA became undetectable after 6 weeks (range, 4 to 12 weeks) of ETV treatment in all HBV reactivators. No hepatitis or liver failure related to HBV was observed during the study.

Studies (21, 22) showed elderly patients are risk factor for HBV reactivation. In our report the patient was 72-year-old, the competency of the adaptive immune and innate immunity function decreased. The weakened ability of immune response leading to disability of viral clearance might contribute to the HBV reactivation. HBV reactivation depends on host related factors, viral factors and factors related to immunosuppressive agent.

In summary, clinicians should be aware of HBV reactivation and late HBV reactivation for HBsAg - negative, HBcAb - positive patients who receiving B-cell monoclonal antibody agents. Eighteen months is not enough for the duration of monitoring. Regular monitoring with a frequency of 1-3 months is the basis for timely diagnosis and treatment of HBV reactivation. Liver biochemistry, HBV DNA and HBsAg can be preferred in monitoring indicators. Notably, ALT abnormalities may be the initial manifestation of HBV reactivation.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

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Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

XG and TJ conducted writing, research, and study revision. SX was responsible for the Figure of the paper and contributed to the writing and study revision. JX and YY helped in revision and final approval of the draft manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Clinical cure rate of inactive HBsAg carriers with HBsAg <200 IU/ml treated with pegylated interferon

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Purpose: HBsAg clearance represents clinical cure for patients with hepatitis B, but remains difficult to obtain for most HBV-infected patients. Recent studies have shown that inactive HBsAg carriers treated with pegylated interferon can achieve higher clinical cure rates, which may imply that the lower the baseline HBsAg quantification, the higher HBsAg clearance rate. Therefore, this study further investigated the HBsAg clearance rate in inactive HBsAg carriers with low level of HBsAg (<200 IU/ml) treated with pegylated interferon.

Methods: This is a prospective cohort study. Inactive HBsAg carriers with HBsAg<200 IU/ml were divided into treatment and control groups. Pegylated interferon was administered to the patients in therapeutic group for 96 weeks. The patients in control group underwent 96 weeks of observation without any anti-viral treatment. All patients were tested for HBsAg, anti-HBs, HBV DNA, liver function, blood count, thyroid function, thyroid antibodies and autoantibodies at baseline, week 12, 24, 36, 48, 60, 72, 84 and 96. Controlled attenuation parameter (CAP) and liver stiffness measure (LSM) were evaluated at baseline and week 96. Patients were classified into no steatosis, mild steatosis, moderate steatosis and severe steatosis according to the value of CAP.

Results: A total of 174 inactive HBsAg carriers with HBsAg<200IU/ml were enrolled, including 84 in the treatment group and 90 in the control group. In the treatment group, HBsAg clearance rate was 30.77% (24/78) at week 48, and increased to 57.69% (45/78) at week 96. HBsAg clearance occurred in 2 patients with a clearance rate of 2.27% (2/88) in control group. The HBsAg clearance rate of the treatment group was significantly higher than that of the control group ($P<0.001$). HBsAg clearance was significantly higher in patients with moderate steatosis than in those without steatosis (74.07% vs. 48.15%, $p=0.008$) at week 96.

Conclusion: High HBsAg clearance rate could be obtained for inactive HBsAg carriers with HBsAg < 200 IU/ml treated with peginterferons. Inactive HBsAg carriers with moderate hepatic steatosis are more sensitive for the treatment.

KEYWORDS

inactive HBsAg carriers, HBsAg clearance, pegylated interferon, hepatic steatosis, clinical cure

Introduction

Approximately 250 million people worldwide are chronically infected with HBV estimated by The World Health Organization (1). Inactive HBsAg Carriers (IHCs) characterized by HBsAg <1000 IU/ml, negative HBeAg, HBV DNA <2000 IU/ml, normal ALT (2) account for about 36% of the whole population infected with hepatitis B virus (HBV) (3). IHCs can convert to HBeAg-positive chronic hepatitis B (CHB), or to HBeAg-negative CHB due to viral mutation and immune escape (4, 5). A long-term follow-up on IHCs found that the cumulative recurrent rates of hepatitis were 10.2%, 17.4%, 19.3%, 20.2% and 20.2% at five, ten, fifteen, twenty, and twenty-five years, respectively (6). Compared to healthy individuals (HBsAg-negative), IHCs have 4.6-fold and 2.1-fold higher mortality associated with liver cancer and liver disease respectively (7, 8). Recent studies have confirmed that some IHCs could achieve HBsAg clearance or seroconversion after pegylated interferon (PEG-IFN) treatment, and there is a correlation between baseline HBsAg quantification and HBsAg clearance, probably the lower level of baseline HBsAg, the higher rate of HBsAg clearance (9). This study further explored the clinical cure rate and safety of IHCs with HBsAg <200 IU/ml treated with PEG-IFN for 96 weeks.

Patients and methods

Patients

All IHCs met the criteria defined in the prevention and treatment guidelines for chronic hepatitis B (2019 edition). (i)

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; IHCs, Inactive surface antigen carriers; ALT, alanine aminotransferase; HBeAg, hepatitis e antigen; PEG-IFN, pegylated-interferon; CAP, controlled attenuation parameter; LSM, liver stiffness measure; SD, standard deviation; HBV, Chronic hepatitis B; TBiL, Total bilirubin; HIV, The human immunodeficiency virus; HCV, Chronic hepatitis C; HEV, Chronic hepatitis E; ULN, upper limit of normal; NAs, nucleos(t)ide analogue; TDF, tenofovir disoproxil fumarate; ETV, entecavir; LdT, telbivudine; ADV, adefovir dipivoxil; LAM, lamivudine.

the history of HBsAg positive was longer than 6 months and the level of HBsAg lower than 200 IU/mL, HBeAg negative, anti-HBe negative/positive; (ii) HBV DNA < 20 IU/mL, Alanine aminotransferase (ALT) normal (male < 50 IU/L, female < 40 IU/L); (iii) white blood cell count > $4 \times 10^9/L$, platelet count > $150 \times 10^9/L$; (iv) Total bilirubin (TBil) < 34 $\mu\text{mol/L}$, albumin > 40 g/L. Exclusion criteria: (i) history of autoimmune diseases; (ii) human immunodeficiency virus (HIV) or hepatitis C virus (HCV) or hepatitis E virus (HEV) coinfecting; (iii) pregnant, lactating women and those who are preparing to be pregnant; (iv) liver cirrhosis or liver cancer; (v) history of severe heart disease; (vi) history of mental illness or psychiatric disorders. (vii) uncontrolled epilepsy. (viii) alcohol or drug abuser. (ix) uncontrolled diabetes mellitus, hypertension, thyroid disease, retinopathy; (x) contraindication to interferon.

Ethics approval

All patients signed an informed consent form before enrollment. The protocol and the consent form for the study were approved by the research ethics committee of Beijing Youan Hospital, Capital Medical University, China ([2017]24).

Treatment and efficacy

This is a prospective cohort study. Patients in treatment group were given PEG-IFN180ug subcutaneously once a week for 96 weeks. Patients in the control group were observed for 96 weeks without anti-viral treatment. Patients in the treatment group were divided into HBsAg clearance group (Responders, R group) and non-HBsAg clearance group (Non-responders, NR group) according to whether HBsAg clearance was achieved within 96 weeks. Patients were divided into four groups according to controlled attenuation parameter (CAP) value at baseline: no hepatic steatosis (CAP < 238dB/m), mild steatosis (238dB/m < CAP < 250dB/m), moderate steatosis (250dB/m ≤ CAP < 292dB/m) and severe steatosis (CAP ≥ 292dB/m).

Laboratory tests

All patients were tested with HBsAg, anti-HBs, HBV DNA, liver function, blood count, thyroid function, thyroid antibodies and autoantibodies at baseline, and at the time point of week 12, 24, 36, 48, 60, 72, 84 and 96. CAP and liver stiffness measure (LSM) were evaluated at baseline and week 96. HBsAg was quantified with HBsAg quantification kit (Roche, Mannheim, Germany), the lower limit of detection of HBsAg was 0.05 IU/mL. HBV DNA was analyzed by cobas® AmpliPrep/cobas® Taqman automated nucleic acid isolation and purification and PCR system (Roche Diagnostics GmbH, Mannheim, Germany), the lower limit of detection of HBV DNA was 20 IU/mL. ALT was measured by OLYMPUS-AU5400 (Japan), normal value < 50 IU/L for men and < 40 IU/L for women. Thyroid antibodies were detected using RocheCobase801, with normal reference values: anti-thyroglobulin antibody (TGAbs) < 115 IU/mL, anti-thyroid peroxidase antibody (TPOAb) < 34 IU/mL, thyrotropin receptor antibody (TRAb) < 1.75 IU/mL. Thyroid function was measured by Architecti2000 (Abbott, Mannheim, USA) with normal reference ranges: FT3 ranged from 3.6 to 6.5 pmol/L, TT3 ranged from 0.92–2.79 nmol/L, FT4 ranged from 11.5 to 22.7 pmol/L, TT4 ranged from 58.1–140.6 nmol/L, and TSH ranged from 0.55–4.78 mIU/L. CAP and LSM were measured by transient elastic scanner (Fibroscan-502, M probe, frequency 3.5M Hz, Echosens, France).

Statistical analysis

Statistical software package SPSS (SPSS Inc., USA) 25.0 software was used for statistical analyses. Normally distributed parameters were expressed as mean $\pm$ standard deviation and compared by t-test. (ANOVA for more than 2 groups) Data that were not normally distributed were expressed as median (25th, 75th range) and non-parametric tests were used for comparison between groups. The Kaplan-Meier survival analysis was used to calculate the cumulative HBsAg clearance rate. P-value < 0.05 was considered statistically significant. In this study, the subjects who completed 96-week trial after enrolment were analyzed without regard of dropped off or loss of follow-up, i.e. using per-protocol analysis (PP analysis).

Results

Therapy outcomes

A total of 174 IHCs were enrolled, 84 patients received PEG-IFN, 90 patients did not receive anti-viral treatment. Two

patients in the treatment group discontinued PEG-IFN at week 24 due to hyperthyroidism and one patient stopped treatment due to hypothyroidism. At week 48, one patient stopped treatment due to hyperthyroidism and one patient discontinued treatment because of an increase of TPOAb (355.3 IU/mL); One patient stopped PEG-IFN due to hyperthyroidism at week 60. 78 patients completed the 96-week course of treatment. 88 patients in the control group were followed up to week 96 and two subjects were lost follow-up at week 24 (Figure 1).

Baseline characteristics

There were no statistical differences in gender, age and HBsAg quantification between the two groups (Table 1).

HBsAg clearance rate in treatment and control group

In the treatment group, the HBsAg clearance rate was 30.77% (24/78) and HBsAg seroconversion rate was 23.08% (18/78) at week 48. HBsAg clearance rate increased to 57.69% (45/78) and HBsAg seroconversion rate was 55.13% (43/78) at week 96. In the control group, spontaneous HBsAg clearance occurred in two patients (one with baseline HBsAg 0.202 IU/mL and HBsAg cleared at week 72, the other with baseline HBsAg 3.67 IU/mL and cleared at week 96, with a clearance rate of 2.27% (2/88) and no patient achieved seroconversion. HBsAg clearance rate was significantly higher in the treatment group than that in the control group ($p < 0.001$) (Figure 2).

HBsAg level in treatment group

A total of 45 patients in the treatment group achieved HBsAg clearance and were classified as responder group (R group). 33 patients did not achieve HBsAg clearance and were classified as non-Responder group (NR group). At baseline, there was no statistically significant difference in HBsAg levels between R and NR group ($p = 0.602$), and HBsAg quantification was significantly lower in R group than that in NR group at 12, 24, 36, 48, 60, 72, 84 and 96 weeks of PEG-IFN treatment ($p = 0.003$, $p = 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$ respectively). In addition, patients in R group had a significantly greater decrease of HBsAg level at each time point during PEG-IFN treatment than those in NR group ($p = 0.031$, $p = 0.019$, $p = 0.124$, $p = 0.002$, $p = 0.059$, $p = 0.017$, $p = 0.002$, $p < 0.001$, respectively). (Table 2; Figure 3).

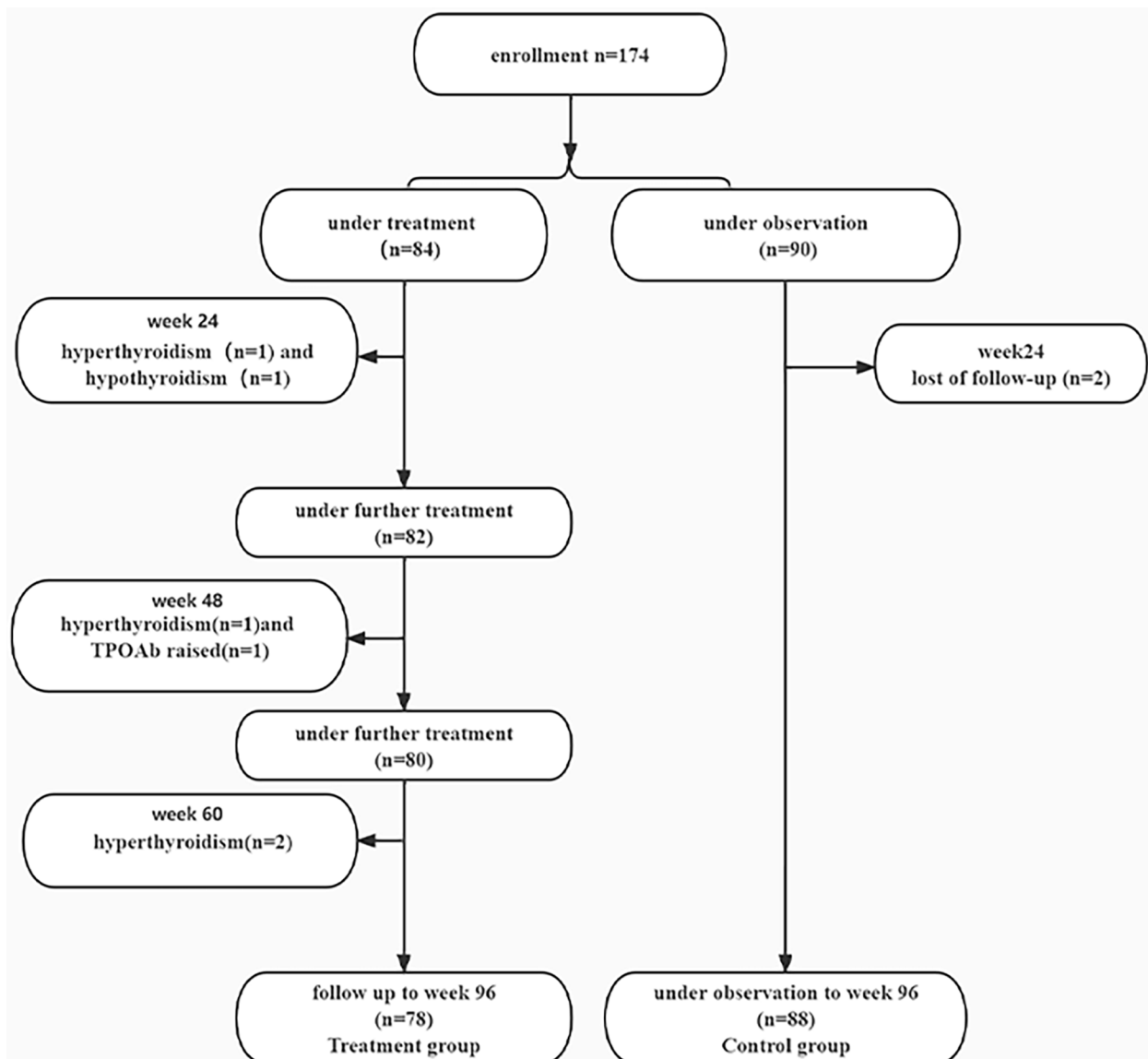


FIGURE 1
Flow diagram of participants enrolled in this study.

ALT level during PEG-IFN treatment in R and NR group

The level of ALT in R group showed significantly higher at week 12 [48.0 (27.0, 72.0)] than baseline [26.0 (15.5, 38.5), $p < 0.001$]. ALT level in NR group also showed significantly higher at week 12 [38.0 (26.5, 64.8)] than baseline [27.0 (18.0, 39.0), $p = 0.001$].

In spite of that the ALT level in R and NR group were not diverse at each time point, the proportion of patients with elevated ALT [1.0–1.5 upper limit of normal (ULN) and ALT

> 1.5 (ULN)] was slightly higher in R group than NR group, especially at week 12. (Table 3; Figure 4).

Association of hepatic steatosis with HBsAg clearance

At baseline, 78 patients in treatment group were divided into four groups according to CAP value, 27 with no hepatic steatosis, 8 with mild steatosis, 26 with moderate steatosis and 17 with severe steatosis. The proportion of patients with moderate

TABLE 1 Baseline characteristics.

Parameter	Treatment group n=78	Control group n=88	P-value
Gender (M/F)	54.00/24.00	48.00/40.00	0.052
Age (years)	42.03 ± 8.16	44.44 ± 9.25	0.078
ALT (U/L)	26.50 (16.00, 32.00)	22 (16.00, 30.50)	0.129
HBsAg (IU/mL)	70.99 (6.19, 161.90)	55.61 (4.31, 113.60)	0.166
Mode of transmission			
Vertical (%)	46.43% (39.00/78.00)	45.56% (41.00/88.00)	0.908
Others (%)	53.57% (45.00/78.00)	54.44% (49.00/88.00)	

steatosis in R group (42.2%) was significantly higher than that in NR group (21.2%) ($p=0.033$). The cumulative clearance rate was 62.47% at week 96 in IHCs with hepatic steatosis (mild, moderate, and severe), compared to 48.15% of those without steatosis ($p=0.058$). The cumulative HBsAg clearance rate was 71.42%, 74.07% and 47.05% at week 96 in patients with mild, moderate, and severe steatosis, respectively. IHCs with moderate steatosis was most sensitive to PEG-IFN, the HBsAg clearance rate in moderate steatosis was significantly higher compared with that in no-steatosis group ($P=0.008$). (Table 4; Figure 5)

Safety

Six of 84 individuals who received PEG-IFN treatment stopped the medication because of side effects. Four patients suffered hyperthyroidism and one patient developed hypothyroidism. The level of TPOAb was increased by at least 10-fold in one patient. HBsAg clearance was obtained in two patients with hyperthyroid when PEG-IFN was discontinued. During the follow-up period, thyroid function and thyroid

antibodies were gradually back to normal in six individuals with aberrant thyroid function (Table 5).

Discussion

PEG-IFN-treated IHCs, especially those with low level of baseline HBsAg, had higher HBsAg clearance rate. In our previous study, 134 IHCs with HBsAg <1000 IU/ml were treated with PEG-IFN alone or in combination with adefovir (ADV), and the clearance rate of HBsAg reached 44.7% at week 96 (10); Dang's study (11) showed that IHCs with HBsAg < 1500IU/ml treated with PEG-IFN for 72 weeks, the HBsAg clearance and seroconversion rates were 37.4% and 29.7%. In addition, the clinical cure rate of PEG-IFN treatment in IHCs with lower HBsAg levels has also been studied. Zeng (12) achieved a high HBsAg clearance rate of 93.8% in IHCs with HBsAg < 20 IU/mL ($n=32$) treated with PEG-IFN for 48 weeks. But it should be pointed out that patients with HBsAg < 20 IU/ml are rare because of too low HBsAg in the clinic and the study sample size was only 32 cases. In order to explore the effect of

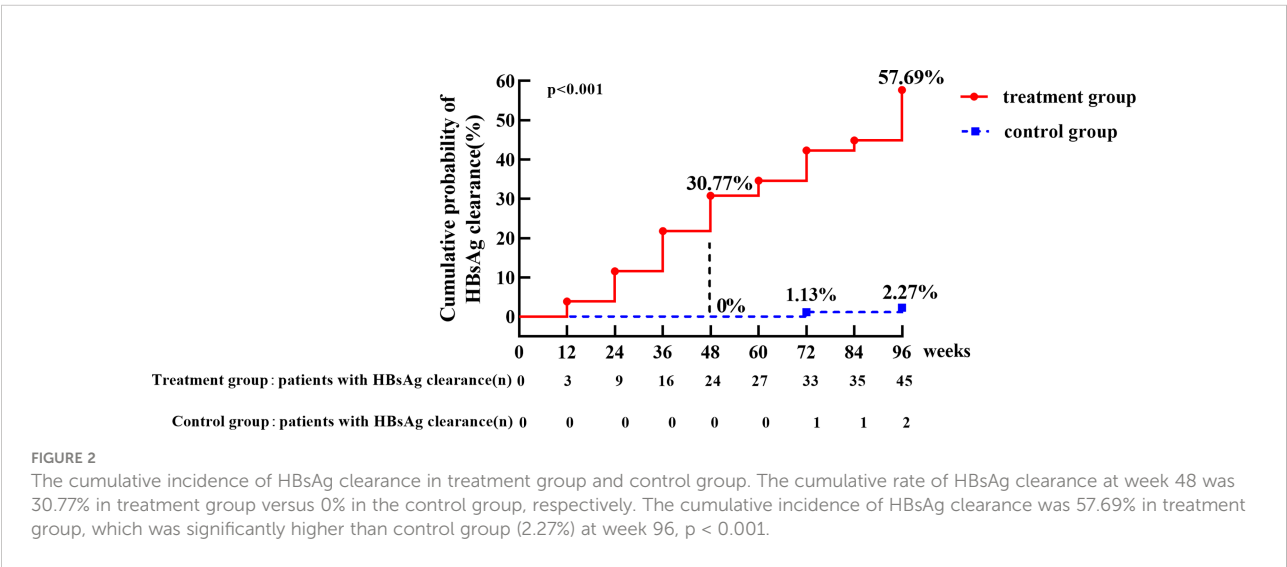


TABLE 2 Longitudinal changes of HBsAg level during treatment.

Parameter	R group n=45	NR group n=33	P-value
HBsAg level (IU/mL)			
Baseline	72.04 (3.45, 164.75)	77.67 (19.16, 167.95)	0.602
Week12	6.75 (0.77, 36.82)	39.52 (7.99, 126.65)	0.003*
Week24	0.96 (0.13, 5.42)	19.83 (0.70, 71.14)	0.001*
Week36	0.40 (3.45, 1.24)	6.86 (0.76, 76.00)	<0.001*
Week48	0.11 (0.00, 0.66)	7.83 (0.58, 64.09)	<0.001*
Week60	0.03 (0.00, 0.57)	3.13 (0.91, 36.16)	<0.001*
Week72	0.00 (0.00, 0.08)	7.74 (1.26, 58.30)	<0.001*
Week84	0.00 (0.00, 0.06)	16.71 (2.29, 76.25)	<0.001*
Week96	0.00 (0.00, 0.00)	10.18 (4.73, 58.01)	<0.001*
HBsAg decline from baseline (log ₁₀ IU/ml)			
Week12	0.60 ± 0.13	0.24 ± 0.10	0.031*
Week24	1.12 ± 0.16	0.58 ± 0.14	0.019*
Week36	1.24 ± 0.22	0.79 ± 0.18	0.124
Week48	1.43 ± 0.15	0.73 ± 0.16	0.002*
Week60	1.31 ± 0.18	0.84 ± 0.16	0.059
Week72	1.38 ± 0.17	0.76 ± 0.17	0.017*
Week84	1.41 ± 0.18	0.50 ± 0.23	0.002*
Week96	1.39 ± 0.14	0.56 ± 0.14	<0.001*

R group (Responder group); NR group (Non-Responder group).
* p < 0.05.

PEG-IFN treatment in more clinical situation, this study explored IHCs with HBsAg < 200 IU/ml. The cumulative clearance rate at week 96 was 57.69% (45/78), suggesting that IHCs with HBsAg < 200 IU/ml at baseline could achieve a high rate of HBsAg clearance with PEG-IFN treatment.

Previous studies have suggested that elevated ALT during PEG-IFN treatment may indicate a better curative effect. A multicenter retrospective study of 121 HBeAg-positive CHB patients treated with PEG-IFN in the United States found that patients with acute ALT elevation after PEG-IFN treatment had

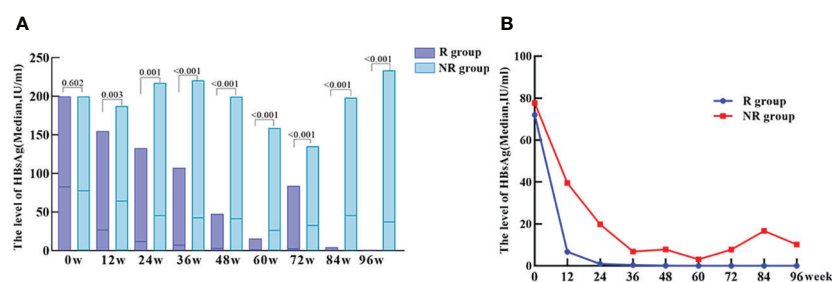


FIGURE 3

The level of HBsAg at baseline and during treatment of PEG-IFN in R and NR groups. **(A)** No significant difference of HBsAg level between R and NR group at baseline ($p = 0.602$). The level of HBsAg in R group was significantly lower than NR group during treatment ($p = 0.003$, $p = 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.001$) **(B)** HBsAg showed a significant downward trend after PEG-IFN treatment in IHCs, especially in R group.

TABLE 3 ALT level at baseline and during treatment.

Parameter	R group n=45	NR group n=33	P-value
Baselines	26.00 (15.50, 38.50)	27.00 (18.00, 39.00)	0.988
12week	48.00 (27.00, 72.00)	38.00 (26.50, 64.75)	0.517
24week	40.00 (25.50, 59.50)	38.50 (22.00, 51.00)	0.338
36week	32.00 (22.25, 51.75)	25.00 (17.75, 42.50)	0.109
48week	32.50 (22.75, 45.75)	32.00 (20.00, 51.00)	0.658
60week	26.00 (20.00, 41.00)	26.00 (19.50, 46.00)	0.735
72week	26.00 (19.50, 43.00)	24.50 (16.75, 39.00)	0.323
84week	28.00 (19.00, 43.00)	22.00 (15.50, 34.75)	0.171
96week	23.00 (17.00, 30.00)	23.00 (18.00, 27.50)	0.913

a sustained HBV DNA response and significantly improved HBeAg and HBsAg clearance (13). A global, multicenter, randomized, controlled study of HBeAg-positive patients treated with tenofovir disoproxil fumarate (TDF) plus PEG-IFN for 48 weeks, patients with acute ALT elevation within 24 weeks had higher HBeAg clearance rate (38.9% vs 10.4%) and HBsAg clearance rate (24.1% vs 1.7%) than those without acute ALT elevation (14). This study also found that IHCs treated with PEG-IFN showed a significant elevation of ALT at week 12 both in R and NR group ($p < 0.001$, $p = 0.001$). Although there was no statistically significant difference of ALT level between the two groups at each time point, it was seen that the proportion of patients with elevated ALT was higher in R group (48.72%) than NR group (34.37%) at early treatment.

Most current studies suggest that hepatic steatosis is conducive to spontaneous clearance of HBsAg. A study by Chu et al. (15) showed that spontaneous HBsAg clearance occurred in 54 of 162 IHCs. IHCs with moderate ($OR = 3.22$, $P = 0.017$) and severe ($OR = 3.87$, $P = 0.041$) steatosis were found to be more than threefold of HBsAg clearance rate than those without hepatic

steatosis, while mild steatosis was not significantly associated with HBsAg clearance ($OR = 1.76$, $P = 0.157$). Li et al. (16) in a retrospective cohort study (including 6,786 Asian CHB patients) found that 10-year cumulative HBsAg clearance was significantly higher in CHB patients with comorbidity of fatty liver than those without fatty liver (15.91% vs 11.84%, $p = 0.003$). However, whether steatosis affects antiviral efficacy remains controversial. Nguyen et al. (17) showed that steatosis was a significant predictor for HBsAg clearance ($HR = 1.84$, 95% CI 1.03–3.29) in CHB patients treated with entecavir (ETV)/TDF. In a Chinese study that enrolled 213 patients with CHB treated with ETV, HBV DNA clearance and HBeAg seroconversion rates were significantly lower in patients with comorbidity of steatosis than those without (18). A study by Lin et al. (19) demonstrated that the HBeAg clearance rate and HBV DNA inhibition rate did not differ in with or without steatosis treated with nucleos(t)ide analogue (NAs) (LAM/ADV/LdT/ETV/TDF) monotherapy. Previous studies on whether steatosis affects antiviral efficacy were mainly based on NAs treatment with HBV DNA suppression and HBeAg seroconversion as indicators. However, it has not been reported whether steatosis affects

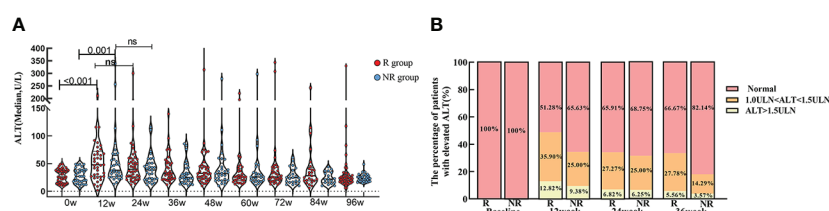


FIGURE 4

ALT level at baseline and during PEG-IFN treatment in R and NR group. (A) ALT level was significant higher both in R and NR group at week 12 than baseline ($p < 0.001$, $p = 0.001$). ALT level were not statistically different at each time point between R and NR group. (B) The proportion of patients with elevated level of ALT in R group was marginally larger than NR group, although there was no statistically significant difference.

TABLE 4 The proportion of patients with different level of CAP in R and NR group at baseline.

CAP (dB/m)	R group n=45	NR group n=33	P-value
<238	28.89 (13.00/45.00)	42.42% (14.00/33.00)	0.214
238-250	11.11% (5.00/45.00)	9.09% (3.00/33.00)	0.771
251-292	42.22% (19.00/45.00)	21.21% (7.00/33.00)	0.033*
>292	17.78% (8.00/45.00)	27.27% (9.00/33.00)	0.316

* p <0.05.

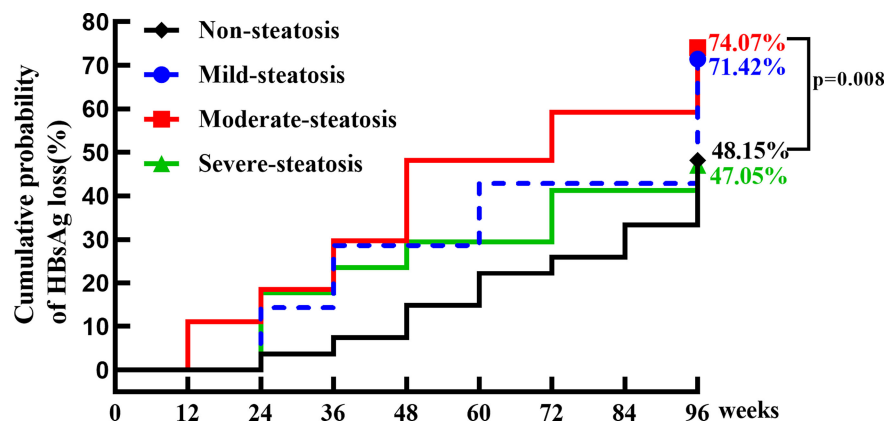


FIGURE 5

The cumulative incidence of HBsAg clearance in IHCs without and with hepatic steatosis (mild, moderate, severe). The cumulative incidence of HBsAg clearance was 48.15% (13/27), 71.42% (5/8), 74.07% (19/26) and 47.05% (8/17) in IHCs without and with mild, moderate, severe hepatic steatosis. The rate of HBsAg clearance was significantly higher in IHCs with moderate hepatic steatosis than in IHCs without hepatic steatosis (74.07% vs 48.15%, $p = 0.008$).

TABLE 5 Adverse effect of the study population.

Patients	Gender	Age	baseline HBsAg(IU/ml)	adverse effect	The time point of treatment discontinuation	EOT HBsAg (IU/ml)	Whether the adverse reaction is improved	96WHBsAg (IU/ml)
1	female	46	142.90	Hypothyroidism	Week24	132.7	YES	135.10
2	male	47	79.63	Hyperthyroidism	Week24	42.25	YES	28.60
3	male	60	23.62	Hyperthyroidism	Week48	<0.05	YES	<0.05
4	male	47	8.41	TPOAb raised	Week48	<0.05	YES	<0.05
5	female	31	45.78	Hyperthyroidism	Week60	0.079	YES	0.44
6	female	37	3.47	Hyperthyroidism	Week60	0.078	YES	0.06

HBsAg clearance for IHCs with PEG-IFN therapy. In this study, the cumulative clearance rate of HBsAg at week 96 in patients with baseline steatosis (CAP>238 dB/m) had higher tendency than that in patients without steatosis. However, moderate steatosis definitively benefits the HBsAg clearance in patients treated with PEG-IFN.

Some adverse effects may occur during the treatment of PEG-IFN, and thyroid dysfunction is one of the common

adverse reactions (20). After discontinuation of interferon therapy, thyroid function and antibodies returned to normal. Therefore, the adverse reactions should be closely monitored during the application of PEG-IFN, and the clinical cure of hepatitis B should be pursued safely and effectively.

In conclusion, PEG-IFN is effective on IHCs with HBsAg< 200 IU/ml. IHCs with moderate hepatic steatosis are more sensitive for the treatment.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by The research ethics committee of Beijing Youan Hospital, Capital Medical University, China ([2017]24). The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZC designed research. HL, LL and SL analyzed the results; YZ and DZ conducted the experiments. HL, LL and SL wrote the manuscript. YL guided statistical analysis. JZ, XC and ZC contributed to the interpretation of the results and critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Postpartum hepatitis and host immunity in pregnant women with chronic HBV infection

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In order to develop immune tolerant to the fetal, maternal immune system will have some modification comparing to the time before pregnancy. Immune tolerance starts and develops at the maternal placental interface. In innate immunity, decidual natural killer (dNK) cells, macrophages and dendritic cells play a key role in immune tolerance. In adaptive immunity, a moderate increase of number and immune inhibition function of regulatory T cells (Treg) are essential for immune tolerance. The trophoblast cells and immune cells expressing indoleamine 2,3-dioxygenase (IDO), the trophoblast cells expressing HLA-G, and Th1/Th2 shifting to Th2 dominant and Th17/Treg shifting to Treg dominant are in favor of maternal fetal immune tolerance. Steroids (estrogen and progesterone) and human chorionic gonadotropin (HCG) also participate in immune tolerance by inducing Treg cells or upregulating immunosuppressive cytokines. Most of the patients with chronic HBV infection are in the "HBV immune tolerance period" before pregnancy, and the liver disease is relatively stable during pregnancy. In chronic HBV infection women, after delivery, the relative immunosuppression *in vivo* is reversed, and Th1 is dominant in Th1/Th2 and Th17 is dominant in Th17/Treg balance. After delivery, the number of Treg decrease and NK cells increase in quantity and cytotoxicity in peripheral blood. Liver NK cells may cause liver inflammation through a non-antigen specific mechanism. After delivery, the number of CD8⁺ T cells will increase and HBV specific T cell response recovers from the dysfunction in pregnancy. Under the background of postpartum inflammation, the rapid decrease of cortisol after delivery, and especially the enhancement of HBV specific T cell response induced by HBV DNA and cytokines, are the main reasons for postpartum hepatitis. HBeAg positive, especially HBeAg<700 S/CO, and HBV DNA>3-5Log₁₀IU/ml are risk factors for postpartum hepatitis. Antiviral treatment in

late pregnancy can reduce the incidence of mother to child transmission (MTCT) in chronic HBV infection women. Chronic HBV infection women have hepatitis both during pregnancy and more often in 12 weeks postpartum. It is generally agreed that postpartum hepatitis is mild symptoms and self-limited. Delaying drug withdrawal to 48 weeks can increase the seroconversion rate of HBeAg in delivery women with elevated alanine aminotransferase (ALT) in pregnancy.

KEYWORDS

pregnant, HBV, immune tolerant, treg, postpartum hepatitis, antiviral therapy (AVT)

In pregnant women with chronic HBV infection, a certain proportion of postpartum hepatitis may occur after delivery. The mechanisms of postpartum hepatitis are complex, involving the changes in the immune system and pregnancy-associated hormone of women in pregnancy and after delivery, the natural stage of chronic HBV infection period, whether preventive antiviral therapy (AVT) and the time of withdrawal AVT after delivery.

Definition and characteristics of postpartum hepatitis

Definition of postpartum hepatitis

Postpartum hepatitis refers to the occurrence of hepatitis after delivery, which is characterized by the elevated ALT with or without fatigue and corresponding digestive tract symptoms. The standards of ALT elevation vary from >1 upper limit of normal range (ULN) or >2 ULN to >5 ULN. Different countries have different ULN standards, most of which are >40 U/L, but >25 U/L or >19 U/L for female in the United States (1). At present, research on postpartum hepatitis has focused on women with chronic HBV infection.

Characteristic of postpartum hepatitis

Postpartum hepatitis mostly occurs within 12 weeks after delivery, but a few also occur in 12-24 weeks. The two peak periods of postpartum hepatitis are 3-4 weeks and 9-12 weeks respectively (2). If postpartum follow-up is only 12 weeks, postpartum hepatitis occurring after 12 weeks is often not monitored. The degree of postpartum hepatitis is mostly asymptomatic or mild to moderate. There is little chance of elevated bilirubin level or clinical decompensation of liver function. Postpartum hepatitis, either without oral AVT in late

pregnancy or withdrawal AVT after delivery, is usually self-limited and does not require medical intervention (2-4). It is occasionally reported that a few patients have severe postpartum hepatitis flare (5). In chronic HBV infection women, the incidence of hepatitis during pregnancy can reach 6-14%, and it is more common after delivery (5). In chronic HBV infection women, percentage of postpartum hepatitis is 3.5%-50% (2, 4). The use of oral AVT to prevent MTCT in late pregnancy can also reduce the incidence of postpartum hepatitis. Postpartum hepatitis associated with MTCT is usually accompanied by a mild flare, usually without the need for AVT again (3).

Changes of host immune system in pregnancy

Innate and Adaptive Immune Responses in Pregnancy

The maternal-fetal interface mainly consists of trophoblast cells from the embryo and decidua from the pregnant women. In order to adapt to those process, the uterus innate immune system is activated in the early pregnancy, then gradually weakens in most of the pregnancy, and reactivates in the late pregnancy (6). The enhancement of innate immunity at the early pregnancy plays an important action in trophoblast growth, angiogenesis and placentation. The maternal placenta is rich in white blood cells, including about 70% decidual natural killer cells (dNK), 20% mononuclear macrophages, and roughly 10% T cells (7).

Changes of innate immunity during pregnancy

NK cells come from bone marrow and circulate to decidual tissue of uterus. During pregnancy, the quantity, phenotype and function of dNK cells at the maternal-fetal interface are different from those before pregnancy. The percentage of dNK cells increased in the first trimester of pregnancy accounting for

70-90% of decidual mononuclear cells, then decreased gradually in the second and third trimesters of pregnancy accounting for 35-40% (8, 9). The function of dNK cells may be influenced by the hormone level during pregnancy (10). High levels of adrenocortical hormone (ACH) during pregnancy directly inhibit NK cell activity (10). The phenotype of dNK cells is different from that of peripheral blood (11, 12). It is CD56^{bright}CD16⁺NK, which can recognize non classical MHC-I molecules, and has weak cytotoxicity (8, 10). dNK cells can produce generous TNF- α and IFN- α which promote the invasion of extravillous trophoblast (EVT) cells and the formation of decidual vessels and spiral arteries (8, 9, 11). dNK cell function is regulated by the balance of inhibitory and active receptor signals (6). During pregnancy, the expression of dNK activated receptor NKG2D decreased, and the expression of inhibitory receptor CD94/NKG2A increased. The basic feature of inhibitory receptor is the structure of immunoreceptor tyrosine inhibitory motif (ITIM) in the cytoplasm. KIR can transmit inhibition signal through ITIM, thus inhibiting NK cells' killing activity against target cells. The dysfunction of dNK is related to abnormal pregnancy, such as recurrent abortion and fetal growth retardation.

In pregnancy, the number of circulating monocytes and granulocytes increased and they were activated by up-regulating activation markers of CD11b and CD14, but the phagocytosis of monocytes decreased (13, 14). The decidual M Φ s mainly remodel the uterine blood vessels, participating in the regulation of immune tolerance and antigen presentation (13, 15). In early pregnancy, M Φ s actively remove apoptotic cells, produce proteases that degrade extracellular matrix, and participate in uterine vascular remodeling (16). M Φ has high plasticity and can polarize into different phenotypes under the stimulation of different microenvironments. Decidual M Φ s are classified into classically activated macrophages (M1) and alternatively activated macrophages (M2) (16). M1 is dominant during both embryo implantation and during labor. M Φ s in decidua can induce Foxp3⁺Treg proliferation *in vitro*. Most decidual M Φ s produce indoleamine 2,3-dioxygenase (IDO), which is conducive to maternal immune tolerance to the half allogeneic fetus. In early pregnancy, most decidual T cells show strong expression of PD-1. Decidual M Φ s express B7 family costimulatory molecules and then inhibit T cells from producing IFN- γ through B7-H1/PD-1 signal pathway (17).

Dendritic cells (DC), derived from bone marrow hematopoietic stem cells, are the most important professional antigen presenting cells in the body. DC also acts as an important immune cell for immune tolerance in pregnancy. DC is divided into mature DC and immature DC. IDC is the main type of DC, and only a few mature CD83⁺DC in endometrium during pregnancy. IDC has strong antigen capture function, but weak antigen presentation function (18). Without the costimulatory signal on the surface, IDC can induce the disability of effector T lymphocytes (Teff), induce the

production of Tregs, and promote immune tolerance in maternal fetal interface. IDC induces antigen specific T cell tolerance by secretes IL-10 and TGF- β . In the mouse model, DC at the maternal and fetal interface is difficult to migrate to the lymphatic vessels of the uterus and lymph nodes even if stimulated by LPS, which can minimize the exposure of fetal or placental antigens to immunogenic T cells and is beneficial to maternal fetal immune tolerance (19).

Other immune cells, such as mast cells. Mast cells were stimulated by cell surface-expressed pattern recognition receptors (PRR) from bacteria, released IL-6, leukotrienes, TNF and histamine. Two receptors of H2R and H4R in mast cells regulate the production of Treg (20, 21). The number of neutrophils from maternal fetal interfaces remained relatively constant during pregnancy and began to increase rapidly before delivery. In the first-trimester, the maternal leukocytes of decidua have significant function in the implantation and early development of embryo and placenta. Neutrophils produce reactive oxygen and lytic enzymes by phagocytosis of bacteria, and promote inflammatory cascade reaction (22).

Changes of adaptive immunity during pregnancy

The absolute counts of T, B lymphocytes and CD4⁺, CD8⁺ T cell subsets in early pregnancy decreased, and the proportion of lymphocytes remained unchanged (9). T cells account for 10%~15% of white blood cells in decidual tissue in early pregnancy, and this proportion increases to 70% in late pregnancy (7).

Treg cells are an important CD4⁺ T lymphocyte subset of immune tolerance in pregnancy (23-25). Treg cells are distributed in iliac and inguinal lymph nodes, spleen and peripheral blood, and later found in uterus (26). The exhaustion of Tregs will lead to pathological pregnancy. At present, the research mainly focuses on the inducing factors of amplification and the molecular action mechanism of Tregs. During early pregnancy, the number of Tregs in the pregnant woman increased and migrated to the surface of the mother and fetus (27). During the third trimester of pregnancy and after delivery, the number of Treg cells decreased significantly (28). CCL22/CCL17 at the maternal fetal interface can chemotactic Treg cells. Treg surface molecules include Foxp3, GITR (glucocorticoid induced TNF receptor), CTLA-4, and TGF- β . Tregs are usually represented by CD4⁺CD25⁺FoxP3⁺Tregs (24, 26). TGF- β can regulate the differentiation of Th17 cells and Treg cell lineage. The interaction between GITR and its ligand can eliminate the inhibition of CD25⁺CD4⁺Tregs and enhance the activation, proliferation and cytokine production of T cells. Treg exerts its immune regulation function mainly through two ways: 1. Direct contact between cells. Treg can inhibit the proliferation of immune cells by directly binding with corresponding receptors of CD4⁺ T, CD8⁺ T and DCs through surface molecules (25, 29). For example, the interaction between

the Treg inhibitory receptor CTLA-4 and its ligand CD80/CD86 induces macrophages or DCs to express indoleamine 2,3-dioxygenase, which inhibits T cell activity and promotes maternal tolerance to the fetus (30, 31). Treg can inhibit the activation and amplification of immune cells such as CD4⁺ T and CD8⁺ T cells. 2. Secretion of inhibitory cytokines. For example, Tregs secrete IL-10 and TGF- β which are also important for self reproduction. In normal pregnancy, Tregs promote Th2 immune tolerance, but do not reduce Th1 cytokines, promoting maternal fetal immune tolerance (28, 32). The interaction between dNK and monocytes can also activate Treg (33). Many cytokines and hormones can regulate Treg production. During the first six months of pregnancy, semen contains TGF- β , IL-10 and prostaglandin E2 (PGE2), paternal antigen, can significantly increase Treg cells in peripheral blood and decidual tissue (34). The number of Treg cells increased significantly with the dramatic increase of human chorionic gonadotropin (HCG) (35).

The most abundant lymphocytes in decidual tissue are CD8⁺ T cells. Exhausted and senescent CD8⁺ T cells exist at the maternal fetal interface (36). In the maternal fetal interface, the inhibition receptors of CD8⁺ T cells such as CTLA4, PD1 and LAG3, suggest a phenotype of T cell depletion or dysfunction. The function of CD8⁺ T cells is temporarily inhibited and can still proliferate and produce IFN- γ , TNF- α , perforin and granzyme when stimulated (37, 38). During early pregnancy, the regulatory CD8⁺ T cell population co-expressing PD-1 and T cell immunoglobulin and mucin domain containing protein 3 (Tim-3) which are enriched in decidua, and can proliferate and produce Th2 type cytokines (39). In the first trimester of pregnancy, apoptosis of T cells which express Fas on the surfaces of cells were induced by FasL from maternal decidua and placental trophoblast cells (40). Antigen presenting cells (APC) which express PD-L1 combined with T cells which express PD1 to induce T cell apoptosis (41). $\alpha\beta$ T cells are the most important T cells in the body. Due to the lack of effective stimulus of MHC molecules, the harm of $\alpha\beta$ T cells to the fetus are almost inhibited in pregnant women. TCR $\gamma\delta$ T cells involved in the immune tolerance of pregnancy (25, 42). By recognizing HLA G and HLA E antigens of trophoblasts, r&T cells are activated and release inhibitory cytokines of IL-10, TGF- β , and inhibit their cytotoxicity in pregnancy (6).

B lymphocyte cells have the ability to produce specific antibodies, in addition, they have the function of secondary antigen presenting cells, and can produce various immune regulatory cytokines. Adaptive cellular immunity was down regulated and humoral immunity was up regulated during pregnancy (43). Regulatory B cells (Bregs) are B lymphocyte subsets with strong immunosuppressive function. Studies have shown that under physiological conditions, the number of Breg is very small. However, in animal experiments, it is found that mature B cells of pregnant rats gather in the abdominal cavity and uterine lymph nodes (44), which may increase the number

of serum immunoglobulins (43). Breg inhibits the toxic activity of Th1, Th17, and CD8⁺ T cells, and they can also induce CD4⁺ T cells to differentiate into FoxP3⁺Tregs (45, 46). Breg also inhibited TNF- α production by monocytes and IFN- α production by plasmacytoid dendritic cells (PDCs). Bregs are negative regulators of the body's immune system through the anti-inflammatory cytokines IL-10, TGF- β . Breg inhibits the immunity through direct contact between cells or the indirect action of cytokines such as IL-35 (45).

Myeloid-derived suppressor cells (MDSCs) are a group of heterogeneous immature cells from bone marrow. MDSCs have immunosuppression function, and become new immune regulating cells at the maternal-fetal interface. MDSCs play an inhibitory role in normal pregnancy. MDSCs are characterized by myeloid origin and immature state, which can inhibit T cells. MDSCs suppress immunity through two ways. One way is through arginase 1 (Arg1), iDO, NO, ROS, and PGE2. Arg1, causing hunger environment by consuming essential amino acids such as arginine and tryptophan, leads to T cell inhibition (47). Another way is through TGF- β which can induce Treg production (48). MDSCs can inhibit NK through NO, ROS and inhibitory receptor TIGIT. MDSCs can also inhibit the activation of T cells by DCs (49).

Natural killer T (NKT) cell is a special T cell subgroup both with T cell receptor and NK cell receptor on the cell. NKT cells can produce a large number of cytokines, and play a similar cytotoxic role to NK cells. The NKT activation process requires the mutual activation of APC and NKT. The decidual NKT has obvious Th2 tendency, which is very important at the maternal fetal interface (50). Under normal conditions, decidual NKT inhibits immune rejection against paternal antigens, which is beneficial to the growth of trophoblast.

Mechanism of immune tolerance to "half allogeneic" fetus during pregnancy

There are a large number of immune cells infiltrating in the uterus which construct the endometrial immune microenvironment and enable the embryo to obtain "immune privilege" (51, 52). At present, it is generally believed that in addition to the placental barrier, maternal fetal immune tolerance maintains mainly by increasing the expression of human leukocyte antigen (HLA) G molecule in the placenta, Th1/Th2 and T17/Treg balance shifting to Th2 and Treg dominant, and trophoblast and immune cells expressing iDO and so on (Figure 1).

(1) Immune cells and iDO mechanism in maternal fetus immune tolerance

Cellular immunity is the central link of maternal immune tolerance to fetus. iDO is involved in maternal immune tolerance to fetus by cellular immunity. The expression level of iDO is low under normal conditions, and lipopolysaccharide

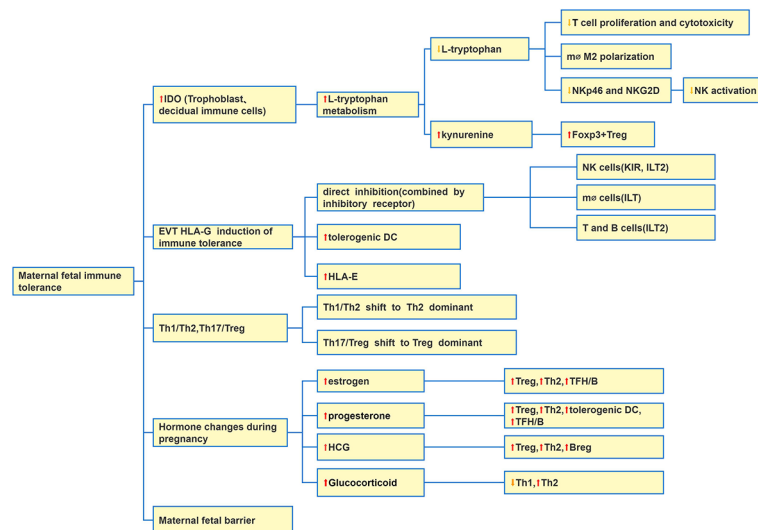


FIGURE 1

Causes of maternal fetal immune tolerance. There are five main reasons: IDO at the maternal-fetal interface increases which induces maternal fetal immune tolerance; EVTs express HLA-G to induce immune tolerance; Changes of Th1, Th2, Th17 and Treg balance; hormone change during pregnancy; maternal-fetal barrier. Abbreviation: Extravillous Trophoblast (EVT).

and IFN- γ can induce the expression of IDO. From the early pregnancy, trophoblasts, NK, macrophages, DC and T cells at the maternal infant interface began to express IDO, and the amount of IDO gradually increased (53). IDO is a monomer enzyme of heme and the only limiting rate enzyme outside the liver that can catalyze tryptophan metabolism. IDO decomposes tryptophan along the kynurenine (KYN) pathway and produce a series of metabolites including quinolinic acid. Tryptophan is an essential amino acid to maintain cell activation and proliferation. IDO mainly suppresses cellular immunity through depletion of tryptophan (53). Firstly, iDO degrades L-tryptophan in local microenvironment, and limits T lymphocyte proliferation. Secondly, iDO produces KYN by metabolizing L-tryptophan (54). KYN is an endogenous ligand of Aromatic hydrocarbon receptor (AhR). KYN combines with AhR to cause immature CD4⁺ T cells to differentiate into inhibitory T cells. In addition, the combination of KYN and AhR can also induce the expression of IDO and further inhibit the immune response of T cells. Macrophages expressing IDO inhibit T cell function and promote the polarization of macrophages to M2 phenotype. IDO also inhibits NK cell activation by down regulating the surface receptors of NKp46 and NKG2D.

(2) HLA-G mainly expressing by trophoblast cells in maternal fetal immune tolerance (55)

Human trophoblast cells do not express MHC II molecule, HLA-A and HLA-B molecules of classical MHC I molecule, but express classical HLA-C and non classical HLA-E, HLA-F, and HLA-G to protect from cellular immune damage. HLA Ib molecules are the key to the generation and maintenance of

maternal fetal immune tolerance. The research mainly focuses on the mechanism of immune escape induced by HLA-G. At present, HLA-C, E is also considered important. In the maternal-fetal interface, HLA class Ib molecules expressed in trophoblast cells were binded to inhibitory receptors of decidual immune cells (DICs), and then shifted Th1/Th2 balance toward Th2 bias. HLA-G as immune tolerance molecules is expressed in EVT of placenta and immune cells *in vivo*. There are two main mechanisms for HLA-G to induce immune tolerance. The first mechanism is that membrane-bound or soluble type of HLA-G can bind to the inhibitory receptors of immune cells (NK, T, B, monocytes and DC), which is a direct inhibition of immune cells. The second mechanism includes inducing tolerance of DC (56). HLA-G specifically inhibits T cell amplification and function in a dose related form (57, 58). The HLA-G antigen specifically combines to the ILT-4 receptor on the surface of DC, activates the downstream IL-6/STAT3 signal pathway, downregulates the expression of MHC-II and costimulatory molecules CD80/CD86, inhibits the maturation and differentiation of DCs, and induces the formation of tolerant DCs (56, 59). HLA-G also mediates the function of MDSC by ILT-4 receptor (58). HLA-C expressed by trophoblast cells can bind with KIRs of dNK cells and take part in trophoblast invasion into the uterine (60). HLA-G and HLA-E molecule expressed by EVTs can combine with the inhibitory receptor CD94/NKG2A of dNK cells, thereby inhibit dNK cell activation (61, 62). Other researchers found that HLA-G molecules could also promote the expression of HLA-E molecules, thereby indirectly reducing the toxicity of dNK cells (63).

(3) Th1/Th2/Th17 and Treg cell models in maternal fetal immune tolerance (64–66) (Figure 2).

The differentiation of CD4⁺ T cells is affected by many factors and cytokines. It is generally believed that Th cell precursor can differentiate into Th0 cells under the stimulation of antigen. In different microenvironments, Th0 cells can selectively differentiate into Th1, Th2, Th17 and Treg (Figure 2). It is traditionally believed that the Th1/Th2 balance at the maternal fetal interface during pregnancy tends to Th2 dominant (67–69). Th1 cells can release cytokines that cause local inflammation and activate MΦS, NK cells and T lymphocytes. Th2 cells can release cytokines and promote humoral immune response. Th2 cells was once considered as the main T cell subgroup that assisted B cells to produce humoral immune response. The latest research suggests that follicular helper T cell (Tfh) belonging to CD4⁺ T is the main subgroup of helper B cells (70), and plays a regulatory role in B cell activation and Ig class conversion.

Treg/Th17 maybe play a much more important immune regulation role than Th1/Th2. At present, it has developed to Th1/Th2/Th17 and Treg cell models (71). Th17 cells have strong proinflammatory effect and can secrete IL-17A, IL-17F, IL-22, IL-21 and other cytokines. Th17 cells are differentiated by TGF-β, which can also induce regulatory T cells (iTreg) (72). IL-17 is the main effector of Th17 cells, which has proinflammatory effect which is in favor of the production of proinflammatory cytokines and chemokines (70, 73). The symptoms of RA usually improve during pregnancy, indicating that Th17 cells may decrease during pregnancy (74). The shift of Th17/Treg balance to Treg dominant may be favor to the outcome of pregnancy (75).

(4) Changes of pregnancy-associated hormone level in the maternal fetal immune tolerance

Hormonal factors are relative to maternal fetal immune tolerance including estrogen and progesterone and HCG. HCG can be detected from maternal serum on the first day after implantation of the fertilized eggs, reaching a peak at 8–10 weeks of gestation, and then dropping rapidly. Estrogen is produced by corpus luteum in early pregnancy, and is mainly synthesized by placental unit after 10 weeks of pregnancy. The concentration of estrogen gradually increases until the third trimester of pregnancy. Progesterone is produced by the corpus luteum of ovarian in the early pregnancy. After 8–10 weeks of pregnancy, placental syncytiotrophoblast cells begin to produce progesterone. The local concentration of the above hormones in the placenta is much higher than that in the whole body, which is mainly a regional effect and has little impact on all systems in the whole body.

During pregnancy, the hypothalamic neuropeptide corticotropin releasing hormone (CRH) mainly comes from endometrium and placenta. CRH induces the expression of Fas ligand (FasL) in DCs at the fetal maternal interface and increases apoptosis of activated T lymphocytes through Fas-FasL induction (76). High levels of adrenocortical hormone during pregnancy inhibit the immune system and directly inhibit NK cell activity. Cortisol secretion during pregnancy increased several times, and the active free cortisol was only 10% (77). Glucocorticoid (GC) can inhibit APC and Th1 cells which produce IFN-α, IFN-γ, TNF-α and IL-12, and promote Th2 cells which produce IL-4, IL-10 and IL-13 (78). The increase of GC level may lead to the maternal fetal local Th1/T2 to Th2 type, but it is not systemic inhibition (78).

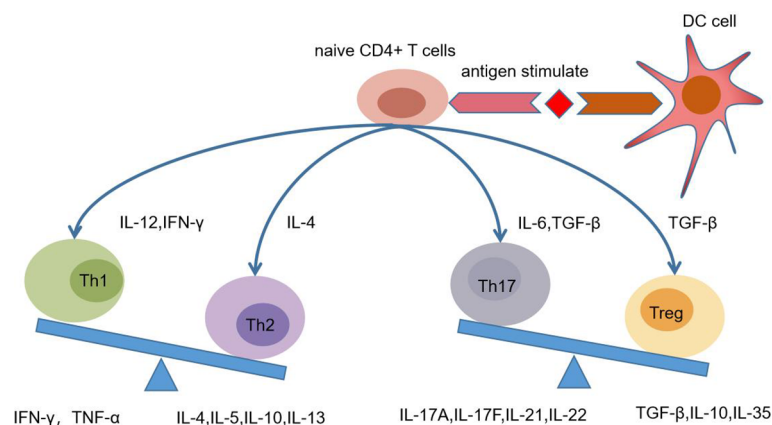


FIGURE 2

Naive CD4⁺ T cells differentiation in the pregnancy. In different microenvironment, naive CD4⁺ T cells can differentiate into Th1, Th2, Th17, and Treg under the antigen stimulation which is presented by dendritic cells (DC). For example, IL12 and IFN-γ can induce the differentiation of Th1 cells, which secrete cytokines IFN-γ and TNF-α. In the Th1/Th2 balance during pregnancy, Th2 is dominant. In the Th17/Treg balance, Treg was dominant.

Estrogen and progesterone levels increase during pregnancy. Research proved that progesterone not only increased the proportion of CD4⁺ CD25⁺ Treg cells and the expression of IL-10, but also enhanced its inhibitory function in mice (79). 17 β -Estradiol (E2) can start immunosuppression through CD4⁺ CD25⁺ Treg cells in early pregnancy, reduce the production of Th1/Th17 inflammatory cytokines and up regulate the production of anti-inflammatory cell factors (80). Progesterone induces Tregs amplification, which is mediated by glucocorticoid receptors (81). Progesterone promotes Th2 cytokine production during normal pregnancy (82). In mice, progesterone directly or indirectly induced DC immune tolerance (83). Progesterone inhibits the activation and proliferation of lymphocytes (84). Progesterone can also inhibit the production of proinflammatory cytokine IL-17 (80). HCG can not only increase the number and activity of Treg, but also promote the migration of Tregs from peripheral blood to the maternal fetal interface (35, 85). In animal experiments, estradiol triggers expansion and activation of regulatory B cells. High level of HCG in pregnant women can induce Breg cells to expand, promote Breg to produce IL-10, and inhibit the proliferation of CD4⁺ T cells (86). During pregnancy, high levels of progesterone and estrogen promote humoral immunity by activating the T follicular helper cells (TFH)/B cell axis (87).

Three hypotheses of immune tolerance in pregnancy

There are three hypotheses about the formation of immune tolerance in pregnancy as follows: Self-non-self models of immunity, danger model and Evolutionary non-self model. Self-non-self models of immunity, that is, if “non self” is recognized, antigen specific cytotoxic T lymphocytes (CTL) will be activated through a series of intermediate links. If it is “self”, antigen specific CTL will not be activated. Maternal fetal immune tolerance during pregnancy is achieved by limiting antigen specific T cell activity (88, 89). For the danger model, if there is no danger signal, the antigen expression from the father will not activate T cells (90). Dangerous signals are related to the activation of DCs or their costimulatory signals in the process of activating T cells. The danger model was validated in adverse pregnancy outcomes. “Evolutionary non-self” model: The presence of fetal antigen on the maternal fetal interface may not activate the immune system, but when infection occurs, the immune response mediated by PRR occurs to protect the maternal function (91). In short, the “Evolutionary non-self” model is more accurate in distinguishing between self and non self, and can accurately distinguish “microbial non self”, “changed self”, etc.

4) Most chronic HBV infected pregnant women in the “HBV immune tolerance period” before pregnancy

In China, most chronic HBV infections are transmitted by MTCT. After chronic infection, there are four periods: immune tolerance period, immune clearance period, immune control period and reactivation period. After acute HBV infection, CD4⁺ T cells secrete cytokines, activate CD8⁺ T cells, and kill HBV from infected hepatocytes through cell lysis and non lysis cell mode. HBV specific CD4⁺ T cells also stimulate B cells to produce antibodies and neutralize free viruses. This antiviral response is often unsuccessful in the immune tolerance period of chronic HBV infected patients, mainly manifested by the dysfunction or exhaustion of HBV specific CD8⁺ T cells (92). In chronic HBV infection, HBV specific CD8⁺ T mediated liver injury is caused firstly by antigen non-specific cells (such as NK cells). Age is an important factor in determining the immune tolerance period (93). It is generally believed that once age exceeds 30 or 40 years old, the immune tolerance is gradually broken (94). Most women of childbearing age (25–35 years old) are in the HBV immune tolerance period, with high HBsAg, HBeAg positive, and HBV DNA >10⁸ IU/ml. During immune tolerance period, the immune cells will not directly or indirectly attack the liver cells, and the liver inflammation is very slight. The condition of chronic HBV infection women during pregnancy is relatively stable, with few HBV related progress or liver failure (95). In 2008, it was reported that in 35 pregnant women without antiviral treatment, abnormal liver function were 34.6% during pregnancy and 50% after delivery (4). There was no statistical difference in the rate of postpartum hepatitis in receiving AVT group in late pregnancy compared with not receiving AVT group (39.4% vs 38.7%, $P=0.942$) in chronic HBV infection pregnant women (96). During pregnancy, high levels of progesterone and estrogen and Glucocorticoid are favour to the Th2 and Treg dominant mode accompanied by the increase of corresponding cytokines. To sum up, chronic HBV infection pregnant women in immune tolerance period before pregnancy remain keep “immune tolerance to HBV” during pregnancy (97).

