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Focusing on the high hepatitis B surface antigen clearance rate in special populations

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Yan-Mei Jiao, Sheikh Mohammad Akbar and Feng Li

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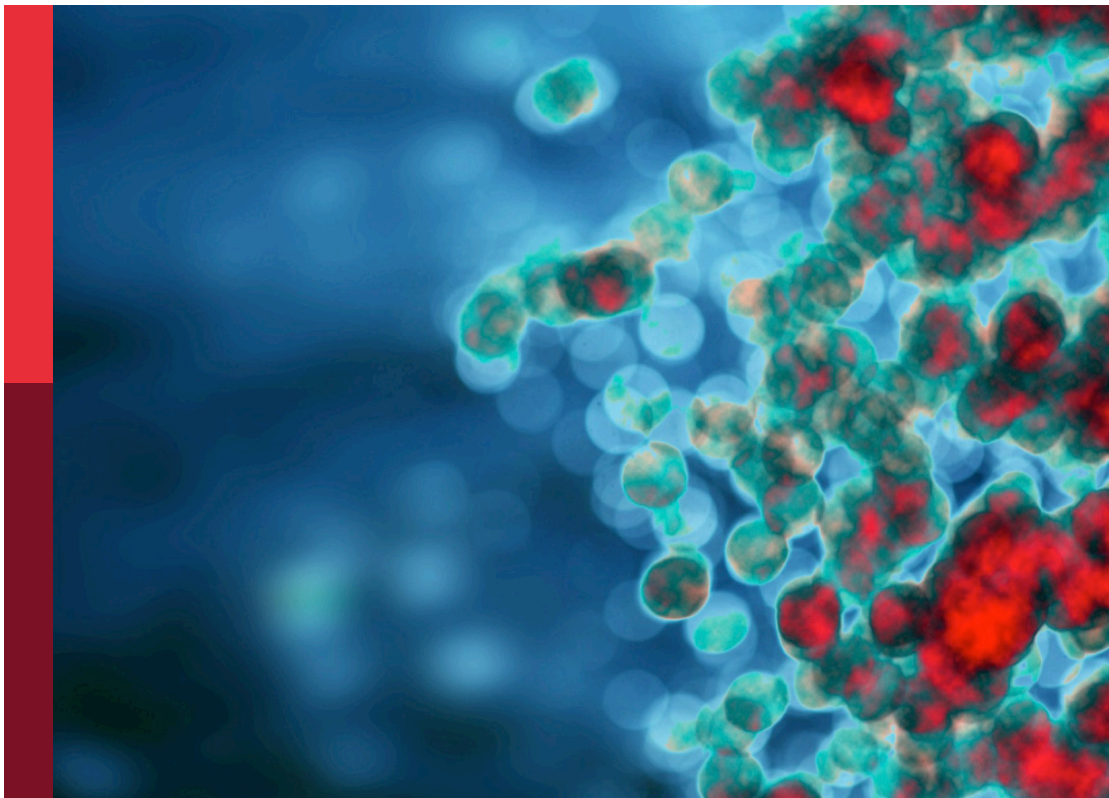

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Focusing on the high hepatitis B surface antigen clearance rate in special populations

Topic editors

Yan-Mei Jiao — Fifth Medical Center of the PLA General Hospital, China

Sheikh Mohammad Akbar — Ehime University, Japan

Feng Li — Guangzhou Eighth People's Hospital, China

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Table of contents

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SYSTEMATIC REVIEW
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Pegylated Interferon Treatment for the Effective Clearance of Hepatitis B Surface Antigen in Inactive HBsAg Carriers: A Meta-Analysis

Aixin Song[1†], Xiao Lin[1†], Junfeng Lu[1], Shan Ren[1], Zhenhuan Cao[1], Sujun Zheng[1], Zhongjie Hu[1], Hong Li[1], Chengli Shen[2] and Xinyue Chen[1*]*

[1] First Department of Liver Disease Center, Beijing Youan Hospital, Capital Medical University, Beijing, China, [2] Division of Surgical Oncology, James Cancer Hospital, The Ohio State University Wexner Medical Center, Columbus, OH, United States

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Edited by:
Yan-Mei Jiao, Fifth Medical Center of the PLA General Hospital, China

Reviewed by:
Tao Dong, University of Oxford, United Kingdom
Joel V. Chua, University of Maryland, United States

***Correspondence:**
Chengli Shen
chengli.shen@osumc.edu
Xinyue Chen
chenxydoc@ccmu.edu.cn

[†]These authors have contributed equally to this work

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Background: Expanding antiviral therapy to benefit more populations and optimizing treatment to improve prognoses are two main objectives in current guidelines on antiviral therapy. However, the guidelines do not recommend antiviral therapy for inactive hepatitis B surface antigen (HBsAg) carriers (IHCs). Recent studies have shown that antiviral therapy is effective with good treatment outcomes in IHC populations. We conducted a systematic review and meta-analysis of HBsAg clearance and conversion in IHCs.

Methods: We searched PubMed, Embase, Medline, and Web of Science to retrieve articles on HBsAg clearance in IHCs published between January 2000 and August 2021. Data were collected and analysed using the random-effects model for meta-analysis.

Results: A total of 1029 IHCs from 11 studies were included in this analysis. The overall HBsAg clearance rate was 47% (95% confidence interval (CI): 31% - 64%), with a conversion rate of 26% (95% CI: 15% - 38%) after 48 weeks of Pegylated interferon (Peg-IFN) treatment. In the control group (including nucleos(t)ide analogue (NA) treatment or no treatment), the overall HBsAg clearance rate was only 1.54% (95% CI: 0.56% - 3.00%), which was markedly lower than that in the Peg-IFN group. Further analysis showed that a low baseline HBsAg level and long treatment duration contributed to a higher HBsAg clearance rate.

Conclusion: This study showed that treatment of IHCs can be considered to achieve a clinical cure for chronic hepatitis B virus (HBV) infection. After Peg-IFN treatment, the HBsAg clearance rate was 47%, and the conversion rate was 26%, which are markedly higher than those reported by previous studies on Peg-IFN treatment in patients with chronic hepatitis B (CHB). A low baseline HBsAg level and long treatment duration were associated with HBsAg clearance in IHCs. Therefore, antiviral therapy is applicable for IHCs, a population who may be clinically cured.

Systematic Review Registration: http://www.crd.york.ac.uk/PROSPERO, CRD): CRD42021259889.

Keywords: HBV, HBsAg, high clearance rate, IHC, meta-analysis

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is an important cause of liver cirrhosis and hepatocellular carcinoma (HCC) (1). To reduce this major threat, two main trends are evident in current Chinese and international guidelines on antiviral therapy for the prevention and treatment of chronic hepatitis B (CHB): 1) the use of de-escalation therapy by relaxing the treatment criteria, allowing more patients to receive treatment and thus improving the prognosis; and 2) optimization of clinical clearance and treatment outcomes in the appropriate populations. However, the guidelines do not recommend antiviral therapy for inactive hepatitis B surface antigen (HBsAg) carriers (IHCs) (2–4), whereas studies (5, 6) in Asian populations have suggested that treating IHC patients is important, mainly because Asian patients usually have a long disease course by the IHC stage, and a risk of developing cirrhosis and HCC remains without treatment.

Immune function can control HBV DNA and maintain HBsAg at a low level (close to a clinical functional cure) in IHCs. Moreover, the cumulative number of patients receiving antiviral therapy has increased over the past decades, and the population of patients with low HBsAg levels has expanded significantly. In the past, few studies have focused on whether IHCs are eligible for antiviral therapy and how such therapy may benefit patients. In this study, we performed a literature review and meta-analysis of recent reports.

METHODS

This review was registered in the International Prospective Register of Systematic Reviews; the protocol is available online (PROSPERO, http://www.crd.york.ac.uk/PROSPERO, CRD): CRD42021259889).

Data Sources and Search Strategy

We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (7) and conducted a search for studies reporting HBsAg clearance and conversion in IHC patients with low HBsAg levels. We searched PubMed, Embase, Medline, and Web of Science to retrieve relevant articles published in the past 20 years, i.e., from January 2000 to August 2021. The keywords included "hepatitis B virus", "hepatitis B surface antigen", "HBsAg", "seroclearance or loss", "seroconversion", "clearance", "undetectable", "inactive hepatitis B surface antigen carrier", "inactive chronic hepatitis B virus carrier", "low surface antigen level", "HBsAg level", "peginterferon", "pegylated-interferon", "treatment", "nucleos(t)ide analogues", and "therapy". The articles retrieved were further screened. Furthermore, all references included in the articles were manually searched to identify additional potentially eligible articles, and authors were contacted for more details if needed. The studies included randomized controlled trials (RCTs) and prospective or retrospective cohort studies published in English or Chinese without any geographical restrictions. Reviews, comments, letters, and case reports were excluded.

Study Selection and Data Extraction

The titles, abstracts, and keywords of eligible articles were screened. Next, the abstracts and the full texts were carefully read for further screening, and duplicate publications were excluded. Two researchers independently completed this process and assessed the relevance of each study and the quality of the methodology. Any discrepancy was resolved with the help of a third researcher.

The inclusion criteria were as follows: (a) studies with more than 20 IHCs with HBsAg clearance data after pegylated interferon (Peg-IFN) treatment, nucleos(t)ide analogue (NA) treatment, or no treatment; and (b) studies with adequate data, including the frequency and rate of HBsAg clearance and observations with at least 24 weeks of Peg-IFN treatment, NA treatment, or no treatment. Studies with inadequate data and studies including patients with liver transplantation or HCC before HBsAg clearance or with human immunodeficiency virus (HIV), hepatitis C virus (HCV), or hepatitis D virus (HDV) coinfection were excluded.

Two researchers independently extracted data from the articles with a standard form. Data collected included the author, year of publication, country/region where the study was conducted, study design, sample sizes of the Peg-IFN group and the control group, patient ages, and baseline HBV DNA levels. Outcome data included the frequency and rate of HBsAg clearance and HBsAg conversion and Peg-IFN treatment courses in different groups.

Outcomes and Definitions

In this meta-analysis, IHC status was defined as HBsAg(+) >6 months, HBsAg <1500 IU/mL, HBeAg (–), anti-HBe(+) or (-), anti-HBc(+), HBV DNA < 2000 IU/mL, normal alanine transaminase (ALT), and no cirrhosis on ultrasound or FibroScan. The treatment group received Peg-IFN, and the control group received NA or no treatment. The outcome measures were HBsAg clearance and conversion after 48 weeks of treatment or follow-up. In addition, subgroup analyses were performed to evaluate the correlations between baseline HBsAg and treatment course and HBsAg clearance.

Assessment of Evidence Quality

Two researchers independently assessed the quality of the articles. The Newcastle-Ottawa Scale (NOS) was used to assess quality and bias (8). The NOS scale has three general areas consisting of eight items, including the selection of study groups, comparability of groups, and assessment of outcomes. The total score is 9; scores of 7 or higher indicate excellent quality, scores from 4 to 6 indicate fair quality, and scores of 4 or lower indicate poor quality (**Table S1**).

Statistical Analysis

The main statistic was rates. A random-effects model was used to summarize HBsAg clearance and conversion rates. Because the rates of outcome measures in the control group may be close to

zero or 100 (if not zero), Freeman-Tukey double-arcsine transformation was used to stabilize the variance, and the Wilson method was used to calculate the 95% confidence intervals (CIs) (9). Finally, the values were reverse-transformed for visualization in figures. The I^2 test and Cochrane's Q test were performed to assess among-study heterogeneity, and a funnel plot and Egger's test were used to assess any publication bias (10). Stata v14.0 was used for the data analysis, P values were two-tailed, and $P<0.05$ was considered statistically significant.

RESULTS

We initially retrieved a total of 833 articles (**Figure 1**), and 141 articles remained after excluding duplicate publications. After screening the abstracts and full texts, 23 articles remained. After final screening, 12 articles were excluded, i.e., one article with a small sample size, three articles with incomplete outcome data, two articles with the same cohort, and six articles with baseline HBsAg <1500 IU/mL but not in compliance with one or more IHC criteria. Finally, a total of 11 articles were included in this meta-analysis (11–21).

Characteristics of the Studies

The characteristics of the included studies are shown in **Table 1**. All 11 studies were conducted in Asia, including 10 in China and one in Singapore (13). Seven articles were published in English (11–15, 20, 21) and four in Chinese (16–19). The baseline HBsAg levels were <20 IU/mL in two studies (14, 16), <100 IU/mL in one study (12), <1000 IU/mL in six studies (11, 13, 17, 18, 20, 21), and <1500 IU/mL in two studies (15, 19). Ten studies used Peg-IFN treatment, with NA treatment (n=1) or no treatment

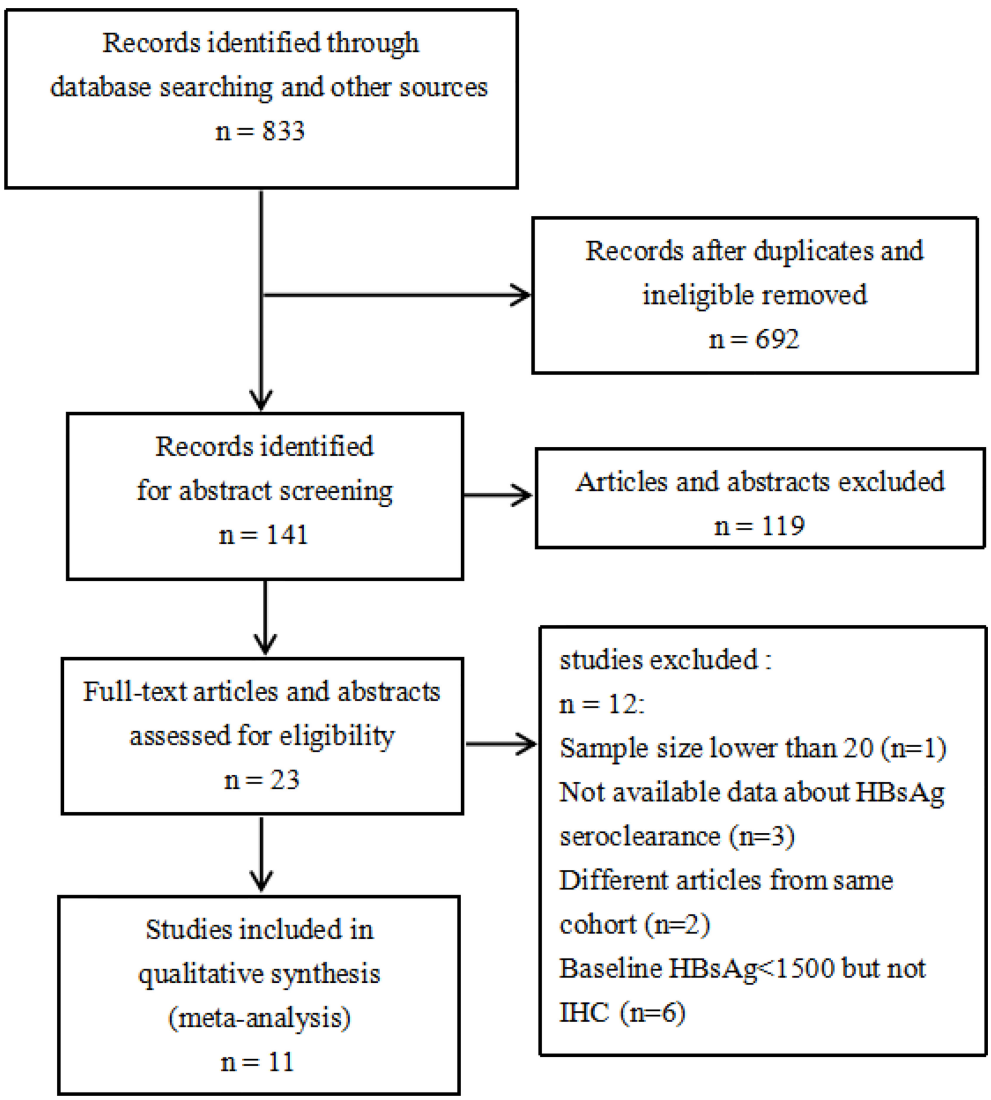

FIGURE 1 | Study selection process.

TABLE 1 | Characteristics of the included studies.

Study	Region	Design	HBsAg level (IU/mL)	Sample size				Treatment period	HBsAg clearance rate (IFN)	HBsAg conversion rate (IFN)	HBsAg clearance rate (control)	Age(mean or median)	HBV DNA (IU/mL)
				Total	IFN	NA	None						
Cao 2017 (11)	Beijing, China	Prospective	<1000	144	102	–	42	96 W	48 W: 29.8% (28/94) 96 W: 44.7% (42/94)	48 W: 20.2% (19/94) 96 W: 38.3% (36/94)	48 W: 2.5% (1/40) 96 W: 2.5% (1/40)	38.8±10.0* 39.8±10.6#	<2000
Li 2016 (12)	Beijing, China	Retrospective	<100	60	20	–	40	72 W	72 W: 60% (12/20) 96 W: 65% (13/20)	72 W: 55% (11/20) 96 W: 60% (12/20)	0	33.80±11.45* 33.85±8.37#	<100
Lim 2019 (13)	Singapore	RCT	<1000	90	60	–	30	24 W 48 W	24 W: 30% (9/30) 48 W: 20% (6/30)	–	0	50.09±10.18* 49.5±10.86#	<2000
Zeng 2020 (14)	Zhengzhou, China	Retrospective	<20	32	16	–	16	48 W	24 W: 68.8% (11/16) 48 W: 93.8% (15/16)	48 W: 31.2% (5/16)	0	34 (32~46.8)* 36 (32~44.8)#	undetectable or <200
Wu 2021 (15)	Xi'an, China	Retrospective	<1500	298	142	–	156	48 W	48 W: 43.3% (58/134) 72 W: 50.7% (68/134)	48 W: 29.9% (40/134) 72 W: 38.8% (52/134)	48 W: 1.4% (2/143) 72 W: 2.1% (3/143)	37.9±10.7* 36.6±10.6#	<2000
Zhao 2020 (16)	Xiamen, China	Retrospective	<15	38	12	–	26	24 W	24 W: 83.3% (10/12)	24 W: 41.6% (5/12)	55 W: 7.7% (2/26)	37.3* 35.9#	<20
Shi 2018 (17)	Jiangsu, China	Retrospective	<1000	80	40 (add-on)	40	–	48 W	24 W: 20% (8/40) 48 W: 32.5% (13/40)	–	24 W: 2.5% (1/40) 48 W: 6.3% (3/40)	34.6±3.2	<3.3lg copies/mL
Zhou 2020 (18)	Chongqing, China	Retrospective	<1000	107	77	–	30	96 W	48 W: 24.7% (19/77) 96 W: 40.3% (31/77)	48 W: 9.1% (7/77) 96 W: 19.5% (15/77)	0	41.3±9.5* 43.4±10.1#	undetectable or <200
Chen 2020 (19)	Jilin, China	Retrospective	<1500	51	51 (add-on for 3 months)	–	–	48 W	48 W: 37.25% (19/51)	48 W: 15.69% (8/51)	–	–	<20
Chen 2021 (20)	Sichuan, China	Retrospective	<1000	90	27	–	63	48 W	24 W: 40.7% (11/27) 48 W: 55.6% (15/27)	–	0	–	–
Huang 2021 (21)	Hunan, China	Retrospective	<1000	39	19	–	20	72 W	48 W: 84.2% (16/19)	48 W: 68.2% (13/19)	0	39.00±11.55* 39.8±8.03#	<2000

*Age: * for the IFN group, # for the control group.*

(n=9) in the control group. One study applied only Peg-IFN treatment, with no control group (19). Eight of the 11 studies reported HBsAg clearance and conversion rates among IHCs after Peg-IFN treatment, and three studies reported only HBsAg clearance rates (13, 17, 20).

Overall 48-Week HBsAg Clearance Rates in IHCs

Among the 11 studies on HBsAg clearance in IHCs after Peg-IFN treatment, the total sample size was 1029 patients, including 566 in the Peg-IFN group and 463 in the control group (NA: n=40, no treatment: n=423). Peg-IFN treatment lasted 24 to 96 weeks [24 weeks: n=1 (16); 48 weeks: n=9, 72 weeks: n=1 (12)]. For the nine studies with 48 weeks of Peg-IFN treatment, 199 of 488 patients achieved HBsAg clearance. Therefore, the overall HBsAg clearance rate was 47% (95% CI: 31% - 64%, I^2 = 94%, random-effects model, **Figure 2**). For publication bias, the shape of the funnel plot (**Figure S1**) was ideal, and Egger's test showed P=0.55, suggesting no significant publication bias. Six of these nine studies reported HBsAg conversion after 48 weeks of Peg-IFN treatment. The overall conversion rate was 26% (92/391) (95% CI: 15% - 38%, **Figure 3**). In addition, eight studies (n=382) reported HBsAg clearance after 48 weeks of follow-up in the control group. Four patients achieved HBsAg clearance, and the overall clearance rate was 1.54% (95% CI: 0.56% - 3.00%, **Figure 4**). Egger's test (P=0.9) suggested no significant publication bias. Eight studies reported HBsAg clearance at 48 weeks in the Peg-IFN group and the control group. The results showed that Peg-IFN treatment significantly increased the HBsAg clearance rate (relative risk (RR)=16.46, 95% CI: 7.60% - 35.66%, P<0.001, I^2 = 0%, **Figure S2**).

HBsAg Clearance Rates for Different Baseline HBsAg Levels

To evaluate the effect of baseline HBsAg on HBsAg clearance and reduce data heterogeneity, we analysed the data of patients who completed 48 weeks of Peg-IFN treatment, with stratification based on HBsAg level. The patients were divided into five groups based on their baseline HBsAg levels: <10 IU/mL, <20 IU/mL, <100 IU/mL, <500 IU/mL, and <1000 IU/mL. Two studies with baseline HBsAg levels <1500 IU/mL were excluded from the analysis due to data bias. The results showed that baseline HBsAg was inversely correlated with the clearance rate. After 48 weeks of Peg-IFN treatment, the HBsAg clearance rates were 92% (95% CI: 79% - 99%) for HBsAg <10 IU/mL, 88% (95% CI: 77% -95%) for <20 IU/mL, 73% (95% CI: 62% - 83%) for <100 IU/mL, 60% (95% CI: 48% - 71%) for <500 IU/mL, and 39% (95% CI: 25% - 53%) for <1000 IU/mL (**Figure 5**).

HBsAg Clearance Rates in Different Treatment Periods

To evaluate the effect of Peg-IFN treatment courses on HBsAg clearance, we analysed the clearance rate among IHCs with baseline HBsAg <1000 IU/mL after Peg-IFN treatment.

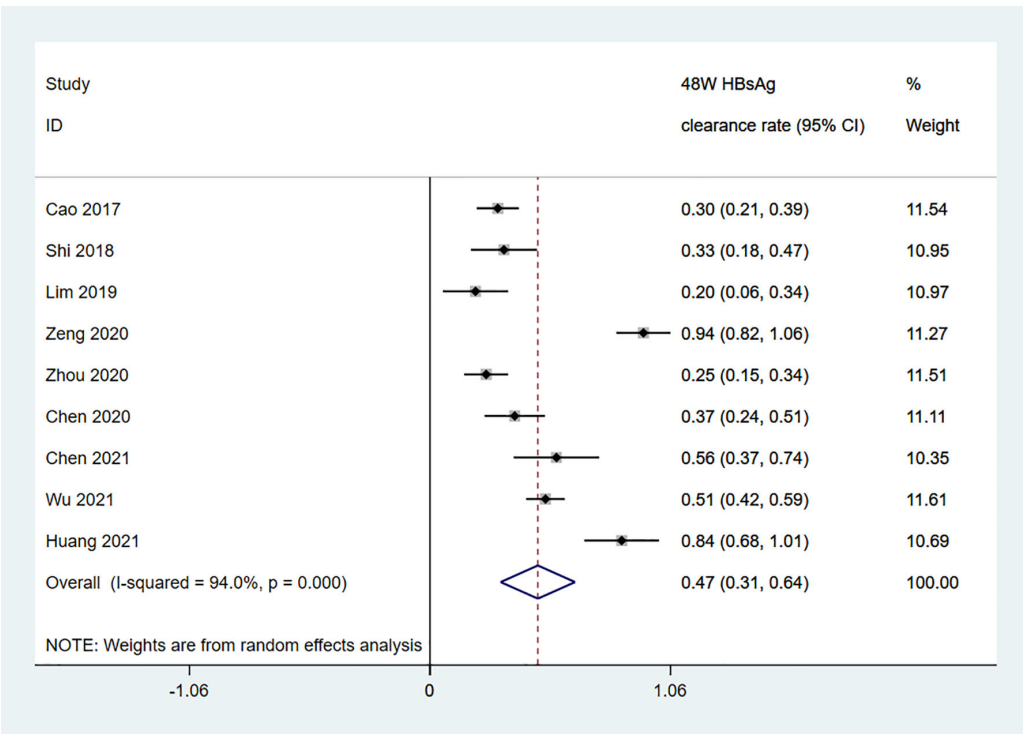

FIGURE 2 | Meta-analysis of the pooled HBsAg clearance rate among IHCs after 48-W Peg-IFN treatment.

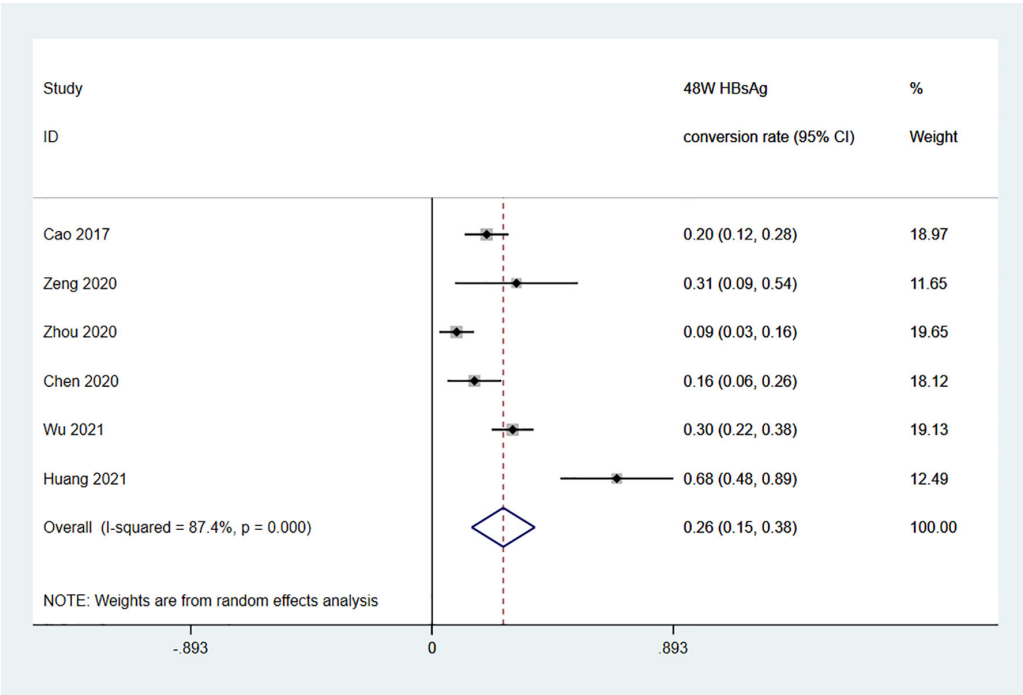

FIGURE 3 | Meta-analysis of the HBsAg conversion rate among IHCs after 48-W Peg-IFN treatment.

The HBsAg clearance rates were 29% (95% CI: 17% - 40%) after 24 weeks of treatment, 39% (95% CI: 25% - 53%) after 48 weeks of treatment, and 43% (95% CI: 35% - 50%) after 96 weeks of treatment (**Figure S3**), suggesting that HBsAg clearance increased with longer Peg-IFN treatment, although the difference did not reach statistical significance (P=0.5). This finding may be related to the small sample sizes and significant heterogeneity in the 24-week and 48-week groups.

Additional Results

In addition to baseline HBsAg, several studies reported that post-Peg-IFN treatment HBsAg and ALT were also strong predictors of HBsAg clearance. Five studies showed that decreased HBsAg after 12 or 24 weeks of Peg-IFN treatment and elevated ALT after 4 or 12 weeks of Peg-IFN treatment were effective predictors of HBsAg clearance (11, 12, 15, 18, 19). One study reported that hepatitis B vaccination contributed to HBsAg clearance (14), and one study reported that baseline HBV DNA <20 IU/mL was a favourable factor for HBsAg clearance (15).

Most studies reported adverse side effects during treatment, including neutropenia, thrombocytopenia, pyrexia, fatigue, hair loss, weight loss, and rash, most of which were mild and resolved after symptomatic care. In addition, a few patients experienced thyroid dysfunction, anxiety disorder, and lipsotrichia, and some patients required treatment suspension or dose adjustment but were able to resume and complete the Peg-IFN treatment course, which is similar to observations during previous CHB treatment. More than half of the patients had increased ALT levels during treatment, which was a strong predictor of HBsAg clearance. Drugs can be used to reduce enzymes and protect the liver. Normalization of ALT levels coincided with HBsAg clearance and was maintained during the follow-up.

DISCUSSION

For CHB patients, HBsAg clearance or conversion is regarded as a "functional cure" in antiviral therapy (2, 3, 22). With HBsAg clearance, patients can safely discontinue treatment with optimal long-term outcomes (23, 24). However, the HBsAg clearance rate is still low (3% to 7%) in CHB patients after 48 weeks of Peg-IFN treatment (25, 26). In this meta-analysis, we focused on the effect of the same treatment and treatment course in IHCs and found that the HBsAg clearance rate increased by more than 10 times to 47%. In the Asia-Pacific region, especially in China, a very large IHC community can benefit from short-term Peg-IFN treatment, suggesting that IHCs should be eligible for antiviral therapy.

This meta-analysis of 1029 IHCs from 11 studies showed that after 48 weeks of Peg-IFN treatment, the overall clearance rate was relatively high at 47% among 488 patients, and the HBsAg conversion rate was 26%. In the control group (n=382), the overall clearance rate was only 1.54%, which was markedly lower than that in the Peg-IFN group, suggesting that IHCs may be clinically cured and that Peg-IFN treatment is far more effective than NA treatment in achieving this goal.

The clearance rate ranged from 20% to 94% across the studies due to different baseline HBsAg levels, Peg-IFN treatment courses, and sample sizes. To minimize data

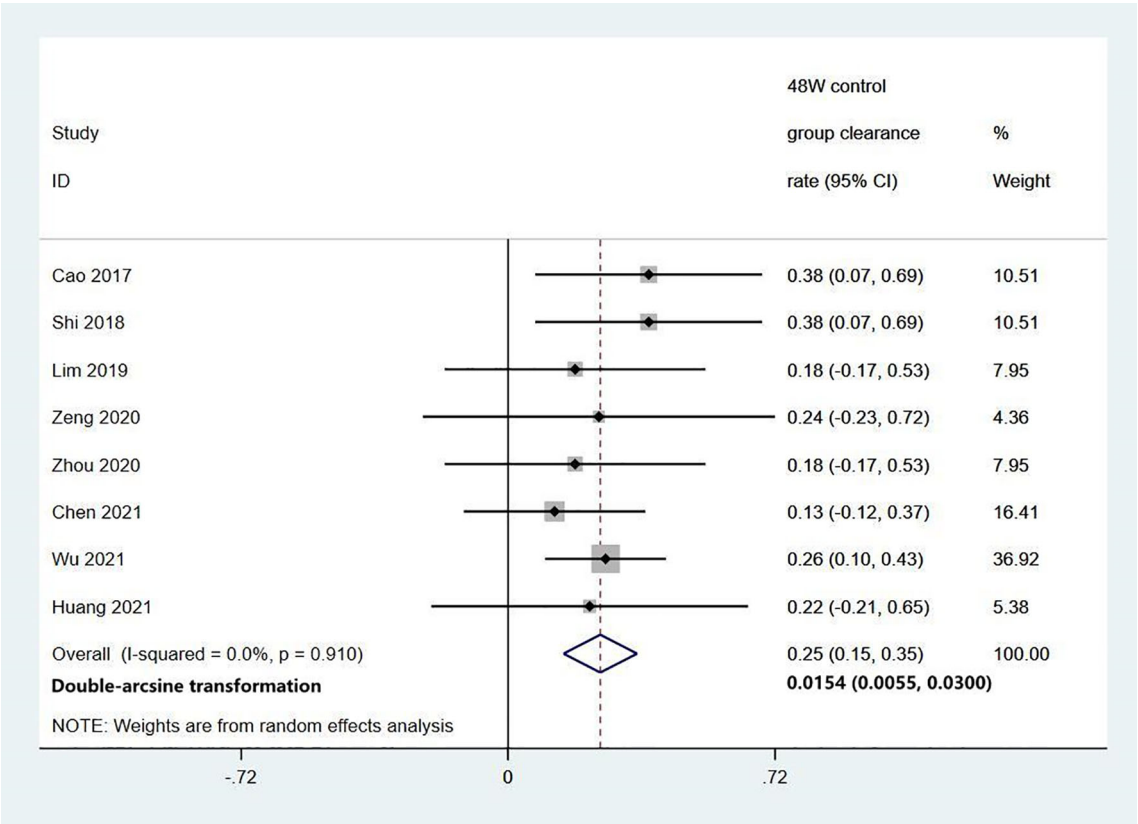

FIGURE 4 | Meta-analysis of the HBsAg clearance rate in the control group.

heterogeneity, we first analysed factors affecting HBsAg clearance in IHCs who completed 48 weeks of Peg-IFN treatment and found that baseline HBsAg was inversely correlated with the clearance rate, which was 92% among IHCs with baseline HBsAg levels <10 IU/mL. Next, we analysed the clearance rate after different Peg-IFN treatment courses in patients with baseline HBsAg levels <1000 IU/mL and found that the HBsAg clearance rates were 29% after 24 weeks of treatment, 39% after 48 weeks of treatment, and 43% after 96 weeks of treatment. These data indicate that a low baseline HBsAg level and longer Peg-IFN treatment are favourable factors for HBsAg clearance with practical value for the clinical treatment of IHCs along with the predictive value of post-Peg-IFN treatment HBsAg and ALT. While the overall HBsAg clearance rate is substantially higher among IHCs than among CHB patients, more precise planning can enable targeted treatment to help IHCs achieve a clinical cure and an optimal outcome as early as possible and at a lower cost. Patients who do not respond to Peg-IFN treatment can discontinue treatment early to prevent adverse drug reactions, which also has pharmacoeconomic value.

This study has some limitations. First, because current Chinese and international guidelines do not recommend treatment for IHCs, few prospective, multicentre cohort studies have been conducted to investigate posttreatment HBsAg clearance in IHCs, and the sample sizes in available studies are usually small. Second, all studies included in this meta-analysis were conducted in the Asia-Pacific region, without any study from Europe or the US, which is due to the regional deviation in the hepatitis B prevalence. The European and American hepatitis B guidelines do not recommend treatment for IHCs. This recommendation is primarily based on studies in Caucasian populations in Europe and the US showing that for IHCs, the HBsAg conversion rate is 15% to 45% over a 10-year follow-up period, without any significant increase in the incidence of HCC compared to that for the general population (27). However, studies in the Asia-Pacific region have reached different conclusions, showing that for IHCs, the HBsAg clearance rate is only 0.5% to 1% over a long follow-up (5). In a long-term follow-up study with 1932 patients, the mortality rates of HCC and liver disease were 4.6-fold and 2.1-fold higher in IHCs than in HBsAg(-) individuals, respectively (6). These data from Asian populations suggest that for IHCs, withholding treatment causes more harm than good, mainly because in Asian populations, HBV infection often occurs in young people, and patients usually have a long disease course by the CHB stage. Therefore, treating IHC patients in the Asia-Pacific region is important. Third, the follow-up time was short in clinically cured IHCs after treatment, and long-term outcome data are lacking. Fourth, sample sizes vary across studies due to different geographic regions and

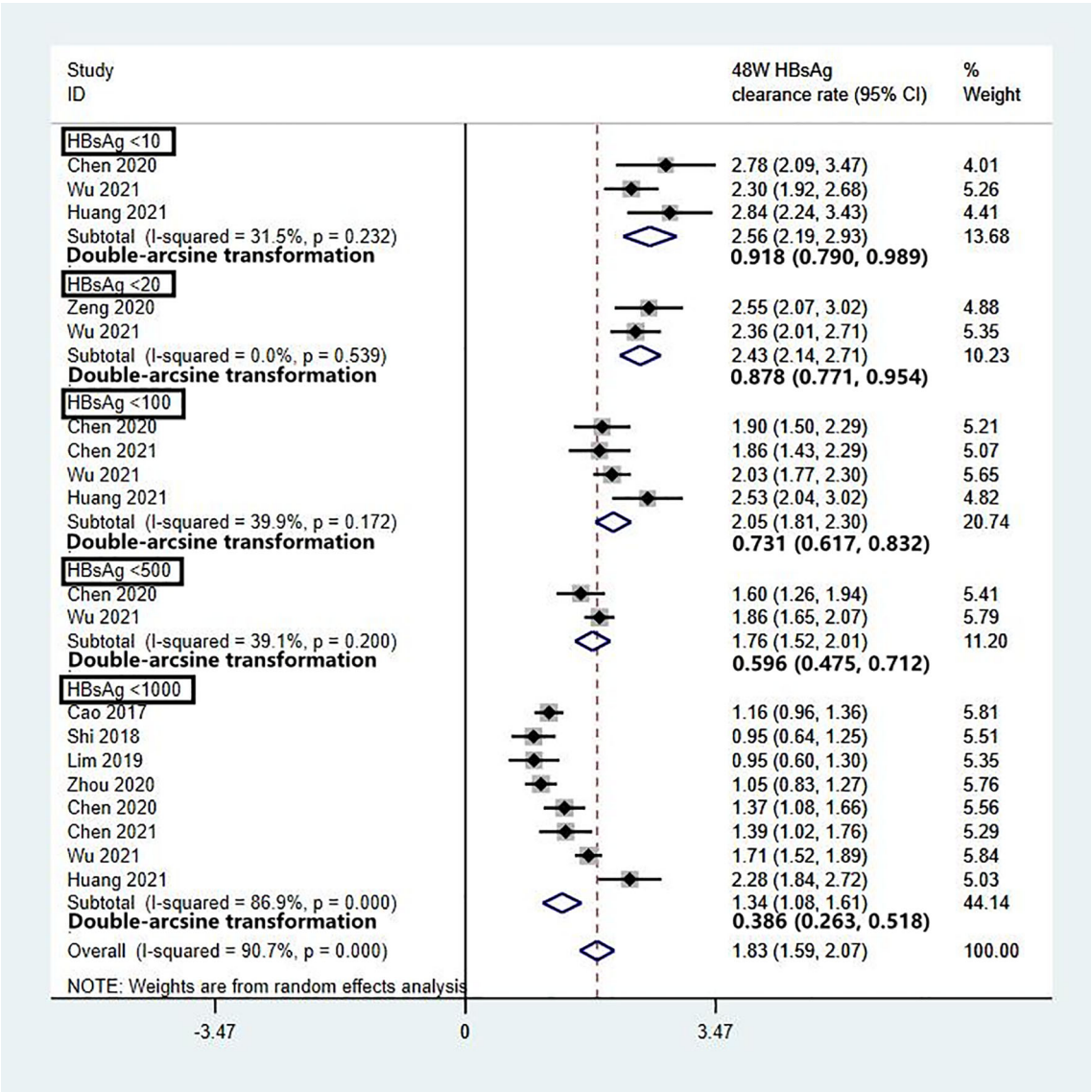

FIGURE 5 | Subgroup analysis of the 48-W HBsAg clearance rate among different baseline HBsAg levels.

subjects. Therefore, the results of this meta-analysis have some inherent heterogeneities.

In short, this meta-analysis shows that IHCs may achieve a high HBsAg clearance rate (47%) after Peg-IFN treatment. Given the very large IHC population and the high response rate, IHCs should be treated with antiviral therapy, as they have the potential to be clinically cured.

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.

AUTHOR CONTRIBUTIONS

AS, XL, and XC conceived and designed the protocol and study. JL and SR identified studies to be screened. ZC and HL identified studies for eligibility, extracted data, and assessed the methodological quality of the included studies. AS performed the analysis with assistance from SZ, ZH, CS, and XC. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Increased HBV Coinfection and Decreased IFN-γ-Producing HBV-Specific CD8+ T Cell Numbers During HIV Disease Progression

Zhiqiang Zhu[1†], Yuanyuan Qin[2†], Qi Liang[3,4†], Wei Xia[5], Tong Zhang[5], Wen Wang[5], Mengmeng Zhang[1], Taiyi Jiang[5], Hao Wu[5*] and Ye Tian[6*]*

[1] Department of Urology, Beijing Youan Hospital, Capital Medical University, Beijing, China, [2] Beijing Key Laboratory for HIV/AIDS Research, Capital Medical University, Beijing, China, [3] Department of Clinical Laboratory, Affiliated Hospital of North Sichuan Medical College, Nanchong, China, [4] Translational Medicine Research Center, North Sichuan Medical College, Nanchong, China, [5] Center for Infectious Diseases, Beijing Youan Hospital, Capital Medical University, Beijing, China, [6] Department of Urology, Beijing Friendship Hospital, Capital Medical University, Beijing, China

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Edited by:
Yan-Mei Jiao,
Fifth Medical Center of the PLA General Hospital, China

Reviewed by:
Liang Cheng,
Wuhan University, China
Yongyin Li,
Southern Medical University, China

***Correspondence:**
Taiyi Jiang
jtyii2004@mail.ccmu.edu.cn
Hao Wu
whdoc@sina.com
Ye Tian
urologisttianye@126.com

[†]These authors have contributed equally to this work

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Objective: To investigate the characteristics and mechanism of the dynamics of HBV infection with the progression of HIV disease and to explore the different responses of T lymphocytes to HBV in HIV patients in different stages of disease.

Methods: We compared the rates and characteristics of HBV coinfection between 372 early HIV-infected and 306 chronically HIV-infected men who have sex with men (MSM) in the Beijing Youan Hospital from October 2006 to November 2014. We further analysed IFN-γ-producing HBV-specific CD8+ T cells in 15 early HIV-infected individuals and 20 chronic HIV-infected individuals with HBV coinfection.

Results: Twenty-three HBsAg-positive cases were detected among the 372 early HIV-infected patients of this cohort, and the coinfection rate was 6.18%, while 35 HBsAg-positive cases were detected among the 306 chronically HIV-infected patients, with a coinfection rate of 11.44%. The coinfection rate of the chronically HIV-infected patients was significantly higher than that of the early-infected patients (p=0.0005). The median CD4+ T cell count in the early HIV infection patients was 445 cells/μL (196-1,030 cells/μL), which was higher than that in the chronic HIV infection patients [358 cells/μL (17-783 cells/μL)] (p<0.001). The proportion of IFN-γ-producing CD8+ T cells in early HIV-infected patients was significantly higher than that in chronically HIV-infected patients.

Conclusion: The coinfection rate of HBV in HIV patients increases with HIV disease progression, which might be related to the decreased IFN-γ-producing HBV-specific CD8+ T cell numbers. The closely monitored HBV serum markers from the early stage of HIV infection are warranted.

Keywords: HIV, HBV, disease progression, co-infection rate, IFN-γ

1 INTRODUCTION

HIV and HBV coinfections are clinically common because they can both be transmitted sexually. HBV can cause early infection with or without clinical symptoms in healthy adults, but the human immune system can fight and clear the HBV in the very early stage to prevent progression to the chronic phase (1). Chronic HBV infection is common in infants and children due to their immature immune systems, which cannot rapidly eliminate the HBV and allow the infection to develop to the chronic stage (2). At present, there are differing perspectives on the impact of HIV and HBV coinfection on the progression of HIV disease (2, 3). Several studies have found that HBV infection has no impact on HIV progression in the clinic, while others have found that it promotes HIV progression (4–6). Generally, HIV combined with HBV infection has a great impact on disease progression in patients (7); for example, the incidence of cirrhosis and hepatocellular carcinoma increases among patients with HIV-HBV coinfection compared with HIV infection only (7). Therefore, it is of great significance to understand the characteristics of HBV coinfection in HIV patients and the ways to manage it as early as possible.

Many studies are focusing on HIV patients with HBV infection, but the conclusions have been inconsistent (1, 3, 7–10). Some studies have shown that HIV infection has a positive role in the elimination of the HBV (11), while others have discovered increased HBV coinfection among HIV patients. These controversial conclusions might be due to the different HIV-infected patients chosen by each study. HIV attacks the human immune system and causes dramatic changes to it as disease develops, and different CD4+ T cell levels might lead to different outcomes of HBV infection in HIV patients.

Since there are no overt clinical symptoms of patients with early HIV infection, and few patients can be recruited for detailed observation, most studies have focused on chronic HIV patients. Our previous research showed that the rate of HBV coinfection in early HIV-infected patients is significantly lower than that in chronically HIV-infected patients. Based on this, we inferred that, with the development of disease, the ability to respond to and clear the HBV dramatically decreases. IFN-γ-producing CD8+ T cells play an important role in HBV viral clearance (12). Although many scientists and physicians have speculated that HBV clearance is higher in the early phase of HIV than in chronic phase patients, no research has compared the T cell response to HBV between early and chronic HIV patients. Therefore, we compared and analysed the T cell response to HBV of HIV-infected patients in different phases in this study.

To study the related factors of HIV patients coinfected with HBV at different disease stages in the MSM group, the MSM follow-up cohort from Beijing Youan Hospital affiliated with Capital University was used in this study (9). We describe the clinical characteristics of HBV infection in early HIV-infected patients and chronic HIV-infected patients in this study, hoping to provide evidence for the prevention of HBV infection in people living with HIV.

2 MATERIALS AND METHODS

2.1 Study Population

The early HIV infection cohort in this study was from an open and prospective cohort of MSM with high-risk behaviours established in the Beijing Youan Hospital, Capital Medical University (9). The cohort recruited MSM who were HIV-negative at the age of 18 years or older and were followed up every 2 months for HIV antibody and HIV RNA testing until a positive HIV antibody conversion occurred from October 2006 to November 2014. The chronic HIV infection cohort was retrospectively collected from consecutive MSM who were diagnosed with chronic HIV infection in the Beijing Youan Hospital, Capital Medical University from October 2006 to November 2014.

The inclusion criteria were as follows: 1) enrolment in either the early HIV infection cohort or the chronic HIV infection cohort; 2) age >18 years; and 3) no history of antiretroviral therapy (ART). The exclusion criteria were as follows: 1) other hepatitis virus (i.e., HAV, HCV, HDV, and HEV) infected patients and 2) patients with opportunistic infections or AIDS-related tumours.

This study was approved by the Ethics Committee of Beijing Youan Hospital. All patients signed an informed consent form before participating in this study.

2.2 Methods

2.2.1 Definition

Early HIV infection: Patients who were HIV antibody negative but HIV RNA-positive, those with suspicious HIV antibody WB bands who were HIV-RNA positive, or those who showed positive HIV antibody conversion within 6 months during the cohort follow-up.

Chronic HIV infection: Patients who showed positive HIV antibody conversion over 6 months.

2.2.2 Study Design

The HBV serum markers, HIV RNA and CD4+ T cell number of all patients in the two phases of HIV infection cohorts were tested at the time of HIV diagnosis and confirmed afterwards. HBV serum markers of patients in the acute HIV infection cohort were collected for testing at weeks 1, 2, 4, 8, and 12 and then every 3 months thereafter.

2.2.3 Detection of Hepatitis B Virus (HBV) Infection

HBV-specific antigens and antibodies in patient plasma were measured by Elecsys® HBsAg Immunoassay kits (Roche Diagnostics GmbH, Mannheim, Germany), immunoassay analyser cobas e411 kits (Roche Diagnostics GmbH, Mannheim, Germany) and specific ELISA test kits (PRECHEK Bio, Anaheim, USA) according to the manufacturer's instructions in the clinical laboratory at Youan Hospital. HBV infection is defined as the presence of HBsAg +/- and detectable HBV DNA (13).

2.2.4 Markers of HIV Disease Progression

Absolute blood CD4+ T cell counts were measured by flow cytometry (BD FACSCanto flow cytometer, BD Bioscience,

San Jose, CA, USA). HIV RNA was tested by the Amplicor HIV monitor ultrasensitive method with a detection limit of 40 copies/mL of plasma.

2.2.5 Liver Function Tests

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in patient plasma were tested by a UV-LDH method test kit (Fortress Diagnostics Limited, United Kingdom).

2.2.6 *In Vitro* Stimulation

The frozen peripheral blood mononuclear cells (PBMCs) collected from healthy individuals, early HIV-infected patients who were HBsAg-positive and chronic HIV-infected patients who were HBsAg-positive were cultured (1×10^6 cells/mL) in 10 mL foetal bovine serum (Gibco Australia Origin, USA) and stimulated with an HBsAg overlapping peptide pool (donated by Prof. Tao Dong, Oxford) for 8 hours. The transport inhibitor Brefeldin A (3 μg/mL, eBioscience) was added into each stimulus condition. The cells were stained with surface antibodies (CD3-PerCP and CD8-FITC, BD Bioscience, San Jose, CA, USA). The surface-stained cells were washed, fixed, and permeabilized using the Permeabilization/Fixation Kit (eBioscience, Waltham, MA, USA) before intracellular cytokine staining with IFNγ-PE, (BD Bioscience, San Jose, CA, USA). The stained cells were fixed with 1% formaldehyde for analysis by a FACS Canto flow cytometer (BD Bioscience, San Jose, CA, USA) within 24 hours.

2.2.7 Statistical Analysis

Data analyses were performed with Statistical Product and Service Solution 16.0 (SPSS for Windows, Version 16.0. Chicago, SPSS Inc.). Data that fit a normal distribution were expressed as the means ± SD, while those that fit a nonnormal distribution were represented by the medians (lower quartile-upper quartile). The comparison of the related data between different groups was analysed by T test or Chi-square test depending on the data type. $P<0.05$ was considered to be statistically significant for all analyses.

3 RESULTS

The general information of 372 early and 306 chronically HIV-infected patients was collected from outpatient clinic-based MSM cohorts established in Beijing Youan Hospital, Capital Medical University from October 2006 to November 2014 (**Figure 1**). Twenty-three patients (6.18%) were HBsAg-positive in the 372-patient early HIV infection group, and 35 patients (11.44%) were HBsAg-positive in the 306-patient chronic HIV infection group. The rate of HBV coinfection in the chronic HIV infection group was significantly higher than that in the early HIV infection group ($p=0.015$, **Figure 2**). The CD4+ T cell counts decreased from an average of 445 in the early HIV infection group to 358 in the chronic HIV infection group ($p<0.001$) (**Table 1**). The distributions of age and HIV viral load

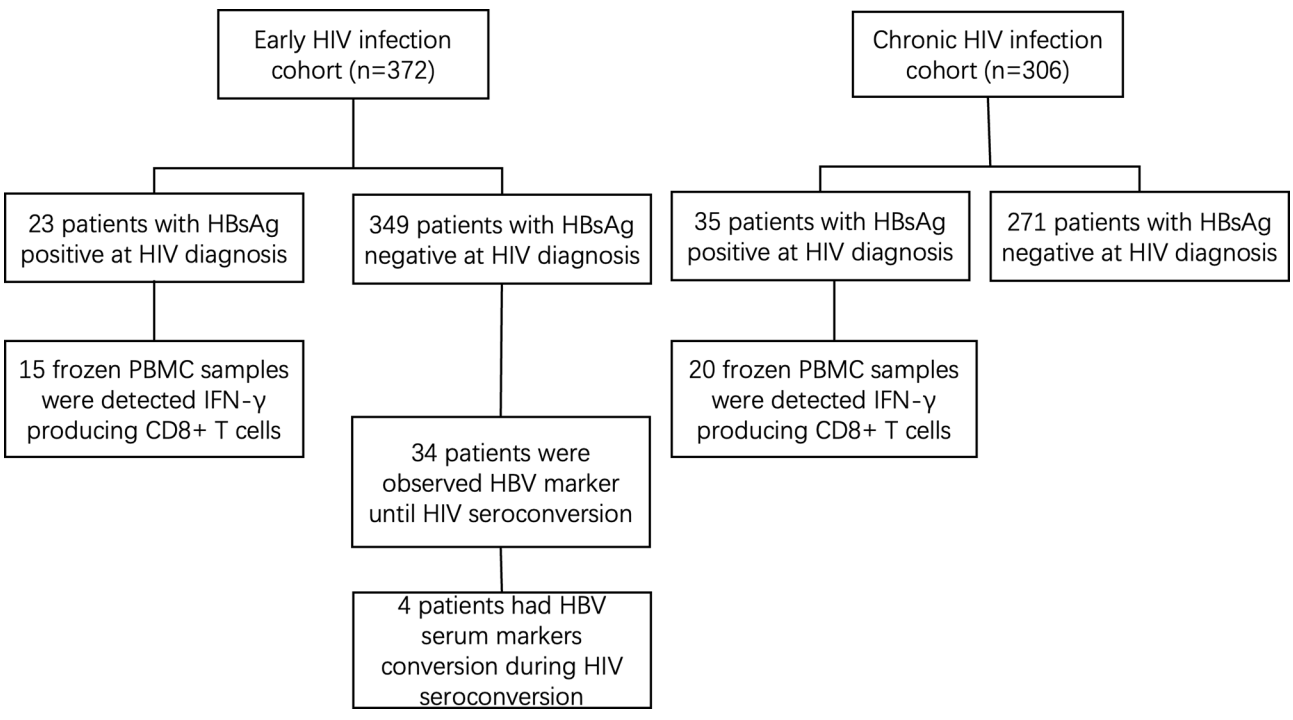

FIGURE 1 | Flow chart of the study procedure.

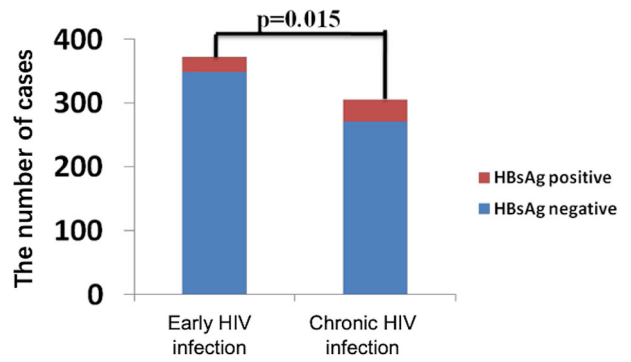

FIGURE 2 | The rate of HBsAg positivity in the early HIV infection and chronic HIV infection groups.

were not significantly different between the two groups. Additionally, the distributions of HBV coinfection and CD4+ T cell counts were also significantly different between the two groups.

Among the other 349 early HIV-infected patients whose HBsAg was negative at diagnosis, 34 were observed to have HBV serum markers until developing to the chronic phase of HIV infection. During the follow-up period, 4 patients showed HBV-specific antigen and/or specific antibody changes (see **Table 2**). The CD4+ T cell counts decreased and the viral load increased in 4 patients from the early phase to the chronic phase of HIV infection. The number of hepatitis B virus-specific antibodies decreased from the early phase to the chronic phase of HIV infection in the four patients, and two patients' HBsAg became positive. The ALT and AST levels were similar, and HIV developed from the early to the chronic stage in the four patients.

Subsequently, the proportion of IFN-γ-producing CD8+ T cells in PBMCs was detected in 15 early HIV-infected patients and 20 chronic HIV-infected patients. After stimulation with HBsAg overlapping peptide, the capacity of CD8+ T cells to secrete IFN-γ was significantly improved in early HIV-infected patients (see **Figure 3A**). Furthermore, the proportion of IFN-γ in CD8+ T cells in the early HIV infection group was significantly higher than that in the chronic group ($p<0.01$) (see **Figure 3B**). The correlation analysis showed that there was no correlation between the proportion of IFN-γ in CD8+ T cells and the CD4+ T cell counts in co-infected patients ($r=0.070$, $p=0.837$).

TABLE 1 | Characteristics of patients in the early and chronic HIV infection cohorts.

	Early HIV infection (n=372)	Chronic HIV infection (n=306)
Age, years, (median, IQR)	32 (22-59)	33 (19-59)
≤20	17 (4.6%)	10 (3.3%)
21-35	224 (60.2%)	169 (55.2%)
36-50	118 (31.7%)	111 (36.3%)
≥51	13 (3.5%)	16 (5.2%)
HBV coinfection, n(%)		
Yes	23 (6.2%)	35 (11.4%)
No	349 (93.8%)	271 (88.6%)
CD4+T counts, cell/ul, (median, IQR)	445 (196-1030)	358(17-783)
≤200	56 (15.1%)	61 (19.9%)
201-349	132 (35.4%)	132 (43.1%)
≥350	184 (49.5%)	113 (36.9%)
HIV RNA load, copies/ml, (median, IQR)	530, 956 (554-7,280,000)	46,363 (<40-630, 000)

4 DISCUSSION

The influence of HIV infection combined with HBV has been reported in many studies, and some studies have suggested that it might be due to susceptibility to HBV at different HIV infection stages (14). In this study, we explored the clinical and immunological characteristics of HIV patients from the MSM cohort coinfected with HBV in a long-term observation. From our retrospective cohort of early and chronic HIV infection studies, we found that the HBV coinfection rate increased significantly during chronic HIV infection compared with the early stage in China. HBV-specific antigens in two of four early HIV infection patients became positive as the disease developed to chronic HIV infection. From the data shown in this study, we found that the rate of HBV infection increased with the development of HIV infection. On the one hand, the lower CD4+ T cell counts and the severer dysfunction make chronic HIV-infected individuals more susceptible to HBV infection. On the other hand, the spontaneous clearance of HBV in chronic HIV-infected individuals might lead the chronic HBV infection to more likely develop due to the CD8+ T cell exhaustion spontaneous clearance ability and the reduced ability of CD8+T cells to secrete IFN-γ.

Most HBV infections are acquired during the perinatal period or due to high exposures in early childhood. In this study,

TABLE 2 | Characteristics of patients who had HBV-specific antigens and/or specific antibody changes from early to chronic phases.

Patient	Follow-up	HBsAg	HBsAb	HBeAg	HBeAb	HBcAb	ALT (IU/L)	AST (IU/L)	CD4 (cells/ul)	HIV RNA (copies/ml)
1	Early	_	+	_	_	+	25.0	23.5	430	41,600
	Chronic	_	+	_	_	_	27.3	27.8	251	121,000
2	Early	_	+	_	+	_	27.5	25.4	500	53,800
	Chronic	_	+	_	_	_	20.5	16.6	190	88,200
3	Early	_	+	_	_	_	22.1	24.1	389	222,000
	Chronic	+	_	_	_	_	43.2	36.2	157	415,000
4	Early	_	_	_	_	+	13.0	19.2	420	295,000
	Chronic	+	_	_	_	_	21.6	24.5	170	410,000

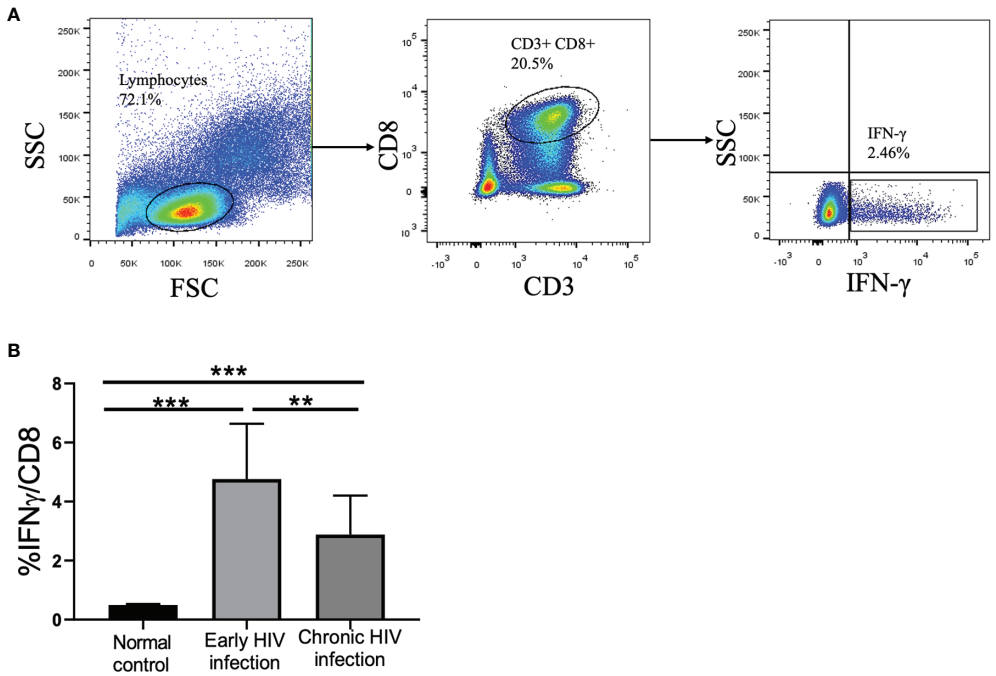

FIGURE 3 | Frequency of IFN-γ-producing CD8+ T cells in normal controls, early HIV infection, and chronic HIV infection groups. **(A)** The gating strategy for flow cytometric analysis of IFN-γ-producing CD8+ T cells. **(B)** Comparison of the proportion of IFN-γ-producing CD8+ T cells. The significance of differences was assessed by calculating P values in Mann–Whitney tests. $^{**}p < 0.01$, $^{***}p < 0.001$.

we observed a clear increase in the HBV infection rate with disease development and immune system damage in adult HIV patients. The rate was significantly higher in the HIV group than in the general population (15). In China, it was reported that only 5%~10% of people above 5 years old could develop chronic HBV infection (16). The reason might be that HIV infection destroys host immune CD4+ T cells, which are resistant to HBV (17). HIV infection can cause CD4+ T cell reduction and dysfunction, making the infected human body vulnerable to HBV (17), subsequently damaging HBV clearance and ultimately aggravating the progression of liver disease (18, 19). cccDNA has been shown to persist even in the liver of patients with successful cellular and humoral control of the infection (20), which suggests that HBV may come from cccDNA in the liver tissue. Other studies have shown the reactivation of HBV after receiving anticancer therapy or rituximab-containing treatment (11, 21). Similar results in our study suggest that HIV patients are more prone to coinfection with HBV. Once infected, the spontaneous clearance ability is low, and chronic HBV infection easily develops. Due to the limitation of the number of cases, this conclusion should be tested and verified in a large-scale prospective study in the future.

Serological changes were detected in the two HIV patients infected with HBV in this study, both of whom were newly infected in the early stage of HIV. The serum of one patient was anti-HBs-positive in the early stage but confirmed as HBV-positive when HIV developed to the chronic stage. The other patient tested anti-HBc-positive only in the early stage of HIV but was HBsAg-positive in the chronic stage, which could be explained by the previous HBV infection history. Many studies have reported that spontaneous seroreversion (HBsAb negative and HBsAg conversion) often occurs in HIV-infected individuals, especially in patients whose CD4+ T cell count is below 200 cells/mm^3 (22–24). Therefore, it suggests that it is particularly important to monitor HBsAb titre changes in HBsAb-positive patients. Vaccination may prevent vulnerable individuals from experiencing HBV reinfection. If liver function is unexplained abnormal, and the reoccurrence of HBV infection cannot be ruled out, clinicians should evaluate the stage of HBV coinfection among HIV patients. Occult Hepatitis B virus infection (OBI) is not common in the HIV-infected population, and the presence of HBV DNA replication in blood and/or liver can be detected, but serum HBsAg is negative (14). The prevalence of OBI in HIV-infected patients varies from 2% to 10% by region, since the ALT and HBV DNA levels usually increase slightly; thus, clinical OBI cannot be confirmed to be very significant. However, some studies have shown that OBI can accelerate the progression of liver disease and have suggested that monitoring HBV markers regularly and vaccination for these populations might be of great importance (25). Therefore, it is worth further exploring whether HBV positivity in these two immunocompromised HIV patients was due to the re-replication of the original HBV infection or a new infection.

Given that HIV/HBV coinfection results in higher morbidity and mortality of liver disease compared to single HBV infection,

greater attention should be given to controlling HBV infection among HIV patients. As immune restoration by HAART treatment can improve HBV modulation, a preventive regimen should be taken into consideration for immune-compromised patients with HIV infection. Currently, sexual transmission among the MSM group has become the main method of HIV spread worldwide (26); the HBsAb-positive rate was less than 70% according to an investigation of the MSM group in China (27), and our study further demonstrated the importance of HBV vaccination and HBsAb detection in the MSM population.

At the early stage of infection, the response of CD8+ T cells specific for HBV plays a key role in viral clearance, but this type of response becomes weakened due to CD8+ T cell exhaustion after the disease develops into the chronic stage (28, 29). IFN-γ and TNF-α secreted by CD8+ T cells possess antiviral properties (12). Based on our observation from the early and chronic HIV patients in this study, we found that, with HIV disease progression, the number and function of CD4+ T cells decreased, and the ability of CD8+T cells to secrete IFN-γ also declined gradually. Altogether, the killing function of these cells is seriously affected. The chance of HBV reinfection is highly increased for HIV patients coinfected by HBV who do not accept ART; with the development of the disease, the HBsAb level decreases precipitously, along with the gradual impairment of immune function.

There are some limitations of this study. First, due to the limitations of our facility, we only detected the ability of CD8+ T cells to secrete IFN-γ instead of testing the killing function of HBV-infected cells directly. Second, the cohort size was relatively small, which limited our capacity to draw a robust conclusion. Third, the markers of cytotoxicity on CD8+ T cells should have been tested. However, as a pilot study, we did not detect other surrogate markers. The results of this study supported us to further explore the relationship between the cytotoxicity of CD8 + T cell after ex vivo stimulation by HBV antigens in follow-up studies. Fourth, it is a pity that the information of HBV vaccine in the two retrospective cohorts was absent. We failed to evaluate the proportion and the efficacy of HBV vaccine in the present study. The results of this study supported us to further explore the benefits in the early HIV-infected patients and chronically HIV-infected patients. In the future, we will explore the mechanism of chronic HBV infection in HIV patients if more appropriate patients become available.

In summary, the coinfection rate of HBV in HIV patients increases with HIV disease progression, which might be related to the decreased IFN-g-producing HBV-specific CD8+ T cell numbers. The closely monitored HBV serum markers from the early stage of HIV infection are warranted.

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 (YAH200605010). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZZ and YQ conceptualized the idea and drafted the manuscript. QL, WX, and MZ performed the experiments. QL, TZ, and WW conducted data analysis. TJ, HW, and YT supervised the manuscript writing. All authors read and approved the final manuscript.

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REVIEW
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HIV Infection Predisposes to Increased Chances of HBV Infection: Current Understanding of the Mechanisms Favoring HBV Infection at Each Clinical Stage of HIV Infection

Silvere D. Zaongo[1,2†], *Jing Ouyang*[2†], *Yaling Chen*[2], *Yan-Mei Jiao*[3], *Hao Wu*[4] *and Yaokai Chen*[1,2*]

[1] Division of Infectious Diseases, Chongqing Public Health Medical Center, Chongqing, China, [2] Clinical Research Center, Chongqing Public Health Medical Center, Chongqing, China, [3] Fifth Medical Center of Chinese PLA General Hospital, Beijing, China, [4] Department of Infectious Diseases, You'an Hospital, Capital Medical University, Beijing, China

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Prathima Institute of Medical Sciences, India

***Correspondence:**
Yaokai Chen
yaokaichen@hotmail.com

[†]*These authors have contributed equally to this work*

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Human immunodeficiency virus (HIV) selectively targets and destroys the infection-fighting CD4+ T-lymphocytes of the human immune system, and has a life cycle that encompasses binding to certain cells, fusion to that cell, reverse transcription of its genome, integration of its genome into the host cell DNA, replication of the HIV genome, assembly of the HIV virion, and budding and subsequent release of free HIV virions. Once a host is infected with HIV, the host's ability to competently orchestrate effective and efficient immune responses against various microorganisms, such as viral infections, is significantly disrupted. Without modern antiretroviral therapy (ART), HIV is likely to gradually destroy the cellular immune system, and thus the initial HIV infection will inexorably evolve into acquired immunodeficiency syndrome (AIDS). Generally, HIV infection in a patient has an acute phase, a chronic phase, and an AIDS phase. During these three clinical stages, patients are found with relatively specific levels of viral RNA, develop rather distinctive immune conditions, and display unique clinical manifestations. Convergent research evidence has shown that hepatitis B virus (HBV) co-infection, a common cause of chronic liver disease, is fairly common in HIV-infected individuals. HBV invasion of the liver can be facilitated by HIV infection at each clinical stage of the infection due to a number of contributing factors, including having identical transmission routes, immunological suppression, gut microbiota dysbiosis, poor vaccination immune response to hepatitis B immunization, and drug hepatotoxicity. However, there remains a paucity of research investigation which critically describes the influence of the different HIV clinical stages and their consequences which tend to favor HBV entrenchment in the liver. Herein, we review advances in the understanding of the mechanisms favoring HBV infection at each clinical stage of HIV infection, thus paving the way toward development of potential strategies to reduce the prevalence of HBV co-infection in the HIV-infected population.

Keywords: HIV, HIV clinical stages, HBV, coinfection, mechanisms

INTRODUCTION

Human immunodeficiency virus (HIV) infection has been a major public health issue for the past four decades. Despite extensive global research and study (1–3), a cure for HIV infection has, thus far, proven elusive. Recently, our research group has proposed novel potential therapeutic options for HIV infection (4, 5) which, we believe, could inspire future clinical trials into curative therapeutic options for HIV. Our first proposition concerns the promotion of P-selectin glycoprotein ligand 1 (PSGL-1), an important receptor from innate immunity, which (i) induces the production of membrane defective virions that are unable to attach to or infect new target cells, and (ii) blocks the HIV reverse transcription process. Our second proposition involves the selective elimination of host cells capable of producing HIV virions *via* the use of a therapeutic cocktail of drugs (latency reversal agents, autophagy inhibitors, apoptosis activators, and antiretroviral drugs).

The World Health Organization (WHO) has proposed that HIV infection may be divided into four clinical stages in adults and adolescents 15 years-of-age and above (6). HIV-positive patients who are asymptomatic or have persistent generalized lymphadenopathy (lymphadenopathy of at least two sites [not including inguinal] for longer than 6 months) are categorized as being in stage 1. Clinical findings included in stage 2 (mildly symptomatic stage) are unexplained weight loss of less than 10 percent of total body weight and recurrent respiratory infections (such as sinusitis, bronchitis, otitis media, and pharyngitis), as well as a range of dermatological conditions including herpes zoster flares, angular cheilitis, recurrent oral ulcerations, papular pruritic eruptions, seborrhoeic dermatitis, and fungal nail infections. Manifestations included in clinical stage 3 (the moderately symptomatic stage) are weight loss of greater than 10 percent of total body weight, prolonged (more than 1 month) unexplained diarrhea, pulmonary tuberculosis, and severe systemic bacterial infections including pneumonia, pyelonephritis, empyema, pyomyositis, meningitis, bone and joint infections, and bacteremia. Stage 4 (the severely symptomatic stage) includes all of the AIDS-defining illnesses, e.g., HIV wasting syndrome, Pneumocystis pneumonia (PCP), recurrent severe or radiological bacterial pneumonia, extrapulmonary tuberculosis, HIV encephalopathy, CNS toxoplasmosis, chronic (more than 1 month) or orolabial herpes simplex infection, esophageal candidiasis, and Kaposi's sarcoma. WHO HIV clinical staging utilizes standardized clinical parameters to direct medical decision making for patients with HIV/AIDS, and can be used based solely on patient clinical features, thus accommodating treatment facilities that may have limited or no access to sophisticated laboratory testing, such as those in low- and middle-income countries and regions (7). There is, also, the existence of the Fiebig staging system of HIV infection (first published in 2003, and comprising 6 stages), which describes the emergence of virological and immunological markers following infection by HIV. Several discrete clinical phases can thus be recognized for HIV infection; however, it has been generally accepted that HIV infection exhibits an acute phase, a chronic phase, and the acquired immunodeficiency syndrome (AIDS) phase (8).

In 2020, it was estimated that 36.7 million people globally were infected by HIV (9), and thus, the global HIV pandemic continues to pose a material threat to the health of mankind. The large majority of new HIV infections occur in low- and middle-income countries (10). Poverty, stigma associated with HIV disease, cultural and social barriers to appropriate testing and treatment, insufficient and inadequate health care infrastructure to support the large patient pool, poor health literacy, limited provider training, inadequate and inappropriate medical equipment, scarcity of appropriately-trained medical manpower to distribute health care throughout specific regions, and an inadequately low number of accredited medical laboratory facilities are some of the numerous factors that contribute to the almost inexorable global propagation of HIV (11).

At the same time, hepatitis B virus (HBV) is also silently spreading amongst the global population, especially in low- and middle-income countries (12). In 2019, the WHO estimated that 296 million people were living with chronic HBV (with 1.5 million new infections each year). More specifically, the WHO Western Pacific Region and the WHO African Region presents the highest chronic hepatitis B infection rates, with 116 million and 81 million people infected, respectively. Lower proportions occur in (i) the WHO Eastern Mediterranean Region (with 60 million people infected), (ii) the WHO South-East Asia Region (with 18 million people infected), (iii) the WHO European Region (with 14 million people infected), and (iv) the WHO Americas Region (with 5 million people infected) (13). Thus, HBV affects hundreds of millions of people worldwide, and is responsible for progressive liver fibrosis and hepatocellular carcinoma, amongst other chronic health sequelae (14, 15) during the chronic phase of HBV disease. Most cases of HBV infection in adults are arrested early, and are defined as an acute infection that is generally successfully limited by the patient's own immune system. Only adults with an immunocompromised immune system tend to progress to chronic HBV (16–18). Unfortunately, most cases of HBV infection acquired in infancy or early childhood however, do become chronic (16–18). According to WHO, around one third of the world's population has been infected by HBV at some point of their lives (16–18). Thus, HIV-HBV coinfection is relatively common (19). Estimations suggest that 10 to 28% of HIV-infected individuals are chronically infected with HBV (20–25). Indeed, the rates of HIV-HBV coinfection vary significantly between regions and risk-based groups. For instance, a study in Vietnam has shown that HIV-HBV coinfection is significantly higher among people who inject drugs (28%) or who are sex workers (15%) (23). Similarly, Xie et al. (26), have reported an estimation of 10% with respect to the existing HIV-HBV coinfection rate in China in general; however, they also state that the prevalence of such HIV-HBV coinfection in China varies between regions from 5% to 15%. In an extensive review on HIV-HBV coinfection, Singh et al. (27), suggested that West Africa and South Africa possess the highest prevalence of HIV-HBV coinfection in the world.

Several past studies have explored the impact of HIV-HBV coinfection on patients' health, and have found that this comorbid association accelerates HBV progression (higher levels of hepatitis B viremia, higher risk of developing cirrhosis and hepatocellular carcinoma) (28), and materially amplifies the complexities related to treatment (27, 29–31). Among the mechanisms triggered by HIV infection which accelerate the progression of HBV infection, we can list (i) HIV replication in the liver, (ii) HIV-associated microbial translocation and immune activation, and (iii) immune exhaustion and tolerance. Each of these mechanisms mediated by HIV pathogenesis has significant effects on liver disease, as noted by Singh et al. (27). However, to the best of our knowledge, there remains a paucity of published research investigation in the literature which critically describes the influence and consequences of HIV clinical staging that potentially favor HBV establishment in HIV-infected individuals.

We therefore propose, herein, to review the appropriate literature to elucidate the potential mechanisms favoring HBV infection at each clinical stage of HIV infection. In the first part, we discuss the transmission routes of both HIV and HBV, and their subsequent life cycles once they have entered the human body. In the second part, we critically discuss the potential influence of each of the HIV acute, chronic, and AIDS phases that either lead to or may potentially lead to HBV infection.

HIV AND HBV: TRANSMISSION ROUTES AND LIFE CYCLE

It is well-established that HIV and HBV share the same transmission routes. Indeed, both viruses are known to be transmitted from person to person through sexual intercourse, *via* contaminated needles used for intravenous drug delivery, from mother to child, and by the therapeutic use of HIV or HBV-infected blood or blood products (32). Thus, individuals who have casual sex in the absence of a condom and those who inject recreational drugs are at a particularly high risk for acquiring not only HIV infection, but also HBV infection (29). Once a person is infected by either HIV or HBV, these viruses exhibit two distinct life cycles within the infected persons body (**Figure 1**).

HIV targets immune cells, preferentially CD4+ T-lymphocytes. Then, a viral envelope glycoprotein molecule (gp120) binds to a host cell receptor or co-receptor, such as CCR5 or CXCR4, responsible for HIV entry into lymphocytes and macrophages. The binding of gp120 to these receptors results in a cascade of molecular conformational changes and the exposure of gp41, bringing the HIV virion in much closer proximity to the target cell. Subsequent fusion of the viral envelope with the host cell membrane is essential for the entry of the inner matrix core of the virus into the intracytoplasmic realm of the host cell (33). Within the viral inner core are two strands of viral RNA held together by two small proteins (P6 and P7), and three of the enzymes essential for viral replication, viz., integrase, protease, and reverse transcriptase. Accessory proteins such as Nef, Vpr, and Vif are also found in the core matrix of the virus. Although these accessory proteins are not essential for viral replication, they play crucial roles in counteracting defensive mechanisms activated by the host cell (34, 35). Once within the host cell cytoplasm, the core matrix of the virion disintegrates, releasing the viral capsid as well as the genome of the virus. The viral RNA, together with the essential viral enzymes, is thus exposed to the host cell cytoplasm. The viral RNA then undergoes reverse transcription into viral DNA through a process mediated by the viral reverse transcriptase. Earlier investigations have revealed that the viral DNA generated by the reverse transcription process within the host cytoplasm is part of a broad nucleoprotein complex known as the pre-integration complex (PIC) (36), which also comprises Vpr and the integrase enzyme. Subsequent migration and entry of the PIC into the nucleus is followed by the process termed integration, which is mediated by the integrase enzyme. The preceding view, that conversion of the HIV RNA genome into DNA occurs in the cytoplasm before nuclear entry has, however, been challenged recently. Indeed, Dharan et al., have provided evidence to support the hypothesis that reverse transcription and uncoating can occur in the nucleus of non-dividing cells, such as macrophages or cells treated with the

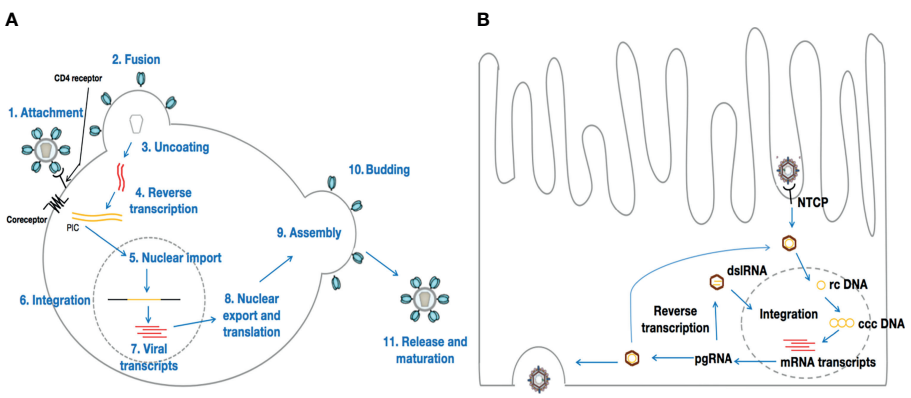

FIGURE 1 | Life cycles of HIV and HBV. **(A)** represents HIV life cycle once in contact with CD4+ T-cells. Although HIV preferentially infects CD4+ T-cells, HIV tropism is not limited to CD4+ T-cells only. Conversely, HBV [the life cycle of which is depicted in **(B)**] infects hepatocytes exclusively.

tetracyclic antibiotic, aphidicolin (37). For integration to occur, the integrase within the PIC acts by slicing through the DNA of the host cell, and thus allowing viral DNA to be inserted at a variety of sites on the host DNA, i.e., integrase catalyzes the insertion of viral dsDNA into the host chromosome (38, 39). For HIV, and most viruses that integrate into the host genome (e.g., murine leukemia virus, Herpes simplex virus-1, Ebola virus), observations from past studies (40, 41) reveal evidence of the DNA splicing and joining steps. It is critical to keep in mind that, usually, two nucleotides are removed from 3' end of the viral DNA. Then, these 3' ends attack a pair of phosphodiester bonds on opposite strands of the target DNA, across the major groove, leading to a bonding of the covalent 3' ends of the viral DNA to the target DNA. Finally, the single-strand gaps and the two-nucleotide overhang at the viral DNA's 5' ends are repaired by cellular enzymes, in order for integration to be complete. For HIV, the sites are five base pairs apart instead of two, resulting in a five base-pair duplication (42). Once HIV DNA is integrated into the host cell genome in this manner, the host cell is considered to be infected for life. Thus, the integrated viral DNA, referred to as provirus, can be used to generate genomic RNA, which can serve as messenger RNA (mRNA) for the synthesis of viral proteins in the host cytoplasm (**Figure 1A**).

HBV targets and replicates solely in the parenchymal cells of the liver (the hepatocytes) (43–46) (**Figure 1B**). Moreover, it has been established that HBV infects only humans, chimpanzees, and to a lesser extent, tree shrews (Tupaia belangeri) (47, 48). Once in contact with the liver, the circulating virion initially attaches to heparan sulfate proteoglycans (HSPGs) (49, 50). Then, the interaction of a specific domain of the HBV L envelope protein with the sodium taurocholate co-transporting polypeptide [NTCP, a hepatocyte-specific transporter of bile acids that is predominantly localized in the basolateral membrane that faces the sinusoidal lumen (51)] on the surface of the hepatocytes contributes to viral entry into the hepatocyte (52). Following entry and uncoating, the nucleocapsid carrying the HBV genome is transported into the nucleus, where it is released as relaxed circular (rc) DNA. There, the rcDNA is converted into an episomal covalently closed circular (ccc) DNA minichromosome by host enzymes (46, 53). Reports suggest that cccDNA is very stable, persisting indefinitely, and is one of the main barriers to cure for hepatitis B disease (46), as it is the template for all HBV RNA transcripts (27, 54) that leave the nucleus unspliced, and produces the viral structural and non-structural proteins (53). Thus, HBV can initiate viral replication with an estimated doubling time of 2-4 days (55, 56). Interestingly, HBV polymerase can encode the pre-genomic RNA (pgRNA) and the reverse transcription of pgRNA can also lead to the formation of double stranded linear HBV DNA (dslDNA). Once in the nucleus, the dslDNA, in a similar manner to HIV, can integrate into the host genome (27). In contrast to HIV, the integrated dslDNA cannot enable viral replication, but it does allow the expression of certain gene products, like the envelope proteins (Env), which are dissimilar to the envelope proteins generated from cccDNA, which coat filamentous and spherical subviral particles (SVPs) (54). In general, acute manifestation of HBV infection occurs within 6 months after a person is exposed to HBV (57). From an acute infection, it can subsequently progress into a chronic infection. Indeed, although most people with healthy immune systems are able to clear the virus at the acute stage, immature immune systems and/or impaired immunity can lead to the establishment of chronic HBV infection in infants and/or adults (58, 59). Once the disease becomes chronic, it becomes a lifelong infection which, in the absence of effective treatment, can cause liver cancer or significant liver damage and scarring, leading to eventual liver failure.

In vitro and *in vivo* reports suggest that HIV can also infect hepatic stellate cells, sinusoidal endothelial cells, Kupffer cells, and the resident macrophages of the liver [as reported by Chamroonkul and Bansal (60), Housset et al. (61) and Cao et al. (62)]. HIV RNA sequences from the livers of untreated HIV-positive individuals show distinct compartmentalized sequences when compared to RNA sequences from other tissue sites (63). Further studies have demonstrated that HIV can persist in the liver even in patients on antiretroviral therapy (ART), primarily in Kupffer cells (64–66). In this review, therefore, we explore and discuss the influence of HIV infection on the establishment of HBV infection, especially being cognizant of the fact that HIV is known to provoke the fundamentally profound immune system impairment necessary for the onset of chronic HBV. Normally, most people with healthy immune systems are able to clear HBV during the acute phase. Utilizing the combined actions of HBV-specific CD4+ T-cells [essential for the induction and the maintenance of both CD8+ T-cells and antibody responses (67, 68)] and HBV-specific CD8+ T-cells [which kill infected hepatocytes and induce local production of proinflammatory cytokines (69–71)], a healthy person can easily overcome acute HBV infection, and thus avoid the chronic and life-threatening phase of the infection. Subsequently, our discussions will consider HIV as the primary infection, and we reflect further on the immunological consequences of HIV infection that favor HBV infection.

The various mechanisms through which liver injury may occur in patients with HIV infection are numerous; a general breakdown of these mechanisms is presented in **Table 1**. A reasonable understanding of these mechanisms is of significant importance to the comprehension of HIV/HBV pathological processes, and any liver injury may further represent an 'open door' for HBV to enter hepatocytes and subsequently establish infection. This preceding assertion is speculative at this stage, and further investigation is required to establish precisely how liver injury induced by HIV infection could facilitate HBV invasion of hepatocytes.

ACUTE AND EARLY HIV INFECTION

Innate Immune Defense Subversion

Acute HIV infection (AHI) is the first stage of HIV infection, occurring soon after viral acquisition and before seroconversion.

TABLE 1 | Summary of reported mechanisms responsible for liver injury in patients with HIV.

Mechanism	Contribution	Details	References
Oxidative stress	Moderate	This is a process whereby free reactive oxygen species (ROS) provoke increased activation of Kupffer cells in the liver. In turn, these activated immune cells promote stellate cell activation *via* nuclear factor kappa-beta (NF-kB) and activator protein 1, leading to increased production of proinflammatory and profibrotic cytokines, resulting in liver damage, fibrosis, and cirrhosis. Nucleoside reverse transcriptase inhibitors (NRTIs) such as didanosine can cause oxidative stress and mitochondrial toxicity.	(21)
Mitochondrial injury	Moderate	As the primary source of energy in the hepatocyte, any process that impairs mitochondrial function may lead to hepatic injury. During HIV, mitochondrial injury can occur through increased stress on the endoplasmic reticulum (ER), initiated by activation of the IRE1/TRAF 2 (Inositol Requiring 1/TNF receptor-associated factor 2) pathway. NRTIs and protease inhibitors (PIs) can directly cause mitochondrial toxicity.	(21, 72, 73)
Immune-mediated injury	Moderate	HIV can interact with hepatic stellate cells (HSCs) *via* gp120, producing inappropriate activation and increased HSC production of collagen and monocyte chemoattractant protein (MCP-1) (a macrophage chemoattractant). HIV decreases the number of Kupffer cells in the liver, decreasing the ability of the liver to clear products of microbial translocation. HIV provokes alterations in cytokine profiles resulting from imbalance between CD4+ and CD8+ T-cells	(21, 74, 75)
Cytotoxicity	Mild	HIV triggers apoptosis *via* the HIV gp120 protein-receptor signaling pathway.	(76)
Systematic inflammation	Significant	The systematic inflammation resulting from HIV infection may induce fibrosis *via* a number of mechanisms, including oxidative stress and mitochondrial dysfunction as a result of ER stress. CD4/CD8 imbalances seen in HIV can lead to underexpression of IFN-gamma (an antifibrotic cytokine), thus favoring induction of apoptosis of activated HSCs, and hepatic progression into a profibrotic state.	(21, 77, 78)
Gut microbial translocation	Significant	This leads to hepatic injury primarily *via* increased hepatic levels of bacterial lipopolysaccharides (LPS), causing hepatic inflammation. More specifically, hepatic inflammation may result from (i) recruitment and activation of inflammatory cells (Kupffer cells and HSCs), (ii) systemic immune responses promoting hepatocyte cell death, or (iii) production of proinflammatory cytokines and acute phase reactants such as transforming growth factor beta 1 (TGFB1), IL-6, and IL-10	(79–81)
Nodular regenerative hyperplasia	Significant	This is a rare condition in which diffuse transformation of the liver parenchyma into micronodules without intervening fibrosis leads to non-cirrhotic portal hypertension in patients with HIV. Pathophysiologically, it is thought that gut bacterial translocation may be responsible for vascular endothelial disruption, vascular and peri-vascular fibrosis and stenosis, and portal hypertension. The epithelial damage observed in the liver isare thought to either be immune-mediated or possibly related to direct viral damage by HIV.	(82–84)

AHI typically lasts 3–4 weeks (**Figure 2**), and is characterized by the presence of HIV RNA and p24 antigen (Ag) (85) in the circulation. During this short period, HIV concentrations in blood and other body fluids (vaginal secretions and semen) are exceptionally high, increasing the likelihood of HIV transmission (85–92). To reach the high levels of HIV observed, HIV-1 subverts dendritic cell and macrophage activities (preferentially CD4+ T-cells) to increase its replication at mucosal locations (93, 94). This strategy also favors HBV, which does not need to use any specific mechanisms to avoid such immune cells (dendritic cells, macrophages, and T-cells) in an HIV-positive individual. Moreover, HIV adopts a variety of strategies to avoid type 1 interferon (IFN-1) control [repression of HIV restriction factors (95–101) and/or blocking of IFN-1 expression by infected cells (102–105)] *via* the infected-cells (dendritic cells, macrophages, and T-cells). Indeed, IFN-1 is an

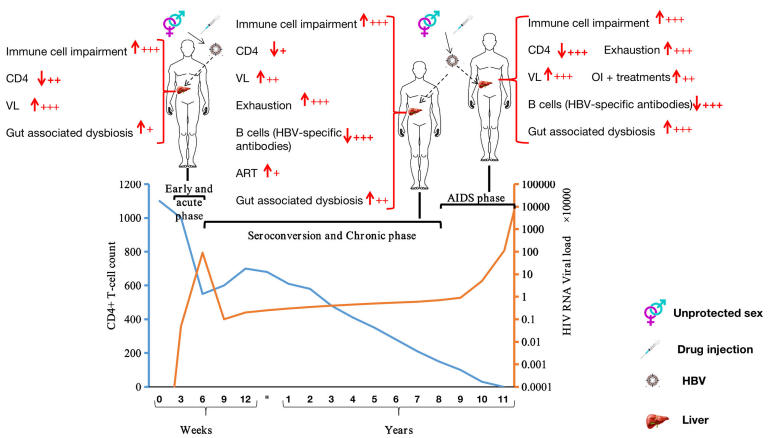

FIGURE 2 | Stages of HIV infection and factors potentially favoring HBV infection at each clinical stage of HIV infection. ↓: depletion; ↑: augmentation; VL, viral RNA load; OI, opportunistic infection; +: Mild; ++: Moderate; +++: Severe.

innate antiviral defense cytokine, and is known as a pleiotropic cytokine that acts by up-regulating transcription of hundreds of IFN-stimulated genes, including HIV restriction factors (106). To illustrate this point, Gondim et al. (107), for instance, have investigated how IFN-1 can control HIV infection and they have shown that IFN-1 (including IFNα2 and IFNβ) administration can reduce viral replication in CD4+ T-cells and macrophages. Furthermore, three major points depicting the interplay between HIV infection and IFN-I are of particular interest, viz., (i) the sensitivity of HIV-1 isolates to IFN-I inhibition consistently changes over time, (ii) HIV-1 isolates obtained during ART therapy were relatively IFN-I sensitive, and (iii) the viruses that rebounded after treatment interruption displayed the highest degree of IFNα2 and IFNβ resistance. Thus, IFN-1 plays an essential role in inflammation, immunoregulation, tumor cell recognition, and T-cell responses. In the absence of effective expression of IFN-1, or in the absence of response resulting from its expression, the immune system becomes vulnerable to viral infections, such as infections by HBV. In addition to IFN-1, HIV avoids INF-gamma (IFN-γ, a type II interferon) control by (i) destroying CD4+ T-cells [also responsible for IFN-γ secretion (67)] or (ii) repressing, for instance, PSGL-1 (an HIV restriction factor) activities, which has been extensively reviewed by our research group (4). Besides, in order to reduce the protective benefits of innate responses, HIV-1 resists well-demonstrated control by natural killer cell (108–113) [stimulated by innate cytokines including IFN-1, IL-15, IL-18 and receptor-ligand interactions (93)], and may disrupt innate regulation of adaptive responses, as suggested by Borrow (93). By utilizing these mechanisms, HIV infection contributes to HBV evasion of immune cells (particularly effector cells) to establish chronic HBV disease, as described by Lannacone and Guidotti (54).

High HIV Viral Load and CD4+ T-Cell Depletion

During AHI, the elevated concentration of viral particles in the systemic circulation facilitates infection of the liver by HIV, which in turn promotes multiple pathways that all converge on activated hepatic stellate cells (HSCs), the primary source of collagen synthesis in the injured liver, which encourages hepatic inflammation and fibrosis (60). For instance, it is known that HIV and its envelope gp120 (i) promote direct pro-fibrogenic effects on HSCs, (ii) promote the production of pro-inflammatory cytokines (such as MCP1, IL-8), and (iii) induce apoptosis in hepatocytes (75, 114). Indeed, HIV glycoproteins induce hepatocyte apoptosis *via* the expression of the TNF-related apoptosis inducing ligand (TRAIL), by stimulation of hepatocytes (115, 116). Furthermore, rapid fibrosis, in addition to causing elevated plasma HIV levels, correlates with reduced CD4+ T-cell counts.

During AHI, there is extensive CD4+ T-cell destruction (HIV-induced CD4+ T-cell depletion) (93). On the one hand, this HIV-induced depletion of CD4+ T-cells relative to CD8+ T-cell recruitment alters the hepatic cytokine profile, establishing a fibrogenic environment. Consequently, an injured liver becomes an ideal target for HBV to establish an acute phase, which progressively metamorphoses into a chronic infection due to the persistence of the systemic inflammation caused by HIV infection. On the other hand, it is recognized that host CD4+ T-cells are essential for the recognition of viral antigens presented by Kupffer cells and the regulation of the activities of (i) CD8+ cytotoxic T-cells, (ii) antibody-producing B-cells, and (iii) cytokine-secreting cells (19, 117–119). When the HIV acute phase leads to drastic depletion of CD4+ T-cells, the immune system is unable to adequately respond to HBV invasion, as HBV antigens presented by Kupffer cells cannot thus be recognized. Moreover, CD8+ T-cells, B-cells, and cytokine-secreting cellular functions are overwhelmed by HIV subvertive activities, which thus facilitates HBV infection establishment.