There are conflicting results about the quantitative changes of HBV DNA during pregnancy. This may be caused by the mix factors such as the natural stage of chronic HBV infection, HBeAg status and the level of HBV DNA before pregnancy. In the Liu J’s study, HBeAg positive pregnant women group was enrolled with baseline HBV DNA >7.0 log₁₀ IU/mL, HBsAg >4.0 log₁₀ IU/mL and HBeAg >3.0 log₁₀ S/CO in the pregnancy (98). It has been found that in pregnant women without AVT in late pregnancy, no matter whether the ALT is normal or abnormal during pregnancy and postpartum, HBV DNA, HBsAg and HBeAg quantification have almost remained stable (98). However, some studies have found that HBV DNA had elevated in some patients during pregnancy. Chang CY found that about 9% of chronic HBV infection pregnant women with low baseline virus load had elevated HBV DNA levels during pregnancy (99). It was also found that there was an increasing trend on HBV DNA level during pregnancy, with average value of 0.4–1 log₁₀ IU/ml (100). Analysis of causes: On the one hand, high levels of adrenocortical hormone during pregnancy

may increase HBV replication (101). The increase of corticosteroid during pregnancy can activate glucocorticoid response elements in HBV gene, further enhance HBV replication and HBV gene expression. But the increase of estrogen level during pregnancy has been proved to reduce HBV replication in animal experiments (102). On the other hand, the predominance of Th2 in chronic HBV infection women during pregnancy is conducive to maintain immune tolerance to HBV *in vivo* (103).

Maternal change on immune system and hormone after delivery

After the fetus is delivered, maternal fetal immune tolerance is no longer required and maternal cell immunity gradually recovers (Figure 3). In the first trimester pregnancy, moderate inflammatory reaction is conducive to placenta implantation, and inflammation is gradually reduced in the second trimester pregnancy. In the third trimester of pregnancy, the uterine is again in an inflammatory state, so as to prepare for the delivery of the fetus and the face of birth canal infection.

Changes of innate immunity and adaptive immunity after delivery.

Both innate and adaptive immunity were activated after delivery. NK cells gathered at the maternal fetal interface during pregnancy returns to the peripheral blood after delivery. The increase of NK cell activity in postpartum mothers was mainly caused by the change of single NK cell activity (104). The percentage and absolute count of NK cells after delivery increased temporarily (9). It was found that the frequency of NK cells increased from 6 weeks after delivery and held plateau at 6-24 weeks (105). Postpartum neutrophils increased. Postpartum monocyte chemoattractant protein-1 (MCP-1), also known as

CCL2 and macrophage derived chemokine (MDC) levels increased after delivery (106). As CC subclass chemokines, MCP-1 and DC can cause chemotactic migration of dendritic cells, monocytes and T cells, especially Th1 cells. During labor, macrophages in the cervix rapidly aggregate, showing M1 proinflammatory phenotype, and participate in regulating cervical maturation and the beginning of labor by producing proinflammatory cytokines (15). In the mouse model, it was found that postpartum cervical macrophages obtained M2 phenotype, which may be related to postpartum tissue repair (107).

Postpartum adaptive immune adjusts accordingly. In not receiving AVT patients with chronic HBV infection, the increase of Th1 cytokines during perinatal period can be slight. Due to the lack of IDO and HLA-G, their inhibition on immune cells is relieved. Th and CTL increased from 1 to 4 months postpartum, while inhibitory T cells increased at 7 months postpartum (108). As an important component of adaptive immunity, B cells can produce specific antibodies, present antigens and regulate immunity. CD5⁻ and CD5⁺ B cells further decreased at 1 month postpartum, but CD5⁺ B cells significantly increased at 7-10 months postpartum (108). Plasma IL-6 and hsC-reactive protein (hsCRP) levels were higher in the early postpartum period. After delivery, IFN- γ , IL-2 and TNF- α were detected to be low level, and recovered 3-4 months later (109).

In a word, after the delivery of the fetus, maternal immunity presents Th1 and T17 dominant. The specific cellular immunity gradually increases in 3-4 months (109). Postpartum humoral immunity also increased after 6 months. The change of immune system lasts for 1 year after delivery.

Estrogen, progesterone, HCG and cortisol levels after delivery

HCG reached its peak at 8-10 weeks of gestation, then decreased rapidly, and disappeared within 2 weeks after

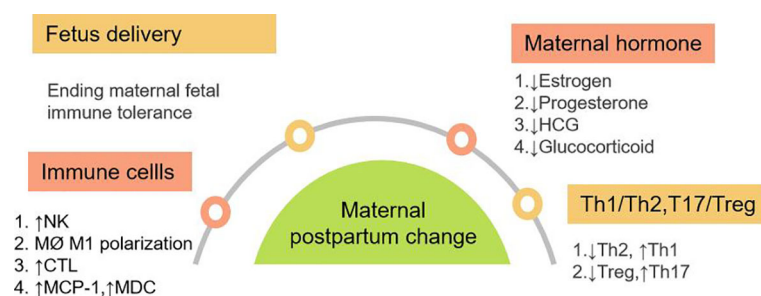


FIGURE 3

Maternal Postpartum changes in immune system and hormone. It mainly includes four aspects: Fetal delivery; changes of immune cells; changes in maternal hormone levels; changes of Th1, Th2, Th17 and Treg balance *in vivo*. Abbreviation: MCP-1: monocyte chemoattractant protein-1, MDC: macrophage derived chemokine.

delivery. Estrogen reached its peak at the end of pregnancy and decreased rapidly after delivery. Progesterone decreased rapidly after delivery. Postpartum progesterone rebound to baseline levels before pregnancy and no longer promotes the production of Th2 cytokine (110). Maternal HCG, estrogen and progesterone levels will recover in one month and the decrease of estrogen, progesterone and HCG promote the transformation from Th2 and Treg dominant in pregnancy to Th1 and Th17 dominant after delivery.

In conclusion, there is no need for maternal fetal immune tolerance after delivery. In the background of uterine inflammation and disappearance of immune tolerance after delivery, the immunosuppression in innate and adaptive immune is reversed. Under the influence of various postpartum factors, there are shifts of Th1/Th2 to Th1 dominant and Th17/Treg to Th17 dominant type. The rapid recovery of hormones of estrogen, progesterone, HCG and cortisol levels aggravate the inflammatory process after delivery. (Figure 3)

Postpartum hepatitis and maternal changes

The causes of postpartum hepatitis are complex. Although immune tolerance to antigens during pregnancy mainly occurs at the maternal-fetal interface, it also affects the systemic immune system. With postpartum uterine inflammation, the body immunity is mainly Th1 and Th17 type. The change of postpartum hormone level does not seem to directly cause

postpartum hepatitis, but it can make Th1 cells predominate. The rapid decrease of postpartum cortisol level is similar to the effect of stopping cortisol, which reduces the inhibition of liver inflammation and may aggravate the liver inflammatory reaction (111, 112). In the above condition, if stimulated by HBV DNA, postpartum hepatitis will flare (Figure 4).

Postpartum hepatitis with innate and adaptive immunity

The changes of innate immunity and adaptive immunity after delivery mainly lead to postpartum hepatitis flare. The immune effect of pregnancy lasts about 1 year after delivery (108). After delivery, the immune tolerance to the fetus disappears rapidly. The number and function inhibition of innate immune cells often recovery earlier after delivery. There are many reasons for postpartum hepatitis, including proinflammatory factor release, the withdrawal of corticosteroids, especially the enhancement of HBV specific T cell response induced by viral HBV DNA, cytokines, etc. Postpartum hepatitis should also exclude various common causes of abnormal liver function, such as non-alcoholic fatty liver disease and drug-induced liver damage.

The largest number of innate immune cells in the liver is NK, followed by macrophages. After delivery, the delivery woman is in an inflammatory state caused by bacteria or other reasons. Postpartum inflammatory cytokines and chemokines may also activate innate immunity or adaptive immunity, causing

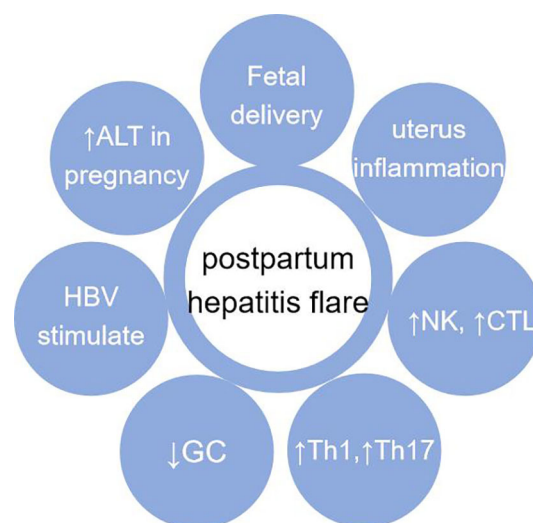


FIGURE 4

Causes of postpartum hepatitis. It mainly includes: fetal delivery (maternal fetal immune tolerance disappears); local inflammation of uterus after delivery; recovery of immune system after delivery; changes of Th1, Th2, Th17 and Treg balance after delivery; after delivery, GC decreases which is similar to "hormone withdrawal" reaction; HBV stimulation. Abbreviation: GC, Corticosteroid.

hepatitis. The number and activity of NK cells in postpartum peripheral blood increased. Liver NK cells may cause liver inflammation through a non-antigen specific mechanism through (TRAIL/TRAIL R) mediated apoptosis, which can be initiated by cytokines such as IFN- α (113). Under the stimulation of inflammation, macrophages derived from Kupffer cells or monocytes in the liver are activated. Macrophages play the role of double swords. On the one hand, they are conducive to controlling infection, on the other hand, they can release chemokines, recruit more monocytes and lymphocytes to the liver, damage liver cells, and promote the occurrence of fibrosis.

In cellular immunity, CD8⁺ T plays a major role in immune cells proliferation and activation. It has been reported that the postpartum ALT and Th1 cytokines in untreated CHB patients increased slightly, and the serum HBsAg and HBV DNA content did not change (95). It is considered that in this study the increase of Th1 cytokines is not strong enough to cause immune suppression to HBV after postpartum (95). Compared with the group without postpartum hepatitis, the number of Treg in delivery woman with postpartum hepatitis was low, the ratio of CD4⁺ T cells expressing activated marker CD69 was significantly higher. In postpartum hepatitis mother, CD4⁺ T cells secreted more IFN- γ , IL-21, IL-2, TNF- α , and secreted more pro-inflammatory/anti-inflammatory cytokines (114). After delivery, CD8⁺ T and its subgroup effector memory T cell (Tem) and terminally differentiated effector memory T cells (TEMRA) in postpartum hepatitis flares group were significantly activated, and the expression levels of perforin and granzyme B increased (108, 115). In postpartum hepatitis flare group, activation of CD8⁺ T cells can produce TNF- α and IFN- α and induce postpartum hepatitis (114, 115). Finally, postpartum hepatitis may be accompanied by the decrease of HBV DNA, and HBeAg or HBsAg.

Postpartum hepatitis and chronic HBV infection

Postpartum hepatitis and the natural course of chronic HBV infection

For the first pregnancy, most HBeAg-positive women of childbearing age with chronic HBV infection are in the HBV immune tolerance period, and a small part are in the immune control period. ALT and HBeAg status can help us distinguish immune tolerance status from immune clearance phase (116). Most pregnant woman are in the period of immune tolerance or immune control before pregnancy. At the early stage, it is believed that pregnancy and childbirth have little impact on the natural process of chronic HBV infection. Women with chronic HBV infection rarely develop severe HBV related liver disease during pregnancy, which may be due to the predominance of Th2 cells in women during pregnancy. Early

research supported that the virology and serology of chronic HBV infection were no significant change in pregnancy and postpartum (98). The quantification of HBV DNA, HBsAg/HBeAg had no significant change from pregnancy to 12 months after delivery in patients with e antigen positive or e antigen negative chronic HBV infection. But now it seems that serological conversion of HBeAg can occur after delivery. As an immune tolerance factor, HBeAg is also a risk factor for postpartum hepatitis. Postpartum hepatitis in HBeAg positive women was 2.56 times higher than that in HBeAg negative delivery women (117). Postpartum immune reactivation can lead to increased ALT, and women with HBeAg positive with low titer can transit to the immune clearance period accompanied by the decline of HBsAg and HBeAg, and even HBeAg serological conversion after delivery (117). Postpartum HBeAg serum conversion rate ranged from 7-17% (117-119). Compared with non pregnant women, the HBeAg seroconversion rate of pregnant women with an average age of 30.7 ± 3.6 years increased from 2.2% to 14.3% (4). The clearance rate of HBeAg in HBeAg positive and NAVT delivery women was closely related to decrease in both HBeAg titer and/or HBV-DNA concentration in 1-2 months after delivery (118). Forty HBeAg positive pregnant women were studied in Taiwan, with an average age of 29.9 ± 5.3 years. During the follow-up period from the beginning of labor to 1 year, it was found that prepartum HBeAg titer <1:650 was associated with high HBeAg seroconversion rate, and there was no significant correlation with genotype B and genotype C (119). Observing the data of 214 HBeAg positive and NAVT delivery woman, HBsAg < 1.0×10^4 IU/mL, and HBeAg < 7.36×10^2 S/CO, HBV DNA level < 1.0×10^7 IU/mL at delivery were independent predictors of HBeAg seroconversion within 12 months postpartum (120). The presence of PC, BCP, and PC and BCP mutations during labor was associated with an increased likelihood of spontaneous postnatal HBeAg seroconversion (all $P < 0.05$) (120). Other studies have similar findings. The NAVT HBeAg positive delivery woman were observed for 6.4 years. The baseline HBV DNA < 3×10^7 IU/mL or HBeAg < 770 S/CO in pregnancy was the predictive factor of HBeAg serological conversion (121).

Relationship between postpartum hepatitis and quantitative of HBsAg and HBV DNA in pregnant women with chronic HBV infection

Because of the interaction between HBV and immune response, postpartum hepatitis is more common in chronic HBV infection women. HBV DNA during delivery is also a predictor of postpartum hepatitis. There is 30% probability for postpartum hepatitis in pregnant women who can detect HBV DNA during delivery, and 20% probability in pregnant women who do not detect viremia in chronic HBV infected patients (2). The study found that hepatitis during pregnancy was 4.6% (2/44) and 7.1% (5/68) respectively for HBV DNA < 2000 IU/mL and HBV DNA > 2000 IU/mL, and hepatitis after delivery was

7.4% (2/28) and 13.0% (3/23) respectively (99). Some studies believe that the cutoff value of postpartum hepatitis is HBV DNA $\geq 5 \log_{10}$ IU/mL at the delivery of untreated chronic HBV infected pregnant women with positive predictive value is 14.4% and the negative predictive value is 98.2% (2). It is speculated that the postpartum hepatitis flare can only be triggered when the HBV DNA load reaches a certain level. Among HBeAg negative pregnant women, about 30% of pregnant women have postpartum hepatitis, which may be related to reactivation in the immune control period. Age is related to HBV immune tolerance. It is believed that some 30 years old HBeAg positive women are in the transition stage from immune tolerance to immune clearance. The study found that once postpartum hepatitis occurred, the quantitative level of HBsAg in delivery women would decrease significantly at 6-8 weeks or 15-18 weeks (114). HBsAg < 100 IU/ml was the predictive factor of HBsAg seroconversion after delivery (121).

High risk factors of postpartum hepatitis

Early articles suggested that HBV DNA, ALT level, HBeAg status or any other characteristics of pregnant women could not predict postpartum hepatitis during pregnancy (51). However, in recent studies, the pregnant women were further differentiated according to the natural period of chronic HBV infection and HBeAg positive, and it was found that there were characteristic indicators to predict the onset of postpartum hepatitis (Table 1). In addition to the mentioned HBeAg positive and < 700 S/CO, HBV DNA > 5 Log₁₀ IU/ml at delivery is a predictor of postpartum hepatitis. Postpartum hepatitis can occur in chronic HBV infected people receiving AVT or NAVT (120–123). The increase rate of hepatitis during pregnancy and after delivery was 14% and 16%, respectively (5). ALT at delivery

is elevated, which is also a high-risk predictor of postpartum hepatitis (2). In 241 pregnant women, 33.6% of the women had elevated serum ALT during pregnancy. Multivariate analysis showed that elevated ALT during pregnancy was associated with postpartum hepatitis after telbivudine withdrawal (124). The cutoff value of predicting postpartum hepatitis in pregnant women was 29 years old, the prenatal ALT was greater than 14.8 U/L, and the postnatal HBeAg level was less than 3.1 log₁₀ S/CO (125). Studies on pregnant women showed that ALT at 32 weeks of gestation and HBcAb were risk factors of postpartum hepatitis (126). HBeAg positivity and gestational diabetes were associated with postpartum hepatitis (123). When oral AVT is used in late pregnancy and stopped after delivery, the prediction model of postpartum hepatitis includes the decrease of HBsAg and HBV DNA from baseline to gestation 32 weeks or delivery, respectively (127).

At first, scholars questioned the correctness of AVT in late pregnancy. According to previous research reports, 62% of women will have postpartum hepatitis after stopping antiviral treatment after delivery, which is significantly higher than that of women who do not receive antiviral treatment (36% vs 62%) (51). Ayres et al. reported that although short-term lamivudine treatment during pregnancy moderately reduced maternal viremia, HBV rtM204I/V and rtA181T mutations were detected (128). With the emergence of powerful and highly resistant barrier drugs, guidelines recommend antiviral treatment for pregnant women in late pregnancy with high viral load of hepatitis B virus infection.

Whether stopping AVT after delivery is a risk factor for postpartum hepatitis in chronic HBV infected women who have received oral nucleoside/nucleotides analogs in late pregnancy? It is believed that even if prophylaxis MTCT treatment continues

TABLE 1 The characteristics of the references on risk factors of postpartum hepatitis flare.

author	Year	(N)	regions	Age(years) ^a	standard ^b (U/L)	Rate	Risk factors	AVT (N) ^c
Giles M (117)	2015	126	75%Asian	31.45 ± 0.43	>2ULN	25%	HBeAg positive at baseline Is 2.56 times	7
Liu J (124)	2017	241	Chinese	27 (20–40)	>1ULN	33.6%	ALT elevation at pregnancy	241
Kushner T (5)	2018	310	African and Asian	30 (25–33)	>2ULN	16%	HBeAg positive	21
Yi We (2)	2018	3367	Chinese	29.15 ± 4.16	>5ULN	3.50%	elevated ALT, HBV DNA $\geq 5 \log_{10}$ IU/mL at delivery	0
Li L (123)	2020	317	Chinese	29.3 ± 4.2	>1.5ULN	16.7%	HBeAg positiv, gestational diabetes	138
Lu J (125)	2021	153	Chinese	28.8 ± 3.88	>2ULN	36.60%	age < 29 years, antepartum ALT > 14.8 U/L, postpartum HBeAg < 3.1 logCOI	153
Quan M (126)	2022	444	Chinese	29 (26, 31)	>1ULN	52.93%	ALT at 32 weeks of pregnancy, HBcAb at intrapartum	444
	2022	170	Chinese	28 (26, 30)	>1ULN	70.59%	–	0

^aData are presented as mean ± Standard Deviation or median, ^bThe standards for postpartum hepatitis, ^cThe number of chronic HBV infection pateints receiving AVT in late pregnancy. ULN, upper limit of normal range.

until 12 weeks postpartum, postnatal hepatitis will still occur during this period. It is believed that postpartum hepatitis is generally not serious and reversible if preventive AVT is stopped after delivery (124). Postpartum hepatitis occurred in 25% of women with elevated ALT during pregnancy after discontinuation of telbivudine (124). In immune related studies, lower IFN- α during pregnancy and higher IFN- α after delivery may be related to postpartum hepatitis (125). In the population receiving antiviral treatment, the number of Treg is relatively reduced in chronic HBV infected women with postpartum hepatitis, the production of pro-inflammatory factors IFN- γ , IL-2 and TNF- α is more, and the production of anti-inflammatory cytokines (IL-10) is less (114).

(4) Postpartum hepatitis and treatment to prevent MTCT

AVT in the third trimester of pregnancy can reduce the incidence of MTCT (129–132). After delivery, the immune reactivation in chronic HBV infection women is the basis of hepatitis flare (106). In addition, many factors are involved in the occurrence of postpartum hepatitis. During AVT, there is a negative correlation between the enhancement of Th1/Th2 immunity and the decrease of HBV DNA load (133). Oral AVT reduces HBV DNA load which may further promote to the recovery of HBV specific T cell function. Treatment of prevention MTCT can obviously increase the frequency of postpartum CD83⁺ pDCs and CD86⁺ pDCs (134). The increase in the frequency of differentiated mature DC means that the immune presentation function is enhanced, which is conducive to activating specific cellular immunity. HBV specific immune enhancement participates the damage of HBV infected liver cells, on the other hand, it is conducive to the HBeAg seroconversion. The incidence of postpartum hepatitis after withdrawal of AVT after delivery ranged from 5% to 62%, which may be related to hepatitis postpartum criteria, the frequency of ALT monitoring, and the withdrawal time of AVT. Postpartum immune effect was maintained for 1 year (108). Professor Xie Yao's team conducted a prospective study on pregnant women with HBeAg positive chronic HBV infection, including 96 cases of withdrawal the drug immediately postpartum, and 37 cases of were delayed withdrawal to six weeks postpartum (135). The conclusion of the study suggests that withdrawal of antiviral drugs immediately or six weeks postpartum does not affect the rate of postpartum hepatitis, but may delay postpartum hepatitis until twelve weeks postpartum (135). AVT in late pregnancy prolonged to 12 weeks postpartum did not decrease the occurrence rate of hepatitis after delivery compared with immediate withdrawal postpartum. In pregnant women who with elevated ALT prolonged AVT to 52 weeks postpartum, HBeAg serum conversion rate increased significantly, and HBsAg titer decreased significantly (124).

Postpartum anti-HBV treatment is often a good time for achieving the seroclearance of HBeAg or HBsAg. In the chronic

HBV infected women who were in immune tolerance period before pregnancy, some people would enter the immune clearance period after delivery. Previous studies have shown that in the pursuit of HBeAg seroconversion or clinical cure, a more effective treatment is the interferon-based treatment. If there is no contraindication of interferon, oral nucleoside/nucleotides analogs combined with pegylated-interferon can often achieve higher HBeAg seroclearance or HBsAg seroclearance. A research was conducted in pregnant women with ALT <1ULN, HBeAg positive and HBV DNA >6log₁₀IU/ml who had received oral AVT in the late pregnancy. If the postpartum women showed the characteristics of ALT >2ULN with HBeAg decreased by more than 20% or HBV DNA decreased by more than 2log₁₀IU/ml compared with baseline of late pregnancy, they were included in the study group which were given pegylated-interferon combined with Adefovir, while the control group stopped taking AVT orally in 12 weeks after delivery. In the study group, 56.7% (17/30) patients achieved HBeAg seroclearance and 26.7% (8/30) patients achieved HBsAg seroclearance. No one in the control group achieved the seroclearance of HBeAg or HBsAg (136).

In conclusion, during pregnancy, maternal fetal immune tolerance makes immunosuppression in specific immunity and non-specific immunity. The elevation of pregnancy related hormones will also affect immunity. The condition of pregnant women with chronic HBV infection seems to be relatively stable during pregnancy. Postpartum human immune system reverses the trend of maternal fetal immune tolerance and were dominated by uterine inflammation. NK's non-specific immunity is enhanced, Th1 cells and corresponding cytokines are increased, DC' antigen presenting function is enhanced. The content of serum cortisol dropped rapidly after delivery, which was equivalent to hormone withdrawal reaction. In delivery women with chronic HBV infection, when HBV DNA can be detected, it can often induce or aggravate the hepatitis flare. There are several indicators to predict postpartum hepatitis, including HBeAg positive and <700 S/CO, childbearing age and <29 years old, and HBV DNA >3-5Log₁₀ IU/ml. When delaying the time for withdrawal AVT to 6-12 weeks postpartum, postpartum hepatitis rate cannot decrease. For pregnant women with elevated ALT during pregnancy, the HBeAg serum conversion rate can be increased by prolonging the AVT to more than 48 weeks if HBeAg tends to decline from the baseline in the pregnant or delivery. If the postpartum biochemical, virological or other indicators indicate that the delivery women have entered the immune clearance period, it is recommended to consider anti-HBV treatment. If there is no interferon contraindication, the interferon-based treatment is more conducive to HBeAg or HBsAg seroclearance after delivery. In rare cases postpartum hepatitis flare would be severe, and oral AVT is recommended.

Author contributions

ML, YLu, YJ and YX contributed to study concept and design. TJ, YY, WD, HL, SW, RL, MC, SWu, YG, HH, GS, XC, LH, LY, XB and YLin collected and sorted out literatures. LZ wrote the first draft and drew pictures. TJ, YY, WD, HL and SW edited the English version. ML, YLu, YJ and YX modify the version to be submitted. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The reviewer XC declared a shared affiliation with the authors LZ, TJ, WD, HL, SW, RL, MC, WS, YG, HH, GS, XM, XC, LH, LY, XB, YL, YJ, ML, YX to the handling editor at the time of review.

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Study on pathological and clinical characteristics of chronic HBV infected patients with HBsAg positive, HBV DNA negative, HBeAg negative

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Aims: Study of clinical characteristics of hepatitis B virus deoxyribonucleic acid (HBV DNA)-negative, hepatitis B surface antigen (HBsAg)-positive, hepatitis B e antigen (HBeAg)-negative patients based on liver histopathology.

Methods: We retrospectively enrolled patients with chronic HBV infection diagnosis at Beijing Ditan Hospital from May 2008 to November 2020. To study the differences between patients with significant hepatic histopathology and those without significant hepatic histopathology. And to study the independent factors of significant hepatic histopathology.

Results: 85 HBV DNA-negative and HBeAg-negative patients were 37.90 ± 10.30 years old, 23.50% of patients with grade of inflammation (G) >1, 35.30% of patients with liver fibrosis stage (S) >1, 44.70% patients were diagnosed with significant hepatic histopathology. Compared to the no significant hepatic histopathology group, another group had older age (41.70 ± 10.70 vs 34.80 ± 8.87 years, $t = -3.28$, $P = 0.002$), higher total bilirubin (TBIL) [$14.9(10.3, 22.4)$ vs $11(8.9, 14.4)$ $\mu\text{mol/L}$, $z = -2.26$, $P = 0.024$], lower cholinesterase (CHE) ($t = -2.86$, $P = 0.005$, 7388.00 ± 2156.00 vs 8988.00 ± 2823.00 U/L) and lower platelet (PLT) ($t = 2.75$, $P = 0.007$, 157.00 ± 61.40 vs 194.00 ± 61.00 $10^9/\text{L}$). Abnormal ALT patients are more likely to have significant hepatic histopathology ($z = 5.44$, $P = 0.020$, 66.70% vs 33.75%). G had significant correlation with CHE ($P = 0.008$, $r = -0.23$), alanine aminotransferase (ALT) ($P = 0.041$, $r = 0.18$), aspartate aminotransferase (AST) ($P = 0.001$, $r = 0.29$). S had significant correlation with TBIL ($P = 0.008$, $r = 0.23$), age ($P < 0.001$, $r = 0.32$), international normalized ratio (INR) ($P = 0.04$, $r = 0.23$), CHE ($P < 0.001$, $r = -0.30$), PLT ($P < 0.001$, $r =$

-0.40) and prothrombin time activity (PTA) ($P = 0.046$, $r = -0.22$). Multivariate logistic analysis indicated only age (95%CI=1.014~1.130, OR=1.069, $P=0.013$) was an impact factor for significant hepatic histopathology. The cutoff point of age was 34.30 years.

Conclusions: A large proportion of chronic HBV infection patients with HBeAg-negative and HBV DNA-negative still have chronic hepatitis. Age is an independent factor for significant hepatic histopathology

KEYWORDS

HBV DNA, HBeAg, chronic HBV-infection, clinical indicators, liver histopathology

Introduction

As reported in the latest (2017) world health organization (WHO) global hepatitis report, in 2015, there were approximately 257 million people infected hepatitis B virus (HBV) worldwide (1). Chronic HBV infection has 5 periods of natural history, which contain hepatitis B e antigen (HBeAg)-positive chronic HBV infection, HBeAg-negative chronic HBV infection, HBeAg-positive chronic hepatitis B, HBeAg-negative chronic hepatitis B, hepatitis B surface antigen (HBsAg)-negative phase (2), and each period has its characteristics.

HBeAg-negative chronic HBV infection is characterized by HBeAb-positive, normal alanine aminotransferase (ALT), and low HBV DNA load (3). In the past, this period was termed as “inactive carriers (IC)”, but as understanding of HBV disease history and immune mechanisms has improved, scholars have replaced the term IC with HBeAg-negative chronic HBV infection (4). Although prognosis for patients in this period is said to be good, some patients may still experience fluctuations in HBV DNA and/or ALT. These fluctuations may then lead to disease progression such as liver fibrosis, liver cirrhosis or even liver cancer (5, 6). Previously, many scholars have studied the clinical features and prognosis of this stage. One limitation of those studies is that they did not use the high-sensitivity HBV DNA quantification assay. Our research was conducted to study the histopathological characteristics and clinical indicators of HBeAg-negative, HBV DNA-negative patients using a high-sensitivity assay in combination with liver biopsy.

Materials and methods

Patients

Chronic HBV-infected patients who underwent liver biopsies were obtained from Beijing Ditan Hospital affiliated to Capital Medical University between May 2008 and November 2020. Information on antiviral therapy, demographic data, HBV

serological test and HBV DNA load results, renal function, liver function, blood routine examination and blood coagulation function were collected.

Admission criteria: 1) HBsAg-positive for over 6 months; 2) 18 to 65 years old; 3) no HBV treatment received; 4) complete viral load and viral serology results, coagulation, liver function, blood routine within 2 weeks after or before biopsy; 5) have clear diagnostic results of liver biopsy.

Exclusion criteria: 1) infected with other viruses (e.g., HIV, cytomegalovirus, etc.); 2) Co-infection with other viral hepatitis (such as hepatitis C, A, D and E), non-alcoholic fatty liver disease, alcoholic liver disease, autoimmune liver disease, drug-induced liver injury, cirrhosis, hepatoma; 3) patients with cardiac or renal dysfunction; 4) patients who have not obtained a clear result of inflammatory grade or liver fibrosis stage.

Study parameters

ALT, AST, HBsAg, HBeAg, HBV DNA, albumin (ALB), international normalized ratio (INR), total bilirubin (TBIL), cholinesterase (CHE), prothrombin time activity (PTA), platelet (PLT).

Liver pathology results included liver fibrosis stage S0-S4, and inflammatory grade G0-G4.

In our study, $S > 1$ indicates significant liver fibrosis, $0 < S \leq 1$ indicates slight liver fibrosis, S0 indicates no liver fibrosis. $G > 1$ indicates significant liver inflammation, and $G \leq 1$ indicates no significant liver inflammation. No significant hepatic histopathology group's patients meet $G \leq 1$ and $S \leq 1$, and significant hepatic histopathology group's patients meet $G > 1$ and/or $S > 1$.

Study contents

To study the correlation factors associated with the pathological grading of liver biopsies in HBeAg-negative

patients with chronic HBV infection and to analyze the characteristics of their inflammatory grading and fibrosis staging. To compare the differences in clinical indicators between the two groups of patients. To study the risk factors (or protective factors) for significant liver histopathology and calculate the cutoff values of the relevant factors.

Statistical analysis

Use the mean $\pm$ standard deviation or quartiles to describe enumeration data and percentages were used to describe categorical data. Categorical data were compared using chi-square test. Student's t-test was used to compare normally distributed data. Using Mann-Whitney test to compare non-normally distributed data. Risk factors (or protective factors) were studied using binary logistic regression analysis, and cutoff values for relevant risk factors (or protective factors) were calculated from youden's index and receiver operating curve (ROC). The software involving figures making was Microsoft Excel 2019 and Graphpad Prism 8.0. Statistical analysis was performed in IBM SPSS 25.0. $P < 0.05$ indicates statistical significance.

Results

Patients

A total of 4421 HBV infected people had liver puncture biopsies. After collecting HBV serologic indicators, HBV DNA load, demographic data, clinical biochemical indicators, and blood routines. We performed screening and excluded 8

patients with hepatitis C, 1870 patients with fatty liver, 219 patients whose liver pathology diagnosis did not clearly show disease grade, 226 patients who received HBV treatment, 165 patients without HBeAg results, 428 patients without HBV DNA load results, 31 patients without ALT results, 613 patients with a detectable HBV DNA load, 776 HBeAg-positive patients. Finally, a total of 85 HBeAg-negative, HBV DNA-negative patients (HBeAg-negative, HBV DNA load below the lower limit of detection) were included in this study. (Figure 1)

In this group of patients, 57.60% were male, age (37.90 ± 10.30) years old, significant hepatic histopathology was observed in 44.70% of patients. Compared with no significant hepatic histopathology group, the other group had older age (41.70 ± 10.70 vs 34.80 ± 8.87 years, $t = -3.28$, $P = 0.002$), higher TBIL levels [$14.9(10.3, 22.4)$ vs $11(8.9, 14.4)$ $\mu\text{mol/L}$, $z = -2.26$, $P = 0.024$], lower CHE levels (7388.00 ± 2156.00 vs 8988.00 ± 2823.00 U/L, $t = -2.86$, $P = 0.005$) and PLT counts (157.00 ± 61.40 vs 194.00 ± 61.00 $10^9/\text{L}$, $t = 2.75$, $P = 0.007$). (Table 1)

Compared with normal ALT group, abnormal ALT group had more patients with significant hepatic histopathology (66.70% vs 337.50%, $z = 5.44$, $P = 0.020$). (Table 1)

Liver histopathology

In liver histopathological diagnosis, liver inflammation grading: 76.50% (65 cases) of patients with $G \leq 1$, 15.30% (13 cases) of patients with $1 < G \leq 2$, and 8.20% (7 cases) of patients with $2 < G \leq 3$. Liver fibrosis grading: S0 patients accounted for 1.20% (1 case), $S \leq 1$ patients accounted for 63.50% (54 cases), $1 < S \leq 2$ patients accounted for 24.70% (21 cases), $2 < S \leq 3$ patients accounted for 8.20% (7 cases), and $3 < S \leq 4$ patients accounted for 2.40% (2 cases) (Table 2).

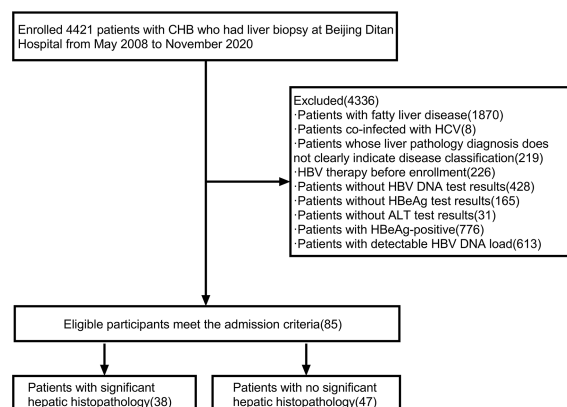


FIGURE 1

Flow chart for screening research patients. Abbreviations: ALT, alanine aminotransferase; CHB, chronic hepatitis B; HBV DNA, hepatitis B virus deoxyribonucleic acid; HBV, hepatitis B virus; HCV, hepatitis C virus; HBeAg, hepatitis B e antigen.

TABLE 1 Characteristics and comparison of clinical indicators in HBeAg-negative, HBV DNA-negative patients.

Characteristics	Total (N=85)	No significant hepatic histopathology (n=47)	Significant hepatic histopathology (n=38)	t/ χ^2/z	P
Male(%)	57.60%	55.30%	60.50%	0.23	0.629
Age(years)	37.90 ± 10.30	34.80 ± 8.87	41.70 ± 10.70	-3.28	0.002
HBsAg(log ₁₀ IU/ml)	2.01 ± 1.49	2.21 ± 1.59	1.81 ± 1.38	0.98	0.33
ALT(U/L)	25.70(17.50, 39.80)	24.00(16.50, 33.50)	30.10(19.60, 54.80)	-1.70	0.09
AST(U/L)	22.80(17.80, 33.20)	22.30(17.60, 28.30)	26.30(17.90, 39.50)	-1.94	0.053
TBIL(μmol/L)	12.30(9.30, 17.50)	11.00(8.90, 14.40)	14.90(10.30, 22.40)	-2.26	0.024
ALB(g/L)	44.90 ± 4.89	45.40 ± 4.01	44.30 ± 5.79	1.04	0.303
CHE(U/L)	8255.00 ± 2649.00	8988.00 ± 2823.00	7388.00 ± 2156.00	2.86	0.005
PLT(10 ⁹ /L)	177.00 ± 63.60	194.00 ± 61.00	157.00 ± 61.40	2.75	0.007
PTA(%)	88.80 ± 14.50	90.90 ± 14.00	86.40 ± 14.90	1.13	0.263
INR	1.02 ± 0.12	1.01 ± 0.11	1.03 ± 0.13	-0.88	0.382
Characteristics	Total (N=85)	normal ALT (n=64)	abnormal ALT (n=21)	t/ χ^2/z	P
Significant hepatic histopathology(%)	44.70%	37.50%	66.70%	5.44	0.020
Male(%)	57.60%	53.10%	71.4%	2.17	0.141
Age(years)	37.90 ± 10.30	37.63 ± 10.52	38.62 ± 9.60	-0.38	0.702
HBsAg(log ₁₀ IU/ml)	2.01 ± 1.49	1.94 ± 1.56	2.18 ± 1.33	-0.52	0.608
ALT(U/L)	25.70(17.50, 39.80)	21.9(14.9, 29.8)	67.8(56.3, 97.5)	-6.85	<0.001
AST(U/L)	22.80(17.80, 33.20)	21.8(17, 24.6)	45.5(35.8, 87.4)	-6.41	<0.001
TBIL(μmol/L)	12.30(9.30, 17.50)	11.3(9.15, 17.5)	14.9(12.3, 17.8)	-1.74	0.083
ALB(g/L)	44.90 ± 4.89	44.83 ± 4.78	45.28 ± 5.32	-0.36	0.721
CHE(U/L)	8255.00 ± 2649.00	8308.97 ± 2754.34	8096.15 ± 2366.06	0.32	0.752
PLT(10 ⁹ /L)	177.00 ± 63.60	180.39 ± 63.67	166.49 ± 63.97	0.84	0.406
PTA(%)	88.80 ± 14.50	88.31 ± 14.4	90.59 ± 15.42	-0.46	0.648
INR	1.02 ± 0.12	1.03 ± 0.12	0.99 ± 0.11	0.90	0.373

ALT, alanine aminotransferase; ALB, albumin; AST, aspartate aminotransferase; TBIL, total bilirubin; HBsAg, hepatitis B surface antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; CHE, cholinesterase; PTA, prothrombin time activity; PLT, platelet; INR, international normalized ratio; /, or.

Correlation between pathological grading and clinical indicators

The correlations of liver inflammation grade with ALT ($P=0.041$, $r=0.18$), AST ($P=0.001$, $r=0.29$), and CHE ($P=0.008$, $r=-0.23$) were significant, while the correlations with HBsAg,

ALB, and PLT were not significant. The correlation between liver fibrosis stage and age ($P < 0.001$, $r = 0.32$), TBIL ($P = 0.008$, $r = 0.23$), CHE ($P < 0.001$, $r = -0.30$), PLT ($P < 0.001$, $r = -0.40$), PTA ($P = 0.046$, $r = -0.22$) and INR ($P = 0.04$, $r = 0.23$) were significant, while the correlation with HBsAg, ALT, AST, and ALB were not significant. (Table 3)

TABLE 2 Histopathological grade distribution table for liver biopsies of HBeAg-negative and HBV DNA-negative patients.

Classification	Degree of Histopathology	Grade	Number of people (%)
G	Insignificant	G0	0
		$G \leq 1$	76.50%
	Significant	$1 < G \leq 2$	15.30%
		$2 < G \leq 3$	8.20%
		$3 < G \leq 4$	0
S	Insignificant	S0	1.20%
		$S \leq 1$	63.50%
	Significant	$1 < S \leq 2$	24.70%
		$2 < S \leq 3$	8.20%
		$3 < S \leq 4$	2.40%

G, inflammation grade; S, liver fibrosis stage.

Logistic regression analysis

We performed logistic regression analysis on the differential indicators (age, TBIL, CHE and PLT) of two groups. The results of univariate logistic regression indicated that significant hepatic histopathology was associated with CHE level (OR = 0.018, 95% CI = 0.005 ~ 0.071, $P < 0.001$), age (OR = 1.077, 95% CI = 1.025 ~ 1.132, $P = 0.003$) and PLT count (OR = 0.988, 95% CI = 0.985 ~ 0.992, $P < 0.001$). Multivariate logistic regression analysis indicated that only age (95% CI = 1.014 ~ 1.130, OR = 1.070, $P = 0.015$) was an independent factor. (Table 4)

The cutoff value was calculated by ROC curve and Jorden index, age 34.30 years, sensitivity 78.90%, specificity 53.20%, AUC=0.688. (Table 4 and Figure 2)

Discussion

During the natural history of chronic HBV infection, HBeAg seroconversion is a very important point, which implies a reduction or even silencing of viral cccDNA transcription activity (7). Also, in clinical practice, HBeAg seroconversion means a better prognosis because patients with HBeAg-negative chronic HBV infection are less likely to progress to cirrhosis or liver cancer. However, if the HBeAg-negative chronic hepatitis B period develops, there is still the possibility of disease progression (3, 8–10). That is to say, effective distinction between HBeAg-negative chronic hepatitis B and HBeAg-negative chronic HBV infection plays a very important role in determining the prognosis of those patients.

TABLE 3 Histopathological grade distribution of the liver biopsy of HBeAg-negative and HBV DNA-negative patients.

Characteristics	G	S
	r/P	r/P
age(years)	0.07/0.417	0.32/<0.001
HBsAg(log ₁₀ IU/ml)	-0.12/0.28	-0.2/0.067
AST(U/L)	0.29/0.001	0.09/0.307
ALT(U/L)	0.18/0.041	0.08/0.382
TBIL(μmol/L)	0.12/0.159	0.23/0.008
CHE(U/L)	-0.23/0.008	-0.3/<0.001
ALB(g/L)	-0.16/0.076	-0.004/0.964
PLT(10 ⁹ /L)	-0.12/0.16	-0.4/<0.001
PTA(%)	-0.09/0.451	-0.22/0.046
INR	-0.06/0.624	0.23/0.04

ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; AST, aspartate aminotransferase; TBIL, total bilirubin; HBV DNA, hepatitis B virus deoxyribonucleic acid; ALB, albumin; INR, international normalized ratio; CHE, cholinesterase; PTA, prothrombin time activity; PLT, platelet.

TABLE 4 Logistic regression analysis of HBeAg-negative, HBV DNA-negative patients with and without significant hepatic histopathology.

Univariate binary logistic regression analysis				
Characteristics	B	P	OR	95% CI
age(years)	0.07	0.003	1.077	1.025~1.132
TBIL($\mu\text{mol/L}$)	-0.96	0.059	1.051	0.998~1.106
CHE($\log_{10}$ U/L)	-5.79	0.006	0.003	0.00005~0.190
PLT($10^9/\text{L}$)	-0.01	0.010	0.990	0.982~0.998
Multivariate binary logistic regression analysis				
Characteristics	B	P	OR	95% CI
age(years)	0.07	0.015	1.070	1.014~1.130
TBIL($\mu\text{mol/L}$)	0.05	0.115	1.051	0.988~1.119
CHE($\log_{10}$ U/L)	-0.0002	0.061	0.9997	0.9995~1.0001
PLT($10^9/\text{L}$)	-0.005	0.240	0.995	0.987~1.003
Cut off value and AUC				
Characteristics	Cut off	sensitivity	specificity	AUC
age(years)	34.30	78.90%	53.20%	0.688
TBIL($\mu\text{mol/L}$)	14.65	55.30%	78.70%	0.643
CHE($\log_{10}$ U/L)	8217.50	66.70%	71.10%	0.679
PLT($10^9/\text{L}$)	178.50	60.90%	70.30%	0.668

TBIL, total bilirubin; CHE, cholinesterase; PLT, platelet; AUC, area under curve.

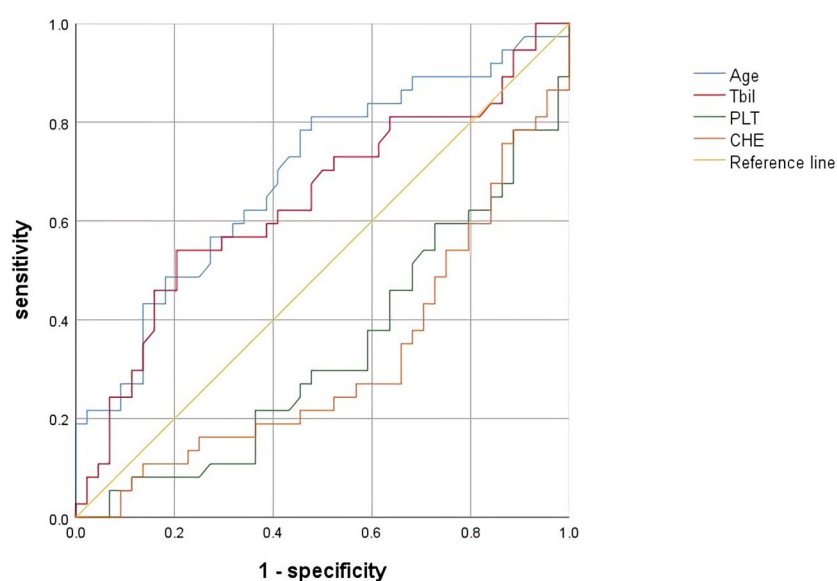


FIGURE 2

ROC of factors associated with significant hepatic histopathology. Abbreviations: CHE, cholinesterase; PLT, platelet; TBIL, total bilirubin; ROC, receiver operating characteristic curve.

Martinot, Park H, Brunett prospectively included HBeAg-negative patients in their respective studies and investigated how to identify HBeAg-negative chronic hepatitis B patients from HBeAg-negative chronic HBV infection patients (11–13). Their results showed that HBsAg could be used as a factor in determining the presence of hepatitis in HBeAg-negative patients (11–13). Martinot and Oliveri, on the other hand, found that HBV DNA could also be used as a predictive factor in their own studies (11, 14). Brunetto found in his subsequent studies that HBcrAg was highly accurate in determining the presence of hepatitis in HBeAg-negative patients (15). However, the grouping methods in all these studies were by ALT and HBV DNA, yet in fact there are times when clinical indicators and liver pathology results do not parallel each other. Liver pathology results are the gold standard for determining the progression of disease. Therefore, we designed this study to investigate the risk factors for inflammation in HBsAg-positive, HBeAg-negative, and HBV DNA-negative patients using liver pathology findings as a grouping method and a more accurate HBV DNA assay.

Chronic hepatitis B is an immune-mediated disease, and age is an important factor in disease progression. Previous studies have shown a gradual decrease in the proportion of HBeAg-positive patients with increasing age, to less than 10% in patients over 40 years old (16). In our study, after comparing the clinical characteristics of significant hepatic histopathology group and no significant hepatic histopathology group, we found that patients with significant hepatic histopathology were older, and the correlation analysis showed that growing age was significantly related to the more severe fibrosis stage. Univariate and multivariate logistic regression showed that age was the independent impact factor of significant hepatic histopathology, meanwhile, the older the patient is, the more likely the patient has significant hepatic histopathology. Hsu Y S conducted a cohort study in HBeAg seroconversion patients and found that the annual incidence of hepatitis relapse was 2.2–3.3% (9). This study proved that with the age growing, more patients would develop hepatitis B. This is consistent with our findings. We calculated the cutoff value of age and it was 34.3 years old, and the AUC was 0.688, a barely satisfactory number.

CHE is an indicator of the synthetic function of the liver, which is affected when hepatitis or fibrosis occurs, and CHE decreases. This is confirmed by our study that patients with significant hepatic histopathology have a lower level of CHE, and with the growth of G and S, CHE decreases. Coagulation is also closely related to liver function, and our study found that PLT count is lower in patients with significant hepatic histopathology. PLT is negatively correlated to S according to the correlation analysis, PTA is also negatively correlated to S and INR is positively correlated to S. While we didn't find similar results when it came to the correlation analysis with G. The findings indicated that liver fibrosis had a greater impact on Coagulation. ALT is a sensitive indicator of liver injury, but there are many causes of liver injury. Since we only included HBV

DNA-negative patients for the study, for HBV DNA-negative patients, it is reasonable to consider more whether there are other causes of liver injury, so we did not consider ALT levels at the time of enrollment. In order to find out the reasons of liver injury, we conducted liver biopsy. In the comparison of the patients with and without significant hepatic histopathology, we did not observe a significant difference between ALT. But the comparison of normal ALT patients and abnormal ALT patients showed that there were more significant hepatic histopathology patients in abnormal ALT patients.

A study has shown that the incidence of hepatic flares ranges from 6% to 33% during the 2 to 7 years of follow-up (17), and intermittent flares may result in liver fibrosis. Therefore, pursuing a better endpoint, HBsAg seroclearance, is important. HBsAg seroclearance, also called “functional cure”, can effectively improve the long-term prognosis of patients and greatly reduce the risk of cirrhosis and liver cancer (18–20). Pegylated interferon α -2a (PEG-IFN α -2a) have a unique advantage over nucleoside (acid) analogues (NAs) in achieving HBsAg seroclearance (21–23).

Our study was a retrospective cross-sectional study. The enrolled patients were grouped by liver pathology results into two groups, significant hepatic histopathology group and no significant hepatic histopathology group. We studied the characteristics of clinical indicators and independent risk factors in HBsAg-positive, HBeAg-negative, HBV DNA-negative patients under high-sensitivity test assay. And we found that age was the independent risk factor. Our study has certain flaws, firstly the retrospective cross-sectional nature makes our findings need to be validated by a more scientifically sound prospective cohort study. Secondly, the volume of patients enrolled in our study was small. In the future, we may design a large-sample prospective cohort study to validate our findings and explore if there are more factors to recognize the presence of significant hepatic histopathology.

Data availability statement

The original contributions presented in the study are included in the article/supplementary materials. Further inquiries can be directed to the corresponding author.

Author contributions

ML and YX contributed to the study design. ZZ, RL, WC, YJL, LY and XB contributed to the data analysis. GS, MC, YL, SLW, MX, XC, LH, LZ and GW contributed to the recruitment, enrolment, and assessment of participants, as well as following up with the patients. TJ, WD, FS, HL and SHW contributed to data collection. ZZ wrote the first draft of the manuscript. YX and ML revised the manuscript and were the guarantor of the

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Conflict of interest

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Serum IL-5 levels predict HBsAg seroclearance in patients treated with Nucleos(t)ide analogues combined with pegylated interferon

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Background: Knowing about cytokine profile contributes to clarify the underlying immune mechanism of HBsAg seroclearance rate increase. This study aims to investigate cytokine changes during nucleos(t)ide analogues (NAs) and peginterferon- α (Peg-IFN α) therapy and their impact on the HBsAg serologic response.

Methods: A total of 78 HBV DNA-negative chronic Hepatitis B (CHB) patients were studied after a lead-in phase of NAs with complete serum cytokines. Serum cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17 and TNF- α) were quantified by flow cytometry (FCM) every 24 weeks, before, during and at the end of NAs and Peg-IFN α treatment. Clinical and laboratory data were also taken at the same time. Analysis was performed between cured and uncured groups characterized by HBsAg seroclearance. PBMCs samples from five patients (two in cured group and three in uncured group) were analyzed by FCM.

Results: HBsAg seroclearance was achieved in 30 (38.5%) patients defined as the cured group. In comparison to uncured individuals, cured patients showed similar expressions of serum IL-1 β , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17 and TNF- α during the treatment of NAs and Peg-IFN α . Compared with the uncured groups, IL-5 was remarkably increased in cured patients. IL-5 at weeks 24 and 48 were associated with HBsAg seroconversion ($p=0.033$ and 0.027 , respectively). PBMCs sample analysis confirmed the predicted value of IL-5 in response to NAs and Peg-IFN α treatment.

Conclusions: IL-5 at weeks 24 and 48 might be used as a biomarker for HBsAg seroclearance in NAs-experienced CHB patients treated with NAs combined

with Peg-IFN α . More importantly, exploiting the expression of this cytokine may help to develop a better understanding of the immune pathogenesis of chronic HBV infection.

KEYWORDS

IL-5, HBsAg seroclearance, cytokines, pegylated interferon, nucleos(t)ide analogues

Introduction

Seroclearance of hepatitis B surface antigen (HBsAg) (“functional cure”) is regarded as the optimal endpoint of antiviral therapy for chronic hepatitis B virus (HBV) infection (1–3), which can stimulate host immune response to induce variable extent of liver injury. Recent studies have emphasized a higher rate of HBsAg loss (4–6) and lower incidence of HCC (7) among individuals treated with nucleos(t)ide analogues (NAs) and peginterferon- α (Peg-IFN α) combination therapy. HBeAg-negative individuals, with lower baseline HBV DNA and quantitative HBsAg levels, had higher rates of HBsAg seroclearance, suggesting that more patients in the inactive phase achieve functional cure (4, 8). Although the use of these two types of available approved treatments has resulted in a remarkable improvement in the rate of HBsAg seroclearance, they are still facing new issues such as immune tolerance/exhaustion and disease progression. It is vital to identify the factors to further improve clinical management.

The progression of chronic hepatitis B virus (CHB) infection is predominantly mediated by persistent intrahepatic immunopathology. Cytokines play an important role in initiating, maintaining, and regulating immunological homeostatic and inflammation of CHB infection. Interleukin (IL)-2 is associated with the evaluation of functions in T cells, especially in the HBV-specific T cells during the natural course of HBV infection, as well as its dynamics in response to the anti-HBV treatment (9, 10). Elevated INF- γ is accompanied by a decrease of IL-4 from the immune tolerance phase to the inactive carrier phase (11), indicating a shift from Th2 to Th1 responses. A sustained high level or dynamic elevated level of serum IL-6 indicates higher mortality in patients with HBV-acute-on-chronic liver failure (ACLF) (12). Furthermore, higher serum levels of IL-12 were accompanied by HBeAg or HBsAg seroconversion in CHB patients (13, 14).

In addition to the cytokines reviewed above, there are still many other cytokines that have directive implications in the possibility of designing immunotherapies for CHB patients. IL-5, mainly produced by T helper-2 (Th2) lymphocytes, can increase antibody secretion by promoting the differentiation and growth of B cells and enhancing the humoral immune response

mediated by Th2 cells. It was also associated with viral response and HBeAg seroconversion after entecavir (ETV) therapy in CHB patients, suggesting serum levels of IL-5 may be an available marker to predict responses to anti-HBV therapy (15). Here, we investigated the expression pattern of cytokines, especially IL-5, in patients with chronic HBV infection, and thereby assessed the relationship between cytokine expression and the effectiveness of NAs and Peg-IFN α combination therapy.

Methods

Patients

The study population recruited a total of 95 HBeAg-negative chronic hepatitis B patients from the third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China from March 2019 to May 2021. All procedures were conducted according to the principles of the Declaration of Helsinki, the protocol and consent forms were approved by the Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University. All participants provided written informed consent. Inclusion criteria included age between 18 and 65 years, NAs treated experience more than 1 year, treatment-naïve for interferon, and low hepatitis B surface antigen levels: HBsAg < 1500 IU/ml. The exclusion criterion for this study included advanced liver cirrhosis, presence of hepatocellular carcinoma or other malignancies, other causes of liver disease or mixed causes, human immunodeficiency virus co-infection, and known serious psycho-pathological disorders.

All patients received peg-interferon α -2b (Pegberon, Amoytop Biotech Co., Ltd., China.) treatment based on the chronic administration of NAs for 48 weeks. The course could be shortened if a patient showed HBsAg seroclearance (HBsAg < 0.05 IU/ML) and lasted more than 12 weeks during the follow-up. The patients who achieved HBsAg seroclearance during the course of treatment was defined as cured group. Conversely, if the patient had detectable serum HBsAg at the end of treatment (EOT), the given patient was attributed to the uncured group. Synchronous serum samples were collected before and during Peg-IFN α treatment at 12-week intervals

for 48 weeks. Blood sample (10 ml) from each patient was collected in the EDTA-treated tube. Plasma was collected after centrifugation, then peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. All samples were stored at -80°C and used for the present study.

Serological parameters

Blood routine indexes were assessed using an automated blood cell analyzer (Sysmex XN-3000, Kobe, Japan). Biochemical indexes of liver function were detected by an automatic biochemical analyzer (HITACHI 7600, Japan). HBeAg, HBeAb, HBcAb, and HBsAb level testing were performed using Roche Cobas E601 automatic electrochemiluminescence assay (Roche Diagnostics, IN, Germany). HBsAg was measured with Elecsys HBsAg II Quant reagent kits (Roche Diagnostics, Indianapolis, IN, USA). Serum HBV DNA was assayed with a Roche COBAS AmpliPrep/COBAS TaqMan HBV Test v2.0 (Roche Molecular Diagnostics, Branchburg, NJ, USA). These analyses were performed at the laboratory department of the third Affiliated Hospital of Sun Yat-Sen University and all procedures were performed following the manufacturer's instructions.

Cytokine assays

Serum samples of patients were collected by centrifugation of blood at 3200 rpm for 10 min and stored immediately at -80°C until use. Using FACS Calibur flow cytometer (BD, San Diego, USA), we detected 10 cytokines (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-17 and TNF- α) with the cytokine detection kit (multiple microsphere flow immunofluorescence method; Changsha Weimi Bio-Tech Co., Ltd., China) by following manufacturer's instructions.

Flow cytometry

Thawed PBMCs samples were taken from five patients (two in cured group and three in uncured group). A total of 15 blood samples were collected at 3 time points: day 0 (the day before), week 24, and week 48 of treatment. Samples were washed 3 times by 1x PBS with centrifugation at 400 g for 6 min and counted for cell numbers. 500,000 cells per tube were then stained for 30 min on ice with antibodies. For flow cytometric analyses, the following antibodies were used: CD3 (CD3-FITC; BioLegend, cat#300305), CD4 (CD4-APC-Cy7; BioLegend, cat#317418), CD8 (CD8-PerCP-Cy5.5; BioLegend, cat#344710), CD19 (CD19-APC; BioLegend, cat#302212), CD20 (CD20-PE; BioLegend, cat#302306), CD25 (CD25-APC; BioLegend, cat#302610), CD127 (CD127-PE; BioLegend, cat#351304), and PD-1 (PD-1-BV421; BioLegend,

cat#329920). Cells stained by antibodies were resuspended in 1x PBS, and analyzed by flow cytometry using BD LSR Fortessa cell analyzer. Flow cytometry data were analyzed using FlowJo (version 10.4.0). Accordingly, B cells and Treg cells were selected as CD19 $^{+}$ CD20 $^{+}$ and CD3 $^{+}$ CD4 $^{+}$ CD25 $^{+}$ CD127 $^{-}$ subsets, independent CD3 $^{+}$ clustering of either CD4 $^{+}$ or CD8 $^{+}$ T cells with PD-1 $^{+}$ used for identifying PD-1 expression respectively from lymphocytes. Flow cytometry gating strategies for CD3+CD4 $^{+}$ /CD3+CD8 $^{+}$ T cell populations see [Supplementary Figure 2](#).

Statistical analyses

Data were analyzed using SPSS (version 21.0 for Windows, Chicago, IL, USA). Continuous variables were shown as medians and interquartile ranges (IQR) unless stated otherwise. Mann-Whitney U test was used for the comparison among groups. The ROC curve was generated to analyze the predictive probability, and the area under the ROC curve (AUC) was calculated. All tests were two-tailed and *P* values of < 0.05 were considered to indicate a significant difference.

Results

Patient characteristics

Seventy-eight out of eighty-five eligible patients with complete serum cytokines were included in this analysis. The demographic and clinical characteristics of patients were shown in [Table 1](#). In all patients, 30 individuals (38.5%) achieved HBsAg seroclearance. The mean age in the cured group was 40.6 (36–45) years old, which was younger than that of the uncured group. There was a comparable gender distribution in both groups with which the majority were male 67 (85.9%): 26 (86.7%) in the cured group and 41 (85.4%) in the uncured group. Baseline HBsAg was significantly lower in the cured group (311.6 (39.0–485.3) than that in the uncured group (460.1 (185.2–741.3), $P = 0.031$). No significant differences in the levels of white blood cell (WBC) ($P = 0.077$), neutrophil ($P = 0.132$), hemoglobin ($P = 0.124$), platelets ($P = 0.204$), albumin ($P = 0.224$), ALT ($P = 0.305$), AST ($P = 0.486$) and total bilirubin (TBIL) ($P = 0.461$) were found between two groups.

Kinetics of cytokine expression in response to the combination therapy with NAs and Peg-IFN α

The kinetics of cytokine expression in two groups were shown in [Figure 1](#). The expressions of IL-1 β , IL-2, IL-4, IL-10 and IL-12 showed similar overall upward trends throughout the course of treatment in all patients, with no significant difference (IL-1 β :

TABLE 1 Baseline demographic and clinical characteristics of both groups.