HIV SEROCONVERSION AND THE CHRONIC PHASE

During the seroconversion phase, which occurs after the acute phase (**Figure 2**), the body starts producing detectable levels of HIV-specific antibodies. A seropositive individual may have flu-like symptoms, such as fever and body aches during this phase. The duration for HIV disease progression with clinical symptoms varies widely across individuals, although it usually progresses slowly (120). Most HIV-positive individuals are diagnosed during or after the seroconversion phase [as HIV diagnostic tests generally target HIV-specific antibodies (121)]. During this period, the earlier detection and earlier initiation of appropriate treatment leads to a reduced risk of onward transmission. Due to HIV-specific antibody production, HIV-infection is stabilized at this stage of the infection, meaning that the plasma viral RNA load, despite being high, remains stable, CD4+ T-cells counts increase slightly, and the immune system activation remains persistent. HIV causes several structural, functional, and immunological impairments, resulting from a persisting underlying chronic inflammatory state (122–124). HBV establishment is likely to be favored by HIV infection during the seroconversion and the chronic phases as HIV infection sustains the immunological impairments present during the acute phase, in conjunction with other mechanisms, as described in the following paragraphs.

HIV-Associated Gut Dysbiosis

It has been reported that the gastrointestinal tract (GI) represents the primary site of HIV replication and reservoir persistence (125). Once HIV infection is established, a rapid loss of GI mucosal integrity is noted. Indeed, HIV disrupts the lymphatic system of the gastrointestinal tract, causing a large loss of CD4+ T-lymphocytes in the gut-associated lymphoid tissue (GALT), which disrupts the tight junctions of the intestinal epithelium. Subsequently, this detrimentally alters the integrity of the intestinal mucosal barrier, leading to intestinal microbiomic disorders (126, 127), which manifest as a decrease in gut microbiotic organism diversity, the augmentation of specific species of potentially pathogenic gut microbiomic

microorganisms (128), and the promotion of an increased permeability (or "leakiness") of the intestinal tract. Consequently, harmful bacteria and their products, such as lipopolysaccharide (LPS), *via* their passage through the portal vein into the liver, may activate the liver's innate immune system by recognition of Toll-like receptors (TLRs, especially TLR2 and TLR4) (129). Some investigators believe that the levels of translocated microbial products, such as LPS, in the portal vein and/or in the liver (which are both difficult to measure) may be more important than these microbial products being present in the systemic circulation (27, 54). This innate immune response, generated by pathogen-associated molecular patterns (PAMPs) produced by intestinal microbes, may be responsible for hepatocyte damage (130). To further illustrate this point, in a study by Evans et al. (131), using SIV-infected macaques, it was demonstrated that increased microbial load in the liver may also trigger chemokine production and an increased infiltration of CXCR6+ activated NK cells, known for their role in the development of liver fibrosis. An HIV-positive individual displaying an HIV-associated gut dysbiosis profile can, thus, readily develop HBV infection, as HIV-associated microbial translocation favors hepatocyte injury. Our group has recently published an extensive review discussing mechanisms whereby gastrointestinal microbiome dysbiosis and a "leaky" gut in PLWH increases susceptibility to HBV infection (132).

Immune Cell Exhaustion

CD8+ T-cells (levels of which remain elevated in the bloodstream during HIV infection), HIV-associated dysbiosis *via* microbial translocation (128, 133), and TRAIL [a proapoptotic ligand with an immune effector function promoting the eradication of infected or malignant cells (134)], are some of the identified factors responsible for CD4+ T-cell depletion. CD4+ T-cell depletion is also responsible for liver injury, which facilitates liver invasion by HBV (as described in the preceding section). Since CD4+ T-cells are important for the recruitment of HBV-specific CD8+ T-cells, a sustained CD4+ T-cell depletion restricts the ability of the immune system to adequately and appropriately respond to HBV invasion. Indeed, in such a context, it is difficult for the immune system to locate and recruit HBV-specific CD4+ T-cells (55), which represents an essential facilitator for the induction and maintenance of both CD8+ T-cells and for B-cell antibody responses (68). Researchers have also noted exhaustion signatures in HIV-infected innate immune cells, rendering them less potent at responding not only to HIV, but also to HBV, which is inherently highly efficient at avoiding recognition by the innate immune system, as reported in several studies (135–138). For example, Wang et al., have identified exhausted CD4+ T-cells and CD8+ T-cells, and then, a closer look at the exhausted CD8+ T-cells has indicated that they present less effector function phenotypes than normal CD8+ T-cells (139). Indeed, Wang et al., have identified key upregulated genes [killer cell lectin-like receptor subfamily G member 1 (KLRG1), cluster differentiation (CD160), and T-cell immunoreceptor with Ig and ITIM domains (TIGIT)] that are associated with T-cell exhaustion. Additionally, Nguyen et al. (140), have demonstrated that HIV-specific CD8+ T-cells from the lymph nodes of HIV chronic progressors preferentially express exhaustion signatures [TIGIT, lymphocyte-activation gene 3 (LAG3), CD244 (recognized as inhibitory receptors), KLRG1, and the transcription factor EOMES (Eomesodermin, also known as T-box brain protein 2, Tbr2)] (141–143). Thus, subsequent to HIV infection, remaining CD4+ T-cells and circulating CD8+ T-cells, should they be exhausted, are potentially less potent at assuming essential protective functions compared to normal CD4 + and CD8+ T-cells. A blockade of PD1 (144), CTL-4 (144), KLRG1 (139), for example, may be potentially helpful in effectively restoring the protective functions of exhausted immune cells, which in turn could promptly respond to HBV invasion.

Antiretroviral Treatment (ART)

Since most HIV-positive individuals are diagnosed during or after the seroconversion phase, most HIV-infected patients often initiate ART during or after this phase of the infection. ART efficiently suppresses HIV-1 replication by targeting key mechanisms in its life cycle (145), which in turn (i) reduces HIV viral RNA load to below detectable levels (146, 147), (ii) increases the circulating number of CD4+ T-cells (148, 149), (iii) reduces the incidence of AIDS-related diseases and/or deaths (148, 150), and (iv) effectively prevents the transmission of HIV to the uninfected population (151). Compared to untreated patients, ART reduces rates of hepatic fibrosis in treated patients by effectively increasing CD4+ T-cell numbers. However, active monitoring for ART-induced liver injury should be considered as it has been reported that some ART therapeutic drugs may be toxic to the liver (152, 153). Moreover, it has also been reported that liver-related death is the leading cause of non-AIDS death in patients whose HIV infection is well-controlled by ART (154). Thus, in ART-treated patients, the risk of liver injury does not originate solely from the prevalent HIV RNA viral load or from CD4+ T-cell depletion, but may also result from toxicity associated with ART drugs. This may also represent a potential additional factor facilitating HBV establishment.

HBV Vaccinated Individuals

In people who have received the HBV immunization, the risk for developing HBV remains, as memory B-cells and long-lived plasma cells, recognized as pivotal for maintenance of serological memory to vaccines and infections, have been shown to be reduced in number during HIV-1 infection (155, 156). Interestingly, their numerical decline correlates with reduction of antibody (Ab) titers against childhood vaccinations (157, 158). It is, therefore, reasonable to speculate on the reduction of HBV-specific antibody titers subsequent to memory B-cell reduction, even if it has been demonstrated that ART initiation shortly after HIV infection may restore memory cell numbers to physiological levels in HIV-1-infected children and adults (159, 160). Moreover, exhausted memory B-cells [activated memory B (AM) and tissue-like memory (TLM) B cells)] are expanded in the circulation during HIV-1 infection (161, 162). From the investigations of Wang et al. (139), and Nguyen et al. (140), it is now known that HIV-related exhausted T-cells become less potent at

accomplishing their full repertoire of immune functions. Although some clarification remains to be elucidated in this specific subject area, we may relatively confidently assume that due to HIV infection, exhausted B-cells do become dysfunctional as well, and are thus, not as immunologically competent as normal B-cells at producing specific antibodies. Chiodi and Scarlatti (163) have proposed that the B-cell dysfunctional profile (inhibition of both B-cell proliferation and antibody production) due to cellular exhaustion caused by HIV infection, could be explained by a specific pathway engaged *via* the expression of inhibitory receptors on the surface of TLM B-cells during HIV-1 infection, which includes the inhibitory receptor Fc receptor-like-4 [FCRL4, which is increased in B-cells during HIV-1 (164) infection, and acts by dampening B-cell receptor (BCR) signaling]. Furthermore, presence of IL-6, known to be increased in B-cells during HIV-1 infection, may lead to aberrant B-cell differentiation (164, 165). In such contexts, the liver is vulnerable to HBV invasion, since the expected specific antibody generation resulting from administration of the HBV vaccine would have been somewhat neutralized *via* B-cell destruction and secondary B-cell functional impairment directly attributable to HIV infection.

ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) PHASE

The global success of ART in treating HIV infection and AIDS has led some to some doubt whether a curative solution to AIDS is necessary. Only patients not on ART or those who are infected with HIV strains resistant to ART can progress to the AIDS phase of HIV infection (166). In general, in untreated people or inadequately treated people, it takes several years to gradually progress from primary HIV infection to the AIDS phase, which is characterized by the onset of symptoms and signs of severe HIV illnesses and profound immunosuppression. The immunological and other issues encountered during the acute and the chronic phases of HIV infection are significantly exacerbated in the AIDS phase. A patient at this stage of the infection may have a substantially high viral load, which may, in addition to a very low CD4+ T-cell count (**Figure 2**), lead to further liver injury, thus favoring HBV infection. The overtly symptomatic stage of HIV illness denotes the late stage of HIV disease (AIDS) in which patients (i) have a CD4+ T-cell count of less than 200 cells/mm^3 and (ii) are vulnerable to additional opportunistic infections (OIs) (167) (such as infections by *Mycobacterium avium* complex, *Mycobacterium tuberculosis*, *Pneumocystis jirovecii*, *Cytomegalovirus*, *Toxoplasma gondii*, and *Candida* species) or the occurrence of aggressive forms of Kaposi's sarcoma or B-cell lymphoma (32). Unfortunately, numerous OIs are known to be associated with liver injury, which is a vital facilitator for HBV invasion of the liver (168–173). The liver is frequently affected by opportunistic infections, most commonly in infections by mycobacteria and *Cytomegalovirus* (174). Compared with non-TB HIV-infected patients, TB-HIV co-infected patients present with more significantly aberrant liver function profiles, with higher serum total bilirubin, alanine transaminase (ALT) and alkaline phosphatase (ALK-P) levels (175). Dey et al., showed that *Mycobacterium tuberculosis* can be an etiological factor for liver abscesses in HIV-infected patients (168). Infection by *Toxoplasma gondii* has also been reported to promote chronic liver disease in HIV-infected individuals (169). Hepatitis C virus infection is also known to act as an opportunistic disease in AIDS patients, directly causing progressive liver damage, which may also result in liver cirrhosis and hepatocellular carcinoma (176, 177).

Moreover, the medications associated with the drug treatment of opportunistic diseases are further contributing factors to persisting liver damage. The current first-line drug treatment for TB is a regimen of four drugs, i.e., isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and pyrazinamide (PZA) (178). However, hepatotoxicity has been frequently observed as a serious adverse reaction following the use of these anti-TB drugs, especially with use of PZA, INH, and RIF, with a 2–28% incidence rate (179–183). Among PLWH, a higher incidence of hepatotoxicity has been seen, and Araújo-Mariz et al., have reported a 30.6% cumulative incidence rate of hepatotoxicity in PLWH following the use of recommended drugs for TB treatment (184). Sulfonamides, including trimethoprim/sulfamethoxazole (TMP/SMZ) and sulfadiazine, are other drugs which have been widely used in AIDS patients, and have been recommended as drugs of first choice for infections by *Pneumocystis jirovecii* and *Toxoplasma gondii* in HIV-infected patients (185). These drugs have also been frequently reported to induce hepatotoxicity (186–189).

Other contributing factors that may occur during the AIDS stage, such as paradoxical and unmasking immune reconstitution inflammatory syndrome (IRIS) and drug-drug interactions, may also result in liver disease or toxicity (190, 191). However, further studies of the baseline liver status of patients (uninfected by HBV) during the AIDS stage and studies of liver enzyme profiles in these patients during the AIDS stage are warranted to further assess other potential influencing factors for HBV establishment in patients with AIDS.

CONCLUSION

It is known that HIV infection induces an immunodeficiency syndrome, rendering the patient vulnerable to infections, including HBV infection. The present review is the first to critically discuss the specific mechanisms leading to HBV establishment in a patient who is already HIV-positive. We report that the acute phase is responsible for a sudden immune system defense subversion, a CD4+ T-cell depletion, and a high viral RNA load, all contributing to increasing the vulnerability of the liver, which subsequently inexorably develops a permissiveness to HBV. During the chronic phase of HIV infection, gut-associated dysbiosis and immune cell exhaustion, compounded by the hepatotoxic phenomena encountered during the acute phase, are two major consequences of HIV infection which are likely to enhance the probability of subsequent HBV invasion of the liver. The other possible facilitatory causes for HBV invasion of the liver in HIV-infected patients that we have discussed herein are the use of

modern ART, and HIV-associated B-cell depletion. Finally, the AIDS phase of HIV infection is often defined by particularly low CD4+ T-cell counts, OIs (and OI-related drug treatments), and extraordinarily high viral RNA loads, all of which, as we have described herein, conspire to inflict further sustained injury to the liver, which also favors HBV establishment.

AUTHOR CONTRIBUTIONS

SZ and JO wrote the first draft of the manuscript. YLC, YJ, and HW provided critical revision of the manuscript. YKC conceived and designed the study. All authors read and approved the manuscript and its submission for publication.

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Clinical Features and T Cell Immune Characteristics of Postpartum Hepatitis Flare in Pregnant Women With HBeAg-Positive Chronic HBV Infection

*Aixin Song†, Yisi Liu†, Zhenhuan Cao, Junfeng Lu, Shan Ren, Sujun Zheng, Lina Ma, Zhongjie Hu, Xiao Lin, Hong Li, Yanhong Zheng and Xinyue Chen**

First Department of Liver Disease Center, Beijing Youan Hospital, Capital Medical University, Beijing, China

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Edited by:
Yan-Mei Jiao,
Fifth Medical Center of the PLA General Hospital, China

Reviewed by:
Yao Xie,
Capital Medical University, China
Xiaoyuan Xu,
Peking University First Hospital, China

***Correspondence:**
Xinyue Chen
chenxydoc@ccmu.edu.cn

†These authors have contributed equally to this work

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Background: The extent of the increase in postpartum alanine transaminase (ALT) varies significantly among pregnant women in the immune tolerance stage of nucleoside analogue (NA) intervention, so this study is an attempt to analyze the clinical features of patients with and without postpartum hepatitis flare and preliminarily explore the differences in their immune functions.

Methods: Pregnant women with a gestational age of 24–28 w and in the immune tolerance stage of NA intervention for hepatitis B virus (HBV) infection were included and divided into a hepatitis group (Group 1) and a nonhepatitis group (Group 2) according to the ALT level at 6–12 w after childbirth. The clinical features were analyzed, and the phenotypes, functions, and cytokines of clusters of differentiation $CD8^+$ T cells in the two groups of patients were detected using flow cytometry before and after childbirth.

Results: A total of 15 patients with postpartum hepatitis flare were enrolled in Group 1, and 10 matched patients were selected as controls for Group 2. Compared with the individuals in Group 2, the postpartum clinical features in Group 1 included a remarkable elevation of the ALT level on the basis of a relatively low HBV DNA level, usually accompanied by a decline in hepatitis B virus surface antigen levels as well as HBeAg levels. In addition, $CD8^+$ T cell activation was enhanced after childbirth in Group 1. In particular, there was a notable difference in the activation of TEMRA subsets, and the frequency of $CD8^+$ T cells expressing perforin and granzyme B increased.

Conclusion: The changes in the immune characteristics of $CD8^+$ T cells may play a certain role in breaking down immune tolerance in patients with postpartum hepatitis flare, and the indexes related to activating and killing functions may help to indicate the population with hepatitis flare after childbirth.

Keywords: HBV, postpartum, alanine transaminase, hepatitis flare, $CD8^+$ T cells

BACKGROUND

Mother-to-child transmission was previously a major transmission route of hepatitis B virus (HBV) infection in China, accounting for about 40% of chronic infections (1, 2). To block mother-to-infant transmission, it is recommended in the Chinese and international guidelines that pregnant women with hepatitis B virus e antigen (HBeAg)-positive chronic HBV (CHB) infection (in the immune tolerance stage) can be treated with nucleoside analogue (NA) intervention combined with conventional immune blocking methods, which can substantially improve the success rate of blocking in the second and third trimesters of pregnancy (2–5). However, new clinical problems are emerging as more patients receive NA intervention in the third trimester of pregnancy; 25–45% patients will suffer postpartum hepatitis flare after drug withdrawal (6–8). The clinical features and the changes in the immune functions of such patients remain unknown at present. Therefore, the clinical features of patients with and without postpartum hepatitis flare were analyzed, and the differences in immune functions were preliminarily explored.

METHODS

Study Population

The women with CHB infection who were pregnant (less than 8 weeks of gestation) and prepared for pregnancy, visited Beijing You'an Hospital, Capital Medical University from July 2017 to July 2018, and agreed on NA intervention in the third trimester of pregnancy to prevent mother-to-child transmission were enrolled. The inclusion criteria were as follows: 1) patients aged 18–40 years old, 2) those with positive hepatitis B virus surface antigens (HBsAgs) for more than 6 months, 3) those with positive HBeAgs, 4) those with an HBV DNA level $\geq 2 \times 10^6$ IU/mL, 5) those with at least two normal test results of alanine transaminase (ALT) within one year before pregnancy, and 6) those without a history of antiviral treatment. The exclusion criteria involved 1) patients with infections of hepatitis A, hepatitis C, human immunodeficiency virus (HIV), or other viruses; 2) those with other chronic liver diseases (autoimmune liver disease, alcoholic liver disease, fatty liver, *etc.*); or 3) those with liver cirrhosis, liver cancer, or obstetrical disease (*e.g.*, intrahepatic cholestasis of pregnancy). All enrolled subjects signed the informed consent, and this study was reviewed by the Ethics Committee of Beijing You An Hospital.

Study Methods

In this retrospective study, the aforementioned patients were followed up at baseline (in the second trimester of pregnancy, without antiviral treatment); before childbirth (at the 35^{th}–38^{th} w of pregnancy); and after childbirth (at 6–12 w after delivery). HBV DNA, HBV serological markers, and hepatic and renal functions were detected at each follow-up. Postpartum hepatitis flare was defined as ALT ≥ 2 ULN (ULN = 40 U/L) during follow-up at 6–12 w after delivery and ALT ≥ 2 ULN in retest within 1 w.

Detection of Clinical Indexes

HBV DNA was examined using a COBAS TaqMan fluorescence quantitative polymerase chain reaction (PCR) system (Roche Diagnostics GmbH, Germany), with a lower limit of detection of 20 IU/mL. HBV serological markers were measured by Elecsys (Roche Diagnostics GmbH, Germany) (lower limit of detection of HBsAg: 0.05 IU/mL and lower limit of detection of HBeAg: 1 COI). Moreover, an OLYMPUS-AU5400 biochemical analyzer was applied to detect the hepatic and renal functions as well as other biochemical indexes.

Collection of Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral venous blood was collected into 5 mL tubes containing anticoagulant EDTA from all subjects before and after childbirth. Then PBMCs (5×10^6) were separated from each sample within 4 h after collection, cryopreserved in the cryopreservation container of a refrigerator at −80°C for 24 h, and transferred into a −130°C refrigerator for long-term storage.

Surface Staining *via* Flow Cytometry

The thawed cells were transferred into 37°C centrifuge tubes containing an RPMI-1640 medium, resuspended with a PBS buffer, and stained by means of such specific surface antibodies as CD3 APC-cy7, CD8 BV510, CD4 FITC, CCR7 Percp, CD45RA PE, CD38 PE-cy7, HLA-DR BV421. Later, the cells were washed and the expressions of markers were determined using a flow cytometer (BD Biosciences, San Jose, CA, USA). Flow cytometry Comp-Beads kits (BD Biosciences, San Jose, CA, USA) were used for compensation.

Intracellular Staining

The PBMCs (1×10^7 cells/mL), CD3 (1 μg/mL), and CD28 (1 μg/mL) were added with GolgiStop and CD107a-FITC at the same time and then incubated at 37°C for 4 h. Next, the cells were collected, washed, and labeled with specific surface antibodies. Subsequently, the cells were collected, washed and fixed, followed by the rupture of membranes and the addition of interleukin-2 (IL-2) BV421, interferon gamma (IFN-γ) PE-Cy7, Perforin AF 647, and PE-GranB antibodies for incubation. Finally, the expressions of markers were measured by the flow cytometer.

Statistical Analysis

The data were expressed as mean ± standard deviation. The data of flow cytometry were analyzed using FlowJo software (Version 10, Tree Star Inc., Ashland, OR, USA), and GraphPad Prism 6.02 (GraphPad software, San Diego, CA, USA) was used for other statistical analyses. The intergroup differences in p values with statistical significance were verified by means of a Mann-Whitney test and a one-way analysis of variance. Spearman's rank correlation test was conducted to assess the statistical correlation between variables. All the tests were two-tailed, and $p < 0.05$ indicated a significant difference.

RESULTS

General Conditions of the Patients

A total of 25 patients with an average age of 28.2 ± 4.3 years old were included in this study, and they started to take tenofovir disoproxil fumarate (TDF) in the second and third trimesters (24–28 w) of pregnancy and stopped the drug at 3 ± 2 w after childbirth. Then the patients were assigned to a hepatitis group (Group 1, n = 15) and nonhepatitis group (Group 2, n = 10), based on the ALT level within 12 w after childbirth. No statistical differences in the age, baseline ALT level, and baseline HBV DNA level were observed when comparing the two groups of patients. The baseline, antepartum, and postpartum clinical features of the patients appear in **Table 1**.

Comparisons of Clinical Features Between the Two Groups

Longitudinal comparisons within groups and horizontal comparisons between groups were adopted for the clinical features of the two groups of patients at baseline, before childbirth, and after childbirth. As for the changes within groups, the ALT level rose markedly after childbirth compared with that at baseline in Group 1 ($p < 0.0001$). It also increased in Group 2 ($p = 0.006$), but the extent was below the standard of a hepatitis flare. The HBV DNA level declined evidently (by up to 3log) before and after childbirth in both groups by contrast with that at baseline ($p < 0.0001$). The HBsAg and HBeAg levels lowered in Group 1 after childbirth by comparison with those at baseline, while the HBeAg level decreased more distinctly, with a statistical difference ($p = 0.004$). However, there were no apparent changes in the HBsAg and HBeAg levels at the three follow-up points in Group 2. Moreover, the ALT and HBsAg levels displayed obvious changes after childbirth in both groups. Specifically, Group 1 had a prominently higher ALT level and a lower HBsAg level than Group 2, showing statistically significant differences ($p < 0.0001$) (**Figure 1**).

Activation of $CD8^+$ T Cells and Their Subsets

The antepartum and postpartum activations of $CD8^+$ T cells and their subsets were compared between the two groups to analyze whether hepatitis flare is associated with immune changes. The gating strategy of $CD8^+$ T cells populations and other markers was shown in **Figure 2**. The results indicated that the frequency of $CD38^+$ HLA-DR^+ $CD8^+$ T cells exhibited an increasing trend after childbirth in Group 1, suggesting enhanced postpartum activation, but such a phenomenon was not observed in Group 2. Besides, there was no statistically significant difference between the two groups ($p = 0.067$, **Figure 3A**). Next, the cell subsets playing key roles in anti-infectious immunity, namely, $CD8^+$ effector memory T cells ($CD45RA^-CCR7^-$, TEM) and effector T cells ($CD45RA^+CCR7^-$, TEMRA), were further analyzed. The activation of TEM and TEMRA subsets was strengthened after childbirth compared with that before childbirth in Group 1, and in particular, there was a statistically significant difference in the enhancement of TEMRA subset activation after childbirth ($p = 0.005$, **Figures 3B, C**). However, Group 2 displayed no such phenomena. To eliminate confounding factors and observe the activation of $CD8^+$ T cells more intuitively, TEM subsets and TEMRA subsets before and after childbirth, along with fold change (postpartum numerical value/antepartum numerical value) were used to evaluate the change degree. According to the results, the fold changes of activation of $CD8^+$ T cells, TEM subsets, and TEMRA subsets after childbirth were markedly higher in Group 1 than in Group 2 ($p = 0.05$, $p = 0.01$, $p < 0.0001$, **Figure 3D**), implying that postpartum hepatitis flare in immune-tolerant HBV patients may be correlated with changes in immune function.

Detection Results of Killing Function and Cytokines of $CD8^+$ T Cells

In light of the significant difference in $CD8^+$ T cell activation between the two groups of patients, whether the killing function and cytokine secretion of $CD8^+$ T cells affected hepatitis flare was

TABLE 1 | Characteristics of all subjects with and without postpartum hepatic flares.

Characteristic	Group 1 Postpartum flares (n=15)	Group 2 No postpartum flares (n=10)	*P*-value
Age (years)	26.8 ± 2.8	30.0 ± 5.4	0.182
ALT (U/L)			
Baseline	26.0 ± 9.6	20.8 ± 7.7	0.510
Gestational 35-38W	20.9 ± 8.2	18.6 ± 8.0	0.376
Postpartum 6–12W	**113.9 ± 40.3**	**32.0 ± 9.8**	**<0.0001**
HBV DNA (log_{10} IU/ml)			
Baseline	7.9 ± 0.7	7.9 ± 0.6	0.713
Gestational 35-38W	4.8 ± 0.8	4.6 ± 0.5	0.999
Postpartum 6–12W	4.7 ± 1.7	5.1 ± 2.3	0.403
HBsAg (log_{10} IU/ml)			
Baseline	4.3 ± 0.5	4.5 ± 0.5	0.249
Gestational 35-38W	4.2 ± 0.4	4.4 ± 0.5	0.077
Postpartum 6–12W	**4.1 ± 0.5**	**4.4 ± 0.5**	**0.022**
HBeAg (log_{10} COI)			
Baseline	3.3 ± 0.1	3.2 ± 0.1	0.113
Gestational 35-38W	3.3 ± 0.1	3.2 ± 0.1	0.673
Postpartum 6–12W	3.0 ± 0.4	3.2 ± 0.2	0.099

The bold values means the difference is statistically significant.

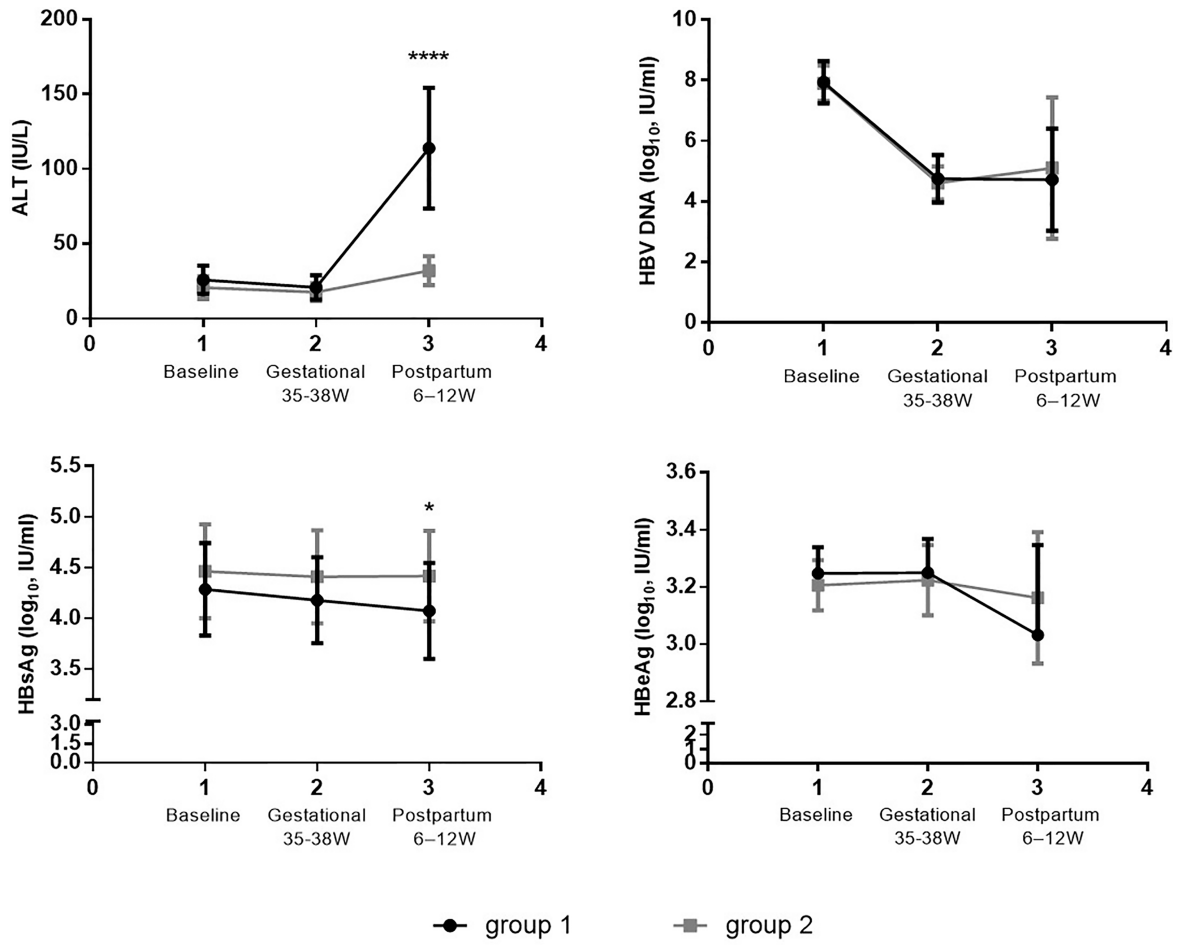

FIGURE 1 | clinical features between the two groups. Compared the difference of the levels of ALT, HBV DNA, HBsAg and HBeAg at each follow-up point between the group 1 (n=15) and group 2 (n=10). *p < 0.05, ****p < 0.0001.

further analyzed. The antepartum and postpartum expressions of CD107a, perforin, and granzyme B in the two groups of patients were measured using the flow cytometer. It was revealed that the expression of granzyme B in $CD8^+$ T cells increased notably after childbirth in Group 1, and the difference was statistically significant (p = 0.008). In addition, the perforin expression increased, but it showed no statistically significant difference (p = 0.074). Moreover, the CD107a expression was unchanged (**Figure 4**). By contrast, the increases in CD107a, perforin, and granzyme B expressions were not observed after childbirth in Group 2. Furthermore, the antepartum and postpartum IFN-γ and IL-2 levels in the two groups were determined, and it was discovered that there were no apparent changes in the levels of the two cytokines between the two groups (**Figure 5**).

DISCUSSION

In recent years, NA intervention, combined with conventional immune blocking methods, has been used for more and more immune-tolerant pregnant women in the second and third trimesters to improve the success rate of blocking, which has substantially increased the success rate of blocking in pregnant women with high HBV load and neonates (2, 4, 5). Nevertheless, multiple researchers have reported that 25–45% patients have ALT elevation and hepatitis flare after childbirth (6, 8), and it was noted in the research that postpartum hepatitis flare is good timing for antiviral treatment (9). The reason the extent of postpartum ALT elevation differs greatly among patients with HBV immune tolerance undergoing NA intervention, the changes in related immunological indexes, and whether these changes can predict hepatitis flare are rarely reported.

In terms of the features of postpartum hepatitis flare, unlike those of an ordinary CHB infection, ALT elevation may occur at a relatively low DNA level before or immediately after drug withdrawal, whereas hepatitis flare emerges in CHB generally at a high HBV DNA level (6, 10, 11). This study showed that obvious HBV DNA decline and ALT elevation were detected in Groups 1 and 2 after childbirth, with statistical differences with those

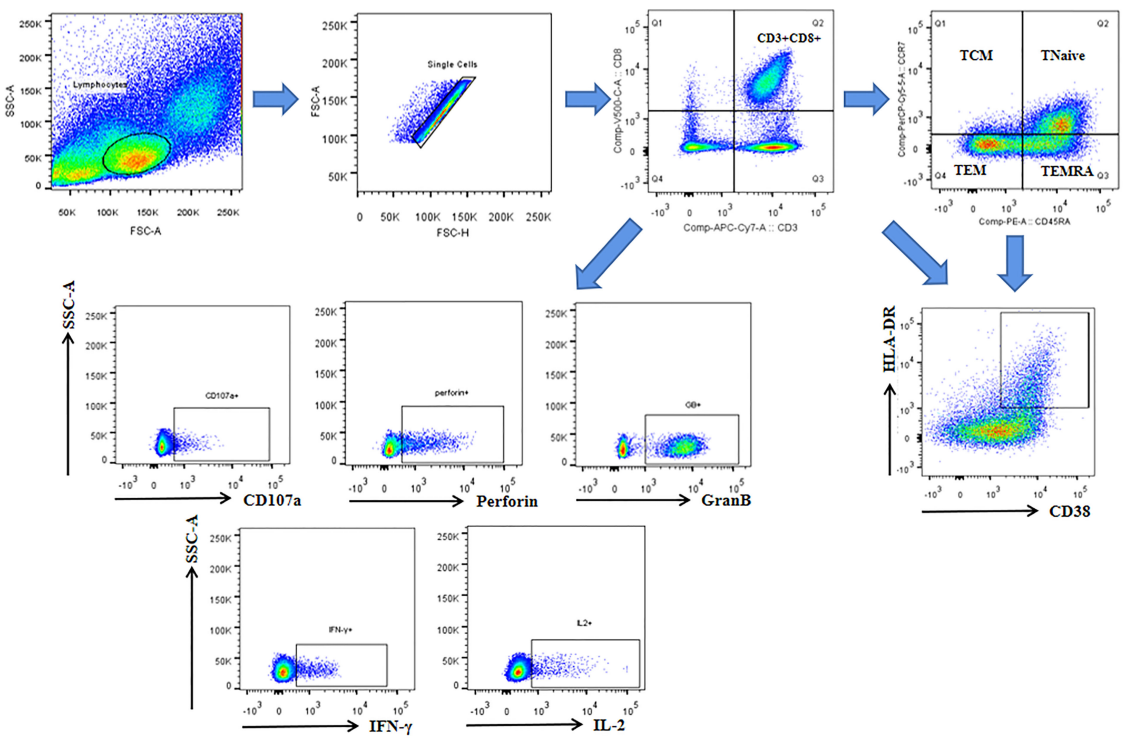

FIGURE 2 | The gating strategy of $CD8^+$ T cells populations and other markers. Lymphocytes and single cells were gated first. Then $CD3^+CD8^+$cells were gated. Central memory (TCM: $CD45RA^-CCR7^+$), Tnaive ($CD45RA^+CCR7^+$), effector memory (TEM: $CD45RA^-CCR7^-$), and terminally differentiated effector (TEMRA: $CD45RA^+CCR7^-$) subsets were gated based on gated $CD8^+$ cells. CD38 and HLA-DR were gated based on gated $CD8^+$ cells and each subsets. CD107a, perforin, GranB, IFN-γ and IL-2 were gated based on gated $CD8^+$ cells.

individuals at baseline, but the ALT elevation in Group 2 did not reach the standard of hepatitis flare. Besides, the HBeAg level in Group 1 was also reduced clearly after childbirth in contrast with the level at baseline, and the difference was statistically significant (p = 0.004), but this phenomenon was not noted in Group 2. Additionally, Group 1 exhibited an overtly higher overall ALT level and an obviously lower HBsAg level after childbirth than Group 2, showing statistically significant differences. However, these changes were representations rather than causes of postpartum hepatitis flare; the specific immunological changes remain unclear and need to be more deeply explored.

Regarding the pathogenesis of CHB, it is generally argued that $CD8^+$ T cell immune responses exert crucial effects on HBV clearance, but the dysfunction or failure of T cells during long-term viral infection can lead to immune tolerance (12). TEM and TEMRA are important $CD8^+$ T cell subsets, and the former is probably a crucial type of effector cell for early elimination after viral infection. It was reported that viruses are gradually removed as the TEM level becomes more elevated in the case of acute hepatitis B, influenza, and other adult viral diseases (13). This phenomenon suggests that the number and function of TEM subsets are critical players in eliminating viruses and preventing chronicity of disease. According to the study of Hess et al. on HIV infection, the fully differentiated TEMRA subsets can express more perforin than TEM subsets, so they can control viral infection more effectively (14). Ma et al. detected $CD8^+$ T cell subsets in different HBV infection states (spontaneous clearance of HBsAg and HBeAg, CHB, *etc.*), finding that the obvious increase in TEMRA subsets could serve as an important mechanism for HBV clearance (including the disappearance of HBV DNA and HBV-related antigens) (15). Similar phenomena were observed in the present study as well. The activation of three cell sets ($CD8^+$ T cells, TEMRA subsets, and TEM subsets) was more prominently enhanced after childbirth in Group 1 than in Group 2. In particular, there was a significant difference among the TEMRA subsets (p = 0.005). Additionally, the fold changes of activation of the three cell sets after childbirth were remarkably higher in Group 1 than in Group 2; TEMRA subsets displayed the greatest difference, implying that TEMRA subsets may play vital roles in breaking down immune tolerance and inducing hepatitis flare.

$CD8^+$ T cells can exert cytotoxic effects by virtue of the cell-killing effect mediated by perforin and granzyme and the non-cell-killing effect mediated by secreted cytokines such as IFN-γ, TNF-α, and IL-2 (16). It was also observed in this study that the frequency of $CD8^+$ T cells secreting perforin and

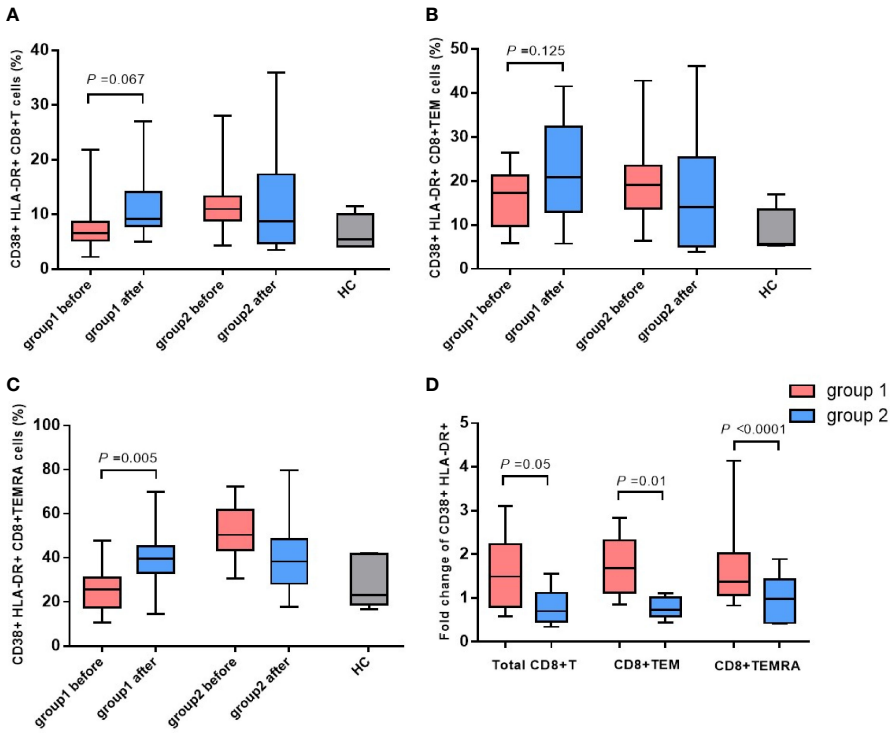

FIGURE 3 | The changes in CD38 and HLA-DR expressed CD8$^+$ T cells and phenotypic subsets. Expression of CD38 and HLA-DR on CD8$^+$ T cells **(A)**, TEM subsets **(B)** and TEMRA subsets **(C)** before and after childbirth between the group 1 and group 2. Fold change of activation of CD8$^+$ T cells, TEM subsets, and TEMRA subsets after childbirth between the group 1 and group 2 **(D)**.

granzyme B clearly increased more after childbirth in Group 1 than in Group 2, and there was a statistical difference (p = 0.008), similar to the findings of Hess et al. (HIV) and Duan et al. Duan et al. discovered that a larger number of CD8$^+$ T cells secreted perforin and granzyme B in CHB patients who had negative HBeAg after TDF treatment (17). There were no obvious changes in cytokines IFN-γ and IL-2 after childbirth in either group. In a study on similar populations to those in this study, the postpartum IL-2 level had no variation, but IFN-γ expression was higher in patients with hepatitis flare than in patients without hepatitis flare (18). The different results between the two studies could be attributed to the differences in time of drug (NA) withdrawal and postpartum follow-up points of enrolled patients. The test results could have been influenced by the varying time points of blood collection, given that corticosteroids withdraw rapidly after childbirth and could be associated with the small sample size in the present study. The relatively small sample size of this study may limit the power to obtain the desired effect, we will expand the sample size and conduct more comprehensive experimental design in future research, such as analysis of CD4$^+$ T cell immunity.

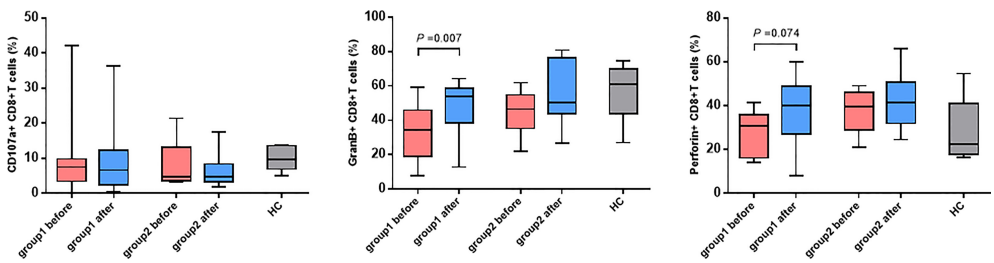

FIGURE 4 | The changes in CD107a, GranB and perforin expressed CD8$^+$ T cells. Expression of CD107a, perforin and GranB on CD8$^+$ T cells before and after childbirth between the group 1 and group 2.

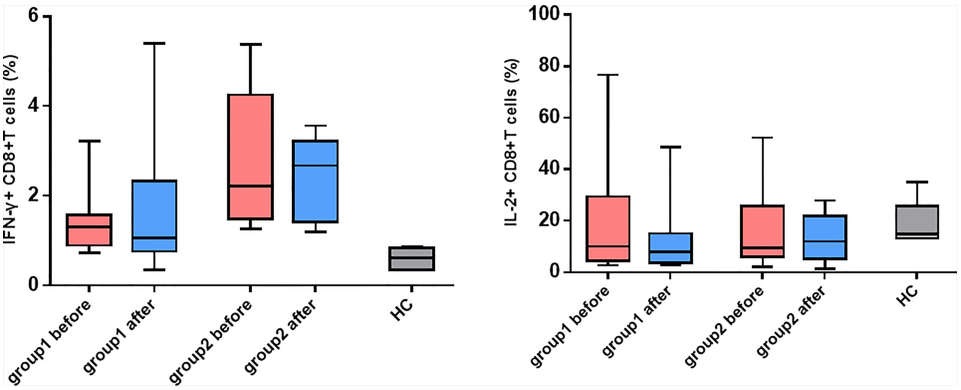

FIGURE 5 | |The changes in IFN-γ and IL-2 expressed $CD8^+$ T cells. Expression of IFN-γ and IL-2 on $CD8^+$ T cells before and after childbirth between the group 1 and group 2.

In conclusion, the clinical features of postpartum hepatitis flare in patients subjected to NA intervention include a remarkable elevation of the ALT level on the basis of a relatively low HBV DNA level, usually accompanied by a prominent decline in HBsAg and HBeAg levels. Patients with hepatitis flare manifest evident activation of $CD8^+$ T cells, and increased frequency of TEMRA subsets and expression levels of perforin and granzyme B may perform crucial functions in breaking down immune tolerance and causing hepatitis flare.

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 Beijing Youan Hospital Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS, YL, and XC conceived and designed the experiments and study. ZC, JL, and SR collected the sample information, contributed to reagents and materials. YL, YZ, XL, and HL performed the experiments and analyzed the data. AS performed the analysis and wrote the manuscript with assistance from SZ, ZH, LM, and XC. All authors read and approved the final manuscript.

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Low Level of Serum Immunoglobulin G Is Beneficial to Clinical Cure Obtained With Pegylated Interferon Therapy in Inactive Surface Antigen Carriers

Hong Li[1†], Xiao Lin[2†], Lili Liu[1], Ling Qin[3], Yanhong Zheng[2], Xiaohui Liu[1], Xinhuan Wei[1], Shan Liang[1], Yali Liu[1], Jing Zhang[1], Xinyue Chen[2] and Zhenhuan Cao[1]*

[1] The Third Unit, Department of Hepatology, Beijing Youan Hospital, Capital Medical University, Beijing, China, [2] The First Unit, Department of Hepatology, Beijing Youan Hospital, Capital Medical University, Beijing, China, [3] Biomedical Information Center, Beijing Youan Hospital, Capital Medical University, Beijing, China

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Edited by:
Yan-Mei Jiao,
Fifth Medical Center of the PLA General Hospital, China

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Anke R. M. Kraft,
Hannover Medical School, Germany
Peng Hu,
Chongqing Medical University, China

***Correspondence:**
Zhenhuan Cao
caozhenhuanyu@mail.ccmu.edu.cn

[†]These authors have contributed equally to this work

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Purpose: Our recent study showed a high rate of HBsAg clearance in inactive HBsAg carriers (IHCs) treated with pegylated IFN (PEG-IFN). To better understand the immune-mediated component of HBsAg clearance, this study investigated the role of serum immunoglobulin G (IgG) and its subclasses in predicting HBsAg clearance in IHCs with PEG-IFN therapy.

Methods: In this study, IHCs received PEG-IFN for 96 weeks. Subjects who achieved clearance of HBsAg were considered responders (R group), and those in whom HBsAg was not cleared were considered non-responders (NR group). The HBsAg, ALT, and serum IgG subtypes (IgG1, IgG2, IgG3, IgG4) were tested at baseline, and at 12 and 24 weeks of treatment. To evaluate the factors in predicting HBsAg clearance, univariate and multivariate logistic regression analyses were performed. The receiver operator characteristic curves and the area under the receiver operator characteristic curve (AUROC) were used to evaluate prognostic values.

Results: Our results showed that 39 cases obtained HBsAg clearance (group R), while 21 cases did not (group NR). There was no significant difference in age, ALT, and AST levels between the two groups. The serum levels of IgG1, IgG2, IgG3 and IgG4 at baseline, and at 12 and 24 weeks were significantly lower in IHC with HBsAg clearance than in the NR group. Univariate logistic regression analysis showed that serum IgG1, IgG2, IgG3, and IgG4 levels at baseline, and at 12, and 24 weeks were all strong predictors of HBsAg clearance. In all indicators, IgG2 had the highest AUROC at baseline and IgG3 the highest AUROC at week 12. A multifactor logistic analysis was performed with y=33.933-0.001*BaselineIgG1-0.002*BaselineIgG2. The area under the curve was 0.941 with 100% sensitivity and 76.19% specificity.

Conclusion: Together, our findings suggest that serum IgG has a higher predictive value compared to the convention predictors of HBsAg and ALT for HBsAg clearance and thus may be a better clinical predictor of HBsAg clearance in IHCs.

Keywords: inactive HBsAg carriers, HBsAg clearance, pegylated interferon, IgG, predictor

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major public health problem in China with approximately 70 million cases (1). Hepatitis B surface antigen (HBsAg) clearance, although a desirable therapeutic goal, is extremely difficult to obtain clinically. Research into the mechanism of HBsAg clearance is severely hampered by the lack of specimens from HBsAg-cleared patients. According to recent reports, Inactive surface antigen carriers (IHCs) are a large population that accounts for approximately 36% of patients with HBV infection (2) and amount to 30 million individuals in total (3). IHCs are defined by normal alanine aminotransferase (ALT), HBV DNA ≤2000 IU/mL, and hepatitis B e antigen (HBeAg)-negative status. Our and other recent studies have shown that IHC treated with pegylated-interferon (PEG-IFN) results in high HBsAg clearance, with HBsAg clearance rates of 44.7% to 65% (4–6). In addition to cellular immunity, humoral immunity also plays an important role in HBV infection and elimination. Immunoglobulin G (lgG) is the main component of serum immunoglobulins and is classified into four isoforms, lgG1, lgG2, lgG3, and lgG4, depending on the amino acid composition and structure of the hinge region (7). Serum lgG and subclasses play a central role in the humoral immune response. IgG1 is the most abundant lgG subclass in the human serum, followed by lgG2, lgG3, and lgG4. IgG1 mediates the immune response to pathogens, binding to soluble and membrane protein antigens through its variable region, while activating the effector mechanisms of the innate immune system, binding to C1q to cause complement-dependent cytotoxicity and binding to each of the different Fc receptors to cause antibody-dependent cell-mediated cytotoxicity (8). lgG3 appears early in the infection and may limit the excessive inflammatory response (9). lgG4 usually appears in a non-infected setting or after prolonged and repeated exposure to antigens and is associated with lgG4-related diseases, involving multiple organs and tissues in chronic progressive autoimmune disorders (10), which were patients excluded from this study. Recent studies have reported that lgG may be involved in the onset and development of HBV infection, that the severity of HBV pathogenesis is related to immune function of the body, and that serum IgG levels are significantly higher in patients with chronic liver failure and severe chronic hepatitis B than in patients with mild to moderate disease and are positively correlated with the severity of the disease (11, 12).

However, the role of IgG in antiviral therapy, particularly in HBsAg clearance, has not been reported. IHCs were recruited in this study and were treated with PEG-IFN for 96 weeks. We detected the levels of serum lgG and its subtypes during treatment and investigated their value in predicting the clearance of HBsAg.

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; IHCs, Inactive surface antigen carriers; ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; PEG-IFN, pegylated-interferon; lgG, immunoglobulin G; SD, standard deviation; ROC, Receiver operator characteristic; AUROC, area under the ROC curve.

MATERIALS AND METHODS

Patients

A total of 60 IHCs were recruited for the study and received regular follow-up visits at Beijing Youan Hospital, Capital Medical University. Five healthy students were recruited as healthy controls.

All IHCs met the criteria defined in the prevention and treatment guidelines for chronic hepatitis B (2019 edition) (1) (i) HBsAg positive > 6 months and HBsAg <1000 IU/mL, HBeAg negative, anti-HBe positive/positive; (ii) HBV DNA <2000 IU/mL, ALT normal (male<50 IU/L, female<40 IU/L); (iii) white blood cell count $>4\times10^9$/L, platelet count $>150\times10^9$/L; (iv) total bilirubin <34 μmol/L, albumin >40 g/L. Exclusion criteria: (i) history of autoimmune diseases; (ii) Human immuno-deficiency virus or Hepatitis C Virus or Hepatitis E Virus coinfection; (iii) Pregnant, lactating women and those who are preparing to have children soon; (iv) liver cirrhosis or liver cancer; (v) history of severe heart disease, including unstable or uncontrolled heart disease within 6 months; (vi) history of mental illness or psychiatric disorders. (vii) Uncontrolled epilepsy. (viii) Unabated alcohol or drug abuser. (ix) Uncontrolled diabetes mellitus, hypertension, thyroid disease, retinopathy; (x) contraindication to interferon. All healthy controls were HBsAg-negative and anti-HBs -positive.

Treatment and Efficacy

The 60 patients enrolled in the study were treated with PEG-IFN 135 μg weekly by subcutaneous injection. Treatment was stopped if neutrophil counts were $<0.50\times10^9$ or platelet count was $<25\times10^9$, or a serious adverse events occurred. The total duration of treatment was 96 weeks. The effect of the treatment was determined by HBsAg clearance. Subjects who achieved HBsAg clearance in 96 weeks were considered responders (group R), and those in whom HBsAg was not cleared were considered non-responders (group NR).

Ethics Approval

The protocol and the consent form for the study were approved by the research ethics committee of the Beijing You'an Hospital, Capital Medical University, China ([2017]24).

Laboratory Tests

Blood samples were collected at baseline, and after 12, and 24 weeks of treatment and were tested for HBV DNA, HBsAg levels, liver function, and routine blood tests. Serum levels of the lgG subtypes (lgG1, IgG2, IgG3, lgG4) were also measured simultaneously. HBV DNA was quantified using the fluorescence quantitative (FQ)-PCR, Cobas Taqman real-time polymerase chain reaction 2.0 system (Roche, Germany), with a lower limit of detection of 20 IU/mL. HBsAg quantification was performed using the HBsAg quantification kit from Roche, having a lower limit of detection of 0.05 IU/mL. Liver function testing was performed by reagents from Shanghai Kehua Dongling Company (China). lgG subtypes were detected by enzyme-linked immunosorbent assay kits from Jianglai Biologicals (China). In order to measure the concentration of IgG subtypes in the sample, this IgG ELISA Kit includes a set of calibration standards. The calibration standards are assayed at the same time as the samples and allow the operator to produce a standard curve of Optical Density versus IgG subtypes concentration. The concentration of IgG subtypes in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Statistical Analysis

Data analysis was performed using SPSS 25 software (IBM SPSS, Chicago, IL, USA), and values were expressed as mean ± standard deviation (SD) and median (25th, 75th), The Mann–Whitney U test or Student's t-test was applied for quantitative variables, and the chi-square or Fisher's exact test was used for categorical variables. Receiver operator characteristic (ROC) curves, which plot sensitivity by 1 – specificity, and the area under the ROC curve (AUROC) were used to evaluate the prognostic values of the quantitative HBsAg, ALT, IgG1, IgG2, IgG3, IgG4 at baseline, and at weeks 12 and 24 as well as the HBsAg change form baseline at weeks 12 and 24 to predict HBsAg clearance. Univariate and multivariate logistic regression analyzes were performed to evaluate the magnitude and significance of the association. A two-sided P-value <0.05 was considered statistically significant.

RESULTS

Levels of Serum IgG1, IgG2, IgG3, and IgG4 in IHCs and Healthy Subjects

Sixty IHCs (43 males and 17 females with a mean age of 38.95 ± 11.23 years) and five healthy controls with a mean age of 27.60 ± 1.67 years were enrolled. lgG1, lgG2, lgG3, and lgG4 levels were 13328.17 μg/mL, 6186.79 μg/mL, 2024.53 μg/mL, and 660.76 μg/mL in IHCs and 9144.00 μg/mL, 4342.61 μg/mL, 1772.73 μg/mL, 395.17 μg/mL, respectively in healthy controls. The levels of lgG1, lgG2, lgG3, and lgG4 were significantly higher in IHC patients than in healthy controls. ($p<0.001$, $p=0.003$, $p=0.079$, $p<0.001$, respectively) (**Figure 1**).

The Level of IgG Subtype at Baseline and During Treatment in R and NR Group

A total of 60 patients enrolled in the study were treated with PEG-IFN for 96 weeks. Of these, 39 cases achieved HBsAg clearance (group R) and 21 cases did not (group NR). There was no statistical difference in age, or in ALT and AST values between the two groups. HBsAg quantification at baseline, at 12 and 24 weeks were all lower in the R group than in the NR group (P=0.067, P=0.001, P<0.001). In addition, the levels of serum immunoglobulin IgG1, lgG2, lgG3, and lgG4 at baseline, 12 and 24 weeks were all significantly lower in patients with HBsAg clearance than in the NR group (**Table 1** and **Figure 1**)

HBsAg Changes in IHC Patients After PEG-IFN Treatment

The baseline HBsAg quantification in group R was not significantly different from group NR. After PEG-IFN treatment, HBsAg showed a significant downward trend, with HBsAg quantification at 12 and 24 weeks significantly lower than baseline, especially in patients in group R. HBsAg quantification at 12 and 24 weeks was significantly lower in group R than in group NR (P=0.001, P<0.001) (**Figure 2**).

The Value of Serum IgG on HBsAg Clearance

To evaluate factors at identifiable at baseline or in early treatment able to predict HBsAg clearance, univariate logistic regression analysis was conducted. The variables included in the analysis were HBsAg, ALT, IgG1, IgG2, IgG3, and IgG4 at baseline, 12, and 24 weeks as well as HBsAg change form baseline at weeks 12 and 24. The results showed that the serum levels of IgG1, IgG2, IgG3, and IgG4 at baseline, 12, and 24 weeks were all strong predictors of HBsAg clearance as well as HBsAg levels at 12 and 24 weeks, HBsAg changes from baseline and at 12 and 24 weeks. Sex, age, baseline HBsAg and ALT levels were not statistically significant (**Table 2**).

Value of Baseline Variables for Predicting HBsAg Clearance

In the univariate analysis, the P-values for baseline HBsAg, lgG1, lgG2, lgG3, and lgG4 were all less than 0.1; the respective ROC curves for predicting HBsAg clearance are shown in **Figure 4**. The results suggest that baseline lgG2 values had the largest area under the curve (AUROC 0.880), followed by the baseline levels of lgG1 (AUROC 0.824), lgG3 (AUROC 0.771), HBsAg (AUROC 0.646), and lgG4 (AUROC 0.596). The areas under the ROC curves were further compared and the

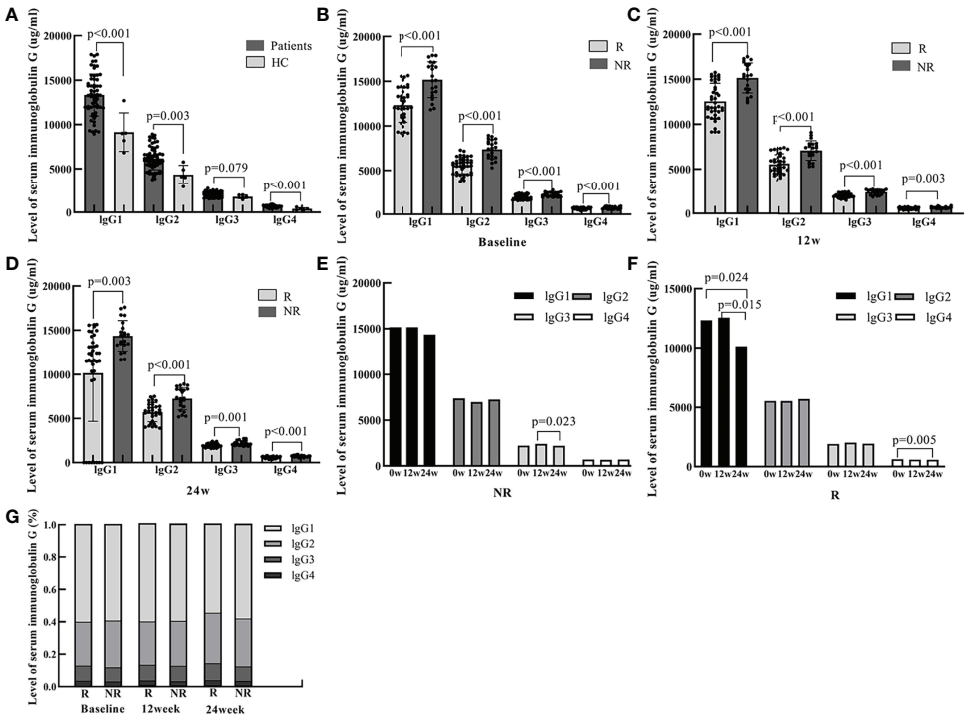

FIGURE 1 | The serum levels of IgG1, IgG2, IgG3, and IgG4 at baseline and 12 and 24 weeks of PEG-IFN treatment in IHCs and healthy controls. **(A)** The level of IgG1, IgG2, IgG3, IgG4 were significantly higher in IHCs than in healthy controls (p<0.001, p=0.003, p=0.079, p<0.001, respectively). **(B)** The levels of serum IgG1, IgG2, IgG3, and IgG4 were significantly lower in the R group than in the NR group at baseline (p<0.001, p<0.001, p<0.001, p<0.001, respectively). **(C)** The levels of serum IgG1, IgG2, IgG3, and IgG4 were significantly lower in the R group than in the NR group at week 12 of PEG-IFN treatment (p<0.001, p<0.001, p<0.001, and p=0.003, respectively). **(D)** The serum levels of IgG1, IgG2, IgG3, and IgG4 were significantly lower in the R group than in the NR group at week 24 of PEG-IFN treatment (p=0.003, p<0.001, p=0.001, and p<0.001, respectively). **(E)** The change in the serum levels of IgG1, IgG2 and IgG4 were not significant in the NR group. The serum levels of IgG3 was lower at week 24 of PEG-IFN treatment than at 12 weeks (p=0.023). **(F)** The serum levels of IgG1 were lower at week 24 of PEG-IFN treatment than at baseline and at 12 weeks (p=0.024 and p=0.015, respectively). **(G)** The proportion of IgG1, IgG2, IgG3 and IgG4 were not significant in the R and NR group.

predictive value of baseline IgG2 was significantly higher than baseline HBsAg (**Figure 3** and **Table 3**).

Value of Variables at 12 Weeks of PEG-IFN Treatment for Predicting HBsAg Clearance

Changes in week 12 IgG1, lgG2, lgG3, lgG4, HBsAg, and HBsAg levels from baseline were all strong predictors of HBsAg clearance. The largest AUC was the week 12 lgG3 (AUROC 0.867), followed by week 12 IgG1 (AUROC 0.852), week 12 lgG2 (AUROC 0.827), which were all better than week 12 HBsAg (AUROC 0.733) and HBsAg change form baseline (AUROC 0.711) (**Figure 4** and **Table 3**).

Value of Variables at 24 Weeks of PEG-IFN Treatment for Predicting HBsAg Clearance

The AUROC for the week 24 HBsAg levels (AUROC 0.891) was greatest in IHC patients treated with PEG-IFN interferon, followed by the HBsAg change from baseline (AUROC 0.813), and week 24 lgG4 (AUROC 0.812), week 24 lgG2 (AUROC 0.804), week 24 lgG1 (AUROC 0.784), and week 24 lgG3 (AUROC 0.780) levels (**Figure 5**). Further comparison of the AUROC indicated that even though the curves for HBsAg and HBsAg showed the changes from baseline were higher than the IgG1, IgG2, IgG3, and IgG4, but were not statistically different (**Table 3**).

Multivariate Logistic Regression Analysis for Predicting HBsAg Clearance

According to the univariate regression analysis, strong predictors included: baseline lgG1, lgG2, lgG3, and lgG4 levels; week 12 IgG1, lgG2, lgG3, and lgG4 levels; and week 24 IgG1, lgG2, lgG3, and lgG4 levels; HBsAg at 12 and 24 weeks, HBsAg changes from baseline at 12 and 24 weeks. A multi-factor logistic analysis was performed with y=33.933-0.001*Baseline lgG1-0.002*Baseline lgG2. The AUROC was up to 0.941 with 100% sensitivity and 76.19% specificity. The optimal cutoff points of Baseline lgG1 was <15100ug/mL and Baseline lgG2 was <6750.67ug/mL respectively. (**Figure 6**).

TABLE 1 | Characteristics of R and NR groups at baseline, and after 12 and 24 weeks of treatment.

Parameter	R group n=39	NR group n=21	*P*-value
Sex (M/F)	27/12	16/5	0.568
Age (years)			0.368
Mean ± SD	38.05 ± 11.25	40.62 ± 11.26	
Median (25th, 75th)	37 (30, 46)	38 (31, 53)	
Baseline			
ALT (U/L)			0.570
Mean ± SD	30.88 ± 14.62	33.17 ± 14.36	
Median (25th, 75th)	27.00 (17.7, 47.00)	37.00 (17.75, 47.25)	
AST (U/L)			0.946
Mean ± SD	27.97 ± 6.89	28.17 ± 8.32	
Median (25th, 75th)	26.50 (24.00, 35.00)	28.00 (20.75, 36.25)	
lg HBsAg (IU/mL)			0.067
Mean ± SD	1.61 ± 0.97	2.08 ± 0.73	
Median (25th, 75th)	1.70 (1.00, 2.38)	2.27 (1.60, 2.72)	
lgG1 (μg/mL)			<0.001
Mean ± SD	12338.97 ± 1930.58	15165.23 ± 1995.97	
Median (25th, 75th)	12180.00 (10970.00, 13830.00)	15100 (13565, 16915)	
lgG2 (μg/mL)			<0.001
Mean ± SD	5550.00 ± 924.60	7369.41 ± 1081.19	
Median (25th, 75th)	5640.00 (4593.96,6219.33)	7503.63 (6452.96, 8349.91)	
lgG3 (μg/mL)			<0.001
Mean ± SD	1915.80 ± 282.70	2226.45 ± 248.62	
Median (25th, 75th)	1866.95 (1655.60, 2225.73)	2190.93 (1972.52, 2430.58)	
lgG4 (μg/mL)			<0.001
Mean ± SD	635.69 ± 105.68	707.32 ± 138.61	
Median (25th, 75th)	653.56 (552.65, 717.16)	666.74 (573.92, 846.55)	
12 week			
ALT (U/L)			0.681
Mean ± SD	82.56 ± 89.52	66.05 ± 49.14	
Median (25th, 75th)	47.00 (34.00, 96.00)	46.00 (34.50, 81.00)	
AST (U/L)			0.545
Mean ± SD	64.89 ± 74.50	47.71 ± 28.05	
Median (25th, 75th)	45.00 (32.00, 60.00)	42.00 (27.50, 56.00)	
lg HBsAg (IU/mL)			0.001
Mean ± SD	0.67 ± 1.21	1.70 ± 0.98	
Median (25th, 75th)	1.10 (-0.47, 1.68)	1.70 (1.34, 2.40)	
lgG1 (μg/mL)			<0.001
Mean ± SD	12,559.74 ± 2002.66	15,156.19 ± 1657.72	
Median (25th, 75th)	11,980.00 (11,030.00, 14,740.00)	15,390.00 (13,725.00, 16,665.00)	
lgG2 (μg/mL)			<0.001
Mean ± SD	5537.06 ± 1083.08	6989.41 ± 1103.60	
Median (25th, 75th)	5380.72 (4573.19, 6395.55)	7284.68 (6084.49, 7731.92)	
lgG3 (μg/mL)			<0.001
Mean ± SD	2027.03 ± 248.77	2417.47 ± 282.86	
Median (25th, 75th)	2001.42 (1824.82, 2225.53)	2489.63 (2141.71, 2651.88)	
lgG4 (μg/mL)			0.003
Mean ± SD	589.99 ± 107.16	679.47 ± 100.57	
Median (25th, 75th)	589.97 (492.85, 675.43)	654.71 (586.96, 743.95)	
24 week			
ALT (U/L)			0.728
Mean ± SD	54.77 ± 50.70	54.45 ± 48.95	
Median (25th, 75th)	37.00 (26.00, 69.00)	37.50 (28.50, 61.50)	
AST (U/L)			0.636
Mean ± SD	46.39 ± 28.24	46.65 ± 45.28	
Median (25th, 75th)	36.00 (28.00, 55.00)	33.50 (31.00, 38.75)	
lg HBsAg (IU/mL)			<0.001
Mean ± SD	-0.29 ± 1.14	1.22 ± 1.27	
Median (25th, 75th)	-0.03 (-0.92, 0.45)	1.50 (0.62,2.35)	
lgG1 (μg/mL)			0.003
Mean ± SD	10146.92 ± 5464.79	14351.90 ± 1744.12	
Median (25th, 75th)	12,190.00 (9430.00, 13,580.00)	14,420.00 (13,325.00, 15,525.00)	
lgG2 (μg/mL)			<0.001

(Continued)

TABLE 1 | Continued

Parameter	R group n=39	NR group n=21	*P*-value
Mean ± SD	5714.71 ± 1079.45	7252.18 ± 12.61.89	
Median (25th, 75th)	5810.81 (4684.04, 6566.44)	7289.20 (6111.19, 8326.01)	
IgG3 (μg/mL)			0.001
Mean ± SD	1943.30 ± 222.42	2202.62 ± 287.04	
Median (25th, 75th)	1903.45 (1771.35, 2109.15)	2089.65 (1940.83, 2485.29)	
IgG4 (μg/mL)			<0.001
Mean ± SD	579.33 ± 110.13	716.37 ± 101.61	
Median (25th, 75th)	567.05 (522.30, 665.69)	713.72 (648.41, 793.03)	

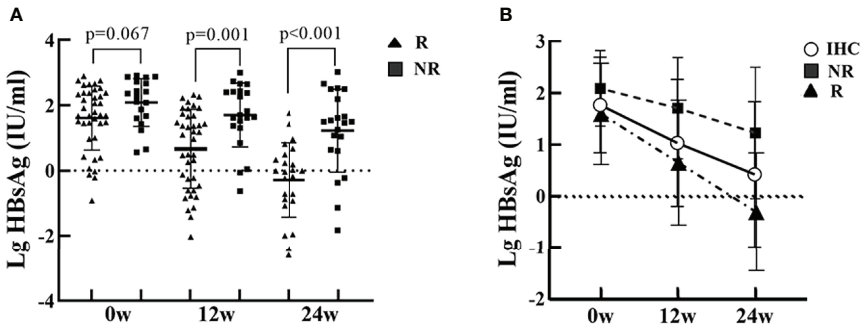

FIGURE 2 | HBsAg at baseline, and at 12 and 24 weeks of PEG-IFN treatment in the R and NR groups. **(A)** The level of HBsAg in the R group was lower than in the NR group, but were not significantly different (P=0.067). The levels of HBsAg in the R group was significantly lower than in the NR group (P=0.001, P<0.001) at 12 and 24 weeks of treatment. **(B)** HBsAg showed a significant downward trend after PEG-IFN treatment in IHCs, with HBsAg quantification at 12 and 24 weeks significantly lower than baseline, especially in the R group.

TABLE 2 | Variables at baseline and early treatment associated with HBsAg clearance (univariable analysis).

Variable	OR	95% CI	*P*-value
Sex	0.32	(0.423, 4.783)	0.569
Age	0.72	(0.934, 1.027)	0.397
Baseline HBsAg	3.17	(0.247, 1.070)	0.075
Baseline ALT	0.82	(0.987, 1.005)	0.366
Baseline IgG1	13.80	(0.999, 1.000)	<0.001
Baseline IgG2	13.98	(0.997, 0999)	<0.001
Baseline IgG3	11.37	(0.994, 0.998)	0.001
Baseline IgG4	4.44	(0.990, 1.000)	0.035
Week 12 HBsAg	8.15	(0.215, 0.752)	0.004
Week 12 HBsAg change from baseline	5.47	(1.237, 11.234)	0.019
Week 12 ALT	0.59	(0.995, 1.012)	0.442
Week 12 IgG1	13.61	(0.999, 1.000)	<0.001
Week 12 IgG2	13.22	(0.998, 0.999)	<0.001
Week 12 IgG3	15.16	(0.992, 0.997)	<0.001
Week 12 IgG4	7.46	(0.986, 0.998)	0.006
Week 24 HBsAg	9.74	(0.175, 0.671)	0.002
Week 24 HBsAg change from baseline	10.72	(1.772, 9.764)	0.001
Week 24 ALT	0.001	(0.989, 1.012)	0.982
Week 24 IgG1	7.68	(0.999, 1.000)	0.006
Week 24 IgG2	11.67	(0.998, 1.000)	0.001
Week 24 IgG3	8.81	(0.993, 0.999)	0.003
Week24 IgG4	10.84	(0.980, 0.995)	0.001

DISCUSSION

The immunological mechanisms underlying HBV infection, particularly HBsAg clearance, are currently unclear. In this study, a cohort of IHCs treated with interferon was established, high HBsAg clearance rates were obtained, and blood specimens were dynamically retained, which provided the necessary basis for further work on mechanisms related to HBsAg clearance.

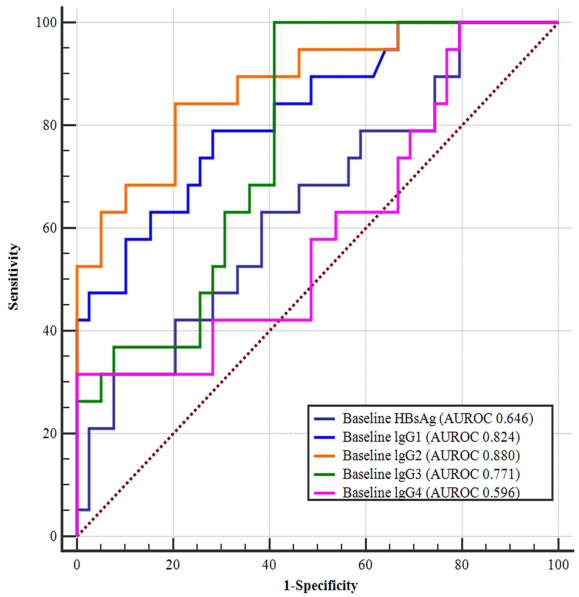

FIGURE 3 | ROC curves of baseline HBsAg, IgG1, IgG2, IgG3, and IgG4 levels for predicting HBsAg clearance. Baseline IgG2 had the largest area under the curve (AUROC 0.880), followed by the baseline IgG1 (AUROC 0.824), IgG3 (AUROC 0.771), HBsAg (AUROC 0.646), and IgG4 (AUROC 0.596) levels.

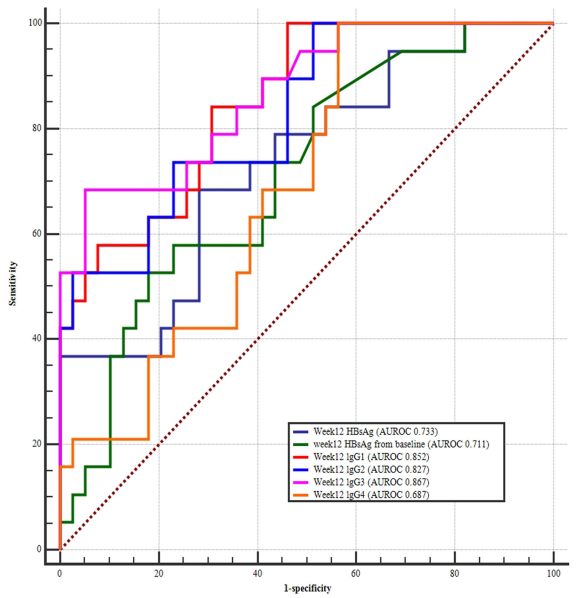

FIGURE 4 | ROC curves at week 12 HBsAg, and week 12 HBsAg changes from baseline, week 12 IgG1, IgG2, IgG3, and IgG4 for predicting HBsAg clearance. The largest area under the curve was week 12 IgG3 (AUROC 0.867), followed by week 12 IgG1 (AUROC 0.852), week 12 IgG2 (AUROC 0.827), week 12 HBsAg (AUROC 0.733), and HBsAg change form baseline (AUROC 0.711).

Persistent HBV infection can lead to impaired immune function in the body, and in addition to a decrease in the number and function of specific T cells, the humoral immune response is severely reduced. Previous studies have analyzed the relationship between the level of immunoglobulins in patients with chronic hepatitis B infection and the activity and severity of the disease and found that lgG levels are positively correlated with the severity of the disease (11, 12). After treatment with nucleoside analogs, HBV DNA levels decreased, and serum immunoglobulin level decreased accordingly (13, 14). However, it has not been reported how the immunoglobulin levels change after interferon treatment, particularly in HBsAg clearance patients. In this study, we investigated the changes of IgG and its subtypes in IHCs treated with interferon and analyzed their correlation with HBsAg clearance.

TABLE 3 | Comparison of the areas under the ROC curves for each indicator at baseline and 12 weeks, 24 weeks.

Indicator of baseline	Difference between areas	95% CI	*P*
Baseline IgG1 *vs*. Baseline HBsAg	0.18	-0.026, -0.381	0.09
Baseline IgG2 *vs*. Baseline HBsAg	0.23	0.053, -0.414	0.01
Baseline IgG3 *vs*. Baseline HBsAg	0.12	-0.067, -0.315	0.20
Baseline IgG4 *vs*. Baseline HBsAg	0.05	-0.179, -0.279	0.67
Week 12 IgG1 *vs*. Week 12 HBsAg change from baseline	0.14	-0.029, -0.311	0.10
Week 12 IgG2 *vs*. Week 12 HBsAg change from baseline	0.18	-0.082, -0.315	0.25
Week 12 IgG3 *vs*. Week 12 HBsAg change from baseline	0.16	0.003, -0.310	0.04
Week 12 IgG4 *vs*. Week 12 HBsAg change from baseline	0.02	-0.164, -0.211	0.80
Week 12 IgG1 *vs*. Week 12 HBsAg	0.12	-0.068, -0.305	0.21
Week 12 IgG2 *vs*. Week 12 HBsAg	0.10	-0.100, -0.289	0.34
Week 12 IgG3 *vs*. Week 12 HBsAg	0.13	-0.027, -0.295	0.10
Week 12 IgG4 *vs*. Week 12 HBsAg	0.05	-0.138, -0.229	0.62
Week24 IgG1 *vs*. Week24 HBsAg change from baseline	0.04	-0.161- 0.233	0.72
Week24 IgG2 *vs*. Week24 HBsAg change from baseline	0.05	-0.168 - 0.258	0.68
Week24 IgG3 *vs*. Week24 HBsAg change from baseline	0.03	-0.135- 0.199	0.71
Week24 IgG4 *vs*. Week24 HBsAg change from baseline	0.02	-0.177 - 0.223	0.82
Week24 IgG1 *vs*. Week24 HBsAg	0.01	-0.194 - 0.215	0.92
Week24 IgG2 *vs*. Week24 HBsAg	0.02	-0.199 - 0.238	0.86
Week24 IgG3 *vs*. Week24 HBsAg	0.01	-0.165 - 0.178	0.94
Week24 IgG4 *vs*. Week24 HBsAg	0.01	-0.205 - 0.210	0.98

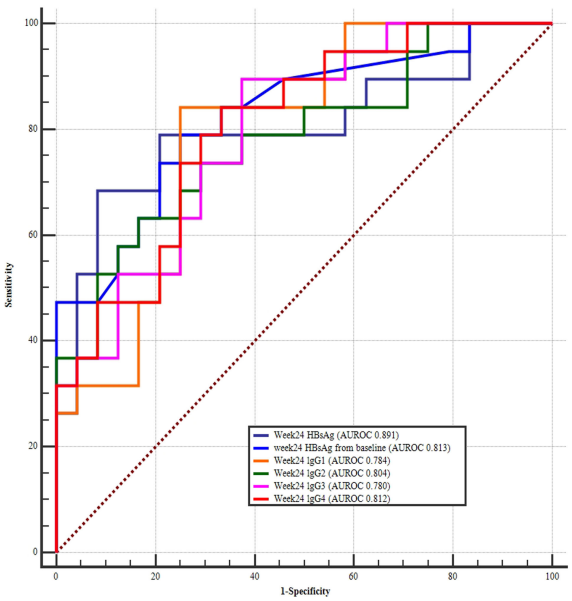

FIGURE 5 | ROC curves of week 24 HBsAg, week 24 HBsAg change from baseline, week 24 lgG1, lgG2, lgG3, and lgG4 for predicting HBsAg clearance. The largest area under the curve was week 24 HBsAg (AUROC 0.891), followed by HBsAg change from baseline (AUROC 0.813), week 24 lgG4 (AUROC 0.812), week 24 lgG2 (AUROC 0.804), week24 lgG1 (AUROC 0.784), and week 24 lgG3 levels (AUROC 0.780).

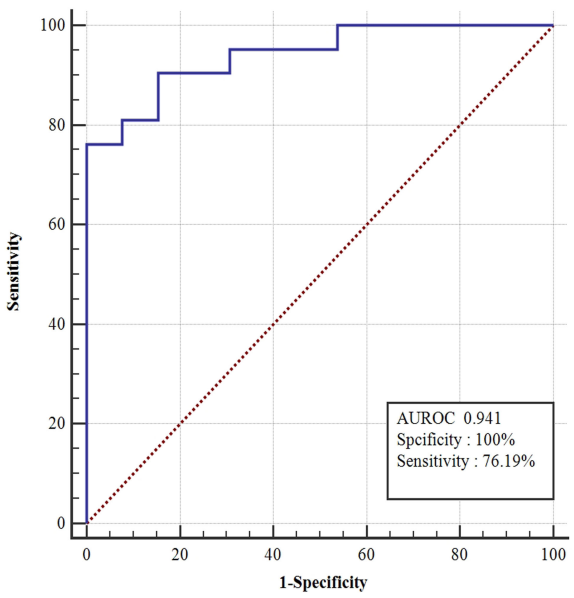

FIGURE 6 | ROC curves of model (y=33.933-0.001*BaselinelgG1-0.002*BaselinelgG2). According to the univariate regression analysis, strong predictors included: baseline lgG1, lgG2, lgG3, and lgG4 levels; week 12 lgG1, lgG2, lgG3, and lgG4 levels; and week 24 lgG1, lgG2, lgG3, and lgG4 levels; HBsAg at 12 and 24 weeks; HBsAg change form baseline at 12 and 24 weeks. A multi-factor logistic analysis was performed with y=33.933-0.001*BaselinelgG1-0.002*BaselinelgG2. The area under the curve was up to 0.941 with 100% sensitivity and 76.19%.

Several studies have confirmed that IHCs receiving IFN-based antiviral therapy can achieve higher HBsAg clearance rates. In this study, 60 IHCs were treated with IFN for 96 weeks, of which 39 patents achieved HBsAg clearance. During treatment, ALT and AST in the NR and R groups were increased, especially in HBsAg clearance group, which was similar to previous reports, suggesting that patients with good response to interferon therapy often experienced elevated ALT (15, 16). After treatment with PEG-IFN, the quantity of HBsAg at 12 weeks and 24 weeks was significantly lower than baseline, especially in the R group, suggesting that patients with rapidly declining HBsAg in the treatment had a higher incidence of HBsAg clearance (17). Therefore, many studies have indicated that changes in ALT and HBsAg at baseline and during treatment were good predictors of HBsAg clearance (15, 18, 19).

In this study, serum lgG1, lgG2, lgG3, and IgG4 levels were measured at baseline, and at 12 weeks, and 24 weeks of PEG-IFN treatment in IHCs. The levels of lgG1, lgG2, lgG3, and IgG4 were significantly lower in patients with HBsAg clearance than in non-cleared patients at all timepoints. Furthermore, we found the AUROC curves of serum IgG were significantly higher than HBsAg and ALT. This result suggests that serum IgG in the early stage of PEG-IFN treatment could be a good predictor of HBsAg clearance, and the decreased level of IgG is beneficial to HBsAg clearance. Multi-factor logistic regression analysis revealed that the combined baseline lgG1 and lgG2 levels had a significantly higher predictive value for HBsAg clearance with an AUROC of up to 0.941, a sensitivity of 100%, and a specificity of 76.19%.

This study provides evidence supporting serum IgG, as a clinical predictor of HBsAg clearance, which has a higher predictive value compared to the conventional predictors HBsAg and ALT levels. However, this study is a single-center exploratory study with a relatively small number of patients, and the exact predictive efficacy needs to be further validated in a larger sample size.

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 protocol and the consent form for the study were approved by the research ethical committee of the Beijing You'an 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 and XL analyzed the results. LQ and YZ conducted the experiments. ZC and HL wrote the manuscript. XHL, XW, SL, YL, JZ, and XC reviewed the data. All authors contributed to the article and approved the submitted version.

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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.2022.864354/full#supplementary-material

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Dynamic Changes of Cytokine Profiles and Virological Markers Associated With HBsAg Loss During Peginterferon Alpha-2a Treatment in HBeAg-Positive Chronic Hepatitis B Patients

Minghui Li[1,2†‡], Luxue Zhang[3‡], Si Xie[4‡], Fangfang Sun[1‡], Zhan Zeng[2], Wen Deng[1], Tingting Jiang[1], Xiaoyue Bi[1], Yanjie Lin[2], Liu Yang[1], Yao Lu[1], Ge Shen[1], Ruyu Liu[1], Shuling Wu[1], Min Chang[1], Leiping Hu[1], Jianping Dong[5], Wei Yi[6*†] and Yao Xie[1,2*†]*

[1] Department of Hepatology Division 2, Beijing Ditan Hospital, Capital Medical University, Beijing, China, [2] Department of Hepatology Division 2, Peking University Ditan Teaching Hospital, Beijing, China, [3] Infectious Disease Department, Xuanwu Hospital, Capital Medical University, Beijing, China, [4] Division of Hepatology, Hepato-Pancreato-Biliary Center, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing, China, [5] Department of Infectious Diseases, Haidian Hospital, Beijing Haidian Section of Peking University Third Hospital, Beijing, China, [6] Department of Gynecology and Obstetrics, Beijing Ditan Hospital, Capital Medical University, Beijing, China

Objective: To explore dynamic changes of cytokines and virological markers associated with hepatitis B surface antigen (HBsAg) loss during peginterferon alpha-2a (PEG-IFN α-2a) treatment in hepatitis B e antigen (HBeAg) positive chronic hepatitis B (CHB) patients.

Methods: It was a single-center prospective cohort study. HBeAg-positive CHB patients were prospectively and consecutively enrolled. Cytokines were detected at baseline, week 12 and 24 of PEG-IFN treatment. HBsAg disappearance rate was the primary evaluation index at 48 weeks of PEG-IFN treatment.

Results: Among 100 patients who completed the 48-week PEG-IFN α-2a treatment, 38 patients achieved serum HBeAg disappearance, 25 patients achieved HBeAg seroconversion, 9 patients achieved functional cure, 37 patients had HBsAg decline of ≥1 log IU/ml, and 8 patients produced hepatitis B surface antibody (HBsAb). Albumin (ALB), fms-like tyrosine kinase 3 ligand (FLT3-L) and interferon-alpha2 (IFN-α2) in the clinical cure group were significantly lower than those in the non-clinical-cure group at baseline. After 12 weeks of treatment, HBsAg in the clinical cure group was significantly lower than that in the non-clinical-cure group (median 1.14 vs. 3.45 log10IU/ml, Z=-4.355, $P < 0.001$). The decrease of HBsAg and hepatitis B virus desoxyribose nucleic acid (HBV DNA) in the clinical cure group was significantly higher than that in non-clinical-cure group (median: HBsAg 1.96 vs. 0.33 log10IU/ml, Z=-4.703, $P< 0.001$; HBV DNA 4.49 vs.3.13 $\log_{10}$IU/ml, Z=-3.053, P=0.002). The increase of IFN-α2 in the cure group was significantly higher than that in the non-clinical-cure group (497.89 vs. 344.74, Z=-2.126, P=0.034). After 24 weeks of treatment, HBsAg,

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Edited by:
Yan-Mei Jiao,
Fifth Medical Center of the PLA General Hospital, China

Reviewed by:
Xinyue Chen,
Capital Medical University, China
Ahmed Abd El Wahed,
Leipzig University, Germany

***Correspondence:**
Jianping Dong
13611351665@163.com
Wei Yi
yiwei1215@163.com
Yao Xie
xieyao00120184@sina.com

†ORCID:
Minghui Li
orcid.org/0000-0003-3233-5473
Wei Yi
orcid.org/0000-0003-4241-8205
Yao Xie
orcid.org/0000-0003-4108-7037

‡These authors have contributed equally to this work

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HBeAg, Flt3-L, and IL-10 in the clinical cure group were significantly lower than those in the non-clinical-cure group (median: HBsAg 0.70 vs. 3.15 log_{10}IU/ml, Z=-4.535, P< 0.001; HBeAg 1.48 vs. 13.72 S/CO, Z = 2.512, *P* = 0.012; Flt3-l 0.00 vs 2.24 pg/ml, Z = 3.137, *P*=0.002; IL-10 0.70 vs. 2.71 pg/ml, Z=-4.067, *P* < 0.001). HBsAg decreased significantly in the clinical cure group compared with non-clinical-cure group (median 3.27 vs. 0.45, Z=-4.463, *P* < 0.001).

Conclusion: Dynamic changes of cytokines and virology markers during early PEG IFN α-2a treatment were associated with HBsAg loss in HBeAg-positive CHB patients.

Keywords: hepatitis B surface antigen, chronic hepatitis B, functional cure, cytokine, interferon

INTRODUCTION

HBsAg loss, with undetectable HBeAg and HBV DNA, known as clinical cure or functional cure (1), is a goal of antiviral treatment for chronic hepatitis B (CHB) and is recommended by many guidelines (2, 3). The disappearance of HBsAg reflects a favorable state of immune control of viral infection in patients with CHB (4). Compared with CHB patients with negative HBV DNA and positive HBsAg, the disappearance of HBsAg can reduce the incidence of liver cancer by 5 times (5). Therefore, it's essential to achieve HBsAg disappearance in patients with CHB. Although HBeAg seroconversion occurs in the state of natural infection, the spontaneous annual disappearance rate of HBsAg in CHB patients is very low (0.5% - 1.0%) due to virus inhibitory effect on the function of host immune cells (6). Regardless of nucleos(t)ide analogues (NA) treatment, disappearance rate of HBsAg is still less than 1% (7).

Interferon (IFN) therapy is the most important way to obtain the disappearance of HBsAg (8, 9). Our previous studies found that changes of virological and serological indexes could predict the disappearance of HBsAg in CHB patients treated with interferon (10, 11). If patients were given consolidation treatment of interferon for 12-24 weeks after functional cure, it was not easy to relapse (10, 12, 13). We also found that incidence of hepatitis B was positively correlated with IFN-α, and negatively correlated with transforming growth factor-beta (TGF-β) and interleukin- 10 (IL-10) (14, 15). Both TGF-β and interferon-gamma (IFN-γ) were found to be associated with efficacy of interferon (16). There are something worth investigating, such as, what are clinical and immunological characteristics of CHB patients who have functional cure after interferon treatment, and what kind of patients are more likely to be clinically cured. In this study we aimed to explore the dynamic changes of cytokine profiles and virological markers associated with HBsAg loss during peginterferon alpha-2a (PEG-IFN α-2a) treatment in HBeAg-positive CHB patients.

MATERIALS AND METHODS

Patients and Follow-up

This was a single-center prospective cohort study. From November 2017 to November 2018, HBeAg-positive CHB patients who were willing to be treated with PEG-IFNα-2a in the Department of Hepatology Division 2 of Beijing Ditan Hospital Affiliated to Capital Medical University were prospectively and consecutively enrolled. Since November 2019, due to the impact of the Corona Virus Disease 2019 (COVID-19), no new patients have been enrolled. Virology markers, serological indicators, cytokine levels and biochemical parameters were detected at baseline and every 12 weeks during following treatment.

The criteria for enrollment were as previously (17): 1) Persistent HBsAg positivity (HBsAg ≥ 0.05 IU/ml) > 6 months; 2) HBeAg positivity (HBeAg ≥ 1.0 S/CO); 3) HBV DNA >10^4 IU/ml; 4) ALT abnormal (≥ 80 IU/L) for more than 3 months or a history of significant liver inflammation (above G2) in examination before this study; 5) Aged from 18-65; 6) Willing to receive interferon treatment; 7) Not receiving immunosuppressants, hormones, or hepatoprotective medicines.

Exclusion criteria were: 1) Co-infection with other hepatitis virus [Hepatitis C virus (HCV), Hepatitis D virus (HDV)]; 2) Autoimmune liver diseases; 3) Other virus infections, such as Epstein-Barr virus, cytomegalovirus, *etc*; 4) Chronic alcohol abuse and/or other liver damaging drugs; 5) Mental illness; 6) Evidence of liver tumor [hepatocellular carcinoma diagnosed in clinical or alpha-fetoprotein (AFP)>100 ng/ml]; 7) Liver fibrosis and cirrhosis confirmed by Fibroscan (18); 8) Serious diseases of brain, lung, heart, kidney and other severe diseases that prevent patients from long-term follow-up; 9) Other liver diseases (metabolic disease, fatty liver, *etc*).

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).

Treatment Plan and Adjustment Strategy

After enrollment, PEG-IFNα-2a was injected subcutaneously at a dose of 180 μg weekly. After 12 weeks of treatment, patients with HBV DNA load above 10^3 IU/ml would receive PEG-IFNα-2a in combination with entecavir (ETV) until the end of week 48. If a patient's HBV DNA load was less than 10^3 IU/ml at week 12, but more than 20 IU/ml at week 24, then ETV was added to PEG-IFNα-2a until week 48.

Detection of Clinical Index, HBV DNA, HBV Serology and Plasma Cytokines

Blood routine (Sysmex Corporation, Japan), kidney function (Sekisui Medical CAL Co, LTD, Japan), liver function (Wako

Pure Chemical Industries, Ltd, Japan), alpha-fetoprotein (Abbott Ireland Diagnostics Division, and Finisklin Business Park, Sligo, Ireland) were detected. HBV DNA load was detected by CobasTaqMan96 real-time quantitative PCR detection reagent (detection of off-line 20 IU/ml) (Roche, Pleasanton, CA, USA), and the detection limit of HBV DNA was <20 IU/ml. HBsAg, anti-HBs, and HBeAg were detected using Abbott Architect i2000 kits (Abbott Laboratories, Abbott Park, IL, USA). HBsAg <0.05 IU/ml was defined as HBsAg disappearance, HBsAb ≥10 mIU/L was defined as positive, HBeAg <1.0 S/CO was defined as HBeAg disappearance, and hepatitis B e antibody (HBeAb) <1.0 S/CO was defined as positive. HBsAg loss with HBeAg-negative and serum HBV DNA <20 IU/ml were defined as functional cure or clinical cure. Clinical indicators were detected at baseline and every 12 weeks during treatment.

The levels of IFN-α2, IFN-γ, IL-10, interleukin-17A (IL-17A), interleukin-6 (IL-6), FLT3-L, TGF-β and tumor necrosis factor-alpha (TNF-α) in plasma were detected with Luminex technique and analyzed by FLEXmap 3D analyzer. Cytokines were detected at baseline, week 12 and 24 of treatment.

Statistical Analysis

All data were analyzed by SPSS 25.0 statistical software. Normal distribution data were expressed as mean ± standard deviation and independent sample *t*-test was used for comparison between two groups. Non-normal distribution data were expressed as median and quartile, while Mann-Whitney *U* nonparametric test was used. The counting data were expressed in frequency and percentage, and chi square test was used. All tests were bilateral tests with $P < 0.05$ as statistically significant. Bonferroni correction method was used to correct the test standard α when comparing the virological indexes, biochemical indexes and cytokines at 12 and 24 weeks, and the difference was statistically significant if $P < 0.025$.

We analyzed the relationship between the early (12 and 24 weeks of PEG-IFN α-2a treatment) response of virology, serology, and immunology indexes and HBsAg loss at 48 weeks of interferon treatment.