Characteristics	All patients (N =78)	Cured (N =30)	Uncured (N =48)	<i>P</i> *
Age, mean median (IQR), years	42.3 (37-48)	40.6 (36-45)	44.3 (38-49)	0.042
Gender				
Male, n (%)	67 (85.9)	26 (86.7)	41 (85.4)	0.592
HBsAg, median (IQR), log10 IU/ml	402.9 (129.1-702.1)	311.6 (39.0-485.3)	460.1 (185.2-741.3)	0.031
Laboratory tests				
White blood cell count, median (IQR), 10 ⁹ /L	6.34 (5.53-7.11)	6.00 (5.10-6.67)	6.57 (5.81-7.37)	0.077
Neutrophil count median (IQR), %	3.57 (2.93-4.01)	3.36 (2.48-3.96)	3.71 (3.02-4.16)	0.132
Hemoglobin, median (IQR), g/L	155 (148-164)	151 (144-161)	157 (150-165)	0.124
Platelets, median (IQR), g/L	226 (185-262)	215 (169-254)	234 (197-263)	0.204
Albumin, median (IQR), g/dL	48.1 (46.8-49.8)	48.4 (47.5-49.7)	47.9 (46.3-49.8)	0.224
ALT, median (IQR), IU/L	26.9 (18.3-34.0)	28.0 (19.5-36.8)	26.2 (18.0-32.5)	0.305
AST, median (IQR), IU/L	23.9 (20.0-26.8)	23.6 (20.1-24.0)	24.1 (20.0-27.3)	0.486
Total bilirubin, median (IQR), μmol/L	14.3 (9.7-17.1)	15.2 (9.8-19.0)	13.7 (9.7-14.9)	0.461
*Comparisons were performed between these two groups using the Mann-Whitney U test.				

*P*0W = 0.307, *P*24W = 0.212, *P*48W = 0.082; IL-2: *P*0W = 0.979, *P*24W = 0.303, *P*48W = 0.124; IL-4: *P*0W = 0.938, *P*24W = 0.781, *P*48W = 0.118; IL-10: *P*0W = 0.845, *P*24W = 0.906, *P*48W = 0.579; IL-12: *P*0W = 0.508, *P*24W = 0.589, *P*48W = 0.459) (Figures 1A, B, C, G, H). The levels of IL-17 decreased at 24 weeks then increased at 48 weeks in the cured group while constantly elevated in the uncured group (IL-17: *P*0W = 0.497, *P*24W = 0.16, *P*48W = 0.126); (Figure 1I). IL-8 exhibited a downward trend before 24 weeks then increased at 48 weeks in both groups (IL-8: *P*0W = 0.704, *P*24W = 0.369, *P*48W = 0.479) (Figure 1F). The constantly elevated IL-6 expression was found in all patients before 24 weeks. Furthermore, IL-6 expression increased further until 48 weeks in the uncured group while it was decreased at 48 weeks in the cured group (IL-6: *P*0W = 0.244, *P*24W = 0.637, *P*48W = 0.246) (Figure 1E). In contrast to the persistently slow increase in the uncured group, TNF-α displayed a significant increase in the cured group from 24 weeks to 48 weeks (TNF-α: *P*0W = 0.618, *P*24W = 0.558, *P*48W = 0.059) (Figure 1J). IL-5 was notably increased in cured group during the whole course of treatment, while it decreased in the uncured patients at 24 weeks and then increased. Interestingly, the difference between the cured and the uncured groups was statistically significant (IL-5: *P*0W = 0.596, *P*24W = 0.033, *P*48W = 0.027) (Figure 1D).

Correlation between IL-5 expression and HBsAg seroclearance

We also investigated the relationship between cytokines expression and HBsAg seroclearance. Serum levels of IL-5

were associated with HBsAg seroconversion between both groups at 24 and 48 weeks of treatment (Figure 1D). We thus performed ROC curve analysis. The results of the diagnostic accuracy of IL-5 expression to predict the rate for HBsAg seroconversion were shown in Figure 2. The AUCs presented by IL-5 expression at 24 and 48 weeks were 0.644 (95% CI, 0.515-0.773) with *P* < 0.05 and 0.649 (95% CI, 0.517-0.781) with *P* < 0.05 respectively.

Immune cell subpopulations at different time points in response to the combination therapy of NAs and Peg-IFNα

As shown in Figure 3A, the proportion of CD4⁺ T cells were increased from the beginning of treatment to 24 weeks in the cured patients, then decreased to the basal level at 48 weeks, however, the uncured group showed an opposite trend, a decrease from 0 to 24 weeks after treatment, which was followed by a slow increase from 24 to 48 weeks. The similar patterns found in the CD8⁺ T cells were shown in Figure 3B. The proportion of CD8⁺ T cells changed less at 24 weeks after treatment in the cured group, and then increased from 24 to 48 weeks. Conversely, the uncured patients showed an elevation of CD8⁺ T cells within 24 weeks after therapy, which was decreased a little from 24 to 48 weeks.

The effects of B cell subsets among CHB patients following combination treatment were also examined and results were

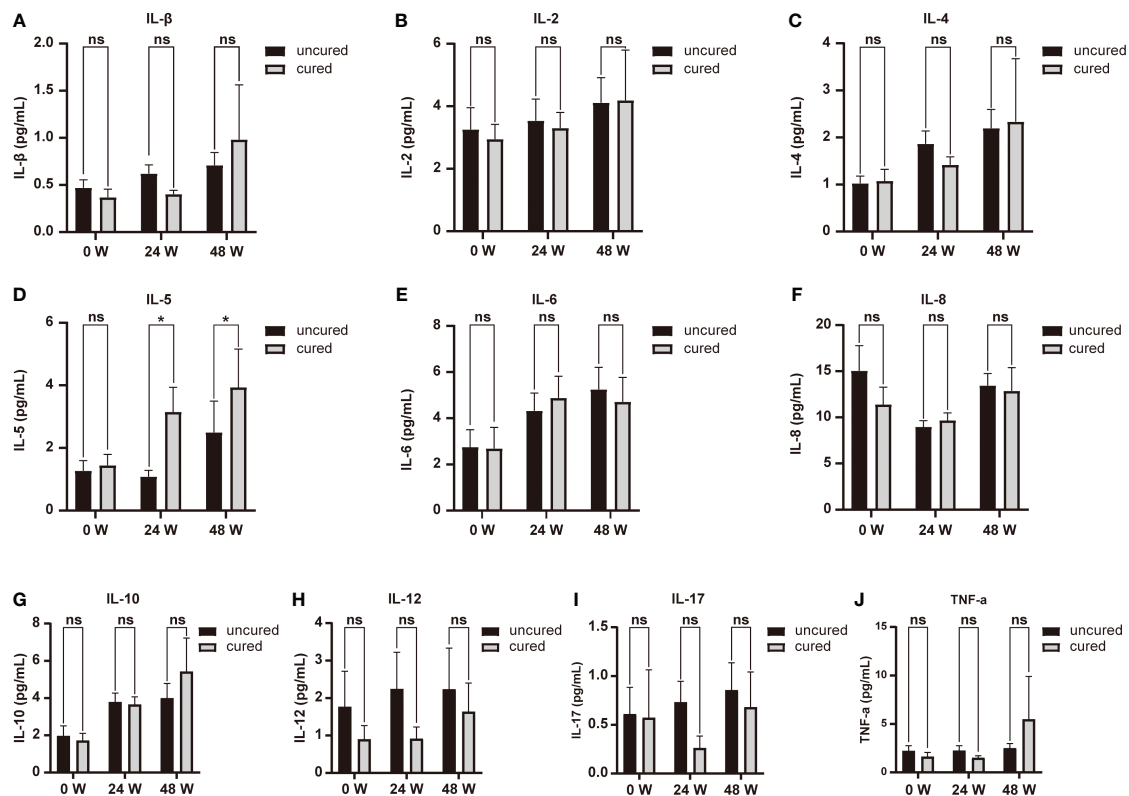


FIGURE 1

Serum cytokines expression during combination therapy with NAs and Peg-IFN α . The expressions of (A) IL-1 β , (B) IL-2, (C) IL-4, (D) IL-5, (E) IL-6, (F) IL-8, (G) IL-10, (H) IL-12, (I) IL-17 and (J) TNF- α were analyzed at 0W, 24W and 48W in both groups. Data were presented as the mean $\pm$ SEM. ns, no significant; *, $P < 0.05$; 0W, the start of treatment; 24W, 24 weeks of treatment; 48W, 48 weeks of treatment.

shown in Figure 4. In both groups, B cell subsets showed similar increases until 24 weeks of treatment, then further increased in the cured group while decreased in the uncured group at 48 weeks.

To explore the mechanisms underlying immunomodulation during combination treatment, we measured Treg population (Figure 5). The proportion of Treg cells in the uncured group was persistently elevated after treatment. In the cured group, this

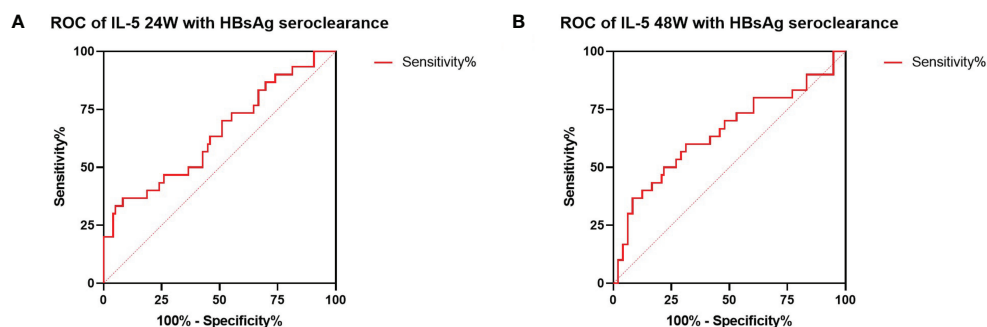


FIGURE 2

ROC analysis between IL-5 expression and HBsAg seroclearance. (A) ROC analysis showing the performance of IL-5 expression in distinguishing HBsAg seroclearance at 24W. AUC = 0.644 (95% CI, 0.515-0.773) ($P = 0.034$). (B) ROC analysis showing the performance of IL-5 expression in distinguishing HBsAg seroclearance at 48W. AUC = 0.649 (95% CI, 0.517-0.781) ($P = 0.028$). ROC, receiver operating characteristic; AUC, area under the curve; CI, confidence interval.

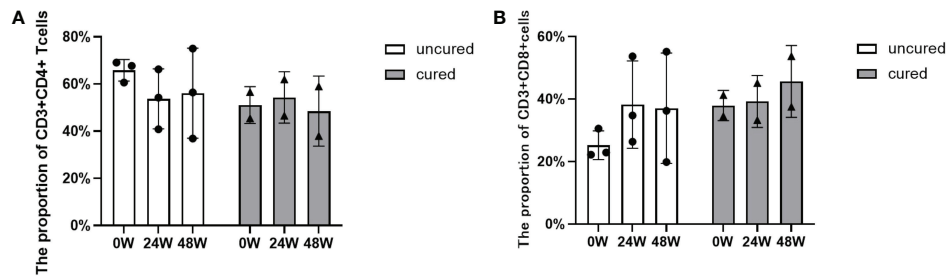


FIGURE 3

Dynamic changes of CD4⁺ T and CD8⁺ T cell subsets during combination therapy with NAs and Peg-IFN α . (A) The proportion of CD3⁺ CD4⁺ T cells at 0W, 24W and 48W. (B) The proportion of CD3⁺ CD8⁺ T cells at 0W, 24W and 48W. Data were presented as the mean $\pm$ SEM. 0W, the start of treatment; 24W, 24 weeks of treatment; 48W, 48 weeks of treatment.

proportion was increased until 24 weeks then decreased at 48 weeks.

In the end, we examined PD-1 expressions in T cells from available patients (Figure 6A). As a result, the expressions reached to similar levels in both groups at 24 weeks, however, at 48 weeks, PD-1 expressions were higher from both CD4⁺ T cells (Figure 6B) and CD8⁺ T cells (Figure 6C) in the uncured group compared to that in the cured group, especially obvious from CD8⁺ subsets (Figure 6C).

Discussion

In our recent study, we observed a high proportion of HBsAg seroclearance in NAs-experienced patients with HBV DNA negative treated with NAs and Peg-IFN α . Our study confirmed the efficacy of this combination therapy for reducing HBsAg level, especially in patients who received a 'switch to' combination therapy, which was consistent with the

findings from previous studies (16, 17). NAs and Peg-IFN α combination therapy has both directly antiviral and indirectly immunomodulatory effects. Peg-IFN α can inhibit HBV transcription and reduce the production of viral antigens by enhancing the degradation of HBV pregenomic RNA and core particles or by modifying the epigenetic regulation of cccDNA. It can purge cccDNA from the nucleus by inducing deaminases and the nuclease ISG20 that modify and subsequently degrade cccDNA (18). Our study involved patients with inhibited HBV DNA and low levels of HBsAg after treatment with NAs. NAs therapy would first lead to strong suppression of viremia, thereby assisting restoration of HBV-specific CD8⁺ T cells (19), Peg-IFN α can then be more effective in enhancing antigen presentation to the immune system, activation of natural killer (NK) cells and other immune cells, increasing the production of cytokines (20). Here, we first performed a longitudinal analysis of cytokines subsets in NAs-experienced CHB patients with low HBsAg levels undergoing NAs and Peg-IFN α combination therapy.

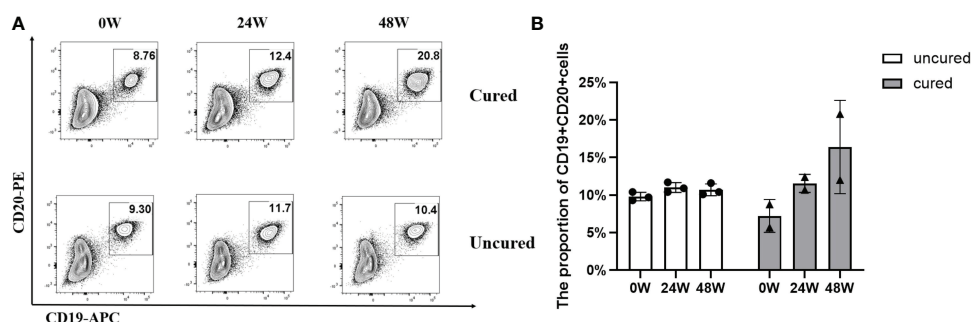


FIGURE 4

Frequency of B cells during combination therapy with NAs and Peg-IFN α . (A) Representative flow cytometric profiles showing the frequency of B cells (CD19⁺ CD20⁺) in PBMCs at different time points in both groups. APC, allophycocyanin; PE, phycoerythrin. (B) The proportion of CD19⁺ CD20⁺ T cells at 0W, 24W and 48W in both groups. Data were presented as the mean $\pm$ SEM. 0W, the start of treatment; 24W, 24 weeks of treatment; 48W, 48 weeks of treatment.

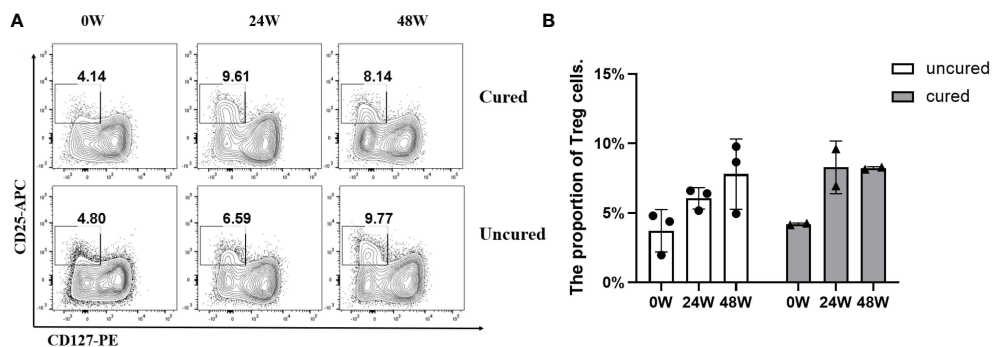


FIGURE 5

Frequency of Treg cells during combination therapy with NAs and Peg-IFN α . (A) Representative flow cytometric profiles showing the frequency of Treg cells in PBMCs at different time points in both groups. CD25⁺ CD127⁻ cells gated from CD3⁺ CD4⁺ cells were treated as Treg cells: CD3⁺ CD4⁺ CD25⁺ CD127⁻. APC, allophycocyanin; PE, phycoerythrin. (B) The proportion of Treg cells at 0W, 24W and 48W in both groups. Data were presented as the mean $\pm$ SEM. 0W, the start of treatment; 24W, 24 weeks of treatment; 48W, 48 weeks of treatment.

In this study, we screened the levels of 10 cytokines in patients under NAs and Peg-IFN α combination therapy and analyzed their associations with HBsAg seroclearance. The present study did not show any differences among these cytokines between the cured and uncured patients throughout the course of treatment, except for IL-5. Serum IL-5 levels increased remarkably in cured group throughout the course of treatment and had a positive correlation with HBsAg seroclearance. IL-5 was originally defined as a T-cell-derived cytokine, mainly produced by activated Th2 cells, that triggers

activated B-1 and B-2 cells for terminal differentiation into antibody-secreting plasma cells (21, 22). IL-5 receptor (IL-5R) comprises α and β c chains. IL-5 specifically binds to IL-5R α and induces the recruitment of β c to IL-5R α (23). B-1 cells constitutively express the IL-5R α and give rise to Ab-producing cells in response to IL-5 stimulation (24). The higher expression of IL-5 found in our patients is in line with the elevated trend of B cells in cured group.

It was reported that HBsAg level at 24 weeks can be used to predict HBsAg loss during Peg-IFN α therapy (25). The present

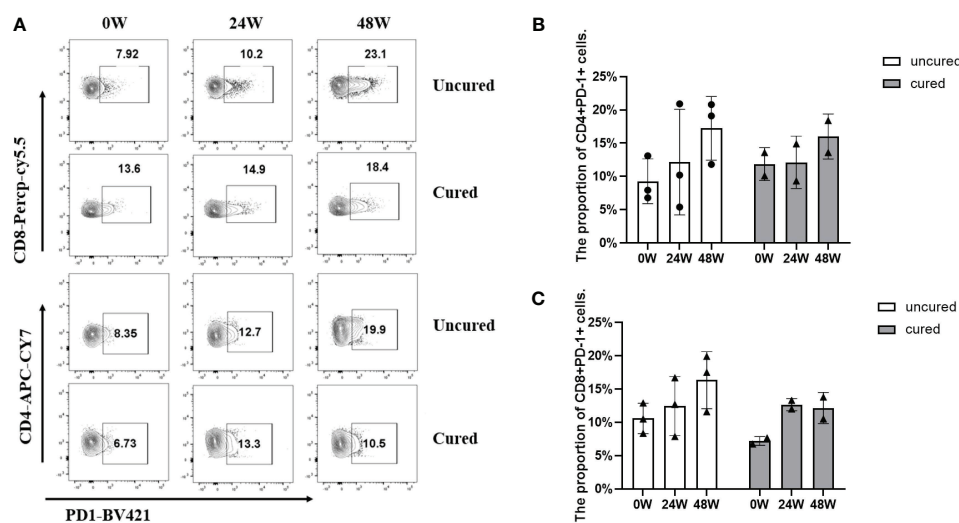


FIGURE 6

Frequency of CD4⁺ PD-1⁺ cells and CD8⁺ PD-1⁺ cells during combination therapy with NAs and Peg-IFN α . (A) Representative flow cytometric profiles showing the frequency of CD4⁺ PD-1⁺ cells and CD8⁺ PD-1⁺ cells in PBMCs at different time points in both groups. (B) The proportion of CD3⁺ CD4⁺ PD-1⁺ cells at 0W, 24W and 48W in both groups. (C) The proportion of CD3⁺ CD8⁺ PD-1⁺ cells at 0W, 24W and 48W in both groups. APC-Cy7, allophycocyanin-cyanine 7; PerCP-Cy5.5, Peridinin-Chlorophyll-Protein Complex-cyanine5.5; BV421, Brilliant VioletTM421. Data were presented as the mean $\pm$ SEM. 0W, the start of treatment; 24W, 24 weeks of treatment; 48W, 48 weeks of treatment.

study also showed the different change timepoint at 24 weeks between the cured and uncured groups. Treatment with Peg-IFN α led to increased expression levels of serum cytokine and immune cell subpopulations before 24 weeks. Our study involved patients with inhibited HBV DNA and low levels of HBsAg after treatment with NAs. Their immune functions were restored after NAs treatment, such as those of CD8 $^{+}$ T cells. Peg-IFN α can then be added to accelerate the decline of circulating and intrahepatic viral antigens and allow activating immune cells and inducing increased production of cytokines (20). Moreover, protracted antigenic stimulation may result in B-cell activation and differentiation to immunoglobulin-producing cells (IPC) (26), potentially leading to a rapid decrease of HBsAg before 24 weeks of treatment.

On the other hand, due to the hyperresponsiveness of immunosuppressive cells (27), the cellular immune response in chronic HBV infection is exhausted (28). Regulatory T cells (Tregs) act as primary immunosuppressive cells in chronic HBV infection and play a critical role in coordinating immunologic tolerance to both self and foreign antigens by inhibiting HBV-specific responses of CD4 $^{+}$ T cells and CD8 $^{+}$ T cells. Depletion of Tregs *in vivo* has been shown to enhance the functionality of HBV-specific CD8 $^{+}$ T cells and accelerate the clearance of HBsAg (29). In the current study, Tregs were also activated before 24 weeks, whereas its expression was downregulated in the cured patients and continued unregulated in the uncured patients after 24 weeks. In terms of the PD-1 CD4 $^{+}$ T cells and PD-1 CD8 $^{+}$ T cells observed in our study, the uncured patients had higher frequencies of PD-1 $^{+}$ CD4 $^{+}$ T cells after 24 weeks of treatment. Higher expression of PD-1 has been demonstrated to confer the exhaustion of HBV-specific CD4 $^{+}$ T cells and CD8 $^{+}$ T cells (30, 31). Blocking the interactions of PD-1 and its ligand PD-L1 *in vitro* has been shown to reverse the exhaustion of viral-specific T cells (32), restoring the functionality of viral-specific B cells (33) and Tregs (34). These findings are similar to the results of the current study. Therefore, we speculate that the cured patients demonstrate a better immune response to Peg-IFN α , especially the immunity activity of CD4 $^{+}$ T cell and B cells, inducing a more rapid increase of serum IL-5 and a decline of HBsAg. Meanwhile, the continued higher expression of Tregs in the uncured patients after 24 weeks of treatment may weaken the immune function of CD8 $^{+}$ T cells and B cells and therefore lead to a higher expression of HBsAg in some uncured patients.

To our knowledge, this is the first study to demonstrate the association between IL-5 and HBsAg seroclearance and provide new insights into the immune mechanisms of “functional cure” in NAs-experienced patients treated with NAs combined with Peg-IFN α . The regulated immune mechanisms involved in HBsAg seroclearance are complex. Due to the limited availability of PBMC samples, in addition to the dynamic change of B cells, CD4 $^{+}$ T cells, CD8 $^{+}$ T cells, and Treg cells during the course of treatment observed in our study, other

immune cell subpopulations, like DC, NK, and T Helper cell and B cell subsets, would be helpful to explore the immune mechanism. Future longitudinal studies are hence needed to address this issue.

In conclusion, we found that serum IL-5 levels were closely associated with HBsAg seroclearance in NAs-experienced CHB patients treated with NAs combined with Peg-IFN α . Serum IL-5 levels after 24 weeks of treatment could predict HBsAg seroclearance and response to combination therapy with NAs and Peg-IFN α .

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

QZ conceived of this study. QZ and PW performed the statistical analyses and wrote the manuscript. QZ, QT, and ZG supervised the writing process and revised the manuscript. PW and ZM contributed to the collection of data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.1104329/full#supplementary-material>

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Immune escaping of the novel genotypes of human respiratory syncytial virus based on gene sequence variation

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Purpose: Immune escaping from host herd immunity has been related to changes in viral genomic sequences. The study aimed to understand the diverse immune responses to different subtypes or genotypes of human respiratory syncytial virus (RSV) in pediatric patients.

Methods: The genomic sequences of different subtypes or RSV genotypes, isolated from Beijing patients, were sequenced and systematically analyzed. Specifically, the antiviral effects of Palivizumab and the cross-reactivity of human sera from RSV-positive patients to different subtypes or genotypes of RSV were determined. Then, the level of 38 cytokines and chemokines in respiratory and serum samples from RSV-positive patients was evaluated.

Results: The highest nucleotide and amino acid variations and the secondary and tertiary structure diversities among different subtypes or genotypes of RSV were found in G, especially for genotype ON1 with a 72bp-insertion compared to NA1 in subtype A, while more mutations of F protein were found in the NH-2 terminal, including the antigenic site II, the target of Palivizumab, containing one change N276S. Palivizumab inhibited subtype A with higher efficiency than subtype B and had stronger inhibitory effects on the reference strains than on isolated strains. However, RSV-positive sera had stronger inhibitory effects on the strains in the same subtypes or genotypes of RSV. The level of IFN- α 2, IL-1 α , and IL-1 β in respiratory specimens from patients with NA1 was lower than those with ON1, while there were higher TNF α , IFN γ , IL-1 α , and IL-1 β in the first serum samples from patients with ON1 compared to those with BA9 of subtype B.

Conclusions: Diverse host immune responses were correlated with differential subtypes and genotypes of RSV in pediatric patients, demonstrating the impact of viral genetics on host immunity.

KEYWORDS

human respiratory syncytial virus, viral genetic differences, antiviral effects, chemokines and cytokines, immune evasion

1 Introduction

Human orthopneumovirus, also called human respiratory syncytial virus (RSV), is a common respiratory virus that affects the lungs and respiratory tract, which can lead to hospitalization in severe cases. Importantly, it is the second largest pathogen that causes acute lower respiratory tract infection (ALRTI) deaths in infants and children under the age of 5 years (1). The global burden of virus-associated acute lower respiratory tract infections in 2019 linked RSV to 25.4 million to 44.6 million infections, 2.9 million to 4.6 million hospitalizations, and 15100 to 49100 deaths in hospitalized children under the age of 2 years (2). In infants, RSV infection is the most common cause of bronchiolitis and pneumonia (3). A clear health burden of RSV and high severity of RSV infection were found even in influenza season (4). Moreover, exacerbated inflammatory damage in the respiratory and nervous systems due to RSV infection was found in both pediatric and elderly populations (5, 6). Thus, RSV infection causes a heavy burden on public health and the social economy worldwide.

RSV belongs to the genus *Orthopneumovirus* of the *Pneumoviridae* family (7). It is a single-stranded and negative-sense RNA-enveloped virus with a 15.2 kb genome containing 10 genes that encode 11 proteins, including envelope spikes fusion protein (F), attachment protein (G), and small hydrophobic protein (SH), inner envelope proteins (matrix M, M2-1, and M2-2), non-structural proteins (NS1 and NS2) and ribonucleocapsid complexes phosphoprotein (P) and nucleoprotein (N). There is no proofreading mechanism for the RNA-dependent genome replication of RSV, which enables RSV to rapidly generate single nucleotide polymorphisms and a high rate of mutations (8).

G and F play very important roles in viral entry since they are the major glycoproteins on the surface of virions and thus may serve as ideal and effective binding targets when using neutralizing antibodies. RSV F and G are the only antigens that induce RSV-neutralizing antibody responses. Based on the antigenic differences of F and G, RSV can be divided into two subtypes: A (RSV-A) and B (RSV-B). Currently, many vaccines under development mostly target F since fusion glycoprotein F has a higher degree of conservation (>90%) among RSV strains (9), which is also more immunogenic and cross-protective than

glycoprotein G (10). The RSV-neutralizing monoclonal antibody, Palivizumab, appears to target the antigenic site II in the prefusion form of F (1). For G, there are two hypervariable regions, HVR1 and HVR2, of the extracellular region of the highly variable G, separated by a highly conserved region with 13 aa (aa 163–189) (11). RSV A and B subtypes can be further divided into various genotypes due to the high variability of the nucleotide sequences of HVR2. The novel genotypes ON1 (subtype A) and BA9 (subtype B) are becoming the dominant genotypes of RSV worldwide. These two novel genotypes harbor a 72- or 60-nucleotide duplicate insertion in HVR2 of the G gene (12–15).

The interaction and combination between host and viral genetics determine the outcomes of virus-induced disease. It has been shown that minimal changes in RSV and human rhinovirus in the viral genome can affect viral virulence and evade host immunity. These changes favor the evasion of antiviral agents and vaccines (16). To determine the impact of duplicate nucleotide insertions of novel genotypes ON1 and BA9 and other viral genetic changes in different subtypes or genotypes of RSV on host immunity, the genomic sequences of different subtypes/genotypes of RSV were obtained in Beijing, and the antiviral effects of F-specific monoclonal antibodies on RSV were evaluated. Human sera from RSV patients were collected to evaluate the cross-reactivity to different subtypes/genotypes of RSV strains. Finally, 38 chemokines and cytokines in respiratory specimens and sera from children with different subtypes/genotypes of RSV were compared and analyzed. Our results suggested that immune escaping of the novel genotypes of RSV existed, which may be explained by the viral genomic variation, including mutations in antigenic site II of F and duplicate nucleotide insertions of G.

2 Materials and methods

2.1 Virus stock preparation and measurement of median tissue culture infectious dose

Reference strains Long, A2 and CH18537 were obtained from the Chinese Center for Disease Control and Prevention. Clinical strains (69438, 61397, 3047, 69104, and 86673) were

isolated from nasopharyngeal aspirates (NPAs) collected from children diagnosed with lower respiratory tract infections and hospitalized in the Affiliated Children's Hospital of the Capital Institute of Pediatrics, Beijing, China, for respiratory virus screening using direct immunofluorescence (DFA) (Diagnostic Hybrids, Athens, OH, USA), and then were identified for the subtype and genotype of RSV by reverse-transcription polymerase amplification (RT-PCR) and sequence analysis (15).

RSV strains were propagated in Hep-2 cells (Chinese Academy of Medical Sciences & Peking Union Medical College). Cells were cultured in Modified Eagle Medium supplemented with 2% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL). When the 100% cytopathic effects of RSV strains on Hep-2 cells were observed, the cells were disrupted by freeze/thawing three times, followed by low-speed centrifugation (500 g for 10 min). Aliquots of supernatants were frozen at -70°C. The 50% tissue culture infective dose (TCID₅₀) of each RSV strain was determined by infecting Hep-2 cells with serial dilutions on a 96-well plate and calculated by the Reed-Muench method.

2.2 Clinical specimens

Nasopharyngeal swabs and serum samples were collected from pediatric patients who met the following criteria (1): diagnosed with “bronchitis”, “bronchiolitis”, or “pneumonia” (2); respiratory specimens positive for RSV tested by DFA (Diagnostic Hybrids, Athens, OH, USA), and further identified for subtype and genotype of RSV by sequence analysis (3, 15). had at least one serum sample: the first was collected on days 0 to 3, and the second was collected on days 5 to 7 after the onset of the disease. Meanwhile, serum samples from healthy children aged 0-5 years were collected as controls.

Nasopharyngeal swabs were pelleted by low-speed centrifugation (500 g for 10 min), then aliquots of clarified supernatants were frozen at -80°C. The sera collected were centrifuged at 500 g for 10 min, then stored at -80°C.

2.3 Amplification and sequencing of RSV genomic fragments

Total nucleic acid was extracted from 140 µL of each collected virus strain using the QIAamp MinElute Virus Spin Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. To amplify the RSV genomic sequences, 15 contigs covering the whole genome of RSV were amplified by high-fidelity long PCR using Platinum[®] Taq DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) and 15 pairs of primers previously described (17). Some were re-designed based on the genomic sequence of RSV A strain ATCC VR26 (AY911262) and RSV B strain (KF826843).

Primers were synthesized by Invitrogen Trading Co., Ltd. (Shanghai, China). Those primers are listed in Table S1. All PCR products were sequenced by Sino Geno Max Co., Ltd. (Beijing, China).

2.4 Bioinformatics analysis

The phylogenetic trees based on viral genomic or gene sequences were constructed using the neighbor-joining method and maximum composite likelihood model of the MEGA version 6.0 software package (18). ProtParam (<https://web.expasy.org/protparam/>) was used to predict the composition and molecular weight of the protein, NPS@SOPMA (<https://pbil.ibcp.fr/>), and five calculation methods (GOR, Levin, dual, PHD and CNRS SOPMA) were used for secondary structure prediction. For the construction of three-dimensional structures of G, F, and SH of RSV-NA1, RSV-ON1, and RSV-BA9, the online SWISS-MODEL service platform (<https://swissmodel.expasy.org/>), in which the tertiary structural models of RSV F were deposited, was used for F, and alpha fold 2.0 was used for G and SH for there was no tertiary structural model built. PyMOL 2.5.1 was used for visualizing and labeling the 3D model.

2.5 Micro-neutralization assays

The F-specific monoclonal antibody, Palivizumab (1mg/mL, received from National Institutes for Food and Drug Control), was diluted to 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, and 1:6400 with 2% fetal bovine serum of Modified Eagle Medium. The sera collected were also diluted to 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640. Viral strains were diluted to 100 TCID₅₀. Add 50 uL of diluted virus and Palivizumab or sera to each 96-well plate, followed by 100 uL of 3×10⁵/mL cell suspension. Cells were routinely propagated in a humidified atmosphere of 5% CO₂ at 33°C, the cytopathic changes were observed daily, and the results were recorded until the fifth day. IC₅₀ was calculated by the Reed-Muench method to indicate the antiviral effect of Palivizumab.

2.6 Microarray analysis

38 cytokines and chemokines in respiratory or serum samples were measured using MILLIPLEX[®] Human Cytokine/Chemokine Magnetic Bead Panel (MERCK, Germany) on the liquid phase micro-array analysis platform (model Luminex100; Luminex, Austin, TX, USA). These included: Fibroblast Growth Factor-2 (FGF-2), Interferon -alpha 2 (IFN-α2), Interferon γ (IFN-γ), Interleukin-10 (IL-10), Interleukin-12 (IL-12), Interleukin-12p40 (IL-12p40), Interleukin-12p70 (IL-12p70), Interleukin-13 (IL-13), Interleukin-15 (IL-15), Interleukin-17A

(IL-17A), IL-1 receptor antagonist (IL-1RA), Interleukin-1alpha (IL-1 α), Interleukin-1 beta (IL-1 β), Interleukin-2 (IL-2), Interleukin-3 (IL-3), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-6 (IL-6), Interleukin-7 (IL-7), Interleukin-8 (IL-8), Interleukin-9 (IL-9), Interferon-inducible protein 10 (IP-10), Monocyte chemotactic protein 1 (MCP-1), Monocyte chemotactic protein 3 (MCP-3), Macrophage-derived chemokine (MDC), Macrophage inflammatory protein-1 alpha (MIP-1 α), Macrophage inflammatory protein-1 beta (MIP-1 β), Transforming growth factor-alpha (TGF- α), Tumor Necrosis Factor-alpha (TNF- α), Tumor Necrosis Factor-beta (TNF- β), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF), Fms-like tyrosine kinase 3 ligand (Flt-3L), Granulocyte colony-stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), Growth Factor (GRO), Eotaxin, and Fractalkine. All samples were repeated in duplicate, and the concentrations were demonstrated as a unit of pg/mL. The plates were analyzed using Luminex xPONENT (Luminex), and the results were analyzed with the Luminex SYNCT Data Analysis Software (Luminex).

2.7 Statistical analysis

The average values and 95% CI of the cytokines and chemokines in duplicate samples for each child were calculated by SPSS. The values below the detection limit were assigned a 0 pg/mL concentration. The chi-square (χ^2) test and rank sum test were used for statistical analyses by SPSS Statistics 22.0 version (IBM, Armonk, NY, USA). For specimens with small sample sizes, “unpaired” in “Experimental design”, “Use nonparametric test in “Assume Gaussian distribution”, then “Mann Whitney test” in “Choose test” was chosen. *P*-values under 0.05 were considered statistically significant. Spearman's rank correlation coefficient in SPSS was used to analyze the relationship between two related variables. GraphPad Prism 8.0.1 was used for data visualization.

3 Results

3.1 Genetic differences in RSV

Two subtypes and several genotypes of RSV were identified by phylogenetic trees based on the HVR2 region of G gene sequences: subtype A (69438-NA1, 61397-ON1, 3047-GA2, Long-GA1, A2-GA1), and subtype B (CH18537-GB2, 86673-BA9, and 69104-BA9) (Figure S1). The nucleotide homologies of genomic sequences in this study were 80.5–81.3% between subtypes A and B and >95% within the same subtype (Table S2).

For the 11 RSV genes, 66.2–86.2% homology in nucleotide and 46.7–96.4% homology in aa were obtained between subtypes

A and B. The highest nucleotide and amino acid variabilities were found in G (66.2–68.5% in nucleotides and 46.7–51.7% in aa), followed by M2-2 (68.6–70.6% in nucleotides and 57.1–60.7% in aa) and SH (76.9–81.0% in nucleotide and 67.2–70.3% in aa).

For protein F, 81.7–82.8% in nucleotide and 89–90.6% in amino acid homology were calculated between subtypes A and B, with more mutations found in the NH-2 terminal of F (Figure S2). Antigenic site II of F (aa 254–277), as the target of Palivizumab, contains one amino acid change N276S, with N in strains long, A2, and 3047 (GA2) and S in strains 61397 (ON1), 69438 (NA1), CH18537 (GB2), 86673 (BA9), and 69104 (BA9). There were several changes in antigenic site ϕ , including D220N, K201N, and K209Q from subtype A to subtype B, and G66E/K (G in long, K in A2, and E in others). There were two changes on the antigenic site V, L172Q (L in subtype A, Q in subtype B) and S173 L (S in subtype A, L in subtype B).

Isolated strains 61397 (ON1) and 69438 (NA1) shared 99% homology in nucleotide and 99.5% in aa in the F region, and 92.3% in nucleotide and 95.6% in aa in G. Strain 61397 (ON1) had a 72-nucleotide (nt) insertion in the HVR2 of G compared with 69438 (NA1) (Figure S3).

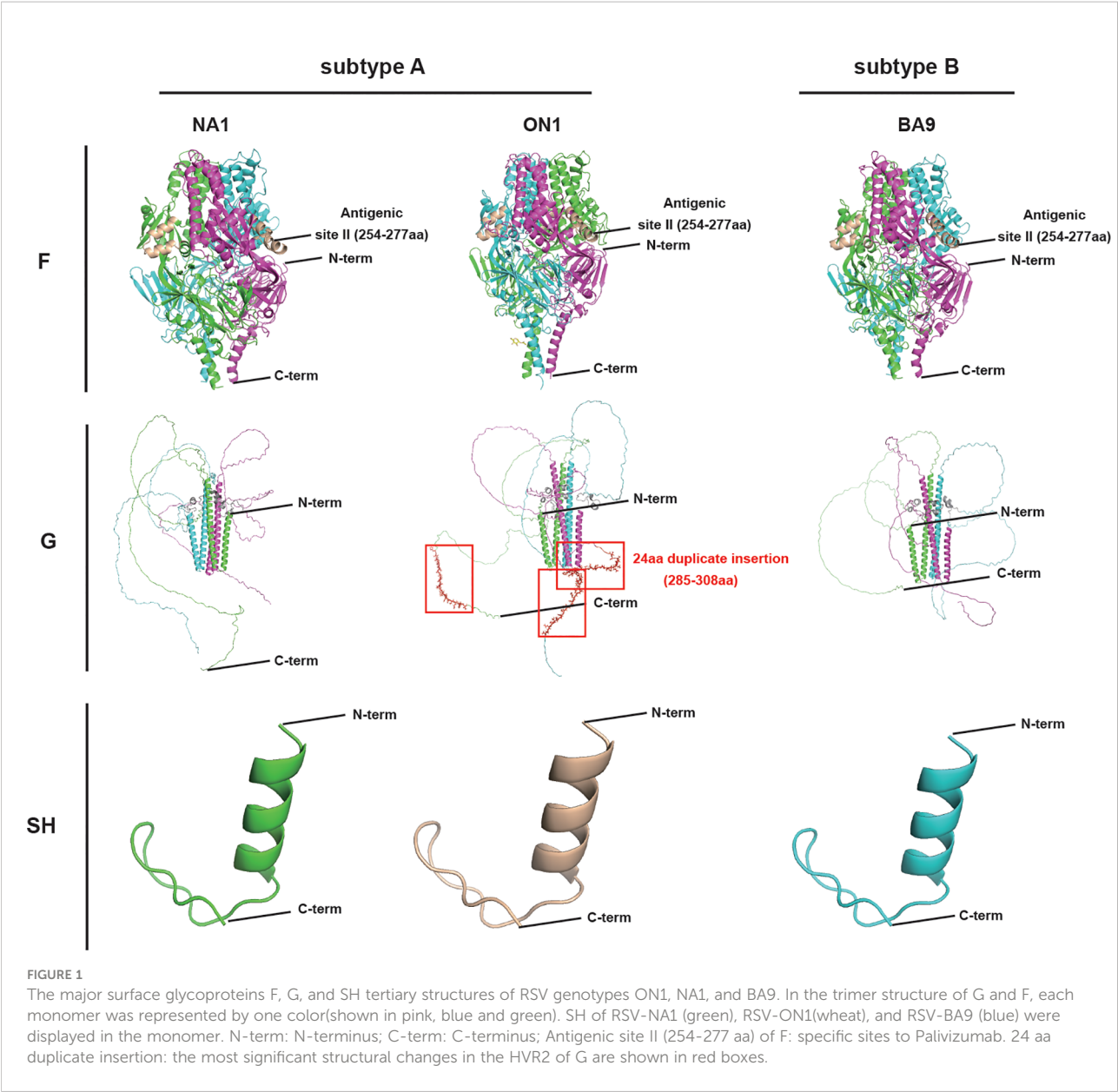
For strains 69104 (BA9) and 86673 (BA9), a 60nt duplicate insertion was found in the HVR2 of G (Figure S4). A 72-nt-insertion in strain 61397 (ON1) and a 60-nt-insertion in strains 69104 (BA9) and 86673 (BA9) in G genes increased the number of aa and molecular weight (Table S3).

The secondary structures of SH and F were an α -helix, accounting for 42.19% (SH) and 38.15% (F) in genotypes ON1 and NA1 and 60% (SH) and 39.2% (F) in genotype BA9. The secondary structure of G is an irregular curl, accounting for 55.14%, 56.23%, and 64.63% in genotypes ON1, NA1, and BA9, respectively (Figure S5).

The results of tertiary structure prediction and three-dimensional (3D) model construction revealed a high similarity in SH between subtypes A and B, whereas a higher similarity in F was found between genotypes ON1 and BA9 than that between ON1 and NA1. However, G's highest diversity was found among genotypes ON1, NA1, and BA9 (Figure 1).

3.2 Antiviral effects of Palivizumab to RSV *in vitro*

Palivizumab is an RSV-specific monoclonal antibody, the specific inhibitor targeting the antigenic site II of RSV F. To compare the differences in immunoreactivity of different subtypes or genotypes of RSV to Palivizumab, we evaluated the neutralization effect of Palivizumab on RSV by micro-neutralization assays. 100 TCID₅₀ was the virus titer used in the procedure, and Palivizumab was diluted from the maximum non-toxic concentration of 1:200. As shown in Table 1, the IC₅₀



of Palivizumab to subtype A reference strain Long (GA1) (0.234 $\mu\text{g/mL}$) was the lowest, followed by the subtype B reference strain CH18537 (GB2) (0.469 $\mu\text{g/mL}$). In addition, among the local strains of subtype A, the IC_{50} of Palivizumab to 69438 (NA1) (1.875 $\mu\text{g/mL}$) was significantly higher than that to 61397 (ON1) (0.938 $\mu\text{g/mL}$) and 3047 (GA2) (0.938 $\mu\text{g/mL}$).

3.3 Cross-reactivity of human sera to RSV

To further determine whether there are differences in the antigenicity of different subtypes or genotypes of RSV, we performed cross-reactivity assays using human sera from

TABLE 1 The IC_{50} ($\mu\text{g/mL}$) of Palivizumab to different subtypes and genotypes of hRSV strains.

Antibody	IC_{50} to strains of subtype A				IC_{50} to strains of subtype B		Average of IC_{50}	
	Long (GA1)	61397 (ON1)	69438 (NA1)	3047 (GA2)	69104 (BA9)	CH18537 (GB2)	subtype A	subtype B
Palivizumab	0.234	0.938	1.875	0.938	1.875	0.469	0.996	1.172

different subtypes or genotype RSV-positive patients. As shown in **Table 2**, serum from a patient positive for RSV ON1 (93158) showed strong antiviral ability against the subtype A and B reference strains, as well as the subtype A isolated strains ($IC_{50} \leq 1:198$) but weak antiviral activity against the subtype B isolated strain 86673 (BA9) ($IC_{50} 1:52$). In addition, the antiviral activities of serum from patient positive for RSV NA1 (22190) against subtype A and B reference strains were similar ($IC_{50} 1:123$), but the antiviral activity against subtype A isolate was significantly better than that of the subtype B isolated strain 86673 (BA9) ($IC_{50} 1:87$). Meanwhile, serum from patient positive for RSV BA9 (92991) showed stronger antiviral activity to subtype B than to subtype A, with the strongest antiviral activity to isolated strain 86673 (BA9) ($IC_{50} 1:276$), and the weakest activity to isolated strain 93158 (ON1) ($IC_{50} 1:60$).

3.4 Diverse immune responses to different subtypes or genotypes of RSV *in vivo*

To evaluate the differences in immune responses to different subtypes or genotypes of RSV, respiratory and serum samples were collected from enrolled patients positive for RSV according to the criteria. There were 40 respiratory specimens genotyped to ON1, BA9, or NA1, including 15 genotyped to ON1, with 15 serum samples collected within 0–3 days (the first serum group) and 14 within 5–7 days after the onset of disease (the second serum group), 17 genotyped to BA9 with 17 serum samples collected within 0–3 days (the first serum group) and 8 within 5–7 days (the second serum group) after the onset of disease, and 18 genotyped to NA1 with no serum samples. In addition, serum samples were collected from 12 healthy children as a control.

The values of cytokines and chemokines in the respiratory specimens from patients were shown in **Figure 2** of the heat map, in which GM-CSF, GRO, IL-1RA, IL-8, IFN- γ -induced IP-10, and MCP-1 were widely detected in all patients. The values of cytokines, GM-CSF, and the soluble cluster of differentiation 40 ligands (sCD40L), markers of innate immunity, IFN- α 2, IL-1 α ,

IL-1 β , IL-2, IL-12p40 and IL-12p70, indicators of the T helper 1 (Th1) cell response, IL-4, IL-5, and IL-13, indicators of the Th2 response, and IL-17A, an indicator of the Th17 response, in patients infected by RSV NA1 were lower than those with ON1 or BA9. Significant differences ($t=-2.136$, $P<0.05$) were shown in the level of IL-12p40 between patients with NA1 and ON1 and IFN- α 2 ($t=-2.091$, $P<0.05$), IFN- γ ($t=-2.013$, $P<0.05$), IL-12p70 ($t=-2.179$, $P<0.05$), IL-2 ($t=-2.339$, $P<0.05$), IL-4 ($t=-0.386$, $P<0.05$), IL-9 ($t=-1.96$, $P<0.05$), IL-17A ($t=-1.995$, $P<0.05$), and FMS-like tyrosine kinase 3 ligand (Flt-3l) ($t=-0.296$, $P<0.05$) between patients with NA1 and BA9 (**Figure 2**).

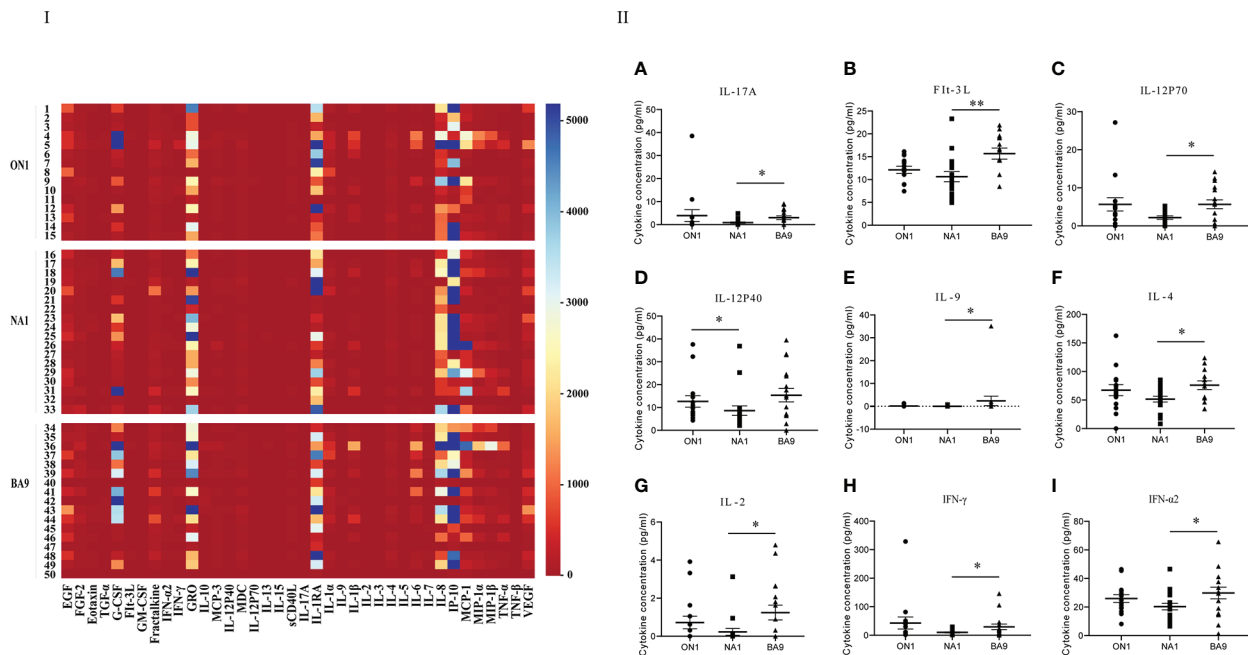
In the first serum group collected from patients of days 0 to 3 after the onset of the disease, GM-CSF, GRO, IL-1RA, IL-8, IP-10, and MCP-1 were different among patients shown in **Figure 3** of the heat map. In the scatter plot, only eotaxin ($t=-0.86$, $P<0.05$) and MDC ($t=-3.342$, $P<0.05$) were significantly different between patients with ON1 and those with BA9 (**Figure 3**). The eotaxin level in healthy children was significantly higher than in patients with ON1 but not significantly different from those with BA9. However, the level of MDC was significantly lower in both ON1 and BA9 groups than in healthy children.

For the second serum group collected from patients on days 5 to 7 after the onset of the disease, the levels of GRO, sCD40L, IP-10, and MCP-1 differed among patients (**Figure 4**). As shown in the scatter plot (**Figure 4**), the levels of cytokines sCD40L ($t=-2.358$, $P<0.05$), IL-7 ($t=-1.644$, $P<0.05$), MIP-1 β ($t=-2.862$, $P<0.05$), GM-CSF ($t=-2.188$, $P<0.05$), eotaxin ($t=-2.03$, $P<0.05$), Flt-3L ($t=-2.257$, $P<0.05$), IL-2 ($t=-2.151$, $P<0.05$), TNF- α ($t=-2.389$, $P<0.05$), IL-1 β ($t=-2.114$, $P<0.05$), and IL-3 ($t=-2.237$, $P<0.05$) were lower in patients with ON1 than those with BA9. GM-CSF, IL-2, IL-3, and Flt-3L levels did not differ significantly between healthy children and RSV-infected individuals. Eotaxin, IL-7, MIP-1 β , and TNF- α levels were lower in RSV-infected individuals than in healthy children, but the level of sCD40L in patients with ON1 was significantly higher than that in healthy children.

Between the first and second groups of serum samples, the levels of fibroblast growth factor-2 (FGF-2) ($t=-1.485$, $P<0.05$), Flt-3L ($t=-2.25$, $P<0.05$), and MIP-1 β ($t=-0.493$, $P<0.05$) were

TABLE 2 The IC_{50} of sera from patients with different genotypes of hRSV strains.

Serum	IC_{50} to strains of subtype A					IC_{50} to strains of subtype B		Average of IC_{50}	
	Long (GA1)	A2 (GA1)	61397 (ON1)	3047 (GA2)	5540 (NA1)	86673 (BA9)	CH18537 (GB2)	subtype A	subtype B
93158 (ON1)	1:241	1:110	1:417	1:247	1:198	1:316	1:52	1:204	1:129
22190 (NA1)	1:123	1:178	1:251	1:115	1:468	1:87	1:123	1:173	1:105
92991 (BA9)	1:117	1:132	1:60	1:132	1:96	1:276	1:263	1:98	1:269



significantly different between patients with ON1 and BA9 (Figure S6), whereas the levels of cytokines GM-CSF ($t = -2.102$, $P < 0.05$), IL-2 ($t = -2.193$, $P < 0.05$), TNF- α ($t = -2.155$, $P < 0.05$), and IL-17A ($t = -2.136$, $P < 0.05$) were lower in the first group of serum samples compared to the second group in patients with BA9 (Figure S7). Positive correlations were found between IFN α and IL-12p70 ($rs = 0.650$, $P < 0.05$), IFN γ and IL-12p40 ($rs = 0.332$, $P < 0.05$), IL-17A and IL-6 ($rs = 0.208$, $P < 0.05$), IL-17A and IL-8 ($rs = 0.277$, $P < 0.05$), eotaxin and IL-5 ($rs = 0.325$, $P < 0.05$), and eotaxin and fractalkine ($rs = 0.033$, $P < 0.05$) (Figure S8).

4 Discussion

RSV has been recognized as the major viral agent causing lower respiratory tract infections (LRTIs) in infants and young children worldwide. The spatial folding, post-transcriptional modification, and antigenic processing of proteins in viral epitopes and regions beyond epitopes were altered due to genetic variation, and the structural and spatial conformation determined the immunity of B and T cells induced by viral infection. Therefore, immune cells that react with one

conformation of a protein fragment may not be able to recognize the altered conformation of a similar fragment of the protein in the virus (19). The frequent emergence of novel genotypes of RSV may be the main consequence of immune evasion, which may be related to the severity of the disease, outbreak, and repeated infections.

The highest difference in viral nucleotide found between subtype A and B was shown in the G gene (66.2–68.5%), followed by the SH and F genes, which might explain the differences in the secondary and tertiary structures found in G, SH, and F proteins, in which G protein with an irregular curl and low bond energy is more prone to a conformational change, leading to highly variable immunogenicity (20). Furthermore, Genotypes ON1 and NA1 of RSV-A had similar 3D modeling structures in F and SH proteins but significant differences in G protein among genotypes ON1, NA1, and BA9 (RSV-B) (Figure 1). Accumulating evidence showed that RSV glycoprotein G has important immune modulatory effects during RSV infection, which may induce an exacerbated Th2 type cytokine expression and affect the antiviral response through the modulation of perforin and granzyme B expression (21). In addition, antibodies induced by the G protein are mainly involved in inhibiting viral

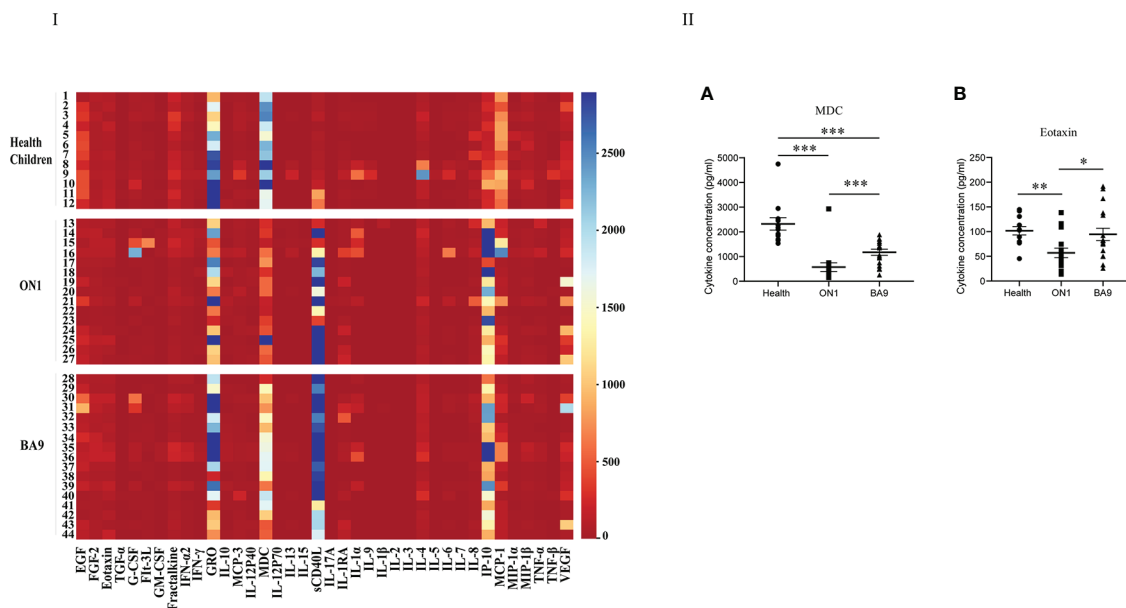


FIGURE 3
Comparison of cytokines and chemokines among the first serum samples from patients infected with genotype ON1, BA9 of RSV, or healthy children. I: the heat map. Each small square in the map represented the specific value (pg/mL) of the cytokines and chemokines of each child. Different colors indicated the range of values of cytokines. For example, purple indicated the highest value, and red was the lowest. II: Significant differences were shown in the level of cytokines and chemokines (A: MDC; B: Eotaxin) among the first serum group from patients infected by genotype ON1, BA9 of RSV, or healthy children. Each spot in the map represented the value (pg/mL) of the cytokines and chemokines of each child. ●: samples from healthy children; ■: samples from patients infected by genotype ON1; ▲: samples from patients infected by genotype BA9; *: significant differences were shown between the two groups ($P < 0.05$). **: significant differences were shown between the two groups ($P < 0.01$). ***: significant differences were shown between the two groups ($P < 0.001$).

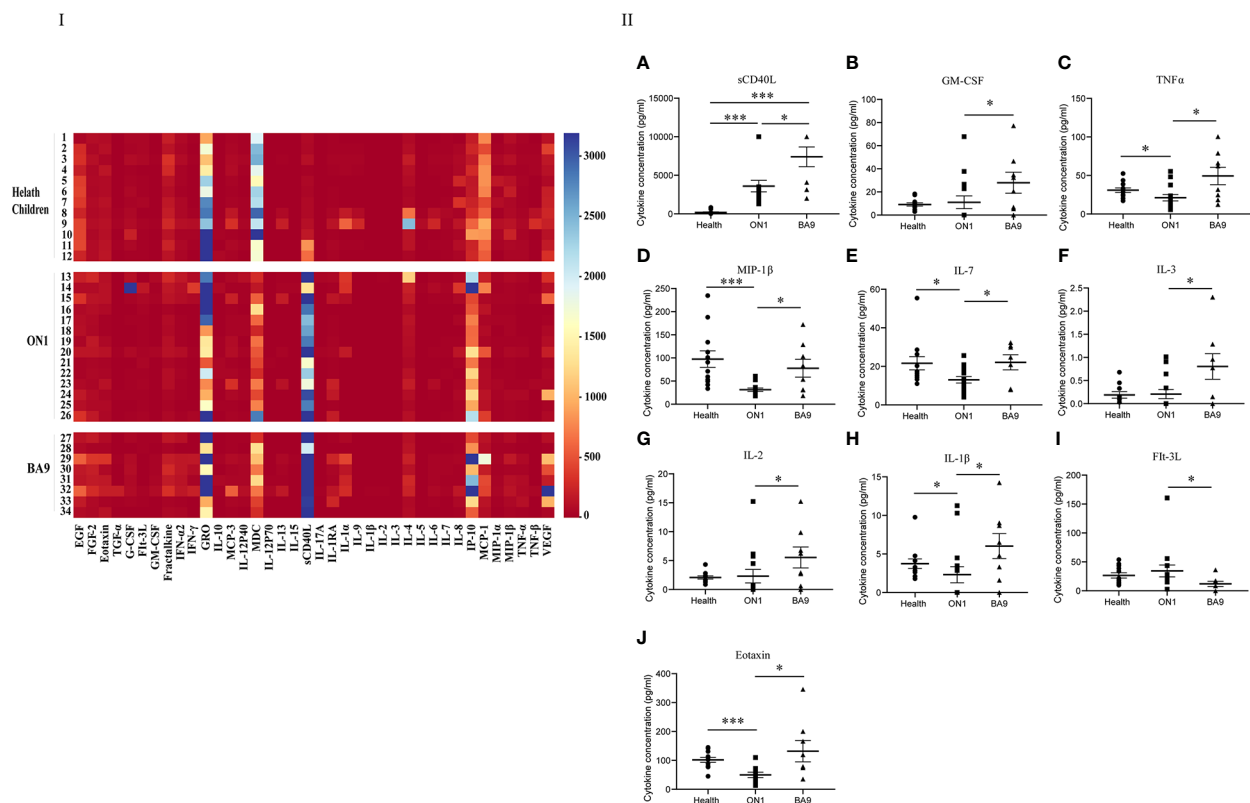
replication and in immune modulation by interfering with the antibody-mediated neutralization response. The differences in G protein sequence and structure among different RSV genotypes indicate the differences in immune responses elicited by RSV *in vivo*, which may be a potential mechanism of viral immune evasion.

In recent years, ON1 with a 72nt-insertion has gradually become the dominant genotype of subtype A, while BA9 with a 60nt-insertion is the dominant genotype of subtype B (15, 22), suggesting that the G protein with the highest structural variation might be a marker of immune escaping from herd immunity (15, 23). Zhang et al. found 10 positive gene selection loci in subtype A and 4 in subtype B when the analysis of RSV strains was collected in Beijing from 2006 to 2016 (15). Most of these loci were within HVR2 of the G gene. Another specific positive selective locus, 274 of the ON1 genotype, was found to be related to the immune evasion of RSV (24). Pretorius et al. postulated that the widespread duplication insertion mutations in ON1 and BA9 might result from immune pressure (25). Thus, under positive selection pressure, the dominant NA1 genotype is eventually replaced by ON1 in subtype A of RSV (20, 25).

For F protein is the major target protein for neutralizing antibodies induced by natural infection, it has been shown that the variability of F protein enables viral escaping from

neutralization by anti-RSV monoclonal antibodies (26). Indeed, Palivizumab has decreased RSV viral loads and RSV-associated immunopathology in mice (27, 28). Our results showed that Palivizumab exerted inhibitory effects on all subtypes or genotypes of RSV. However, Palivizumab had stronger inhibitory effects on the reference strain than on the isolated strains, suggesting that the mutations of isolated strains may evade the antiviral effects of Palivizumab. The results that strain 61397-ON1 (IC_{50} 0.938 μ g/mL) was more sensitive to Palivizumab than 69438-NA1 (IC_{50} 1.875 μ g/mL) revealed that the immune evade of ON1 was not solely dependent on mutations at antigenic site II of the F protein, for mutation N276S at antigenic site II was with N in strains long, A2, and 3047 (GA2) and S in strains 61397 (ON1), 69438 (NA1), CH18537 (GB2), 86673 (BA9), and 69104 (BA9). Therefore, although F proteins are relatively conserved among different subtypes and genotypes, minor differences in nucleotides still exist in the region, which might account for some differences in the efficiency of the host immune responses (29).