RESULTS

Patients Characteristics and Outcome

Except 29 patients who returned to local area for treatment, 221 of 250 eligible HBeAg-positive patients with CHB infection signed the informed consent from November 2017 to November 2018. Among them, 116 were treated with PEG-IFNα-2a, while 105 were treated with NAs. 3 cases quitted from PEG-IFNα-2a group because of side effects, 5 withdrew because of fertility plan, and 8 lost to follow-up. Finally, 100 patients receiving PEG-IFNα-2a treatment completed the whole 48-week follow-up, including 60 males and 40 females, with a median age of 31 (28-36) years (**Figure 1**). 55 patients were combined with ETV therapy after peG-IFNa-2a for 12 weeks, and 10 patients were combined with ETV after PEG-IFNa-2a for 24 weeks.

After 48-weeks, 72 patients achieved virological response and 64 gained ALT normalization; 38 got serum HBeAg disappearance, and 25 achieved HBeAg seroconversion; 37 got a HBsAg decline ≥1 log10 IU/ml, 9 patients' HBsAg disappeared, and 8 patients' HBsAb became positive. 9 patients achieved clinical cure (HBsAg disappeared, HBeAg negative, and HBV DNA <20 IU/ml).

Patients Grouping and Baseline Characteristics

The patients were divided into clinical cure group (n=9) and non-clinical-cure group (n=91) according to whether they obtained clinical cure (HBsAg loss, with undetectable HBeAg and HBV DNA) after 48 weeks of interferon therapy. The median levels of HBV DNA, HBeAg and HBsAg in the Clinically cure group were 6.55 $\log_{10}$IU/ml, 671.46 S/CO, 3.88 $\log_{10}$IU/ml, and those in the non-clinical-cure group were 6.69 $\log_{10}$IU/ml, 878.93 S/CO, 3.88 $\log_{10}$IU/ml, respectively, but there was no significant difference between the two groups at baseline.

At baseline, the median ALB [40.90 (39.50, 45.60) vs. 45.50 (43.10, 47.40) g/L, Z=-2.030, P=0.042], Flt3-L[0.02 (0, 28.8) vs. 29.26 (0.18, 87.92) pg/ml, Z=-2.080, P=0.037], IFN-α2 [9.13 (0.89, 52.7) vs. 43.75 (20.57, 97.83) pg/ml, Z=-2.187, P=0.029] was significantly lower in the Clinically cure group than that in the non-clinical-cure group (**Table 1**).

Virology, Biochemical Indices and Cytokines in Patients With or Without Clinical Cure

At 24 weeks, in clinical cure group the median HBsAg [0.70 (0.00, 1.17) vs. 3.15 (2.69, 3.74) $\log_{10}$ IU/ml, Z=-4.535, P <0.001], HBeAg [1.48 (0.32, 8.63) vs. 13.72 (2.57, 64.64) S/CO, Z= -2.512, P=0.012], Flt3-L [0.00 (0.00, 0.00) vs. 2.24 (0.04, 24.53) pg/ml, Z=-3.137, P=0.002], IL-10 [0.70 (0.22, 0.92) vs. 2.71 (1.96, 8.64) pg/ml, Z=-4.067, P<0.001], IL-17A [2.39 (0.76, 2.91) vs. 3.62 (1.9, 8.15) pg/ml, Z=-2.356, P=0.018],and IL-6 [0.57 (0.39, 1.1) vs. 1.19 (0.71, 2.29) pg/ml, Z = -2.464, P = 0.014] were significantly lower than those in the non-clinical-cure group (**Figure 2**).

At 12 weeks, the median HBsAg [1.14 (0.00, 2.37) $\log_{10}$ IU/ml vs. 3.45 (2.96, 3.85) $\log_{10}$ IU/ml, Z=-4.355, P <0.001)], HBeAg level[1.66 (0.36, 75.20) S/CO vs.51.97 (9.60, 382.71) S/CO, Z=-2.295, P =0.022], and TBIL level [9.00 (7.25, 10.45) μmol/L vs. 12.10 (9.80, 14.70) μmol/L, Z=-3.108, P =0.002] in the clinical cure group were significantly lower than those in the non-clinical-cure group. The negative conversion rate of HBV DNA [5(55.60%) vs. 15(16.50%), c^2 = 5.563, P =0.018] was significantly higher than that in the non-clinical-cure group (**Figure 3** and **Table S1**).

Changes of Cytokines and Virological Indexes and HBsAg Response

During PEG-IFN treatment, the levels of cytokines and virological indexes changed dynamically. HBsAg, HBeAg and HBV DNA decreased significantly. At 12 weeks of treatment, the decline of HBsAg and HBV DNA in the clinical cure group was significantly higher than that in the non-clinical-cure group (HBsAg: 1.96 (1.86, 2.67) vs. 0.33 (0.01, 0.81), Z=-4.703, P <0.001; HBV DNA: 4.49

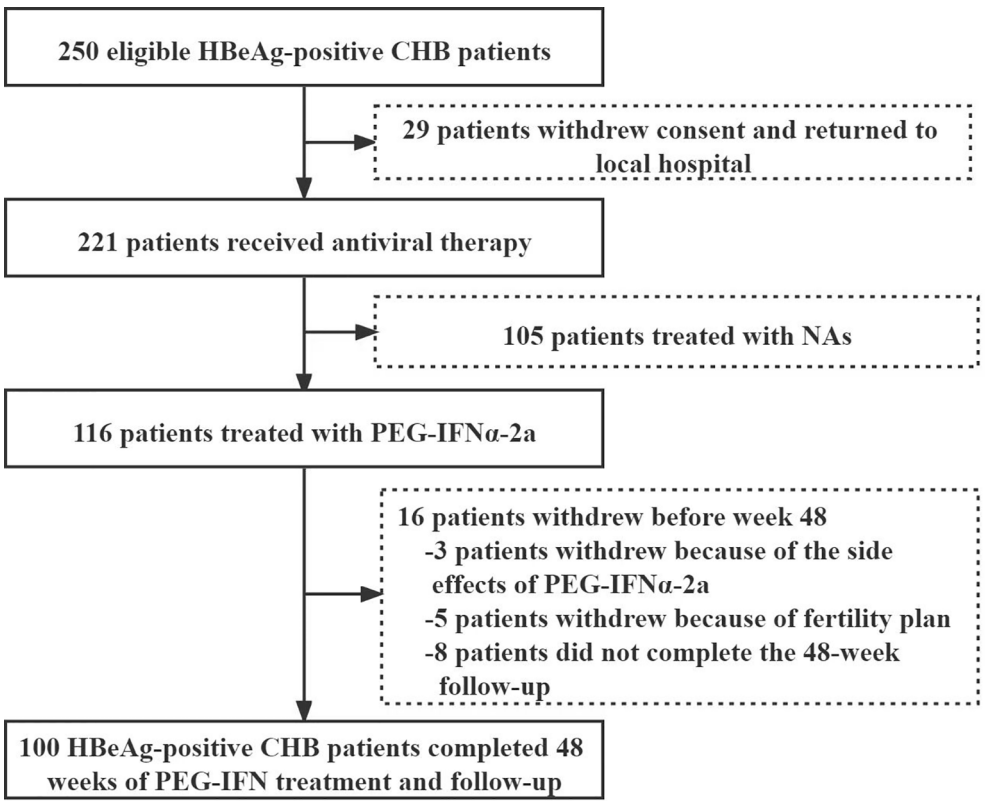

FIGURE 1 | Patient enrollment and deposition.

TABLE 1 | Comparison of virology indicators, biochemistry indicators, and cytokines at baseline between Clinical cure and non-clinical cure patients.

	Clinical cure (n=9)	Non-clinical-cure (n=91)	$Z/\chi^2/P$
Age	33 (27, 43.4)	31 (28, 36)	-0.646/0.518
Male	3 (33.33%)	57 (62.60%)	1.837/0.175
HBsAg (log_{10} IU/ml)	3.88 (2.91, 4.20)	3.88 (3.61, 4.09)	-0.403/0.687
HBeAg (S/CO)	671.46 (266.45, 923.73)	878.93 (566.90, 1196.38)	-1.331/0.183
HBV DNA (log_{10} IU/ml)	6.55 (5.15, 7.29)	6.69 (6.29, 7.31)	-1.415/0.157
ALT (U/L)	271.10 (191.50, 855.01)	232.80 (123.50, 353.70)	-1.524/0.128
AST (U/L)	172.70 (63.05, 464.28)	121.50 (65.20, 168.10)	-0.753/0.452
TBil (μmol/L)	12.70 (9.80, 14.10)	14.10 (11.80, 19.00)	-1.657/0.098
ALB (g/L)	40.90 (39.50, 45.60)	45.50 (43.10, 47.40)	-2.03/0.042
Flt3-L (pg/ml)	0.02 (0, 28.8)	29.26 (0.18, 87.92)	-2.081/0.037
IFN-α2 (pg/ml)	9.13 (0.89, 52.7)	43.75 (20.57, 97.83)	-2.187/0.029
IFN-γ (pg/ml)	16.8 (8.39, 74.87)	22.75 (8.39, 63.01)	-0.416/0.678
IL-10 (pg/ml)	16.41 (1.17, 35.23)	8.9 (3.39, 18.61)	-0.163/0.871
IL-17A (pg/ml)	5.11 (2.25, 29.19)	8.64 (3.29, 39.56)	-0.596/0.551
IL-6 (pg/ml)	3.5 (0.39, 7.51)	2.03 (1.01, 8.39)	-0.271/0.786
TGF-β1 (pg/ml)	4587 (2248, 7557)	4072 (2911, 7308)	-0.633/0.527
TGF-β2 (pg/ml)	425.81 (308.41, 837.69)	499.84 (368.61, 716.21)	-0.729/0.466
TGF-β3 *Z/P Z/P*(pg/ml)	135.16 (118.63, 183.12)	155.32 (131.69, 196.55)	-0.789/0.430

(4.05, 5.95) vs. 3.13 (1.75, 4.22) log_{10} IU/ml, Z =-3.053, P =0.002). At 24 weeks, the decline of HBsAg in the clinical cure group was significantly higher than that in the non-clinical-cure group [3.27 (2.24, 3.44) vs. 0.45(0.15, 1.06), Z=-4.463, P <0.001], as shown in **Figure 4** and **Table s2**.

The changes of cytokines in the two groups after 12 weeks and 24 weeks of treatment were also compared. There was only significant difference in the IFN-α2 change range at 12 weeks [-497.89(-785.83, -422.21) vs. -344.74 (-542.14, -101.69), Z=-2.126, P =0.034] (**Figure 4** and **Table S2**). The decline rate of

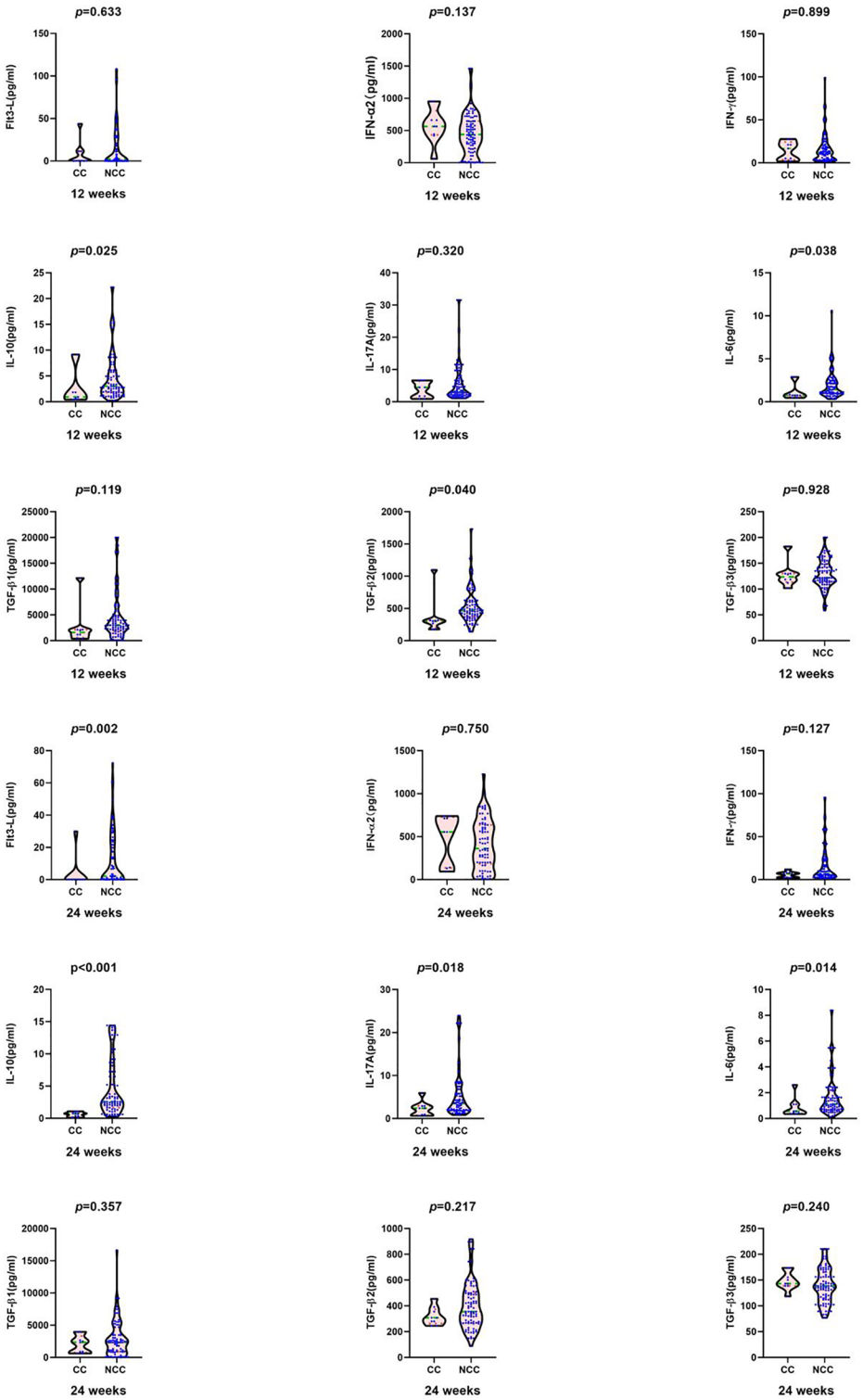

FIGURE 2 | Comparison of cytokines between clinical cure group (CC: n=9) and non-clinical-cure group (NCC n=91) at week12 and 24 during interferon therapy. P < 0.025 is regarded as statistically significant. In clinical cure group, the median Flt3-L, IL-10, IL-17A and IL-6 were significantly lower than those in the non-clinical-cure group at week 24.

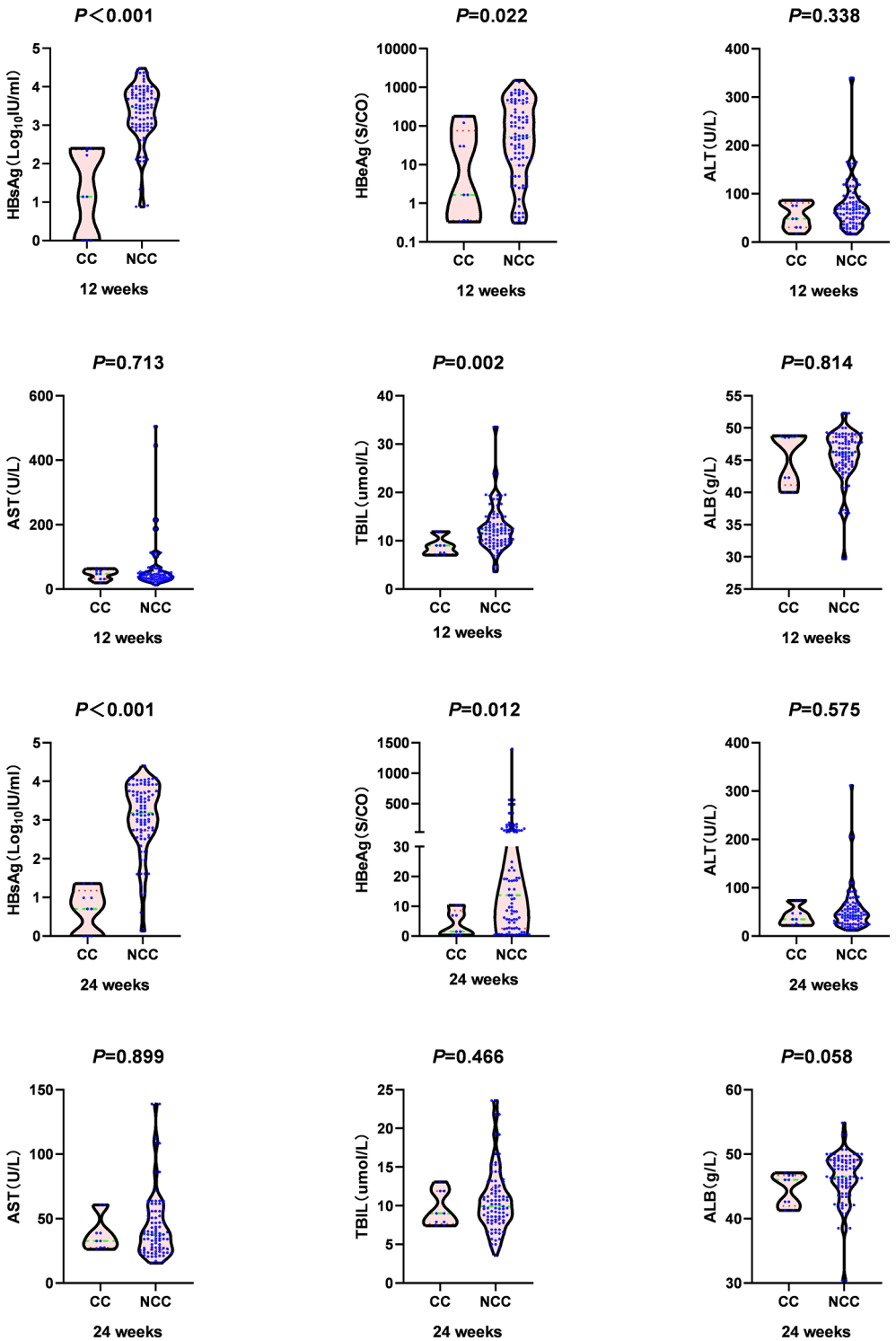

FIGURE 3 | Comparison of virology indicators and biochemistry indicators at week 12 and week 24 between clinical cure (CC: n=9) and non-clinical cure (NCC: n=91) patients. In clinical cure group, the median HBsAg and HBeAg were significantly lower than those in the non-clinical-cure group at week 12 and week 24. P < 0.025 is regarded as statistically significant.

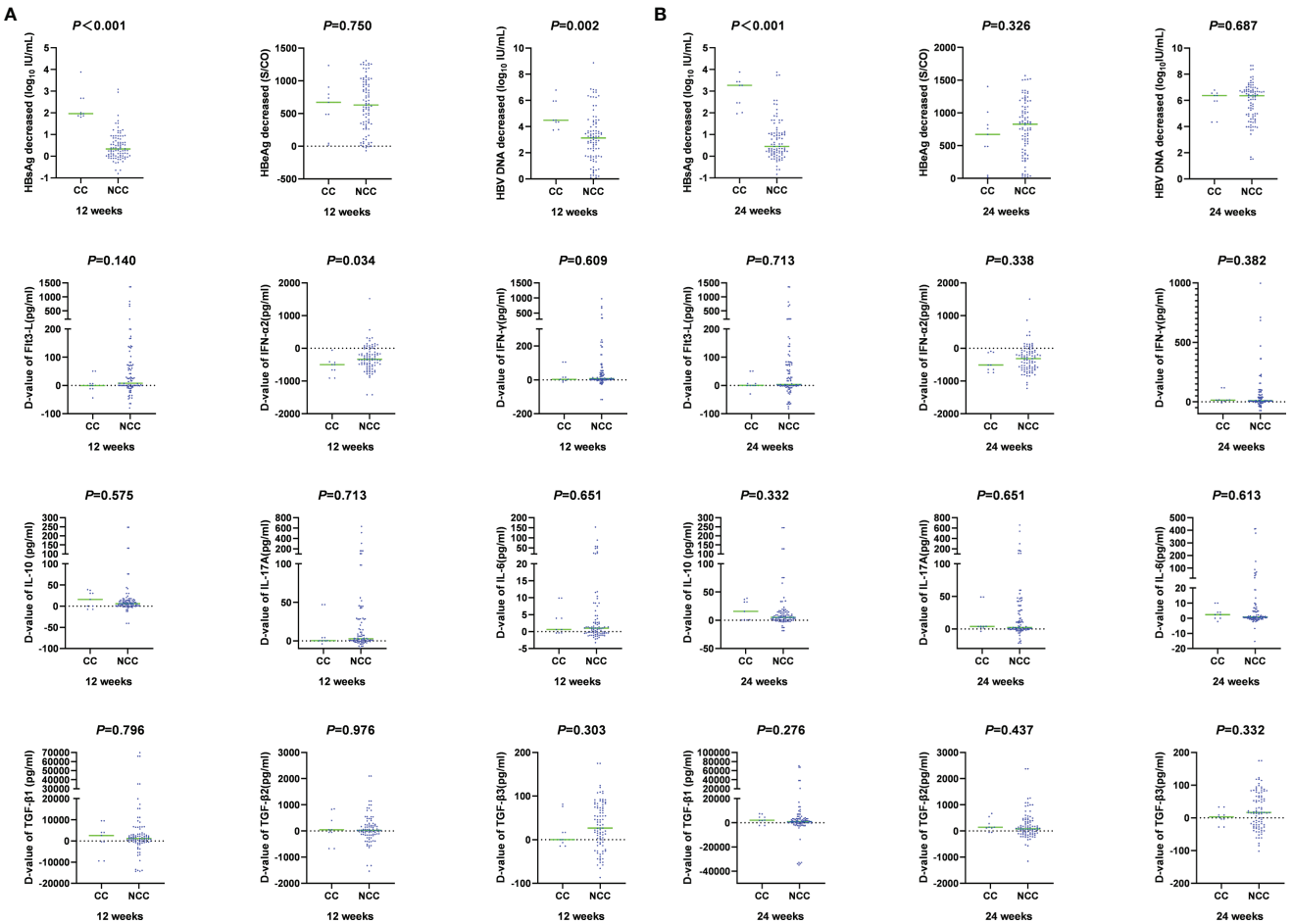

FIGURE 4 | Comparison of magnitude of changes in cytokines and virological indicators at week 12 and week 24 between clinical cure (CC: n=9) and non-clinical cure (NCC: n=91) patients. A: at week 12.B: at week 24. During PEG-IFN treatment, the levels of cytokines and virological indexes changed dynamically. HBsAg, HBV DNA decreased significantly, and IFN-α2 increased significantly.

cytokines between the two groups at different times were similar ($P > 0.05$, **Figure 5** and **Table S3**).

DISCUSSION

With the wide application of antiviral therapy in clinic, more and more patients get HBsAg disappearance through IFN antiviral therapy (8–13). Interferon (IFN) can induce the maturation and activation of dendritic cells (DCs), stimulate the expression and cytokine secretion of natural killer cells (NK), $CD4^+$ helper T lymphocytes (TH), $CD8^+$ cytotoxic T cells (CTL) and monocytes, resulting in clearance of virus infected cells (16, 19). A large number of clinical data show that PEG-IFN α treatment can help 8.5%-16.2% of patients get HBsAg disappearance, especially for patients with HBsAg < 1500 IU/ml after NA treatment (11, 20–22), and the disappearance rate of HBsAg increases year by year after stopping treatment (23). Therefore, currently interferon treatment is considered as one of the most important means to get clinical cure (24). The purpose of this study was to investigate the correlation between the disappearance of HBsAg and changes of cytokine level and virological markers associated with HBsAg loss in PEG-IFN treatment.

HBsAg, HBeAg, HBV DNA polymerase and virus particles can inhibit the function of immune cells and antigen-presenting cells (25, 26), which present difficulty to obtain good curative effect through IFN antiviral therapy. Our study showed that the baseline median levels of HBV DNA, HBeAg and HBsAg in the clinical cure group were lower than those in the non-clinical-cure group. There was no significant difference between the two groups, which might be caused by the small sample size of the cure group. At baseline, the ALB level in the clinical cure group was significantly lower than that in the non-clinical-cure group. The main manifestations of liver inflammation are hepatocyte necrosis and inflammation of liver tissue. The severer the liver inflammation, the lower the ALB level. What's more, liver inflammation leads to the reduction in HBV replication and the production site of virus antigen, i.e., clinically showing the decline of HBV DNA, HBeAg and HBsAg levels. In our study,

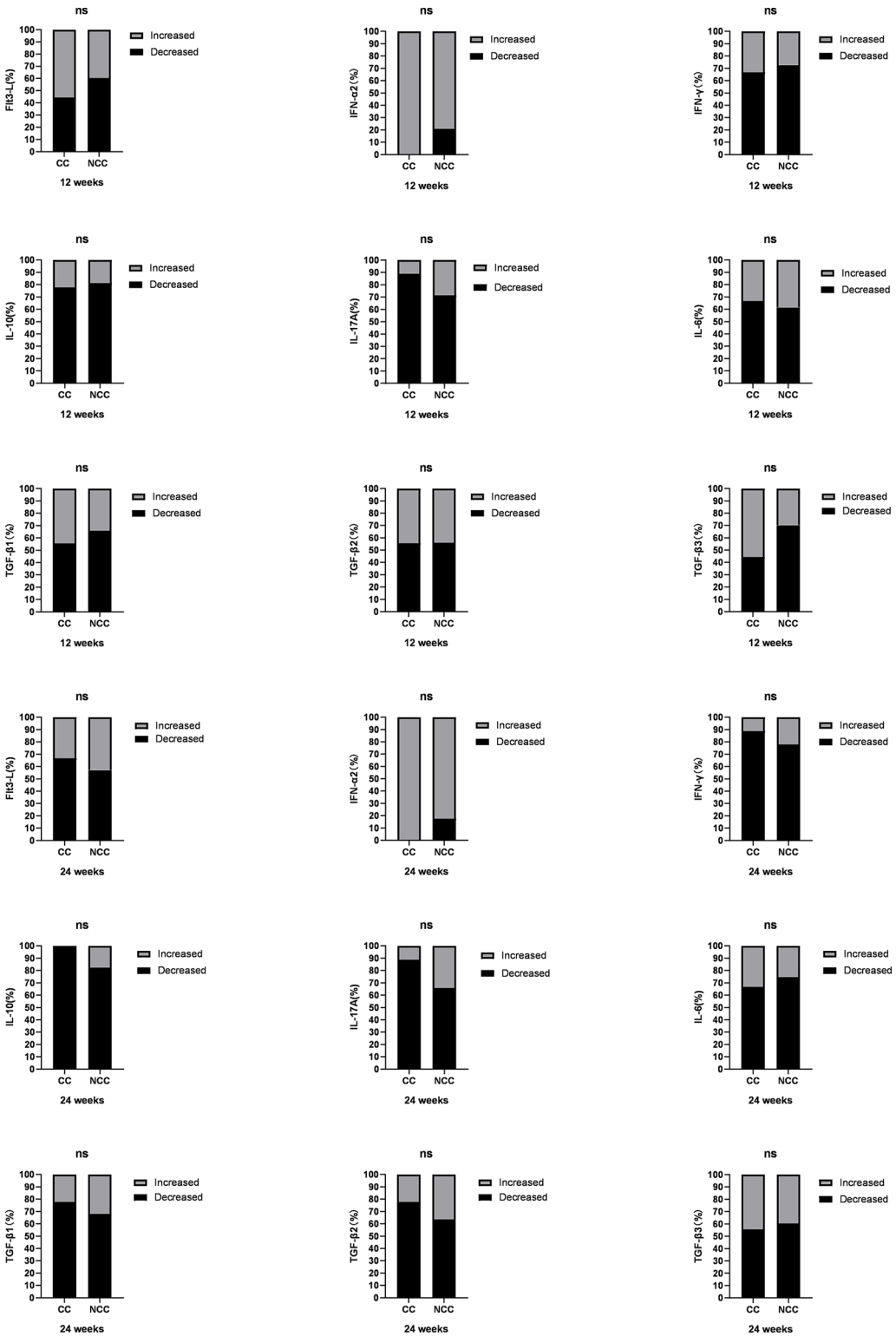

FIGURE 5 | Comparison of different cytokine decline rates at week 12 and week 24 between clinical cure (CC: n=9) and non-clinical cure (n=91) patients. The decline rate of cytokines between the two groups at different times were similar. ns, no significance.

HBsAg, HBeAg and HBV DNA decreased significantly during interferon treatment. At 12 and 24 weeks, HBsAg and HBeAg in the clinical cure group were significantly lower than those in the non-clinical-cure group, and the negative conversion rate of HBV DNA was significantly higher than that in the non-clinical-cure group. The results suggest that the more the decrease in HBV DNA load, HBeAg and HBsAg levels, the better the curative effect of interferon.

We further analyzed of HBsAg disappearance after interferon treatment by stratification to explore the correlation between immunosuppressive and immunostimulatory cytokines and the disappearance of HBsAg. Many immune cells are involved in the occurrence of CHB and interferon antiviral treatment, including $CD4^+$ T lymphocytes ($CD4^+$ T), $CD8^+$ T lymphocytes ($CD8^+$ T), myeloid dendritic cell (mDC) and plasmacytoid dendritic cell (pDC), NK, natural killer T cells (NKT) cells, monocytes, liver Kupffer cells, regulatory T cells, regulatory B cells (24). In this study we examined the cytokines with immunosuppressive effect (IL-6, IL-10 and TGF- β), cytokines with immune function stimulation (IFN -α), cytokines with virus clearance effect (IL-17A, IFN- γ), and FLT3-L stimulating proliferation of DC cells and NK cells (27–29). The expression of inhibitory cytokines (IL-6, IL-10 and TGF-β) was up-regulated, which weakened the immune response of the host. If these inhibitory cytokines are down-regulated, the host immune response will be enhanced (27–30). IFN-α is derived from pDC. IFN-α is up-regulated, and its role in the activation of NK, $CD4^+$ helper cells and $CD8^+$ effector cells is enhanced, and vice versa (30–32). Virus-clearing cytokines (IL-17A and IFN-γ) exert cytotoxic effects by regulating CTL cells and mediating cellular immunity. The up-regulated expression of IL-17A and IFN-γ was beneficial to elimination of the virus itself; however, if being down-regulated, it is unfavorable to the elimination of the virus (30–32). FLT3-L is an important stimulator of DC cell proliferation and can also induce NK cell proliferation *in vivo* (27, 28). Upregulation of FLT3-L enhances virus-clearance by activated immune cells, and vice versa (33).

IL-6, IL-10, and TGF-β were similar at baseline in patients with or without HBsAg disappearance, but after 24 weeks, they were significantly higher in patients without HBsAg disappearance as shown in **Figures 2**, **3** and **Table s1**. If untreated, the two groups had similar levels of inhibitory cytokines (IL-6, IL-10, and TGF-β), suggesting that the spontaneous disappearance of HBsAg is hard to occur in CHB without interferon treatment. At 24 weeks of interferon treatment, the levels of IL-10 and IL-6 in the no-HBsAg disappearance group were significantly higher than those in the HBsAg disappearance group (**Figures 2**, **3** and **Table s1**). Compared with baseline, the median decline range of IL-10 (5.21) and TGF-β2 (79.56) in no-HBsAg disappearance group was lower than that in the HBsAg disappearance group (15.71 and 145.67). As for stimulating effector cytokines (IFN-α, IL-17A, IFN-γ, and FLT3-L), the baseline FLT3-L and IFN-α2 levels in patients with HBsAg disappearance were lower than those in the no-HBsAg disappearance. At the 24th week of interferon treatment, FLT 3-L and IL-17A in the no-HBsAg disappearance were significantly higher than those in the HBsAg disappearance group (**Figures 2**, **3** and **Table s1**). Therefore, the failure of HBsAg disappearance after interferon treatment may be related to the small decline range of immunosuppressive cytokines, such as IL-10, which can inhibit the function of NK cells, PDC and HBV specific CTL cells (34).

The presence of HBsAg and HBeAg can damage immune cells, induce production of regulatory T cells, and inhibit the function of immune effector cells (14, 15, 25, 26). NA treatment cannot effectively reduce viral antigen, so it is difficult to achieve clinical cure (7). Interferon can reduce the production of viral antigens and restore the function of damaged immune cells, resulting in a higher HBsAg disappearance rate than NA treatment (12, 13, 21, 35). Our study showed that the baseline IL-10, IL-6 and TGF-β2 levels were not different in the two groups, but after 24 weeks of interferon treatment, the level of IL-10 in the clinical cure group was significantly lower than that in the non-clinical-cure group, and the clinical cure group had a greater decline range of IL-10 and TGF-β2. Our study suggests that, although interferon can stimulate immune effector cells with function of virus infected cell clearance, the clinical cure may be dependent more on the direct effect on virus replication and virus antigen reduction.

At baseline, there was a significant difference in IFN-α2 between the cured group and non-cured group, indicating that there was a difference in immune basis. But there was no significant difference between the two groups at 12 and 24 weeks of treatment owning to PEG-IFN treatment.

In conclusion, our research shows that dynamic changes of cytokine profiles and virology markers during early interferon treatment were associated with HBsAg loss in HBeAg-positive CHB patients. There was significant difference in the magnitude of changes in IFN-α2 between the two groups after 12 weeks of interferon treatment. The levels of IL-10 and IL-6 in the clinical cure group were significantly lower than those in the non-clinical-cure group after 24 weeks of interferon treatment. These characteristics help provide a basis for early screening of target population treated with interferon. Clinical cure is difficult to achieve through antiviral therapy in CHB patients. Therefore, the deviation of sample size between the cured group and the non-cured group is obviously inevitable. The bias of sample size between the two groups may affect the results of the study. It must be pointed that the study has some limitations, such as a small sample size and discrete detection range of cytokines, which needs to be further verified in future studies. For CHB patients with HBV DNA>10^4 IU/ml, we will further explore the initial combination of PEG-IFN and NAs in the future.

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 University of Medical Sciences (JDL-2017-034-01). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ML, JD, WY, and YX contributed to the study design. ML, LZ, SX, and YX contributed to the data analysis. ML, LZ, SX, LY,YL, GS, RL, SW, MC, LH, and YX contributed to the recruitment, enrolment, and assessment of participants, as well as data collection. FS, WD, TJ, and XB contributed to following up with the patients. ZZ, YL, and LY managed all aspects of laboratory support. 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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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.892031/full#supplementary-material

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REVIEW
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Immune Mechanisms Underlying Hepatitis B Surface Antigen Seroclearance in Chronic Hepatitis B Patients With Viral Coinfection

Shuling Wu[1‡], Wei Yi[2†‡], Yuanjiao Gao[1‡], Wen Deng[1], Xiaoyue Bi[1], Yanjie Lin[3], Liu Yang[1], Yao Lu[1], Ruyu Liu[1], Min Chang[1], Ge Shen[1], Leiping Hu[1], Lu Zhang[1], Minghui Li[1,3*†] and Yao Xie[1,3*†]*

[1] Department of Hepatology Division 2, Beijing Ditan Hospital, Capital Medical University, Beijing, China, [2] Department of Gynecology and Obstetrics, Beijing Ditan Hospital, Capital Medical University, Beijing, China, [3] Department of Hepatology Division 2, Peking University Ditan Teaching Hospital, Beijing, China

It is considered that chronic hepatitis B patients have obtained functional cure if they get hepatitis B surface antigen (HBsAg) seroclearance after treatment. Serum HBsAg is produced by cccDNA that is extremely difficult to clear and dslDNA that is integrated with host chromosome. High HBsAg serum level leads to failure of host immune system, which makes it unable to produce effective antiviral response required for HBsAg seroclearance. Therefore, it is very difficult to achieve functional cure, and fewer than 1% of chronic hepatitis B patients are cured with antiviral treatment annually. Some chronic hepatitis B patients are coinfected with other chronic viral infections, such as HIV, HCV and HDV, which makes more difficult to cure. However, it is found that the probability of obtaining HBsAg seroclearance in patients with coinfection is higher than that in patients with HBV monoinfection, especially in patients with HBV/HIV coinfection who have an up to 36% of HBsAg 5-year-seroclearance rate. The mechanism of this interesting phenomenon is related to the functional reconstruction of immune system after antiretroviral therapy (ART). The quantity increase and function recovery of HBV specific T cells and B cells, and the higher level of cytokines and chemokines such as IP-10, GM-CSF, promote HBsAg seroclearance. This review summarizes recent studies on the immune factors that have influence on HBsAg seroconversion in the chronic hepatitis B patients with viral coinfection, which might provide new insights for the development of therapeutic approaches to partially restore the specific immune response to HBV and other viruses.

Keywords: hepatitis B virus, hepatitis B surface antigen, functional cure, coinfection, immune

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Yan-Mei Jiao,
Fifth Medical Center of the PLA General Hospital, China

Reviewed by:
Huiying Rao,
Peking University People's Hospital, China
Bo Feng,
Peking University People's Hospital, China

***Correspondence:**
Yao Xie
xieyao00120184@sina.com
Minghui Li
wuhm2000@sina.com
Lu Zhang
zhanglu1218@126.com

***ORCID:**
Minghui Li
orcid.org/0000-0003-3233-5473
Wei Yi
orcid.org/0000-0003-4241-8205
Yao Xie
orcid.org/0000-0003-4108-7037

These authors have contributed equally to this work

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INTRODUCTION

There are more than 250 million hepatitis B virus (HBV) carriers in the world, and about 600 000 patients die of HBV-related liver diseases every year (1, 2). The pathogenesis of hepatitis B is considered to be related to the host immune response, but the underlying mechanism is not completely clear at present. In the immune tolerance state, the virus replicates a lot, and the levels of

serum hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) are very high. After entering the stage of immune clearance, the virus replication decreases, and the levels of HBsAg and HBeAg decrease as well. HBV DNA can be inhibited by effective antiviral treatment, but it is hard to clear covalently closed circular DNA (cccDNA) and double stranded linear DNA (dslDNA) which integrated with the host chromosome. As a result, it is quite difficult to completely clear serum HBsAg. Clearance of serum HBsAg with or without anti-HBs is defined as hepatitis B functional cure (3, 4). In order to improve the rate of functional cure, some new drugs have been developed and entered the stage of clinical trials. In the long process of chronic hepatitis B virus infection, the human body may also be coinfected with other hepatophilic or non-hepatophilic viruses. These viruses inhibit or activate human immunity, making the immune clearance mechanism of HBsAg more complex.

THREE FORMS OF HBSAG DERIVED FROM TWO SOURCES

The total length of HBV genome is about 3.2 kb, containing four partially or completely overlapping open reading frames (ORF) C, S, P and X. HBsAg is encoded by the S ORF, which contains PreS1, PreS2 and S. The production of HBsAg comes mainly from cccDNA. The 2.4 kb and 2.1 kb S mRNA of cccDNA transcripts, that is PreS1/S and PreS2/S respectively, then translates into three sizes of proteins: L-HBs (PreS1+PreS2+S), M-HBs (PreS2+S), and S-HBs (S). The three S proteins differ in their N- terminus but share a common S domain with 4 putative transmembrane (TM) domains on their C-terminus. PreS1 has 108-109 amino acid residues, PreS2 has 55 amino acid residues and S has 226 amino acid residues (see **Figure 1**).

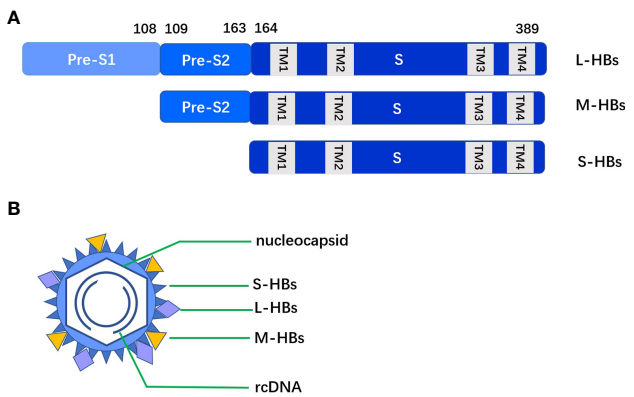

FIGURE 1 | **(A)** The three S proteins (L-HBs, M-HBs and S-HBs) differ in their N- terminus but share a common S domain with 4 putative TM domains on their C-terminus. **(B)** Intact HBV particles contain a large amount of S-HBs and the same amount of M-HBs and L-HBs, with a composition ratio of about 4:1:1.

Another source of HBsAg is dslDNA integrated with host genes. The cccDNA transcript pregenomic RNA (pgRNA) is reverse transcribed into negative strand DNA, of which about 90% is synthesized relaxed circular DNA (rcDNA) and about 10% is synthesized dslDNA. After shelling, dslDNA enters the hepatocyte nucleus and integrates in the host gene chromosome. This integration can occur in the early stage of HBV infection, but the integration level in HBeAg positive stage is low, and the integration in HBeAg negative stage is frequent (5). HBsAg production pathways are shown in **Figure 2**.

The integrated DNA is no longer involved in the formation of virus particles, but can translate into HBsAg. Due to the deletion of some PreS in dslDNA, the proportion of M-HBs and L-HBs in HBsAg from integrated HBV DNA is low. HBsAg translated through the above two pathways accumulates in the endoplasmic reticulum (ER) and forms agglomerates with different cysteines in the S region through covalent disulfide bonds. Intact HBV particles contain a large amount of S-HBs and the same amount of M-HBs and L-HBs, with a composition ratio of about 4:1:1 (6). HBsAg in virus particles accounts for about 1/3 of the total amount of HBsAg, other HBsAg exists in small spherical subvirus particles and filamentous particles.

A recent study found that the proportion of M-HBs in HBeAg positive patients is the best predictor of early HBsAg clearance before nucleoside analogue (NA) treatment. The median level of M-HBs in patients with HBsAg clearance before treatment is significantly lower than that in patients without HBsAg clearance. The proportion of M-HBs and L-HBs decreases rapidly during treatment, and M-HBs cannot be detected after half a year of treatment. In patients with HBsAg clearance treated with pegylated interferon (PEG-IFN), the proportion of M-HBs and L-HBs also shows similar dynamic changes (7). The mechanisms underlying the change in HBsAg composition prior to HBsAg loss is unknown. It is assumed that the structural arrangement of the integrated dslDNA form does not necessarily affect the expression of S-HBs, but parts of PreS may be missing. HBsAg derived from integrated dslDNA contains a low proportion of M-HBs and L-HBs. M-HBs and L-HBs mainly derived from cccDNA. Consequently, the decrease in M-HBs and L-HBs before HBsAg loss might reflect a progressive shutdown of cccDNA activity, but more research is needed to verify this hypothesis.

HBSAG IS RELATED TO HBV SPECIFIC IMMUNE DYSFUNCTION

After human body is infected by HBV, the virus is jointly cleared by innate and specific immune responses. However, the simultaneous nonspecific immune response will cause liver inflammation and necrosis, and even occurrence of liver cirrhosis and liver cancer (8). The immune function of patients with chronic hepatitis B (CHB) is impaired. On the one hand, the immune function of HBV specific T cells is low. such as the increase of immune negative regulatory components [regulatory T cells (Treg), myeloid derived suppressor cells (MDSC), programmed death receptor 1 (PD1), transforming growth factor (TGF-β), interleukin (IL)-10, *etc*], depressed effector

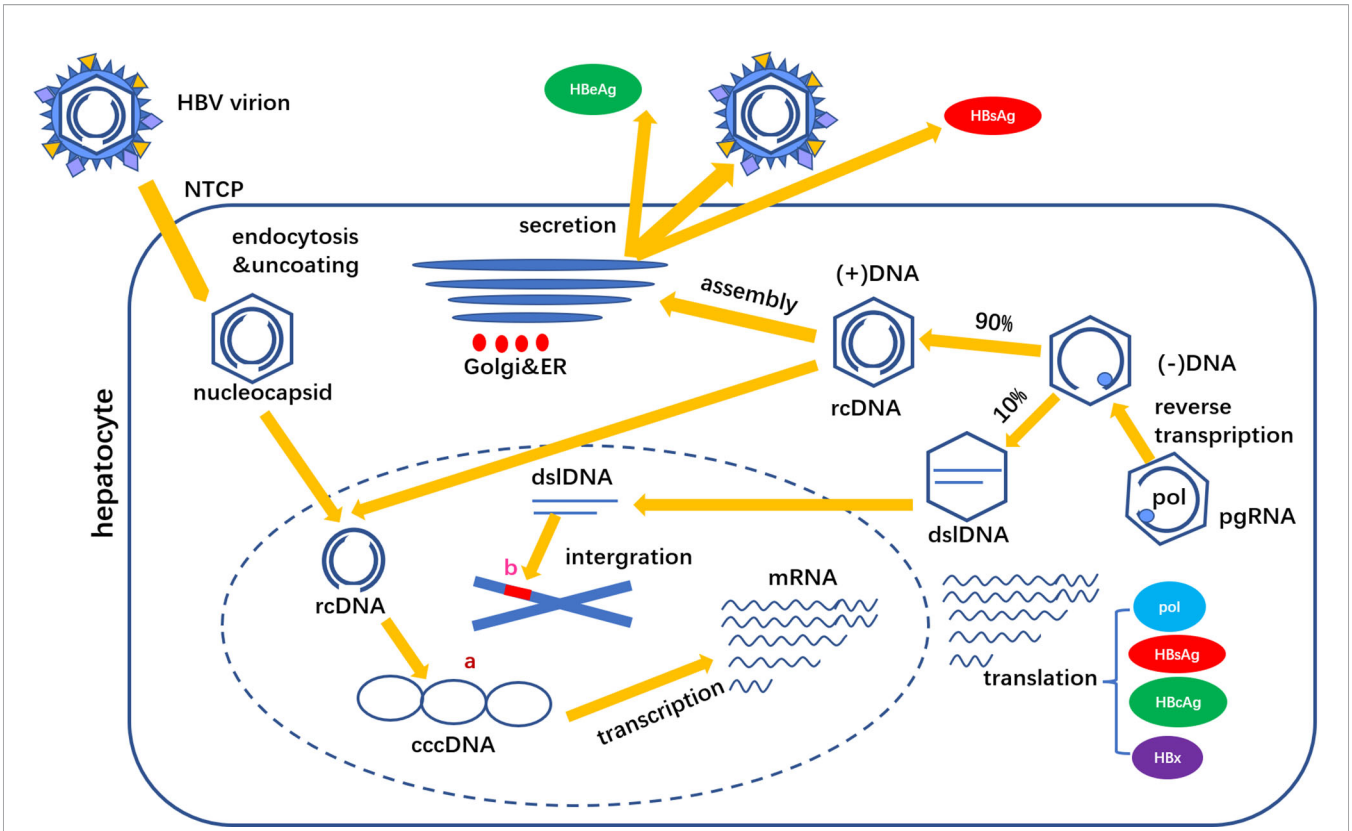

FIGURE 2 | HBsAg is derived from two sources **(A)** cccDNA and **(B)** dslDNA. cccDNA is the main source of HBsAg production.

function and proliferation ability, imbalance of cytokine network. As a result, the body is unable to eliminate effectively the virus, leading to continuous replication of HBV in the human body.