In this study, cross-reactions were found between subtypes and genotypes of RSV. However, the immune response to RSV was subtype or genotype-specific. Serum from patient 22190, positive for RSV NA1, exhibited the strongest inhibition to strain 5540 (NA1) (IC_{50} 1:468) and the weakest inhibition to 86673



(BA9) (IC_{50} , 1:87). The serum from patient 93158 was positive for ON1 had the strongest inhibition to the strain 61397 (ON1) (IC_{50} , 1:417), and the weakest activity to CH18537 (GB2) (IC_{50} , 1:129). The serum from the patient (92991 positives for BA9) had the strongest inhibition to strain 86673 (BA9) (IC_{50} , 1:276) and the weakest inhibition to the 61397 (ON1) (IC_{50} , 1:60). These results indicate that those differences of inhibition might be induced by gene mutations of G, other than F which were conserved among subtypes and genotypes, of clinical RSV strains (30, 31).

The upper respiratory tract is the initial spot where the innate immune response to RSV infection (32) is induced by immune cells recruited to recognize pathogens and produce cytokines, such as eotaxin, FGF-2, and fractalkine. The final elimination of the virus depends on both innate and subsequent adaptive immune responses. Specifically, during infection, $CD4^+$ T lymphocytes can be activated by toll-like receptor signaling pathways. Other signaling pathways induce Th1, Th2, and Th17

or regulatory T cells (Tregs) to drive immune responses (33). The level of cytokines was different from sample to sample, and they had different expression patterns. Almost all cytokines and chemokines correlate positively with disease severity in RSV infection. High levels of chemokines secreted from the airway epithelium were found in this study, which might be involved in the recruitment and activation of immune cells (31), leading to varying degrees of immune response and subsequently affecting the severity of the disease.

Surfactant viral protein genes, host cell receptor genes, and Th1/Th2 genes are involved in RSV infection (34). In RSV infections, Th2-biased immunity is related to the enhanced severity of respiratory diseases (35), although some studies have revealed an imbalance of Th17/Treg responses (36). A balance between Th1 and Th2 immune responses is required for effective viral clearance (37). The levels of Th1 cytokines (IFN- α 2, IL-1 α , and IL-1 β) in respiratory specimens in patients with NA1 were lower than those with ON1, which explained that

children with NA1 are more likely to have longer hospitalization and a more frequent incidence of severe disease than those with ON1 (38). The higher expression of Th1 cytokines such as TNF α , IFN γ , IL-1 α , and IL-1 β in the first group of serum samples from patients with ON1 compared to those with BA9 may explain why patients with ON1 are more likely to show symptoms of fever (39). Those patients with ON1 who had lower Th1 cytokines IL-2 and TNF α in the second group serum samples may explain a more rapid recovery than those with BA9.

IL-3 and IL-33 are associated with the prognostic value of RSV infections; their overexpression suggests enhancing inflammatory response (37). IL-3 and IL-33 indicate the severity of the disease to a certain extent. Lower levels of IL-3 in respiratory specimens and the first and second group of serum samples in patients with ON1 compared to BA9 were found in this study, suggesting that patients with ON1 are more likely to have that milder disease (38).

During infection, the secretion of many cytokines shows two peaks: the early stage (days 1–3) and the late stage (days 6–8). The cytokines produced by T cells in the second peak may lead to the aggravation of RSV infection (38). There were significant differences in FGF-2, Flt-3L, and MIP-1 β between the first and second serum samples in patients with ON1. These data suggest that innate immunity is the first line of defense in the early stage of infection. Lower Th1 and Th17 reactions in the first serum samples of patients with BA9 suggest that adaptive immunity is involved in the later stage of infection.

It has been suggested that the F protein of RSV mainly induces Th1-type responses and secretes IL-12 to induce IFN- α production, thereby facilitating Th1 cell differentiation (40), which is consistent with our data showing that IFN α and IL-12p70 are positively correlated. IL-12 and IFN- γ can promote the differentiation of Th cells into Th1 cell subsets. Antigen-presenting cells can secrete IL-12 after stimulation by IFN- γ and CD40L, which can induce natural killer cells to secrete a larger amount of IFN- γ , consistent with our data showing that IFN γ and IL-12p40 are positively correlated. IFN- γ can further enhance the expression of T-bet, which promotes the expression of IFN- γ itself in a positive feedback manner; this so-called “waterfall effect” can further promote the differentiation of T cells towards Th1 (41). In addition, there is also a positive correlation between eotaxin and fractalkine.

RSV G protein can induce CD4⁺ T cells, basophils, and monocytes to secrete Th2 cytokines (42). The later Th2 response can produce fractalkine and guide IL-5-activated eosinophils to traffic to the lungs, leading to the infiltration of eosinophils (43). Mice with RSV infection can produce Th2 cytokines (IL-4, IL-5, and IL-13), leading to airway remodeling and mucus overproduction (44, 45). It has been suggested that Th2 and Th17 responses induce allergic asthma by enhancing airway hyperresponsiveness, airway remodeling, and mucus production (20), which can promote the progression of RSV infectious diseases. IL-8, IFN- α , IL-6, TSLP, IL-3, and IL-33 are associated with the severity of RSV infections (37).

This study found a significant positive correlation between IL-17A and IL-6. IL-6 can activate Th17 lymphocytes by activating Th0 cells through the signal transducer and activator of the transcription 3 pathway. Th17 lymphocytes can recruit neutrophils and promote the development of lymphoid structures in the lungs, thereby leading to serious autoimmune inflammation (20). A significant positive correlation between IL-17A and immune cells was found in this study, which might result from the cross-talk and cooperation among IL-17, IL-1 β , and TNF- α . Specifically, IL-17 may act on stromal cells to recruit neutrophils and promote the proliferation of fibroblasts and epithelial cells, among others. Then those cells may secrete inflammatory cytokines such as FGF-2, VEGF, GM-CSF, IL-8, eotaxin, MIP-1 α , and MIP-1 β to promote inflammation (46).

There were several limitations in this study. Due to the absence of NA1 strains in recent years, NA1 strains in this study were only collected from retrospective research from 2014 to 2015. Secondly, for the small sample size of clinical specimens used in the comparison of cytokines and chemokines, only “unpaired” in “Experimental design”, and “Use nonparametric test in “Assume Gaussian distribution” were used in statistical analysis. More data should be accumulated. However, the data collected in the study will be helpful for future studies when novel genotypes appear.

In conclusion, the immunology results indicated that the monoclonal antibody Palivizumab, specific to antigen site II of F, had stronger inhibition to subtype A than to subtype B and to reference strains than to isolated strains in Beijing. In contrast, RSV-positive human sera had higher inhibitory effects on the same origin subtype or genotype of RSV. The inductions of innate and adaptive immunity were diverse among different subtypes or genotypes of RSV, with higher adaptive responses in patients infected with genotype ON1. All these results may be explained by the viral genomic variation, including mutations in antigenic site II of F and the duplicate nucleotide insertions of G. The duplication insertions were found in ON1 and BA9. G protein had the highest variation, leading to substantial conformational and major immunogenicity changes. These results might explain why reinfection of RSV occurs and why there are differences in severity related to the subtype and genotype of RSV. These results provide useful references for developing antibodies, vaccines, and therapeutic drugs to treat RSV.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Capital Institute of Pediatrics (SHERLLM2015012). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

LZ conceived and designed the experiments. XZ, MJ, FJW, and YX performed the experiments. LZ, YQ, and RD analyzed the data. XZ, FW, YS, and RZ handled clinical samples and isolated viruses. XZ, MJ, F(J)W, YX, and RD wrote the paper. LZ and RD reviewed and revised the manuscript. QS, DQ, LC, and LM collected clinical data and samples. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.1084139/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Maximum likelihood phylogenetic trees of the HVR2 region in RSV G, in which subtypes A and B and different genotypes of RSV were identified. Sequences obtained from the study were labeled with black dots. Others were sequences downloaded from GenBank.

SUPPLEMENTARY FIGURE 2

Homology and variability analysis of nucleotide and amino acid shown in A and B, respectively, between subtype A and B; in C and D, respectively, between genotype ON1 and NA1 of RSV-A.

SUPPLEMENTARY FIGURE 3

72nt-insertions in HVR2 of G in 61397 (ON1) of RSV.

SUPPLEMENTARY FIGURE 4

60nt-insertions in HVR2 of G in 86673 (BA9) and 69104 (BA9) of RSV

SUPPLEMENTARY FIGURE 5

Secondary structures of surface glycoproteins of RSV ON1, NA1 and BA9, including SH of 61397-ON1 (A), 69438-NA1 (B), 86673-BA9 (C), G of 61397-ON1 (D), 69438-NA1 (E), 86673-BA9 (F), and F of 61397-ON1 (G), 69438-NA1 (H), and 86673-BA9 (I).

SUPPLEMENTARY FIGURE 6

Maximum likelihood phylogenetic trees of the HVR2 region in RSV G for the subtyping of respiratory specimens collected to evaluate immune response. Sequences obtained from the study were labeled with black dots. Others were sequences downloaded from GenBank.

SUPPLEMENTARY FIGURE 7

Cytokines showed a significant difference between the first and second serum groups from patients positive for RSV ON1.

SUPPLEMENTARY FIGURE 8

Cytokines showed a significant difference between the first and second serum groups from patients positive for RSV BA9.

SUPPLEMENTARY FIGURE 9

Cytokines showed positive pairwise correlations among patients with RSV.

SUPPLEMENTARY TABLE 1

Primers designed for genomic sequencing of RSV.

SUPPLEMENTARY TABLE 2

Homology and divergence analysis of whole genomic sequences from isolates of RSV.

SUPPLEMENTARY TABLE 3

Physicochemical properties of F, G, and SH of RSV dominant genotypes ON1, NA1, and BA9 were analyzed by ProtParam.

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Diagnostic and prognostic value of *STAP1* and *AHNAK* methylation in peripheral blood immune cells for HBV-related hepatopathy

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Introduction: Although we had identified that the methylation of *AHNAK* was a good diagnostic marker for hepatopathy, here we speculate that there was also another marker, *STAP1*, whose methylation also involved in the detection of hepatopathy.

Methods: We investigated the methylation levels of the *AHNAK* and *STAP1* in peripheral blood mononuclear cells of chronic hepatitis B (CHB) patients, compensatory liver cirrhosis (CLC) patients, decompensated liver cirrhosis (DCLC) patients, hepatocellular carcinoma (HCC) patients and healthy controls by methylation-specific PCR. We also evaluated the differences and changes of methylation and expression of *AHNAK* and *STAP1* at different stages of liver disease using the TCGA and GEO public datasets.

Results: Methylation level of *STAP1* in PBMC was positively correlated with the course of liver cancer. The combination of *AHNAK* and *STAP1* methylation was able to predict different HBV related hepatopathy. The GEO datasets also supported that the methylation of *AHNAK* and *STAP1* was associated with different types of hepatopathy. The TCGA data showed that the levels of methylation and expression of *STAP1* were down-regulated in HCC. We also found the *STAP1* methylation level in PBMC and T cells was associated with age, gender, alcohol drinking and anti-HBe. Hyper-methylation of *STAP1* was correlated with the poor prognosis of patients but its expression had no association.

Conclusion: We concluded that combination of *AHNAK* and *STAP1* methylation in peripheral blood immune cells can be used as a diagnostic marker for HBV related hepatopathy and *STAP1* methylation may be a potential prognostic marker for HBV related HCC. Our clinical study registration number was ChiCTR2000039860.

KEYWORDS

STAP1, *AHNAK*, DNA methylation, hepatopathy, diagnosis, prognosis, HBV

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common neoplasm and the third most common cause of cancer-related deaths in the world (1–3). Almost all patients with liver cirrhosis (4) and a large portion of those with chronic liver disease eventually progress to liver fibrosis and HCC (5). Because the majority of people with liver cirrhosis are asymptomatic and because HCC patients frequently come with hepatitis and HCC symptoms at an advanced stage, the effectiveness of treatment is frequently limited (6, 7). Therefore, finding efficient biomarkers for early diagnosis detection, therapy evaluation, and prognosis prediction of liver cancer is therefore very valuable.

Emerging evidence suggests that many genetic and epigenetic abnormalities are involved in the development of HCC (8). DNA methylation is recognized as one of the first epigenetic modifications discovered, which can regulate gene expression by influencing the chromatin structure, DNA conformation, DNA stability, and DNA–protein action mode (9). A growing number of studies have shown that DNA methylation can be used as a diagnostic and prognostic molecular marker for multiple diseases (10–12). Our previous study has reported that the promoter methylation level of *AHNAK* gene in peripheral blood mononuclear cells (PBMC) was associated with the increase of the severity of liver diseases, and *AHNAK* can be employed as an early diagnostic marker for HCC (13). However, a few patients did not conform to the *AHNAK*-based rule. In addition, our previous research also showed that *STAP1* methylation in PBMC is also associated with the progression of hepatitis B virus (HBV)-related liver diseases. Therefore, in this study, we collected new patient samples to investigate the correlation between the methylation of another gene, *STAP1*, as well as *AHNAK*, and the progression of hepatitis and HCC and to assess the diagnostic effect of the combination using the genes *STAP1* and *AHNAK*.

The protein *STAP1* is a substrate of tyrosine-protein kinase Tec, participating in a positive feedback loop by upregulating the activity of tyrosine-protein kinase Tec. Although *STAP1* is reported to be involved in B cell receptor signaling (14) and in the prognosis gene signature of lung adenocarcinoma (15), the role of *STAP1* in HCC is not clear. We here used methylation-specific polymerase chain reaction (MSP) to study the *STAP1* and *AHNAK* methylation level in hepatitis B patients and its correlation to the progress of HCC from BCLC stage 0 to stage C. We also explored the association of methylation level and expression level of *STAP1* and *AHNAK* to the different stages of HCC and survival of patients using several public datasets. The current study aimed to explore the diagnostic and prognostic value of *STAP1* methylation in liver cirrhosis and HCC combined with *AHNAK*.

Materials and methods

Patients and collection of samples

A total of 302 HBV-infected patients in Beijing YouAn Hospital, Capital Medical University were recruited during June 2014 to August 2020, including 46 cases of chronic hepatitis B (CHB), 46 cases of compensatory liver cirrhosis (CLC), 53 cases of decompensated liver cirrhosis (DCLC), 157 cases of HCC (stage 0 = 34, stage A = 42, stage B = 19, and stage C = 62). In total, 22 healthy volunteers were recruited as controls during a routine physical examination, and none of them had hepatobiliary diseases before the peripheral blood sample collection. The whole PBMC suspension was prepared by taking 3–5 ml peripheral blood of the patients into EDTA anticoagulant tubes. T cells were also isolated from the PBMC by using the magnetic activated cell sorter (MACS[®]) T-cell isolation kit following the manufacturer's instructions. All the participants signed informed consents, and the study was approved by the medical ethics committee of Beijing YouAn Hospital (BJYACE-201721).

DNA extraction, bisulfite modification, and MSP

Genomic DNA was extracted from PBMC or T cells of patients and healthy controls using the QIAamp DNA mini kit (Qiagen NV, Venlo, The Netherlands) in line with the manufacturer's protocol. The quality and the quantity of the isolated DNA were measured by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). The bisulfite treatment of genomic DNA was processed using an EpiTect Fast DNA Bisulfite Kit (Qiagen NV). The bisulfite-treated DNA samples were next kept at -20°C for further use. After analyzing a 2,000-bp region upstream of the transcription start site (*AHNAK* or *STAP1* promoter region), one CpG island was identified (Figure 1A). With bisulfite-treated DNA as the template, the methylation pattern in the CpG island within *AHNAK* or *STAP1* promoter was measured by MSP, and the MSP primers are presented in Supplementary Table S1. The MSP primers were designed according to the principle described previously (16). The reaction condition for MSP was 10 min at 95°C, followed by 40 cycles of 30 s at 94°C and 30 s at 72°C, and then 5 min of extension at 72°C. After having been separated on 1.5% agarose gels, the MSP products were then stained by ethidium bromide and visualized under a UV spectrophotometer. Water blanks served as the negative control. We obtained the methylation levels of *AHNAK* or *STAP1* in PBMC or T cells.

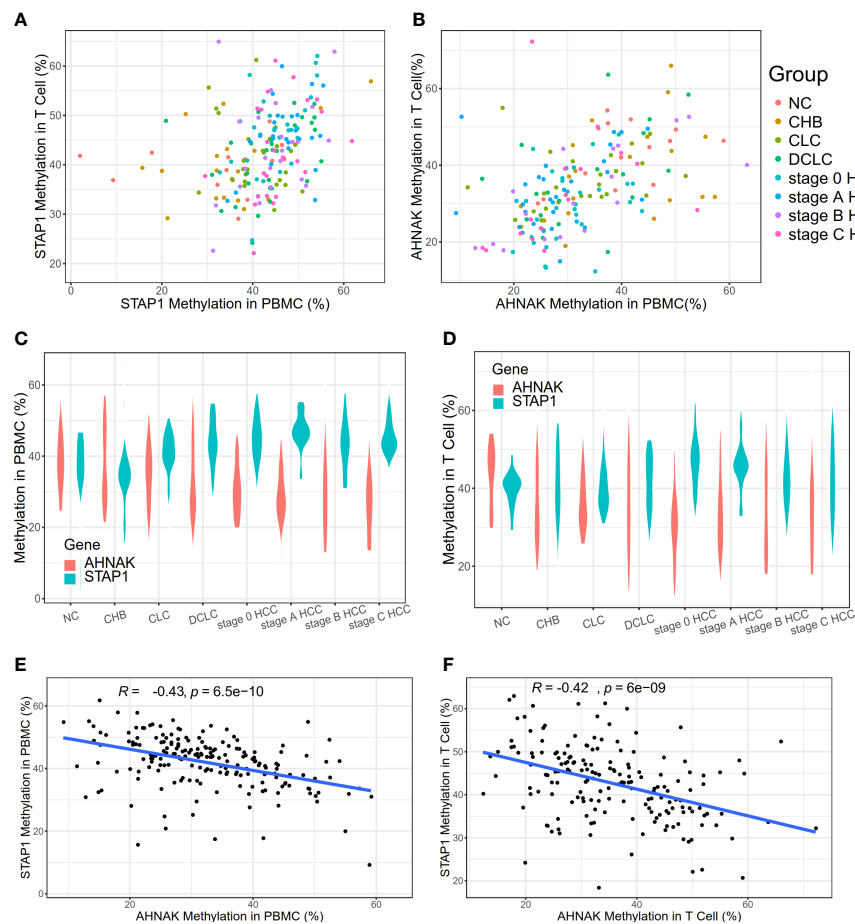


FIGURE 1

Methylation level of *AHNK* and *STAP1* in peripheral blood mononuclear cells (PBMC) and T cells in different liver diseases. (A) Methylation levels of *STAP1* in PBMC and T cells. (B) Methylation levels of *AHNK* in PBMC and T cells. (C) Methylation levels of *AHNK* and *STAP1* in PBMC at different disease stages. (D) Methylation level of *AHNK* and *STAP1* in T cells at different disease stages. (E, F) Negative correlation of methylation of *AHNK* and *STAP1* in PBMC and T cells.

Public datasets used

The expression and methylation data of HCC in the LIHC project in The Cancer Genome Atlas (TCGA) (17) was used to confirm the correlation of *AHNK* and *STAP1* with the HCC and the overall survival of patients. DNA methylation data from the Gene Expression Omnibus (GEO) database were employed to investigate the methylation profile of *AHNK* and *STAP1* in the different types of liver cirrhosis or HCC: (1) methylation profiling of 1,204 HCC patients, 392 patients with CHB or liver cirrhosis, and 958 healthy individuals and patients with benign liver lesions (GSE112679); (2) methylation profiling of 34 healthy liver tissues and 122 primary liver disease tissues arising in the setting of chronic HBV or C viral infection, alcoholism (EtOH) (GSE60753); and (3) methylation profiling

of 48 HBsAg carriers who developed HCC and 48 HBsAg carriers without HCC during follow-up (GSE78904). The normalized data of those GEO datasets were downloaded through the GEOquery package in R.

Statistical analysis

All 302 HBV-infected patients in Beijing YouAn Hospital were included in the statistical analysis. Student's *t*-test and one-way analysis of variance (ANOVA) were used to compare the differences of *AHNK* or *STAP1* methylation between groups. Spearman's correlation was carried out to compare the difference of continuous variables. Linear regression was utilized to compare the difference of discrete variables. Correlations

between the methylation levels of *STAP1* or *AHNAK* and the clinical characteristics (including ages, sex, alcohol drinking, and anti-HBeAb) were determined *via* Spearman rank correlation analysis. Using the data of patients with information of overall survival (OS), we investigated the association of methylation or expression of *STAP1* as well as *AHNAK* to the patients' OS. We grouped the patients into uppers and lowers according to the median of methylation or the expression level of *STAP1* or *AHNAK*. Kaplan–Meier curves with log-rank test were used for the overall survival. Statistical significance was defined as two-tailed $P < 0.05$ for all analyses. To assess the diagnostic value of *STAP1* and *AHNAK* methylation levels as markers for CHB, CLC, DCLC, or HCC, respectively, we performed receiver operating characteristic (ROC) analysis based on their methylation levels and used the area under the curve (AUC) as an assessment of diagnostic accuracy. The ROC curve analysis was performed using SPSS19.0 statistical software. Other data were analyzed using statistical software R3.1.1 or GraphPad Prism version 6.0.

Results

The methylation level of *STAP1* in PBMC was positively correlated with the course of liver cancer, while *AHNAK* had the reverse correlation

We found out that there was no significant difference in the methylation level of *STAP1* or *AHNAK* between the PBMC and T cells (Figures 1A, B). The *STAP1* methylation level was positively correlated with the severity of the liver disease not only in the PBMC but also the T cells, while *AHNAK* showed a negative correlation (Figures 1C, D). Thus, there was a reverse correlation between *AHNAK* and *STAP1* in the PBMC and T cells (Figures 1E, F). The ANOVA test suggested that the methylation levels of *AHNAK* and *STAP1* both showed significant differences among different groups whether in the PBMC or T cells (Table 1). A further two-paired comparison of *STAP1* methylation levels demonstrated that there existed

significant differences between NC and DCLC and HCC (stage 0, A, B, C), between CHB and DCLC and HCC, and between CLC and DCLC, stage 0 HCC, and stage C HCC in the PBMC. There were significant differences between NC, CHB, CLC, and HCC stage 0 and HCC stage A in the T cells (Supplementary Tables S2–S5).

Diagnostic value of *STAP1* and *AHNAK* methylation level in patients with different liver diseases

To further verify whether *STAP1* or *AHNAK* methylation in PBMC can be used as an indicator for the diagnosis of liver diseases, the ROC curve was drawn for the analysis (Figure 2). The *STAP1* and *AHNAK* methylation levels were not highly specific for identifying CHB patients. *STAP1* methylation was also not specific for predicting patients with CLC (AUC = 0.65 in PBMC and AUC = 0.54 in T cells); however, *AHNAK* methylation in T cells was predictive of CLC (AUC = 0.7). The methylation of either *STAP1* or *AHNAK* in PBMC may be predictive of DCLC, with AUCs of 0.74 and 0.70, respectively. Either *STAP1* or *AHNAK* demonstrated pretty high specificity in predicting HCC, where the AUC of methylation of either *STAP1* or *AHNAK* in PBMC was mostly greater than 0.70.

Methylation level of *AHNAK* and *STAP1* also showed significant differences among different types of liver diseases in the public datasets

In order to confirm the association of *STAP1* and *AHNAK* with liver diseases, we further analyzed the *AHNAK* and *STAP1* methylation levels in the different types of liver cirrhosis or HCC using the GEO datasets. In the GSE60753 data, there was a significant difference in *STAP1* methylation level among liver cirrhosis and HCC (Kruskal–Wallis test, $p = 1.2 \times 10^{-6}$) (Figure 3A). In the GSE112679 data, the methylation level of *STAP1* was also significantly higher than in healthy controls and

TABLE 1 *AHNAK* and *STAP1* methylation level among different groups in peripheral blood mononuclear cells (PBMC) and T cells.

Gene	Sample	df	Sum square	Mean square	F-value	Pr (>F)	Significance
<i>STAP1</i>	PBMC	7	3,527	503.8	7.928	1.34E-08	***
	T cell	7	1,141	163.04	2.387	0.0229	*
<i>AHNAK</i>	PBMC	7	2,932	418.9	4.251	0.000204	***
	T cell	7	2,005	286.4	2.37	0.0239	*

* $P < 0.05$, *** $P < 0.001$.

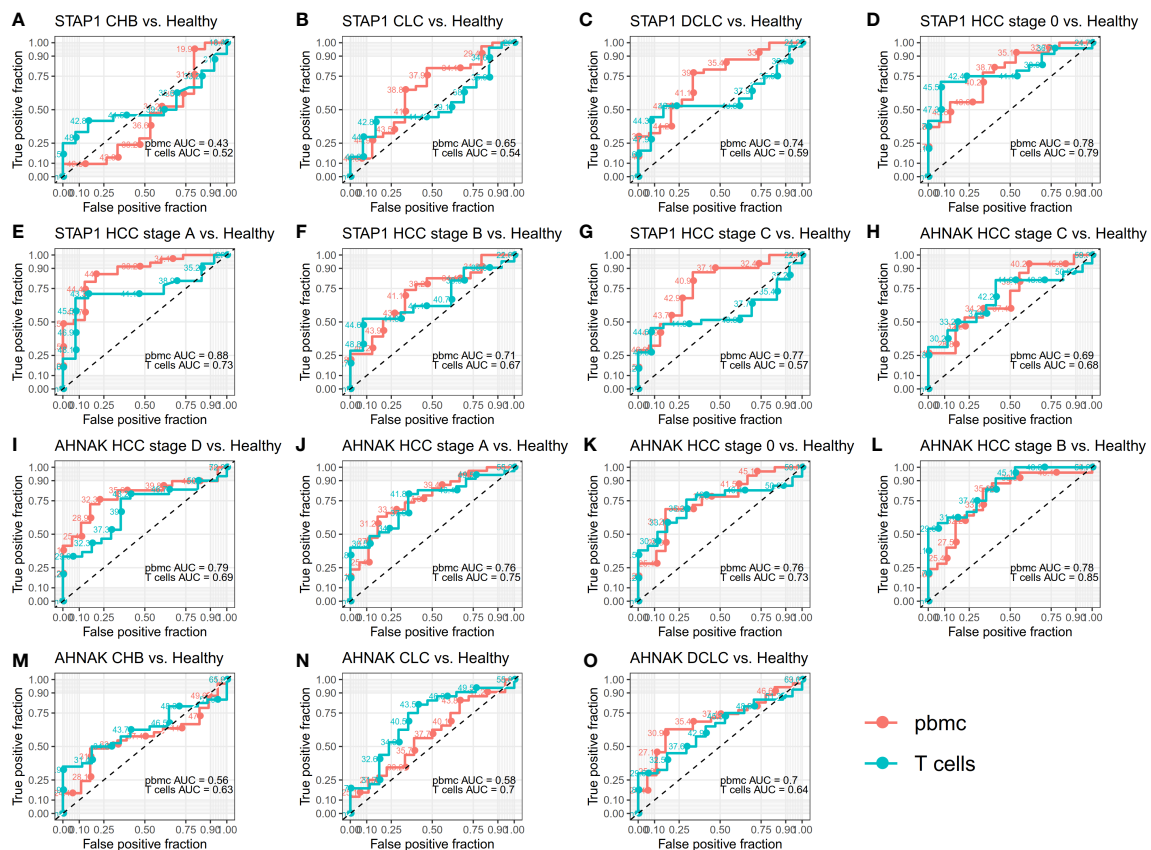


FIGURE 2

Receiver operating characteristic (ROC) curves for whether *STAP1* or *AHNAK* methylation in peripheral blood mononuclear cells (PBMC) can be used as an indicator for the diagnosis of liver disease. (A–G) ROC curves for methylation of *STAP1* in PBMC and T cells. (H–O), ROC curves for methylation of *AHNAK* in PBMC and T cells.

CHB and HCC patients (Figure 3B). In the GSE112679 data, *AHNAK* also showed a significantly high methylation level in liver cirrhosis than other types, and it was significantly higher in HCC compared with benign liver lesions and CHB (Figure 3C).

Lower methylation level and expression level of *STAP1* and *AHNAK* in liver cancer tissues

We found that the cancer tissues displayed a lower methylation level of *STAP1* than the adjacent tissues (by *t*-test, $P < 0.05$) (Table 2). However, there was no significant difference between HBsAg carriers who developed HCC and HBsAg carriers who did not during follow-up in the GSE78904 data (Figure 4A).

As gene methylation is able to regulate the gene expression of itself, we analyzed the expression levels of *STAP1* between

primary tumor and solid normal tissue in TCGA data. We found that *STAP1* had a lower expression in primary tumor, while *AHNAK* showed no significant difference (Wilcoxon rank sum test, $P < 0.05$) (Figure 4B).

STAP1 methylation level was pertinent to age, sex, alcohol drinking, and anti-HBe

In our new collection of patients with HBV, we found that the *STAP1* methylation level was positively correlated with age not only in the PBMC ($R = 0.289$, $p = 1.9 \times 10^{-5}$) but also in T cells ($R = 0.243$, $p = 6.2 \times 10^{-4}$) (Figures 5A, B). The *STAP1* methylation level in the PBMC was significantly higher in male than in female patients, while there was no significant difference in T cells (Figure 5C). Similarly, the *STAP1* methylation level in the PBMC was significantly higher in the alcohol-drinking patients than in the non-alcoholic patients and higher in the anti-HBe+

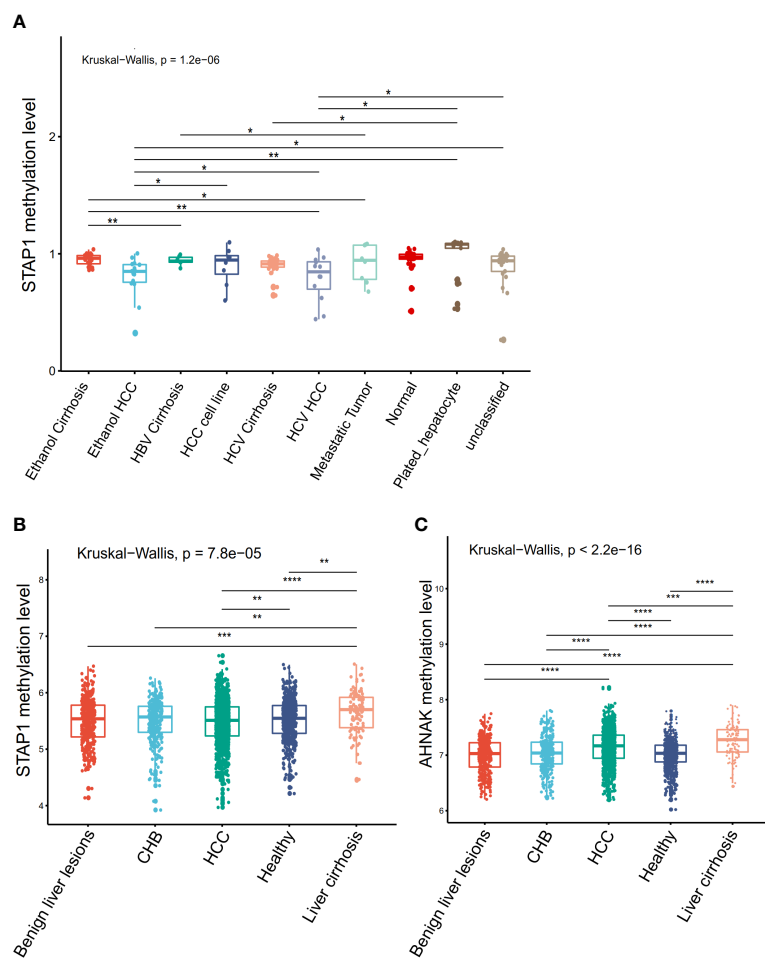


FIGURE 3
Comparison of the methylation of *AHNK* and *STAP1* among different types of liver disease in the Gene Expression Omnibus datasets. **(A)** Comparisons in *STAP1* methylation among the different types of liver cirrhosis and hepatocellular carcinoma (HCC) patients (GSE60753 data). **(B)** Comparisons in *STAP1* methylation among the chronic hepatitis B (CHB), liver cirrhosis, and HCC patients and benign liver lesions (GSE112679 data). **(C)** Comparisons in *AHNK* methylation among the CHB, liver cirrhosis, and HCC patients and benign liver lesions (GSE112679 data). * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

patients than in the anti-HBe- patients, but it showed no significant difference in T cells (Figures 5D, E). In the same set of patients, the *AHNK* methylation level, as it was negatively correlated with *STAP1* methylation, was lower in the older patients and was significantly higher in the female patients, in the non-alcoholic patients, and in non-smoking patients (Supplementary Figure S1).

On the other hand, we verified the relationship between the clinical characteristics and *STAP1* and *AHNK* in the HCC patients using the TCGA data. The *STAP1* methylation level was associated with postoperative rx tx (Figure 6A), gender (Figure 6B), and age (Figure 6C), while the *AHNK* methylation level was associated with additional pharmaceutical therapy (Figure 6D) and sex (Figure 6B).

TABLE 2 Methylation level of *STAP1* between cancer tissues and adjacent tissues.

CpG	Location	median_pt	median_pn	P_pt	FDR_pt
cg04398282	chr4:67558538-67558539	0.845	0.9005	1.70E-05	7.11E-05
cg09632271	chr4:67575504-67575505	0.6945	0.776	0.000357387	0.00110967
cg12879425	chr4:67558777-67558778	0.4535	0.6875	1.63E-06	8.68E-06

Pt, hepatocellular carcinoma tumor; Pn, normal tissue.

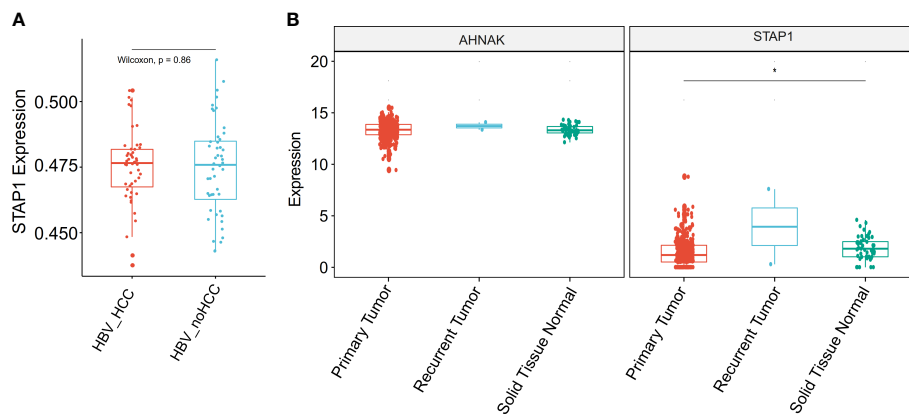


FIGURE 4

Comparison of the methylation and expression of *AHNK* and *STAP1* between hepatocellular carcinoma (HCC) and normal patients. (A) Comparison of the methylation level of *STAP1* between HBsAg carriers who developed HCC and HBsAg carriers who did not. (B) Comparison of the expression level of *AHNK* and *STAP1* between tumor and normal tissues in The Cancer Genome Atlas data. * $P < 0.05$.

The combination of *AHNK* and *STAP1* methylation levels was associated with the overall survival of patients

The result suggested that methylation of *STAP1* was significantly associated with OS, and it was a risk factor (HR = 1.49, $P = 0.0282$), but the expression level of *STAP1* was not significantly associated with OS (HR = 0.75, $P = 0.0908$) (Figures 7A, B). Neither the methylation nor the expression of *AHNK* was significantly associated with OS (HR <1, $P > 0.05$) (Figures 7C, D). However, there was a significant distinction in OS (HR = 0.98, $P = 0.0396$) when using the combination of methylation of *STAP1* and *AHNK* with the patients who carried lower *AHNK* and upper *STAP1* having poor OS (Figure 7E).

Discussion

AHNK, as a good diagnostic marker [with AUC as 0.883 ($P < 0.001$) in the diagnosis of CHB, 0.885 ($P < 0.001$) in the diagnosis of compensatory liver cirrhosis, 0.955 ($P < 0.001$) in the diagnosis of decompensated liver cirrhosis, and 0.981 ($P < 0.001$) in the diagnosis of hepatocellular carcinoma], has been identified in our previous research (13). We here employed a new set of patients to investigate the methylation level of a novel marker, *STAP1*, as well as *AHNK*, in the PBMC and T cells also in different course samples of hepatitis B. We obtained a similar result for *AHNK*. In this study, we investigated the methylation of *STAP1* and found that the methylation of *STAP1* was negatively correlated with the methylation of *AHNK*. Although *AHNK* can distinguish hepatitis B patients to a certain extent in the methylation, the combination of these two markers can achieve better performance. It is reported

that *STAP1* was associated with hypercholesterolemia (18–20). However, there were a few reports about *STAP1* in cancer (14, 15) and no publication for hepatopathy. Therefore, our study is the first to discover the relationship of methylation of *STAP1* with hepatopathy and HCC. The combination of methylation of *STAP1* and *AHNK* indeed achieved a better performance in distinguishing the different types of hepatopathy patients, which suggests the diagnostic value of *STAP1*.

On the other hand, using the expression data and the methylation data of public datasets, we investigate the relationship between the methylation and the expression of *STAP1* and *AHNK*. For *AHNK*, there was hypo-methylation shown in the HCC than in adjacent tissues in TCGA dataset but conversely in the GEO data. In our data, we found a negative correlation of the methylation of *AHNK* and *STAP1* in the PBMC. However, in the public data, there was hyper-methylation of both *AHNK* and *STAP1* in liver cirrhosis and hypo-methylation both in the HCC and adjacent tissues. The expression of the gene can be downregulated by the hyper-methylation of its promoter region through inhibition of the binding of transcription factors or recruitment of methyl-CpG-binding proteins to silence the gene (21, 22). However, with the data including expression and methylation simultaneously in TCGA, we found decreases in both the methylation and the expression levels of *STAP1* in HCC. We think that the contradiction mentioned above is due to the difference between tissue and blood or the difference between the detected positions of methylation.

Similar to *AHNK*, *STAP1* methylation was also related to alcohol drinking, which suggests that these bad habits do affect the occurrence of cancer. Liver cancer is often diagnosed at an advanced stage and has a high mortality rate (23, 24). Therefore, early diagnosis is very important. The combination of these two genes can be used in the early screening and prognostic

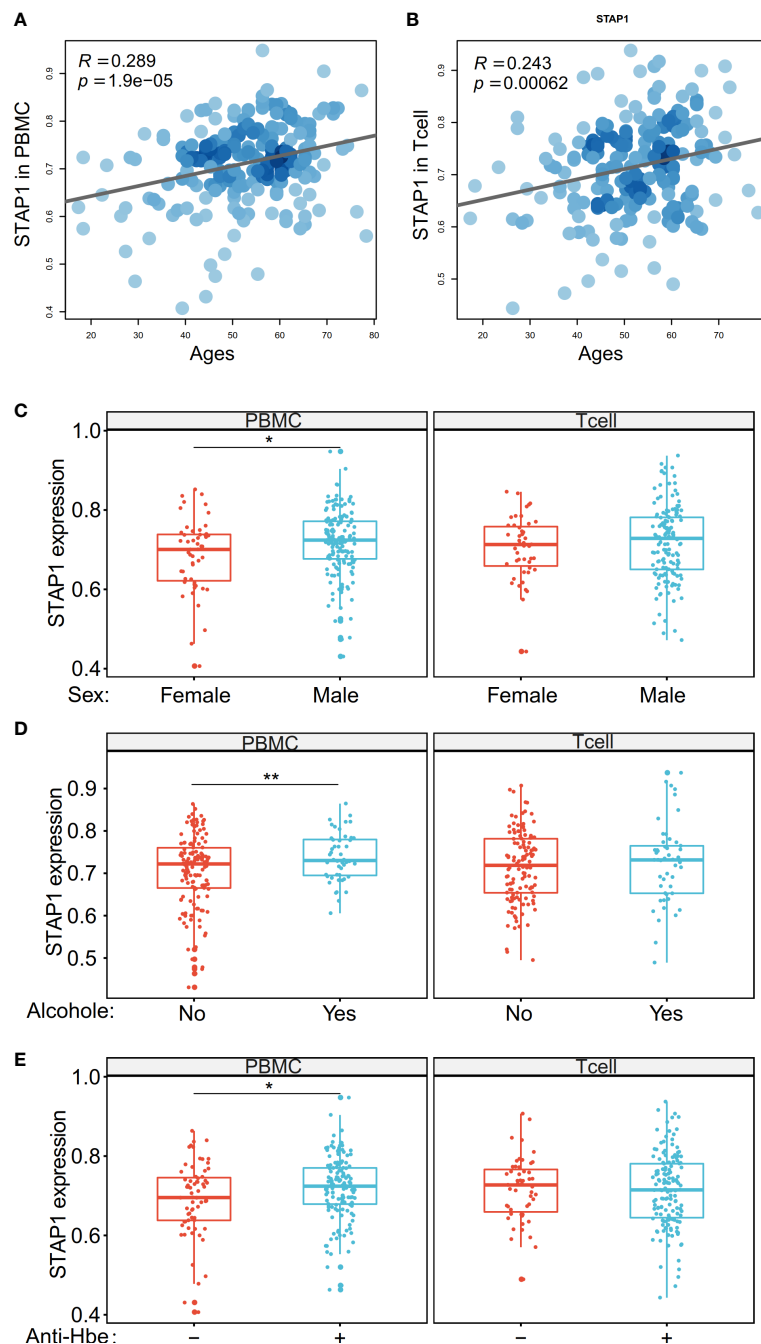


FIGURE 5

The methylation level of *STAP1* was correlated with age, sex, alcohol drinking, and anti-HBe in peripheral blood mononuclear cells (PBMC) and T cells. (A, B) Correlation of *STAP1* methylation level with ages in PBMC and T cells. (C) Difference of *STAP1* methylation in different genders in PBMC and T cells. (D) Difference of *STAP1* methylation between PBMC and T cells relative to alcohol abuse. (E) Difference of *STAP1* methylation between anti-HBe+ and anti-HBe- patients in PBMC and T cells. * $P < 0.05$, ** $P < 0.01$.

judgments and provide a basis for the formulation of treatment strategies.

To date, little is known about the role of *STAP1* in hepatocarcinogenesis. There are several intracellular signaling pathways that derive from the *STAP-1* and *STAP-2* members of

the signal-transducing adaptor protein (STAP) family. These proteins have Pleckstrin homology in their N-terminal sections and SRC homology 2 domains in their middle regions, which are common architectures for adaptor proteins. During carcinogenesis and inflammatory/immune reactions, STAP

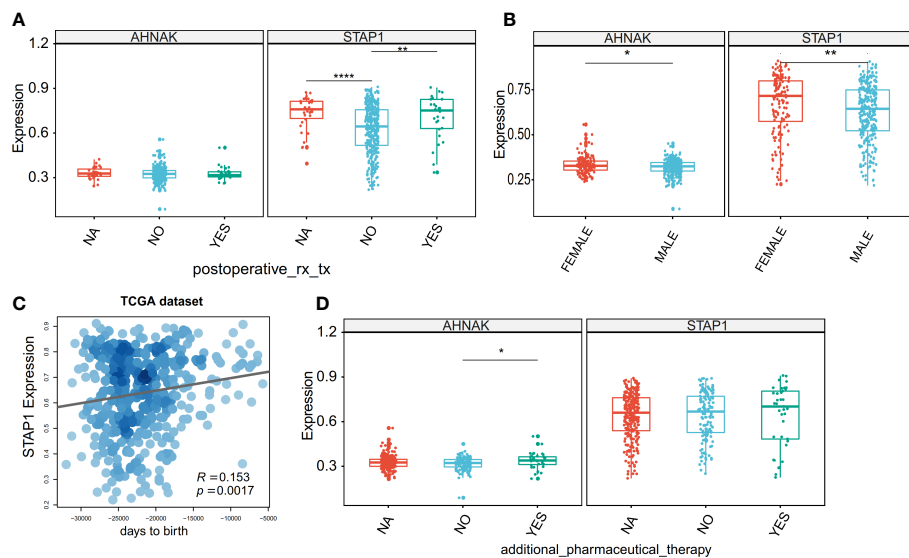


FIGURE 6

Association between the methylation level of *STAP1* or *AHNK* and clinical characteristics in The Cancer Genome Atlas data. (A) Difference of *STAP1* and *AHNK* methylation between whether postoperative rx tx. (B) Difference of *STAP1* and *AHNK* methylation between different genders. (C) Correlation of *STAP1* methylation with ages. (D) Difference of *STAP1* and *AHNK* methylation between whether additional pharmaceutical therapy was used or not. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

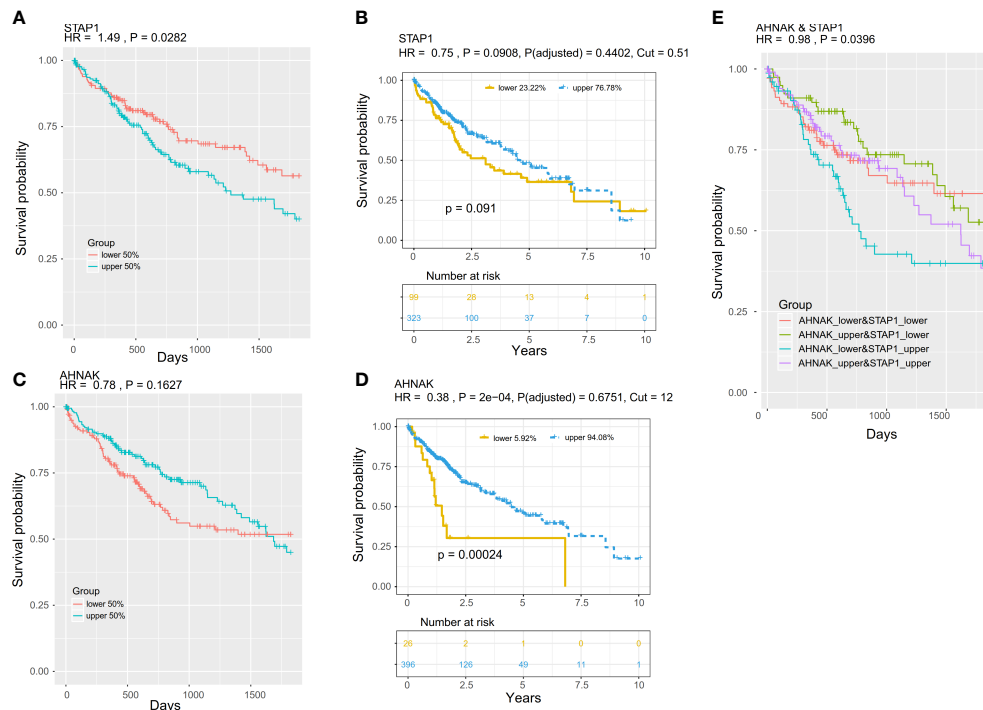


FIGURE 7

Prognostic value of the methylation and expression of *AHNK* and *STAP1*. The K-M survival curves show the overall survival of patients in The Cancer Genome Atlas project. The patients were stratified (upper and lower) according to the median of the methylation or expression level. (A, B) K-M survival curves for *STAP1* methylation (A) and expression (B). (C, D) K-M survival curves for the *AHNK* methylation (C) and expression (D). K-M survival curves for the combination of methylation of *AHNK* and *STAP1*. (E) OS of different *AHNK* & *STAP1* combined groups.

proteins interact with the inhibitor of $\text{nf-}\kappa\text{b}$ kinase complex and activator of transcription 3. In hepatocellular carcinoma, the aberrant methylation of *STAP1* may also contribute to hepatocarcinogenesis *via* these signaling pathways. However, there are some limitations in the present study. First, only PBMC and T cell specimens were used in this study, although the methylation and the expression status in liver tissues were investigated using the public data. The finding in the PBMC and T cell for those two genes was not overall consistent with that in the tissues, and even the different datasets showed different status. Thereby, the intrahepatic methylation of *AHNAK* and *STAP1* still needs to be studied in the future. However, the main purpose of our study was to identify the potential clinical diagnostic marker for HBV-related patients, and it is beneficial to use peripheral blood immune cells in the clinical application. In future research, we should investigate the methylation status of *STAP1* in different tissues. Second, the MSP approach that we used here only tests whether methylation occurs; other approaches such as gene sequencing would be more helpful. However, it is undeniable that MSP is of excellent specificity, sensitivity, and operability in frequent detection. Finally, the mechanisms underlying *STAP1* and *AHNAK* have not been studied here. Especially for *STAP1*, little is known about its function, and thus in the future, an experimental investigation about it should be carried out to understand its functional role in HBV-related hepatopathy.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/[Supplementary Material](#).

Ethics statement

The studies involving human participants were reviewed and approved by the medical ethics committee of Beijing YouAn Hospital (BJYACE-201721). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

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Author contributions

LS contributed to sample and data acquisition and manuscript drafting. JL, KL, HZ, and XZ provided sample and data acquisition and technical support. GL and NL made substantial contributions to the conception and design, funding, and supervision of the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.1091103/full#supplementary-material>

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A nomogram based on HBeAg, AST, and age to predict non-minimal liver inflammation in CHB patients with ALT <80 U/L

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Objective: Precise assessment of liver inflammation in untreated hepatitis B e antigen (HBeAg)-positive patients with chronic hepatitis B virus (HBV) infection can determine when to initiate antiviral therapy. The aim of this study was to develop and validate a nomogram model for the prediction of non-minimal liver inflammation based on liver pathological injuries combined with age and alanine aminotransferase (ALT), aspartate aminotransferase (AST), hepatitis B surface antigen (HBsAg), HBeAg, and HBV DNA quantification.

Methods: We retrospectively included 735 HBeAg-positive chronic hepatitis B (CHB) patients with ALT < 80 U/L as the primary cohort and prospectively enrolled 196 patients as the validation cohort. Multivariate logistic regression analysis identified independent impact factors. A nomogram to predict significant liver inflammation was developed and validated.

Results: Multivariate logistic regression analysis showed that HBeAg, AST, and age were independent risk factors for predicting non-minimal liver inflammation in untreated CHB patients. The final formula for predicting non-minimal liver inflammation was $\text{Logit}(P) = -1.99 - 0.68 \times \text{Log}_{10}\text{HBeAg} + 0.04 \times \text{Age} + 0.06 \times \text{AST}$. A nomogram for the prediction of non-minimal liver inflammation was established based on the results from the multivariate analysis. The predicted probability of the model being consistent with the actual probability was validated by the calibration curves, showing the best agreement in both the primary and validation cohorts. The C-index was 0.767 (95%CI = 0.734–0.802) in the primary cohort and 0.749 (95%CI = 0.681–0.817) in the prospective validation cohort.

Conclusions: The nomogram based on HBeAg, AST, and age might help predict non-minimal liver inflammation in HBeAg-positive CHB patients with ALT < 80 U/L, which is practical and easy to use for clinicians.

KEYWORDS

HBV, nomogram, aspartate aminotransferase (AST), hepatitis B e antigen (HBeAg), liver inflammation

Introduction

Some patients with chronic hepatitis B (CHB) can progress to hepatocellular carcinoma directly without hepatic cirrhosis because of hepatitis B virus (HBV) integration and specific HBV mutants, among others (1–3). The pathogenesis of hepatitis B is very complex, involving changes in immunity and cytokines (4, 5). With the advent of new antiviral drugs, such as tenofovir (TDF), tenofovir alafenamide (TAF), and the combination of interferon with nucleos(tide) analogues for the treatment of CHB, the HBV virological response or the rate of clinical cure has increased significantly (6–9). Currently, it is recommended in many guidelines that CHB patients with a family history of liver cirrhosis and hepatocellular carcinoma should be considered for antiviral treatment after the age of 30–40 years (10–12).

It is essential to evaluate whether liver inflammation has reached the status necessitating antiviral therapy. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), HBV DNA, hepatitis B surface antigen (HBsAg), and hepatitis B e antigen (HBeAg) can be used to predict significant liver inflammation (13–16). After chronic HBV infection, with increasing age, liver inflammation becomes gradually obvious, which means that the immune tolerance has been broken and that it is time to start antiviral therapy. There are different criteria to determine the timing of the breakdown of immune tolerance in patients with CHB infection, two of which are obvious inflammation in the liver biopsy, which is considered the gold standard, and ALT > 2 upper limit of normal (ULN, 80 U/L) lasting 6 months, excluding common diseases such as drugs and fatty liver, among others. Liver biopsy is hindered by poor patient compliance and low reproducibility, which limited its application value for dynamic monitoring during follow-up. Therefore, the use of more serum markers is needed to help determine the severity of liver inflammation.

The main purpose of the present study was to develop a new nomogram for better prediction of non-minimal liver inflammation in CHB patients with ALT < 2 ULN. The study has great importance for clinicians in determining whether treatment-naïve patients with CHB infection and with minimal ALT elevation (<80 U/L) need to start antiviral therapy.

Materials and methods

Patients

We retrospectively included 735 HBeAg-positive CHB patients in the primary cohort from January 2008 to December 2017. The prospective cohort of 196 patients was enrolled from January 2018 to December 2019 for external validation. Patients underwent liver biopsy at Beijing Ditan Hospital of Capital Medical University. All patients gave written informed consent. This study has been registered in ClinicalTrials.gov (NCT: 04032275).

Enrollment criteria

The criteria for enrollment were: 1) with CHB infection for more than 6 months; 2) HBeAg positive; 3) not receiving anti-HBV treatment; 4) had liver biopsy; 5) had sustained ALT < 2 ULN; and 6) with HBV DNA $\geq 10^3$ IU/ml. Patients with HIV infection, other viral hepatitis infections (hepatitis A, C, D, and E), metabolic or drug-associated liver injury, alcoholic liver disease or non-alcoholic fatty liver disease (NAFLD), autoimmune hepatitis, and liver cancer or liver cirrhosis were excluded from the study. Pregnant women were also excluded.

The Knodell scoring system (0–18) of pathology was used to determine liver inflammation from stained specimens by liver biopsy. The enrolled patients were divided into two groups: the minimal liver inflammation (Knodell score, <4 points) group and the non-minimal liver inflammation (Knodell score, ≥ 4 points) group (17, 18).

Serological detection

Serum HBsAg and HBeAg were detected using Abbott Architect i2000 test kits (Abbott Park, IL, USA). The detection range of HBsAg was 0.05–250 IU/ml. The sample was diluted to 1:50–1:500 if the HBsAg concentration was more than 250 IU/ml. HBeAg positivity was defined as a detection level >1 sample/cutoff (S/CO). The reference range for ALT or AST was 0–40 U/L.

Liver biopsy

Liver biopsy was performed using a Max-Core puncture needle (BARD, Peripheral Vascular, Inc., Tempe, AZ, USA). A liver biopsy core about 1.5 cm long with 11–15 portal tracts is appropriate for the evaluation of inflammation. Liver biopsy specimens were read independently by three pathologists, and the average score was used as the final score.

Statistical analysis

SAS 9.2 and R3.02 software were used for statistical analyses. Logistic regression analysis was performed for the analysis of liver inflammation-related factors, and the nomogram method was used for scoring. The bilateral test was adopted for all analyses. A $p < 0.05$ signifies a statistically significant difference. Bootstraps with 200 resamples were used for validation. The concordance index (C-index) was used to evaluate the accuracy of the nomogram. A calibration index was drawn to evaluate the predicted and observed probabilities.

Results

Baseline data of the primary and external validation cohorts

The characteristics of the enrolled patients are listed in Table 1. The age (33.22 ± 9.33 vs. 36.65 ± 8.68 years, $p < 0.001$), platelet (PLT) (202.17 ± 52.57 vs. $215.74 \pm 60.09 \times 10^9/L$, $p = 0.004$), and

prothrombin activity (PTA) ($86.76 \pm 8.97\%$ vs. $96.22 \pm 10.31\%$, $p < 0.001$) of the primary cohort were lower than those of the validation cohort.

Univariate and multivariate statistical analyses

The results of the univariate analysis showed that nine influencing factors were correlated with non-minimal liver inflammation in the primary cohort (Table 2). When age was over 30 and 40 years, non-minimal inflammation was respectively found in 43.24% and 53.07% of patients with CHB infection and ALT < 80 U/L. Age, Log₁₀HBsAg, Log₁₀HBeAg, Log₁₀HBV DNA, ALT, AST, albumin (ALB), and PLT as continuous variables and sex as the binary variable were introduced into the multivariate logistic regression analysis (Table 3). The final formula was as follows: $\text{Logit}(P) = -1.99 - 0.68 \times \text{Log}_{10}\text{HBeAg} + 0.04 \times \text{Age} + 0.06 \times \text{AST}$.

Establishing a simple scoring system for non-minimal liver inflammation

The scoring system for non-minimal liver inflammation was based on the logistic regression analysis. Each variable was converted into a corresponding score. The total score was the sum of the scores from three independent influencing factors. The total score corresponded to a probability of non-minimal liver inflammation. Details of the point assignment for liver inflammation are shown in Table 4.

TABLE 1 Characteristics of the HBeAg-positive chronic hepatitis B (CHB) patients with ALT < 80 U/L in the primary and external validation cohorts.

Characteristics	Total ($n = 931$)	Primary cohort ($n = 735$)	Validation cohort ($n = 196$)	Statistics	p -value
Sex					
Male, n (%)	586 (62.94)	475 (64.63)	111 (56.63)	4.24(χ^2)	0.040
Female, n (%)	345 (37.06)	260 (35.37)	85 (43.37)		
Age (years)	33.95 ± 9.30	33.22 ± 9.33	36.65 ± 8.68	$-4.64(t)$	<0.001
Log ₁₀ HBsAg (IU/ml)	3.57 ± 0.99	3.55 ± 0.97	3.63 ± 1.03	$-1.04(t)$	0.299
Log ₁₀ HBeAg (S/CO)	2.63 ± 0.91	2.66 ± 0.88	2.51 ± 1.02	$1.96(t)$	0.051
Log ₁₀ HBV DNA (IU/ml)	6.95 ± 1.46	6.96 ± 1.38	6.93 ± 1.72	$0.24(t)$	0.811
ALT (U/L)	42.4 (29.6–54.6)	42.4 (30.1–54.6)	42.1 (28.0–54.0)	0.48(Z)	0.629
AST (U/L)	28.0 (23.0–35.7)	27.9 (22.9–36.1)	29.0 (24.0–34.0)	0.55(Z)	0.581
TBIL ($\mu\text{mol/L}$)	12.6 (9.5–16.4)	12.8 (9.6–16.5)	12.0 (9.5–16.0)	1.25(Z)	0.212
ALB (g/L)	46.1 (44.0–48.1)	46.2 (44.1–48.2)	46.0 (43.0–48.0)	0.55(Z)	0.579
WBC ($10^9/L$)	5.7 (4.8–6.7)	5.7 (4.8–6.7)	5.7 (4.7–6.7)	0.539(Z)	0.589
PLT ($10^9/L$)	205.03 ± 54.49	202.17 ± 52.57	215.74 ± 60.09	$-2.88(t)$	0.004
PTA (%)	89.13 ± 10.18	86.76 ± 8.97	96.22 ± 10.31	$-11.30(t)$	<0.001

HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; S/CO, sample/cutoff; HBV, hepatitis B virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; ALB, albumin; WBC, white blood cells; PLT, platelet; PTA, prothrombin activity.

TABLE 2 Univariate logistic regression analysis of the factors of non-minimal liver inflammation in patients from the primary cohort.

	<i>b</i>	SE	Wald	<i>p</i> -value	OR	95%CI for OR	
						Lower	Upper
Sex	0.34	0.16	4.71	0.030	1.41	1.03	1.92
Age (years)	0.05	0.01	33.32	0.000	1.05	1.03	1.07
Log ₁₀ HBsAg (IU/ml)	−0.21	0.08	6.59	0.010	0.81	0.69	0.95
Log ₁₀ HBeAg (S/CO)	−0.77	0.10	64.46	0.000	0.46	0.38	0.56
Log ₁₀ HBV DNA (IU/ml)	−0.33	0.06	31.76	0.000	0.72	0.64	0.80
ALT (U/L)	0.02	0.00	14.25	0.000	1.02	1.01	1.03
AST (U/L)	0.06	0.01	50.30	0.000	1.06	1.04	1.08
TBIL (μmol/L)	−0.02	0.01	3.69	0.055	0.98	0.95	1.00
ALB (g/L)	−0.08	0.02	14.26	0.000	0.92	0.88	0.96
WBC (×10 ⁹ /L)	0.01	0.01	0.60	0.437	1.01	0.99	1.03
PLT (×10 ⁹ /L)	−0.01	0.00	20.98	0.000	0.99	0.99	1.00
PTA (%)	0.00	0.01	0.07	0.798	1.00	0.98	1.02

HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; S/CO, sample/cutoff; HBV, hepatitis B virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; ALB, albumin; WBC, white blood cells; PLT, platelet; PTA, prothrombin activity. SE, standard error.

Establishing a prognostic nomogram for non-minimal liver inflammation

A nomogram was established based on the results obtained from the multivariate analysis. Each factor, including age, AST, and HBeAg, was assigned to a specific value in the corresponding scale axis and a line drawn upward to determine its score. The sum of the three scores resulted in a total score, and then a line was drawn downward to determine the probability of non-minimal liver inflammation in the risk axis for each patient (Figure 1).

TABLE 3 Multivariate logistic regression analysis of the factors of liver inflammation in patients from the primary cohort.

	<i>b</i>	SE	Wald	<i>p</i> -value	OR	95%CI for OR	
						Lower	Upper
Intercept	−1.99	0.54	13.69	0.000			
Age (years)	0.04	0.01	17.55	0.000	1.04	1.02	1.06
Log ₁₀ HBeAg (S/CO)	−0.68	0.10	46.70	0.000	0.51	0.42	0.62
AST (U/L)	0.06	0.01	53.80	0.000	1.07	1.05	1.08

HBeAg, hepatitis B e antigen; S/CO, sample/cutoff; AST, aspartate aminotransferase. SE, standard error.

Validation of the prognostic nomogram for non-minimal liver inflammation

The C-index and calibration curves were used to evaluate the predictive accuracy of the nomogram. The C-index of the nomogram from the primary cohort for predicting liver inflammation was 0.767 (95%CI = 0.734–0.802) (Figure 2), while that from the prospective validation cohort was 0.749 (95%CI = 0.681–0.817) (Figure 3). The calibration curves showed the best agreement between the predictive and observed probabilities in both the primary and prospective validation cohorts.

The predicted probability of the nomogram was plotted on the *x*-axis, while the actual probability was plotted on the *y*-axis. A dashed line along the 45° line through the point of origin represented the perfect calibration model, where the predicted probabilities were identical to the actual probabilities.

Discussion

Accurate assessment of liver inflammation in untreated patients with CHB infection has always been one of the focal points in HBV research. The purpose of this study was to establish a noninvasive liver inflammation diagnostic model mainly based on blood indicators.

In previous research studies, a decrease in the quantitative HBsAg and HBeAg to a certain cutoff value often indicates the onset of an immune clearance period (16, 19–21). Immune tolerance is gradually weakened with age (22, 23). The higher the ALT, the more obvious the liver inflammation. However, for patients with conflicting indicators, such as high ALT and HBsAg or low ALT and HBsAg, neither ALT nor HBsAg alone could make correct predictions (24, 25). Therefore, there is a need to develop a multifactorial scoring system for estimating liver inflammation that is simple and easy to use in untreated patients with CHB infection.

For patients with ALT > 2–5 ULN or above, liver inflammation was obvious or active, which marks the breakdown of immune tolerance. Deciding whether or not to start antiviral therapy in CHB patients with ALT < 2 ULN is a challenge for clinicians. There are pieces of evidence in favor of antiviral therapy. The results of liver biopsy can confirm that some of these patients have met the indication for antiviral therapy with obvious liver inflammation or liver fibrosis (21, 26–28). A subset of patients can progress from CHB directly to liver cancer even if ALT is normal (1–3). A timely antiviral treatment can reduce the HBV DNA load and decrease the incidence of liver cancer. Therefore, there is a need to establish a nomogram to identify patients with obvious liver inflammation and who need treatment from those HBeAg-positive CHB patients with ALT < 2 ULN.

The aim of this study was to identify non-minimal liver inflammation in treatment-naïve CHB infection patients with ALT < 2 ULN with the help of a number of clinical noninvasive markers. Our results showed that AST, HBeAg, and age were independent variables for predicting noninvasive liver inflammation. The correlation between ALT or AST and liver inflammation has been well recognized by clinicians for a long time. Age affects the natural course of CHB infection, and it has

TABLE 4 Point assignment for the nomogram and the prognostic probability of non-minimal liver inflammation in HBeAg-positive chronic hepatitis B (CHB) patients with ALT < 80 U/L.

Age (years)	Points	AST (U/L)	Points	Log ₁₀ HBeAg (S/CO)	Points	Total points	Risk
10	0	5	0	0	6	3	0.1
15	0	10	1	0.5	5	5	0.2
20	1	15	1	1	4	6	0.3
25	1	20	2	1.5	3	8	0.4
30	2	25	3	2	2	8	0.5
35	2	30	4	2.5	2	9	0.6
40	3	35	4	3	1	10	0.7
45	3	40	5	3.5	0	12	0.8
50	4	45	6			14	0.9
55	4	50	6				
60	5	55	7				
65	5	60	8				
		65	9				
		70	9				
		75	10				

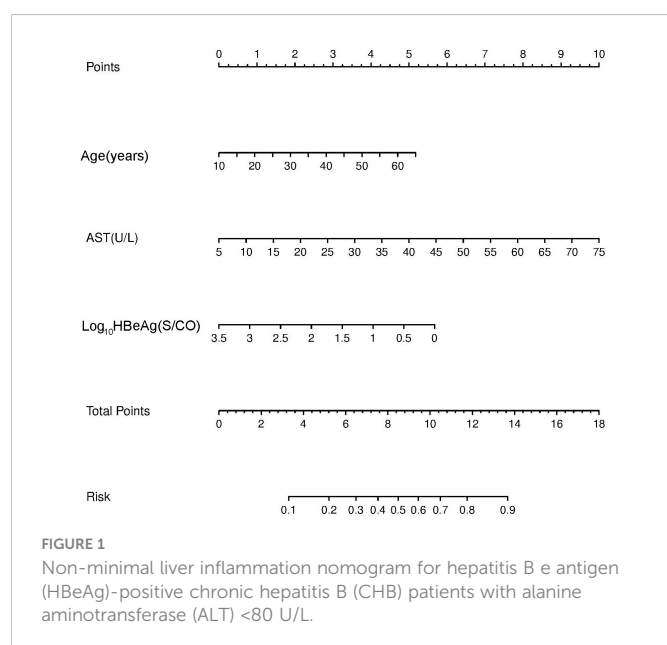
HBeAg, hepatitis B e antigen; S/CO, sample/cutoff; HBV, hepatitis B virus; ALT, alanine aminotransferase.

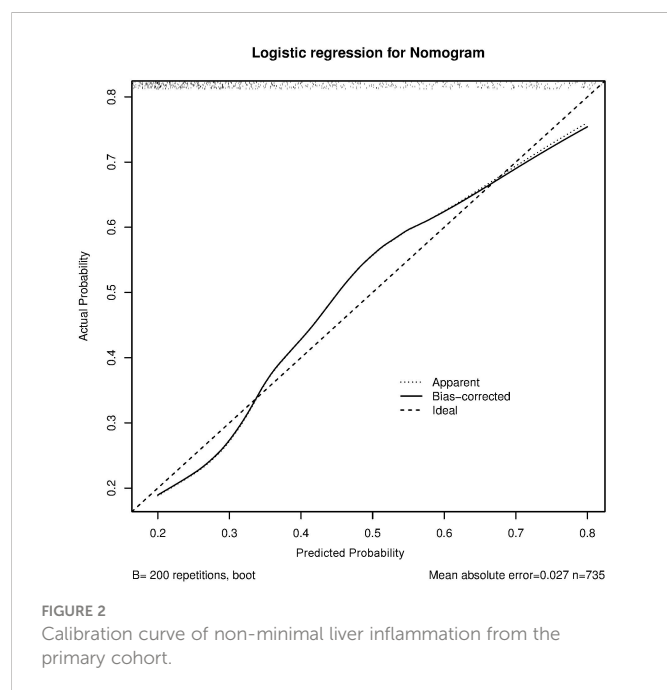
been found that immune tolerance may be broken and that liver inflammation may occur when patients are over 30–40 years old. In our study, when age was over 30 and 40 years, non-minimal inflammation was found in 43.24% and 53.07% of patients with CHB infection and ALT < 2 ULN. There are many similar findings in the literature of quantitative HBeAg and HBsAg being both associated with HBV DNA or replicative covalently closed circular DNA (cccDNA) in HBeAg-positive patients (13, 21). In our research, we

included AST instead of ALT into the prediction formula, considering that AST is a more sensitive factor for obvious liver inflammation of ALT < 2 ULN. Coincidentally, in the APRI (AST-to-PLT ratio index) formula for cirrhosis, AST was used, but not ALT. Similarly, HBeAg, but not HBsAg, was entered into the prediction formula. A possible explanation is that a decrease in HBeAg can better reflect the replication status of HBV or the breakdown of immune tolerance in untreated HBeAg-positive CHB patients.

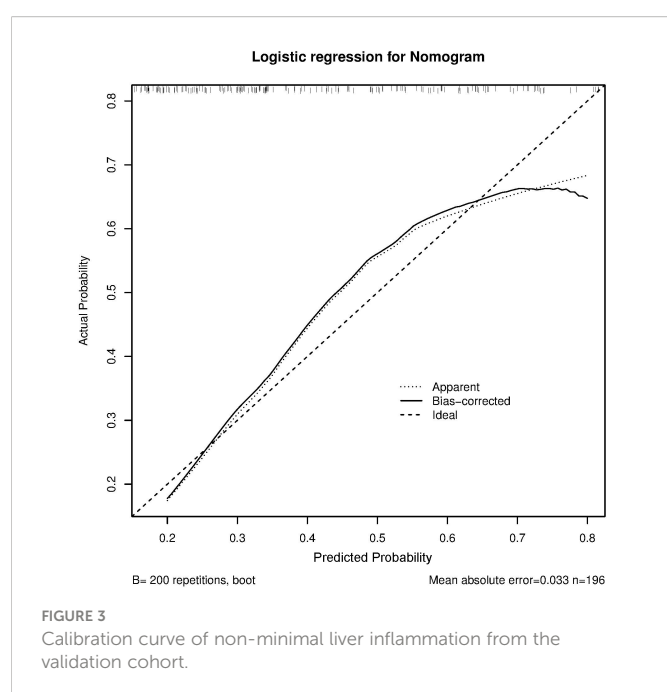
To confirm the effect of the non-minimal liver inflammation model, we evaluated the predictive accuracy and discriminative ability of the nomogram using the C-index and calibration curves. In HBeAg-positive CHB patients, the calibration curve showed good agreement between the model predictions and the actual observations in both the primary and external validation cohorts. The C-index values were 0.767 (95%CI = 0.734–0.802) and 0.749 (95%CI = 0.681–0.817), respectively, indicating good predictive accuracy.

A nomogram is a visual prediction model. The predictors of liver inflammation in CHB patients with ALT < 2 ULN were shown in the nomogram. Nomograms were first used to evaluate the prognosis of cancer patients (29). They have been extended to other fields because of their reliability, visual intuition, and convenience (30, 31). A nomogram based on multivariate logistic regression analysis allows prediction of the diagnosis, stage, and prognosis of tumor or other diseases (32). Nomograms have been proven to be superior to risk stratification and artificial neural network models in predicting the prognosis of prostate cancer (33). The nomogram in this study revealed that liver inflammation becomes more obvious with age, corresponding to 2 at 30 years and 3 at 40 years. HBeAg of 1,000 S/CO represented 1, 100 S/CO represented 2, and 10 S/CO represented 4, indicating that the degree of liver inflammation increased with the





decrease of quantitative HBeAg. When AST increased to 2 ULN, the effect on liver inflammation was more obvious. The probability of liver inflammation in CHB infection could be predicted by combining AST, age, and HBeAg. The total score was 10, corresponding to 0.7 in the nomogram, which means that the predicted probability of non-minimal liver inflammation was 70%. The effect of AST on the score was greater than that of age and HBeAg. AST > 2 ULN alone corresponded to a score of 10 and predicted the need for antiviral therapy. On the other hand, single indicators such as age and HBeAg could not exactly predict the need for antiviral treatment. The influence of the predictors age, HBeAg, and AST on liver inflammation was clearly reflected using this nomogram.



For patients with CHB infection, more effort has to be made on non-minimal liver inflammation model research. At present, there are many non-invasive prediction models for chronic hepatitis, most of which are primarily for predicting liver fibrosis, such as APRI and FIB-4 (Fibrosis-4) (34), with a few models for predicting liver inflammation (24). Liver inflammation usually occurs earlier than liver fibrosis; therefore, early diagnosis of liver inflammation in CHB infection is more important and is difficult. Chang et al. and other researchers have reported a prediction model of CHB infection whose scope is the histological progression of liver diseases, i.e., significant liver fibrosis and/or liver inflammation (25, 28, 35). The model developed in this study is a noninvasive prediction model for the identification of non-minimal liver inflammation in patients with CHB infection. This study did not involve fibrosis evaluation and is therefore not fit for comparison with APRI or FIB-4. In untreated HBeAg-positive CHB patients, the liver inflammation caused by CHB is related to the balance between the immune system and HBV infection. Quantitative changes in several indicators, such as HBsAg and HBeAg, can signal the breakdown of immune tolerance and the start of immune clearance in CHB infection. It is necessary to include immune-related indicators in the inflammatory prediction model for CHB infection. In general, ALT or AST is included in most models of liver inflammation or fibrosis. It has been found that the predictive ability of AST is better than that of ALT. HBV DNA, HBsAg, and HBeAg can reflect the replication ability of HBV in untreated CHB patients. HBsAg and HBeAg have been previously found to be negatively correlated with liver inflammation. In this study, HBsAg, HBeAg, and HBV DNA were all included, and HBeAg was found to have better predictive ability. Compared with other models, the significance of this non-minimal liver inflammation model is that it confirmed the importance of HBeAg in the identification of inflammation. On the one hand, HBeAg can reflect the replication level of HBV in HBeAg-positive CHB patients; on the other hand, a decline in HBeAg is often an early sign of the breakdown of immune tolerance (36).

The advantages of the non-minimal liver inflammation nomogram developed in this study are as follows: 1) it can be calculated and predicted repeatedly at different time points of the disease, which is convenient for long-term prospective assessment of liver inflammation in HBeAg-positive CHB patients; 2) the alignment chart of the nomogram is visual and practical, which is beneficial for doctors to make decisions on when to start antiviral treatment for individual patients, and it is also suitable for multifactor comprehensive prediction of liver inflammation; 3) this nomogram has been verified in the prospective validation cohort; and 4) the nomogram included HBeAg, a replication- and an immune-related marker of HBV. This study has several limitations. Firstly, the data were from Chinese CHB patients, whose genotypes are mostly of B and C types. Secondly, the sample size was relatively small, and the nomogram was unable to predict liver inflammation in patients with CHB infection according to gender.

In conclusion, the non-minimal liver inflammation nomogram that included age, AST, and HBeAg index has been well established and verified in HBeAg-positive CHB patients with ALT < 2 ULN. The nomogram could help in predicting the probability of non-minimal liver inflammation. The predictive accuracy of this nomogram was confirmed using the C-index and calibration curves.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Beijing Ditan Hospital. The patients/participants provided written informed consent to participate in this study.

Author contributions

ML and YX contributed to the study concept and design. XB, QX, LuZ, XC, and WC conducted the experiments and collected the data. YanL, GS, TJ, WD, and SW collected patient information. ShiW, LuxZ, LY, ShuW, MX, and YG ordered the reagents and materials. YaoL performed the statistical analyses and wrote the first draft. HH, SW, RL, MC, MX, LH, and XC edited the English version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Serum cytokine change profile associated with HBsAg loss during combination therapy with PEG-IFN- α in NAs-suppressed chronic hepatitis B patients

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Objective: The aim of this study was to explore the profile of cytokine changes during the combination therapy with pegylated interferon alpha (PEG-IFN- α) and its relationship with HBsAg loss in nucleos(t)ide analogs (NAs)-suppressed chronic hepatitis B patients.

Methods: Seventy-six patients with chronic hepatitis B with HBsAg less than 1,500 IU/ml and HBV DNA negative after receiving ≥ 1 -year NAs therapy were enrolled. Eighteen patients continued to take NAs monotherapy (the NAs group), and 58 patients received combination therapy with NAs and PEG-IFN- α (the Add-on group). The levels of IFNG, IL1B, IL1RN, IL2, IL4, IL6, IL10, IL12A, IL17A, CCL2, CCL3, CCL5, CXCL8, CXCL10, TNF, and CSF2 in peripheral blood during treatment were detected.

Results: At week 48, 0.00% (0/18) in the NAs group and 25.86% (15/58) in the Add-on group achieved HBsAg loss. During 48 weeks of combined treatment, there was a transitory increase in the levels of ALT, IL1RN, IL2, and CCL2. Compared to the NAs group, CXCL8 and CXCL10 in the Add-on group remain higher after rising, yet CCL3 showed a continuously increasing trend. Mild and early increases in IL1B, CCL3, IL17A, IL2, IL4, IL6, and CXCL8 were associated with HBsAg loss or decrease >1 log, while sustained high levels of CCL5 and CXCL10 were associated with poor responses to Add-on therapy at week 48.

Conclusions: The serum cytokine change profile is closely related to the response to the combination therapy with PEG-IFN- α and NAs, and may help to reveal the mechanism of functional cure and discover new immunological predictors and new therapeutic targets.

KEYWORDS

chronic hepatitis B, cytokine, CXCL-10, HBsAg loss, combined treatment, Peg-IFN- α

Introduction

Approximately 290 million people are chronically infected with the hepatitis B virus (HBV) worldwide (1). More than 650,000 people die each year from end-stage liver disease associated with HBV, including liver failure, cirrhosis, and hepatocellular carcinoma (1–3). Achieving the functional cure, meaning both HBV DNA and hepatitis B surface antigen (HBsAg) undetectable (4–6), can significantly improve the disease progression of chronic hepatitis B (CHB) and is considered the ideal endpoint for antiviral treatment (4–7). Nucleos(t)ide analogs (NAs) and interferon (IFN) are the main first-line agents for CHB, which can significantly inhibit HBV replication. However, it is hard to achieve a functional cure with either drug alone. Although many studies have shown that sequential or combination therapy with the two drugs can significantly improve the probability of functional cure in some specific populations, it is still not satisfactory (8–12). Therefore, it is particularly important to explore the mechanisms behind HBsAg loss and to identify predictive markers to expand the population suitable for treatment.

The development of HBV infection is mainly affected by the host's immune response. In acute infection, 95% of adult patients showed an adequate immune response and eventually cleared the virus. Otherwise, patients can become chronically infected when the host immune response is inadequate or inappropriate (13, 14). As important immune system components, cytokines may introduce immune dysregulation or tolerance and be associated with progression in CHB (15–18). Inflammatory cytokines, such as IFN- α , CXCL8, CXCL9, and CXCL10, can induce inflammatory immune cell recruitment and promote hepatocyte apoptosis in CHB (15, 19–21). For instance, an elevated serum IFN- α and CXCL8 could promote NK-cell-mediated liver cell injury (20, 21), high serum CXCL9 and CXCL10 levels were reported to correlate with the development of hepatitis flares (20–22), IL-2 and IFN- γ were upregulated with high ALT levels (21). The changes of multiple cytokines can more comprehensively and accurately reflect the immune network in the liver to guide clinical treatment better.

It was also demonstrated that in the case of antiviral therapy, changes in serum cytokines were related to the response to antiviral therapy. Li M. et al. reported that an early rise in IFN- α 2 levels during pegylated interferon alpha (PEG-IFN- α) treatment was related to functional cure in hepatitis B e antigen (HBeAg) positive CHB patients (23, 24). In addition, CXCL10 has also shown a predictive role in PEG-IFN- α treatment of chronic hepatitis C (25). Still, the detailed relationship among virological and biochemical markers, and cytokines behind different responses to anti-HBV treatment remains unclear.

In this study, we investigated the dynamic changes of serum cytokines levels during PEG-IFN- α add-on therapy in NA-suppressed CHB patients with HBsAg levels < 1,500 IU/ml, and

explored the relationships between the changes of cytokines, the virological response, and the fluctuation of liver inflammation.

Materials and methods

Study population and design

This was an open-label, clinical controlled, observational study. The CHB patients were recruited from the Fifth Medical Center of Chinese PLA General Hospital. Subjects aged 18–65 years who had taken NAs drugs for ≥ 1 year and achieved serum HBsAg levels < 1,500 IU/ml and HBV DNA < 20 IU/ml were eligible. Exclusion criteria for recruited patients were co-infected with other hepatitis viruses or human immunodeficiency virus; liver cirrhosis, liver transplantation, or other liver diseases or severe systemic diseases; and IFN, glucocorticoids, or other immunomodulatory therapy in the six months before enrollment. Enrolled subjects were divided into two groups (the NAs group and the Add-on group) according to their choices after being informed of the benefits and risks of PEG-IFN- α therapy. NAs group continued to receive entecavir (ETV) or tenofovir disoproxil fumarate (TDF). The Add-on group was treated with PEG-IFN- α -2b (180 μ g once a week) in addition to ETV or TDF. The primary outcome was HBsAg loss or decline > 1 log after 48 weeks of treatment. Patients were divided into three subgroups according to the responses to 48 weeks of the combination therapy. At week 48, patients who achieved HBsAg loss were defined as complete responders (the CR group); patients who achieved HBsAg decreased > 1 log from baseline but remained positive were classified as partial responders (the PR group); patients who achieved HBsAg decreased < 1 log from baseline and still positive were defined as non-responders (the NR group). The study protocol was approved by the Ethics Committee of the Fifth Medical Center of Chinese PLA General Hospital.

Clinical and laboratory evaluation

Peripheral blood samples were collected from all enrolled patients during the screening period. Samples were then continuously collected after enrollment every 24 weeks (the NAs group) or every 12 weeks (the Add-on group). Serological and biochemical markers of HBV were routinely tested in the central clinical laboratory. Serum HBV DNA levels were determined by the COBAS AmpliPrep/COBAS TaqMan HBV Test (Roche Molecular Systems, Inc, Branchburg, USA). The lower limit for HBV DNA detection was 20 IU/ml. Serum HBsAg levels were quantified by Elecsys HBsAg II quant II (Roche Diagnostics GmbH, Mannheim, Germany). The lower limit for HBsAg detection was 0.05 IU/ml. COBAS e602 (Roche Diagnostics GmbH, Mannheim, Germany) was used to detect HBsAb, HBeAg, and HBeAb levels. Serum levels of IFNG, IL1B, IL1RN, IL2, IL4, IL6, IL10, IL12A, IL17A, CCL2, CCL3, CCL5, CXCL8, CXCL10, TNF, and CSF2 were determined by flow-cytometer using AIMPLEX kit (Aimplex Biosciences, Inc., Beijing, China) according to the manufacturer's instructions.

Abbreviations: Anti-HBs, antibody to hepatitis B surface antigen; CHB, chronic hepatitis B; ETV, Entecavir; HBeAb, hepatitis B e antibody; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBeAg, hepatitis B e antigen; HBsAb, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen; IFN, interferon; NAs, nucleos(t)ide analogs; PEG-IFN- α , pegylated-interferon-alpha; TDF, tenofovir disoproxil fumarate;

Statistical analysis

SPSS version 25.0 and R version 4.1.2 were used for statistical analyses. Search Tool for the Retrieval of Interacting Genes (STRING) platforms was utilized for protein-protein interaction network (PPIN) analysis (26). Median (quartiles) were reported for continuous variables. The statistical significance of the difference between the two groups was determined using the Mann-Whitney U test, while among three groups using the Kruskal-Wallis H test. Categorical variables were analyzed using the Chi-squared test. Spearman correlation was adopted to determine the correlation between continuous variables. In the bilateral test, $P < 0.05$ was considered to have a statistical difference.

Results

Characteristics of enrolled patients

In this open-label, observational, clinical controlled study, seventy-six patients were enrolled. The characteristics of patients were shown in Table 1. At baseline, HBsAg levels were comparable between the NAs and Add-on groups. At week 48, 0.00% (0/18) in the NAs group and 25.86% (15/58) in the Add-on group achieved HBsAg loss, respectively.

After 48 weeks of combination therapy, 15 patients with HBsAg loss were assigned to the CR group, 9 patients with HBsAg decreased > 1 log from baseline but still positive were assigned to the PR group, and 34 patients with HBsAg decreased < 1 log from baseline and remain positive were assigned to the NR group.

The dynamic change of HBsAg and ALT in the NAs and Add-on groups

During 48 weeks of treatment, HBsAg levels declined significantly in the Add-on group, barely dropping in the NAs group. ALT increased more than twofold from baseline at week 12 in the Add-on group and then gradually declined, but the ALT level was still

higher than that of the NAs group. ALT in the NAs group was not significantly increased (Figure 1).

The dynamic changes of cytokines induced by PEG-IFN- α are different from those of NAs

The levels of most cytokines, except IFNG and IL6, were lower in the NAs group than in the Add-on group. During 48 weeks of combined treatment, there was a transient spike in the levels of IL1RN, IL2, and CCL2. Compared with the NAs group, CXCL8 and CXCL10 in the Add-on group remain higher after rising, and yet CCL3 showed a continuously increasing trend, and all were significantly higher at week 24 and week 48. (Figure 1)

Mild and early increases in IL1B, CCL3, and IL17A were associated with HBsAg loss or decrease > 1 log during combination therapy

In the Add-on group, ALT peaked at week 12 regardless of the response. Non-responders had the highest ALT peak, with no statistical difference, and then it continued to decline. ALT decreased in the CR and PR groups and then showed a slight upward trend at week 48 (Figure 2).

In the CR group, higher baseline levels of IFNG and an early elevation of IL2, IL6, and CXCL8 were observed. The levels of IFNG (week 0), IL2 (week 24), IL6 (week 12), and CXCL8 (week 12) in the CR group were weakly higher than those in the PR and NR group, though with no statistical difference (IFNG [week 0]: $H=4.282$, $P=0.118$; IL2 [week 24]: $H=1.255$, $P=0.534$; IL6 [week 12]: $H=0.233$, $P=0.890$; CXCL8 [week 12]: $H=0.822$, $P=0.663$). IL17A levels increased significantly at week 12 in the CR group than in the PR and NR groups ($H=9.466$, $P=0.009$). Typically, at week 12, the CR group showed a transient increase in IL1B and CCL3. IL1B levels increased more in the CR group than in the other patients ($H=6.542$, $P=0.038$). At week 24, the PR group showed a transient increase in IL1B and CCL3, with no significant difference. However, no visible

TABLE 1 Characteristics of the Enrolled Patients.

Indicators	NAs group (n=18)	Add-on group (n=58)	P
Male/female, n	14/4	49/9	0.763
Age(years), median (quartiles)	38.0(34.0, 47.8)	38.5(32.0, 46.8)	0.859
Baseline HBsAg(log ₁₀ IU/ml), median (quartiles)	2.79(2.28, 3.03)	2.59(1.97, 2.95)	0.328
Baseline HBeAg positive, n (%)	8(47.06)	17(30.91)	0.222
Baseline HBeAb positive, n (%)	7 (41.18)	23 (41.82)	0.963
Baseline ALT(U/L), median (quartiles)	19.5(13.0, 25.0)	22.0(17.8, 27.0)	0.409
Baseline AST(U/L), median (quartiles)	24.0(17.5, 27.3)	21.0(19.0, 24.0)	0.363
HBsAg loss at week 48, n (%)	0 (0)	15(25.86)	0.039

HBeAg/HBeAb data were not available for one patient in the NAs group, for three patients in the Add-on group.

Abbreviations: NAs, nucleos(t)ide analogs; Add-on, nucleos(t)ide analogs combined with pegylated-interferon-alpha; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBeAb, hepatitis B e antibody; ALT, alanine aminotransferase; AST, aspartate amino transferase.

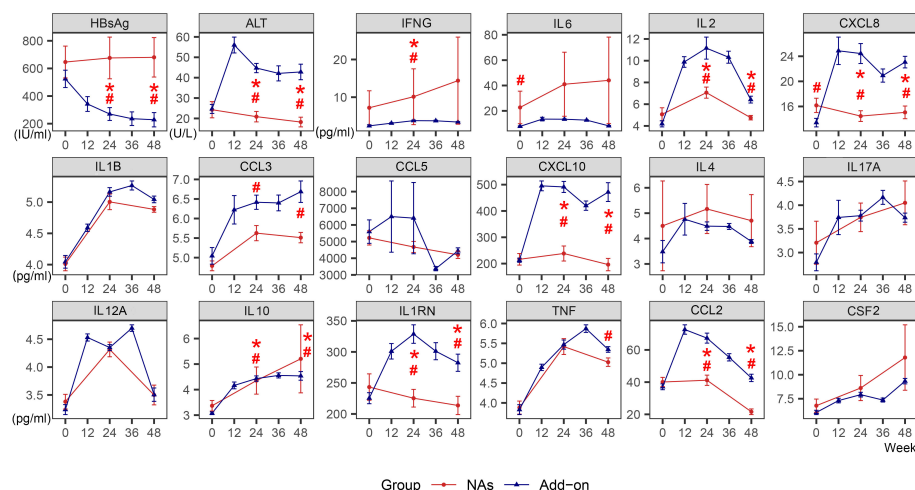


FIGURE 1

Dynamic changes of HBsAg, ALT and cytokines in the NAs group and Add-on group. The line represents the mean and the bar represents the standard error. #, significant differences in cytokine levels between the two groups, $P < 0.05$; *significant differences in the amplitude of cytokine change from baseline between the two groups, $P < 0.05$. HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; NAs, nucleos(t)ide analogs; Add-on, NAs combined with pegylated interferon alpha therapy.

increase in CCL3 was observed in the NR group. Besides, there was an increase in IL-4 in the CR group at week 12 ($H=1.225$, $P=0.542$).

In the NR group, the levels of CCL5 and CXCL10 remained high after an obvious increase. At week 12, 24, and 48, the CCL5 level in NR group was significantly higher than that in CR group and PR group (week 12: $H=6.361$, $P=0.042$; week 24 $H=6.409$, $P=0.041$; week 48: $H=7.584$, $P=0.023$). At week 12 and 36, CXCL10 levels increased significantly in the NR group than in the CR and PR group (week 12: $H=8.530$, $P=0.014$; week 36: $H=6.210$, $P=0.045$).

Other cytokines, such as IL12A, IL10, TNF, CCL2, and CSF2, showed similar trends regardless of the response to the combination therapy.

The correlations between the dynamic changes of cytokines, HBsAg, and ALT varied according to the response to combination therapy

We analyzed the correlation between the dynamic changes of cytokines with HBsAg and ALT. A PPIN was constructed among 16 cytokines involved by the STRING database (Supplementary Figure 1A). Cytokines with statistically different expression levels in CR, PR, and NR groups were used to reconstruct a PPIN and included in the correlation analysis (Supplementary Figure 1B; Figures 3A–C).

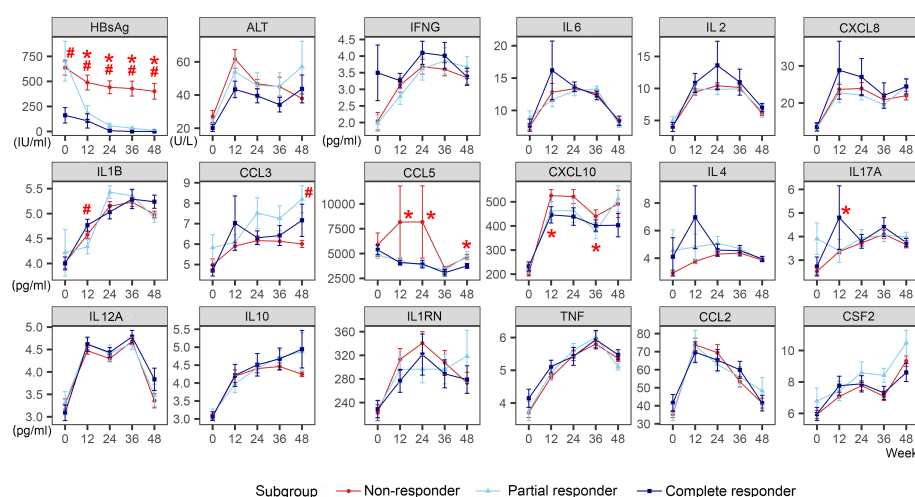


FIGURE 2

Dynamic changes of HBsAg, ALT, and cytokines of different response subgroups in the Add-on group. The line represents the mean and the bar represents the standard error. #, significant differences in cytokine levels among the three subgroups, $P < 0.05$; *significant differences in the amplitude of cytokine change from baseline among the three subgroups, $P < 0.05$. HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; Add-on, nucleos(t)ide analogs combined with pegylated interferon alpha therapy; complete responder, achieving HBsAg loss at week 48; partial responders: achieving HBsAg decreased by > 1 log from baseline but HBsAg remains positive at week 48; non-responder, achieving HBsAg decreased by < 1 log from baseline and HBsAg remain positive at week 48.

The presence of all cytokines in one cluster indicated a strong interaction among each other cytokines. Existing databases and text mining showed that most of the cytokines involved in this study had co-expression relationships. There were laboratory-confirmed interactions between IFNG and TNF, CXCL8 and CCL5 and CCL2, IL2 and IL17A, and CXCL10 and CXCL5. There may be protein homology between CCL5 and CCL3 (Supplementary Figures 1A–B). The existing database showed that CXCL10 was co-expressed with IL1B, CCL5, and CCL3. Text mining showed that CXCL10 and IL17A were co-expressed. (Supplementary Figure 1B)

In the CR subgroup, the increases of IL1B, IL17A, and CCL3 were positively correlated ($P < 0.05$). No correlation existed between the increases of other cytokines, HBsAg, and ALT (Figure 3A).

In the PR group, the increase of CCL5 was positively correlated with the increase of CXCL10, and the increase of IL17A was also positively correlated with CCL3 ($P < 0.05$). There was no correlation between the dynamics of other cytokines, HBsAg and ALT. Of note, the increase in IL1B at week 48 was significantly inversely related to the magnitude of the increase in CCL5 from baseline ($r = -0.867$, $P = 0.002$) (Figure 3B).

In the NR group, the decline of HBsAg level was correlated positively with the rise of IL1B and CXCL10 ($P < 0.05$). The early

elevation of ALT was correlated positively with the elevation of CXCL10 ($P < 0.05$). The increase of IL1B was correlated negatively with the increase of CCL5 ($P < 0.05$) and was correlated positively with the increase of IL17A and CCL3 ($P < 0.05$). In addition, the increase of IL17A was correlated negatively with the increase of CCL5 and positively correlated with the increase of CCL3 ($P < 0.05$) (Figure 3C).

Discussion

CHB is an immune-related disease; immune cells and cytokines play an essential part in disease progression. Several studies have demonstrated that baseline quantitative HBsAg $< 1,500$ IU/ml can effectively predict HBsAg loss after a finite course of IFN-based therapy (27, 28). However, HBsAg alone as a predictor could not fully predict the efficacy (15, 29) nor reflect the liver's immune status (30). Cytokines in serum can indirectly reflect intrahepatic immune response and inflammation (10). Dynamic detection of multiple cytokines can reflect the immune changes more thoroughly to reveal the mechanism of antiviral therapy. However, the association of cytokines with the response to antiviral treatment is yet to be entirely clear. In this study, we found that the slight early increase of

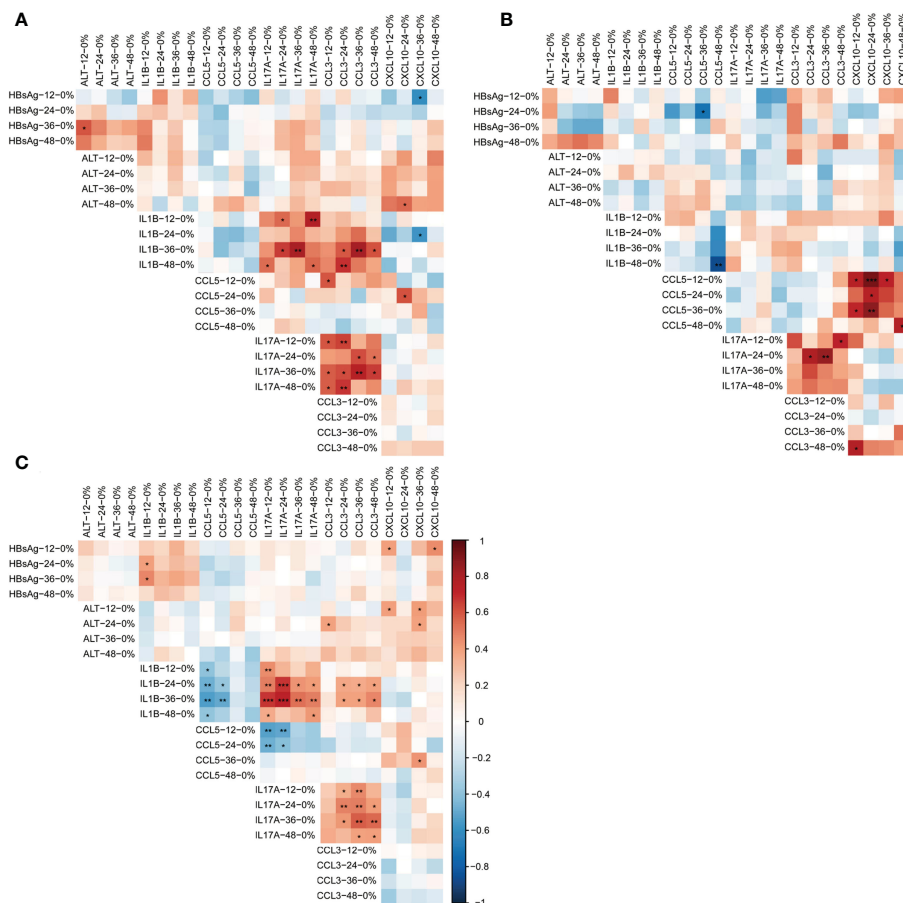


FIGURE 3

Correlation between dynamic changes of HBsAg, ALT and cytokines in different response to the add-on therapy. (A) Correlation between dynamic changes of HBsAg, ALT and cytokines in complete responders (B) Correlation between dynamic changes of HBsAg, ALT and cytokines in partial responders (C) Correlation between dynamic changes of HBsAg, ALT and cytokines in non-responders. * $P < 0.05$; ** $P < 0.01$. HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; Add-on, nucleos(t)ide analogs combined with peg-interferon alpha therapy; complete responder, achieving HBsAg loss at week 48; partial responders: achieving HBsAg decreased by > 1 log from baseline but HBsAg remain positive at week 48; non-responder, achieving HBsAg decreased by < 1 log from baseline and HBsAg remain positive at week 48.

Th1 cytokine (IL2), Th2 cytokine (IL4), Th17 cytokine (IL17A and IL6), and proinflammatory cytokines (IL1B, CCL3, and CXCL8) was associated with HBsAg loss or decrease >1 log in the Add-on group, and sustained high levels of proinflammatory chemokines (CCL5 and CXCL10) were associated with poor response during combination therapy of NAs and PEG-IFN- α .

The incidence of liver cirrhosis and hepatocellular carcinoma is significantly reduced after HBsAg loss, which is the treatment goal of CHB (4, 31, 32). An expert consensus recommends a 1log drop in HBsAg in early treatment as an indication for continued PEG-IFN- α therapy (27). Multiple studies have confirmed that a 1log drop in HBsAg predicts functional cure (33–37). Therefore, we defined good response and partial response as HBsAg loss and decreasing > 1 log, respectively.

In CHB, Th1/Th2 and Th17/Treg were in immune imbalance (30, 38, 39), and antiviral treatment partially restored immune cell function (30). IL2 (Th1 cytokine) activated CD8⁺T cells and NK cells and promoted their proliferation and cytotoxic activity (40–42). NK cells can indirectly regulate T cells by releasing or consuming cytokines (43, 44). Peg-IFN- α increased IL2 activity *in vitro* (45) and enhanced the inhibition of NK cells on regulatory T cell proliferation and differentiation through IFNG (46). IL4 (Th2 cytokine), produced by NKT cells, inhibits HBV RNA and HBsAg production (47). Activated Th17 cells primarily secrete cytokines such as IL17A, IL6, and TNF and also recruit macrophages, neutrophils, and lymphocytes to induce local inflammation (48–51). IL6 (Th17 cytokine) exerts direct antiviral effects by activating the NF- κ B pathway in infected hepatocytes (52, 53). A cross-sectional study found that high levels of IL17A were associated with spontaneous HBsAg loss (48), which is consistent with our study. IL-17 could effectively inhibit HBV replication in a noncytopathic manner (54). IL4 has been confirmed to inhibit the secretion of IL6 and TNF *in vivo* and *in vitro* (55). In this study, IL2, IL4, IL6, and IL17A (Th17 cytokine) in the Add-on group showed the same change trend, increasing at the early stage and decreasing at the later stage, especially in patients with good response, suggesting that the dynamic balance between Th1/Th2/Th17 immunity may play a key role in functional cure under combination therapy.

Especially, the timing of CCL3 and IL1B elevation may be associated with the response of antiviral therapy. During the combination therapy, patients with an increase in IL1B and CCL3 at week 24 did not respond as well as those with an increase at week 12, but the non-responders had almost no significant increase in CCL3. IL1B is a pro-inflammatory and antiviral cytokine (52, 56), produced mainly by inflammatory macrophages in the liver (52). HBV modulates liver macrophage function to impair the production of IL1B to maintain the infection status (52). It is speculated that the late or insignificant increase in IL1B may be a manifestation of an inhibitory immune microenvironment, which is not conducive to the functional cure of CHB.

Compared with the CR and PR groups, CCL5 and CXCL10 were maintained at high levels after the early increase in the NR group during combined treatment. CXCL10 is a potent chemoattractant of activated T cells, leading to immune activation (48). Sonneveld MJ et al. observed that higher levels of CXCL10 before PEG-IFN therapy may be beneficial for obtaining HBsAg loss (57). However, Wong, G.L. et al. reported that lower levels of CXCL10 can predict HBsAg

loss (58), which was consistent with our studies. In fact, 41 HBeAg-positive and 45 HBeAg-negative patients were enrolled and received 48 weeks of PEG-IFN- α plus adefovir. And S B Willemse et al. figured that higher baseline CXCL10 levels appeared to be associated with favorable responses in HBeAg-positive patients but not in HBeAg-negative patients (13). Therefore, the relationship between cytokines and the response may be influenced by HBV replication levels. However, in this study, CXCL10 did not show significant changes during NAs treatment, which may be a manifestation of the lack of an effective immune response.

We noted that the level of IFNG was higher in patients with good responses than in patients with partial responses and poor responses under combination therapy. But patients treated with NAs had higher levels of IFNG and worse responses. This phenomenon may be related to the duration of high levels of IFNG. It has been reported that the presence of long-term IFNG is associated with chronic inflammation and contributes to the occurrence of tumors (59). In the Add-on group, the IFNG level increased and then decreased. It is suggested that long-term high levels of pro-inflammatory cytokines are not conducive to the functional cure, and the dynamic balance between pro-inflammatory and anti-inflammatory cytokines plays a vital role in treating CHB.

CHB is characterized by immunosuppression, decreased anti-inflammatory cytokines expression, and increased anti-inflammatory cytokines expression. The ability of NK cells to secrete cytokines and the HBV-specific immune response was partially restored after the immune tolerance was broken (60). The PEG-IFN- α treatment restored immunity and suppressed the production of immunosuppressive cytokines (61). Li, M. H. et al. (61) enrolled patients with CHB and treated them with NAs and PEG-IFN- α , respectively. It was found that the level of IL10 in both groups increased with time, and the increase was more evident in the NAs group, which was consistent with our study. In our study, the level of IL10 increased in both the NAs and Add-on treatment, especially during NA treatment.

The different correlations of cytokines among patients with different responses to PEG-IFN- α treatment are due to different profiles of cytokine change. Ning Qin et al. found that PEG-IFN- α enhanced the inhibition of NK cells on Treg cells, and this inhibition was associated with a significant decline in HBsAg (46). Nishio, A. et al. found that patients with early NK cell activation after PEG-IFN- α treatment had greater HBsAg decline (62). Our study suggests that PEG-IFN- α treatment induces different immune microenvironment changes, which may be the mechanism of different outcomes. But the underlying mechanism needs further exploration. There was a significant inverse correlation between IL1B and CCL5 in nonresponsive patients. IL1B is a proinflammatory cytokine associated with innate immunity. CCL5 is mainly produced by CD8⁺T cells and can chemotaxis and activate T lymphocytes, dendritic cells, and natural killer cells (63). The negative correlation between the two cytokines in non-responders may indicate that the coordinated interaction between innate and specific immunity contributes to viral clearance.

In summary, our study found that the pattern of serum cytokine dynamics correlates with the response to the sequential combination therapy of PEG-IFN- α and NAs. A mild and harmonious interaction of Th1/Th2/Th17 cytokines is associated with a favorable virological

response, and sustained high levels of certain inflammatory cytokines are not conducive to HBsAg clearance. There were some deficiencies in this study. First, we did not detect the variation in the number and function of cytokine-related immune cells. Second, cytokines and HBsAg in liver tissue were not detected. However, these preliminary results provide clues for further revealing the immune mechanism of antiviral therapy and developing new therapeutic targets.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The study protocol was approved by the Ethics Committee of the Fifth Medical Center of Chinese PLA General Hospital. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JF contributed to conception and design of the study. X-YJ, XL, S-NZ, X-NZ, C-BZ participated in data collection. W-XW performed the statistical analysis. W-XW, RJ wrote the first draft of the manuscript. F-SW and JF revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1121778/full#supplementary-material>

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Dynamic changes of the proportion of HLA-DR and CD38 coexpression subsets on T lymphocytes during IFN-based chronic hepatitis B treatment

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Background: To investigate the changes of human leukocyte antigen DR (HLA-DR) and CD38 coexpression subsets on T lymphocytes following interferon (IFN) therapy for those who have chronic hepatitis B (CHB).

Methods: A prospective cohort of CHB patients participated in this study. CHB patients without IFN treatment (including naïve and nucleoside [nucleotide] analogs [NAs]-treated patients) were given pegylated interferon alfa (Peg-IFN α) treatment. Peripheral blood samples were taken at baseline, 4 weeks and 12–24 weeks of Peg-IFN α treatment. For the patients who entered the Peg-IFN α plateau phase due to the stagnation of the decrease in HBsAg, and Peg-IFN α was discontinued and Peg-IFN α therapy was resumed after an interval of 12–24 weeks. During the interval, they received first-line NAs treatment. Peripheral blood samples were collected at the baseline of the plateau phase, 12–24 weeks of intermittent treatment, and 12–24 weeks of Peg-IFN α retreatment. The peripheral blood samples were taken to determine virological, serological and biochemical indices of hepatitis B virus (HBV), and T lymphocyte related phenotypes were detected using flow cytometry.

Results: In the process of long-term treatment of Peg-IFN α , the percentage of HLA-DR⁺CD38^{dim} subsets increased significantly at first, then decreased gradually, while the percentage of HLA-DR⁺CD38^{hi} subsets markedly increased. During long-term Peg-IFN α treatment, there was a considerable negative correlation between HBsAg and the HLA-DR⁺CD38^{hi} subset

percentage. The persistent high proportion of HLA-DR⁺CD38^{hi} subsets was related to the occurrence of Peg-IFN α plateau phase. After Peg-IFN α intermittent treatment, the percentage of HLA-DR⁺CD38^{hi} subsets decreased significantly. After Peg-IFN α retreatment, the level of HBsAg began to decrease again. At the same time, the percentage of HLA-DR⁺CD38^{hi} subsets significantly increased, but it was still lower than that at the baseline level.

Conclusions: The spectrum of HLA-DR and CD38 coexpression subsets on T lymphocytes changed during the long-term treatment of IFN. The establishment of the IFN plateau phase was linked to the persistence of a considerable proportion of HLA-DR⁺CD38^{hi} subsets on T lymphocytes. IFN intermittent treatment could significantly reduce the proportion of HLA-DR⁺CD38^{hi} subsets, helping regain the antiviral efficacy of IFN during IFN retreatment.

KEYWORDS

chronic hepatitis B, interferon, HLA-DR, CD38, plateau phase, intermittent therapy

Introduction

Millions of people, especially those in China, are infected with the chronic hepatitis B virus (HBV). Around 86 million people in China have chronic HBV infection, including 30 million who have chronic hepatitis B (CHB), and 400 thousand people die from HBV-related causes per year (1, 2). In addition, HBV infection accounts for 77% and 84% of patients with liver cirrhosis and liver cancer in China, respectively (3). The most crucial step in slowing the disease's course and enhancing these patients' long-term prognosis is antiviral medication (4, 5). Two efficient antiviral medications for the treatment of CHB are interferon (IFN) and nucleoside (nucleotide) analogs (NAs). In comparison to NAs, IFN regulates the immune system in addition to acting as a direct antiviral, which plays a more and more important role in antiviral therapy (6–9).

However, although many studies have indicated that long-term IFN antiviral therapy increases hepatitis B surface antigen (HBsAg) clearance, which helps to achieve long-lasting immune control and clinical cure (10, 11). There are still a considerable number of patients who are unable to clear HBsAg from the long-term treatment of IFN. After the patient's HBsAg level plateaued during IFN therapy, the continued use of IFN for antiviral therapy could not further reduce the HBsAg level (9). It is only after intermittent treatment with IFN that HBsAg levels begin to decrease again (12).

Natural killer (NK) lymphocytes, dendritic lymphocytes, and T lymphocytes all participate in the immune system's ability to eliminate HBV, with T lymphocyte activation

playing the most significant role in virus clearance (13–15). Immature T lymphocytes express the transmembrane glycoproteins human leukocyte antigen DR (HLA-DR) and CD38, which are reexpressed during the T lymphocyte immunological response. Therefore, HLA-DR and CD38 expression on T lymphocytes' surface, especially the coexpression of both, can well reflect the state of immune activation (16–18). According to the study, the absolute number of CD8⁺ T lymphocytes declined dramatically while receiving long-term IFN therapy, and they primarily lost subsets that were in the stage of terminal differentiation (19). However, this study did not further explore the dynamic changes of phenotypes and functions related to T lymphocyte activation and depletion during long-term treatment of IFN. In addition, there are few reports on the dynamic changes of T lymphocyte-related phenotypes in IFN intermittent therapy for patients reaching plateau phase.

This study's major goal is to investigate how HLA-DR and CD38 coexpression subsets proportion on T lymphocytes fluctuates dynamically throughout IFN-based treatment of CHB. The findings of this study will aid in explaining why IFN antiviral therapy's efficiency varies at different phases.

Materials and methods

Study design

CHB Patients hospitalized at Beijing Ditan Hospital's Second Department of Hepatology between June 2021 and

August 2022 made up the prospective cohort. According on their course of therapy, the patients were categorized into three groups: The Naïve group consisted of CHB patients who had never undergone antiviral medication, while patients who had only ever undergone first-line NAs antiviral medication made up the NA-treated group. Patients who had previously undergone pegylated interferon alfa (Peg-IFN α) therapy were enrolled in the Plateau group due to the failure of HBsAg to continuously decrease.

In the Naïve group, patients began to receive subcutaneous injection of Peg-IFN α -2a 180 μ g/week, and selected first-line NAs antiviral drugs (entecavir [ETV] 0.5 mg/day or tenofovir disoproxil fumarate [TDF] 300 mg/day or tenofovir alafenamide fumarate [TAF] 25 mg/day) according to their clinical data and personal wishes for combined treatment. Peg-IFN α -2a 180 μ g/week was administered subcutaneously to patients in NA-treated group on the basis of NAs antiviral drugs. Blood samples were taken from patients in Naïve group and NA-treated group at baseline, 4 week and 12-24 weeks after IFN treatment. The HBV virological and serological indices, biochemical indices, and phenotypes associated with T lymphocytes were identified. In the Plateau group, when HBsAg decreased to the plateau phase, the treatment of IFN was suspended and entered into intermittent treatment for 12-24 weeks, during which only ETV (0.5 mg/day) or TDF (300 mg/day) or TAF (25 mg/day) was used to maintain the antiviral efficacy, and after the interval, IFN was retreated on the basis of NAs. Blood samples were taken from patients in Plateau group at the baseline of plateau, during intermittent treatment of 12-24 weeks and during 12-24 weeks of IFN retreatment. The HBV virological and serological indices, biochemical indices, and phenotypes associated with T lymphocytes were identified. In addition, liver ultrasonography was performed every 12-24 weeks at baseline and during the follow-up period.

In addition, the comparison between the Plateau group and its corresponding control group (that is, patients who continued to receive IFN treatment when HBsAg dropped to a plateau) could more accurately portray the advantages of IFN intermittent therapy and the relationship between the changes of T lymphocyte-related surface markers and clinical efficacy during this period. However, patients who HBsAg levels decreased < 1 lg IU/mL after 24 weeks of Peg-IFN α therapy should stop using IFN (3). Even if they remained receiving IFN therapy, patients whose levels of HBsAg plateaued found it challenging to experience a continuous fall in the level of HBsAg, according to our earlier studies (9, 12, 20). Therefore, we did not create a control group for the Plateau group in this study.

The Ethics Committee of Beijing Ditan Hospital, which is a part of Capital Medical University, granted approval for this study (Jing Di Lun Ke Zi 2018 NO. 023-01), and it was registered as a clinical trial (NCT04028856). It was designed and

implemented according to Helsinki standards. All selected patients have signed informed consent.

Inclusion and exclusion criteria

Inclusion criteria (1): 18-65 years old; (2) HBsAg positive lasting more than 6 months (≥ 0.05 IU/mL). In addition, it was also necessary for Naïve group: (1) never received antiviral therapy; it was also necessary for NA-treated group: (1) so far and only take first-line oral antiviral therapy more than 6 months. For Plateau group, it also needed to be satisfied: (1) the level of HBsAg decreased to the plateau period after IFN antiviral treatment for more than 6 months. In order to be considered in a plateau period, the level of HBsAg must have dropped by 0.5 lg IU/mL from the preceding detection time point (more than 3 months) (12).

Exclusion criteria: (1) previously known viral hepatitis (except hepatitis B) and Epstein-Barr virus, cytomegalovirus, human immunodeficiency virus and other non-hepatitis virus infections; (2) complicated with other liver diseases, for instance metabolic-related fatty liver disease, alcoholic hepatitis, autoimmune hepatitis, hepatolenticular degeneration, decompensated cirrhosis and liver cancer; (3) mental illness; (4) severe heart, brain, lung, kidney or other system diseases; (5) usage of other liver-damaging drugs and/or chronic alcoholism; (6) usage of hormones and/or immunosuppressants.

Peripheral blood mononuclear lymphocytes isolation

The peripheral venous blood of the patient was collected at the specified time point and put into the EDTA anticoagulation tube. Using lymphocyte separation solution, peripheral blood mononuclear lymphocytes (PBMC) were isolated by density gradient centrifugation. All samples were processed and analysed within 24 hours after sample collection.

Flow cytometry analysis

PBMCs and direct coupling antibody were incubated together in dark environment at 4°C for 20 minutes. Then 2 mL of 1 $\times$ PBS were added to wash lymphocytes. After rotating 1200 rpm for 5 minutes, the supernatant was discarded. Finally, 200 mL of 1 $\times$ PBS was added to make the pre-test sample. The monoclonal antibodies were anti-human CD4-APC-Fire750 (Clone SK3), CD8-BV510 (Clone SK1), HLA-DR-PE-Cy7 (Clone L243), and CD38⁺APC (Clone HB-7) (BioLegend, San Diego, CA, USA). FACSCanto flow cytometry (BD Biosciences, San Diego, CA, USA) and FlowJo software (Tree Star, Ashland, OR, USA) was used to gather and analyze the data, respectively.

Clinical indicators detection

Roche CobasTaqMan 96 (Roche, Pleasanton, CA, USA), an automatic real-time fluorescence quantitative polymerase chain reaction (PCR) detector, was utilized to detect serum HBV DNA load. Serum HBsAg and hepatitis B e antigen (HBeAg) levels were measured by Abbott Architect i2000 kits (Abbott Diagnostics, Abbott Park, IL, USA), and liver function indexes were detected by Hitachi 7600 automatic biochemical analyzer (Wako Pure Chemical Industries, Tokyo, Japan).

Statistical analysis

SPSS 21.0 (IBM Corporation, Chicago, IL, USA) and GraphPad5 (GraphPad Software, La Jolla, CA, USA) software were used for statistical analysis. The categorical variables were expressed by frequency and percentage, and the chi-square test was used to compare the differences between groups. The normality of continuous variables was evaluated by Shapiro-Wilk test. Continuous variables that were normally distributed were represented by mean $\pm$ standard deviation (SD), and continuous variables that were not normally distributed were represented by the median and inter-quartile range (median, Q1-Q3). According to the normality of the baseline data, One-way ANOVA test and Kruskal-Wallis test were respectively used to compare the multiple groups at baseline, and then Tukey's multiple comparisons test and Dunn's multiple comparisons test were utilized to conduct pairwise comparisons between the groups. To make pairwise comparisons between the groups. The linear mixed effect model (restricted estimation maximum likelihood) was used to analyze the changes of parameters at different time points in each group, and the multiplicity of P value was adjusted by Bonferroni method. Patients were taken as random effects, groups and measurement time points were taken as fixed effects. The standard model was selected by likelihood ratio test. It did not violate the standard model hypothesis. The adjusted model took age and sex as covariables to exclude potential confounding factors. The Spearman correlation test was used to get the P values and correlation coefficient. All meaningful analyses were double-tailed, and $P < 0.05$ was statistically significant.

Result

Patient cohort

This study comprised 150 patients with CHB in total, including 53 patients in Naïve group, 51 patients in NA-treated group and 36 patients in Plateau group. [Table 1](#) displayed the initial demographic data of patients. Then,

according to the wishes of the patients, 37 patients in the Naïve group were willing to start IFN treatment, collecting a total of 93 samples at 3 time points for follow-up detection and analysis, while 13 patients in the NA-treated group were willing to start using IFN on the basis of the original NA treatment, and a total of 33 samples were gathered at 3 different time points for follow-up analysis. 13 Plateau group patients were unable to complete the follow-up examination in our hospital, so a total of 56 samples were gathered from 23 patients at 3 time points for follow-up analysis.

Baseline clinical characteristics of patients

In comparison to both the Naïve group and the NA-treated group, HBsAg amount in the Plateau group was much lower ($P < 0.0001$; $P = 0.0122$). Although the HBsAg level in the NA-treated group was marginally less than that in the Naïve group, there was no markedly significant variation ($P = 0.0680$). HBV DNA load was considerably higher in the Naïve group compared to the NA-treated group and Plateau group, respectively ($P < 0.0001$; $P < 0.0001$), but there was no remarkable variation in HBV DNA level between NA-treated and Plateau group ($P = 0.9019$). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in NA-treated group were notably lower than those in Naïve group and Plateau group respectively (ALT: $P = 0.0015$; $P < 0.0001$; AST: $P = 0.0106$; $P = 0.0395$), but there was no discernible variation in terms of ALT and AST levels between Naïve group and Plateau group (ALT: $P = 0.3170$; AST: $P > 0.9999$), as shown in [Table 1](#).

Comparison HLA-DR and CD38 coexpression subsets on CD4⁺ and CD8⁺ T lymphocytes between groups at baseline

In order to determine the level of T lymphocyte activation under different treatment conditions, we analyzed the coexpression of HLA-DR and CD38, representing the key T lymphocyte activation indicator. We defined three subsets in activated HLA-DR⁺ CD4⁺ T and CD8⁺ T lymphocytes based on CD38 expression: HLA-DR⁺CD38⁻ subgroup, HLA-DR⁺CD38^{dim} subgroup and HLA-DR⁺CD38^{hi} subgroup ([Figure 1](#)). The percentage of HLA-DR⁺CD38⁻ CD4⁺ T lymphocytes in Plateau group was much less than that in Naïve group and NA-treated group ($P = 0.0075$; $P < 0.0001$). In the NA-treated group, more HLA-DR⁺CD38⁻ CD4⁺ T lymphocytes were present than those in the Naïve group ($P = 0.0289$). HLA-DR⁺CD38^{dim} CD4⁺ T lymphocyte percentage in Plateau group was considerably greater than that in Naïve and

TABLE 1 Comparison of baseline characteristics of the study population^a.

Variables	Naïve group (n=53)	NA-treated group (n=51)	Plateau group (n=36)	<i>P</i>	<i>P</i> (Naïve vs. NA-treated)	<i>P</i> (Naïve vs. Plateau)	<i>P</i> (NA-treated vs. Plateau)
Age (years)	34.00 (31.50, 40.50)	42.00 (30.00, 50.00)	36.00 (32.25, 44.50)	0.0710	/	/	/
Man (%)	35 (66.04)	34 (66.67)	24 (66.67)	0.9971	/	/	/
Patient of HBeAg positive (%)	32 (60.38)	25 (49.02)	17 (47.22)	0.3747	/	/	/
HBsAg (log10 IU/mL)	3.65 ± 1.21	3.10 ± 0.86	2.34 ± 1.42	<0.0001	0.0680	<0.0001	0.0122
HBV DNA (log10 IU/mL)	4.94 ± 2.97	1.04 ± 1.28	0.84 ± 0.60	<0.0001	<0.0001	<0.0001	0.9019
ALT (U/L)	45.70 (31.40, 50.30)	22.00 (16.25, 25.50)	48.50 (45.00, 51.00)	<0.0001	0.0015	0.3170	<0.0001
AST (U/L)	31.30 (18.95, 50.90)	23.00 (19.00, 28.00)	28.95 (21.63, 39.58)	0.0060	0.0106	>0.9999	0.0395
HLA-DR ⁺ CD38 ⁻ CD4 ⁺ T lymphocytes (%)	4.16 (2.57, 6.35)	5.46 (3.82, 6.79)	3.04 (2.59, 3.37)	<0.0001	0.0289	0.0075	<0.0001
HLA-DR ⁺ CD38 ^{dim} CD4 ⁺ T lymphocytes (%)	2.79 (2.04, 4.15)	2.66 (1.91, 3.84)	5.90 (3.52, 9.49)	<0.0001	>0.9999	<0.0001	<0.0001
HLA-DR ⁺ CD38 ^{hi} CD4 ⁺ T lymphocytes (%)	0.38 (0.25, 0.62)	0.38 (0.26, 0.55)	3.11 (2.70, 3.67)	<0.0001	>0.9999	<0.0001	<0.0001
HLA-DR ⁺ CD38 ⁻ CD8 ⁺ T lymphocytes (%)	5.90 (2.64, 8.14)	11.70 (7.70, 15.20)	1.55 (0.93, 2.89)	<0.0001	0.0002	<0.0001	<0.0001
HLA-DR ⁺ CD38 ^{dim} CD8 ⁺ T lymphocytes (%)	9.05 (5.70, 13.35)	9.58 (5.93, 12.80)	5.02 (3.49, 7.41)	<0.0001	>0.9999	0.0003	<0.0001
HLA-DR ⁺ CD38 ^{hi} CD8 ⁺ T lymphocytes (%)	1.87 (0.95, 3.10)	1.51 (1.03, 2.37)	21.00 (16.07, 38.08)	<0.0001	>0.9999	<0.0001	<0.0001

NA, nucleoside (nucleotide) analogs; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HLA-DR, human leukocyte antigen DR; SD, standard deviation; Q1, lower quartile; Q3, upper quartile.

^aValues are expressed as No. (%) or mean ± SD or median (Q1, Q3).

NA-treated group ($P < 0.0001$; $P < 0.0001$), but no discernible variation was observed between Naïve and NA-treated group ($P > 0.9999$). The proportion of HLA-DR⁺CD38^{hi} CD4⁺ T lymphocytes in Plateau group was considerably greater than that in Naïve and NA-treated group ($P < 0.0001$; $P < 0.0001$), but there was no discernible variation in the percentage of HLA-DR⁺CD38^{hi} CD4⁺ T lymphocytes between Naïve group and NA-treated group ($P > 0.9999$). HLA-DR⁺CD38^{dim} CD8⁺ T lymphocyte percentage in Plateau group was noticeably less than that in Naïve group and NA-treated group, respectively ($P = 0.0003$; $P < 0.0001$), however there was also no discernible variation in HLA-DR⁺CD38^{dim} CD8⁺ T lymphocyte level between Naïve group and NA-treated group ($P > 0.9999$). The difference trend of HLA-DR⁺CD38⁻ CD8⁺ T lymphocytes and HLA-DR⁺CD38^{hi} CD8⁺ T lymphocytes in different groups was similar to that of CD4⁺ T lymphocytes. HLA-DR⁺CD38⁻ CD8⁺ T lymphocyte percentage in Plateau group was considerably lower than that in Naïve and NA-treated group ($P < 0.0001$; $P < 0.0001$). In NA-treated group, HLA-DR⁺CD38⁻ CD8⁺ T lymphocyte percentage was noticeably more than that in Naïve

group ($P = 0.0002$). In comparison to the Plateau group, the HLA-DR⁺CD38^{hi} CD8⁺ T lymphocyte percentage was considerably less in Naïve group and NA-treated group ($P < 0.0001$; $P < 0.0001$). HLA-DR⁺CD38^{hi} CD8⁺ T lymphocyte proportion was not markedly different between Naïve group and NA-treated group ($P > 0.9999$), as shown in Table 1. Our data initially showed that the phenomenon that the antiviral efficacy of patients with long-term use of IFN could not continue was related to the change of HLA-DR and CD38 coexpression subsets percentage on CD4⁺ and CD8⁺ T lymphocytes after long-term IFN treatment.