On the other hand, the immune response of non-HBV specific $CD8^+$ T cells, natural killer (NK) cells and T helper (Th) 17 cells is enhanced, which can cause liver damage. A large number of non-HBV specific $CD8^+$T cells infiltrate in the liver of patients with CHB, and their ability to proliferate and produce IL-2 is significantly reduced, but the function of other proinflammatory factors is not damaged, such as interferon (IFN)-γ and tumor necrosis factor (TNF)- α, resulting in nonspecific inflammatory injury (9, 10).

For those HBeAg positive patients in immune activation stage, there is a significant increase in NK cell activity because of increased expression of IL-12, IL-15 and IL-18 in liver and decreased expression of IL-10, NK cells enhance the killing ability, but the ability of secreting IFN-γ is not enhanced, causing liver damage but not clearance of virus. In addition, NK cells can also mediate hepatocyte apoptosis through the upregulation expression of TNF related apoptosis inducing ligand (TRAIL). A large number of Th17 cells infiltrate in the liver of patients with CHB, and is positively correlated with viral load (HBV DNA), alanine aminotransferase (ALT) level and histological activity. IL-17 secreted by Th17 cells mainly promotes the secretion of IL-1β, IL-6, TNF-α and IL-23 inflammatory factors by myeloid dendritic cells (MDC) and monocytes to mediate liver injury (11, 12).

What role does HBsAg play in immune disorder in patients with chronic hepatitis B? It is found that a large amount of HBsAg is the main factor associated with low anti HBV specific immune function. Although there is no strong evidence to support that HBsAg can directly inhibit HBV specific immune response, some studies suggest that HBsAg is related to HBV specific immune dysfunction (13, 14). High level of HBsAg is associated with the impairment of anti-HBV specific T and B cell immune function. Reducing the HBsAg level should promote the recovery of specific immune function, and in turn promote the clearance of HBsAg. When HBsAg clearance or HBsAg seroconversion is realized, the anti-HBV immune response function of the body nearly returns to normal. The incidence of HBsAg variation increases when there is an excessive immune response, such as slow plus acute liver failure (15). HBsAg can inhibit monocyte activity by binding to specific receptors on monocytes, and HBsAg can cause dysfunction of MDC and plasma cell like dendritic cells (PDC). HBsAg can also increase the response of IL-23/IL-17 axis and mediate liver immune injury (16).

HBsAg and Innate Immunity

Innate immunity is the first defense line against microbial infection, which relies on different pattern recognition receptors (PRRS) to recognize nucleic acids. Hepatitis B virus

infection can activate inflammatory factors through two main types of PRR i.e., Toll like receptor (TLR) signal pathway (17–19), and retinoic acid induced gene 1 (RIG-1) signaling pathway (19–21). The expression levels of TLR3, RIG-1 and melanoma differentiation associated gene 5 (MDA5) in peripheral blood of patients with chronic hepatitis B are significantly decreased, which may account for the chronic state of HBV infection. HBsAg can inhibit innate immunity by inhibiting TLR-mediated signaling pathway and inducing IL-10 in Kupffer cells (KCS) and sinusoidal endothelial cells (LSEC) (17). In the presence of HBsAg, the function of myeloid dendritic cells (MDC) is also impaired, which stimulates T cell response (16). In another study, DC isolated from CHB patients is functional, and DC stimulates autologous HBV specific T cell expansion through the cross presentation of circulating HBsAg (22). Most experiments of HBsAg mediated innate immunity are carried out *in vitro*, which is related to the difference of these results. A recent study shows that HBsAg suppressed the activation of the nuclear factor kappa B (NF-κB) pathway *via* interaction with the TAK1-TAB2 complex, leading to downregulation of innate immune responses (23).

HBsAg and Cellular Immunity

Dysfunction and failure of HBV specific $CD8^+$ T cell response are markers of chronic HBV infection (24, 25). High levels of HBsAg in circulation and liver may lead to impaired HBsAg specific $CD8^+$ T cell response through continuous antigen stimulation. In addition, HBsAg can inhibit T cell response and enhance regulatory T cell response by promoting the differentiation of monocytes into MDSC (26). In the woodchuck hepatitis virus (WHV) transgenic mouse model, high levels of viral replication and protein expression in male mice induces the expansion of regulatory T cells in the liver, resulting in impaired WHV specific $CD8^+$ T cell response and gender related differences in virus infection results (27). Compared with healthy persons, the proportion of myeloid dendritic cells (MDC) and plasma like dendritic cells (PDC) in patients with chronic hepatitis B is by and large normal, but MDC has the decreased ability of providing costimulatory signals to T cells and secreting cytokines such as TNF-α. The main function of these cytokines is to promote the maturation of DC and proliferation of DC induced T cells (28). In this process, the HBsAg and HBV DNA levels are high, suggesting that the presence of these two viral components may damage the function of MDC. Other studies have shown that in transgenic mouse models, circulating HBsAg clearance does not improve HBV specific CD8+ T cell response *in vivo* (29).

HBsAg and Humoral Immunity

B cell response may play an important role in controlling HBV infection. For example, the clinical application of rituximab, which consumes B cells, can lead to the reactivation of HBV in controlled patients. This suggests that the response of B cells to HBV is essential for maintaining effective host immune control of HBV (30–32). In chronic hepatitis B patients, the antigen presenting function of HBsAg and the dysfunction of $CD4^+$ T cells due to the high level of DC can affect the secretion of anti-HBs by HBV specific B cells. This may also lead to insufficient affinity or no function of anti-HBs, so it can't play the role of neutralizing antibody.

HBs-ELISPOT and flow cytometry fluorescence sorting (FACS) technology were used to detect HBsAg specific memory B cells (CD19 cells) in HBV vaccine inoculation staff and CHB patients. These two methods detected a small number of HBsAg specific B cells in HBV vaccine inoculation staff, but none was detected in CHB patients (33–35). Other studies have shown that the frequency of HBsAg specific B cells in blood is similar in patients with acute, chronic and cured HBV infection, and has no relationship with the serum levels of HBsAg, HBV DNA or ALT (36, 37).

HBsAg specific B cells from patients with chronic hepatitis B are atypical B cells, characterized by low expression of CD21 and CD27, but high expression of inhibitory markers such as PD-1 and T-bet. In addition, HBsAg specific B cells from patients with chronic hepatitis B are not able to mature into anti-HBs secreting cells *in vitro*. However, their function can be partially restored by specific culture conditions, such as PD-1 blocking or adding IL-2, IL-21 and CD40L (38). Le Bert (39) et al. found that in CHB patients, HBcAg specific B cells were more frequent than HBsAg specific B cells. The phenotypic and functional differences between HBsAg and hepatitis B core antigen (HBcAg) specific B cells in a same patient suggest that high levels of HBsAg may lead to programming obstacles of HBsAg specific B cells through continuous stimulation. Follicular helper T cell can improving HBsAg-specific B cell response in chronic hepatitis B patients targeting by TLR8 signaling (40).

In some CHB patients, the presence of anti-HBs and HBsAg coexists. Only the presence of anti-HBs may eliminate HBsAg in peripheral blood, but it will not terminate chronic HBV infection in the liver. Therefore, HBsAg specific B cell response contributes to HBV pathogenesis, clearance and protective immunity, but it is not enough to control HBV infection alone (41).

IMMUNE MECHANISMS OF CURRENT DRUGS AND EMERGING THERAPIES TARGETING FUNCTIONAL CURE OF HEPATITIS B

Persistent HBsAg seroclearance after treatment, and with or without anti-HBs serologic conversion, is the ideal end point of antiviral therapy for chronic hepatitis B, which represents sustained virological inhibition and immunological control. Current hepatitis B antiviral treatments include two main classes of drugs, one is the oral NAs and the other is injected IFN/PEG-IFN.

It is difficult to achieve the goal of HBsAg seroclearance with standard antiviral treatments. Functional cure occurs at an average annual rate of 0.22% in CHB patients during first-line oral NAs antiviral treatment (42). PEG-IFN treatment can acquire an average HBsAg clearance annual rate of 3% (43–45), 5-year cumulative rate of 14% and 10-year cumulative rate of 32% (46) in CHB patients. But in inactive HBsAg carriers, the HBsAg clearance rate can reach to 47% after 48 weeks of PEG-IFN treatment (47). New treatment strategies such as combination therapy (initial combination therapy

of NAs and IFN/PEG-IFN, continuous combination therapy of NAs and IFN/PEG-IFN) and new therapeutic drugs may help patients improve the negative conversion rate of HBsAg and even the seroconversion rate of HBsAg.

NAs and IFN/PEG-IFN play different roles in host immune response. IFN mainly regulates innate immune response, especially NK cell activity. Micco et al. (48) found that PEG-IFN can induce the production of IL-15 and promote the activation and expansion of $CD56^{bright}$ NK cells, so as to enhance its antiviral activity and promote IFN-γ expression of apoptosis inducing ligand related to soluble TNF. PEG-IFN may lead to the continuous consumption of effector $CD8^+$ T cells, and has limited repair effect on the function of HBV specific $CD8^+$ T cells. NAs cannot resume the antiviral ability of NK cells, but temporarily repair the function of damaged T lymphocytes. In patients with virological inhibition after long-term NAs treatment, the damaged function of HBV specific T lymphocytes is partially restored *in vitro* (49–55). These studies showed that NAs might promote the recovery of T cell function mainly through inhibiting HBV replication. Besides, increased NK cell function is associated with active hepatitis and HBsAg seroclearance following structured NAs cessation (56). Host immune function repair is a key step to achieve chronic hepatitis B functional cure. The rationality of the combined treatment strategy of NAs and IFN/PEG-IFN lies in that the two kinds of antiviral mechanisms play different roles in innate immunity and adaptive immunity. The inhibition of HBV replication by NAs can enhance the activation of IFN on innate immunity (57, 58).

New drugs targeting at the functional cure of hepatitis B mainly include two main categories: direct antiviral drugs and indirect antiviral drugs. The former directly targets viral diseases and interfere with the replication process of HBV DNA, and the latter targets the host immune system to attack HBV. Direct antiviral drugs include siRNA (ARC-520 and JNJ-3989) (59, 60), HBV entry inhibitor (Bulevirtide, formerly known as Myrcludex B) (61), core protein allosteric regulator (NVR 3-778, JNJ-56136379, RO7049389 and ABI-H0731) (62–65), antisense RNA (IONIS-HBVRx and IONIS-HBVLRx) (66), cccDNA inhibitor (not yet in clinical trial), HBsAg release inhibitor (REP 2139) (67), HBsAg neutralizer (lenvervimab) (68), *etc.*

Indirect antiviral drugs include Toll like receptor (TLR) agonists (vesatolimod, selgantolimod) (69), immune checkpoint inhibitors (anti-PD-L1) (70), therapeutic vaccines (GS-4774) (71), engineering T cells, *etc.* Among them, the effect of therapeutic vaccine is disappointing (72). The combination of existing and new antiHBV drugs may improve HBsAg seroclearance rate (73), and the elimination of HBV requires a treatment scheme based on a combination of multiple drugs.

IMMUNE MECHANISMS AND HBSAG SERUM LEVEL CHANGES IN HBV COINFECTION PATIENTS

HBV infection here only refers to chronic HBV infection, that is, HBsAg positive lasts for more than half a year. HBV coinfection only includes HBV and other viruses, excluding bacteria, fungi, parasites, protozoa and other infections. According to the tropism of coinfected viruses, they can be divided into coinfected hepatophilic viruses such as hepatitis C virus (HCV), hepatitis D virus (HDV), hepatitis E virus (HEV), hepatitis A virus (HAV), and non-hepatophilic viruses such as human immunodeficiency virus (HIV).

HBV/HAV Coinfection

HAV is often transmitted through fecal-oral route and mostly leads to acute and self-limiting infection. In a few of cases, it can cause severe liver function damage or even liver failure (74). Acute HAV infection used to occur in adolescents who were not vaccinated against hepatitis A. Due to the emergence of hepatitis A vaccine, hepatitis A has become increasingly a disease of adults in many parts of the world. The pathogenesis of acute hepatitis A tends to be dominated by host immune response, and HAV causes a weak interferon response in the liver of infected chimpanzees (75). Compared with $CD8^+$ T cell response, immune control of HAV may be more directly related with $CD4^+$ T cells (76). The frequency of HAV specific $CD8^+$ T cells in blood and liver of patients with jaundice may decrease with the clearance of infection (77).

In $Ifnar1^{-/-}$ transgenic mice, HAV induced hepatocyte apoptosis and inflammatory response are activated by innate immunity (78). Innate cytotoxic cells and Treg cells are transformed into inflammatory phenotypes in symptomatic infected individuals (79, 80). In HBV-infected PXB cells superinfected with HAV, HBV replication was reduced as compared to that in PXB cells infected with HBV alone, which means to a certain extent, HAV infection inhibits HBV replication (81). Earlier study also found that infection with HBV downregulated the expression of the two HBV proteins (HBsAg and PreS2) in PLC/PRF/5 cells (82). The sharp rise in IFN-γ production mediated by the acute HAV infection may be pivotal in the suppression of HBV replication in chronic hepatitis B (83). Fu et al. (84) retrospectively analyzed 211 HBV coinfection patients in a tertiary teaching hospital in China from 2005 to 2014, and 35 patients were coinfected with HAV. Patients with HAV coinfection generally had better outcomes than those with other viruses coinfection. Sagnelli et al. (85) reported that 3 of 9 patients with HBV/HAV coinfection became negative for HBsAg after 6-month follow-up. Beisel et al. (86) reported a 47-year-old patient with HBV-related compensated cirrhosis who had an acute HAV superinfection. The spontaneous HBsAg seroconversion occurred and the non-specific immunity of HAV led to functional cure of hepatitis B. Acute HAV superinfection may trigger sustained clearance of HBsAg in patients with chronic HBV infection.

HBV/HEV Coinfection

HEV is transmitted usually through fecal-oral pathway and occasionally through blood transfusion pathway (87). HEV is also a zoonotic virus in some genotypes. Acute HEV infection generally occurs in adults. Wong et al. (88) found that the seropositive rate of antiHEV-IgG was 19.86% among HBV infected patients by using the data of the National Health and Nutrition Examination Survey from 2011 to 2018. In a cross-

sectional study in Vietnam from 2012 to 2013, the seropositive rate of antiHEV-IgM was 11.6% among HBV infected patients (89).

In acute hepatitis E infection patients, the percentage of NK and NKT cells in peripheral blood monocytes decreased significantly, while the ratio of activated NK and NKT cells was higher than that in the uninfected group (90). The expression of activated NK cell markers Granzyme B and CD69 also increased significantly (91). The severe condition of pregnant women infected with HEV is related to the decrease of NK cell activity (92). In pregnant women with HEV infection, the inflammatory cytokine TNF-α, IL-6 and IFN-γ level increased significantly (93).

A large amount of evidence shows that TNF-α and NF-κB signaling pathways play an important role in stimulating inflammatory response in HEV. In cell culture, TNF-α has been shown to moderately inhibit HEV replication. Interestingly, it can cooperate with IFN-α anti HEV effect through NF-κB cascade inducing a subset of IFN-stimulated gene (ISG) (94).

HEV coinfection can accelerate the disease progression of patients with chronic HBV infection and increase the mortality of patients with liver cirrhosis. Acute HEV superinfection was associated with a 1-year mortality rate of 2.4% in non-cirrhotic patients with chronic HBV infection. The 1-year mortality rate increased to 35.7% in patients with compensated liver cirrhosis after HEV superinfection. HEV superinfection increased the long-term risk of cirrhosis, hepatocarcinoma, and liver-related death in patients with chronic HBV infection (95).

Compared with HBV monoinfection, the expression of cytokines related to hepatocyte necrosis such as IL-6, IL-10 and TNF-α increased in HBV/HEV coinfection patients (96). There are scarce and conflicting data regarding the replication of viruses in coinfection patients. The median level of HBV DNA in HBV/HEV coinfection patients is lower than that in HBV monoinfected patients. However, due to the small number of samples, it is not clear whether this difference is statistically significant. In addition, baseline HBV DNA are not available to compare with HBV DNA levels after HEV superinfection (97). The higher HBV DNA level in patients with HBV monoinfection may be explained by that HEV is an RNA virus, which may play a role of ribozyme in HBV DNA replication (96). There is no significant difference in HBV DNA levels between CHB/HAV coinfection patients and CHB/HEV coinfection patients (98). Yeh et al. (99) reported the disappearance of HBsAg in a renal transplant patient with chronic HBV/HEV coinfection. However, we cannot draw a conclusion about the effect of HEV on HBsAg from a single case, and further studies are required to evaluate this hypothesis.

HBV/HCV Coinfection

HCV is a single stranded RNA virus, which mainly leads chronic infection. Innate immune response is very important for HCV infection. It limits virus transmission by inducing apoptosis of infected hepatocytes and stimulates antigen specific adaptive immune response. NK cells destroy infected hepatocytes and cytokine release through cytolysis, which plays a vital role in the innate immune response to acute HCV infection.

IFN produced by NK cells can directly inhibit HCV replication. IFN-γ and TNF-α lead to maturation of dendritic cells, release of IL-12 and differentiation of CD4 and $CD8^+$ T cells. Specific $CD8^+$ T cells destroys HCV infected hepatocytes through human leukocyte antigen (HLA) class I antigen presenting cells and induces cytokines (TNF-α and IFN-γ) secretion. Helper $CD4^+$ T cells support this function through IL-2 to stimulate activation of $CD8^+$ T cells and NK cells (100).

During chronic HCV infection, the production of IL-2 by HCV specific CD4+ T cells decreases, resulting in impaired activation of CD8+ T cells. HCV core protein and PD-1 are also associated with T cell inhibition (101). The strong CD4+ T cell response during acute HCV infection is associated with virus clearance. The lack of strong CD4+ T cell response during acute infection and the decline of CD4+ T cell response after acute infection are related to chronic progression (102). Regulatory T cells such as CD25+ T cells can inhibit CD8+ cells and cytokines (such as IL-10 and transforming growth factor TGF-β) release to inhibit immune response during chronic HCV infection (103, 104).

HBV and HCV share the same transmission mechanism, thus coinfection of HBV and HCV is common, particularly in high endemic areas where individuals have a high risk of parenteral infection. The prevalence of HBV/HCV coinfection is approximately 5%-20% in HBsAg positive patients and 2%-10% in HCV-positive patients (105). A prevalence of overt HBV coinfection in HCV positive patients was reported at 1.4% in the United States (106).

Both HBV and HCV complete their life cycle in hepatocytes, and HCV core protein strongly inhibits HBV replication during HBV/HCV coinfection (107). A recent study found that HCV core protein inhibits HBV replication by downregulating HBx levels *via* Siah-1-mediated proteasomal degradation during coinfection (108). HCV core gene also inhibits the induction of an immune response to HBsAg. The observed interference effect of the HCV core occurs in the priming stage and is limited to the DNA form of the HBsAg antigen, but not to the protein form (109). HCV plays a dominant role, so high HCV RNA and low HBV DNA levels are observed in most cases with HBV/HCV coinfection. The cure of HCV infection may lead to HBV reactivation, and a meta-analysis showed that the pooled proportion of patients who had HBV reactivation was 24% in patients with chronic HBV infection and 1.4% in those with resolved HBV infection (110). HBV reactivation is the result of the weakening of hepatocyte IFN response after HCV clearance. Higher serum TNF-α at baseline and lower IFN-γ at week 4 were associated with mild clinical reactivation of HBV in HBV/HCV-coinfected patients receiving direct-acting antiviral agents (DAAs) (111). Chemokine ligand CXCL-10 (another name is interferon induced protein-10, IP-10), CCL5 and ALT have predictive value for HBV reactivation after HCV clearance (112). On the other hand, exogenous HBsAg stimulated NKG2D expression on NK cells from CHB patients, which inhibits HCV replication, suggesting that HBsAg may facilitate the clearance of HCV in HBV/HCV-coinfected patients (113).

In HBV/HCV coinfection patients, the HBsAg level is usually lower than that in HBV monoinfection patients, and the decrease of HBsAg production is also related to the increase of CXCL-10 level (114). A 5-year follow-up study in HBV/HCV coinfection patients showed that the cumulative HBsAg seroclearance rate was 30.0%, with 33.1% in the 48-week PEG-IFN plus ribavirin combination therapy group, and 24.3% in the 24-week therapy group (115). DAAs-treated HBV/HCV-coinfected patients had significantly higher rate of HBV seroclearance, particularly among those with low pre-treatment HBsAg titer; on the contrary, those with higher pre-treatment HBsAg titer were at greater risk of HBV reactivation (116).

HBV/HDV Coinfection

HDV is a defective virus, which relies on HBV for packaging, release and transmission. The global total prevalence of hepatitis D varies greatly in various literatures. The rate of HBsAg positive patients complicated with HDV infection ranges from 4.5% to 13.02% (117–121). This difference may be related with the inconsistent diagnostic criteria of hepatitis D. HBV/HDV coinfection can cause the most severe viral hepatitis.

Due to severe inflammation and necrosis of hepatic lobules, liver biopsy showed that the degree of liver injury during coinfection was almost twice that of HBV or HCV monoinfection (122). Due to HDV-induced interferon response (123), pronounced induction of innate immune responses (such as elevated cytokine levels of ISGs, TGF-β, IFN-γ, IP-10, etc.) may lead to a higher degree of liver inflammation compared with HBV monoinfection, resulting in a more severe infection process (124).

In Huh7 and HEK293 cells, large hepatitis D antigen (L-HDAg) can interfere with TNF-α-NF-κB signal transduction axis (125). L-HDAg can enhance TGF-β-c-Jun induced signal cascade, while TGF-β is the main regulator of liver fibrosis and cirrhosis (126). L-HDAg can also induce oxidative stress and activate NF-κB and signal transducer and activator of transcription-3 (STAT-3), leading to liver cirrhosis and cancer (127). HBsAg may increase the translocation of L-HDAg from nucleus to ER, and the translocation is accompanied by an increase in NF-κB activity (128). Compared with HBV monoinfection, the upregulation of antigen processing mechanism leads to higher efficiency of HBV epitope presentation in HBV/HDV coinfected cells, which can promote the recognition of infected cells by T cells (129).

Although HDV has been shown to inhibit HBV replication in many studies, serum HBsAg levels in patients with HBV/HDV coinfection are higher or equal than those in patients with HBV monoinfection (130, 131). HBV/HDV coinfected sequences exhibited certain unique mutations in HBsAg genes. Some of these mutations affected the generation of proteasomal sites, binding of HBsAg epitopes to MHC-I and -II ligands, and subsequent generation of T- cell epitopes. Selective amplification of these mutations at certain strategic locations might not only enable HBV to counteract the inhibitory effects of HDV on HBV replication, but also facilitate its survival by escaping the immune response (132). The percentage of conserved HBsAg-positions was significantly higher in HBV/HDV coinfection than HBV monoinfection. HDV can constrain HBsAg genetic evolution to preserve its fitness (133).

HBV/HIV Coinfection

HIV is a non-hepatophilic virus that mainly invades lymphocytes. The human immune function gradually loses and eventually leads to acquired immune deficiency syndrome (AIDS) by HIV infection. Host and virus jointly determine the disease progression after HIV infection, in which the activation level of innate immunity plays an important role (134).

Evolution during primary HIV infection does not require adaptive immune selection (135). It is found that DC, NK cells, macrophages and NKT cells play an important and irreplaceable role in innate immunity in long-term nonprogressors and elite controllers (that means with HIV-1 infection for many years, long-term asymptomatic, normal $CD4^+$ T cell count and no antiretroviral therapy) of HIV infection. Other innate immune cells are inefficient or even ineffective.

The progression of HIV infection may be related to the number and phenotypic function of DC. In patients with typical progression of HIV-1 infection, the number and phenotype of DC change with the progression of the disease (136), while DC in elite controllers can enhance and expand the ability to stimulate HIV specific $CD8^+$ T cell response by improving the internal immune recognition of HIV infected cells. Type I IFN secreted by DC plays an important role in inducing effective HIV specific $CD8^+$ T cell immunity (137).

The dysfunction of DC after HIV infection contributes to the persistence of the virus (138). NK cell activity is in the normal range in long-term nonprogressors, but decreased in patients with disease progression, indicating that NK cell activity is an important factor in controlling the progression of HIV infection (139). The levels of macrophage inflammatory protein (MIP), IP-10, monocyte chemoattractant protein-1 (MCP-1) and TGF decrease in elite controllers of HIV infection (140). Increased expression of CD224 on NKT cells is associated with HIV disease progression (141), and NKT cells in non-progression patients secrete more IFN-γ, IL-2 and TNF-α than those in progressive patients. These cytokines can significantly reduce HIV viral load and maintain a high number of $CD4^+$ T cells (142).

In addition to the difference of innate immunity, adaptive immunity, such as $CD8^+$ T cell function, has also been enhanced in HIV elite controllers. HIV-1 specific $CD8^+$ T cells of elite controllers can reduce HIV-1 replication in infected $CD4^+$ T cells by 60%-80%, and can also recognize resting infected $CD4^+$ T cells and kill these cells without virus activation (143–146). There are a small amount of HIV-1 specific $CD57^+$ $CD4^+$ T cells in elite controllers, which may play a direct role in killing virus infected cells and supplement the cytotoxic activity of HIV-1 specific $CD8^+$ T cells (147). Elite controls can produce effective anti-HIV-1 antibodies, and the frequency of preserved memory B cells is higher (148, 149).

A meta-analysis showed that the global rate of combined HBV infection in HIV patients was 7.6% (150). HIV coinfection has a negative impact on the progress of HBV infection, which

can lead to rapid progression to liver fibrosis and cirrhosis (151). Enhanced production of CXCL10 following coinfection of hepatocytes with both HIV and HBV may contribute to accelerated liver disease in the setting of HIV/HBV coinfection (152). It is known that HBV Pre-S deletion is closely related to HBV-associated terminal liver disease in HBV monoinfection. High-frequency Pre-S quasispecies deletions are predominant in HIV/HBV coinfection patients, providing a reference for the pathogenesis of the accelerated progression of liver disease in HIV/HBV coinfection (153). Even after effective antiretroviral therapy, the chronic immune activation of patients with HIV/HBV coinfection is higher than that of patients with HIV momoinfection. Chronic immune activation may lead to hepatic steatosis and cirrhosis, increase the risk of liver cancer (154). At the same time, the presence of active HBV infection will affect the viral immunological status of patients with HIV/HBV coinfection, which is characterized by the low number of $CD4^+$ T cells at the onset and the slow recovery of $CD4^+$ T cell count after antiretroviral treatment (155, 156).

HBsAg levels and HBsAg production were significantly higher in untreated HIV/HBV coinfection patients compared to HBV monoinfection patients. The highest HBsAg concentrations were observed in patients with more advanced HIV disease (157). Compared with patients with HBV monoinfection, successful long-term tenofovir dipivoxil (TDF) inclusive ART can increase the HBsAg seroclearance rate in HIV/HBV coinfection patients, reaching 3.2%-36% (158–173) (see **Table 1**). The longer the follow-up time, the higher the HBsAg seroclearance rate. Higher HBsAg seroclearance rate is associated with increased $CD4^+$ T cells. The sudden recovery of adaptive immunity causes immune reconstitution inflammatory syndrome, and then accelerates the production of protective antibodies. Therefore, immune reconstitution under antiretroviral therapy may affect the HBsAg serum conversion rate.

TABLE 1 | HBsAg seroclearance rate in HBV/HIV coinfection patients with TDF inclusive ART.

Publication year	Country or region of patients	Number of patients included	Main ART drugs	Mean follow-up time or therapy duration time	Number or rate of HBsAg seroclearance
2005 (158)	Germany	31	TDF	48 weeks	1/31 (3.2%)
2007 (159)	France	92	LAM	ART: 65 (1-155) months HARRT: 43 (1-93) months LAM: 36 (1-83) months	5/92 (5.4%)
2010 (160)	Dutch	102	TDF LAM ETV	5 years	10/102 (9.8%)
2012 (161)	Dutch	104	TDF	57 (34-72) months	8/104 (7.7%)
2012 (162)	Austria	110	LAM TDF FTC	5 years	HBeAg+: LAM: 8% TDF: 25% TDF+FTC: 27% HBeAg-: LAM: 11% TDF: 27% TDF+FTC: 36%
2013 (163)	Zambia South Africa	92	TDF LAM	12 months	LAM: 4/20 (20%)* TDF:3/17 (17.6%)*
2013 (164)	Thailand	47	LAM FTC TDF	168 weeks	6/47 (12.7%)
2014 (165)	France	111	TDF LAM FTC	74.7 (33.7-94.7) months	No detail data
2015 (166)	USA	99	TDF	5 years	18/99 (18.1%)
2015 (167)	Austria	111	TDF	74.2 (33.1-94.7) months	4/111 (3.6%)
2019 (168)	Taiwan, China	366	TDF LAM	5 years	15/366 (4.1%)
2020 (169)	Zambia	284	TDF	2 years	29/284 (10.2%)
2020 (170)	Australia Thailand	92	TDF	5 years	11/92 (12.0%) 11/72 (15.3%)#
2020 (171)	Germany	359	TDF TAF	11 years	66/359 (18.3%)
2021 (172)	France	165	TDF	15 years	13/165 (7.8%)
2022 (173)	USA	88	TDF FTC LAM	144 weeks	TDF+FTC: 30% FTC or LAM: 10%

TDF, tenofovir dipivoxil; FTC, emtricitabine; LAM, lamivudine; ETV, entecavir; TAF, tenofovir alafenamide; ART, antiretroviral therapy; HARRT, highly active antiretroviral therapy
**Because stored samples were unsuitable or not available, they only calculated documented data.*
#The data of 72 patients was available to year 5.

CONCLUSIONS

The pathogenesis of chronic hepatitis B is mainly related with immune mechanisms. The functional cure of hepatitis B with HBsAg seroclearance as the therapeutic target mainly depends on the immune response. When coinfected with HBV and other viruses, the body immune state becomes more complicated. In some cases, coinfection can improve the seroclearance rate of HBsAg. The specific mechanism needs to be further elaborated for better guiding the clinical application.

AUTHOR CONTRIBUTIONS

ML, LZ, and YX contributed to study concept and design. SW, WY, YG, WD, XB, YJL, LY, YL, RL, MC, GS, and LH collected and sorted out literatures. SW and LZ drew pictures. SW, WY, and YG wrote the first draft. ML and YX edited the English version. YX approved the submitted version after modification. All authors contributed to the article and approved the submitted version.

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Expression of Functional Molecule on Plasmacytoid Dendritic Cells Is Associated With HBsAg Loss in HBeAg-Positive Patients During PEG-IFN α-2a Treatment

Weihua Cao[1,2†], Si Xie[3†], Lu Zhang[1†], Xiaoyue Bi[1†], Yanjie Lin[4], Liu Yang[1], Yao Lu[1], Ruyu Liu[1], Min Chang[1], Shuling Wu[1], Ge Shen[1], Jianping Dong[5], Yao Xie[1,4*] and Minghui Li[1,4*]*

[1] Department of Hepatology Division 2, Beijing Ditan Hospital, Capital Medical University, Beijing, China, [2] Department of Infectious Diseases, Miyun Teaching Hospital, Capital Medical University, Beijing, China, [3] Division of Hepatology, Hepato-Pancreato-Biliary Center, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing, China, [4] Department of Hepatology Division 2, Peking University Ditan Teaching Hospital, Beijing, China, [5] Department of Infectious Diseases, Haidian Hospital, Beijing Haidian Section of Peking University Third Hospital, Beijing, China

Objective: The ideal endpoint of antiviral therapy in chronic hepatitis B (CHB) patients is to clear hepatitis B surface antigen (HBsAg). This study aimed to evaluate whether the expression of functional molecules on plasmacytoid dendritic cells (pDCs) is associated with HBsAg loss in HBeAg-positive patients during peginterferon alpha-2a (PEG IFN α-2a) therapy.

Methods: A single-center prospective cohort study was performed in HBeAg-positive CHB patients who were treated with PEG-IFN α-2a and followed up for 4 years. HBsAg clearance, HBeAg loss and undetectable HBV DNA achieved by PEG-IFN α-2a therapy was considered as functional cure. The frequencies of pDC and $CD86^+$ pDC in peripheral blood, and the mean fluorescence intensity of CD86 (CD86MFI) on the surface of pDC were measured at starting therapy, after 12 and 24 weeks of therapy.

Results: Of 63 patients enrolled, 17 patients achieved HBsAg loss. The baseline HBV DNA load in Non-functional-cure group was significantly higher than that in Functional cure group, and the $CD86^+$ pDC% was significantly lower in patients without functional cure. HBV DNA load (OR=0.146, P = 0.002) and $CD86^+$ pDC% (OR=1.183, P = 0.025) were independent factors associated with functional cure confirmed by binary logistic regression analysis. In the Functional cure group, HBsAg, HBeAg, and HBV DNA loads decreased remarkably after 12 weeks and 24 weeks of treatment compared to baseline. In Non-functional-cure group, $CD86^+$ pDC% and CD86MFI increased significantly from baseline after 12 weeks of treatment. In the Functional cure group, compared with baseline, pDC% increased significantly at 24 weeks, while CD86MFI increased significantly after 24 weeks of treatment.

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Feng Li,
Guangzhou Eighth People's Hospital, China

Reviewed by:
Lu Junfeng,
Capital Medical University, China
Chengli Shen,
The Ohio State University, United States

***Correspondence:**
Minghui Li
wuhm2000@sina.coml
Yao Xie
xieyao00120184@sina.com
Jianping Dong
13611351665@163.com

†These authors have contributed equally to this work

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Conclusion: The lower the baseline HBV DNA load and the more the baseline $CD86^+$ pDC%, the easier it is for patients to obtain functional cure.

Keywords: hepatitis B surface antigen, chronic hepatitis B, interferon, functional cure, plasmacytoid dendritic cells

INTRODUCTION

About 250-300 million people are chronically infected with HBV (1, 2). Chronic hepatitis B (CHB) remains an important worldwide public health problem, and long-term chronic infection can result in liver cirrhosis, hepatic failure and hepatocellular carcinoma (3). There is an urgent need to explore optimized therapeutic strategy for CHB. Moreover, antiviral therapy is the most effective treatment for slowing down the progression of CHB. Currently, "functional cure" defined as a sustained HBsAg loss and undetectable HBV DNA, with or without HBsAg seroconversion in serum, is the endpoint of antiviral treatment for CHB (4, 5). In the natural process of chronic HBV infection, HBsAg loss is as rare as 1.15% per year (6). Several studies indicated that degradation or permanent inactivation of cccDNA can contribute to achieving the "functional cure" of CHB (7–9). In the current antiviral therapies, peginterferon alpha-2a (PEG IFN α-2a), as a first-line antiviral drug, has direct antiviral as well as immunomodulatory activity (10, 11). PEG-IFN-a therapy for CHB can result in HBeAg clearance, HBsAg loss and even HBsAg seroconversion in about 10%-30% patients within a certain period of time (12–15).

In addition to the direct antiviral activity, the immunomodulatory effect of IFN-α may eventually induce an immunological control of hepatitis B infection (10, 11). As a vital component of early antiviral innate immune response, plasmacytoid dendritic cells (pDCs) are the main effector cells producing IFN α *in vivo* in response to viral infection (16). In chronic HBV infection, pDCs' function is markedly impaired by hepatitis B virology (17–19). HBsAg blocks the IRF7 phosphorylation signal pathway induced by TLR9, resulting in decrease of IFN-α generation and impaired expression of costimulatory molecules (such as CD80, CD86, CD83) on the surface of pDCs (17, 20, 21). Martinet et al. have proven that pDCs with defective responses to stimulation with Toll-like receptor 9 ligand (TLR9-L) did not induce cytolytic activity of NK cells in hepatitis B patients (22). Other study revealed that, acting as an immune tolerant protein, HBeAg may promote DCs differentiation into regulatory dendritic cells (DCregs) which can induce naive T cells to differentiate into Tregs to impair virus-specific cytotoxic T lymphocyte (CTL) response (23). Therefore, pDCs dysfunction may be a crucial cause for the persistence of hepatitis B infection. Active immunotherapy based on pDCs may become a prospective method to treat CHB.

Previous studies suggested that PEG-IFN-α can activate pDCs in the early stage of chronic hepatitis B (24–26). These studies illustrated the changes of immune cell function in the process of interferon treatment of CHB, but little is known about the frequency and functional changes of pDCs in CHB patients with functional cure after PEG-IFN α treatment. Therefore, this study aims to explore whether the frequency of pDCs and the expression of functional molecules on pDCs are associated with HBsAg loss in HBeAg-positive CHB patients during PEG-IFN α-2a therapy.

MATERIALS AND METHODS

Patients

This prospective study was performed in the Liver Disease Center of Beijing Ditan Hospital, Capital Medical University between October 2014 and October 2017, and 63 HBeAg-positive CHB patients as naïve therapy were enrolled. The ethical approval of this study was obtained from the Ethics Review Committee of Beijing Ditan Hospital of Capital Medical University. All participants provided written patients' informed consent for sample collection and subsequent follow-up. This study was registered at clinicaltrials.gov (Clinical Trials.gov ID: NCT03210506).

The enrollment criteria were: patients with HBsAg and HBeAg positivity for more than 6 months, detectable HBV DNA, and abnormal alanine aminotransferase (ALT) levels lasting for at least 3 months, or liver biopsy revealing significant inflammation. The exclusion criteria were: 1) patients co-infected with other viruses such as HIV, HDV, HCV; 2) carrying HIV or syphilis antibodies; 3) accompanied with other liver diseases such as alcoholic liver disease, fatty liver, metabolic liver disease, autoimmune hepatitis, liver cirrhosis, or hepatic cancer; 2) using hormone or immunomodulatory medication during the 4-year follow-up period.

These patients were assigned to receive personalized PEG-IFN α-2a 180 μg subcutaneous injection weekly and complete a 4-year follow-up. These patients received PEG-IFN α-2a monotherapy for 24 weeks. If HBV DNA turned negative, patients continued to receive PEG-IFN α-2a monotherapy. Otherwise, patients received PEG-IFN α-2a combined with entecavir or tenofovir therapy according to the patients' past medication history or physical condition. In the subsequent treatment, if HBsAg and HBeAg declined continuously and even reached functional cure, patients continued to receive PEG-IFN α-2a consolidation treatment for 12-24 weeks; however, if HBsAg and HBeAg did not continue to decline and remain in the plateau phase, PEG-IFN α-2a was stopped and ETV or TDF single-agent maintenance therapy was continued. Serum HBV DNA loads, HBsAg/anti-HBs, HBeAg, and HBe antibody (HBeAb) levels were detected before treatment and thereafter every 12 weeks until HBsAg loss. The frequency and function of pDCs were measured before treatment as the baseline and at weeks 12 and 24 of treatment.

Definition of Functional Cure

The American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL) set the hepatitis B therapy endpoints as functional cure, which is defined as sustained HBsAg loss assayed with a lower limit of HBsAg detection of 0.05 IU/mL, with or without HBsAg seroconversion, undetectable HBV DNA and the persistent transcriptional inactivation of cccDNA after the end of the treatment.

Clinical Indicators Detection

The Roche Cobas AmpliPrep/Cobas TaqMan 96 full-automatic real-time fluorescent quantitative PCR detection reagent (Roche, Pleasanton, CA) was applied for the detection of serum HBV DNA loads; the Abbott Architect i2000 Detection Reagent (Abbott Diagnostics, Abbott Park, IL) was used to test Serum HBsAg/anti-HBs, HBeAg, and HBeAb levels; and Hitachi 7600 full-automatic biochemical analyzer was applied to measure the parameters of liver function.

Analysis of pDCs by Flow Cytometry

Peripheral venous blood samples from these patients were collected into EDTA anticoagulant tubes to analyze pDCs within 4 h. Three monoclonal antibodies (mouse anti-human leukocyte antigen (HLA)-DR (Clone L243), mouse anti-human CD123 (Clone 7G3) and mouse anti-human lineage cocktail 1 (Lin 1) (CD3, Clone SK7; CD14, Clone MøP9; CD16, Clone 3G8; CD19, Clone SJ25C1; CD20, Clone L27; CD56, Clone NCAM16.2) from BD Biosciences (San Jose, CA) were added into blood to label pDCs, then mouse anti-human CD86 (Clone 2331 (FUN1) from BD Biosciences (San Jose, CA) was added to label the costimulatory molecules CD86 on the surface of pDCs in the dark at room temperature for 20 min. Then 2 mL red blood cell lysis buffer (BD Biosciences) was evenly mixed into the above samples and left aside for 5 min in the dark at room temperature. Cell debris in the supernatant was removed by centrifugation at 1200 rpm for 5 min. Then, the supernatant was discarded and cells were washed with 2 mL of 1 x PBS which were spun (1200 rpm, 5 min). Finally, after discarding the supernatant and adding the 200 μL of 1 x PBS, pDCs were detected. pDCs were quantified by 4-color FACSCalibur (BD Biosciences) and FlowJo 7.6.1 software, as shown in **Figure 1**. The proportion of pDC in peripheral blood mononuclear cells was regarded as the frequency of pDC (pDC%). pDCs which express costimulatory molecule CD86 on the surface were regarded as $CD86^+$ pDCs. The proportion of $CD86^+$ pDC in pDCs was regarded as the frequency of $CD86^+$ pDC ($CD86^+$ pDC%). $CD86^+$ pDC% and CD86MFI were used to reflect the function of pDC.

Statistical Analysis

Data were performed using SPSS for Windows software, version 26 (SPSS Inc., Chicago, IL, USA), and Prism software (GraphPad Software version 5.01). All data were described as mean ± standard deviation (SD) or median (Q1, Q3). Changes of the parameters at different time points were analyzed by repeated-measures analysis of variance; Bonferroni was used to adjust *P* values for multiplicity, and the correction level was α. There were three observation time points for pDC/PBMC%, $CD86^+$ pDC% and CD86MFI. α was set as 0.016, and $P<0.016$ was considered to be statistically significant. Comparisons between two groups were carried out Using the Mann-Whitney nonparametric *U* test. The association of variables was assessed by Spearman's correlation. Independent factors of functional cure were analyzed by binary logistic regression analysis. All analyses of significance were two-tailed, and *P* value of less than 0.05 was considered as statistically significant.

RESULTS

Characteristics of Enrolled Patients

In this study, 63 HBeAg positive CHB patients received PEG-IFN-α-2a personalized treatment and completed the 4-year follow-up. There were 38 males and 25 females, with an average age of 30 years (20-51 years). After PEG-IFN α-2a treatment, 17 cases achieved functional cure. Two patients in the Functional cure group and 6 patients in the Non-Functional-cure group were lost to follow-up at 12 or 24 weeks of treatment.

There was no significant difference in serum HBsAg, HBeAg, or ALT levels between the two groups at baseline, but HBV DNA load in Non-functional-cure group was significantly higher than that in Functional cure group ($P = 0.007$, **Table 1**). At the 12^{th} week and the 24^{th} week, HBsAg levels (12w $P=0.000$, 24w $P=0.000$), HBeAg levels (12w $P=0.036$, 24w $P=0.016$) and HBV DNA loads (12w $P=0.014$, 24w $P=0.001$) in Functional cure group were significantly lower than those in Non-functional-cure group as shown in **Table 1**. We analyzed the relationship between the dynamic changes of clinical indicators and HBsAg clearance by binary logistic regression, but found no correlation. For the frequency of pDC and the expression of costimulatory molecule CD86, the pDC% ($P = 0.620$) and CD86MFI ($P = 0.114$) were not different between the two groups, while Functional cure group had higher $CD86^+$ pDC% ($P = 0.037$) than Non-functional-cure group (**Table 2**).

Binary logistic regression was used to analyze the correlation of baseline serological and virological indexes, pDC frequency and CD86 expression with the occurrence of HBsAg loss after PEG-IFN therapy. Results showed that HBV DNA load (OR = 0.146, 95% CI (0.044, 0.489), $P = 0.002$) and $CD86^+$ pDC% (OR = 1.183, 95% CI (1.021, 1.370), $P = 0.025$) were two independent factors associated with functional cure.

Correlations Between pDCs and HBV Virological Characteristics, ALT Before PEG-IFN Therapy

Before treatment, there was no correlations between the frequency of pDC and the expression of surface costimulatory molecule CD86 with the baseline virological markers, including HBV DNA load, HBsAg level and HBeAg concentration (**Figure 2**).

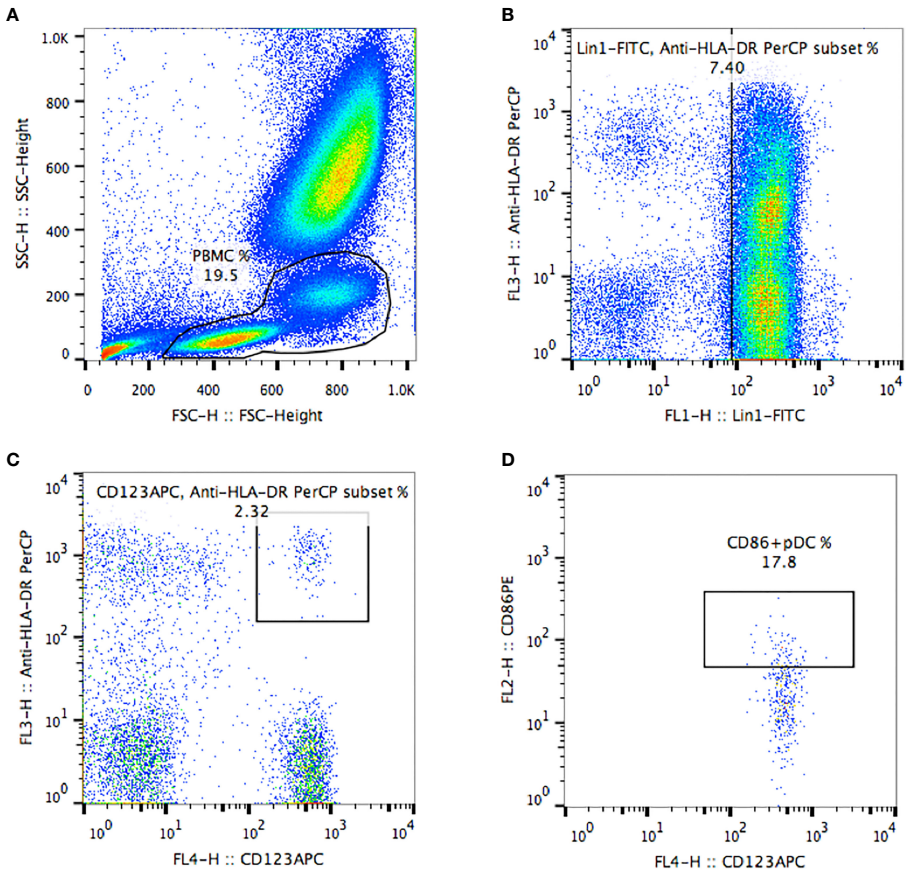

FIGURE 1 | The frequency of peripheral blood pDCs and expression of costimulatory molecule CD86 were analyzed by FlowJo software. **(A)** Peripheral blood mononuclear cells were delineated according to forward scatter and side scatter. **(B, C)** pDCs were delineated based on anti-human HLA-DR, anti-human lineage cocktail, and anti-human CD123 in mononuclear cells. **(D)** Delineation of $CD86^+$ pDCs was based on the CD86-PE marker in pDCs. HLA-DR+ CD123+ lineage-cells were defined as pDCs. The pDC proportion among peripheral blood mononuclear cells was defined as pDC frequency (pDC%). pDCs expressing costimulatory molecule CD86 on the surface were defined as $CD86^+$ pDCs. The $CD86^+$ pDC proportion among pDCs was defined as $CD86^+$ pDC frequency ($CD86^+$ pDC%). Quantification of pDCs included the frequencies of pDCs and $CD86^+$ pDCs and the mean fluorescence intensity of CD86 (CD86MFI).