Comparison HLA-DR and CD38 coexpression subsets on CD4⁺ and CD8⁺ T lymphocytes during IFN treatment

In order to further explore the dynamic changes in HLA-DR and CD38 coexpression subsets on CD4⁺ and CD8⁺ T lymphocytes during IFN therapy, we followed up these patients who chose to

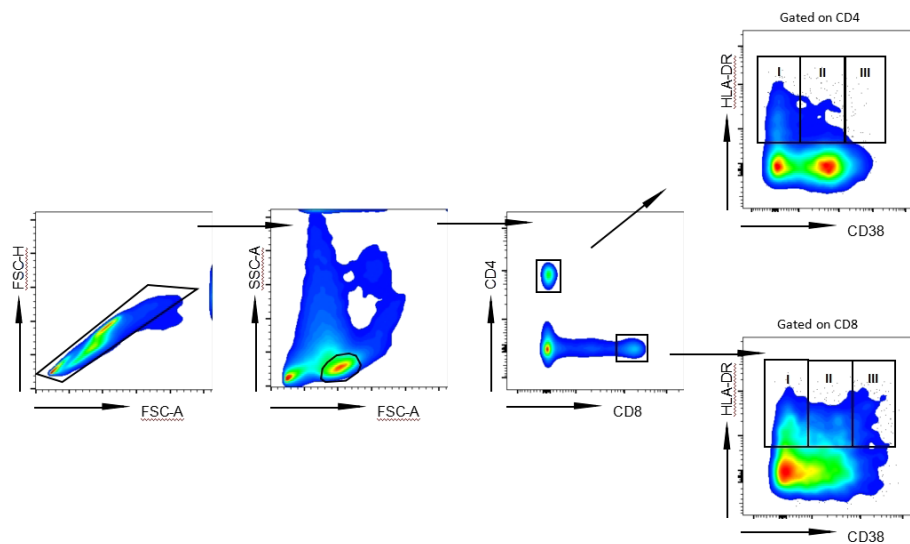


FIGURE 1

Representative flow cytometry gating strategy for HLA-DR⁺ subgroups on CD4⁺ and CD8⁺ T lymphocytes. Representative FACS pseudocolor showed three subpopulations of HLA-DR⁺ according to the expression of CD38 on CD4⁺ T lymphocytes and CD8⁺ T lymphocytes from CHB patient: HLA-DR⁺CD38⁻ (I), HLA-DR⁺CD38^{dim} (II) and HLA-DR⁺CD38^{hi} (III).

start antiviral therapy with IFN in Naïve group and NA-treated group. Compared with those before IFN treatment, there was no noteworthy variation in the percentage of HLA-DR⁺CD38⁻ CD4⁺ T lymphocytes in Naïve group and NA-treated group after IFN treatment (Naïve group: 0 week vs. 4 week $P > 0.9999$; 0 week vs. 12-24 week $P = 0.7210$; NA-treated group: 0 week vs. 4 week $P > 0.9999$; 0 week vs. 12-24 week $P > 0.9999$). In both Naïve group and NA-treated group, HLA-DR⁺CD38^{dim} CD4⁺ T lymphocytes proportion increased considerably after 4 weeks of IFN treatment ($P = 0.0020$; $P = 0.0010$). HLA-DR⁺CD38^{dim} CD4⁺ T lymphocyte percentage in the Naïve group after 12-24 weeks of IFN treatment did not differ dramatically from that after 4 weeks of IFN treatment ($P = 0.2020$), but the HLA-DR⁺CD38^{dim} CD4⁺ T lymphocyte percentage in NA-treated group was dramatically less than that in IFN treatment for 4 weeks ($P = 0.0090$). The change trend in HLA-DR⁺CD38^{hi} CD4⁺ T lymphocyte percentage was consistent in Naïve group and NA-treated group during the treatment of IFN. At the beginning of treatment, HLA-DR⁺CD38^{hi} CD4⁺ T lymphocyte percentage in both groups increased compared with the baseline, but there was no discernible variation ($P = 0.3500$; $P = 0.5110$). In the late stage of IFN treatment, HLA-DR⁺CD38^{hi} CD4⁺ T lymphocyte percentage in both groups was dramatically higher than that at baseline ($P < 0.0001$; $P < 0.0001$). In Naïve group and NA-treated group, the proportion of HLA-DR⁺CD38⁻ CD8⁺ T lymphocytes decreased gradually with the increase of IFN treatment time (Naïve group: 0 week vs. 4 week $P = 0.0170$; 0 week vs. 12-24 week $P < 0.0001$; NA-treated group: 0 week vs. 4 week $P = 0.0010$; 0 week vs. 12-24 week $P < 0.0001$). In both Naïve group and NA-treated group, the proportion of HLA-DR⁺CD38^{dim} CD8⁺ T

lymphocytes increased notably after 4 weeks of IFN treatment ($P < 0.0001$; $P < 0.0001$). After 12-24 weeks of IFN treatment, the percentage of HLA-DR⁺CD38^{dim} CD8⁺ T lymphocytes decreased notably compared with that of IFN treatment for 4 weeks in both groups ($P < 0.0001$; $P < 0.0001$). Moreover, with the increase of IFN treatment, the change trend of HLA-DR⁺CD38^{hi} CD8⁺ T lymphocyte percentage in Naïve group and NA-treated group was similar, which increased significantly with the duration of treatment (Naïve group: 0 week vs. 4 week $P < 0.0001$; 0 week vs. 12-24 week $P < 0.0001$; NA-treated group: 0 week vs. 4 week $P < 0.0001$; 0 week vs. 12-24 week $P < 0.0001$) (Table 2).

The aggregate data showed that there was no significant change in HLA-DR⁺CD38⁻ on CD4⁺ T lymphocytes in both groups during the treatment of IFN, but the proportion of CD8⁺ T lymphocytes decreased gradually. Except that the percentage of HLA-DR⁺CD38^{dim} CD4⁺ T lymphocytes in Naïve group did not change significantly at the later stage of IFN treatment, the changes of other HLA-DR⁺CD38^{dim} subsets on CD4⁺ and CD8⁺ T lymphocytes in both groups increased markedly first and then decreased gradually. Meanwhile, the proportion of HLA-DR⁺CD38^{hi} on both CD4⁺ and CD8⁺ T lymphocytes increased significantly as the increase of IFN treatment time.

Comparison of clinical characteristics during IFN treatment

In Naïve group and NA-treated group, the HBsAg levels of all samples collected at 12-24 weeks after IFN treatment

TABLE 2 The changes of indicators in each group during IFN treatment^a.

Variables	Naïve group			<i>P</i> (0 week vs. 4 week)	<i>P</i> (0 week vs. 12-24 week)	<i>P</i> (4 week vs. 12-24 week)	NA-treated group			<i>P</i> (0 week vs. 4 week)	<i>P</i> (0 week vs. 12-24 week)	<i>P</i> (4 week vs. 12-24 week)
	0 week (Baseline) (n=37)	4 week (IFN treat- ment) (n=34)	12-24 week (IFN treat- ment) (n=22)				0 week (Baseline) (n=13)	4 week (IFN treat- ment) (n=10)	12-24 week (IFN treat- ment) (n=10)			
HBsAg (log10 IU/ mL)	3.60 ± 1.29	/	2.79 ± 1.26	/	0.0010	/	3.48 ± 1.05	/	2.47 ± 1.06	/	0.0270	/
HBV DNA (log10 IU/mL)	5.19 ± 2.92	/	1.05 ± 0.66	/	<0.0001	/	1.98 ± 1.33	/	1.28 ± 0.94	/	0.3240	/
ALT (U/L)	48.86 ± 37.83	72.16 ± 45.35	47.53 ± 34.32	0.0250	>0.9999	0.0380	29.89 ± 14.27	72.39 ± 43.46	31.80 ± 8.28	0.0310	>0.9999	0.0420
AST (U/L)	33.19 ± 20.56	49.84 ± 26.34	43.05 ± 31.17	0.0080	0.8370	0.3090	24.04 ± 6.50	50.90 ± 17.35	34.00 ± 17.68	0.0420	>0.9999	0.4570
HLA-DR ⁺ CD38 ⁻ CD4 ⁺ T lymphocytes (%)	4.01 ± 2.19	4.02 ± 2.45	4.62 ± 2.14	>0.9999	0.7210	0.5740	4.52 ± 2.20	4.33 ± 2.38	4.07 ± 1.83	>0.9999	>0.9999	>0.9999
HLA-DR ⁺ CD38 ^{dim} CD4 ⁺ T lymphocytes (%)	3.42 ± 2.03	4.66 ± 2.03	5.50 ± 1.79	0.0020	<0.0001	0.2020	3.00 ± 1.41	5.34 ± 3.09	3.23 ± 0.79	0.0010	>0.9999	0.0090
HLA-DR ⁺ CD38 ^{hi} CD4 ⁺ T lymphocytes (%)	0.44 ± 0.27	0.79 ± 0.47	2.32 ± 2.03	0.3500	<0.0001	<0.0001	0.36 ± 0.23	0.87 ± 0.60	2.07 ± 0.12	0.5110	<0.0001	0.0110
HLA-DR ⁺ CD38 ⁻ CD8 ⁺ T lymphocytes (%)	5.59 ± 4.42	3.71 ± 2.50	2.22 ± 0.59	0.0170	<0.0001	0.0640	11.14 ± 7.12	6.10 ± 2.72	4.62 ± 2.98	0.0010	<0.0001	0.0160
HLA-DR ⁺ CD38 ^{dim} CD8 ⁺ T lymphocytes (%)	10.40 ± 7.94	14.89 ± 6.04	7.79 ± 3.35	<0.0001	0.0940	<0.0001	9.61 ± 4.57	17.62 ± 2.27	8.06 ± 3.12	<0.0001	>0.9999	<0.0001
HLA-DR ⁺ CD38 ^{hi} CD8 ⁺ T lymphocytes (%)	2.23 ± 1.95	7.69 ± 6.39	18.57 ± 9.74	<0.0001	<0.0001	<0.0001	2.08 ± 1.11	11.8 ± 2.98	20.02 ± 4.23	<0.0001	<0.0001	0.0020

NA, nucleoside (nucleotide) analogs; HBsAg, hepatitis B surface antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HLA-DR, human leukocyte antigen DR; SD, standard deviation.

^aValues are expressed as mean ± SD.

decreased more than 0.5 lg IU/mL compared with their respective treatment baselines. In Naïve group, compared with the baseline, HBsAg and HBV DNA levels declined considerably at 12–24 weeks after IFN treatment ($P = 0.0010$; $P < 0.0001$). At 12–24 weeks after IFN treatment, the level of HBsAg in NA-treated group declined markedly when compared to the baseline ($P = 0.0270$), but HBV DNA load had no significant change compared with the baseline ($P = 0.3240$). ALT and AST levels in Naïve group and NA-treated group were markedly higher than their respective baselines early in the course of IFN treatment (ALT: $P = 0.0250$; $P = 0.0310$; AST: $P = 0.0080$; $P = 0.0420$), but there was no discernible change compared with the baseline in the late stage of IFN therapy (ALT: $P > 0.9999$; $P > 0.9999$; AST: $P = 0.8370$; $P > 0.9999$), as shown in Table 2.

Correlations between HBsAg and HLA-DR and CD38 coexpression subsets on CD4⁺ and CD8⁺ T lymphocytes during IFN treatment

Then, we used the longitudinal data collected from all patients in both groups to analyze the correlation between HBsAg and HLA-DR and CD38 coexpression subsets on CD4⁺ and CD8⁺ T lymphocytes (Figure 2). The proportion in HLA-DR⁺CD38^{hi} CD4⁺ T lymphocytes and HLA-DR⁺CD38^{hi} CD8⁺ T lymphocytes was adversely linked with HBsAg level, respectively ($R = -0.3298$, $P = 0.0025$; $R = -0.3495$, $P = 0.0013$). HLA-DR⁺CD38⁻ and HLA-

DR⁺CD38^{dim} subset proportions on CD4⁺ and CD8⁺ T lymphocytes were not linked with HBsAg level. Collectively, these findings suggested that when the patients did not enter the plateau phase, the proportion of HLA-DR⁺CD38^{hi} CD4⁺ T lymphocytes and HLA-DR⁺CD38^{hi} CD8⁺ T lymphocytes increased gradually with the decrease of HBsAg level during IFN treatment.

Changes in HLA-DR and CD38 coexpression subsets on CD4⁺ and CD8⁺ T lymphocytes during IFN intermittent treatment

In order to further explore whether the persistent high proportion of HLA-DR⁺CD38^{hi} subset on CD4⁺ and CD8⁺ T lymphocytes played a role in the plateau phase following long-term treatment of IFN, we continued to analyze the data of patients in the Plateau group after IFN intermittent treatment and IFN retreatment. Our data showed that after 12–24 weeks of IFN intermittent therapy, the proportion of HLA-DR⁺CD38⁻ CD4⁺ T lymphocytes ($P < 0.0001$) and HLA-DR⁺CD38^{dim} CD4⁺ T lymphocytes ($P < 0.0001$) increased significantly compared with the plateau baseline, while the HLA-DR⁺CD38^{hi} CD4⁺ T lymphocyte percentage decreased significantly compared with the plateau baseline ($P < 0.0001$). After 12–24 weeks of IFN intermittent treatment, the corresponding subsets of CD8⁺ T lymphocytes also had a similar pattern. Compared with the plateau baseline, the proportion of HLA-DR⁺CD38⁻ CD8⁺ T

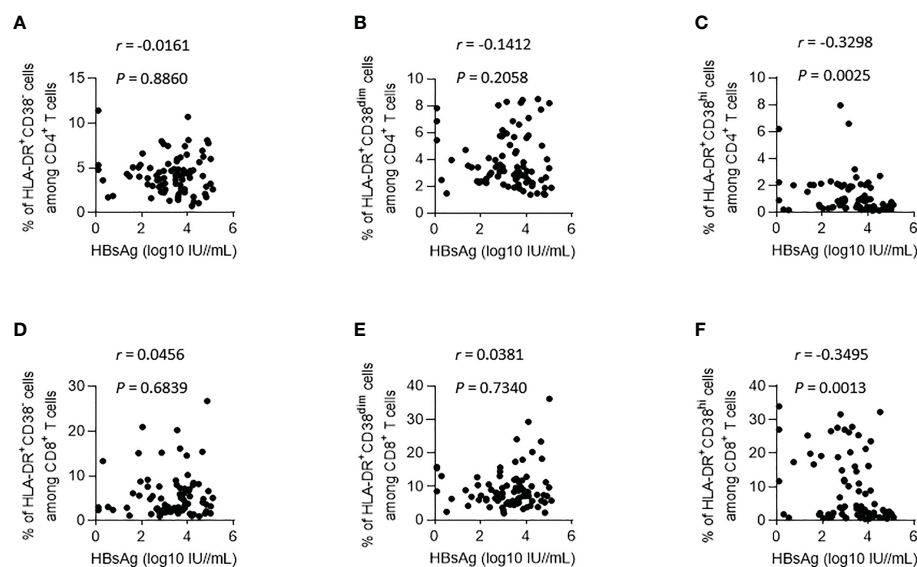


FIGURE 2

Correlations between HBsAg and HLA-DR⁺ subgroups (according to the expression of CD38) on CD4⁺ and CD8⁺ T lymphocytes during IFN Treatment. (A–C) Correlations between HBsAg and HLA-DR⁺CD38⁻ (A), HLA-DR⁺CD38^{dim} (B) and HLA-DR⁺CD38^{hi} (C) on CD4⁺ T lymphocytes. (D–F) Correlations between HBsAg and HLA-DR⁺CD38⁻ (D), HLA-DR⁺CD38^{dim} (E) and HLA-DR⁺CD38^{hi} (F) on CD8⁺ T lymphocytes.

lymphocytes ($P < 0.0001$) and HLA-DR⁺CD38^{dim} CD8⁺ T lymphocytes ($P < 0.0001$) increased significantly, while the proportion of HLA-DR⁺CD38^{hi} CD8⁺ T lymphocytes decreased markedly ($P < 0.0001$) after IFN intermittent treatment with 12-24 weeks. In comparison to IFN intermittent treatment, the proportion of HLA-DR⁺CD38^{dim} CD4⁺ T lymphocytes and HLA-DR⁺CD38^{dim} CD4⁺ T lymphocytes decreased markedly after 12-24 weeks of IFN retreatment ($P = 0.0010$; $P < 0.0001$). However, compared with plateau baseline, there were no noteworthy variation in both the proportion of HLA-DR⁺CD38^{dim} CD4⁺ T lymphocytes and HLA-DR⁺CD38^{dim} CD4⁺ T lymphocytes after IFN retreatment ($P > 0.9999$; $P = 0.6840$). After 12-24 weeks of IFN retreatment, HLA-DR⁺CD38^{hi} CD4⁺ T lymphocyte percentage was considerably more than that after IFN intermittent treatment ($P = 0.0130$), but dramatically lower than that at the plateau baseline ($P = 0.0180$). Likewise, HLA-DR⁺CD38^{dim} and HLA-DR⁺CD38^{hi} subsets proportion on CD8⁺ T lymphocytes after 12-24 weeks of IFN retreatment was greatly lower than that after IFN intermittent treatment, respectively ($P < 0.0001$; $P < 0.0001$), but there was no discernible change compared with the plateau baseline ($P > 0.9999$; $P > 0.9999$). Furthermore, HLA-DR⁺CD38^{hi} CD8⁺ T lymphocyte proportion increased considerably following IFN retreatment compared to

IFN intermittent treatment. ($P = 0.0060$), but considerably lower than that at plateau baseline ($P = 0.0160$) (Table 3).

Changes of clinical characteristics during IFN intermittent treatment

There was no significant variation in HBsAg level in Plateau group before and after IFN intermittent treatment ($P > 0.9999$). HBsAg level of each sample collected after IFN retreatment in Plateau group declined more than 0.5 lg IU/mL compared with that after IFN intermittent treatment, which was statistically noteworthy ($P = 0.0030$), and considerably lower than that at plateau baseline as well ($P = 0.0060$). During the IFN intermittent treatment and IFN retreatment, HBV DNA load in Plateau group had no significant change (0 week vs. 12-24 week IFN treatment interval $P = 0.6950$; 12-24 week IFN treatment interval vs. 12-24 week IFN retreatment $P > 0.9999$). In comparison to the baseline of plateau phase, ALT ($P < 0.0001$) and AST ($P < 0.0001$) levels in Plateau group decreased significantly after IFN intermittent treatment, while compared with those after IFN intermittent treatment, ALT ($P < 0.0001$) and AST levels ($P < 0.0001$) increased considerably in Plateau group after IFN retreatment, as shown in Table 3.

TABLE 3 The changes of indicators in Plateau group during IFN intermittent treatment and IFN retreatment^a.

Variables	0 week (Baseline) (n=23)	12-24 week (IFN treatment interval) (n=15)	12-24 week (IFN retreatment) (n=18)	<i>P</i> (0 week vs. 12-24 week IFN treatment interval)	<i>P</i> (0 week vs. 12- 24 week IFN retreatment)	<i>P</i> (12-24 week IFN treatment interval vs. 12-24 week IFN retreatment)
HBsAg (log10 IU/mL)	2.25 ± 1.46	2.32 ± 1.46	1.25 ± 1.29	>0.9999	0.0060	0.0030
HBV DNA (log10 IU/mL)	0.77 ± 0.65	0.52 ± 0.50	0.60 ± 0.57	0.6950	>0.9999	>0.9999
ALT (U/L)	50.39 ± 8.52	21.61 ± 6.95	52.00 ± 11.60	<0.0001	>0.9999	<0.0001
AST (U/L)	33.72 ± 12.56	15.68 ± 1.54	36.25 ± 4.22	<0.0001	>0.9999	<0.0001
HLA-DR ⁺ CD38 ^{dim} CD4 ⁺ T lymphocytes (%)	5.18 ± 3.22	9.11 ± 6.90	6.48 ± 3.77	<0.0001	>0.9999	0.0010
HLA-DR ⁺ CD38 ^{dim} CD4 ⁺ T lymphocytes (%)	6.95 ± 4.45	9.35 ± 3.45	6.86 ± 3.83	<0.0001	0.6840	<0.0001
HLA-DR ⁺ CD38 ^{hi} CD4 ⁺ T lymphocytes (%)	3.35 ± 0.80	0.32 ± 0.25	2.00 ± 2.59	<0.0001	0.0180	0.0130
HLA-DR ⁺ CD38 ^{dim} CD8 ⁺ T lymphocytes (%)	2.02 ± 2.02	6.46 ± 3.16	1.90 ± 1.36	<0.0001	>0.9999	<0.0001
HLA-DR ⁺ CD38 ^{hi} CD8 ⁺ T lymphocytes (%)	5.18 ± 2.77	26.93 ± 21.05	4.94 ± 1.85	<0.0001	>0.9999	<0.0001
HLA-DR ⁺ CD38 ^{hi} CD8 ⁺ T lymphocytes (%)	25.73 ± 15.58	3.13 ± 1.73	15.89 ± 7.53	<0.0001	0.0160	0.0060

HBsAg, hepatitis B surface antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HLA-DR, human leukocyte antigen DR; SD, standard deviation.

^aValues are expressed as mean ± SD.

Discussion

The coexpression of CD38 and HLA-DR was associated with T lymphocyte activation. Many studies have shown HLA-DR⁺CD38⁺ T lymphocytes played an effective role in immune activation and virus clearance (21, 22). However, some studies (23, 24) have illustrated that activated HLA-DR⁺CD38⁺ T lymphocytes were closely related to the severity of the disease. Du et al. (25) further explored the HLA-DR⁺CD38⁺ on T lymphocytes in patients with acute coronavirus disease 2019 infection, and it was found that there were two heterogeneous subsets of HLA-DR⁺CD38⁺ on T lymphocytes: HLA-DR⁺CD38^{dim} and HLA-DR⁺CD38^{hi}. HLA-DR⁺CD38^{dim} subsets expressed low level of inhibition checkpoints and strong cytotoxic potential, and were less sensitive to apoptosis. HLA-DR⁺CD38^{hi} subsets have been proved to be in a state of immune disorder of overactivation or depletion, and the cytotoxic function was impaired. Although patients with CHB had a chronic virus infection, IFN could activate immune lymphocytes, enhancing their secretion of cytokines and killing function (26). Therefore, we also focused on how HLA-DR and CD38 coexpression subsets changed dynamically on T lymphocytes following IFN-based CHB therapy.

CD4⁺ T lymphocytes helped activate B lymphocytes to produce neutralizing antibodies and induced cytotoxic T lymphocytes (CTL) responses, while CD8⁺ T lymphocytes contributed to the generation of antiviral cytokines, such as IFN- γ and tumor necrosis factor- α , and could differentiate into CTL lymphocytes to exert cytotoxic activity to clear virus-infected hepatocytes (27). Through the comparison of the proportion of CD4⁺ T and CD8⁺ T subsets in three groups of patients under different treatment background at the baseline, it could be found that for both CD4⁺ T and CD8⁺ T lymphocytes, long-term IFN treatment significantly decreased the proportion of HLA-DR⁺CD38^{dim} subsets and dramatically increased the proportion of HLA-DR⁺CD38^{hi} subsets. However, the results of HLA-DR⁺CD38^{dim} subsets in CD4⁺ and CD8⁺ T lymphocytes were slightly different. At this time, the dynamic changes of subsets in Naïve and NA-treated group during IFN treatment were analyzed, and it was found that the proportion of HLA-DR⁺CD38^{dim} CD8⁺ T lymphocytes decreased gradually during IFN treatment, but the change in HLA-DR⁺CD38^{dim} CD4⁺ T lymphocyte percentage during treatment was not significant. Actually, this abnormal phenomenon might be associated with the following reasons: it could be seen from the data of this study that the percentage change of each subgroup in CD4⁺ T lymphocytes was slighter than that in CD8⁺ T lymphocytes during IFN treatment. In the course of IFN treatment, HLA-DR⁺CD38^{dim} CD4⁺ T lymphocyte proportion in NA-treated group decreased gradually, although there was no statistical difference. In addition, it might also be related to the large dispersion of samples. In the course of IFN treatment, the change trend of HLA-DR⁺CD38^{dim} subsets which played a positive function of virus clearance was found to be significantly increased at first, and then gradually decreased. This trend was most typical in

CD8⁺ T lymphocytes, which was consistent with earlier studies (26, 28). During IFN treatment in Naïve group, and the comparison between three groups at first at the baseline, the abnormal phenomenon of HLA-DR⁺CD38^{dim} CD4⁺ T lymphocyte percentage could be explained as the long-term treatment of IFN eventually led to the decrease of HLA-DR⁺CD38^{dim} subgroup percentage on T lymphocytes, which was based on the increase of the percentage of this subgroup in the early stage of IFN treatment. At this time, reduced HLA-DR⁺CD38^{dim} CD4⁺ T lymphocyte subsets percentage was still higher than that at baseline. The proportion of overactivated and exhausted HLA-DR⁺CD38^{hi} subsets increased significantly during IFN treatment for both CD4⁺ and CD8⁺ T lymphocytes.

Moreover, HBsAg level in Naïve group and NA-treated group decreased significantly during IFN treatment. ALT and AST levels increased significantly in the initial stage of IFN treatment, which was linked with the enhancement of T lymphocyte immunological performance and NK lymphocyte activity after IFN treatment, indicating a better antiviral effect (29). The correlation between virological index and the proportion of HLA-DR and CD38 coexpression subsets on T lymphocytes was analyzed. The results revealed that when the patients did not reach the plateau stage of IFN, the level of HBsAg was significantly linked with the proportion of HLA-DR⁺CD38^{hi} subsets. With the extension of IFN treatment time, the proportion of HLA-DR⁺CD38^{hi} subsets of both CD4⁺ and CD8⁺ T lymphocytes would gradually increase as the HBsAg level fell. In summary, our research showed that in the process of IFN treatment, the percentage of HLA-DR⁺CD38^{dim} subsets that played a positive virus clearance function was significantly increased in the initial stage of IFN treatment, which was helpful to quickly eliminate the virus and reduce the level of HBsAg. At the same time, HLA-DR⁺CD38^{dim} subsets gradually differentiated into HLA-DR⁺CD38^{hi} subsets, which represented depletion. In the late course of IFN treatment, HLA-DR⁺CD38^{dim} subset percentage decreased, while the percentage of HLA-DR⁺CD38^{hi} subsets increased gradually.

The plateau phase occurred after long-term treatment of IFN, which meant that the antiviral effect of IFN could not be continued. Some studies have shown that it might be related to the gradual decrease of IFN receptor expression (30), the production of endogenous IFN antibodies (31) which decreased or eliminated IFN efficacy or affected the normal clearance and degradation of IFN, and the significant decrease of the absolute number of CD8⁺ T lymphocytes (19). Whether the occurrence of the IFN plateau phase and the effectiveness of intermittent therapy can also be explained by the dynamic changes of HLA-DR and CD38 coexpression subsets during the long-term treatment of IFN is worthy of further discussion. Surprisingly, compared with the baseline of IFN plateau, after pausing the use of IFN for 12-24 weeks, the subsets of HLA-DR⁺CD38^{dim} and HLA-DR⁺CD38^{hi} on CD4⁺ and CD8⁺ T lymphocytes elevated significantly, while HLA-DR⁺CD38^{hi} subsets decreased significantly. After IFN retreatment with 12-24 weeks, HLA-DR⁺CD38^{dim} and HLA-DR⁺CD38^{hi} subsets decreased significantly, while HLA-DR⁺CD38^{hi} subsets increased

significantly, which was consistent with the above demonstration. Before and after retreatment of IFN, the level of HBsAg declined significantly, while the level of ALT and AST elevated significantly, which also meant that IFN began to exert its antiviral effect and further enhance the function of immune lymphocytes (6, 29). Meanwhile, even if IFN began to restore antiviral efficacy, there was no noticeable variation in the percentage of HLA-DR⁺CD38⁺ and HLA-DR⁺CD38^{dim} subsets on CD4⁺ and CD8⁺ T lymphocytes after IFN retreatment compared with the baseline of IFN plateau phase. At this time, the proportion of HLA-DR⁺CD38^{hi} subsets on CD4⁺ and CD8⁺ T lymphocytes was dramatically lower than that at the baseline of plateau phase, respectively. In a word, the persistent high proportion of HLA-DR⁺CD38^{hi} subsets on CD4⁺ and CD8⁺ T lymphocytes was related to the occurrence of IFN plateau phase. After IFN intermittent treatment, the proportion of HLA-DR⁺CD38^{hi} subsets decreased, suggesting that the immune disorder of T lymphocyte overactivation may be restored. During the IFN retreatment, the proportion of HLA-DR⁺CD38^{hi} subsets increased gradually, but it did not reach the higher level compared with the baseline of plateau phase. Therefore, IFN could continue to play an antiviral role and HBsAg began to decline again.

Our study had several drawbacks, including a small sample size and lack of enrollment of patients who participated in the whole period from initial IFN treatment to the IFN plateau stage. More importantly, the exact functional properties of HLA-DR⁺CD38^{dim} and HLA-DR⁺CD38^{hi} subsets on T lymphocytes could not be determined without further functional verification. Therefore, more evidence is needed to verify the role of HLA-DR and CD38 coexpression subsets on T lymphocytes when treating CHB using IFN.

Conclusion

In summary, our study demonstrated that the HLA-DR and CD38 coexpression subsets on T lymphocytes dynamically changed over the course of receiving long-term IFN treatment. The appearance of IFN plateau phase was related to the persistent high proportion of HLA-DR⁺CD38^{hi} subsets on T lymphocytes. IFN intermittent treatment could significantly reduce the proportion of HLA-DR⁺CD38^{hi} subsets, helping regain the antiviral efficacy of IFN during IFN retreatment.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of Beijing Ditan Hospital

Affiliated with Capital University of Medical Sciences. The patients/participants provided their written informed consent to participate in this study.

Author contributions

RH, ML and YX contributed to study concept and design. YLi, XB and LY conducted experiments and collected the data. YLi, GS, SX, HL, XB and LY collected the information of the patients. TJ, WD, SWa, LZ, YLu and YG ordered laboratory materials. YLi performed the statistical results and wrote the first draft. HH, SWu, RL, MC, MX, LH and XC edited the English version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Changes of natural killer cells' phenotype in patients with chronic hepatitis B in intermittent interferon therapy

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Background: To investigate the changes of natural killer (NK) cell phenotype in the interferon alpha (IFN- α) treatment of chronic hepatitis B (CHB) and its relationship with clinical indicators.

Methods: The CHB patients who did not receive any antiviral treatment were set as initial treatment group and used pegylated interferon alpha (PEG-IFN α). Peripheral blood samples were collected at baseline, 4 weeks, and 12-24 weeks. For IFN-treated patients who entered the plateau were set as plateau group, and PEG-IFN α was discontinued and resumed after an interval of 12-24 weeks. Besides, we also enrolled some patients who had received oral drug for more than 6 months as oral drug group without follow up. Peripheral blood was collected during the plateau period, which was set as baseline, and after 12-24 weeks of intermittent treatment, and after 12-24 weeks of additional treatment with PEG-IFN α . The aim of the collection was to detect hepatitis B virus (HBV) virology, serology and biochemical indicators, and the NK cell related phenotype was detected by flow cytometry.

Results: In the plateau group, subgroup of CD69⁺CD56^{dim} was higher with statistical significance when comparing with the initial treatment group and oral drug group [10.49 (5.27, 19.07) vs 5.03 (3.67, 8.58), $Z = -3.11$, $P = 0.002$; 10.49 (5.27, 19.07) vs 4.04 (1.90, 7.26), $Z = -5.30$, $P < 0.001$]. CD57⁺CD56^{dim} was significantly lower than that in initial treatment group and oral drug group respectively [68.42 $\pm$ 10.37 vs 55.85 $\pm$ 12.87, $t = 5.84$, $P < 0.001$; 76.38 $\pm$ 9.49 vs 55.85 $\pm$ 12.87, $t = -9.65$, $P < 0.001$]. The CD56^{bright}CD16⁻ subgroup in the plateau group was higher with statistical significance compared with initial treatment group and oral drug group respectively [11.64 (6.05, 19.61) vs 3.58 (1.94, 5.60), $Z = -6.35$, $P < 0.001$; 11.64 (6.05, 19.61) vs 2.37 (1.70, 4.30), $Z = -7.74$, $P < 0.001$]. CD57⁺CD56^{dim} in the plateau group had a significant higher percentage than that at baseline after IFN discontinuation for 12-24 weeks (55.85 $\pm$ 12.87 vs 65.95 $\pm$ 12.94, $t = -2.78$, $P = 0.011$).

Conclusion: During the long-term treatment of IFN, the killer subgroup of NK cells is continuously depleted, leading to the differentiation of the regulatory subgroup into the killer subgroup. In the killing subgroup, although the number is continuously depleted, the activity of the subgroup is continuously increased. In the plateau phase, after stopping IFN for a period of time, the number of NK cell subsets would gradually recover, but was still lower than that in the initial treatment group.

KEYWORDS

natural killer cell, chronic hepatitis B, antiviral treatment, intermittent therapy, PEG-IFN α

Introduction

At present, about 257 million people are suffered from HBV infection and according to a survey in 2015, there was a number of 887 thousand deaths happened because of HBV infection-related diseases, including liver cirrhosis and hepatocellular carcinoma (HCC), which comprised about 52% and 38% respectively (1). It is of great necessity for CHB patients to receive timely therapy because of the huge threat and damage brought by this disease. One method with great effectiveness to decrease the risk of HCC is eliminating hepatitis B surface antigen (HBsAg), which is also an indicator attached with great importance to test the long-term prognosis of CHB patients, as well a key result of functional cure (2, 3). Yeo et al. found in a recent meta-analysis that in CHB patients without any antiviral treatments, the disappearance rate of HBsAg was only 1.02% yearly. Based on the above researches, antiviral treatment is considered to be necessary for CHB patients (4).

The recommended antiviral drugs in guidelines are nucleoside (nucleotide) analogues (NAs) and IFN- α . In the researches we have conducted before, the disappearance rate of HBsAg could maintain at 15% in CHB patients treated by PEG-IFN α combined therapy or extended therapy, which was better than using NAs alone (5). However, in the process of HBsAg elimination, it is not achieved easily through one-time combined therapy or extended therapy, but through multiple stages, which is called intermittent therapy. When received long-term IFN treatment, some patients' HBsAg decline level may come to a plateau, which is defined as HBsAg level decreases $< 0.5\text{lg IU/ml}$ compared to last detection. Even continuing with previous IFN treatment in plateau period, it is not easy to achieve sustained virological response for patients. Meanwhile, it is also recommended in guidelines that if the decrease level of HBsAg $< 1\text{lg IU/ml}$ after 24-week IFN therapy, the antiviral therapy should be replaced by NAs (6, 7). Therefore, for patients stalling in plateau period, we conducted intermittent therapy for them. About 20% of the patients achieved HBsAg disappearance through this mode and no HBsAg disappearance happened in the patients who stopped interferon in the plateau period and did not receive interferon re-treatment (8). This indicates that interferon intermittent therapy has an ideal outlook that can improve the HBsAg conversion rate in patients with CHB. However, the molecular mechanism of

intermittent therapy and the causes of the plateau need to be further explored.

NK cells can be defined as an crucial component of antiviral immune response and can eliminate infected cells through cytotoxicity or other mechanisms (9). The role of NK cells in chronic HBV infection has also been reported, saying that HBV infection may alter the activation state of NK cells and the expression of relevant receptors on the cell surface (10). A research about CHB patients' cessation of long-term nucleoside analogue therapy showed that HBsAg clearance was closely related to NK cells' phenotype and function (11). In patients treated with IFN, long-term exposure to IFN- α can increase the expression level of STAT1, promote its phosphorylation and activation, and lead to phenotypic changes in NK cells and increased cytotoxicity (12, 13). In CHB patients who gained functional cure through IFN therapy, dynamic changes of NK cells were also associated with HBsAg disappearance (14). In line with the above studies, we believe that the changes and variety of NK cell phenotype during intermittent treatment are worth exploring.

Materials and methods

Patients

Patients diagnosed as CHB by Hepatology Center of Beijing Ditan Hospital from November 2021 to August 2022 were enrolled as subjects and were divided into three subgroups: initial treatment group, Plateau group, and oral drug group. CHB was defined as HBsAg positive ≥ 24 weeks, with or without hepatitis B e antigen (HBeAg) positive, and abnormal ALT ($> 120\text{U/L}$) more than 12 weeks. Patients who had never received any treatment before and began to use IFN at the first time were initial treatment group; who had received first-line oral drug more than 6 months were oral drug group; and who had used IFN for a period then the level of HBsAg decreased $< 0.5\text{lg IU/ml}$ were plateau group. Exclusion criteria: 1) combined-infection with other viral, such as hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (15), etc.; 2) long-term use of immunosuppressive drugs or drugs that can easily cause liver damage; 3) other diseases which are liver-related, such as alcoholic hepatitis, autoimmune hepatitis, metabolic liver disease, cirrhosis,

liver tumors, etc.; 4) mental illness, such as depression; 5) serious cardiovascular and cerebrovascular diseases or other systemic diseases with greater harm.

The oral drug group was set up to make a cross-sectional comparison with the initial treatment group and the plateau group, aiming to explore the regulatory effects of oral drug and PEG-IFN α treatment on NK cells. Meanwhile, the results also showed that the percentages of NK cell modulation subsets and killing subsets did not change significantly after oral drug treatment compared with the initial treatment group. To further investigate the regulation of NK cell subsets by oral medication, regular follow-up should be made. However, this study mainly discussed the changes of NK cell subgroup during interferon treatment, so the dynamic changes during follow up in oral group was not included in the study.

Setting up corresponding control group (IFN treatment continued after HBsAg plateau) can better reflect the advantages of intermittent therapy and the relationship between NK cells and treatment strategy. However, according to the Chinese Guidelines for the Prevention and Treatment of Chronic Hepatitis B (2019 edition) (1), IFN should be discontinued after 24 weeks of PEG-IFN α treatment when HBsAg drops to $< 1 \text{ IU/ml}$. Therefore, in this study, we did not establish such a control group.

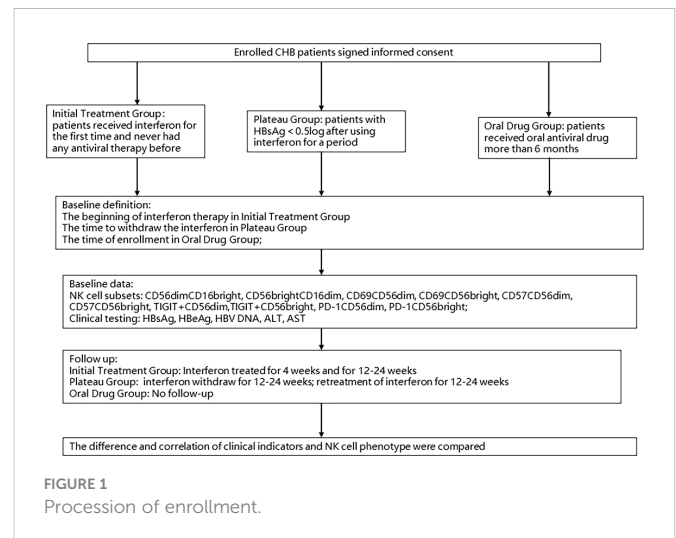
This was a prospective study approved by the Ethics Committee of Beijing Ditan Hospital (Jing Di Lun Ke Zi 2018 no. 023-01), with registration of Clinical Trial (NCT04028856). All subjects have signed informed consent before enrollment.

Sample collection

In the initial treatment group, patients began to receive subcutaneous injection of PEG-IFN α -2a 180 μg weekly, combined with first-line antiviral drugs (entecavir [ETV] 0.5 mg/d or tenofovir disoproxil fumarate [TDF] 300 mg/d or tenofovir alafenamide fumarate [TAF] 25 mg/d) according to their clinical characteristic and personal willing. The beginning time of IFN- α was set as baseline and the 4th week and the 12th to 24th week were follow-up points. The plateau group was based on interferon discontinuation at baseline and followed up 12 to 24 weeks after discontinuation and 12 to 24 weeks after re-introduction. During the intermittent, first-line antiviral drugs (ETV 0.5 mg/d or TDF 300 mg/d or tenofovir alafenamide fumarate [TAF] 25 mg/d) were applied to maintain antiviral effect. In the oral drug group, baseline was set as the time of enrollment and no need to follow up. At baseline and each follow-up time point, we used two EDTA purple tubes with a capacity of 9 ml respectively to collect peripheral venous blood to test clinical indicators such as HBsAg, HBeAg, HBV DNA and liver function. Peripheral blood mononuclear cell (PBMC) determination was extracted within 4 hours of peripheral blood collection for subsequent staining and flow cytometry. The process of enrollment and detection is shown in Figure 1.

Peripheral blood NK cell phenotype

The PBMC was extracted and resuspended with phosphate buffered saline (PBS). Anti-human antibodies CD3/CD19-Fluorescein Isothiocyanate (FITC), CD14-phycoerythrin (PE) anti-



human antibody, CD16-peridinin green protein (PerCP) anti-human antibody, CD56-Brilliant Violet 510 (BV510) anti-human antibody, CD57-BV421 anti-human antibody, CD69-allophycocyanin (APC) anti-human antibody, TIGIT-PE anti-human antibody, PD-1-APC anti-human antibody were added to the 100 μl PBMC cell suspension sequentially according to instructions. After full vortex mixing, leave samples avoid light at room temperature for 15-20min to dye. After staining, 2ml PBS was added, and after 1200 $\times$ 5min centrifugation, the supernatant was discarded, and 200 μl PBS was added to homogenize the cell mass at the bottom of the tube. Canto flow cytometry was used for detection. Voltage and fluorescence compensation were adjusted to the optimum, cells were collected, and data were derived for ringgate analysis using Flowjo software. The strategy of subgroup gate is shown in Figure 2.

Detection of clinical indicators

HBsAg and HBeAg were detected by chemiluminescent particle method (Abbott Diagnostics, Abbott Park, IL, USA). If the concentration of HBsAg was larger than 250U/ml, the sample was diluted to 1:500 and the specific concentration was calculated. Real-time quantitative polymerase chain reaction (PCR) was used to detect HBV DNA concentration in serum (Piji Co, Ltd, Shenzhen, China). ALT and AST were detected by Wako Pure Chemical Industries, Ltd., Japan. All clinical indicators were tested by Ditan Hospital Inspection Center.

Statistical analysis

All data were analyzed by SPSS25.0 (IBM Corporation, Chicago, IL, USA). The measurement data were first tested by Shapiro-Wilk test for normality. Those meeting the normality test were represented by mean $\pm$ standard deviation, while those not meeting the normality distribution were represented by median (Q1, Q3). Data were compared between groups by t test, Wilcoxon test or Mann-Whitney test. Linear regression was used to analyze the correlation between subsets of NK cells and clinical indicators (ALT, AST,

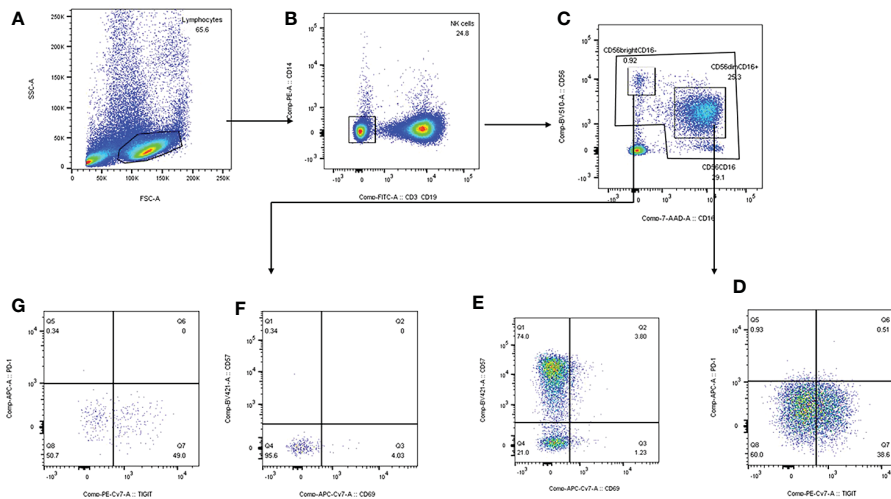


FIGURE 2

Strategy of subgroup gate and analysis on FlowJo software. (A) mononuclear cells were separated according to side scatter and forward scatter. (B) We draw the gate of NK cells based on CD3/CD19-FITC and CD14-PE. (C) CD56^{dim}CD16⁺ and CD56^{bright}CD16⁻ were separated by CD16-7-AAD and CD56-BV510. (D, E) CD69⁺CD56^{dim}, CD57⁺CD56^{dim}, TIGIT⁺CD56^{dim}, PD-1⁺CD56^{dim} were delineated by TIGIT-PE-Cy7, PD-1-APC, CD69-APC-Cy7 and CD57-BV421. (F, G) CD69⁺CD56^{bright}, CD57⁺CD56^{bright}, TIGIT⁺CD56^{bright}, PD-1⁺CD56^{bright} were delineated by TIGIT-PE-Cy7, PD-1-APC, CD69-APC-Cy7 and CD57-BV421.

HBsAg, HBsAg, HBV DNA). If $P < 0.05$, it was considered statistically significant. For repeated measurements, α segmentation method was used, and we set $P < 0.017$ as significant statistically.

Result

Baseline clinical features and NK cell phenotype of patients

176 patients with CHB were enrolled totally, including 51 in the initial treatment group, 50 in the plateau group and 75 in the oral drug group. The mean age was 34.24 (31.22, 41.16) years in initial treatment group, 34.24 (31.22, 41.16) years in plateau group, 48.00 (41.13, 58.01) in oral drug group. Between initial treatment group and oral drug group, CD56^{dim}CD16⁺ subgroup [80.90 (69.41, 86.40) vs 79.30 (66.70, 84.82), $Z = -1.03$, $P = 0.305$] and CD56^{bright}CD16⁻ subgroup [3.58 (1.94, 5.60) vs 2.37 (1.70, 4.30), $Z = 11.78$, $P = 0.075$] had no significant differences, but when compared with the plateau group, the result was significantly different [CD56^{dim}CD16⁺: 80.90 (69.41, 86.40) vs 46.51±18.51, $Z = -7.08$, $P < 0.001$ (initial treatment group vs plateau group); 79.30 (66.70, 84.82) vs 46.51±18.51, $Z = -6.44$, $P < 0.001$ (oral drug group vs plateau group); CD56^{bright}CD16⁻: 3.58 (1.94, 5.60) vs 11.64 (6.05, 19.61), $Z = -6.35$, $P < 0.001$ (initial treatment group vs plateau group); 2.37 (1.70, 4.30) vs 11.64 (6.05, 19.61), $Z = -7.74$, $P < 0.001$ (oral drug group vs plateau group)]. When comparing the subgroup of CD69⁺CD56^{dim} in plateau group, initial treatment group and oral drug group, the former was significantly higher than the two others [10.49 (5.27, 19.07) vs 5.03 (3.67, 8.58), $Z = -3.11$, $P = 0.002$; 10.49 (5.27, 19.07) vs 4.04 (1.90, 7.26), $Z = -5.30$, $P < 0.001$], while CD57⁺CD56^{dim} was significantly lower than that in initial treatment group and oral drug group [68.42±10.37 vs 55.85±12.87, $t = 5.84$, $P < 0.001$; 76.38±9.49 vs 55.85±12.87, $t = -9.65$, $P < 0.001$]. The expression of CD56^{bright}CD16⁻ in plateau group was significantly higher than that in initial treatment group and oral administration group [11.64

(6.05, 19.61) vs 3.58 (1.94, 5.60), $Z = -6.35$, $P < 0.001$; 11.64 (6.05, 19.61) vs 2.37 (1.70, 4.30), $Z = -7.74$, $P < 0.001$]. The baseline characteristic was shown in Table 1.

Changes of NK cell phenotype during treatment

In the initial treatment group, CD56^{dim}CD16⁺ subgroup, CD57⁺CD56^{dim} and TIGIT⁺CD56^{dim} all decreased gradually with the extension of treatment time, and the differences were statistically significant compared with baseline, respectively [80.90 (69.41, 86.40) vs 51.18 (34.34, 60.00), $Z = -3.81$, $P < 0.001$; 68.42±10.37 vs 57.14±10.92, $t = 3.74$, $P = 0.001$; 49.94±21.19 vs 30.04±14.22, $t = 3.29$, $P = 0.004$]. The expression of CD56^{bright}CD16⁻ subgroup increased with the extension of treatment time, and the difference was statistically significant compared with baseline [3.58 (1.94, 5.60) vs 10.70±5.95, $Z = -3.17$, $P = 0.002$].

As for plateau group, CD57⁺CD56^{dim} expression increased significantly higher after interferon discontinuation for 12-24 weeks compared to baseline (55.85±12.87 vs 65.95±12.94, $t = -2.78$, $P = 0.011$). There was no significant difference between baseline and untreated group (65.95±12.94 vs 68.42±10.37, $t = 1.12$, $P = 0.266$). The dynamic changes of NK cells' subsets were shown in Table 2 and Figure 3.

Correlation between NK phenotype and clinical indicators

In the initial treatment group, HBV DNA was positively correlated with CD69⁺CD56^{dim} ($\beta = -0.35$, $t = -2.27$, $P = 0.029$) and on the other hand, had negative correlation with CD56^{bright}CD16⁻ ($\beta = 0.43$, $t = 2.15$, $P = 0.038$), as shown in Table 3.

TABLE 1 Characteristics of initial treatment group, plateau group and oral drug group at baseline.

	Initial treatment group(n=51)	Plateau group (n=50)	Oral drug group (n=75)	Z/t			P		
				C0 vs P0	C0 vs N0	P0 vs N0	C0 vs P0	C0 vs N0	P0 vs N0
Age (years) (median[Q1,Q3])	34.24(31.22, 41.16)	36.17(32.88, 44.04)	48.00(41.13, 58.01)	-1.519	-4.97	-3.99	0.129	<0.001	<0.001
Gender(male/female)	31/20	19/31	27/48	5.24	7.51	0.05	0.022	0.006	0.820
HBsAg (log10IU/ml)	3.80(3.28, 4.25)	2.62(0.96, 3.42)	2.94(2.24, 3.57)	-4.57	-3.68	-1.74	<0.001	<0.001	0.082
HBeAg (S/CO)	15.86(0.35, 1256.44)	0.39(0.32, 4.90)	0.53(0.40, 29.31)	-3.231	-1.47	-1.92	0.001	0.142	0.055
HBV DNA (log10IU/ml)	4.53(2.28, 8.11)	1.00(0.04, 1.00)	0.52(0.04, 1.00)	-6.22	-5.69	-1.50	<0.001	<0.001	0.133
ALT (U/L)	35.00(25.38, 77.98)	34.00(25.60, 47.80)	25.50(20.75, 39.50)	-0.861	-2.63	-2.26	0.389	0.009	0.024
AST (U/L)	27.05(18.78, 64.80)	29.60(22.60, 39.50)	23.00(19.00, 27.50)	-0.01	-1.92	-3.15	0.992	0.055	0.002
CD56 ^{dim} CD16 ⁺	80.90(69.41,86.40)	46.51±18.51	79.30(66.70,84.82)	-7.08	-1.03	-6.44	<0.001	0.305	<0.001
CD69 ⁺ CD56 ^{dim}	5.03(3.67, 8.58)	10.49(5.27, 19.07)	4.04 (1.90, 7.26)	-3.11	-2.43	-5.30	0.002	0.015	<0.001
CD57 ⁺ CD56 ^{dim}	68.42±10.37	55.85±12.87	76.38±9.49	5.84	-3.88	-9.65	<0.001	<0.001	<0.001
TIGIT ⁺ CD56 ^{dim}	49.94±21.19	43.2(30.8, 56.1)	44.71±19.48	-0.60	1.42	-0.64	0.423	0.157	0.521
PD-1 ⁺ CD56 ^{dim}	1.97(0.964, 3.9)	1.5(0.728, 5.16)	0.39 (0.17, 0.71)	-0.65	-6.54	-5.59	0.548	<0.001	<0.001
CD56 ^{bright} CD16 ⁻	3.58(1.94,5.60)	11.64(6.05,19.61)	2.37(1.70,4.30)	-6.35	-1.78	-7.74	<0.001	0.075	<0.001
CD69 ⁺ CD56 ^{bright}	3.10(1.95, 5.07)	2.97(2.14, 4.2)	1.57 (0.00, 2.24)	-0.11	-4.70	-4.81	0.913	<0.001	<0.001
CD57 ⁺ CD56 ^{bright}	0.48(0.00, 1.41)	0.51(0.24, 1.16)	1.56 (0.42, 3.96)	-0.61	-3.21	-2.81	0.545	0.001	0.005
TIGIT ⁺ CD56 ^{bright}	8.10(5.79, 12.3)	9.04(6.72, 14.8)	7.67 (3.20, 30.71)	-1.20	-0.26	-0.95	0.232	0.794	0.343
PD-1 ⁺ CD56 ^{bright}	0.24(0.00, 0.79)	0.033(0, 0.991)	0.00 (0.00, 0.00)	-0.34	-4.10	-3.68	0.735	0.001	<0.001

HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV DNA, hepatitis B virus deoxyribonucleic acid; ALT, alanine transaminase; AST, aspartate aminotransferase.

Discussion

In the process of antiviral treatment for CHB, the therapeutic effect is closely related to the virus type, treatment strategy and immune status (16). During the use of IFN, our previous research found that 75% of the disappearance of HBsAg occurred after 48 weeks, and prolonged treatment was helpful for the disappearance of HBsAg and maintenance of antiviral effect (17). However, not all patients can obtain the ideal effect through prolonged treatment, and the decline of HBsAg in some patients stagnates with the extension of treatment time. Based on this phenomenon, we have conducted clinical studies and found that about 20% of patients achieved the disappearance of surface antigens through the intermittent treatment mode of initial - intermittent - retreat, which proved the effectiveness of this strategy (8). But the molecular mechanisms behind intermittent therapy, and why platform of HBsAg level occurs, remain to be explored. In this study, by observing the changes of NK cell-related phenotypes in patients at different treatment stages in different times, we explored the molecular mechanism of the emergence of the plateau, providing a theoretical basis for intermittent therapy.

NK cells, as a component of innate immune cells with significant importance in the human body, and play a role in eliminating viruses

through cytotoxicity or secretion of cytokines which can induce inflammation reaction (18). Chronic HBV infection results in decreased NK cell count and function. IFN, as an antiviral drug recommended by the guidelines, induces ISG transcription through the JAK/STAT pathway and targets to interfere with different stages of viral replication, including virus invasion, uncoating, gene recombination, and assembly machine release, acting as a resistance power in viral infection (15, 19–21). Interferon has been reported to significantly amplify CD56^{bright}CD16⁻ cells and promote the activation of CD56^{dim}CD16⁺ cells (22). CD56^{dim}CD16⁺ and CD56^{bright}CD16⁻ are the main subgroups of NK cells, and CD56^{dim}CD16⁺ plays the key role of killing, accounting for about 90% of NK cells, and exerts cytotoxic effect through the secretion of granzyme and perforin. CD56^{bright}CD16⁻ mainly works with regulatory function, accounting for about 10% of the total NK cells, and secretes TNF- α , IFN- γ and other cytokines (23). In this study, in addition to the determination of CD56^{bright}CD16⁻ and CD56^{bright}CD16⁻ subgroups, we also continued to gate the two subgroups to detect the expressions of CD69, CD57, TIGIT and PD-1 in the two subgroups respectively. CD69 is an activating molecule of NK cells and a killer receptor (24, 25). CD57 is expressed in mature and differentiated NK cells, marking the maturity of NK cells (26). Both TIGIT and PD-1 are co-inhibitory

TABLE 2 Comparison of initial treatment group and plateau group during follow up.

	Initial Treatment Group									Plateau Group								
	W ₀ (n=51)	W ₄ (n=29)	W ₁₂₋₂₄ (n=20)	Z/t			P			W ₀ (n=51)	Withdraw 12-24w (n=)	Retreat 12-24w (n=)	Z/t			P		
				W ₀ vs W ₄	W ₀ vsW ₁₂₋₂₄	W ₄ vs W ₁₂₋₂₄	W ₀ vs W ₄	W ₀ vs W ₁₂₋₂₄	W ₄ vs W ₁₂₋₂₄				W ₀ vs Withdraw	W ₀ vs Retreat	Withdraw vs Retreat	W ₀ vs Withdraw	W ₀ vs Retreat	Withdraw vs Retreat
CD56 ^{dim} CD16 ⁺	80.90 (69.41,86.40)	71.58 (63.19, 77.34)	51.18 (34.34, 60.00)	-1.44	-3.81	-2.69	0.150	<0.001	0.007	0.4651 ±0.1851	0.5322±0.1807	5.63 (1.30,14.60)	2.11	-1.35	-1.25	0.047	0.177	0.210
CD69 ⁺ CD56 ^{dim}	5.03(3.67, 8.58)	9.1 (6.12,11.65)	4.99 (2.99,10.68)	-2.00	-0.075	-1.09	0.045	0.940	0.278	10.49(5.27, 19.07)	10.68 (4.07,17.30)	5.97 (3.16,11.27)	-0.85	-0.83	-0.78	0.398	0.407	0.435
CD57 ⁺ CD56 ^{dim}	68.42±10.37	67.80 ±10.30	57.14 ±10.92	-1.02	3.74	3.63	0.315	0.001	0.002	55.85±12.87	65.95±12.94	63.58±10.14	-2.78	-2.37	-0.36	0.011	0.031	0.722
TIGIT ⁺ CD56 ^{dim}	49.94±21.19	49.23 ±14.57	30.04 ±14.22	0.66	3.29	2.86	0.516	0.004	0.012	43.2(30.8, 56.1)	45.49±21.01	23.25 (20.87,39.75)	-0.85	-2.01	-2.30	0.398	0.044	0.022
PD-1 ⁺ CD56 ^{dim}	1.97(0.964, 3.9)	1.83 (0.9,2.78)	0.72 (0.29,1.73)	-0.97	-2.35	-2.17	0.331	0.019	0.030	1.5(0.728, 5.16)	0.84(0.20,4.09)	0.40(0.21,1.22)	-1.52	-2.11	-1.73	0.128	0.035	0.084
CD56 ^{bright} CD16 ⁻	3.58 (1.94,5.60)	4.10 (3.06,6.57)	10.70±5.95	-1.46	-3.17	-3.26	0.144	0.002	0.001	0.1164 (0.0605, 0.1961)	0.0580 (0.0396,0.1477)	0.0924 (0.0590,0.1828)	-2.37	-0.50	-0.17	0.018	0.619	0.868
CD69 ⁺ CD56 ^{bright}	3.10(1.95, 5.07)	3.46 (2.36,4.02)	1.72 (0.33,4.52)	-0.25	-0.52	-0.16	0.804	0.601	0.877	2.97(2.14, 4.2)	3.41 (1.92,12.17)	2.26 (1.32,18.42)	-2.37	-0.45	-0.17	0.018	0.653	0.868
CD57 ⁺ CD56 ^{bright}	0.48(0.00, 1.41)	0.58(0.1,48)	0.82 (0.43,1.25)	-0.75	-0.11	-1.82	0.454	0.911	0.069	0.51(0.24, 1.16)	0.60(0.1,54)	1.51(0.54,2.18)	-0.41	-2.01	-1.97	0.686	0.044	0.049
TIGIT ⁺ CD56 ^{bright}	8.10(5.79, 12.3)	9.57 (6.84,16.06)	6.28 (3.43,11.16)	-0.77	-1.20	-1.76	0.443	0.232	0.079	9.04(6.72, 14.8)	8.26 (5.66,19.43)	5.34 (1.55,13.55)	-0.17	-1.59	-1.73	0.866	0.113	0.084
PD-1 ⁺ CD56 ^{bright}	0.24(0.00, 0.79)	0.00 (0.00,0.3)	0.00 (0.00,0.36)	-1.68	-1.70	-1.53	0.094	0.088	0.126	0.033(0, 0.991)	0(0, 0.34)	0(0,0.41)	-0.37	-0.09	-0.16	0.715	0.925	0.875

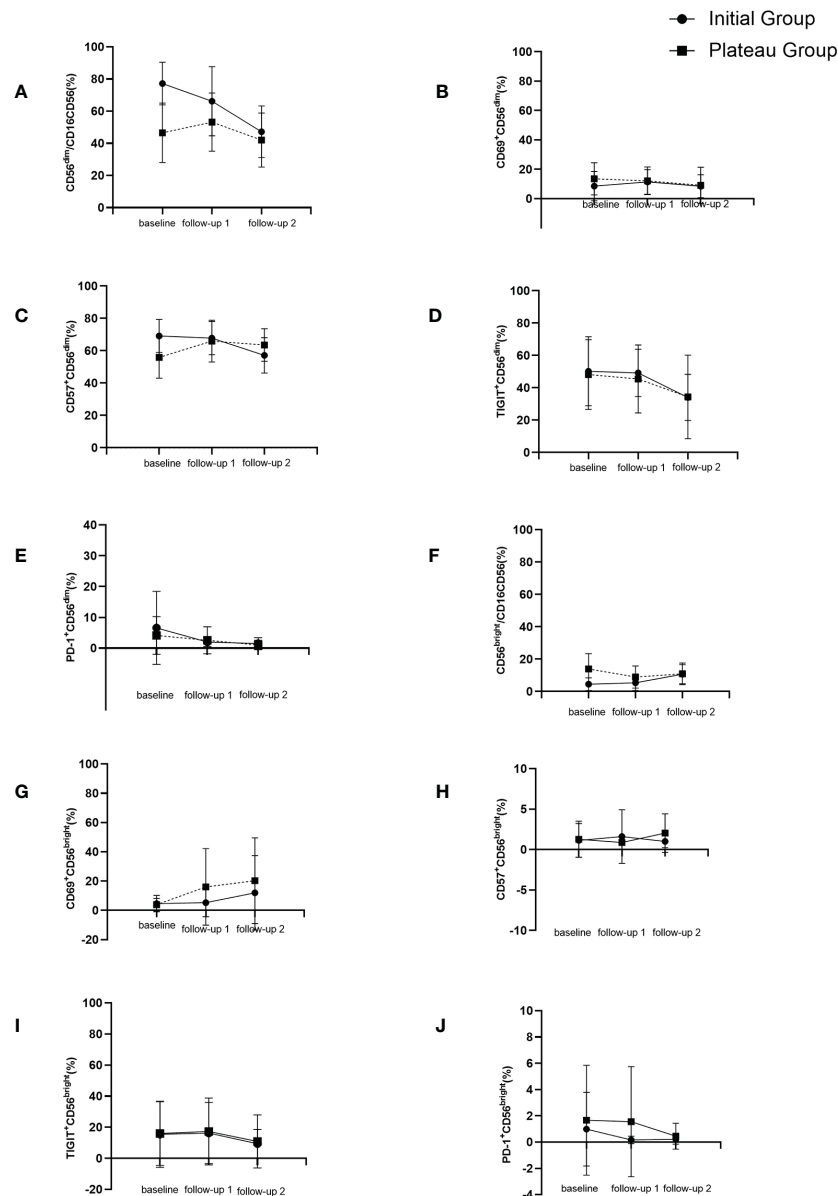


FIGURE 3

Dynamic changes of NK cell subgroups with follow-up time. (A) changes of $CD56^{dim}CD16^{+}$ (%) during follow up. (B) changes of $CD69^{+}CD56^{dim}$ (%) during follow up. (C) changes of $CD57^{+}CD56^{dim}$ (%) during follow up. (D) changes of $TIGIT^{+}CD56^{dim}$ (%) during follow up. (E) changes of $PD-1^{+}CD56^{dim}$ (%) during follow up. (F) changes of $CD56^{bright}CD16^{-}$ (%) during follow up. (G) changes of $CD69^{+}CD56^{bright}$ (%) during follow up. (H) changes of $CD57^{+}CD56^{bright}$ (%) during follow up. (I) changes of $TIGIT^{+}CD56^{bright}$ (%) during follow up. (J) changes of $PD-1^{+}CD56^{bright}$ (%) during follow up.

receptors, which are specifically expressed on the surface of NK cells and T cells, playing a co-inhibitory role. Studies have reported that TIGIT acts on liver cytotoxic T lymphocyte (CTL), leading to immune tolerance and making HBV difficult to clear. Blocking TIGIT pathway or inhibiting TIGIT expression can restore immune function (27, 28). In an animal experiment, PD-1 deficient mice had an advantage in liver clearance of adenovirus, but they were also prone to immune damage, which was speculated to be due to the excessive autoimmune reaction caused by PD-1 deficiency (29). Therefore, the moderate expression of TIGIT and PD-1 is also extremely important in innate immunity.

Our research found that patients treated with IFN for a period of time had significant differences in the percentage of $CD56^{dim}CD16^{+}$, $CD69^{+}CD56^{dim}$, and $CD57^{+}CD56^{dim}$ compared with the initial

treatment group. The expression of $CD56^{dim}CD16^{+}$ and $CD57^{+}CD56^{dim}$ decreased in patients treated with IFN, indicating that the subgroup which played a killing role was weakened and the number of mature cells decreased after IFN treatment for a period of time. The frequency of $CD69^{+}CD56^{dim}$ increased, indicating that although the killing subsets decreased, the activity of NK cells in the killing subsets increased in patients treated with IFN. Studies have shown that the elevation of transaminase during interferon trial is associated with the upregulation of NKCD69. This study can further confirm that in patients treated with IFN, the killing subgroup $CD69^{+}CD56^{dim}$ is significantly up-regulated, which enhances the killing activity and may cause abnormal transaminase. During the follow-up of patients initially treated with IFN, the frequency of $CD56^{dim}CD16^{+}$ gradually decreased with the extension of IFN use

TABLE 3 Correlation between NK phenotype and clinical indicators.

	Initial Treatment Group				Plateau Group				NA			
	CD56dim	CD56dimCD69	CD56dimCD57	CD56bright	CD56dim	CD56dimCD69	CD56dimCD57	CD56bright	CD56dim	CD56dimCD69	CD56dimCD57	CD56bright
HBsAg	t=-0.144 P=0.886	t=0.426 P=0.672	t=1.251 P=0.218	t=0.818 P=0.418	t=-1.168 P=0.249	t=0.209 P=0.836	t=-0.181 P=0.858	t=0.832 P=0.41	t=-0.546 P=0.59	t=-0.425 P=0.675	t=-0.933 P=0.36	t=-0.744 P=0.464
HBcAg	t=1.164 P=0.251	t=-1.253 P=0.218	t=1.192 P=0.24	t=2.34 P=0.024	t=-0.051 P=0.96	t=-0.712 P=0.481	t=-0.525 P=0.602	t=0.071 P=0.944	t=-0.486 P=0.631	t=0.958 P=0.346	t=-0.092 P=0.928	t=-0.198 P=0.845
HBV DNA	t=0.209 P=0.835	t=-2.273 P=0.029	t=1.326 P=0.193	t=2.153 P=0.038	t=-1.757 P=0.087	t=0.976 P=0.335	t=-0.459 P=0.649	t=0.849 P=0.401	t=0.079 P=0.937	t=0.006 P=0.995	t=-1.208 P=0.238	t=1.316 P=0.2
ALT	t=-0.057 P=0.955	t=-0.5 P=0.62	t=1.683 P=0.102	t=1.563 P=0.128	t=-0.704 P=0.486	t=0.285 P=0.777	t=-1.143 P=0.26	t=0.872 P=0.388	t=0.125 P=0.901	t=-1.149 P=0.261	t=-0.581 P=0.566	t=-0.442 P=0.662
AST	t=-0.314 P=0.756	t=-0.366 P=0.717	t=1.7 P=0.099	t=1.515 P=0.14	t=-0.528 P=0.6	t=0.373 P=0.711	t=-1.208 P=0.235	t=0.796 P=0.431	t=0.013 P=0.99	t=-1.201 P=0.24	t=0.247 P=0.807	t=0.872 P=0.391

time, indicating that the killing function of NK cells was gradually weakened with the application of IFN, which may lead to the emergence of a plateau period. At the same time, the frequency of CD56^{bright}CD16⁻ increased gradually, because the differentiation direction of NK cells was from regulated subgroup to killing subgroup, that is, from CD56^{bright}CD16⁻ to CD56^{dim}CD16⁺, and finally to exhaustion. Therefore, it can be considered that the change of CD56^{bright}CD16⁻ frequency is due to the compensatory increase due to the decrease of CD56^{dim}.

It has been shown that HBsAg decline of less than 0.5lgIU/ml from baseline after 12 weeks of PEG-IFN treatment is indicative of difficulty in achieving sustained virological remission after cessation of treatment (30). This suggests that if a significant and sustained decline of HBsAg does not occur during treatment, it is difficult to obtain a good follow-up response. Therefore, we took the HBsAg decline range $\leq 0.5\text{lgIU/ml}$ as the standard of the plateau period.

In plateau group, with the extension of IFN discontinuation period, the frequency of NK cell-related subsets had gradually recovered. The frequency of CD57⁺CD56^{dim} increased significantly after 12-24 weeks of IFN suspension compared to baseline, indicating the maturation of the killing subgroup. Compared with CHB patients who had not received any antiviral drugs (baseline in the initial treatment group), the frequency of CD57⁺CD56^{dim} returned to the ideal level after 12 to 24 weeks of discontinuation of IFN during the plateau period. However, the frequency of some subgroups is still different from that of untreated patients, which needs to be further discussed with additional follow-up data.

Studies have shown that reducing viral load through antiviral therapy can partially reshape the function of NK cells. Reducing viral load is beneficial to improve NK cell function (31). In pregnant women infected with HBV, antiviral therapy can significantly increase the frequency of NK cells (32). In this study, through multiple linear regression analysis of baseline clinical indicators and NK subpopulation rows, it was found that the HBV DNA level in the initial treatment group was negatively correlated with the frequency of CD69⁺CD56^{dim}, suggesting that viral load may inhibit the activation of NK cells.

In conclusion, this study increased the difference in the expression of NK cell subsets in patients without any treatment and IFN-treated, and provided part of the theoretical basis for the emergence of a plateau in IFN treatment. From the perspective of NK cells, it provides theoretical support for intermittent treatment of interferon. The results showed that during IFN treatment, the killing subsets of NK cells decreased gradually, the compensatory subsets increased, and the activity of killing subsets increased. After interferon was discontinued, the frequency and function of NK cell subsets gradually recovered. High virus levels affect the function of NK cells and affect the activation of NK cells.

It is recommended according to the guideline that treatment modification should not be considered until the desired results were not achieved after 24 weeks of interferon therapy (1). For the initial treatment group, our follow-up time nodes were 4 weeks and 12-24 weeks, respectively. During the 24 weeks, we will not consider changing the treatment regimen, so no patients from the initial treatment group will enter the platform group. However, with the extension of follow-up time, it is not excluded that some patients in the initial treatment group will enter the platform group due to the

stagnation of surface antigen decline. This is also the limitation of this study. We will continue to follow up and focus on those patients who entered the platform group from the initial treatment group.

As a novel serological marker, HBV RNA contains HBV pre-genomic RNA and its shear variants, which can reflect the activity of hepatic cccDNA to a certain extent (33). Therefore, it is also of great significance in reflecting the response to antiviral therapy and predicting relapse after drug withdrawal. However, a number of recent studies have confirmed that HBV RNA cannot be used as an indicator of drug withdrawal in patients with CHB (34, 35). Compared with classical serological indicators such as HBsAg, HBV DNA, ALT, etc., the clinical guiding significance of HBV RNA still needs to be studied.

Interferon-gamma-induced protein10 (IP-10), also known as C-X-C ligand 10 (CXCL10), belongs to the CXC family of chemokines and plays an important role in the pathogenesis of immune diseases and inflammatory diseases. Serum IP-10 expression levels were higher in patients with higher levels of liver inflammation and fibrosis (36). In addition, intrahepatic IP-10 levels were positively correlated with oral efficacy in patients with CHB (37). However, its response to the degree of fibrosis and prediction of antiviral efficacy are also correlated with other clinical indicators, such as ALT, AST, HBV DNA, and HBsAg levels. There is currently a lack of large sample size studies to confirm the relationship between IP-10 and antiviral efficacy. Compared with classical clinical indicators such as HBV DNA, IP-10, as an emerging clinical indicator, needs further research. In summary, we did not conduct correlation analysis of HBV RNA, IP-10 and NK cell phenotypes in this study. However, in subsequent studies, we will include more clinical indicators and more cytokines for analysis.

The analysis of clinical characteristic in plateau group at the beginning use of IFN was in absence. We have studied in our previous article that HBsAg response at 12 weeks and 24 weeks of initial treatment was significantly correlated with the effect of retreatment after plateau. Compared with patients who did not respond to initial treatment, patients who developed an antiviral response early in treatment had a higher rate of response after intermittent treatment during the plateau period. In the platform group, HBsAg level at first use of IFN, early treatment response, HBsAg level at discontinuation of IFN, and duration of IFN use were all significantly correlated with functional recovery by intermittent therapy (8). Moreover, in this study, with the extension of follow-up time, some patients in the initial treatment group will be transferred to the plateau group later, and their data is more comprehensive and representative. We have been working on the follow-up work, and we believe it will be elaborated in a future article.

There are also several limitations in this research, such as small sample size, lack of whole period data from initial treatment to plateau stage. Besides, counting NK cell subsets can more accurately explain the effect of interferon treatment on NK cells. Therefore, more evidence and work are need for the further exploration.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Beijing Ditan Hospital (Jing Di Lun Ke Zi 2018 no. 023-01). The patients/participants provided their written informed consent to participate in this study.

Author contributions

ML and YX contributed to study concept and design. MC, SX and LY conducted experiments and collected the data. YLu, GS, SX and LY collected the information of the patients. TJ, WD, SWa, LZ, YLi and YG provided reagents to help the experimental work. YLi analyzed the statistical results. HH, SWu, RL, MX, LH and XC edited the English version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Decreased granzyme-B expression in CD11c⁺CD8⁺ T cells associated with disease progression in patients with HBV- related hepatocellular carcinoma

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Introduction: CD11c⁺CD8⁺ T cells are an unconventional CD8⁺ T cell subset that exerts antiviral activity in infectious diseases. However, its characteristics in hepatocellular carcinoma (HCC) have not been elucidated.

Methods: Twenty-six patients with hepatitis B virus (HBV)-related HCC and 25 healthy controls (HC) were enrolled. The frequency and phenotype of CD11c⁺CD8⁺ T cells in peripheral blood and tumors in situ were detected by flow cytometry and immunohistochemistry.

Results: Both the HCC group and HC group had similar frequency and phenotype characteristics of CD11c⁺CD8⁺ T cells in the periphery. CD11c⁺CD8⁺ T cells were mainly composed of effector T cells, most of which were CD45RA⁺CCR7⁻. Compared with CD11c⁻CD8⁺ T cells, CD11c⁺CD8⁺ T cells had a higher proportion of CD38 and HLA-DR double positive, and expressed high levels of granzyme-B (GB) and degranulation marker CD107a, and produced high levels of interleukin-2 (IL-2), tumor necrosis factor alpha (TNF- α) and interferon-gamma (IFN- γ). However, the ability of degranulation and TNF- α production of CD11c⁺CD8⁺ T cells in patients with HCC were significantly lower than that in healthy controls. The GB expression level of peripheral CD11c⁺CD8⁺ T cells in patients with advanced stage of HCC was significantly lower than that in patients with early stage of HCC, and the GB expression level of liver-infiltrating CD11c⁺CD8⁺ T cells in tumor tissues was lower than that in non-tumor tissues. More importantly, the GB expression level of peripheral CD11c⁺CD8⁺ T cells was negatively correlated with tumor volume.

Conclusions: These findings indicate that CD11c⁺CD8⁺ T cells may have potential anti-tumor activity and that GB⁺CD11c⁺CD8⁺ T cells are associated with disease progression in patients with HBV-related HCC.

KEYWORDS

CD11c, CD8 T cells, hepatocellular carcinoma, hepatitis B virus, anti-tumor

Introduction

Chronic hepatitis B virus (HBV) infection can lead to liver cirrhosis and liver failure and accounts for more than 80% of hepatocellular carcinoma (HCC) cases worldwide (1, 2). Viral clearance and tumor cell clearance are largely dependent on robust CD8⁺ T cell responses (3); however, T cell exhaustion occurs in various situations, leading to the loss of their ability for cytokine production or cytotoxicity against tumor cells (4–6). Moreover, cell exhaustion is also closely related to increased levels of exhaustion-related immune molecules, such as T cell Ig and ITIM domain and programmed cell death 1 (PD-1), as well as disease progression (5). Based on recent advances in CD8⁺ T cell anti-tumor therapy, some unconventional CD8⁺ T cell subsets have been identified to help reduce the tumor burden and inhibiting tumor progression (7).

CD11c is a member of the β 2 integrin family, is highly expressed in dendritic cells, and is normally expressed in some B lymphocytes, CD8⁺ T cell subsets, and natural killer cells (8, 9). Previous studies have found that CD11c plays an important regulatory role in the proliferation and function of CD4⁺ and CD8⁺ T cells (10). Acute viral, bacterial, and parasitic infections lead to upregulation of the frequency of CD11c⁺CD8⁺ T cells, and this CD8⁺ T cell subset displayed characteristics of effector cells, including high activation and cytotoxic capacity to eliminate pathogenic microbes, whereas CD11c⁺CD8⁺ T cells did not (11–13).

The secretion of cytokines, such as interleukin-2 (IL-2), tumor necrosis factor alpha (TNF- α), interferon-gamma (IFN- γ), and cytolytic granules, such as granzyme B (GB), are the main functional attributes of CD8⁺ T cells that exhibit anti-tumor activity (14, 15). Moreover, tumor-infiltrating CD8⁺ T cells in colorectal cancer and lymphoma with CD11c upregulation reflect the tumoricidal efficacy (8, 16–18). The expression level of CD11c on CD8⁺ T cells could be a potential marker for evaluating tumoricidal cytotoxic T lymphocytes to predict the efficacy of anti-tumor immunotherapies (16). Although CD11c⁺CD8⁺ T cells may be potential therapeutic targets for tumors, their roles in patients with HCC remain unclear.

In this study, the phenotype and function of CD11c⁺CD8⁺ T cells in HCC patients and healthy controls (HCs) were evaluated, and the

relationship between the number of CD11c⁺CD8⁺ T cells in both peripheral tissues and tumors *in situ* and HCC disease progression was further explored.

Materials and methods

Study participants

Twenty-six HCC patients and 25 HCs from the Fifth Medical Center of the Chinese PLA General Hospital were included in this study (detailed information of the participants was shown in Table 1). Peripheral blood samples were obtained from all participants, and tumor and non-tumor tissues (relatively normal liver tissue surrounding the tumor with no obvious tumor tissue observed on macroscopic observation) were obtained from nine HCC patients. Patients with concurrent hepatitis C virus and human immunodeficiency virus infections, and autoimmune or alcoholic liver disease were excluded from the study. Informed consent was obtained from all the participants in accordance with the Declaration of Helsinki. This study was approved by the Institutional Review Board of the Fifth Medical Center of the Chinese PLA General Hospital (KY-2022-4-16-1).

Sample processing and storage

Peripheral blood mononuclear cells were isolated using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ, USA) density gradient centrifugation and used directly in flow cytometry experiments. Freshly excised tumor tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and stored in a refrigerator at -20°C.

Flow cytometry

Peripheral blood mononuclear cells or mononuclear cells isolated from liver tissue were stained with the following antibodies for phenotypic staining: CD3-APC/Cy7, CD8-BV510, CD8-Percp,

TABLE 1 Clinical characteristics of the enrolled patients at baseline.

Variables	HCC (n=26)	HC (n=25)	P
Age (year)	45 (39–52.5)	33 (31–43)	<0.01
Sex (men/women)	22/4	15/10	0.049
ALT (U/L)	29 (18–43.75)	23 (15–30)	0.058
AST (U/L)	34 (24–51.75)	23 (16–29)	0.001
TBIL (umol/L)	12.65 (9.92–19.6)	12.9 (10.5–16.4)	0.558
AFP (ng/mL)	7.35 (2.25–508.4)	–	–
HBsAg (log ₁₀ IU/mL)	3.58 (2.07–3.78)	–	–
HBeAg (S/Co)	0.1 (0.08–0.2)	–	–
HBV DNA (log ₁₀ copies/mL)	1.6 (1.59–2.1)	–	–
HCC stages (Ia/Ib/IIa/IIb/IIIa)	4/2/6/5/9	–	–

HCC, hepatocellular carcinoma; HC, health control; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; AFP, alpha fetoprotein; HBsAg, hepatitis B virus surface antigen; HBeAg, hepatitis B virus e antigen; HBV, hepatitis B virus. Variables were defined as non-normally distributed variables based on the results of the Kolmogorov–Smirnov test, and results are expressed as the median and interquartile range (IQR). P values were generated using the Mann-Whitney U-test (for quantitative variables) and χ^2 test (for classified variables).

CD11c-FITC, CD11c-PE, PD-1-APC, HLA-DR-BV421, CCR7-FITC, and CD45RA-BV510. After permeabilization using a Cytofix/Cytoperm Kit (BD Biosciences, Franklin Lakes, NJ, USA), cells were used for intracellular cytokine staining using an anti-GB-PE antibody. Anti-IL-2, anti-IFN- γ , and anti-TNF- α antibodies were used to stain intracellular cytokines after stimulation with ionomycin (1 μ M) and phorbol-12-myristate-13-acetate (PMA) (100 ng/mL) for 6–8 h at 37°C in the presence of 5% CO₂. The anti-CD107a antibody was added at the same time as the stimulation. The flow cytometry antibodies were obtained from BioLegend (San Diego, CA, USA).

Immunohistochemical staining

Paraffin-embedded and formalin-fixed liver tissue specimens was cut into 5 μ m sections and placed on polylysinecoated slides. Then, the liver tissue sections were chosen to bake, deparaffinize, block the endogenous peroxidase activity with 0.3% H₂O₂ and heat-induce epitope retrieval. Following a brief wash and rinse in phosphate buffered saline, sections were stained with primary antibodies including mouse-anti-human CD11c and rabbit-anti-human CD8 (ZSGBBIO, Beijing, China) separately overnight at 4°C. Double staining was performed by using the HRP secondary antibody (ZSGB-BIO, China) for CD8 (blue color) and CD11c (brown color). The nuclei was stained with hematoxylin and the cytoplasm was stained with eosin. Images (100 $\times$ and 400 $\times$) were acquired using Olympus CX31 microscope and Aperio VERSA 8 scanning system (Leica Microsystems, Wetzlar, Germany).

Statistical analysis

Categorical variables were expressed as frequencies or proportions. Quantitative variables were expressed as medians and quartiles. Chi-square tests (for classified variables) and nonparametric tests (for quantitative variables) were used to compare the characteristics between the two groups (Table 1). The Wilcoxon paired test and Mann-Whitney U test were used to compare the median values between the two groups. Spearman's correlation coefficient was used to assess the correlation between the two variables. The data were analyzed using SPSS 25.0 and GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $P < 0.05$.

Results

Characteristics of peripheral CD11c⁺CD8⁺ T cells

To determine the differentiation characteristics of CD11c⁺CD8⁺ T cells, CD45RA and CCR7 were stained in the CD8⁺ T cell populations. In both the HCC and HC groups, CD11c⁺CD8⁺ T cells mainly showed an effector phenotype (CD45RA⁺CCR7⁺), while CD11c⁺CD8⁺ T cells were mostly effector memory phenotypes (Figure 1A). We further analyzed the activation and exhaustion phenotype, intracellular cytokine expression, cytolytic granule GB expression, and degranulation marker CD107a in CD11c⁺CD8⁺ and CD11c⁺CD8⁺ T cells. Compared with CD11c⁺CD8⁺ T

cells, CD11c⁺CD8⁺ T cells exhibited a highly activated and cytotoxic phenotype, with upregulated expression of CD38&HLA-DR, GB, and CD107a (Figures 1B, D, E). There were no significant differences in PD-1 expression between CD11c⁺CD8⁺ and CD11c⁺CD8⁺ T cells (Figure 1C). We also found that CD11c⁺CD8⁺ T cells produced higher levels of IL-2, IFN- γ , and TNF- α after PMA stimulation than the CD11c⁺CD8⁺ T cells did (Figures 1F–H). Representative flow cytometry plots of CD11c⁺CD8⁺ and CD11c⁺CD8⁺ T cells were shown in Supplementary Figure 1.

Comparison of CD11c⁺CD8⁺ T cells between the HCC and HC groups

There was no significant difference in the frequency of CD11c⁺CD8⁺ T cells and the levels of CD38&HLA-DR, PD-1, and GB expression of CD11c⁺CD8⁺ T cells between the HCC and HC groups (Figures 2A–D). We then compared the degranulation and cytokines production of CD11c⁺CD8⁺ T cells between the two groups. We found that the CD107a and TNF- α produced by CD11c⁺CD8⁺ T cells in HCC group were significantly decreased compared with that in HC group, while IL-2 and IFN- γ levels between the groups were comparable (Figures 2E–H).

The distribution characteristics of CD11c⁺CD8⁺ T cells in tumor tissues

To compare the characteristics of CD11c⁺CD8⁺ T cells in peripheral blood and liver tissue, mononuclear cells from the peripheral blood, tumor tissues, and non-tumor tissues of nine HCC patients were isolated. We found that the frequency of CD11c⁺CD8⁺ T cells in tumor and non-tumor tissues was significantly higher than that in the peripheral blood, and the difference between tumor and non-tumor tissues was not significant (Figure 3A). Notably, CD11c⁺CD8⁺ T cells in tumor tissues had increased expression of CD38&HLA-DR, and PD-1, and decreased expression of GB, compared with those in peripheral blood and non-tumor tissues (Figures 3B–D). These data indicate that CD11c⁺CD8⁺ T cells in tumor *in situ* may be in a state of high activation, exhaustion, and decreased killing capacity. The distribution of CD11c⁺CD8⁺ T cells in the liver tissue of patients with HCC was further examined by immunohistochemical staining. As shown in Figures 3E, F, CD11c⁺CD8⁺ T cells (black arrow) were mainly distributed at the boundary between the tumor and para-tumor tissues.

The GB expression level of CD11c⁺CD8⁺ T cells correlates with disease progression of HCC

According to the standard for diagnosis and treatment of primary liver cancer (2022 edition) (19), a total of 26 HCC patients were divided into an early-stage group (stages Ia, Ib, and IIa; n = 12) and an advanced-stage group (stages IIb and IIIa; n = 14). There was no significant difference in the frequency of CD11c⁺CD8⁺ T cells and the levels of CD38&HLA-DR, and PD-1 expression on CD11c⁺CD8⁺ T cells between the early- and advanced-stage groups (Figures 4A–C). Meanwhile, we found that the GB expression level of CD11c⁺CD8⁺ T cells in the early-stage group was significantly higher than that in the

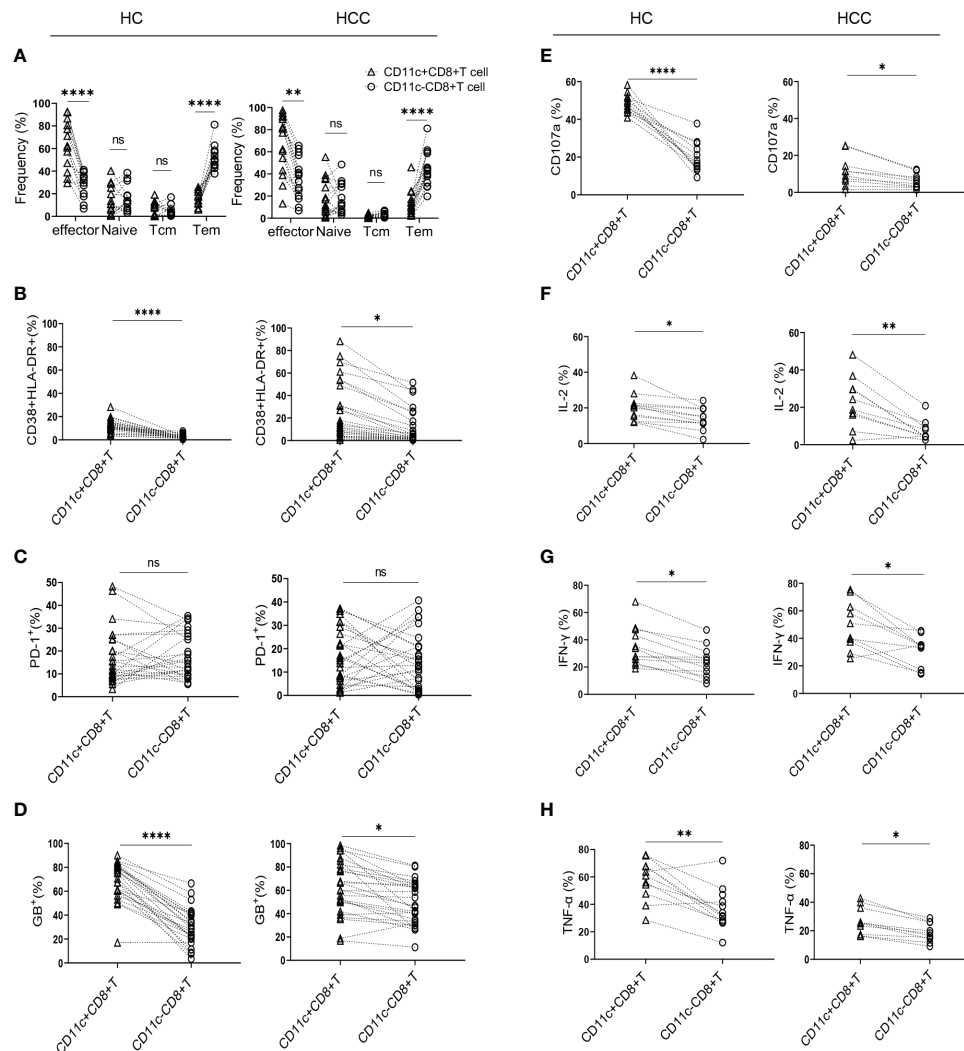


FIGURE 1

Characteristics of CD11c⁺CD8⁺ T cells in HCC and HC groups. (A) The frequency of effector memory (CCR7⁺CD45RA⁺) (Tem), central memory (CCR7⁺CD45RA⁻) (Tcm), naive (CCR7⁻CD45RA⁺), and effector (CCR7⁻CD45RA⁻) subsets of CD11c⁺CD8⁺ and CD11c⁻CD8⁺ T cells in HCC (n = 16) and HC (n = 13) groups. (B–D) The levels of CD38&HLA-DR, PD-1, and GB expression of CD11c⁺CD8⁺ and CD11c⁻CD8⁺ T cells in HCC (n = 26) and HC (n = 25) groups. (E–H) After PMA stimulation, the level of CD107a expression and IL-2, IFN-γ, and TNF-α production of CD11c⁺CD8⁺ and CD11c⁻CD8⁺ T cells in HCC (n = 10) and HC (n = 12) groups. P values were generated using the Wilcoxon paired test between the two groups. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not significant.

advanced-stage group ($P = 0.001$) (Figure 4D). We further explored the relationship between tumor volume and the GB expression level of CD11c⁺CD8⁺ T cells in patients with HCC. Referring to a previous study, tumor volume was calculated using the equation: volume = $0.52 \times \text{width}^2 \times \text{length}$ (20). We found that the GB expression level of CD11c⁺CD8⁺ T cells in peripheral blood was negatively correlated with tumor volume ($r = -0.581$, $P = 0.002$) (Figure 4E). However, there was no significant correlation between the GB expression level of CD11c⁺CD8⁺ T cells in tumor and non-tumor tissues and tumor volume (Figures 4F, G).

Discussion

Tumoricidal cytotoxic T lymphocytes play an important role in viral eradication and elimination of tumors (7, 21). The effector

CD8⁺ T cells are characterized by low exhaustion and high activation and proliferation (22). Previous studies have reported that CD11c⁺CD8⁺ T cells have a high proportion of antigen-specific effector cells with increased frequency following acute or chronic viral infections, such as human immunodeficiency virus type 1 or herpes simplex virus type 1 infection, and may have antiviral effects (13, 16, 23, 24). However, there are few studies on CD11c⁺CD8⁺ T cells in HBV-related HCC. In this study, we found that CD11c⁺CD8⁺ T cells of HCC patients mostly belong to effector cell subsets with higher expression levels of activated markers CD38&HLA-DR. Additionally, in both HCC and HC groups, CD11c⁺CD8⁺ T cells showed higher level of GB expression, as well as stronger ability of degranulation and IL-2, IFN-γ, and TNF-α production than CD11c⁻CD8⁺ T cells. Collectively, these findings indicate that CD11c⁺CD8⁺ T cells may be a multifunctional effector subset.

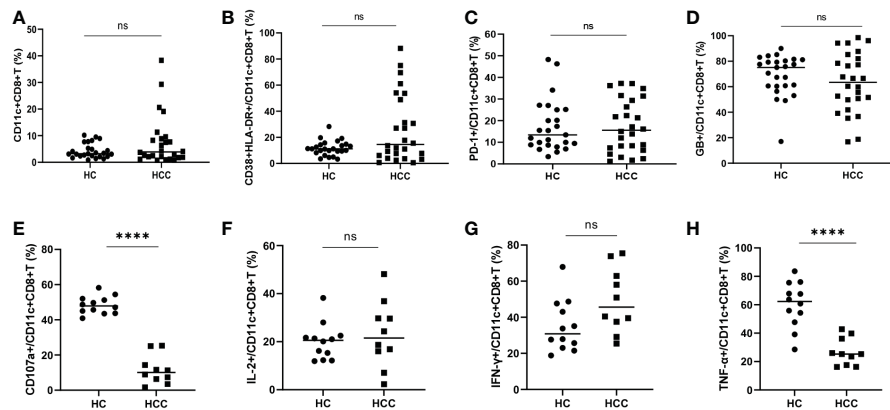


FIGURE 2

Frequency of CD11c⁺CD8⁺ T cells in HCC and HC groups. (A) The frequency of CD11c⁺CD8⁺ T cells in HCC (n = 26) and HC (n = 25) groups. (B–D) The levels of CD38&HLA-DR, PD-1, and GB expression of CD11c⁺CD8⁺ T cells in HCC (n = 26) and HC (n = 25) groups. (E–H) After PMA stimulation, the level of CD107a expression and IL-2, IFN-γ, and TNF-α production of CD11c⁺CD8⁺ T cells were detected in HCC (n = 10) and HC (n = 12) groups. The Mann-Whitney U test was used to compare the median values between the two groups. **** *P* < 0.001; ns, not significant.

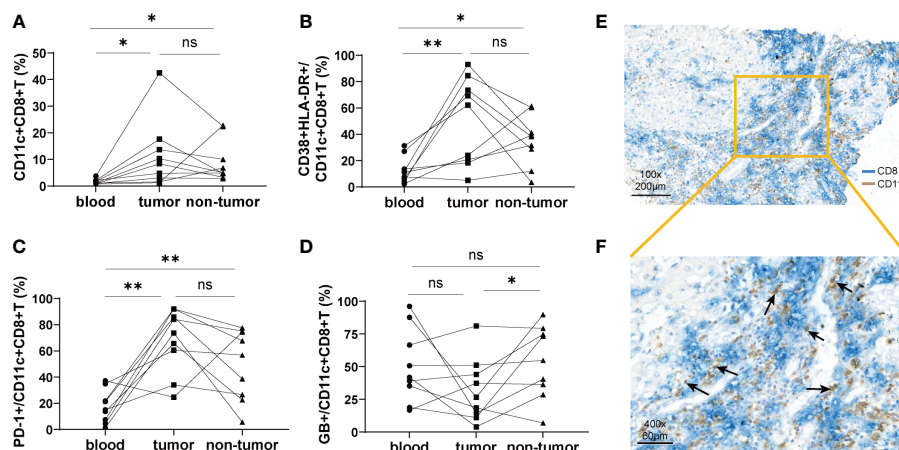


FIGURE 3

The distribution of CD11c⁺CD8⁺ T cells in tumor tissue and paired non-tumor tissue. (A) The frequency of CD11c⁺CD8⁺ T cells in the peripheral blood, tumor, and non-tumor tissues. (B–D) Paired analysis of the CD38&HLA-DR, PD-1, and GB expression on the CD11c⁺CD8⁺ T cells in the peripheral blood, tumor, and non-tumor tissues. (E, F) Representative results of immunohistochemical double staining of CD11c (brown) and CD8 (blue) from a patient with HCC. The orange square indicates the demarcation area between cancer and para-cancerous tissue. The black arrows point to the CD11c⁺CD8⁺ T cells. The data were from 9 HCC patients who have matched peripheral blood, tumor and non-tumor tissues. *P* values were generated using the Wilcoxon paired test between the two groups. **P* < 0.05; ***P* < 0.01; ns, not significant.

In this study, we found that the frequency of CD11c⁺CD8⁺ T cells in both tumor tissues and non-tumor tissues were significantly higher than that in peripheral blood. Combined with the data from phenotypic studies, we hypothesized that the accumulation of a large number of CD11c⁺CD8⁺ T cells in tumors is conducive to tumor control. A previous study found that CD8⁺ T cells in the tumor microenvironment have high expression levels of exhausted molecules, such as PD-1 and ITIM domains, were prone to exhaustion (25), and influence disease progression and clinical outcome (26). However, PD-1 expression is also significantly increased in activated T cells, which could be a functional effector T cell molecule, representing its active functional state (27, 28).

Interestingly, our data showed that activation markers, such as CD38&HLA-DR, were highly expressed in CD11c⁺CD8⁺ T cells in tumor tissues, as well as PD-1, compared to that of CD11c⁺CD8⁺ T cells in peripheral blood. This suggests that CD11c⁺CD8⁺ T cells in the liver tumor tissues may be in a state of overactivation and exhaustion. The overactivation of immune cells usually results in increased apoptosis and decreased function. Consistent with this hypothesis, we found that the expression of GB in CD11c⁺CD8⁺ T cells in tumor tissues was significantly lower than that in non-tumor tissues.

CD107a is a marker for degranulation of intracellular cytotoxic granules. We found that the levels of CD107a expression of

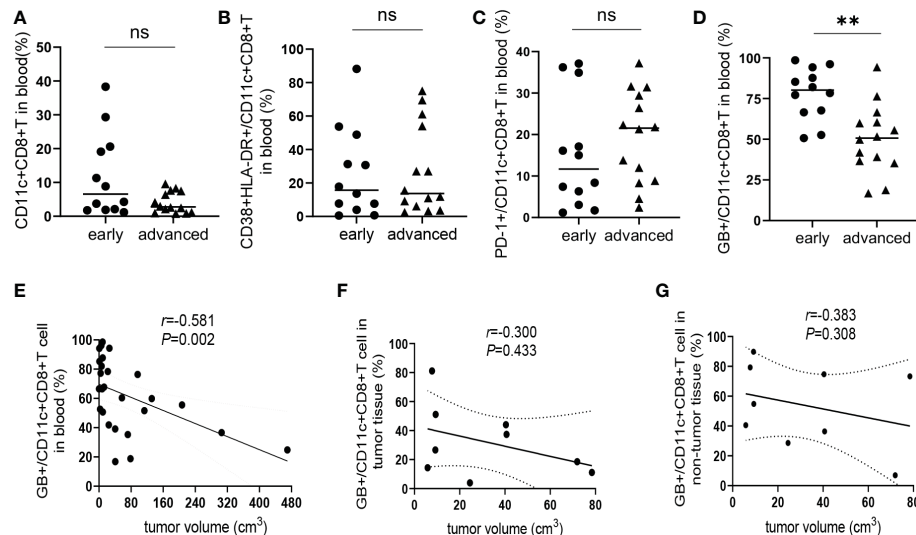


FIGURE 4

Correlation between CD11c⁺CD8⁺ T cells with estimated tumor volume. (A) The frequency of CD11c⁺CD8⁺ T cells in the peripheral blood of early-stage group (n = 12) and advanced-stage group (n = 14) of HCC patients. (B–D) The levels of CD38&HLA-DR, PD-1, and GB expression of peripheral CD11c⁺CD8⁺ T cells of early-stage group (n = 12) and advanced-stage group (n = 14) of HCC patients. (E) The correlation analysis between GB expression level in peripheral CD11c⁺CD8⁺ T cells and tumor volume of HCC patients (n = 26). (F, G) The correlation analysis between tumor volume and GB expression level in tumor and non-tumor infiltrating CD11c⁺CD8⁺ T cells of HCC patients (n = 9), respectively. The Mann-Whitney U test was used to compare the median values between the two groups. Spearman's correlation coefficient was used to assess the correlation between the two variables. *r*, correlation coefficient. ***P* < 0.01; ns, not significant.

CD11c⁺CD8⁺ T cells after PMA stimulation in HCC group were significantly lower than that of HC group, suggested that the capacity of GB-secreting of CD11c⁺CD8⁺ T cells in HCC patients was decreased. Consistently, the capacity of TNF- α production of CD11c⁺CD8⁺ T cells was also significantly decreased in patients with HCC than in healthy controls. Most importantly, we found that the GB expression level of peripheral CD11c⁺CD8⁺ T cells in patients with advanced HCC was significantly lower than in patients with early-stage HCC. Moreover, GB⁺CD11c⁺CD8⁺ T cells in peripheral blood were negatively correlated with tumor volume. These findings suggest that the decline in the number and function of CD11c⁺CD8⁺ T cells may be an important factor promoting the progression of HCC. In addition, our study is the first to observe that CD11c⁺CD8⁺ T cells were mainly distributed over the boundary of cancerous and para-cancerous tissues in the livers of patients with HCC.

This study has some limitations. First, the small sample size and lack of long-term follow-up in this cross-sectional study did not validate the role of CD11c⁺CD8⁺ T cells in predicting HCC progression. Second, there is a lack of antigen-specific tests for the number and function of CD11c⁺CD8⁺ T cells, as well as *in vivo* validation. Third, the exact mechanisms of the changes in the number and function of tumor-infiltrating CD11c⁺CD8⁺ T cells needs to be further explored.

In summary, we demonstrated that CD11c⁺CD8⁺ T cells were highly activated effector cells, and showed increased PD-1 and decreased GB expression in tumor tissues. Moreover, we found that the GB expression level of CD11c⁺CD8⁺ T cells was negatively correlated with tumor volume and associated with HCC progression. Therefore, CD11c⁺CD8⁺ T cells may be a potential prognostic marker and therapeutic target for patients with HBV-related HCC.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding authors.

Ethics statement

This study was approved by the institutional review board of the Fifth Medical Center of Chinese PLA General Hospital (KY-2022-4-16-1). The patients/participants provided their written informed consent to participate in this study.

Author contributions

LG and ZH performed the literature search, conducted laboratory experiments, analyzed data, produced the figures, wrote the manuscript. GL and A-LG conducted laboratory experiments and analyzed data. F-SW assisted with data interpretation and edited the manuscript. Y-MJ and JF designed the hypothesis, analyzed data, produced the figures and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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SUPPLEMENTARY FIGURE 1

Gating strategy for flow cytometry analyses. Effector memory (CCR7⁻CD45RA⁻) (Tem), central memory (CCR7⁺CD45RA⁻) (Tcm), naïve (CCR7⁺CD45RA⁺), and effector (CCR7⁻CD45RA⁺) subsets, and CD38&HLA-DR, PD-1, GB expression were identified on CD11c⁺ and CD11c⁻CD8⁺CD3⁺ T cells. The production of intracellular IFN-γ, and TNF-α, and IL-2 and CD107a expression on cell membrane after PMA stimulation were identified based on CD11c⁺ and CD11c⁻CD8⁺CD3⁺ T cells. The numbers indicate the percentages of cells within the gates.

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Effects of hepatitis B virus infection and strategies for preventing mother-to-child transmission on maternal and fetal T-cell immunity

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One of the most common routes of chronic hepatitis B virus (HBV) infection is mother-to-child transmission (MTCT). Approximately 6.4 million children under the age of five have chronic HBV infections worldwide. HBV DNA high level, HBeAg positivity, placental barrier failure, and immaturity of the fetal immune are the possible causes of chronic HBV infection. The passive-active immune program for children, which consists of the hepatitis B vaccine and hepatitis B immunoglobulin, and antiviral therapy for pregnant women who have a high HBV DNA load (greater than 2×10^5 IU/ml), are currently two of the most important ways to prevent the transmission of HBV from mother to child. Unfortunately, some infants still have chronic HBV infections. Some studies have also found that some supplementation during pregnancy can increase cytokine levels and then affect the level of HBsAb in infants. For example, IL-4 can mediate the beneficial effect on infants' HBsAb levels when maternal folic acid supplementation. In addition, new research has indicated that HBV infection in the mother may also be linked to unfavorable outcomes such as gestational diabetes mellitus, intrahepatic cholestasis of pregnancy, and premature rupture of membranes. The changes in the immune environment during pregnancy and the hepatotropic nature of HBV may be the main reasons for the adverse maternal outcomes. It is interesting to note that after delivery, the women who had a chronic HBV infection may spontaneously achieve HBeAg seroconversion and HBsAg seroclearance. The maternal and fetal T-cell immunity in HBV infection is important because adaptive immune responses, especially virus-specific CD8 T-cell responses, are largely responsible for viral clearance and disease pathogenesis during HBV infection. Meanwhile, HBV humoral and T-cell

responses are important for the durability of protection after fetal vaccination. This article reviews the literature on immunological characteristics of chronic HBV-infected patients during pregnancy and postpartum, blocking mother-to-child transmissions and related immune mechanisms, hoping to provide new insights for the prevention of HBV MTCT and antiviral intervention during pregnancy and postpartum.

KEYWORDS

hepatitis B virus, effects, pregnancy, mother-to-child transmission, immunological characteristics, T-cell immunity

1 Introduction

The presence of the hepatitis B virus (HBV) in the body for a period of more than 6 months is considered to be a chronic HBV infection. Chronic HBV infection is the primary factor that leads to liver cirrhosis and hepatocellular carcinoma (HCC). In 2019, there were roughly 316 million persons in the world who were living with chronic HBV infection, and the chronic HBV infection rate was 4.1% across the globe. The number of deaths that can be attributed to HBV rose by 5.9% between the years 1990 and 2019, and it rose by 2.9% between 2015 and 2019 (1). In countries with a high prevalence rate of chronic HBV infection, the most prevalent form of chronic HBV infection is mother-to-child transmission (MTCT). About 75 million women of childbearing age worldwide are infected with chronic HBV, accounting for about 25.3% (2, 3). In the absence of HBV preventive measures, the MTCT incidence of HBV infection is 31.3%; in addition, about 95% of cases infected during infancy or early childhood almost always result in chronic hepatitis (2, 3). Therefore, preventing HBV MTCT and the success of preventative measures taken for neonates are of great importance to human health.

Several guidelines recommend that pregnant women with a high level of HBV DNA should take anti-HBV drugs (like tenofovir

disoproxil fumarate (TDF)) during the second or third trimester (4–7) to decrease the amount of HBV DNA in the mother, which in turn will lower the chance of HBV MTCT. The guidelines for HBV-infected pregnant women during pregnancy and postpartum are shown in Table 1. After birth, children should take preventive measures, namely regular injections of the hepatitis B vaccine and hepatitis B immunoglobulin (HBIG). The use of antiviral drugs during pregnancy and the injection of the hepatitis B vaccine and HBIG into the newborn greatly reduce the failure rate of HBV MTCT. In children younger than 5 years of age, in 2019, the hepatitis B surface antigen (HBsAg) prevalence rate was 1% across the world, which was 76.8% lower than the prevalence rate in 1990 (1). However, there are still some newborns that are afflicted with HBV, particularly those born to pregnant women who either have a high HBV DNA load or are positive for the hepatitis B e antigen (HBeAg) (4, 8). Therefore, some studies pointed out that the implementation of strategies for blocking MTCT of HBV should not only consider HBV DNA load but also combine HBeAg status to achieve a higher degree of precision in determining the risk of HBV MTCT. In addition, the HBeAg test can be used as an indicator to determine whether or not antiviral treatment is required during pregnancy in regions where the HBV DNA test is not available (9). In 2016, the

TABLE 1 Guidelines for HBV-infected pregnant women during pregnancy and postpartum.

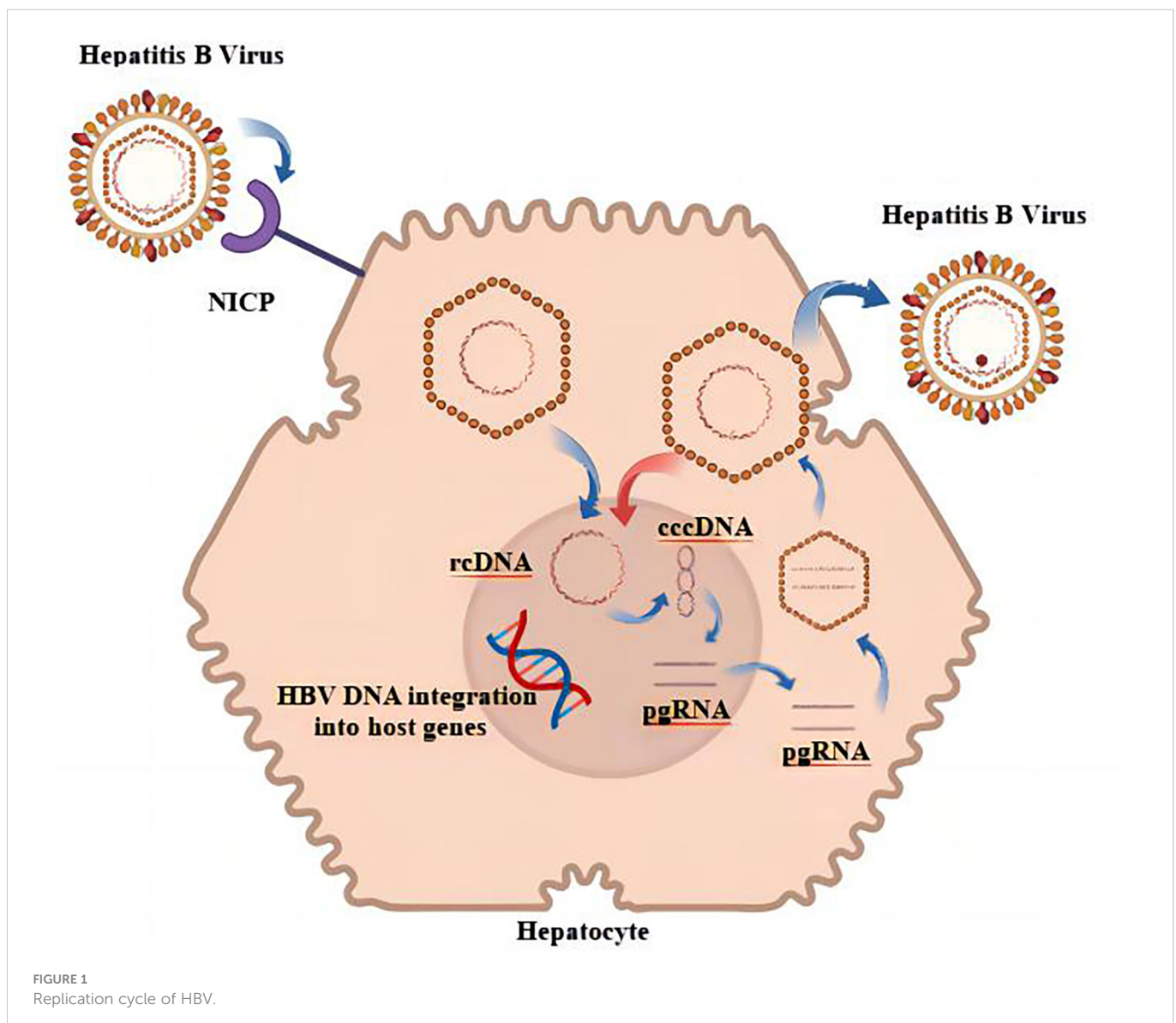
Guideline	Year of publication	Pregnant women who should receive antiviral intervention	Start antiviral intervention gestational week	First-choice antiviral drug	Puerpera when should discontinue the antiviral intervention	Postpartum monitoring
European Association for the Study of the Liver (EASL) (7)	2017	HBV DNA >200,000 IU/ml or HBsAg >10,000 IU/ml	24–28 weeks	TDF	Continue for up to 12 weeks after delivery	–
American Association for the Study of Liver Diseases (AASLD) (4)	2018	HBV DNA >200,000 IU/ml	28–32 weeks	TDF	Immediately after delivery to 3 months postpartum	Postpartum 6 months for hepatitis flares and seroconversion
Chinese Society of Infectious Diseases, Chinese Medical Association (6)	2019	HBV DNA >200,000 IU/ml	24–28 weeks	TDF	Immediately after delivery or continue up to 1–3 months postpartum	Up to postpartum 6 months
Asian Pacific Association for the Study of Liver (APASL) (5)	2022	HBV DNA >200,000 IU/ml	24–28 weeks	TDF	Immediately after delivery or continue up to 12 weeks postpartum	At least 24 weeks for hepatitis flare and rebound of HBV DNA

World Health Organization (WHO) made a proposal to reduce the number of new cases of hepatitis by 90% in the year 2030 (10). Therefore, aggressive and safe antiviral and immunological trials based on pregnant women are required to lower the risk of HBV-related unfavorable consequences for both the mother and the offspring.

2 Biological characteristics of HBV

As a hepatotropic virus, HBV can be integrated into the genome of host hepatocytes after infecting humans, and the integrated HBV is a factor in the continuous generation of viral proteins as well as the induction of HCC (11, 12). HBV releases its genome into the host liver nucleus, where it establishes a DNA pool that is full of covalently closed circular DNA (cccDNA); the pool is characterized by persistence and stability (13). In addition, a number of studies came to the conclusion that the sources of HBsAg are different in various

phases of chronic HBV infection and that cccDNA was a primary source of HBsAg in HBeAg-positive persons (14). When HBeAg was negative, it is possible that HBV DNA integration was the main source of HBsAg in HBV-infected individuals (15). It is also one of the reasons why it is difficult to achieve serological disappearance of HBsAg in CHB treatment. The replication cycle of HBV is shown in Figure 1. The likelihood of developing HCC would drop dramatically after HBsAg was no longer present (16). Unfortunately, this risk has not disappeared entirely. Studies had shown that patients who had achieved functional cures are still at the risk for HCC, which might be related to the influence of continuous HBV integration on the promotion of HCC (17). HBV integration plays a similar biological function in promoting HCC, regardless of whether HBsAg is positive or negative (18). The biological characteristics of HBV that have been discussed imply that one of the essential steps toward decreasing the number of worldwide HBV-related adverse events is the complete and total elimination of HBV MTCT, as well as the reduction in the number of new cases.



3 Adverse maternal outcomes and HBV infection

Whether HBV infection can affect maternal immunity and lead to adverse maternal outcomes is still controversial. According to the findings of a retrospective cohort study, having a positive HBsAg was associated with an increased risk of a number of adverse maternal outcomes during pregnancy; these adverse maternal outcomes included gestational diabetes mellitus (GDM), postpartum hemorrhage (PPH), cesarean delivery, and intrahepatic cholestasis (19). Zhang et al. (20) retrospectively analyzed the pregnancy outcomes of 9,699 HBsAg-positive and 73,076 HBsAg-negative pregnant women, and the result found that pregnant women who were HBsAg-positive were more likely to undergo PPH and intrahepatic cholestasis of pregnancy; in addition, HBsAg-positive pregnant women had a higher risk of placental abruption and premature birth in the vaginal delivery group. According to the findings of another meta-analysis, the risk of preterm birth in pregnant women who had a chronic HBV infection was significantly higher than the risk of preterm birth in pregnant women who did not have an HBV infection; furthermore, the risk of preterm delivery rose by 16% (21). Moreover, the presence of HBV was an independent risk factor for early preterm delivery, but the level of HBV DNA did not affect the increase in risk associated with early preterm delivery (22). Peng et al. (23) demonstrated that baseline HBeAg status and HBV DNA level were not associated with GDM, despite the fact that maternal carrying of HBsAg was an independent risk factor for GDM. The occurrence of GDM may be related to age (> 35 years old) and abnormal liver function (24). However, advanced age is a risk factor for GDM (25). According to the findings of another study, chronic HBV infection was linked to a marginally increased risk of low birth weight and preterm birth, but HBeAg positivity led to an increased risk of low birth weight, preterm birth, and GDM (26).

According to some studies, there was no difference in the risk of preterm delivery or GDM between the HBsAg-positive group and the non-HBV-infected group; however, the abortion rate of the non-HBV-infected group was significantly less than that of the HBsAg-positive group before or after using the multivariate model to adjust the confounding factors (sociodemographic variables and obstetric complications) (27). Bajema et al. (28) believed that there was no correlation between having a positive HBV and an increased risk of adverse pregnancy outcomes in a country that had a low epidemic situation (the USA). In addition to the adverse outcomes of preterm birth, GDM, and abortion, alanine aminotransferase (ALT) flare in HBV-infected women during pregnancy or postpartum is also of great concern for the reason that the maternal immunity may change after delivery (29, 30). A prospective study involving 158 chronic HBV-infected pregnant women showed that during pregnancy or postpartum, spontaneous elevation of ALT in HBV-infected women was infrequent, mild, and self-limited; after birth, the group of women discontinuing antiviral medication was more likely to have ALT flare (AF, defined as ≥ 100 U/L), and the AF was also self-limiting and mild (31). Another study of untreated women with HBV infection during pregnancy and postpartum, which excluded women who started antiviral therapy during pregnancy for postnatal analysis, found a sudden increase in ALT and HBV DNA during the third trimester

and early postpartum period in women with CHB; however, baseline ALT, HBV DNA, HBeAg-positive status, gestational age, and delivery times were not predictors of undesirable clinical outcomes, including spontaneous abortion, hepatic flare, premature delivery, GDM, pre-eclampsia, and liver failure (32). Another Chinese study involving 869 HBV-infected patients who did not receive antiviral intervention and 3,367 pregnant women without HBV infection showed that elevated ALT levels were more common in the CHB group than those in the HBV-free group; women with HBV DNA levels $\geq 5 \log_{10}$ IU/ml or increased ALT during delivery are more likely to have a sudden increase or worsening of ALT (33). Therefore, compared with other adverse consequences, elevated ALT after delivery is common in pregnant women with HBV infection, and they should be followed up closely after delivery regardless of whether they have taken antiviral drugs during pregnancy. Mild elevations in ALT are usually self-limiting. If the ALT flares ($\geq$ five times ULN), it should be combined with other viral markers (such as HBV DNA level and HBeAg state) to determine the need for antiviral therapy. With antiviral therapy, ALT can usually be controlled and rarely leads to serious consequences such as liver failure.

4 T cells and HBV infection

4.1 Maternal T-cell immunity and HBV infection

HBV infection affects maternal immunity, resulting in adverse maternal outcomes that may be related to pregnancy and T-cell mechanisms. T-cell-induced immune mechanisms also play an important role in the phenomenon of allograft tolerance zygote formation, embryo implantation and development, and fetal allograft tolerance. The fetus is a combination of chromosomes of both parents, and for the mother, the zygote is an allograft. The success of pregnancy is bound to be accompanied by the successful suppression of immune rejection and the establishment of maternal immune tolerance, and the dynamic balance of immune cells controls the whole process from the successful implantation of the embryo to the delivery of the fetus (34). The inhibition of Th1 response and activation of Th2 immunity would impair maternal immune reaction to HBV and reduce CD8 T cells; then, the impaired immune led to virus activation and immune escape, resulting in increased vertical transmission for the reason that CD8 T cells were the main effector cells in T-cell response (35). Progesterone has the ability to prevent threatened abortion (36). Th2-related cytokines such as interleukin-4 (IL-4) and interleukin-10 (IL-10) can promote progesterone production by luteal cells, and the promoting effect is more significant in luteal cells in early pregnancy (37). The study by Piccinni et al. also found a significant increase in decidual T cells and the contents of leukemia inhibitory factor (LIF), IL-4, and IL-10 in women who were normal gestation compared with decidual cells in women with recurrent miscarriage (38). At the fetal-maternal interface, IL-4 and LIF mediated by progesterone contributed to the success and maintenance of pregnancy. Furthermore, LIF expression by T cells was positively correlated with IL-4 production, implying that progesterone could promote LIF expression by T cells by

inducing the production of IL-4, which could be one of the mechanisms by which progesterone worked to maintain normal pregnancy (38). According to the findings of another study, both IL-4 and IL-10 could suppress the maturation and function of Th1 cells as well as macrophages, which helped to prevent allograft rejection (39). The changes in decidual memory T-cell subsets and related cytokines may also be related to the occurrence of pre-eclampsia (40). Chronic HBV infection changed the immune response of women with reproductive failure by increasing the frequency of B cells, decreasing the number of CD3⁺CD4⁺ helper T cells, and reducing the activity and toxicity of peripheral NK cells (41). Although there is no clear conclusion on the adverse influences of HBV infection on mother and fetus in different studies, in the era of precision medicine, it is necessary to closely detect HBsAg-positive pregnant women during pregnancy and postpartum. As an immune-involved disease, T-cell immunity plays an important role in the elimination of HBV. The adverse events induced by HBV infection may be because HBV disrupts the maternal–fetal immune balance by interfering with the activation of T cells or the secretion of related cytokines, resulting in a series of pregnancy and postpartum complications. In contrast, it is possible that HBV infection in those uninfluenced by HBV infection is in equilibrium with T-cell immunity.

Gao et al. (42) analyzed the immune microenvironment of pregnant women in their second trimester (22–25 weeks), who were either healthy pregnant women without HBV infection or pregnant women with a positive state of HBeAg and HBV DNA $\geq 1.0 \times 10^7$ IU/ml; the results by analyzing T-cell receptor sequencing in mononuclear cells taken from peripheral blood showed a more active immune response among the immune cell subsets in HBV-related samples; more specifically, HBV-infected pregnant women had a higher percentage of effector/memory CD8⁺ T cells, and both CD4⁺ and CD8⁺ effector/memory T cells of HBV-infected pregnant women increased expression of genes related to metallothionein at higher levels, activated more metal ion pathways, and caused a variety of inflammatory responses. Another interesting thing was that even women with a normal pregnancy (non-HBV infection) also had immune effects, which might last until approximately 1 year after delivery. During early pregnancy, for instance, there is a rise in suppressor T cells that help the mother accept the fetus and a rise in helper T cells that aid in pregnancy maintenance; furthermore, the postpartum aggravation of autoimmune diseases may be associated with the activation of helper T cells and cytotoxic T cells between 1 and 4 months postpartum and the activation of suppressor T cells between 7 and 12 months postpartum (39). The changes of pregnancy to the immune system, particularly T-cell-associated immunity, can lead to elevated ALT levels during pregnancy or the postpartum period in women who have a chronic infection with HBV.

4.2 Fetal immunity and HBV infection

4.2.1 Placental immunity and viral infection

As a link between mother and fetus, the placenta has the functions of material exchange, barrier, immunity, and others (43). For some substances, however, the placental barrier does not completely block MTCT. In the case of viral infections, some viruses have developed

innate immune mechanisms to evade the placental barrier. The most common “TORCH” testing includes *Toxoplasma gondii*, other viruses (such as *Treponema pallidum*, parvovirus B19, varicella-zoster virus), herpes simplex virus 1 and 2, rubella, and cytomegalovirus (44). These viruses might cause a series of adverse events, such as early abortion, fetal organ malformations (such as congenital heart disease (45)), functional defects of organs (such as sensorineural hearing loss in children (46)), and so on. Unfortunately, some defects in fetal organ function are not detected until after childbirth, for the reason that prenatal testing (such as ultrasound during pregnancy) is unable to identify them (such as amentia) before delivery. Therefore, the TORCH examination is one of the recommended examinations for women who are ready for pregnancy or who are in early pregnancy. In addition to those effects during pregnancy and the perinatal period, viral infections can also make babies more susceptible to a number of diseases. Some scholars believed that this observation was related to the impact that early-life virus infection has on the immune system. In their studies, they found that infants infected with cytomegalovirus within the first year of life had a higher risk of tuberculosis in childhood (47). Similar to the above viruses, HBV or HBV-related markers can cross the placental barrier to cause intrauterine infection, which is one of the possible mechanisms of HBV intrauterine infection, which ultimately leads to the failure of MTCT treatment for HBV and undesirable outcomes. Failure to block MTCT of HBV is usually associated with HBeAg positivity and/or high HBV DNA levels, which are important virological factors, and the intrauterine infection rate of children born to such mothers is 1%–9% (48). In addition, infants who were younger with gestational age at birth or who have not received three complete doses of the hepatitis B vaccine were also prone to failure of MTCT of HBV (49).