TABLE 1 | Characteristics of subjects.

Item	All patients N=63	Baseline		*P** value	12w		*P** value	24w		*P** value
		Non-functional-cure group N = 46	Functional cure group N=17		Non-functional-cure group N = 40	Functional cure group N=15		Non-functional-cure group N = 40	Functional cure group N = 15	
Male/Female	38/25	30/16	8/9		25/15	6/9		25/15	6/9	
Age (yrs)	30 (20–51)	30 (20–51)	32 (24–49)	0.625	30 (20–51)	32 (24–49)	0.857	30 (20–51)	32 (24–49)	0.857
HBsAg level (log10 IU/mL)	3.857 ± 0.692	3.905 ± 0.687	3.727 ± 0.710	0.345	3.377 ± 0.702	1.797 ± 1.460	0.000	3.196 ± 0.802	1.502 ± 1.654	0.000
HBV DNA load (log_{10} IU/mL)	7.059 ± 1.161	7.330 ± 0.935	6.324 ± 1.405	0.007	4.095± 1.555	2.922 ± 1.447	0.014	2.084± 0.760	1.233 ± 0.879	0.001
HBeAg concentration (PEIU/mL)	902.260 (411.970, 1403.620)	916.210 (371.640, 1396.338)	880.570 (441.715, 1409.685)	0.975	59.895 (16.438, 406.53)	29.94 (0.36, 124.35)	0.036	18.92 (5.728, 82.75)	1.43 (0.33, 32.36)	0.016
ALT level (U/L)	253.300 (129.600, 361.100)	240.700 (128.350, 361.300)	273.000 (135.250, 371.150)	0.361	67.15 (49.45, 82.05)	48.30 (20.70, 86.80)	0.186	44.25 (28.15, 56.425)	26.90 (22.40, 64.50)	0.177

**Non-functional-cure group vs. Functional cure group.*

TABLE 2 | Baseline pDC frequency and expression of costimulatory molecule CD86 between two groups.

Item	All patients (n = 63)	Non-functional-cure group (n = 46)	Functional cure group (n = 17)	*P* value*
Male/Female	38/25	30/16	8/9	
pDC/PBMC	23.601 ± 11.116	24.414 ± 12.001	21.767 ± 8.276	0.620
$CD86^+$ pDC (%)	22.543 ± 8.203	22.109 ± 10.599	25.948 ± 6.542	0.037
CD86MFI	61.000 (49.600, 79.900)	62.600 (49.900, 80.300)	56.500 (47.000, 65.400)	0.114

**Non-functional-cure group vs. Functional cure group.*

PDC Function and Treatment Response During PEG-IFN- α Treatment

The frequency of pDC were not significantly different between two groups at baseline ($P = 0.524$), 12 weeks ($P = 0.282$), or 24 weeks ($P = 0.441$) after treatment. The baseline $CD86^+$ pDC% in Non-functional-cure group was significantly lower than that in Functional cure group ($P = 0.018$); however, there were no difference at the 12^{th} week and the 24^{th} week between two groups. The CD86MFI on the pDC surface were similar between two groups throughout the treatment (**Figure 3**). The baseline *P* values in **Table 2** and **Figure 3** were different due to the number of patients. **Table 2** showed the data of total number

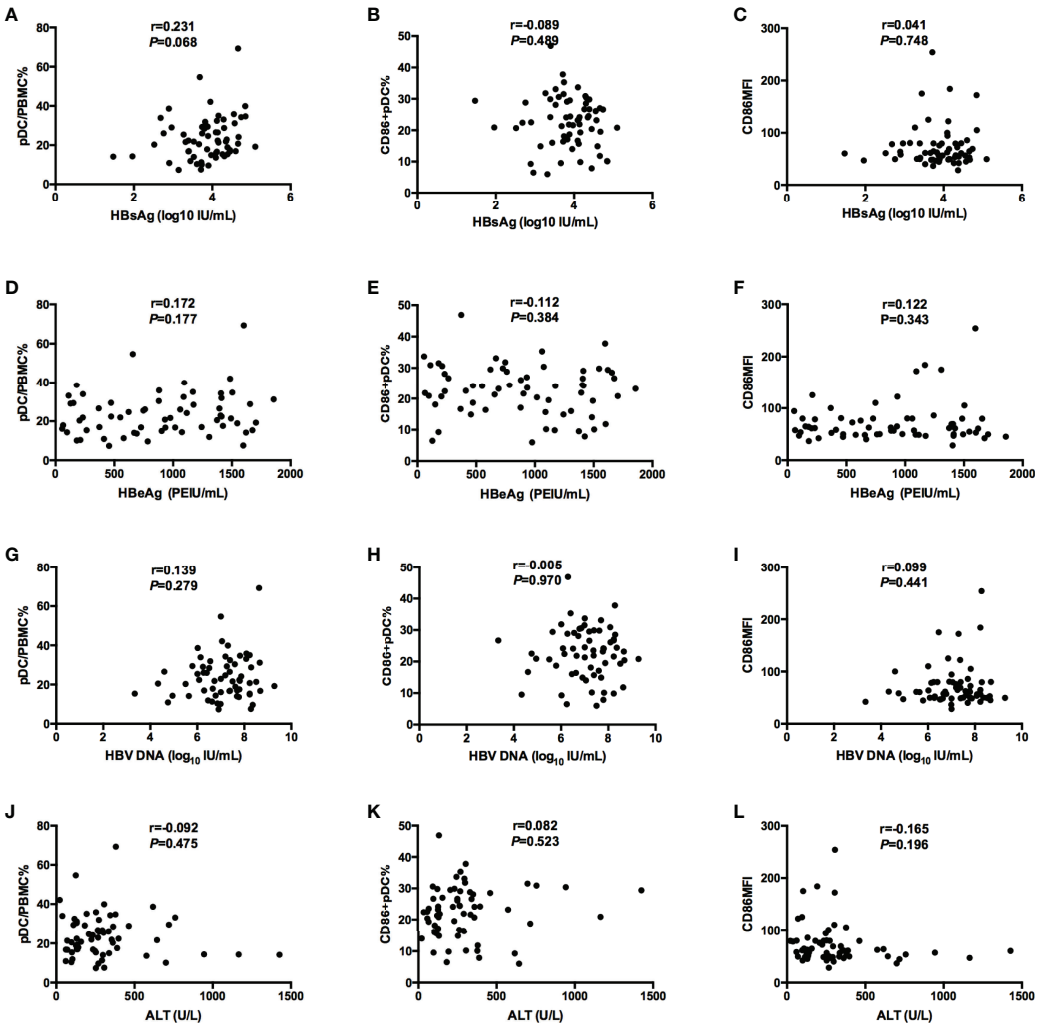

FIGURE 2 | Correlations between pDC frequency or function (pDC%, CD86+ pDC%, and CD86MFI) and serological and virological indicators (HBsAg, HBeAg, and HBV DNA, ALT). **(A–C)** Correlations between pDC frequency or function [pD% **(A)**, CD86+ pDC% **(B)**, and CD86MFI **(C)**] and HBsAg. **(D–F)** Correlations between pDC frequency or function [pDC% **(D)**, CD86+ pDC% **(E)**, and CD86MFI **(F)**] and HBeAg. **(G–I)** Correlations between pDC frequency or function [pDC% **(G)**, CD86+ pDC% **(H)**, and CD86MFI **(I)**] and HBV DNA. **(J–L)** Correlations between pDC frequency or function [pDC% **(J)**, CD86+ pDC% **(K)**, and CD86MFI **(L)**] and ALT.

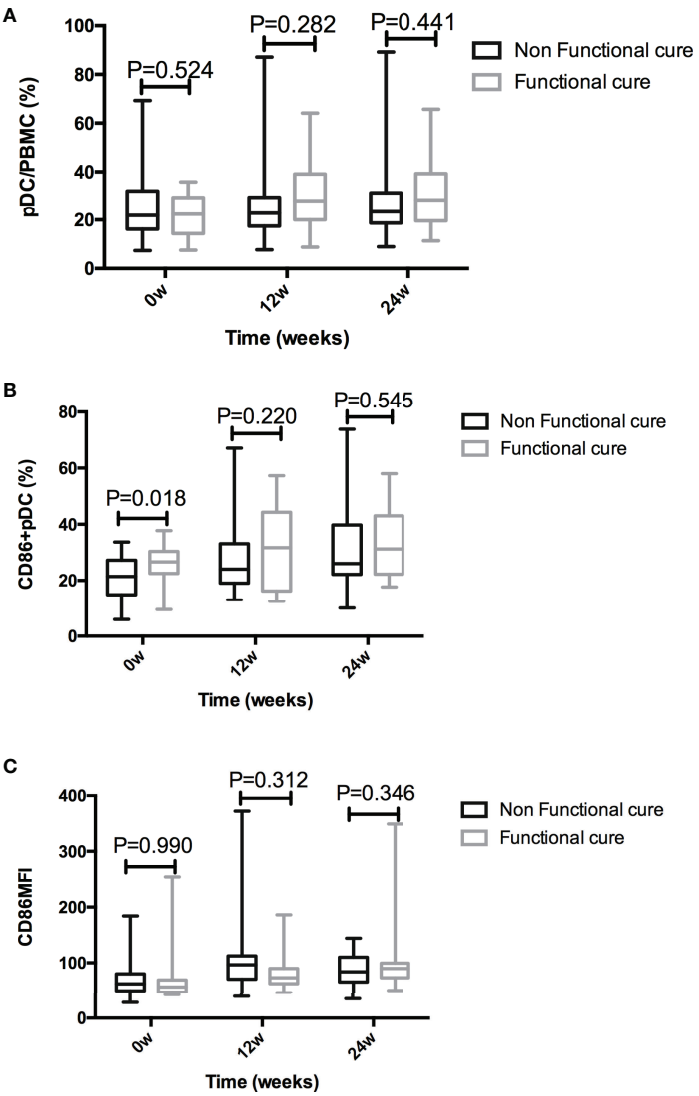

FIGURE 3 | Comparison of pDC% **(A)**, CD86+ pDC% **(B)**, and CD86MFI **(C)** between the Functional cure group and Non-functional-cure group at baseline and after PEG-IFN-a-2a therapy for 12 and 24 weeks.

of patients. **Figure 3** showed the statistical analysis after excluding the lost follow-up patients (2 in the Functional cure group, 6 in the Non-functional-cure group).

In addition, compared with baseline, the frequency of pDC in Non-functional-cure group did not change after PEG-IFN α-2a treatment, while $CD86^+$ pDC% and CD86MFI increased significantly after 12 and 24 weeks of treatment. In Functional cure group, the frequency of pDC and $CD86^+$ pDC, and CD86MFI increased significantly from baseline after treatment (**Figure 4**).

We analyzed the correlations between pDC frequency or function and serological and virological indexes respectively in Non-functional-cure group and Functional cure group in 12 weeks and 24 weeks, but found no correlations between them (**Figures S1**, **S2**). There were no correlations between the levels of $CD86^+$ pDC% and HBV viral load at week 12 and 24 for the Functional cure and Non-functional-cure group either (**Figure S3**).

DISCUSSION

Although pDCs exist as a small proportion of the total cells in blood, they are the main effector cells producing type I interferon *in vivo* and play a vital role in HBV infection. However, functional defects in pDCs have been reported in patients with chronic HBV infection (18, 19, 27). At present, the ultimate goal of CHB treatment is to achieve functional cure, which requires antiviral immune response. PEG-IFN-α has not only antiviral effect, but also immunomodulatory function (10, 11). Based on the above theory, we dynamically described the changes of pDCs

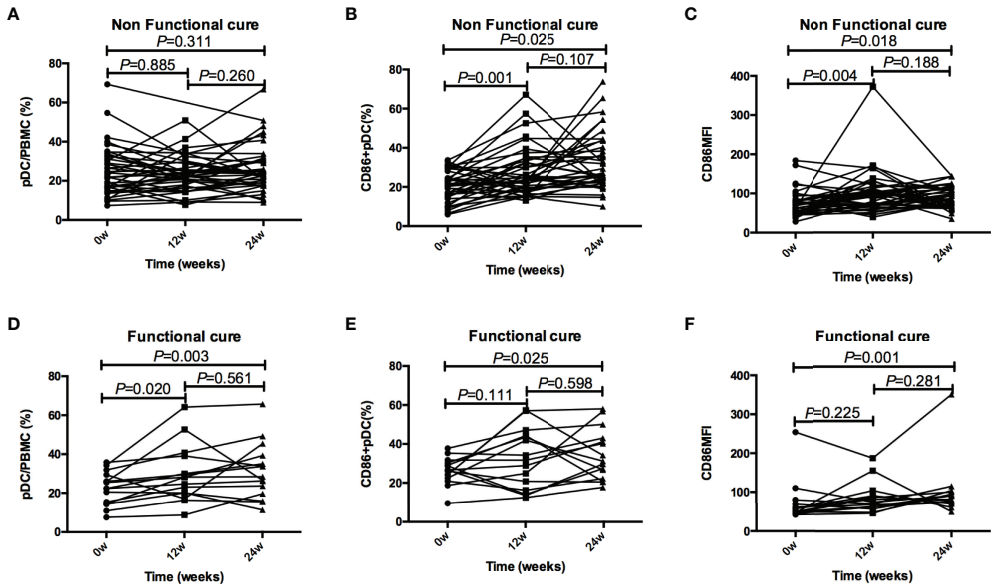

FIGURE 4 | Tendencies of pDC frequency and function, including pDC%, CD86+ pDC%, and CD86MFI, at baseline and after 12 and 24 weeks of treatment in the Functional cure group and Non-functional-cure group. **(A–C)** Tendencies of pDC% **(A)**, CD86+ pDC% **(B)**, and CD86MFI **(C)** at baseline and after 12 and 24 weeks of treatment in the Non-functional-cure group. **(D–F)** Tendencies of pDC% **(D)**, CD86+ pDC% **(E)**, and CD86MFI **(F)** at baseline and after 12 and 24 weeks of treatment in the Functional cure group. The correction level a was set as 0.016, $P < 0.016$ was considered to be statistically significant.

in patients with CHB in the early stage of PEG-IFN-α-2a treatment, and explored whether the frequencies of pDCs and expression of functional molecules on the surface of pDCs are correlated with functional cure of CHB patients with HBeAg positivity during PEG-IFN α-2a therapy. Our study highlighted the function of pDCs in achieving functional cure and brought a fresh perspective of immunologic changes triggered by PEG-IFN α therapy in HBeAg positive CHB patients.

Our data showed that baseline HBV DNA load in patients with functional cure was significantly lower than that in patients without functional cure. The results of binary logistic regression demonstrated that baseline HBV DNA load was an independent influencing factor of functional cure, which is in line with reports indicating that the lower the viral load, the easier to obtain functional cure (28, 29). The viral load is at a relatively low level, and its transcription and replication activity is low, resulting that the production of viral protein is reduced, which is consistent with our result: virological indicators in patients with functional cure significantly decreased during PEG-IFN α treatment. Nevertheless, previous studies revealed that low baseline HBsAg was more reliable than serum HBV DNA levels for predicting good response to antiviral treatment in HBeAg-positive patients (30–34). Actually, the decrease of HBsAg indicates the decrease of cccDNA (35, 36), which is important for predicting the outcome of PEG-IFN α treatment in CHB patients. Theoretically, low baseline HBsAg is more likely to achieve HBsAg loss, but our data suggested that there was no significant difference in baseline HBsAg levels between the two groups, which may be due to the small sample size of our study.

HBV itself impairs the function of pDCs. As a bridge linking innate immunity and adaptive immunity, pDCs play a critical role in the immune response to HBV infection, such as producing type I interferon, antigen-presenting, and stimulating NK cells, T cells and other immune responses mediated by adaptive T cells (37–39). The function defect of pDCs may lead to deficiency of HBV immune elimination and persistent chronic infection. Actually, an effective antiviral immune response requires the involvement of functional pDCs. These functions of pDCs depend on maturation and activation of pDCs. A variety of costimulatory molecules are highly expressed on the surface of pDCs. CD86, as one of the costimulatory molecules, promotes the maturation and activation of pDCs. We analyzed the expression of CD86 during the early antiviral therapy in HBeAg (+) CHB patients to indirectly evaluate pDCs function. Our data suggested that the baseline $CD86^+$ pDC% in patients with functional cure was higher than that in patients without functional cure, and the baseline $CD86^+$ pDC% was an independent influencing factor of functional cure. At baseline, there was a significant difference in $CD86^+$ pDC% between Functional cure group and Non-functional-cure group, indicating a difference in immune basis between them. The differences between the two groups at 12 and 24 weeks of treatment became insignificant owning to PEG-IFN treatment. The more the baseline $CD86^+$ pDC%, the easier it is for patients to obtain functional cure. Moreover, after PEG-IFN α treatment, CD86 expression increased significantly, especially in patients with functional cure. Based on these results, it may be concluded that the increase in CD86 expression manifested the enhancement of pDCs function, which in turn promotes HBV clearance and even HBsAg loss.

In summary, our data shed new light on the correlation between HBsAg loss and functional changes of pDCs in the early PEG-IFN α treatment of HBeAg positive CHB patients. We observed that the

lower the HBV DNA load, the more the frequency of $CD86^+$ pDC before PEG-IFN-α treatment. The increase in frequency of pDC and the higher expression of functional molecules CD86 after PEG-IFN α treatment are more helpful for HBsAg loss. Generally, HBsAg loss/functional cure depends on the low titer HBV DNA load, the increase in the number of pDCs, and the recovery of function in the early stage of PEG-IFN α therapy. The findings could help provide a new therapeutic strategy for chronic hepatitis B in the pursuit of functional cure.

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 Beijing Ditan Hospital Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WC, SX, ML, YX, and JD conceived and designed this research. LZ, XB, YLi, and LY conducted experiments and collected the data. YLu, LZ, RL, MC, SW, and GS collected the sample information of the patients and ordered reagents and materials. WC performed the statistical results and wrote the manuscript with assistance from YX, ML, and SX. The final manuscript was approved by all authors.

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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.2022.891424/full#supplementary-material

Supplementary Figure 1 | The correlations between pDC frequency or function and serological and virological indexes in Functional cure group in 12 weeks and 24 weeks. **Δ**: the dynamic changes of virological and clinical indicators from baseline to 12 weeks or from baseline to 24 weeks.

Supplementary Figure 2 | The correlations between pDC frequency or function and serological and virological indexes in Non-functional-cure group in 12 weeks and 24 weeks. **Δ**: the dynamic changes of virological and clinical indicators from baseline to 12 weeks or from baseline to 24 weeks.

Supplementary Figure 3 | The association between the levels of $CD86^+$ pDC% and HBV viral load at week 12 and 24 for the Functional cure and Non-Functional cure group.

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Effect of Antiviral Therapy During Pregnancy on Natural Killer Cells in Pregnant Women With Chronic HBV Infection

Fuchuan Wang[1‡], Si Xie[2‡], Chongping Ran[3‡], Hongxiao Hao[3‡], Tingting Jiang[3], Wen Deng[3], Xiaoyue Bi[3], Yanjie Lin[4], Liu Yang[3], Fangfang Sun[3], Zhan Zeng[4], Yao Xie[3,4†], Minghui Li[3,4*†] and Wei Yi[1*†]*

[1] Department of Gynecology and Obstetrics, Beijing Ditan Hospital, Capital Medical University, Beijing, China, [2] Division of Hepatology, Hepato-Pancreato-Biliary Center, Beijing Tsinghua Changgung Hospital, School of Clinical Medicine, Tsinghua University, Beijing, China, [3] Department of Hepatology Division 2, Beijing Ditan Hospital, Capital Medical University, Beijing, China, [4] Department of Hepatology Division 2, Peking University Ditan Teaching Hospital, Beijing, China

Objective: To study the effect of antiviral therapy during pregnancy on the frequency of natural killer (NK) cells in peripheral blood of women with HBV DNA positive chronic hepatitis B (CHB).

Method: In total 124 female subjects were divided into four groups: 11 healthy non-pregnant women (Normal group), 26 non-pregnant women in immune tolerance period of chronic hepatitis B virus (HBV) infection (CHB group), 41 pregnant CHB women without antiviral treatment during pregnancy (Untreated group), and 46 pregnant CHB women receiving antiviral treatment during pregnancy (Treated group). The frequency of NK cells in peripheral blood were detected by flow cytometry.

Result: The frequency of NK cells in healthy women [15.30 (12.80, 18.40)] was higher than that in women with HBV infection, but there was no significant statistical difference (p=0.436). The frequency of NK cells in CHB group [10.60 (6.00, 18.30)] was higher than those in pregnant CHB women [Untreated: 6.90 (4.89, 10.04), P=0.001; Treated: 9.42 (6.55, 14.10), P=0.047]. The frequency of NK cells in treated group was significantly higher than that in untreated group (P = 0.019). The frequencies of NK cells, $CD56^{bright}$ NK cells and $NKp46^{dim}$ NK cells at 12 and 24 weeks postpartum in the untreated group were increased significantly than those before delivery. In treated group, the frequencies of NK cells, $CD56^{bright}$ NK cells, $NKp46^{+}$ NK cells and $NKp46^{dim}$ NK cells were significantly increased at 6 and 12 weeks than those before delivery. The frequencies of NK cells and $CD56^{bright}$ NK cells postpartum were increased significantly in treated group than those in untreated group. The frequencies of $CD56^{dim}$ NK cells decreased significantly after delivery in treated than those in untreated patients. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) significantly increased after delivery than those before

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Shanghai General Hospital, China

***Correspondence:**
Wei Yi
yiwei1215@163.com
Minghui Li
wuhm2000@sina.com
Yao Xie
xieyao00120184@sina.com

†ORCID ID:
Minghui Li
orcid.org/0000-0003-3233-5473
Wei Yi
orcid.org/0000-0003-4241-8205
Yao Xie
orcid.org/0000-0003-4108-7037

‡These authors have contributed equally to this work

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delivery. The results showed that the postpartum ALT level was weak positive correlated with NKp46^{high} frequency (r=0.199) and was weak negative correlated with NKp46^{dim} frequency (r= -0.199).

Conclusion: Antiviral treatment during pregnancy could significantly increase the frequency of NK cells postpartum. Postpartum hepatitis may be related to the immune injury caused by change of NK cell frequency and HBV infection.

Keywords: hepatitis B virus, natural killer cells, chronic hepatitis B, antiviral treatment, postpartum

INTRODUCTION

Mother-to-child transmission of hepatitis B virus (HBV) is the main cause of chronic HBV infection in China (1, 2). For HBV DNA positive pregnant women, antiviral treatment in the third trimester of pregnancy can further reduce the mother-to-child transmission of HBV. Postpartum hepatitis is common in women with chronic hepatitis B (CHB). Our previous research suggests that postpartum hepatitis mainly occurs in pregnant women with HBV DNA > 5.01 log10 IU/ml before delivery, while abnormal postpartum liver function in patients with HBV infection mainly occurs at 3-4 weeks and 9-12 weeks postpartum (3).

Natural killer (NK) cells are one of the important effector cells in antiviral immunity. The amount and function of NK cells decrease in course of chronic HBV infection. To achieve immune recovery by antiviral therapy, recovering of the number and function of NK cells is of great significance. At present, there are few studies on the effect of antiviral therapy on immunological characteristics of postpartum NK cells in CHB patients.

In the present study, we designed a prospective case cohort study in HBV DNA positive CHB pregnant women. We detected the frequencies of NK cell in peripheral blood before delivery and at 6, 12, and 24 weeks after delivery to explore the correlation of NK cells with antiviral therapy in pregnant women with HBV infection.

MATERIALS AND METHODS

Patients

From January 2017 to January 2018, 124 patients from the Department of Gynecology and Obstetrics, Beijing Ditan Hospital, Capital Medical University were included in this study, including 11 healthy cases with hepatitis B surface antigen (HBsAg) negative and normal liver function (Normal group). All patients with hepatitis B virus were immune tolerant patients (HBV DNA> 2.0×10^7 IU/ml, hepatitis B e antigen and HBsAg continued to be positive more than six months, biochemical examination, imaging examination and other noninvasive examinations showing no clinical characteristics of hepatitis attack). 26 cases of non-pregnant women in immune tolerance period of HBV infection (CHB group), 41 cases of HBV infected pregnant women without antiviral treatment (Untreated group), and 46 pregnant women with HBV infection who received tenofovir disoproxil fumarate (TDF) antiviral therapy at 32 week of pregnancy and stopped immediately after delivery (Treated group). All subjects signed 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).

Pregnant women have regular prepartum examination. Chronic hepatitis B was diagnosed according to the EASL Clinical Practice Guidelines (4). Exclusion criteria were: combined with hepatitis C virus and other viral infections; autoimmune liver disease, idiopathic cholestasis of pregnancy and other liver diseases; other systemic diseases such as systemic lupus erythematosus, diabetes, hypertensive disorder, etc.

Blood Specimen Collection

EDTA anticoagulant purple tube was used to collect 2 ml of peripheral venous blood from all pregnant women at the time of prepartum (baseline), 6, 12 and 24 weeks postpartum, while blood samples were taken from non-pregnant women at the time of enrollment. The cells were collected by BD FACS Calibur within 4 h after collection.

Peripheral Blood NK Cells Were Measured by Flow Cytometry

Anticoagulant whole blood was drawn from the subjects, 3 μL PerCP Mouse anti-Human CD3, 3 μL APC Mouse anti-Human CD56 and 5 μL PE anti-Human CD335 was added to 100 μl anticoagulant whole blood, incubated at room temperature avoid light for 15 min after vortex mixing, and make the same type control, add 2 ml of erythrocyte lysate, vortex and mix well, incubated 3-5 min avoid light at room temperature and centrifuged cells, washed with PBS, resuspended cells, NK cells were detected by flow cytometry. According to the forwards cattering (FSC) and lateral sides cattering (SSC) gates the lymphocyte population, and more than 10000 lymphocytes were obtained from each sample. NK cell image analysis was performed using FlowJo software.

Relevant Index Detection

HBV serological markers (HBsAg and HBeAg) were measured by chemiluminescent microparticle immunoassay (Architect i2000 analyzer; Abbott Diagnostics, Abbott Park, IL, USA). Serum HBV DNA was tested by real-time quantitative PCR (Piji Co, Ltd, Shenzhen, China) with a detection range of 100 - 2.0×10^9 IU/ml. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) level were tested using a Hitachi 7600

fully automatic biochemical analyzer with the ULN set at 40 U/L (Wako Pure Chemical Industries, Ltd, Osaka, Japan). Mouse anti-human-CD3-PerCP, mouse anti-human CD56-APC (Becton Dickinson, USA) and anti-human CD335 (NKp46, Biolegend, USA) were used to detect NK cells by flow cytometry (Becton Dickinson, USA). Data was analyzed using FlowJo7.6 software.

Statistical Analysis

Data were analyzed by SPSS 19.0 software. All data were presented as mean ± standard deviation (mean ± SD) or median, interquartile interval [median (Q1, Q3)] or percentage. The comparison of data between groups of normal distribution data was completed by analysis of ANNOVA or *t*-test. LSD method was used for multiple comparison if the data between four groups were statistically significant. The comparison of non-normal distribution measurement data was conducted by Kruskal Wallis *H* analysis. Mann Whitney nonparametric *U*-test was used for pairwise comparison if the data between the four groups were statistically significant. Spearman rank correlation test was used to analyze the correlation of variables. $P < 0.05$ was considered statistically significant.

RESULTS

Frequency of NK Cells at Baseline

The baseline characteristics of participants are summarized in **Table 1**. At baseline, the frequency of NK cells in pregnant CHB patients were significantly lower than that in normal group [15.30 (12.80, 18.40)], both P < 0.05. Frequency of NK cells in pregnant CHB patients [untreated: 6.90 (4.89, 10.04); treated: 9.42 (6.55, 14.10)] was lower than that in non-pregnant CHB patients (CHB group: 10.60 (6.00, 18.30), P=0.001, P=0.047, respectively].

Compared with the normal group, pregnancy may increase the frequency of $NKp46^+$ NK cells in untreated group and treated group (both P<0.05). Antiviral therapy during pregnancy could significantly increase the antepartum frequency of NK cells and $NKp46^+$ NK cells (both P<0.05, untreated vs. treated) (**Table 1**).

Postpartum ALT, AST and Frequency of NK Cells Before and After Delivery

The levels of ALT and AST increased significantly at 6 and 12 weeks after delivery and decreased at 24 weeks after delivery in HBV infected pregnant women (Intra group comparison, P<0.05), but there was no significant difference in the levels of ALT and AST postpartum between the treated and untreated group (P>0.05).

In untreated group, the frequencies of NK cells, CD56bright NK cells were significantly increased at 6 and 12 weeks than those before delivery (Intra group comparison, P<0.05). In treated group, the frequencies of NK cells, $CD56^{bright}$ NK cells, $NKp46^+$ NK cells and $NKp46^{dim}$ NK cells were significantly increased at 6 and 12 weeks than those before delivery (Intra group comparison, P<0.05). The frequencies of NK cells and $CD56^{bright}$ NK cells postpartum were increased significantly in treated group than those in untreated group (P<0.05). The frequencies of $CD56^{dim}$ NK cells decreased significantly after delivery in treated than those in untreated patients (P<0.05). ALT and AST significantly increased after delivery than those before delivery (Intra group comparison, P<0.05) (**Table 2**).

Correlation Between Postpartum ALT Level and NK Frequency in Pregnant CHB Women

In order to explore the relationship between postpartum hepatitis and NK cells in pregnant women, 87 pregnant women were further divided according to the level of ALT (54 cases in group A with ALT < 80 U/L and 33 cases in group B with ALT 80≥U/L). The correlation between ALT level and frequency of NK cells was analyzed with Spearman rank correlation test. The $NKp46^{high}$ frequency in patients with postpartum ALT ≥ 80 U/L was higher than that in patients with ALT<80 U/L (P = 0.021), and the $NKp46^{dim}$ frequency in patients with postpartum ALT ≥ 80 U/L was lower than that in patients with ALT<80 U/L (P = 0.021). The results showed that the postpartum ALT level was weak positive correlated with $NKp46^{high}$ frequency (r=0.199) and was weak negative correlated with $NKp46^{dim}$ frequency (r= -0.199) (**Figure 1**).

DISCUSSION

NK cells, one of the important effector cells in the body's antiviral immunity, account for 5%-10% of the total number of lymphocytes in human peripheral blood. Chronic HBV infection leads to decrease in the number of NK cells and function (5). NK cells can be divided into two categories according to the number of CD56 molecules expressed on the cell surface. More than 90% are $CD56^{dim}$ NK cells, which mainly mediates the cytotoxicity and killing effect of NK cells, while less than 10% are $CD56^{bright}$ NK cells, which secretes some important immunomodulatory cytokines and has weak cytotoxic activity (6).

Our results suggested that the frequency of NK cells was reduced under the condition of pregnancy. Studies have reported that NK cells play a major role in the occurrence of CHB (7–10). HBV virus can damage and inhibit the function of NK cells (11). The frequency of $CD56^{bright}$ NK cells in peripheral blood of patients with CHB treated with nucleoside increased after reaching HBV-DNA inhibition and low serum HBsAg level (12). Stelma et al. (13) found that polyethylene glycol α interferon combined with adefovir dipivoxil antiviral treatment could increase the frequency and absolute number of $CD56^{bright}$ NK cells in peripheral blood, and decrease the frequency and absolute number of $CD56^{dim}$ NK cells, which is consistent with our results.

NKp46 is a specific cytolytic receptor expressed in mature NK cells, which is divided into $NKp46^{high}$ and $NKp46^{dim}$ subtypes according to the expression of NKp46 receptor. NKp46 can activate NK cells and participate in the recognition and

TABLE 1 | Baseline characteristics of the study population.

Varies	Normal group (n=11)	CHB group (n=26)	Untreated group (n=41)	Treated group (n=46)	*F/t/P* value (Normal vs. CHB)	*F/t/P* value (Normal vs. Untread)	*F/t/P* value (Normal vs. Treated)	*F/t/P* value (CHB vs. Untreated)	*F/t/P* value (CHB vs. Treated)	*F/t/P* value (Untreated vs. Treated)
Age (years)	26.36±1.96	30.42±6.56	29.07±3.46	30.39±4.27	12.775/-2.868/0.013*	5.939/-3.381/0.002*	5.789/-4.660/0.000*	14.096/0.968/0.232	7.678/0.022/0.977	0.938/-1.569/0.174
ALT (U/L)	9.20 (7.90,13.10)	25.20 (20.60, 30.90)	15.40 (12.10, 21.15)	19.40 (14.97, 24.02)	3.119/-3.002/0.006*	1.863/-1.389/0.132	4.625/-7.274/0.000*	0.012/1.205/0.233	5.353/2.149/0.042	3.669/0.444/0.650
AST (U/L)	17.20 (12.70,18.30)	20.70 (16.40, 24.40)	18.80 (15.50, 22.25)	21.20 (19.30, 24.37)	1.767/-1.284/0.074	1.362/-1.106/0.274	0.230/-3.077/0.003*	0.726/0.828/0.272	6.395/0.842/0.246	4.302/0.057/0.962
HBVDNA (log_{10} IU/ml)	0	8.15±0.37	7.91±0.94	4.31±0.99				0.496/1.118/0.319	18.087/23.029/0.000*	6.751/17.351/0.000*
HBsAg (log_{10} IU/ml)	0	4.77±0.22	4.43±0.59	4.30±0.46				1.034/3.096/0.009*	4.183/5.059/0.000*	1.031/2.147/0.035*
HBeAg(S/CO)	0	1596.64 (1552.68,1637.09)	1640.00 (1523.40,1779.93)	1313.42 (1000.64,1521.29)				5.328/0.198/0.899	17.820/5.224/0.000*	3.375/3.926/0.000*
NK/PBMC(%)	15.30 (12.80,18.40)	10.60 (6.00,18.30)	6.90 (4.89,10.04)	9.42 (6.55,14.10)	4.234/0.716/0.436	0.129/5.352/0.000*	0.710/2.624/0.021*	16.475/3.741/0.001*	7.494/1.515/0.047*	3.937/-2.965/0.019*
$CD56^{dim}$ (%)	92.52 (90.23, 93.63)	92.81 (87.61, 97.08)	93.03 (87.20, 95.62)	91.33 (87.20, 94.65)	2.945/-0.345/0.749	1.673/0.509/0.535	3.806/0.544/0.774	0.005/1.025/0.199	2.927/1.072/0.398	1.996/-0.508/0.603
$CD56^{bright}$ (%)	3.94 (2.50, 7.46)	5.88 (2.60,11.70)	6.97 (4.38,12.80)	8.67 (5.35, 12.80)	4.392/-1.696/0.372	1.565/-1.478/0.054	3.418/-2.803/0.007*	0.431/1.064/0.188	1.177/-1.281/0.394	1.959/0.500/0.581
NKP46 +(%)	88.40 (65.50, 94.30)	91.40 (84.05, 94.75)	93.50 (91.75, 95.40)	95.90 (94.40, 97.20)	0.298/-0.702/0.244	30.721/-2.377/0.000*	50.216/-2.913/0.015*	15.943/-2.835/0.002*	23.199/-4.012/0.000*	1.654/-2.395/0.019
$NKp46^{high}$ (%)	9.21 (7.22,12.60)	7.17 (5.52,13.81)	20.30 (15.85, 29.65)	23.90 (17.00, 33.20)	2.853/-0.508/0.627	8.292/-6.433/0.000*	7.503/-7.641/0.000*	0.535/-3.971/0.000*	0.054/-4.657/0.000*	0.517/-0.394/0.690
$NKp46^{dim}$ (%)	76.10 (56.80, 79.00)	80.00 (68.20, 86.29)	79.70 (70.35, 84.15)	76.10 (66.80, 83.00)	0.096/-1.060/0.192	0.958/-1.695/0.101	2.505/-1.616/0.153	2.602/-0.319/0.731	5.186/-0.040/0.964	0.517/0.394/0.728

**P < 0.05 was considered statistically significant.*

cytotoxicity of target cells without antigen pre-stimulation (14–18). NKp46 is the first way to resist virus infection in the early stage line of defense. In this study, the frequency of $NKp46^{+}$ NK cells was significantly increased in pregnant women, indicating that pregnant may stimulate the expression of NKp46 molecules. We further found that antiviral therapy in the third trimester of pregnancy can significantly increase the expression of NKp46 molecules.

We further investigated the changes of NK cells in pregnant women with CHB and observed that the frequency of NK cells increased significantly at 6 weeks postpartum and maintained a steady state during 6-24 weeks postpartum. Our previous retrospective study (3) showed an increase of ALT level in normal pregnant women soon after delivery, which is consistent with the increase of NK cell frequency. It speculates that the recovery of postpartum immune function will produce a certain immune response to liver cells and lead to the emergence of liver inflammation, which may be the reason for the increase of ALT level in normal women after delivery. However, for HBV infected pregnant women, the recovered immune function further produces immune response to HBV virus, resulting in the re increase of ALT level in 6-12 weeks postpartum, which may be the reason for the bimodal change of ALT level in HBV infected pregnant women in our retrospective study (3). This study also showed that antiviral treatment could significantly improve the frequency of NK cells in postpartum women. The frequency of NK cells in the treated group was higher than that in the women without antiviral treatment. It also reminds us that the decrease of HBV DNA level and HBeAg level may reduce the effect of HBV virus and HBeAg on NK cells.

In conclusion, the results of this study indicate that HBV infection and pregnancy may reduce the frequency of NK cells, while antiviral treatment during pregnancy could significantly increase the frequency of NK cells. Postpartum hepatitis may be

TABLE 2 | Changes in NK cell frequency and functional molecular expression after delivery in both pregnant groups.

Varies	Untreated group (n = 41)					Treated group (n = 46)		F value/t value/P value				
	0 week	6 week	12 week	24 week	z value/ P value	0 week	6 week			12 week	24 week	z value/ P value
ALT	15.40 (12.10,21.15)	48.20 (27.10,18.45)	66.20 (34.00,137.70)	31.20 (23.50,50.10)	a1=-4.530/0.000* a2=-3.619/0.000* a3=-3.771/0.000*	19.40 (14.97,24.02)	39.60 (26.62,59.05)	44.60 (31.15,74.35)	28.45 (23.63,36.73)	a1=-5.583/0.000* a2=-5.012/0.000* a3=-3.070/0.002*	b1 = 3.669/0.444/0.650 b2 = 8.795/-1.592/0.120	b3 = 4.720/-1.469/0.153 b4 = 4.505/-1.529/0.139
AST	18.80 (15.50,22.25)	29.80 (21.70,58.60)	34.90 (24.70,80.50)	25.50 (17.00,33.85)	a1=-4.494/0.000* a2=-3.559/0.000* a3=-3.094/0.002*	21.20 (19.30,24.37)	27.30 (22.43,41.37)	33.50 (23.00,45.85)	22.0 (18.57,28.67)	a1=-4.389/0.000* a2=-4.824/0.001* a3=-1.328/0.184	b1 = 4.302/0.057/0.962 b2 = 8.650/-1.562/0.126	b3 = 4.768/-1.309/0.201 b4 = 4.958/-1.428/0.166
NK/ PBMC(%)	6.90 (4.89,10.04)	10.60 (7.14,16.20)	10.55 (6.23,16.15)	9.43 (6.62,11.80)	a1=-4.684/0.000* a2=-4.114/0.000* a3=-2.580/0.010*	9.42 (6.55,14.10)	13.35 (10.73,17.95)	13.20 (10.80,20.70)	13.40 (9.82,19.25)	a1=-4.437/0.000* a2=-3.942/0.000* a3=-1.682/0.093	b1 = 3.937/-2.965/0.019* b2 = 1.701/-2.177/0.033*	b3 = 0.185/2.118/0.039* b4 = 1.402/2.669/0.013*
$CD56^{dim}$ (%)	93.03 (87.20,95.62)	90.82 (85.90,93.30)	90.19 (84.60,94.53)	91.09 (88.75,93.99)	a1=-2.704/0.007* a2 = 4.286/0.000* a3=-3.622/0.000*	91.33 (87.20,94.65)	87.90 (84.38,92.31)	87.50 (80.20,90.50)	87.20 (81.80,90.77)	a1=-3.824/0.000* a2=-4.130/0.000* a3=-1.886/0.059	b1 = 1.996/-0.508/0.603 b2 = 3.100/-1.302/0.197	b3 = 0.144/40.093/0.000* b4 = 1.478/43.034/0.000*
$CD56^{brigh}t$ (%)	6.97 (4.38,12.80)	9.18 (6.70,14.10)	9.00 (5.47,15.40)	8.91 (6.01,11.25)	a1=-2.704/0.007* a2=-4.286/0.000* a3=-3.622/0.000*	8.67 (5.35,12.80)	12.60 (9.66,17.15)	12.50 (9.50,19.80)	12.80 (9.23,18.20)	a1=-4.659/0.000* a2=-4.130/0.000* a3=-1.886/0.059	b1 = 1.959/0.500/0.58 b2 = 2.293/2.330/0.023*	b3 = 0.144/-40.093/0.000* b4 = 1.478/-43.034/0.000*
NKP46 +(%)	93.50 (91.75,95.40)	95.20 (91.40,97.30)	93.90 (87.08,97.15)	95.90 (93.00,97.55)	a1=-0.617/0.537 a2=-0.171/0.864 a3=-2.296/0.022*	95.90 (94.40,97.20)	97.10 (95.43,97.80)	97.30 (95.60,97.80)	96.95 (94.83,97.58)	a1=-2.109/0.035* a2=-2.584/0.010* a3=-1.735/0.083	b1 = 1.654/-2.395/0.019* b2 = 5.015/1.805/0.081	b3 = 27.361/3.224/0.003* b4 = 1.642/0.922/0.365
$NKp46^{high}$ (%)	20.30 (15.85,29.65)	18.20 (12.60,24.70)	19.15 (14.63,25.73)	17.40 (15.35,21.50)	a1=-1.921/0.055 a2=-	23.90 (17.00,33.20)	18.40 (12.05,22.63)	16.70 (11.40,21.50)	14.10 (11.43,17.93)	a1=-4.095/0.000* a2=-	b1 = 0.517/-0.394/0.690	b3 = 0.461/-1.271/0.210

(Continued)

TABLE 2 | Continued

Varies	Untreated group (n = 41)					Treated group (n = 46)		F value/t value/P value				
	0 week	6 week	12 week	24 week	z value/ P value	0 week	6 week			12 week	24 week	z value/ P value
NKp46^{dim} (%)	79.70 (70.35,84.15)	81.80 (75.30,87.40)	80.85 (74.28,85.38)	82.60 (78.50,84.65)	3.043/ 0.002* a3=-2.533/ 0.011* a1=-1.921/ 0.055 a2=-3.043/ 0.002* a3=-2.533/ 0.011*	76.10 (66.80,83.00)	81.60 (77.38,87.95)	83.30 (78.50,88.60)	85.90 (82.08,88.58)	3.740/ 0.000* a3=-2.701/ 0.007* a1=-4.095/ 0.005* a2=-3.740/ 0.000* a3=-2.701/ 0.007*	b2 = 2.281/- 1.313/ 0.194 b1 = 0.517/ 0.394/ 0.728 b2 = 2.281/ 1.313/ 0.194	b4 = 0.489/- 1.720/ 0.098 b3 = 0.461/ 1.271/ 0.210 b4 = 0.489/ 1.720/ 0.098

1. 0 week=before delivery 6 week=6 week after delivery 12 week=12 week after delivery 24 week=24 week after delivery.

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

3. Comparsion among groups: b1: 0 week vs 0 week b2: 6 week vs 6 week b3: 12 week vs 12 week b4: 24 week vs 24 week.

*4. *P < 0.05 was considered statistically significant.*

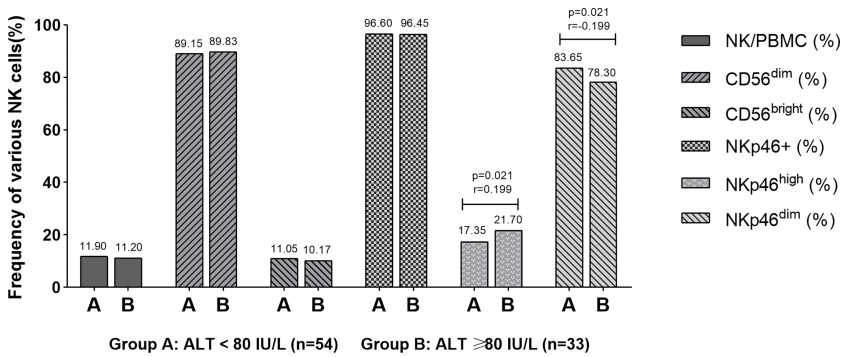

FIGURE 1 | Correlation between postpartum ALT level and NK frequency. The results showed that the postpartum ALT level was weak positive correlated with NKp46^{high} frequency (r = 0.199) and weak negative correlated with NKp46^{dim} frequency (r = -0.199).

related to the immune injury caused by change of NK cell frequency and HBV infection. However, due to the small number of cases in this study, the conclusions need be further verified.

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 (JDL-2017-004-01). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WY, ML, and YX contributed to the study design. CR, SX, and HH contributed to the data analysis. CR, SX, HH, WY, ML, and YX contributed to the recruitment, enrolment, and assessment of participants, as well as data collection. TJ, WD, FS, and XB contributed to following up with the patients. FW, CR, YL, LY, and ZZ managed all aspects of laboratory support. FW wrote the first draft of the manuscript. WY, ML, and SX revised the manuscript and is the guarantor of the article. All authors approved the final version of the manuscript.

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Correlation of HBV DNA and Hepatitis B Surface Antigen Levels With Tumor Response, Liver Function and Immunological Indicators in Liver Cancer Patients With HBV Infection Undergoing PD-1 Inhibition Combinational Therapy

Shida Pan[1,2†], Yingying Yu[2,3†], Siyu Wang[1,2†], Bo Tu[2], Yingjuan Shen[2], Qin Qiu[2], Xiaomeng Liu[2], Nan Su[2,4], Yanmei Zuo[1,2], Junqing Luan[2], Ji–Yuan Zhang[2], Ming Shi[2,3‡], Fanping Meng[1,2*‡] and Fu-Sheng Wang[1,2*‡]*

[1] Chinese People's Liberation Army (PLA) Medical School, Beijing, China, [2] Department of Infectious Diseases, The Fifth Medical Center of Chinese People's Liberation Army (PLA) General Hospital, Beijing, China, [3] Peking University 302 Clinical Medical School, Beijing, China, [4] The Second School of Clinical Medicine, Southern Medical University, Guangzhou, China

Background: Thus far, few studies have investigated the safety and efficacy of programmed death-1 (PD-1) immune checkpoint inhibitors (ICIs) and tyrosine kinase inhibitors (TKIs) antibodies in patients with hepatitis B virus (HBV)-related liver cancer.

Objective: To investigate the effect of combination therapy with programmed death-1 (PD-1) immune checkpoint inhibitors (ICIs) and tyrosine kinase inhibitors (TKIs) on HBV-related liver cancer.

Methods: Until January 31, 2022, liver cancer patients with hepatitis B surface antigen (HBsAg) or HBV DNA positivity, treated with PD-1 ICIs and TKIs combined with nucleoside analogs (NAs), were retrospectively reviewed. The correlation between the change in HBV DNA and HBsAg levels and tumor response was analyzed using the χ^2 test. Cox univariate and multivariate survival analyses and Kaplan–Meier curves were used to identify and compare risk factors and overall survival (OS).