4.2.2 Placental immunity and HBV markers

Intrauterine transmission (IUT) is one of the ways of MTCT of HBV. At present, little is known about the clear mechanism of IUT caused by HBV. There are many factors that lead to IUT with HBV. In addition to the positive status of HBeAg and/or high levels of HBV DNA, these negative factors also include the poor immune status of the mother, the presence of minor thalassemia, HBV mutation, disruption of placental barriers, and fetal susceptibility (50–53). Some research on the mechanism of intrauterine HBV infection has shown that the levels of HBV or its related markers decrease from the maternal surface to the fetal surface of the placenta. However, whether HBV or its related markers replicate in the placenta remains controversial. Some scholars have studied the derivatives of HBV. Derivatives such as HBsAg-positive and HBV DNA-positive were markers of HBV infection. When HBsAg is negative, HBeAg or HbcAb is important evidence of contact with HBV. Zhang et al. (54) found that both HBsAg and HbcAg can be found in placentas that come from mothers who were positive for HBsAg using ABC immunohistochemical method. They also detected that the level of two antigens decreased from the maternal side of the placenta to the fetus' side. It also indicated that the placenta might act as a partial barrier to HBV. *In vitro* experiments, HBV translocated across a trophoblastic barrier by transcytosis of infectious hepadnavirus and HBV DNA, which were detected in placentas after the co-culture of HBV DNA-positive serum and trophoblast cells (55, 56). Garima

et al. (57) also observed that even at relatively low levels, receptors and HBV markers could replicate in the placenta. As the placenta developed, trophoblast cells differentiated and fused into multinucleated cells that formed the syncytiotrophoblast, and the maturity of the placenta and specific maternal antibodies, such as maternal immunoglobulin G (IgG), had the ability to reduce the risk of HBV transmission (55). The placenta usually matures with gestational age, but in premature infants, the placenta may not mature due to gestational age or other factors (58). This could explain why premature babies are more likely to succumb to MTCT.

4.2.3 Fetal immunity and HBV markers

HBeAg and HBsAg are two significant secretory proteins of HBV and have the function of affecting host immunity to maintain persistent HBV infection. The point had been confirmed in many studies. Another thing to consider is that maternal-derived HBeAg can spread throughout the placenta and be detected in newborns. In addition, such HBeAg altered macrophage function in infants and induced immunologic tolerance *in utero* to drive the persistence of HBV infection after vertical transmission in a mouse model (59, 60). However, another study believed that the source of the HBeAg-positive mother did not lead to T-cell tolerance toward products related to the HBV-related gene, and this result was based on the research about a mice model born to HBeAg-transgenic mothers. Fortunately, if HBV MTCT is successful, HBeAg in an infant will not always be in the body and cannot be detected before the first 6 months of life; anti-HBe becomes undetectable before the age of 1 year (61).

The HBsAg exposure *in utero* has not been completely investigated to determine whether or how it affects the continued presence of HBV replication and the fetal immune response. Through the research on a mouse model of only HBsAg exposure *in utero*, Ning et al. came to the first conclusion that, contrary to what others had previously thought, HBeAg played an immunosuppressive effect on the offspring; only HBsAg exposure *in utero* did not develop the immune tolerance to HBV on the fetus; however, when re-exposed to HBV, the offspring could speed up the elimination of HBV in the body; and this beneficial effect may be related to only HBsAg exposure *in utero*, which could activate antigen-presenting dendritic cells and then induce an increase of the HB-specific CD8⁺ T cells and secretion of interferon (IFN)- γ in the hepar of mice (62). Another research by cord blood analysis also showed that immunity of newborn infants can be beneficially affected due to the HBV-infected state of their Asian mothers; it was mainly manifested as the enhancement of innate immune cell maturation and the increasement of Th1 development, and specially showed that low levels of IL-10 (Th2-related cytokines) and high levels of IL-12p40, IFN- α 2 (Th1-related cytokines); importantly, such enhancement effect led to the robust response of the neonatal immune cells when they were exposed *in vitro* to pathogens that were unrelated to their condition, and the pathogens were *Pseudomonas aeruginosa*, *Salmonella typhimurium*, uropathogenic, *Escherichia coli*, *Acinetobacter baumannii*, and *Listeria monocytogenes*; however, this beneficial effect cannot be demonstrated in the baby Caucasian HBV-infected mother's delivery, which was explained by different HBV genotypes; patients of Asian descent had HBV genotypes B/C, while patients of Caucasian descent had HBV genotype D (63). From the result of their important

experiments, we can infer that compared to the offspring of HBeAg-positive females, those born to HBeAg-negative females are less adversely affected by HBV infection and may be beneficially affected. A number of researchers had also conducted research on how chronic HBV infection altered the immune response at the maternal interface of the placenta. A study supported enrolled chronic HBV-infected pregnant women (HBV group) and pregnant women without HBV infection as a healthy control group (HC group), and showed that hyperactivated decidual CD8⁺ T cells in the HBV group generated a lower level of IFN- γ than those in the HC group, indicating that the ability of hyperactivated decidual CD8⁺ T cells had been impaired in the HBV group; it concretely demonstrated that the activation molecules of CD69 and HLA-DR in the HBV group were low; they suggested that in order to stop the spread of HBV through MTCT, antiviral intervention in the HBV group was necessary and important (64).

A function of CD8⁺ T cells was found to be associated with persistent HBV infection in newborns, as was discovered in other studies that investigated the mechanism behind chronic HBV infection. A study from the USA showed that there was not a significant difference between the three groups when it came to the frequency of CD8 and CD4 T cells in peripheral blood and cord blood that was collected at birth; two groups (positive for both HBV DNA and HBsAg: group 1; negative for both HBV DNA and HBsAg: group 2) were born to HBsAg-positive mothers, and another group (HBV DNA and HBsAg negative: group 3) was born to healthy mothers; but in comparison to groups 2 and 3, it was found that CD8⁺ T cells of group 1 had a functional defect that was characterized by decreased IFN- γ production and decreased CD107A expression. In addition, significant differences were found among the three groups with regard to the frequency of regulatory T cells expressing FoxP3 in the peripheral blood, and the frequency of regulatory T cells expressing FoxP3 in group 1 was the highest of all three groups (group 1: 63.79%, group 2: 8.12; group 3: 11.06%, $p < 0.05$). In other words, CD8⁺ T cells in newborns who tested positive for HBV DNA and HBsAg were dysfunctional. It was possible that the immune tolerance state of the adaptive immune system in neonates who had chronic HBV infection was related to the expansion of regulatory T cells and the impairment of the TCR signal in these infants (65). Another study also showed that HBV or HBV-related markers could be exposed to the fetus *in utero*; when the HBV-related markers had enough concentration, they could stimulate fetal/neonatal immune responses; after HBcAg stimulation, increased IFN production was observed in 30.4% of infants with both negative HBsAg and HBeAg, and the infants were born to HBsAg-positive mothers (66).

5 T cells and measures of HBV-MTCT prevention

Anti-HBV intervention for the appropriate population and timely and standardized injection of HBIG and hepatitis B vaccine after birth are important measures of HBV-MTCT at present. The safety of anti-HBV drugs and hepatitis B vaccine for mothers and offspring has been confirmed in many clinical studies (67–70). It is suggested that the hepatitis B vaccine should be given at the first 24 h, 1 month, and 6

months of neonatal life. Moreover, the WHO also recommended one dose of HBIG combined with the hepatitis B vaccine to improve the success of HBV-MTCT prevention within 24 h of birth. The effect of anti-HBV drugs on maternal and offspring immune systems is also one of the issues worth exploring.

5.1 Maternal immune and measures of HBV-MTCT prevention

5.1.1 Maternal immune and antiviral intervention

Huang et al. (71) demonstrated that T-cell immunity got involved in AF after delivery, and their study showed that compared with pregnant women who did not use antiviral intervention (NAF group), pregnant women who received the antiviral intervention (AF group) during pregnancy had significantly lower HBsAg levels at 6–8 or 15–18 weeks after delivery. In addition, CD4⁺ T cells or CD8⁺ T in the AF group produced fewer anti-inflammatory cytokines, such as IL-10, or more proinflammatory cytokines (tumor necrosis factor- α (TNF- α), IFN- γ , IL-2, IL-21) before, during, and after antiviral therapy; furthermore, the proportion of IFN- γ to IL-10 was higher in the AF group than that in the NAF group during pregnancy or postpartum. Another study enrolled HBV-infected pregnant women in the immune-tolerant phase of NA intervention at 24 to 28 weeks of gestation. According to the ALT levels at 6–12 weeks after delivery, the pregnant women were divided into the postpartum hepatitis group (group 1) and nonhepatitis group (group 2), and the results showed that compared to baseline or group 2, group 1 after childbirth usually had relatively lower levels of HBsAg, HBeAg, and HBV DNA. In addition, ALT flare after childbirth in immune-tolerant HBV patients may be associated with alterations in immune system performance. The result also showed that CD8⁺ T cells included CD8⁺ effector memory T cells and effector T cells that were significantly activated after childbirth in group 1 (72). A study found that compared with the group not taking antiviral drugs, the postpartum NK cell count in the group receiving antiviral therapy during pregnancy increased significantly, and postpartum hepatitis may also be related to the change of NK cell frequency and immune damage caused by HBV infection (73). Li et al. (74) conducted a prospective study to investigate whether antiviral therapy and the timing of postpartum drug withdrawal affected the risk of developing postpartum hepatitis among pregnant women infected with HBV, and the study showed that the timing of drug withdrawal (immediately or $\geq$ 6 weeks postpartum) did not have any impact on the rate at which postpartum hepatitis occurred in CHB women. Meanwhile, within 12 weeks after delivery, hepatitis occurred in more than 90% of the women who did not take antiviral drugs and those who stopped antiviral drugs immediately after delivery, but drug withdrawal at 6 weeks postpartum and more than 6 weeks postpartum may delay the onset of postpartum hepatitis. Another study revealed that 45% of women who had a chronic HBV infection showed activity of liver disease after delivery, and liver disease activity was defined as ALT within 6 months postpartum $\geq$ three times the lowest ALT value during pregnancy. Compared with those not treated with lamivudine, pregnant women receiving lamivudine intervention during pregnancy had a higher rate of liver disease activity after delivery (75).

T cells play an important role in anti-HBV therapy. HBV produces and secretes a large number of related markers and depletes B cells and specific T cells, resulting in impaired cytokine activation and continuous expression of a variety of inhibitory receptors, which enable it to evade the host's innate immune system and continue to replicate in hepatocytes. Le et al. (76) analyzed the immune cells of patients with chronic HBV infection and found that the number of HBS-specific T cells in patients with chronic HBV infection negatively increased with age, and no correlation was found between serum HBsAg levels and the phenotype or function of T cells or NK cells. A study had also found that the escape mechanism might be connected to the manipulation of cell metabolome by HBV (77). CHB is an immune response-mediated disease, and nucleo(t)ide analog (NA) or IFN is an effective and widely used therapy for CHB patients. IFN has a stronger ability to clear HBsAg than NAs, which may be related to the fact that IFN can significantly promote the expression of a variety of immune cells, including NK cells and plasmacytoid dendritic cells (pDCs) (78, 79). In addition, CD8⁺ T-cell-mediated immunity has the function of anti-HBV, and NK cells have been found to contribute to the induction of CD8⁺ T-cell-mediated immunity in animal models (80). Therefore, IFN induces the expression of a variety of immune cells and indirectly induces host T-cell immunity, which may be the key to achieving HBsAg seroconversion. In addition, dynamic changes in cytokines are observed when CHB patients receive antiviral therapy (81). CHB patients receiving IFN antiviral therapy can achieve HBeAg serological conversion when the frequency of inhibitory cells (such as FoxP3(+) Tregs) is reduced (82). Overexpression of some chemokine ligands can also accelerate HBV clearance by activating depleted T cells. Yan et al. (83) observed that overexpression of C-C motif chemokine ligand 19 (CCL19), a leukocyte chemoattractant, could rapidly eliminate HBV in primary mouse hepatocytes. Their research also suggested that overexpression of CCL19 could increase the number of CD8⁺ T cells, reduce the frequency of PD-1⁺ CD8⁺ T cells in the blood, and weaken the inhibition of hepatic antigen-presenting cells (APCs). *In vitro* models, it was found that the T-cell conjugation antibodies were used to drive T cells to adsorb specifically to tumor tissues expressing HBV envelope proteins, and then by activating the antiviral activity of T cells, it could eliminate HBV-infected hepatocytes and specifically kill tumor cells (84). In conclusion, anti-HBV therapy and the elimination of HBV-related tumors both rely heavily on the contributions of T cells.

5.1.2 The effect of HBIG implementation during pregnancy

The results on the effectiveness of HBIG implementation during pregnancy for MTCT are conflicting, and so far, this treatment has not been adopted by other countries. A meta-analysis included 36 trials of pregnant women receiving HBIG for the prevention of MTCT of HBV infection during the third trimester of pregnancy; the result showed that adverse events of the HBIG in newborns had not increased and HBIG could reduce HBsAg-MTCT in newborns compared with no intervention, but the investigator believed that the quality of the evidence found in this review was very low, so the result could not prove that HBIG was beneficial in preventing MTCT of HBV (85). Another study using immunohistochemical staining of the

placentas of HBsAg-positive pregnant women found that HBsAg positivity was mostly found in trophoblastic cells and villous mesenchymal cells, while HBIG deposition mostly distributed in Hofbauer cell areas. There was no colocalization of HBIG and HBsAg in the placenta, and HBV DNA, HBeAg, and HBcAb load in peripheral venous blood from mothers receiving prenatal HBIG treatment did not differ significantly from that of rejected HBIG mothers. As a result, HBIG had no effect on virus replication; however, HBIG had the potential to form an immune barrier in the placenta, blocking HBV transmission from mother to fetus (86). Overall, rigorously designed, randomized, and prospective clinical trials should be conducted to determine the benefits and drawbacks of using HBIG in the prevention of MTCT of HBV.

5.2 Fetal immune and measures of HBV-MTCT prevention

5.2.1 Fetal immune and antiviral intervention

Little information is available on the influence of antiviral drugs on neonatal immunity. A study from China showed that the mother used LDT for the prevention of HBV-MTCT, and in this condition, LDT had effect on CD4⁺CD25⁺ regulatory T cells of neonate. The researchers enrolled HBsAg-positive pregnant women who were divided into HBeAg-negative group (viral load < 10⁶ IU/ml) and HBeAg-positive group (viral load ≥ 10⁶ IU/ml). According to the willingness to receive LDT intervention, HBeAg-positive group was divided into the LdT-treated group and the nontreated group during the third trimester of pregnancy. Analyzing CD4⁺CD25⁺ Tregs in the neonatal peripheral blood within 6 h postpartum by flow cytometric techniques, they found that the frequency of CD4⁺CD25⁺ T cells in the LdT-treated group was the lowest in contrast to the other categories and highest in nontreated group (LdT-treated group vs. nontreated group vs. HBeAg-negative group: 2.8% vs. 7.0% vs. 4.2%, $p < 0.05$). This phenomenon proved that the ability of CD4⁺CD25⁺ Tregs to stimulate virus multiplication and infant CD4⁺ T cell and CD8⁺ T-cell ratios may be affected by the maternal HBV reproductive status. In addition, the enhancement of neonatal cellular immune function appeared in the pregnant women who were being treated with LDT treatment (87).

5.2.2 Fetal immune and hepatitis B vaccine

The purpose of infant injection hepatitis B vaccine is to boost immunity, resulting in loss of HBsAg and long-term control of HBV replication. According to current research, the recipient's response to the hepatitis B vaccine may be influenced by their T-cell immunity. For instance, the edited T-cell receptor-chain variable was linked to the immune response of healthy vaccinees to recombinant hepatitis B surface antigen (rHBsAg) as well as the production of hepatitis B surface antibody (HbsAb) (88). Circulating follicular helper T cells (cTfh) and subsets were shown to be involved in the immune response to hepatitis B vaccine injection *in vitro*, and the CXCR3-Tfh cell subset was preferentially activated when HBsAg stimulated PBMCs. One of the reasons for reduced antibody responses in the immune response to hepatitis B vaccination could be a decrease in cTfh cells and subset skewing (89, 90). Approximately 5%–10% of healthy recipients of the hepatitis B vaccine did not produce HbsAb;

nonresponse was associated with other factors such as age, gender, body mass, and route of injection, in addition to impaired Th cell response (91).

The HbsAb of those who were effective in the hepatitis B vaccine usually lasted a long time. A study of infants born to HBsAg- and HBeAg-positive mothers found that with or without HBIG inoculation, the fetal HbsAb and immune memory were still persistent 20 years later. Furthermore, if the infants received an interim booster dose of hepatitis B vaccine at age 5, compared with those in the unboosted group, the rate of HbsAb concentration ≥ 10 mIU/ml and the mean HbsAb concentration were higher in the boosted group at the age of 20 (92). In addition, maternal supplementation, such as folic acid, can improve the persistence of protective antibodies; this improvement was linked to the fact that folate deficiency may reduce iron absorption, resulting in anemia, and iron deficiency anemia could influence cell-mediated and nonspecific immunity. Moreover, folic acid may also affect the persistence of HbsAb by providing methyl groups (93). Another interesting question is whether maternal antiviral drugs or HBV markers influence infant immunity response to the hepatitis B vaccine. In order to investigate whether telbivudine exposure during pregnancy influences offspring's immune response to the hepatitis B vaccine, the researchers enrolled HBsAg-positive mothers and their neonates. All infants had completed a three-dose vaccine schedule at 0, 1, and 6 months. Anti-HBs, immune cells, and cytokines were detected when they were followed up on 11–13 months after birth. The results showed that there was no significant difference between the LdT group and control group in infant anti-HB GMC, positive rate of anti-HBs, strong positive rate of anti-HBs, proportion of myeloid dendritic cells, B cells, helper T cells, cytotoxic T cells, and plasmacytoid dendritic cells, and the concentrations of TNF- α , IFN- α , IFN- γ , IL-2, IL-4, IL-6, IL-10, and IL-12; hence, LdT exposed *in utero* could affect infant immune response to hepatitis B vaccine (94). There was a point where, regardless of dosage, infants were born to HBsAg-positive mothers, and their immune response to the hepatitis B vaccine would not be affected by maternal anti-HB serostatus in the case of successful HBV MTCT. However, HBeAg state had an effect on vaccine efficacy in the lower 5 μ g dose; as a result, the infant born to an HBsAg-positive and HBeAg-positive mother had a lower estimated vaccine efficacy value than those born to an HBsAg-positive and HBeAg-negative mother; thus, it was suggested that a higher-dose (10 μ g) hepatitis B vaccine was used to prevent HBV MTCT, especially for the infant born to an HBsAg-positive and HBeAg-positive mother (95). Some scholars advised that mothers of infants born HBsAg-positive with HBV DNA loads > 6 log₁₀ IU/ml inject 20 μ g hepatitis B vaccine, and that increasing the dose of hepatitis B vaccine could protect this high-risk population from occult HBV infection (OCI) (96). A hepatitis B intervention study from Qidong, China, showed that adults benefited from neonatal hepatitis B vaccination, which could protect them from OCI (97). However, a 20- μ g hepatitis B vaccine did not reduce the rate of immunoprophylaxis failure compared to a routine dose (10 μ g) of the hepatitis B vaccine (96, 98).

5.2.3 Fetal immune and HBIG

HBIG is used to prevent the invasion and replication of HBV, and the recipient can quickly develop passive protective immunity, neutralize

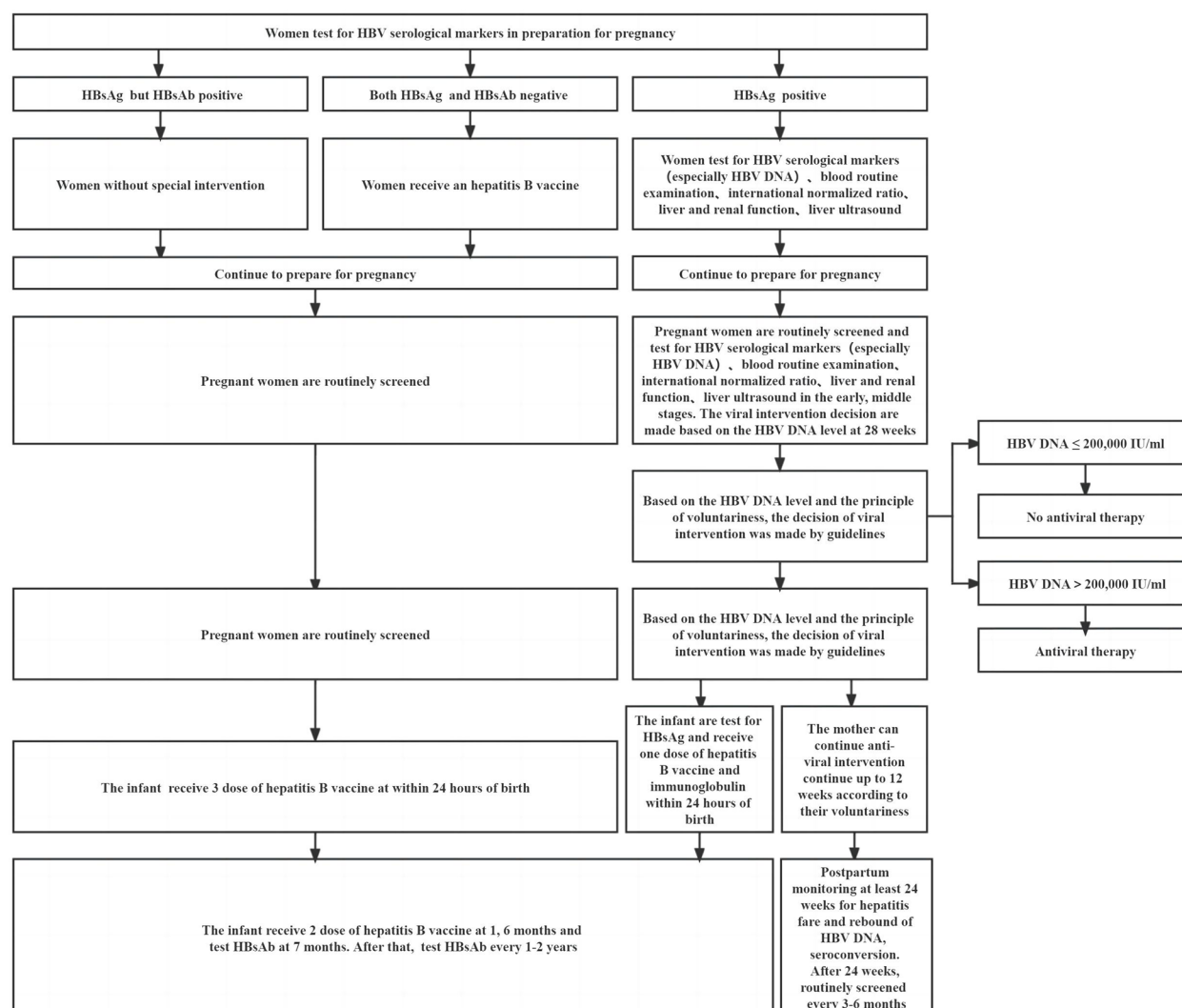


FIGURE 2

Recommendations for a woman of reproductive age during preparation for pregnancy, pregnancy, and postpartum.

and clear the HBV, and avoid HBV infection (99). Compared with the hepatitis B vaccine alone, the immunization schedule of the hepatitis B vaccine combined with HBIG improved the success rate of preventing HBV MTCT (100), and the effect was more pronounced among infants born to HBeAg-positive and HBsAg-positive mothers (101). However, this effect may not be evident in infants born to HBsAg-positive but HBeAg-negative mothers, and some researchers argue that HBIG for an infant who was born to an HBsAg-positive and HBeAg-negative mother was unnecessary for preventing HBV MTCT (102–104). In our opinion, this phenomenon may be related to the low HBV transmission rates between HBeAg-negative and HBsAg-positive mothers and children, but HBIG did not play a role in them. In addition, a number of studies concluded that administering HBIG injections to infants who were born to mothers who were HBsAg-positive but HBeAg-negative did not appear to lower the rate of HBV-MTCT, but it may be helpful in preventing infantile fulminant hepatitis (8, 105). Therefore, evidence that children born to HBeAg-negative and HBsAg-positive mothers are free of HBIG is not sufficient. In addition, in both high and low HBV endemic areas, considering the cost and economic efficiency of vaccination, HBIG treatment for infants whose mothers had HBsAg positivity is a cost-

effective addition to universal hepatitis B vaccination, regardless of whether their mothers had HBeAg positivity or not (106–108). Compared to a higher dosage (200 IU) of HBIG, 100 IU HBIG may be enough to block HBV MTCT (109).

6 Conclusion and perspective

HBV infection can affect human immunity, and the immune environment also changes after pregnancy. Therefore, women who are pregnant and have a chronic HBV infection appear to be at a higher risk of complications during pregnancy compared to women who do not have HBV infection. When maternal immunity is balanced, complications may not occur. Precision medicine in resource-allowable settings requires close monitoring of the mother during pregnancy and postpartum. In addition, people at high risk of HBV transmission can be identified earlier, and intervention to prevent HBV MTCT can begin as soon as possible. Postpartum complications such as ALT flare may not be all bad. Immune-mediated ALT flare may be accompanied by clearance of HBV and may rarely cause hepatic decompensation or even death

under close monitoring (31, 110). Hence, chronic HBV pregnant women who use antiviral drugs correctly when postpartum hepatitis occurs and their specific immune environment may favor a good outcome from chronic HBV infection. Some studies indicated that approximately 12%–55% of pregnant women who were HBeAg and HBsAg positive achieved spontaneous HBeAg seroconversion after delivery, which may be related to the presence of mutations in the pre-core, the basal core promoter, or both of those locations combined (111, 112). Timely and appropriate antiviral intervention and standardized vaccination of hepatitis B vaccine and HBIG are particularly important for children born to HBsAg-positive mothers. The recommendations for a woman of reproductive age during preparation for pregnancy, pregnancy, and postpartum care are shown in Figure 2. An infant's immune function is not mature, making them susceptible to certain diseases, slow to recover from diseases, and prone to sequelae. Compared with those infected by horizontal transmission, adult chronic HBV patients with MTCT were susceptible to severe liver diseases and had a poor therapeutic effect (110). In addition to liver cirrhosis and HCC, people with chronic HBV infection also had a higher risk of developing non-Hodgkin lymphoma, pancreatic cancer, and other nonhepatocellular carcinoma malignancies (113, 114). Prenatal HBV exposure alters the epigenome profile (such as DNA methylation) in infants, and the differences in DNA methylation have been linked to the development of hepatocellular and colorectal carcinoma, as well as fatty acid oxidation (115). There are many things we do not know about HBV infection. It is clear that a combining scheme of screening positive mothers, administering antiviral therapy in a high-risk group (HBV DNA $> 2 \times 10^5$ IU/ml, rather than HBeAg positive or negative) during pregnancy, and implementing infant immunization schedule for administering the hepatitis B vaccine in conjunction with HBIG are required to prevent HBV MTCT. For the sake of postpartum complications and occult HBV infection in children, it is recommended to extend their follow-up time after birth. It is not appropriate to conduct double-blind, randomized trials on pregnant women or infants.

In order to prevent HBV MTCT more successfully, we should conduct feasibility prospective studies, relying on good data retrospectively and immunoassay *in vitro*. For example, observational studies with high confidence are needed to determine whether HBV infection increases maternal and fetal adverse outcomes. In addition, relevant studies of maternal serology are required to prove whether maternal DNA is associated with the failure of HBV blockade. More immune tests and placental pathology *in vitro* should be carried out to clarify the mechanism of HBV MTCT.

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Author contributions

HL, ML, and YX contributed to the study concept and design. HL, WC, LuxZ, LY, XB, YLin, WD, TJ, FS, ZZ, YLu, LuZ, RL, YG, SW, HH, XC, LH, MX, QX, JD, and RS collected and sorted out literature. HL wrote the first manuscript and drew pictures. WC, LuxueZ, LY, XB, and YLin edited and modified the English manuscript. JD, RS, ML, and YX submitted the modified version. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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LAMP3 expression in the liver is involved in T cell activation and adaptive immune regulation in hepatitis B virus infection

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Background: The disease burden caused by chronic hepatitis B virus (HBV) infection is still heavy, and the current treatment scheme has not achieved a complete cure. Changes in natural and adaptive immunity usually accompany chronic HBV infection. As a marker expressed on dendritic cells (DCs), whether lysosome-associated membrane glycoprotein 3 (LAMP3) participates in chronic HBV infection deserves further analysis.

Methods: We retrieved chronic HBV infection transcriptional information from the Gene Expression Omnibus (GEO) database. The LAMP3 expression in the liver of patients with chronic hepatitis B (CHB) was analyzed in three GEO datasets and confirmed in our validation cohort (27 patients with CHB). Differentially expressed genes were obtained from one CHB cohort by comparing LAMP3^{high} and LAMP3^{low} expression subgroups. These genes underwent Gene Ontology, Kyoto Encyclopedia of Genes and Genomes analysis, and Gene Set Enrichment Analysis to decipher the influence of LAMP3 on the biological process and immunity changes in HBV infection. Furthermore, we investigated the potential relationship between LAMP3 levels, the abundance of infiltrating immune cells, and liver dysfunction.

Results: Compared to healthy controls, LAMP3 expression was upregulated in the transcriptional profiles of the liver in patients with CHB. The high LAMP3 expression was related to T cell activation and the chemokine signaling pathway. The LAMP3 gene was positively linked to marker sets of infiltrating activated regulatory T cells (Treg), T cell exhaustion, monocytes, and DCs. Moreover, CHB patients with high LAMP3 expression had unfavorable liver dysfunction.

Conclusions: LAMP3 is a gene related to HBV infection, which might be involved in HBV infection by regulating T cell activation and adaptive immune response.

KEYWORDS

LAMP3 1, T cell 2, immune regulation 3, liver 4, hepatitis B virus

1 Introduction

Chronic hepatitis B (CHB) infection is a globally recognized public health problem, estimated to affect approximately 300 million people worldwide (1–4). Despite the successful introduction and practical application of an HBV vaccine, the disease burden of HBV-related liver disease is still heavy (5). CHB is directly related to the occurrence and development of cirrhosis, liver cancer, and the long-term morbidity associated with liver disease. Additionally, as the second most lethal cancer in the world, liver cancer accounts for 600,000 deaths annually (5, 6).

Previous basic and clinical studies have analyzed chronic HBV infection's characteristics, natural history, and immunological pathogenesis (4, 6, 7). Currently, the primary therapeutic strategy against chronic HBV infection is suppressing viral replication, reducing liver injury caused by the virus, and improving patients' life quality (8). The treatment of CHB infection mainly focuses on nucleoside (nucleotide) analogs that inhibit HBV DNA replication, reverse transcription, and pegylated interferon that activates the adaptive immune response. These treatment schemes can achieve a partial or functional cure for chronic HBV infection. However, these schemes are still unable to achieve a complete or sterilizing cure (9). Recently, researchers presented various ideas on hepatitis B immunotherapy, including the TLR 7/8 agonist and retinoic acid-inducible gene I, immune checkpoint inhibitors, therapeutic vaccines, and peginterferon lambda (10–15). However, these are all trial treatments without adequate clinical evidence.

Recent studies have provided a new perspective on immunological markers as therapeutic agents for chronic liver diseases. Lysosome-associated membrane glycoprotein 3 (LAMP3) is a glycosylated membrane protein located on the 3q chromosome (16, 17). As a member of the lysosomal-associated membrane protein family, LAMP3 is usually expressed in lymphoid organs (18). At the cellular level, LAMP3 is a marker for mature dendritic cells (DCs) in humans, and it is upregulated upon DC activation and maturation (19). However, it is unclear whether LAMP3 is involved in chronic HBV infection. In this study, we analyzed the Gene Expression Omnibus (GEO) databases and our cohort, explored the correlation between LAMP3 and HBV infection, and investigated related immunological changes.

2 Material and methods

2.1 Data resources

We collected three independent transcriptional profiles from the GEO database, including the liver samples of healthy control

and patients with CHB. Furthermore, as a representative of non-viral hepatitis, one RNA-seq dataset of patients with nonalcoholic steatohepatitis (NASH) was also used for validation in other hepatitis. Table 1 showed detailed information on these four cohorts. The LAMP3 expression between patients with CHB and healthy controls was analyzed through the “edgeR” package.

2.2 Differentially expressed genes analysis

According to the median counts of LAMP3 expression, patients with CHB were divided into LAMP3^{high} and LAMP3^{low} expression groups. DEGs (Log2 [Fold change] >1, adjusted p value <0.05) between these two groups were extracted and analyzed using R 4.1.2 (R Foundation for Statistical Computing, Vienna, Austria) for Gene Ontology (GO) function, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and Gene Set Enrichment Analysis (GSEA).

2.3 Gene correlation analysis

Pearson correlation analysis were applied to evaluate the genes related to LAMP3 expression. The associations of LAMP3 expression with the adaptive immune response-related genes were analyzed by the “cor.test” function of R studio. The “ggscatterstats” function in the “ggstatplot” package generated the dot plot. Finally, the analyzed results were visualized in Glue modules by Cytoscape 3.9.1.

2.4 Validation cohort

We collected liver tissues of 27 patients with CHB and 5 healthy controls (normal liver tissue of patients with hepatic hemangioma) to validate the bioinformatic results. The detailed information of all participants showed in Table 2. All paraffin-embedded formalin-fixed liver biopsy samples were obtained from the Peking University People's Hospital Hepatology Department. This study was approved by the Ethical Committees of Peking University People's Hospital (no. 2020PHE081), and all participants signed the written informed consent forms.

2.5 Staining and histological evaluation

Subsequently, immunohistochemistry (IHC) was performed using primary antibody anti-LAMP3 (1:200, cat# ab271053, Abcam) to validate the expression in our cohort. Five fields (×200) were randomly selected and captured for analysis in each sample. The number of cells in the portal tract (per portal tract area) was quantified.

2.6 Statistical analysis

All statistical analysis was completed by SPSS 20.0 (Chicago, IL, USA) and R 4.1.2 (Vienna, Austria). The differences of transcriptional levels between control and CHB patients, or LAMP3^{high} and LAMP3^{low} expression groups were analyzed by the Mann–Whitney U test. Two paired p < 0.05 was assumed significant statistically.

Abbreviations: HBV, hepatitis B virus; DCs, dendritic cells; CHB, chronic hepatitis B; DEGs, differentially expressed genes; GO, gene ontology; NA, nucleoside (nucleotide) analogues; GEO, Gene Expression Omnibus; NASH, nonalcoholic steatohepatitis; GSEA, Gene set enrichment; FFPE, paraffin embedded formalin fixed; IHC, immunohistochemistry; NK, natural killer; ALT, alanine aminotransferase; AST, aspartate aminotransferase. and DBIL, direct bilirubin; ISG, interferon stimulated gene; LNMTs, lymph node metastasized tumors.

3 Results

3.1 LAMP3 expression upregulated in transcriptional profiles of patients with CHB

In total, three GEO RNA-seq profiles (GSE83148, GSE159413, and GSE166759) were used to compare the transcriptional expression of LAMP3 between patients with CHB and healthy controls. **Table 1** presented detailed information on these GEO datasets. Analyzed results showed that the mRNA levels of LAMP3 in the livers of patients with CHB increased significantly compared to that in healthy controls (all $p < 0.05$, **Figures 1A–C**). Furthermore, using our cohort, we

validated the upregulated LAMP3 protein level in our CHB liver tissues, the representative images showed in **Figure 1D**. We also explored mRNA levels in a transcriptional profile of patients with NASH, representing a sterile inflammatory and immune-activated state. Similarly, the levels of LAMP3 mRNA in the liver of patients with NASH was significantly higher than that of healthy controls (**Figure 1E**).

3.2 LAMP3 upregulation was associated with T cell activation and adaptive immune regulation in patients with CHB

To explore the potential function of LAMP3 in CHB infection, we equally divided patients from one CHB cohort (GSE83148) into two

TABLE 1 Details of four GEO cohorts used in this study.

GEO series	Contributors	Year	Control (N)	CHB/NASH (N)	Platform
GSE83148	Zhou W, Ma Y, Wang J, Zheng H, Liu J	2017	6	122	Affymetrix Human Genome U133 Plus 2.0 Array
GSE159413	Nishio A, Rehmann B	2020	19	12	NanoString nCounter GX Human Immunology v2
GSE166759	Yao J, Diao H	2021	3	3	Agilent-079487 Arraystar Human LncRNA microarray V4
GSE89632	Allard JP, Arendt BM, Comelli EM, Ma DW, Lou W, Teterina A, Kim T, Fung SK, Wong DK, McGilvray I, Fischer SE	2016	19	24	Illumina HumanHT-12 WG-DASL V4.0 R2 expression beadchip

TABLE 2 Clinical information of 27 patients with CHB.

	Control (n=5)	All CHB (n=27*)	CHB (IT, n=3)	CHB (IA, n=5)	CHB (LR, n=11)	CHB (RA, n=3)
Sex (M/F)	3/2	22/5	3/0	5/0	9/11	1/2
Age (y)	49	43	47	40	45	41
ALT (U/L)	35	94	49	294	30	192
AST (U/L)	30	99	40	334	26	185
ALP (U/L)	56	105	85	111	87	112
GGT (U/L)	38	78	20	177	33	68
TBIL	15.3	31.2	13.4	134.6	16.5	19.1
HBsAg	–	+	+	+	+	+
HBsAb	–	–	–	–	–	–
HBeAg	–	+/-	+	+	–	–
HBeAb	–	+/-	–	–	+	+
HBcAb	–	+	+	+	+	+
HBV DNA (IU/ml)	–	6.29 10E+06	3.94 10E+07	3.91 10E+06	4.73 10E+02	2.84 10E+04
LAMP3+ cells (n, ×200)	1	7	4	10	5	17

Ps: Sex showed the number of males/females of CHB patients, ALT, AST, ALP, GGT, HBV DNA and the LAMP3+ cells showed the mean of each item. IT, Immune tolerant phase; IA, Immune activation; LR, Low replication; RA, Reactivation; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, γ -glutamyl transpeptidase; y, year; CHB, chronic hepatitis B. *There were some missing data from certain samples.

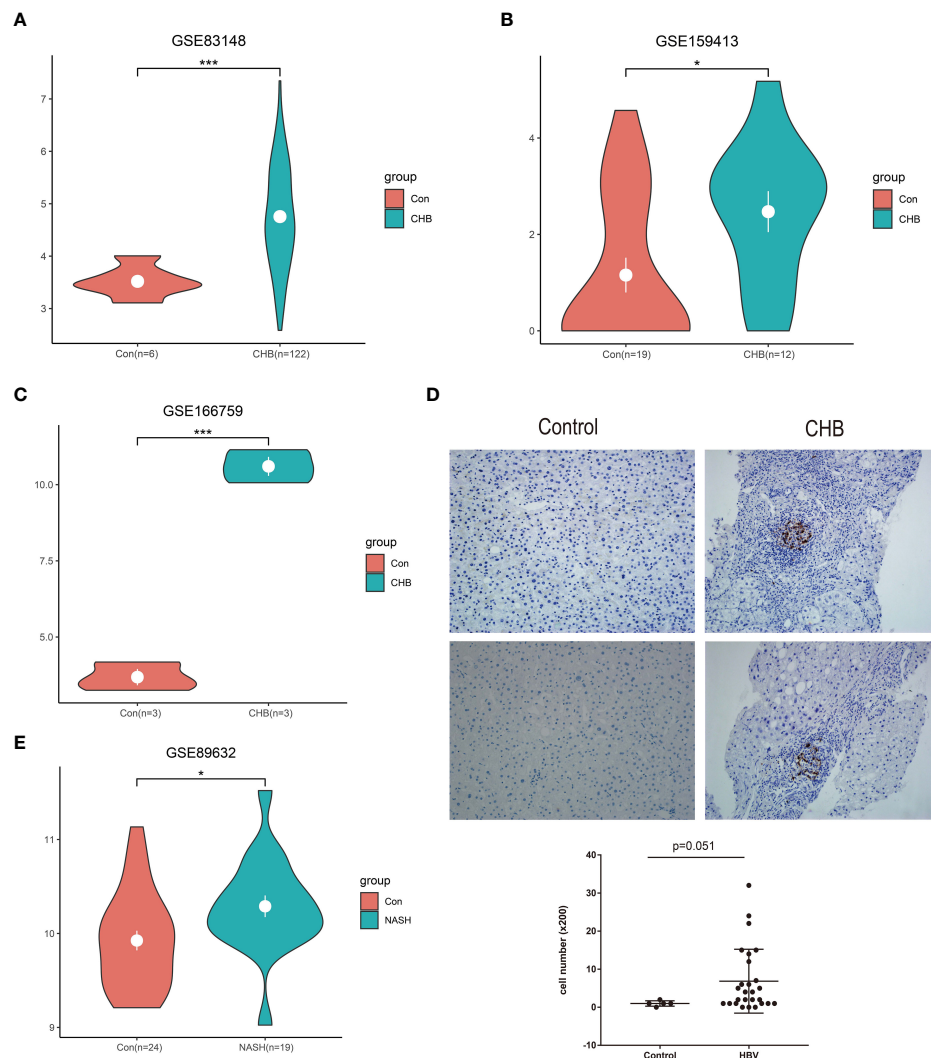


FIGURE 1

Upregulated LAMP3 expression in patients with CHB. (A–C) The comparison of LAMP3 transcriptional levels between patients with CHB and healthy controls in three GEO databases. (D) The expression of LAMP3 in our validation cohort. (E) LAMP3 expressions in patients with NASH and controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. con, Control; CHB, chronic hepatitis B; GEO, Gene Expression Omnibus; NASH, nonalcoholic steatohepatitis; LAMP3, lysosome-associated membrane glycoprotein 3.

groups (LAMP3^{high} and LAMP3^{low}) according to the median expression levels of LAMP3. DEGs between LAMP3^{high} and LAMP3^{low} subgroups were then analyzed. Biological processes and molecular functions in GO enrichment showed that the upregulated DEGs were mainly associated with T cell activation, cell response to chemokine, cytokine, and chemokine receptor binding (Figures 2A–C). KEGG enrichment analysis indicated that the upregulated genes were primarily distributed in the cytokine-cytokine receptor interaction and chemokine signaling pathway (Figure 2D). GSEA analysis explored the signaling pathways associated with upregulated LAMP3 mRNA levels. The analyzed results showed that genes in the LAMP3^{high} cohort were mainly enriched in the cytokine signaling pathway. For example, IL2-STAT5, IL6-JAK-STAT signaling, and interferon α and γ response were enriched, similar to that observed in GO and KEGG analyses (Figures 2E–H).

Co-expression analysis was performed to characterize the genes associated with the expression of LAMP3 in the CHB cohort (Figure 3A). These genes were strongly associated with LAMP3 expression and

analyzed further ($r > 0.8$, $p < 0.05$, Figure 3B). We found that the genes related to T cell activation, DCs, macrophage maturation, and cytokine release were significantly related to the expression of LAMP3. The CCND2, CSF2RB, STK17B, and TNFAIP8 genes were primarily expressed on T- and DC cells (all $r > 0.83$, $p < 0.05$) (Figure 3B). Additionally, using GlueCo, we could visualize the interactive network of genes that were in the same biological processes and functionally related. The significantly related biological functions were leukocyte proliferation, migration, and T cell activation ($p < 0.001$, Figure 3C).

3.3 Relationship between LAMP3 expression and marker genes sets of various immune cells

We explored the relationship of LAMP3 mRNA levels to the abundance of infiltrating immune cells, including T cells, B cells,

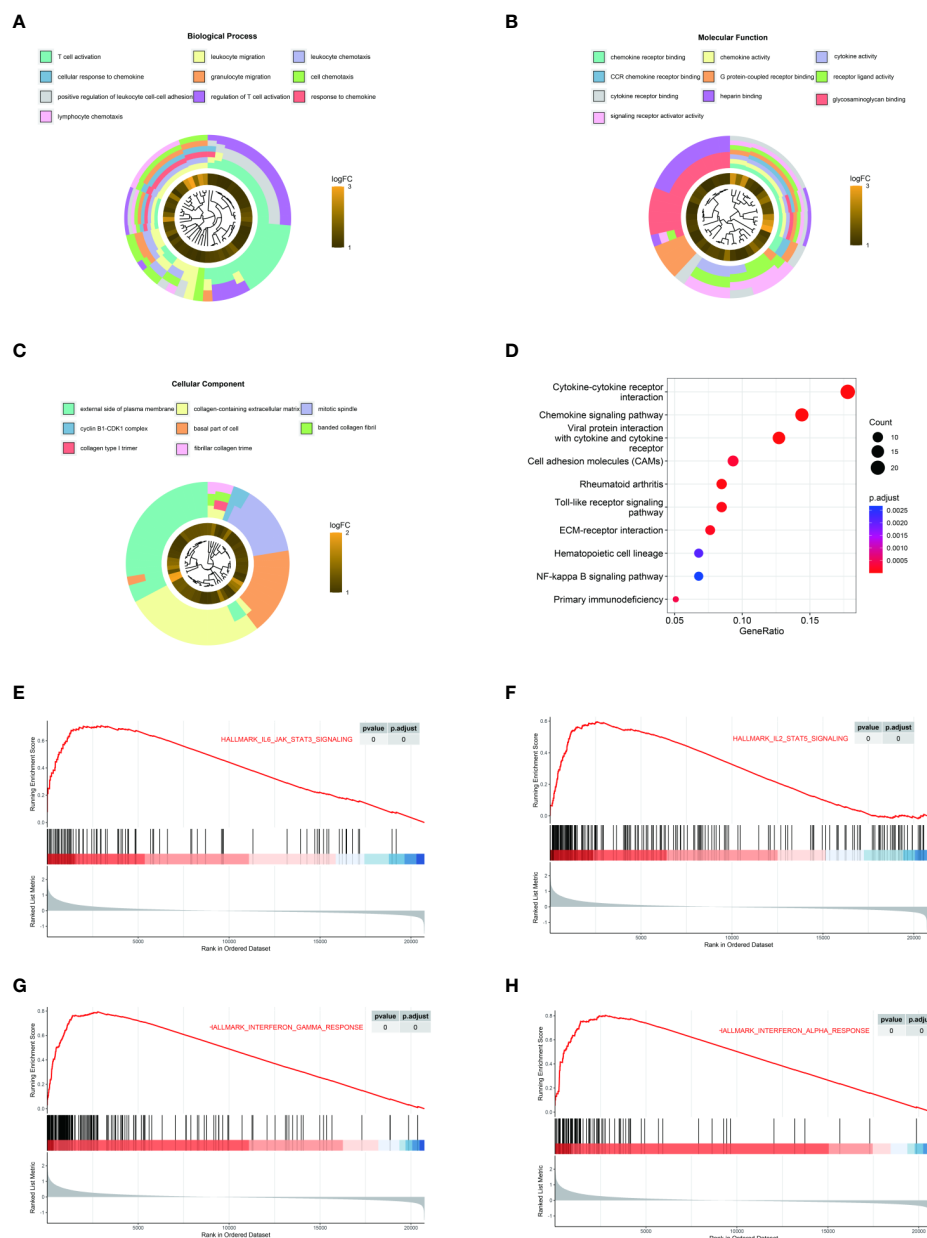


FIGURE 2

LAMP3 upregulation is associated with T cell activation and adaptive immune regulation. (A–D) GO analysis and KEGG pathway enrichment of 216 upregulated DEGs. (E–H) GSEA showed that LAMP3 is positively associated with various bioprocesses of T cells. DEGs, differentially expressed genes; KEGG, Kyoto encyclopedia of genes and genomes; GSEA, Gene Set Enrichment Analysis; LAMP3, lysosome-associated membrane glycoprotein 3.

neutrophils, natural killer cells, monocytes, macrophages, and DCs (Figure 4A). Remarkably, there was a close relationship among markers of Treg cells (TGF β), T cell exhaustion (TIM3), and Th1 cells (STAT1, IFN- γ). Moreover, the mRNA levels were positively associated with general T cell markers and CD4 $^{+}$ T cells. In addition, the DC markers (CD1C, HLA-DPB1), macrophages, monocytes (CD86, CSF1R), and neutrophils (ITGAX, CCR7) showed a close correlation with LAMP3 levels (Figure 4A). After T cell was divided into CD4 $^{+}$ T cells and CD8 $^{+}$ T cells, the mRNA levels of RGS1 and TRAT1 in CD4 $^{+}$ T cells and CD69 and LCK in CD8 $^{+}$ T cells were correlated with LAMP3 level significantly (Figure 4B).

3.4 LAMP3 expression was associated with liver dysfunction in HBV infection

We counted LAMP3 $^{+}$ cells in each portal area under a 200 $\times$ visual field and analyzed the correlation between LAMP3 $^{+}$ cells and serum markers of liver function injury. The results indicated that the number of LAMP3 $^{+}$ cells was positively correlated with the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and direct bilirubin (DBIL) (Figures 5A–C). In addition, there were differences in the expression level of LAMP3 in patients at different natural history stages of HBV infection. The expression level of

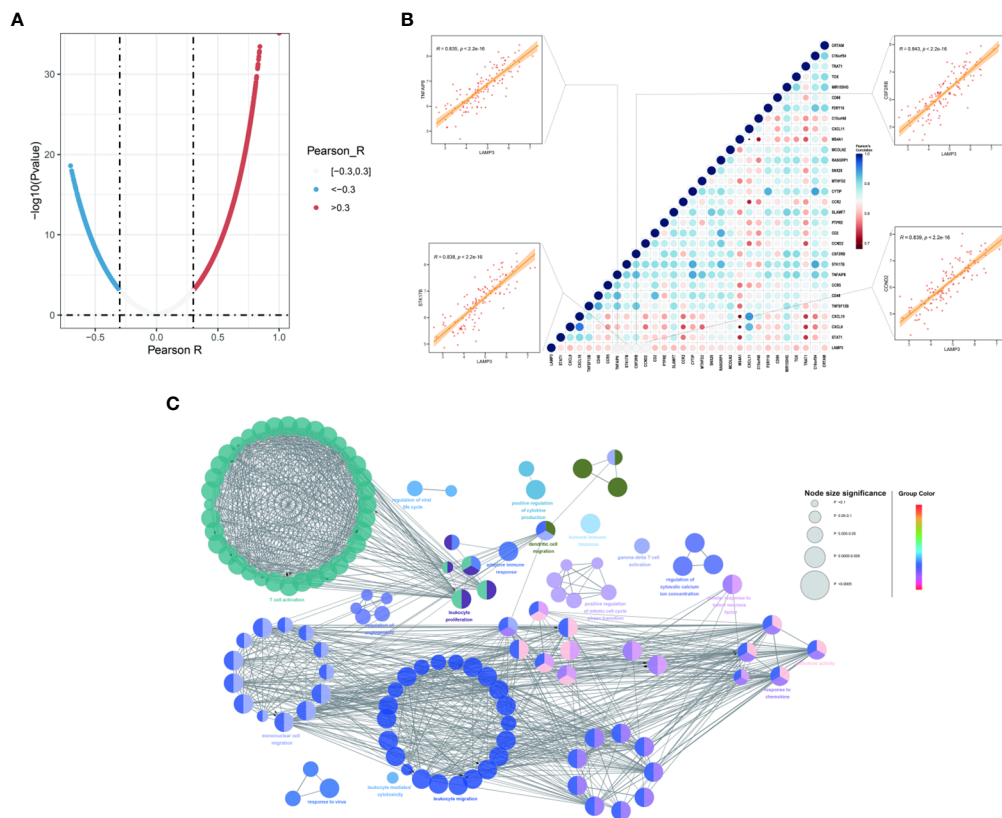


FIGURE 3

Associations between LAMP3 and key genes related to immune cell regulation. (A) The spoon-shaped plot shows genes related to LAMP3 expression calculated by Pearson correlation analysis. (B) Correlation between immunocyte regulatory genes and LAMP3, and their impact on HBV infection. (C) Visualization of the interaction network of genes strongly associated with LAMP3 by Cytoscape (ClueGO module). Node size, p value; Node color, gene groups. HBV, Hepatitis B virus; LAMP3, lysosome-associated membrane glycoprotein 3.

LAMP3 in patients in immune activation (IA) and reactivation (RA) stage is relatively high (Table 2; Supplementary Figure 2).

4 Discussion

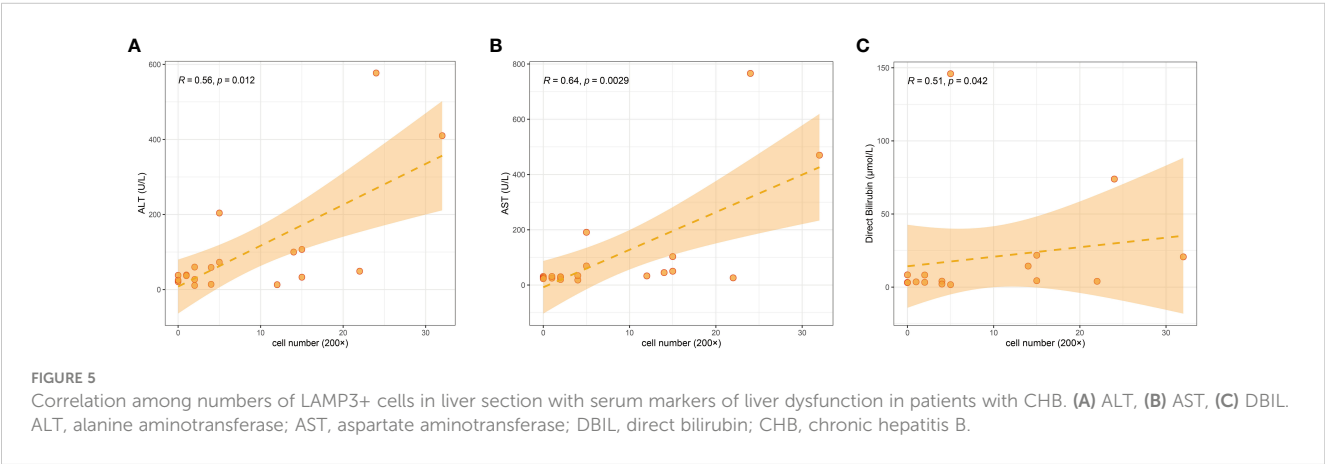
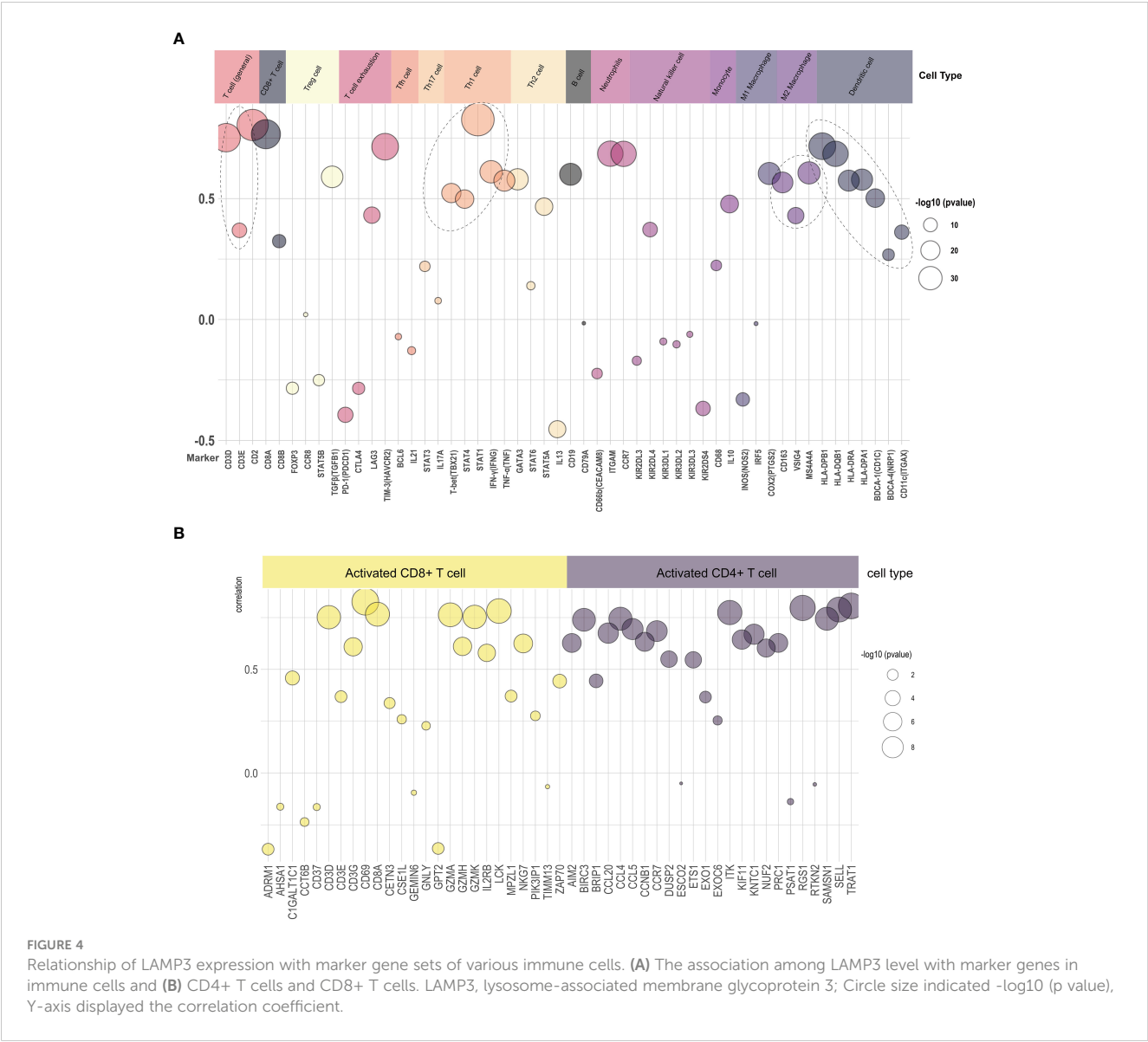
Chronic HBV infection and persistence are usually accompanied by changes in natural and adaptive immunity, particularly in T- and DC cells. In this study, by analyzing GEO datasets, we found that the LAMP3 gene was associated with chronic HBV infection, and the upregulation of LAMP3 expression was mainly involved in T cell activation and adaptive immune response. Additionally, the increased LAMP3 expression was positively associated with liver dysfunction caused by HBV infection.

In our research, LAMP3 mRNA and protein levels increased in the liver of patients with CHB compared to healthy controls. Previous studies showed that LAMP3 was involved in viral infection and the formation of various biological processes. For instance, in nonalcoholic fatty liver disease (NAFLD), the overexpression of LAMP3 substantially increased the lipid accumulation of hepatocytes (20), in our study, we also found that the expression of LAMP3 in the liver of NASH patients was significantly higher than that in healthy controls. From the perspective of virus infection, LAMP3 also participated in the

replication of the influenza virus. Influenza viral infection could induce the upregulation of LAMP3 expression (21).

Furthermore, in other viral infections, e.g., hepatitis C virus and human papillomavirus, LAMP3 is also specifically induced as a classical interferon-stimulated gene (22, 23). Moreover, LAMP3 is overexpressed in various tumors and is related to a poor prognosis and tumor metastasis (24–26). Therefore, the expression of LAMP3 is involved in various viral infections and tumor prognoses.

We also analyzed the biological mechanism of LAMP3 participation in HBV infection. The GEO database was analyzed, and we found that the upregulated LAMP3 expression in HBV-infected patients mainly participated in T cell activation and adaptive immune response. Previous studies showed that LAMP3 was primarily expressed in DC cells, and LAMP3 participated in major histocompatibility complex class II-restricted antigen presentation and the processing of exogenous antigens (27). LAMP3 is involved in HBV infection, possibly because LAMP3 on DC cells promoted T cell activation and adaptive immunity changes. Zhang et al. and Oh et al. proposed that LAMP3+ DCs expressed many ligands to interact with receptors on T cells, possibly being the most active immune regulators of lymphocytes (28, 29).



Currently, research on the interaction between LAMP3+ DCs and T cells is mainly focused on the tumor field, which might provide a basis for the understanding that LAMP3 participates in T cell activation and immune regulation during HBV infection. For example, in hepatocellular carcinoma, LAMP3+ DCs were positively correlated with the infiltration of exhausted CD8+ T cells and Tregs (30). In gastric cancer, LAMP3+ DCs were predicted to deliver attracting and activating signals to lymphocytes. However, LAMP3+ DCs inhibited anti-tumor T cell activity through a high expression of PD-L1 (31). In lymph node metastasized tumors, LAMP3+ DCs showed a stronger interaction with Tregs to enhance immunosuppression (32). In pancreatic adenocarcinoma, urothelial bladder carcinoma, and cutaneous T-cell lymphoma, LAMP3+ DCs also promote immune tolerance and immunosuppression through interacting with CD8+T or tumor-infiltrating Tregs (33–35). LAMP3+ DCs highly expressed CD80 and CD86, through interaction of CD80-CD28, CD80-CTLA4, CD86-CD28, and CD86-CTLA4, which might modulate CXCL13+/CD4+, and FOXP3+/CD4+ Tregs activities (36, 37). This indicated a close relationship among markers of Treg cells, T cell exhaustion, and Th1 cells, which illustrated the tight association between LAMP3 levels and the exhausted status of T cells. These results suggested that LAMP3+ DCs triggered T cell activation and exhaustion signaling simultaneously. Although there was no direct evidence, this might be the molecular mechanism of LAMP3 involvement in liver immunity changes and liver function during HBV infection.

5 Conclusion

Although no previous studies have reported on the association of LAMP3 with HBV infection, our study adds knowledge to this research gap. Our results revealed that the increased LAMP3 expression could enhance T cell activation and adaptive immune regulation. However, this study had several limitations. First, our explanation of the role of LAMP3 in HBV infection was based on pre-existing data from the GEO database and verified by our cohort; however, we did not confirm the function of LAMP3 in HBV infection by conducting *in vivo* and *in vitro* experiments. Second, the validation sample in this study was a cross-sectional cohort. In the future, we need to explore the application value of LAMP3 in HBV treatment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

This study was approved by the Ethical Committees of Peking University People's Hospital (no. 2021PHB122). The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZW, XW and BF contributed to conception and design of the study. RJ, FL and HR performed the experiments. LW, HC, and BF analyzed and interpreted of data. ZW and XW wrote the first draft of the manuscript. BF made critical revision of the manuscript for important intellectual content. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer XP declared a shared parent affiliation with the author LW at the time of review.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1127572/full#supplementary-material>

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