Results: A total of 48 patients were enrolled in the study, with an objective response rate (ORR) of 31.3%, a disease control rate (DCR) of 66.7%; the incidence of adverse events was mostly mild. A significant decrease in HBV DNA and HBsAg levels was observed at 12 and 24 weeks compared with the baseline ($p < 0.05$). Compared to patients with progressive disease (PD), patients with disease control showed a more significant decrease in HBV DNA and HBsAg levels at 12 and 24 weeks ($p < 0.001$). Eleven patients showed elevations in HBV DNA level and one of them showed HBV reactivation; however, the reactivation was not associated hepatitis. Moreover, eight patients showed elevation in HBsAg. Elevation in HBV DNA level was associated with poor tumor response

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Feng Li,
Guangzhou Eighth People's Hospital, China

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Jin Hou,
Second Military Medical University, China
Xiaoyong Zhang,
Southern Medical University, China

***Correspondence:**
Fu-Sheng Wang
fswang302@163.com
Fanping Meng
drmengfanping@126.com
Ming Shi
shiming302@sina.com

†These authors have contributed equally to this work and share first authorship

‡These authors share senior authorship

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(P=0.001, OR=18.643 [95% CI: 3.271–106.253]). Cox survival analysis suggested that HBV DNA increase (P=0.011, HR=4.816, 95% CI: 1.439–16.117) and HBsAg increase (P=0.022, HR=4.161, 95% CI: 1.224–16.144) were independent risk factors associated with survival time. Kaplan–Meier curves suggested that patients who exhibited an increase in HBV DNA (6.87 months *vs* undefined, log-rank test: p= 0.004) and HBsAg (8.07 months *vs* undefined, log-rank test: p= 0.004) levels had a shorter median survival time (MST). Patients without increased HBsAg showed better baseline liver function and routine blood tests ($p<0.05$) than patients with increased HBsAg. An increase in C-reactive protein (CRP) and interleukin-6 (IL-6), and a decrease in T lymphocytes, CD4+ T lymphocytes, and B lymphocytes at 1-week post-treatment associated with HBsAg well-controlled.

Conclusion: HBV-related liver cancer patients treated with combination therapy showed improved efficacy and safety profiles. Combination therapy has some effect on HBV infection, and a correlation between tumor response and antiviral efficacy was found. Elevation of HBV DNA and HBsAg levels may indicate poorer tumor response and survival time. Better baseline liver function and early immune activation may be associated with decline in HBsAg levels.

Keywords: hepatitis B virus, hepatitis B surface antigen, HBV reactivation, median survival time, C-reactive protein, interleukin-6

INTRODUCTION

According to a status report on the cancer burden worldwide, published by the International Agency for Research on Cancer (IARC), liver cancer was predicted to be the sixth most commonly diagnosed cancer and the fourth leading cause of cancer-related deaths worldwide in 2018, with approximately 840,000 new cases and 780,000 deaths per year (1). However, more than half of the new cases and deaths occurred in China (2). Until 2017, liver cancer was the sixth most commonly diagnosed cancer and the fourth leading cause of cancer in China (3).The common causes of liver cancer include hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, aflatoxin contamination, alcohol abuse, obesity, smoking, and type 2 diabetes (4). Common causes of liver cancer vary with region. In developed countries, HCV infection and alcohol abuse contribute the most to liver cancer; in China, the key etiology is chronic HBV infection, with nearly 85% of liver cancer patients having previous or current HBV infection (5). The 5-year survival rate of liver cancer patients worldwide is approximately 18%, while that in China is only 12.1% (6, 7). Most liver cancers are always detected in the middle or late stages, resulting in a poor prognosis.

Currently, tyrosine kinase inhibitors (TKIs) such as sorafenib and lenvatinib are recommended as first-line treatments for advanced liver cancer (8, 9). However, based on the existing studies, the survival benefits of sorafenib administration are limited (10, 11). Immune checkpoint inhibitors (ICIs), such as PD-1 and PD-L1, provide a new option for the treatment of patients with liver cancer. Several ICIs, such as nivolumab, pembrolizumab, and camrelizumab, have been approved for monotherapy in liver cancer cases due to the promising results in clinical trials (12–14). Although phase I/II trials have shown good efficacy and safety, phase III trials were less favorable. Although some patients have durable response to monotherapy, the benefit remains low, with an objective response rate (ORR) ranging from 15% to 20%. Therefore, combination therapies have been developed to improve ORR. Commonly used combination therapies include ICIs, targeted drugs, radiotherapy, chemotherapy, and interventional therapies (15–19). The efficacy and safety of the combination of atezolizumab and bevacizumab used in phase III IMbrave150 trial were higher compared to those obtained with sorafenib, with the results indicating a higher 12-month survival rate (67.2% *vs*. 54.6%) and median progression-free survival (PFS) (6.8 months *vs* 4.3 months); however, the incidence of immune-related adverse events (irAEs) was similar (20).

Although ICI treatment has shown better efficacy than sorafenib in liver cancer, there are few reports on its safety and efficacy in patients with HBV-associated liver cancer, especially in those with high viral loads. This is due, firstly, to the HBV viral load that is associated with carcinoma recurrence and prognosis. Patients with HBV DNA > 10^5 IU/mL before transplantation showed frequent HCC (hepatocellular carcinoma) recurrence after transplantation (21). Patients with a HBV DNA load of < 2000 IU/mL have longer recurrence-free survival (RFS) rate, and a complete virologic response after operative therapy is associated with a lower risk of cancer recurrence (22). High serum levels of HBV DNA before transarterial chemoembolization (TACE) are associated with poor overall survival (OS) and rapid progression of HCC after TACE (23). Except surgical treatment, the HBV viral load was also associated with poor prognosis in patients receiving systemic

therapy. Poor OS was recorded for patients with detectable HBV DNA levels after antiangiogenic therapy; the baseline HBV DNA level was an independent predictor of poor OS (24). High baseline HBV load was an adverse prognostic factor for survival in patients treated with sorafenib (25). A high viral load before treatment was an exposure risk factor for survival during systemic chemotherapy and was associated with a higher incidence of severe hepatitis during treatment (26). In most clinical trials for PD-1/PD-L1 ICIs, liver cancer patients with HBV infection were required to have a baseline HBV load <100 IU/mL for eligibility; therefore, the effect of baseline viral load on clinical outcomes could not be assessed. However, several studies have enrolled patients with a high viral load, suggesting an absence of correlation between baseline HBV viral load and patient OS and PFS during PD-1/PD-L1 treatment; however, the presence or absence of antiviral therapy during immunotherapy could affect tumor prognosis (27, 28).

Secondly, HBV reactivation could affect the use of PD-1/PD-L1 ICIs in HBV-related liver cancer patients. In HBV-related liver cancer patients, common triggers for HBV reactivation include surgery, radiation therapy, chemotherapy, and immunosuppressive drugs. For example, in patients with HBV reactivation and HBV reactivation-associated hepatitis after chemotherapy, use of prophylactic antiviral drugs were independent risk factors for HBV reactivation (29, 30). In patients treated with partial hepatectomy, radiofrequency ablation, and interventional therapy, HBV reactivation and HBV reactivation-related hepatitis may be associated with the immunosuppressive state caused by surgery and anesthesia as well as surgical injury of the liver (31–33). HBV reactivation has also been reported in PD-1PD-L1 antibody therapy. In an Asian cohort of CheckMate 040, patients were subjected to antiviral therapy to achieve an HBV DNA load of ≤ 100 IU/mL; however, five patients exhibited an increase of > $1\log_{10}$ IU/mL of HBV DNA after PD-1 ICI immunotherapy but did not develop hepatitis (34). Studies that included patients with an HBV DNA load of >100 IU/mL found that the baseline viral load had no effect on HBV reactivation, but antiviral therapy was an independent risk factor for HBV reactivation (35, 36).

Although PD-1PD-L1 ICIs exhibit good efficacy and safety in patients with HBV-related liver cancer, there are a few shortcomings that need to be addressed. For example, the load of HBV in patients enrolled in studies is usually low (< 2000 IU/mL), and the efficacy and safety of the drugs against high viral loads (> 2000 IU/mL) remains unclear. For patients with high viral loads, is it necessary to keep the viral load level low before initiating PD-1/PD-L1 antibody immunotherapy? Does the presence of HBV re-replication or reactivation indicate a poor prognosis for patients compared with those with a decrease in HBV DNA? To address these questions, further studies are required.

For patients with HBV-related liver cancer, PD-1/PD-L1 antibodies not only showed good tumor response efficacy but also played a role in antiviral treatment of HBV infection (37, 38). In some pre-clinical studies, PD-1 was highly expressed on HBV-specific CD8+ T cells in virally persistently infected mice than in HBsAg-clear mice; PD-1 antibody treatment restored the function of HBV-specific CD8+ T cells and promoted HBsAg clearance (39–41). Nivolumab, with and without therapeutic vaccination, when used on serum-positive chronic hepatitis B (CHB) patients, resulted in HBsAg reduction and clearance in some patients (42). Although PD-1/PD-L1 ICIs are effective in both tumor control and HBV infection, no study has investigated whether there is an association between tumor response and viral reduction. It is also unknown whether baseline characteristics and dynamics of clinical parameters during PD-1/PD-L1 treatment are associated with decrease HBsAg levels.

In our study, liver cancer patients did not need to maintain lower HBV DNA levels when subjected to combined treatment with PD-1 ICIs and TKIs, but concurrent initiation of nucleoside analog (NA) antiviral therapy was required. The aim of the study is to investigate the efficacy, safety (incidence of adverse events and hepatitis related to HBV reactivation) and impact on HBV DNA and HBsAg levels of combination therapy, and to determine the correlation between HBV replication or reactivation with patient response and survival.

MATERIALS AND METHODS

Patients' Inclusion

Between March 2020 and January 2022, patients diagnosed with liver cancer through histological or imaging examination, with HBV DNA or HBsAg seropositivity and treated with at least one cycle of PD-1 ICIs combined with TKIs, were retrospectively reviewed. All patients were treated in the Department of Infectious Diseases, Fifth Medical Center of PLA General Hospital. Sintilimab (Innovent Biologics and Eli Lilly and Company) and camrelizumab (Jiangsu Hengrui Medicine Co. Ltd.), at a fixed dose of 200 mg, and toripalimab at 240 mg were prescribed for a three week cycle. Sorafenib (Bayer Schering Pharma AG) was administered at 400 mg/day, lenvatinib (Eisai Co. Ltd) at 8 to 12 mg/day and regorafenib (Bayer) at 80 mg/day, according to body weight. Treatment options for patients who met the criteria for TACE treatment were decided together by the patient and the physician before or after systemic combination therapies. NAs including entecavir (ETV) (0.5 mg/day), tenofovir (TDF) (300 mg/day), and adefovir dipivoxil (ADV) (10 mg/day) were provided simultaneously with immunotherapy.

Radiological response was recorded using computed tomography (CT) or magnetic resonance imaging (MRI) initially as the baseline and then at 3 month intervals. Tumor response was assessed by the modified Response Evaluation Criteria in Solid Tumors (mRECIST) (43, 44). Taking the baseline sum of the diameters of target lesions as a reference, complete response (CR) was defined as the disappearance of any intratumoral arterial enhancement in all target lesions; at least a 30% decrease in the sum of diameters of target lesions was defined as a partial response (PR); an increase in the sum of the diameters of at least 20% was defined as progressive disease (PD); and no change in the sum of the diameters between PR and PD was defined as stable disease (SD). IrAEs were documented according to Version 5 of the Common Terminology Criteria

for Adverse Events (CTCAE) (45). Patients with spontaneous bacterial peritonitis, a complication of primary liver cancer, were excluded from this study. Depending on the toxicity grade, TKI dose was reduced, suspended, discontinued, or switched to other TKIs in patients who developed irAEs associated with TKI treatment. PD-1 ICIs were suspended or discontinued, and immunosuppressant agents were administered based on the severity of irAEs.

HBV reactivation was defined according to the American Association for the Study of Liver Diseases (AASLD) hepatitis B guidance in 2018 for HBsAg-positive and anti-hepatitis B core (HBc)-positive patients, including (1) an increase in HBV DNA ≥ 2 log (100-fold) compared to the baseline level, (2) HBV DNA ≥ 3 log (1000) IU/mL in a patient with previously undetectable levels, or (3) HBV DNA ≥ 4 log (10,000) IU/mL if the baseline level was not available. HBsAg-negative and anti-HBc-positive patients who met the following criteria were considered to have HBV reactivation hepatitis: detectable HBV DNA or reverse serological conversion of HBsAg or HBV reactivation accompanied with an increase in alanine aminotransferase (ALT) ≥ 3 times the baseline level and absolute value > 100 U/L (46).

The cut-off date for follow-up was January 31, 2022, and all data were obtained from patient medical records. The baseline data included patient demographics, such as Child-Pugh stage, Barcelona Clinic Liver Cancer (BCLC) stage, serum alpha-fetoprotein (AFP) level, type of combination therapy, and TACE treatment. The data pertaining to the levels of HBV DNA and HBsAg, routine blood tests, liver function, lymphocyte subsets, C-reactive protein (CRP), and interleukin-6 (IL-6) at baseline and after 1 week were collected. HBV DNA was quantified using real-time fluorescence PCR (Roche COBAS, USA) with a lower limit of 20 IU/mL. HBsAg was quantified using a Beckman AU5800-03 with a lower limit of 0.05 IU/ml. Serum CRP and liver function parameters were measured using an automatic biochemical analyzer (AU5400; Beckman Coulter, Brea, CA), with the upper limit of the normal value for serum CRP set at 8.2 mg/L. Serum IL-6 was examined using a Roche Cobas 8000 (Roche Diagnostics GmbH, Mannheim, Germany), and the upper limit of the normal value for serum IL-6 was set at 7 pg/mL. Lymphocyte subsets and counts were measured using a FACSCalibur flow cytometer (BD Biosciences, Becton, NJ). Routine blood parameters were measured using an automatic hematology analyzer (HN-2000 series; SYSMEX, Kobe, Japan).

Statistical Analysis

All statistical analysis were conducted using the IBM SPSS Statistics software application (version 25.0; IBM, Armonk, NY, USA). The baseline data and adverse events were summarized using descriptive statistics. HBV DNA and HBsAg levels below the lower limit were recorded as the lower limit of detection. Continuous variables that conformed to a normal distribution were represented as mean ± SD, non-normal distribution data were presented using median (quartiles), and categorical data by variable numbers (percentages). The student t test was used to compare continuous variables that conformed to a normal distribution. The χ^2 test or Fisher's exact test were used to compare categorical data. Comparisons between the two groups were performed using the nonparametric Mann–Whitney U test. Comparisons within groups were analyzed using nonparametric Wilcoxon's paired test. The cut-off date for survival analysis was January 31, 2022, and OS was estimated using Kaplan–Meier curves and compared using the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were analyzed using Cox univariate and multifactorial survival analyses to identify any independent predictive factors that were associated with OS. All figures were generated using GraphPad Prism statistical software (version 9.0; GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$.

RESULTS

Baseline Characteristics of Patients

A total of 48 patients were enrolled in this study: 41 with HCC and 7 with cholangiocarcinoma. Thirty-four patients were treated with a combination of sintilimab and lenvatinib, while 14 patients received other combination therapies. Twelve patients underwent TACE during the treatment period. Thirty-three patients had received antiviral therapy prior to combination therapy and 15 patients had never received antiviral therapy. Fifteen patients had baseline HBV DNA levels below the lower limit of detection, 16 patients had HBV DNA levels >1000 IU/mL, and other patients had HBV DNA levels between 20 IU/mL and 1000 IU/mL. The baseline HBsAg level of five patients was not available; one patient was seronegative for baseline HBsAg but with HBV DNA > 20 IU/mL, and 45 patients had HBsAg levels > 0.5 IU/mL. Other details are listed in **Table 1**.

Immune-Related Adverse Events and Tumor Response Profiles

According to mRECIST, two (4.2%) patients had CR, 13 (27.1%) had PR, 17 (35.4%) had SD, and 16 (33.3%) had PD, with an ORR of 31.3% and Disease control rate (DCR) of 66.7%, as shown in **Table 1**.

There were 22 (45.8%) patients who experienced at least one adverse event of any grade, and 12 patients (25%) developed grade 3 (G3)/grade 4 (G4) irAEs. The most common adverse events in G3/G4 irAEs were fever (n = 9, 18.8%), fatigue (n = 7, 14.6%), lymphopenia (n = 2, 4.2%), bacterial infection (n = 2, 4.2%), diarrhea (n = 2, 4.2%), and hepatitis (n = 2, 4.2%). All patients who experienced severe irAEs improved after receiving glucocorticoids according to clinical guidelines. The overall occurrence of adverse events in the patients is shown in **Table 2**.

During treatment, four patients permanently discontinued sintilimab, three due to irAEs, and one due to tumor progression; five patients had their TKI dose reduced due to adverse AEs; six patients discontinued TKIs, two due to AEs, and four due to complications such as upper gastrointestinal bleeding. Twelve patients switched to regorafenib, three due to AEs, and nine due to tumor progression.

TABLE 1 | Baseline characteristics of patients enrolled in the study.

	n = 48
Age (year)	55.96 ± 9.72
Sex	
Male	41 (85.4%)
Female	7 (14.6%)
Types of tumors	
hepatocellular carcinoma	43 (89.6%)
Cholangiocarcinoma	5 (10.4%)
BCLC stage	
B	12 (25.0%)
C (PVTT)	22 (45.8%)
C (M)	14 (29.2%)
Child-Pugh stage	
A	26 (54.2%)
B	22 (45.8%)
AFP	
<400 (IU/ml)	27 (56.2%)
≥400 (IU/ml)	21 (43.8%)
Previous antiviral therapy	
Yes	33 (68.7%)
No	15 (31.3%)
HBV DNA	
≤20 (IU/ml)	15 (31.2%)
>20 and ≤10^3 (IU/ml)	17 (34.5%)
>10^3 (IU/ml)	16 (33.3%)
HBsAg	
≤0.05 (IU/ml)	1 (2%)
>0.05 (IU/ml)	42 (87.6%)
No available	5 (10.4%)
HBeAg state	
Seropositive	9 (18.8%)
Seronegative	38 (79.2%)
No available	1 (2%)
Combination treatment	
Sintilimab+Lenvatinib	34 (70.8%)
Sintilimab+Sorafenib	7 (14.6%)
Camrelizumab+Lenvatinib	3 (6.3%)
Toripalimab+Lenvatinib	4 (8.3%)
Combinated TACE therapy	
Yes	12 (25.0%)
No	36 (75%)
Combination treatment as systemic	
First line	21 (43.8%)
Second line	5 (10.4%)
Third line	16 (33.3%)
Fourth line	6 (12.5%)
Antiviral therapy	
ETV	38 (79.2%)
ADV	1 (2%)
TDF	4 (8.3%)
ETV+TDF	4 (8.3%)
Tumor response	
CR	2 (4.2%)
PR	13 (27.1%)
SD	17 (35.4%)
PD	16 (33.3%)
OR (CR+PR)	15 (31.3%)
DC (CR+PR+SD)	32 (66.7%)

ADV, Adefovir dipivoxil; AFP, Alpha-Fetoprotein; BCLC, Barcelona Clinic Liver Cancer; CR, complete response; DC, disease control; ETV, Entecavir; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B viral; OR, objective response; PD progressive disease; PR, partial response; SD, stable disease; TACE, transcatheter arterial chemoembolization; TDF, Tenofovir.

Antiviral Efficacy of Combination Therapy

Patients were treated with a combination therapy of PD-1 ICIs and TKIs. All patients received antiviral therapy with NAs and were followed up for more than 24 weeks. One patient was HBsAg negative and HBV DNA positive at the start of treatment. One patient died within 12 weeks. The follow-up time of 12 patients was more than 12 weeks but not more than 24 weeks, during which time, seven patients died, two were lost to follow-up, and three were still subject to follow-up. Fifteen patients had baseline HBV DNA levels below the lower limit of detection, of which three patients had positive HBV DNA after 24 weeks of treatment, and one of three patients reached HBV reactivation. Twelve patients with baseline HBV DNA positivity had HBV DNA clearance at 12 weeks and five at 24 weeks. HBsAg changes could not be assessed in nine patients at 12 or 24 weeks due to lack of baseline data. Compared with the established baseline levels, overall HBV DNA and HBsAg levels showed a statistically significant difference at 12 and 24 weeks ($p < 0.05$) (**Figure 1**).

To investigate the correlation between patient tumor response and virus control, we analyzed the changes in HBV DNA and HBsAg levels in patients with different tumor responses. The results suggested that there was no statistically significant difference in HBV DNA and HBsAg levels between patients with tumor control (including CR, PR, SD) and tumor progression (PD), and no significant difference was observed among patients with CR plus PR, SD, and PD ($p > 0.05$) (**Figures 2A, D**). Compared to patients with PD, patients with tumor control showed a significant decline in HBV DNA and HBsAg levels at baseline, 12 weeks, and 24 weeks ($p<0.05$) (**Figures 2B, C**). In addition, we divided patients with DCR into CR plus PR and SD groups and analyzed the changes in HBV DNA and HBsAg levels in the two groups. The results showed that patients with CR plus PR and SD had similar changes in HBV DNA levels at baseline, 12 weeks, and 24 weeks. However, the change in HBsAg levels was more significantly different in CR plus PR patients than in SD patients (**Figures 2E, F**).

Correlation Between Elevated HBV DNA, HBsAg Levels, Tumor Response, and Survival Time

After 24 weeks of treatment, 11 patients had elevated HBV DNA and ALT or aspartate aminotransferase (AST) levels, one of whom had HBV reactivation; however, this patient did not develop HBV reactivation-related hepatitis. Two patients developed hepatitis, but it was not associated with HBV reactivation. The characteristics of the 11 patients with elevated HBV DNA levels are shown in **Table S1**. In addition, 8 patients suffered from elevated HBsAg levels, the characteristics of which are shown in **Table S2**.

Nine of the 11 patients who suffered from HBV DNA elevation had PD, one patient had SD, and one patient had PR. Four of the eight patients with elevated HBsAg levels had SD and four patients had PD. The results of the χ^2 test suggested that HBV DNA elevation was significantly associated with poor tumor response

TABLE 2 | Occurrence of adverse events.

	G1/G2	G3/4
Fever	9 (18.8%)	0
Fatigue	7 (14.6%)	0
Hypertension	6 (12.5%)	0
Diarrhea	6 (12.5%)	2 (4.2%)
Rash	6 (12.5%)	0
Hypothyroidism	4 (8.3%)	0
Pruritus	2 (4.2%)	0
Proteinuria	3 (6.3%)	0
Renal dysfunction	2 (4.2%)	0
Pneumonia	1 (2%)	1 (2%)
Lymphopenia	1 (2%)	2 (4.2%)
Thrombocytopenia	1 (2.1%)	1 (2.1%)
Hyperthyroidism	1 (2.1%)	0
Cardiotoxicity	1 (2.1%)	0
hoarseness	1 (2.1%)	0
hepatitis	0	2 (4.2%)
Bacterial infection	0	2 (4.2%)
fungal infection	0	1 (2.1%)
Herpes virus infection	0	1 (2.1%)
Intestinal infections	0	1 (2.1%)

(p=0.001, OR=18.643 [95% CI: 3.271–106.253]), but elevated HBsAg was not associated with tumor response (p=0.270, OR=2.440 [95% CI: 0.499–11.965]) (**Table 3**).

The results of Cox univariate and multivariate survival analyses suggested that increase in HBV DNA (P=0.011, HR=4.816, 95 CI%: 1.439–16.117) and HBsAg (P=0.022, HR=4.161, 95 CI%: 1.224–16.144) levels were independent risk factors associated with patient survival time (**Table 4**). Patients who had increased HBV DNA (6.87 months *vs* undefined, log-rank test: p= 0.004) and HBsAg (8.07 months *vs* undefined, log-rank test: p= 0.004) levels had a shorter median survival time (MST) than patients without an increase in HBV DNA and HBsAg levels (**Figure 3**).

Correlation Between Changes in Clinical Parameters and Elevated HBsAg

To identify the biomarkers associated with elevated HBsAg levels, we analyzed the baseline characteristics and early change trends in some clinical parameters. The results suggested that there were higher baseline levels of albumin (ALB), AST/ALT ratio, cholinesterase (CHE), prothrombin activity (PTA), and platelets (PLT), and lower baseline levels of direct bilirubin (DBIL) and absolute value of monocytes (AMC) in patients without increased HBsAg than in patients with increased HBsAg. Compared to patients with increased HBsAg, other patients showed an increase in CRP, IL-6, AST/ALT ratio, and DBIL and a decrease in T lymphocytes, CD4+ T lymphocytes, B lymphocytes, and CHE one week after the initiation of treatment (**Figure 4**).

DISCUSSION

In our study, we investigated the safety and efficacy of a combined treatment with PD-1 ICIs and TKIs in liver cancer patients with

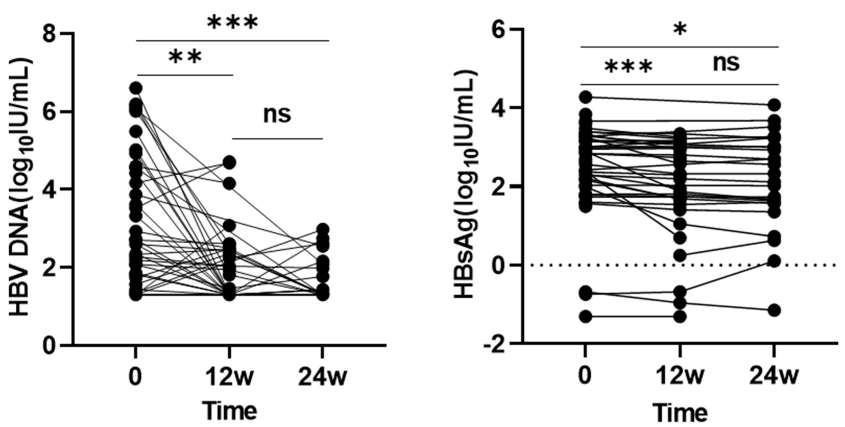

FIGURE 1 | The overall changes in HBV DNA and HBsAg levels at different time point. ns: >0.05; ***: ≤0.005; **: ≤0.01; *: ≤0.05.

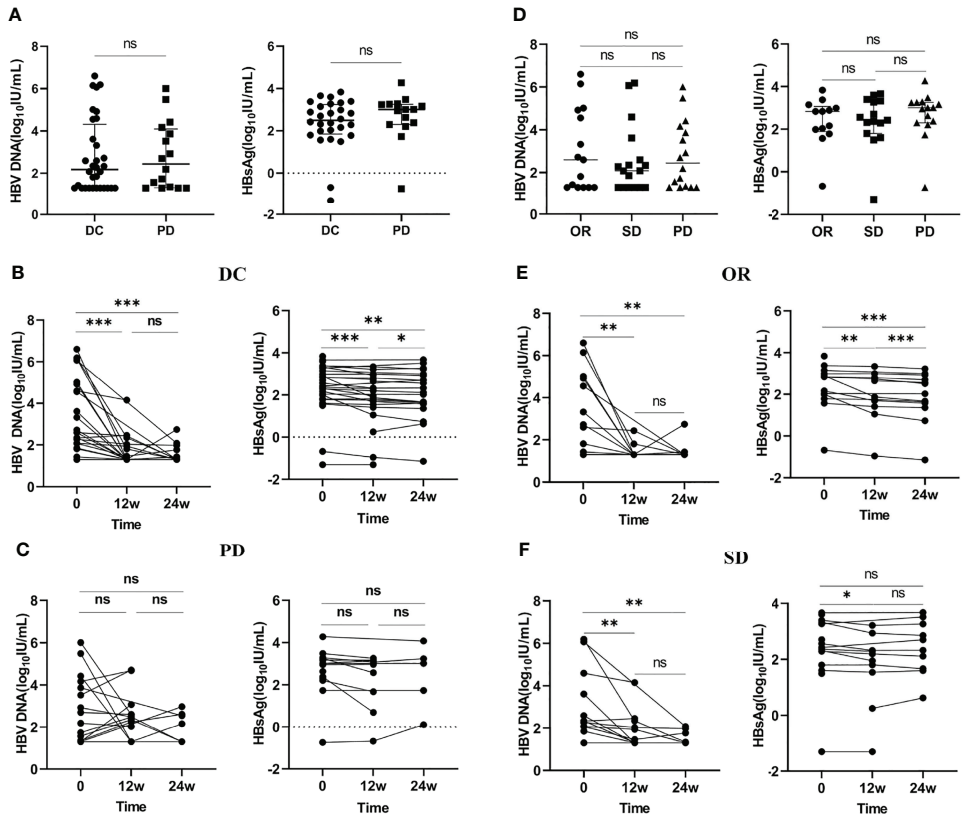

FIGURE 2 | The change in HBV DNA and HBsAg levels in patients with different tumor responses. **(A)** Comparison of baseline levels of HBV DNA and HBsAg between patients with DC and PD. **(B)** The changes in HBV DNA and HBsAg levels at different time points in patients with DC. **(C)** The changes in HBV DNA and HBsAg levels at different time points in patients with PD. **(D)** Comparison of baseline levels of HBV DNA and HBsAg among patients with OR, SD and PD. **(E)** The changes in HBV DNA and HBsAg levels at different time points in patients with OR. **(F)** The changes in HBV DNA and HBsAg levels at different time points in patients with SD. DC, disease control; OR, objective response; PD, progressive disease; SD, stable disease. ns: >0.05; ***: ≤0.005; **: ≤0.01; *: ≤0.05.

HBV infection, the effect of combination therapy on HBV DNA and HBsAg levels, and biomarkers associated with HBsAg elevation.

Combination therapy showed good safety and efficacy; the ORR and DCR was 31.5% and 66.7%, respectively. There were 22 (45.8%) patients with irAEs, of which 10 patients suffered from grade 3/4 irAEs, but their symptoms improved upon the administration of glucocorticoids, and no deaths were associated with irAEs. Patients suggested a decrease in overall HBV DNA and HBsAg levels. Compared with PD patients, patients with DCR (including CR, PR and SD) showed a more significant decline in HBV DNA and HBsAg levels. Eleven patients showed an increase in HBV DNA level within 24 weeks; one of these patients met the criteria for HBV reactivation, but did not develop HBV-related hepatitis. The baseline level of HBV DNA between patients with and without elevated HBV DNA was not statistically different, and univariate regression analysis suggested that the baseline level of HBV DNA was not a risk factor for HBV DNA elevation. The χ^2 test showed an association between elevated HBV DNA level and tumor response, and patients with elevated HBV DNA level were more likely to experience tumor progression. Survival analysis

TABLE 3 | χ^2 test for change trends of HBV DNA and HBsAg levels according to tumor response.

	CR+PR+SD	PD	p value	OR
The change of HBV DNA			<0.001	18.643 (3.271-106.253)
Without increase	29	7		
With increase	2	9		
The change of HBsAg			0.270	2.44 (0.499-11.965)
Without increase	22	9		
With increase	4	4		

AFP, Alpha-Fetoprotein; BCLC, Barcelona Clinic Liver Cancer; HBsAg, hepatitis B surface antigen; HBV, hepatitis B viral; HBsAg, hepatitis B surface antigen; MST, median survival time; TACE, transcatheter arterial chemoembolization.

TABLE 4 | Cox univariate and multifactorial survival analysis to identify any independent predictive factor associated with OS.

	Cox univariate survival analysis			Cox multivariate survival analysis		
	HR	95%CI	p value	HR	95%CI	p value
Age	0.979	0.925-1.036	0.461			
Sex	0.785	0.420-1.465	0.446			
Types of tumors	1.062	0.141-8.030	0.953			
BCLC stage	1.165	0.644-2.105	0.614			
Child-Pugh stage	0.140	0.160-1.294	0.140			
Options of combination therapy	1.154	0.426-3.125	0.778			
TACE treatment	0.329	0.075-1.439	0.140			
Line of treatment	0.9103	0.579-1.440	0.696			
AFP	2.312	0.855-6.257	0.099			
The occurrence of irAEs	0.491	0.185-1.298	0.152			
Prior antiviral therapy	1.176	0.406-3.411	0.765			
Baseline level of HBV DNA	1.012	0.753-1.358	0.939			
HBV DNA increase	4.034	1.456-11.179	0.007	4.816	1.439-16.117	0.011
HBsAg increase	4.869	1.476-16.064	0.009	4.161	1.224-16.144	0.022

AFP, Alpha-Fetoprotein; BCLC, Barcelona Clinic Liver Cancer; HBsAg, hepatitis B surface antigen; HBV, hepatitis B viral; TACE, transcatheter arterial chemoembolization.

revealed that patients who developed elevated HBV DNA levels had a shorter survival than those who did not, with a median OS of only 6.87 months. To the end, eight patients developed elevated HBsAg levels within 24 weeks, and elevated HBsAg level showed a shorter median OS (8.07 months). Furthermore, patients with increased HBsAg level showed lower levels of ALB, CHE, PTA, monocytes, and PLT and higher levels of DBIL and AST/ALT ratio at baseline. There was a decrease in CHE, PTA, LYM, T lymphocytes, CD4+ T cells, and B cells, and an increase in DBIL, AST/ALT ratio, CRP, and IL-6 in patients without increased HBsAg one week after treatment initiation, but these results were not observed in patients with increased HBsAg.

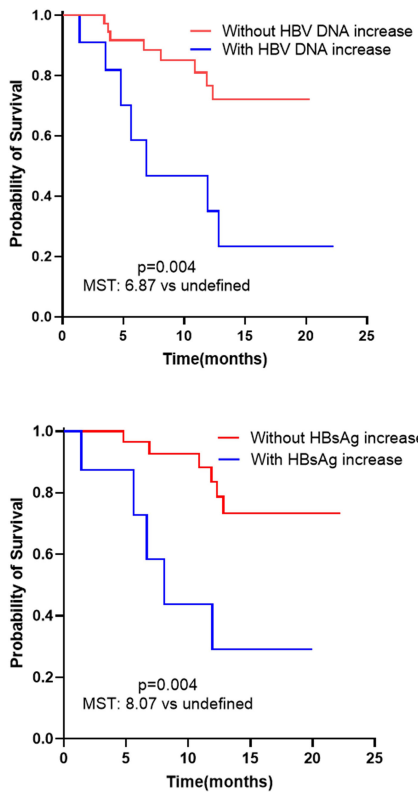

FIGURE 3 | Correlation analysis between tumor response, survival time and the elevation of HBV DNA and HBsAg levels. Kaplan–Meier overall survival estimate curves in patients with increasing HBV DNA and HBsAg and patients exhibiting no increase in HBV DNA and HBsAg. HBsAg, hepatitis B surface antigen; HBV, hepatitis B viral; MST, median survival time.

HBV DNA is often associated with poor prognosis in the local or systemic treatment of liver cancer (21–28). However, during PD-1/PD-L1 ICI treatment, patients with HBV-related liver cancer had a similar tumor response to that of all patients. For example, in the CheckMate 040 study, the ORR of nivolumab monotherapy was 19% and 14% in all patients and the HBV subgroup, respectively, and the ORR of nivolumab and ipilimumab was 31% in both the groups (12, 15). In the KEYNOTE-224 study, the DCR was 60% and 52% in all patients and HBV infection group, respectively (13). The ORRs were 36% and 33% in patients treated with atezolizumab and bevacizumab, respectively. However, these patients started immunotherapy with a low viral load (less than 500 IU/mL), which may have contributed to the impact of HBV DNA being overlooked. In our study, 16 patients had HBV DNA levels > 1000 IU/mL, and 11 patients had HBV DNA levels less than 1000 IU/mL but more than 100 IU/mL. The overall ORR and overall DCR were 31.3% and 66.7%, respectively, most of the irAEs were mild, and no patient died from adverse events. In patients with HBV DNA levels > 100 IU/mL, one patient had CR, seven patients had PR, and ten patients had SD; the ORR and DCR were 29.6% and 66.7%, respectively. From the results of the mate analysis, the overall ORR and DCR were 20% and 60%, respectively, for PD-1/PD-L1 ICI monotherapy in liver cancer patients, while the ORR was 29% and DCR was 77% for combination therapy with anti-VEGF drugs; the overall rate of irAE was 63%, and the occurrence of severe adverse events was 11% (47). Our results suggest that regardless of the HBV DNA levels, there were similar tumor responses and irAEs between patients with HBV-associated liver cancer and other liver cancer patients. Therefore, HBV infection

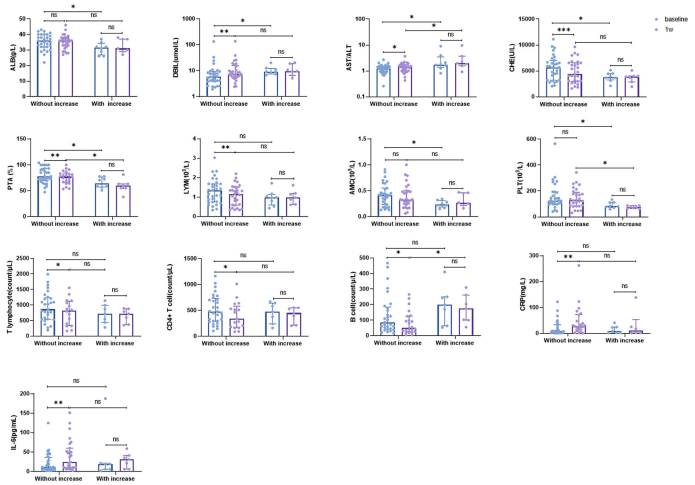

FIGURE 4 | Baseline characteristics and trends of some clinical parameters in patients with or without increasing HBsAg level. ALB, albumin; AMC, absolute monocyte count; CHE, cholinesterase; CRP, C-reactive protein; DBIL, direct bilirubin; IL-6, interleukin-6; LYM, absolute lymphocytes; PLT, platelets; PTA, prothrombin activity. ns: >0.05; ***: ≤0.005; **: ≤0.01; *: ≤0.05.

may not be a promoter during PD-1/PD-L1 therapy in patients with liver cancer. However, the sample size considered in this study was small; future studies require larger sample sizes to confirm this observation.

CD8+ T cells play an important role in virus clearance and are critical for controlling the progression of CHB (48–50). CD8+ T lymphocytes play an important role in tumor immunity and are also crucial in virus clearance and regulation of CHB infection (48–50). Exhaustion of CD8+ T cells could be found in CHB patients, characterized by upregulated expression of PD-1 accompanied by a reduction in secretion and killing functions. Therefore, blocking the PD-1/PD-L1 signaling pathway can restore the function of CD8+ T cells and promote virus clearance. In our study, we investigated the effect of PD-1 antibodies on HBV, and the results suggested that patients showed a decrease in overall HBV DNA and HBsAg levels when using a prophylactic antiviral. Furthermore, patients with DCR showed better efficacy of antiviral therapy and more pronounced decrease in HBV DNA and HBsAg levels compared to those with PD. We hypothesized that this may be related to the reactivation of exhausted CD8+ T cells (51). However, a recent study indicated that tumor-infiltrating HBV-specific CD8 T cells have been proven to be associated with prolonged patient relapse-free survival. More HBV-specific CD8 T cells could be detected in both liver cancer tissue and healthy tissue compared to their levels in the tissues of relapsed patients (52). This indicates that restoring the function of HBV-specific CD8 T cells may be important for improving both the tumor response and virus clearance in patients with HBV-related liver cancer. However, in our study, patients treated with both PD-1 antibodies and NAs did not show HBsAg clearance. Although the non-clearance could be related to the immunosuppressed state, it suggests that when choosing PD-1/PD-L1 antibodies for antiviral therapy, the efficacy of single-agent or combined NA therapy may not be satisfactory, and combination therapy with other treatment options should be considered to improve HBsAg clearance.

HBV reactivation is a key issue that requires close attention during the treatment of patients with HBV-infected liver cancer. HBV reactivation has been observed in both local and systemic treatments, irrespective of HBV DNA levels, and the use of prophylactic antiviral drugs is an independent risk factor for HBV reactivation (29–36). Although HBV reactivation can also occur during PD-1/PD-L1 ICI treatment, it is less frequent than during surgery, radiation therapy, and other treatments. Treatment such as surgery and radiation therapy can lead to immunosuppression accompanied by dysfunctional HBV-specific T and B cells, resulting in HBV DNA replication and HBV reactivation (53). However, PD-1/PD-L1 antibodies promote the reactivation of the immune system by blocking inhibitory signaling pathways to inhibit viral replication (41). At present, the precise mechanism of HBV re-replication or reactivation in patients treated with PD-1/PD-L1 antibodies is unknown. In a recent study, researchers reported that virological breakthrough(VB) was an indicator of poor outcome in patients with HBsAg positive HCC under ICIs therapy (54). This finding is similar with our study. In our research, all patients underwent PD-1 combined with targeted drug therapies. We found that some patients who had elevated HBV DNA did not meet the requirements for VB or HBV reactivation. We included this group of patients in the study and found similar results, expanding the applicability of our conclusions. Besides HBVDNA, most patients underwent a regular post-treatment HBsAg assay, which enabled us to assess the impact of ICIs on HBsAg. Liver function and immunological characteristics were evaluated based on the change trend of HBsAg in our study. We discussed the relationship between the changes of HBsAg and inflammatory factors, lymphocyte subsets and other

immunological indicators. In our study, 11 patients showed HBV DNA elevation, resulting in HBV reactivation in one of them, but it did not lead to HBV reactivation-related hepatitis. In all patients with HBV DNA elevation, one patient had baseline HBV DNA > 10000 IU/mL, one patient had baseline HBV DNA >1000 IU/mL, two patients had baseline HBV DNA > 100 IU/mL, and other patients had HBV DNA < 100 IU/mL. Univariate regression analysis suggested that the baseline HBV DNA level was not a risk factor for HBV DNA elevation. This result suggests that there is no correlation between baseline HBV DNA levels and HBV DNA increase. Keeping viral load at a lower level when starting PD-1 therapy is therefore not necessary if antiviral therapy is also available, and a high viral load should not be a contraindication to PD-1/PD-L1 ICI therapy in patients with HBV-related liver cancer. In addition, in our study, we found that elevated HBV DNA levels were associated with tumor response and patient survival. These results suggest that elevated or reactivated HBV DNA may occur due to poor tumor control, or that elevated HBV DNA may imply poor prognosis; however, due to the small sample size, the relationship between the two remains unclear. The existence of this correlation may explain why HBV reactivation occurred during treatment with PD-1/PD-L1 ICIs, a class of immune activators. It is possible that patients with PD experience immunosuppression and lack of activated immune cells, such as depleted CD8 T cells, which is consistent with the immunosuppressed state caused by other treatments (53). However, this hypothesis and its exact mechanism require further study.

Along with an increase in HBV DNA level, we also observed elevated HBsAg levels during treatment. Up to 24 weeks, eight patients did not show a decrease in HBsAg, and patients who developed HBsAg elevation had shorter OS. Although our results did not suggest a correlation between HBsAg decline and tumor response, this may be due to missing HBsAg data after treatment in some patients, or there may not be an intrinsic correlation. The exact reasons need to be explored considering a larger sample size. Furthermore, we investigated the correlation between changes in clinical parameters and HBsAg levels. These results suggested that better baseline liver function was associated with changes in HBsAg levels. As an immune organ, the liver can mediate adaptive immune tolerance, and better liver function implies better immune regulation, which may play a role in HBsAg clearance (55). Furthermore, an early increase in CRP and IL-6 levels and a decrease in lymphocyte subsets are associated with a decline in HBsAg levels. Although the exact mechanisms responsible for the changes in immune markers are not known, there is an association between changes in CRP and IL-6 levels and changes in lymphocyte subsets. This may be related to the systemic inflammatory response caused by a cytokine storm, which has also been seen in COVID-19 patients. Compared with mild-type COVID-19 patients, severe-type patients who experienced cytokine storms had higher levels of CRP and IL-6, which are characterized by high levels of granulocyte colony stimulating factor, interferon-inducible protein-10, monocyte chemotactic Protein-1, macrophage inflammatory protein-1α/β, IL-8, and other cytokines, which can promote chemotaxis or apoptosis of peripheral blood lymphocyte subsets, leading to a decrease in cell numbers (56–59).

However, this study had several limitations. First, this was a single-center retrospective study with a relatively small sample size. Second, because fewer patients presented with elevated HBsAg, the baseline and changing characteristics of liver function, blood tests, CRP, IL-6, and lymphocyte subsets were not reflected in this group of patients. Large prospective clinical trials might elaborate the association between CRP, IL-6, lymphocyte subsets, liver function, blood routine, and HBsAg increase, as well as lead to the discovery of other markers.

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 Chinese Ethics Committee of Registering Clinical Trials. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SP, YY, F-SW, FM, and MS conceived the study. SP and YY wrote the manuscript. SP, YY, and SW collected data and performed the data analysis. SW, BT, YS, XL, NS, and YZ participated in the clinical treatment. QQ and JL supervised the clinical treatment. F-SW, FM, MS and J-YZ directed the writing and revision of the manuscript. All authors contributed to the article and approved the submitted version.

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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.2022.892618/full#supplementary-material

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B10 Cells Are Associated With Clinical Prognosis During Adult Symptomatic Acute HBV Infection

Yali Liu[1], Xiaofei Du[1], Junfeng Lu[2], Lina Ma[2], Yi Jing[2], Haijing Ben[3], Xinyue Chen[2] and Jing Zhang[1]**

[1] The Third Unit, Department of Hepatology, Beijing Youan Hospital, Capital Medical University, Beijing, China, [2] The First Unit, Department of Hepatology, Beijing Youan Hospital, Capital Medical University, Beijing, China, [3] Beijing Institute of Hepatology, Beijing Youan Hospital, Capital Medical University, Beijing, China

There are few reports about the role of B10 cells in acute hepatitis B (AHB) infection. In this study, based on 48 acute hepatitis B infected patients, we analysis the correlation of B10 cells with HBV clinical prognosis. The results showed that B10 was positively correlated with HBsAg and HBeAg and inversely correlated with anti-HBs. The level of B10 in one week before HBsAg clearance was significantly lower than 2 weeks prior to HBsAg clearance and after 1-2 weeks of HBsAg clearance. B10 cell frequency displayed no correlation with HBV DNA; however, it showed significant temporal synchronization with hepatic inflammatory markers such as ALT. B10 level also associated with hospitalization time. These results indicated that B10 is closely related to the clinical prognosis of acute HBV infection.

Keywords: hepatitis B, acute, interleukin-10, regulatory B cell, cytokine, prognosis, B10

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Yan-Mei Jiao,
Fifth Medical Center of the PLA General Hospital, China

Reviewed by:
Tao Dong,
University of Oxford, United Kingdom
Ping Ma,
Tianjin Second People's Hospital, China

****Correspondence:***
Xinyue Chen
chenxydoc@ccmu.edu.cn
Jing Zhang
zjyouan@ccmu.edu.cn

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INTRODUCTION

Although adult acute hepatitis B is mostly self-limited, more than 100,000 people still die from the disease each year, especially in areas with restricted health resources, according to Global Burden of Disease Study (1). There are significant differences in immunological pathogenesis and clinical outcome between acute and chronic HBV infection. For spontaneous clearance of the HBV in the acute setting within several months, both the innate and the adaptive immune system have been proven to be critical (2–4). Otherwise, inflammation will aggravate or turn into chronic persistent infection. The main function of regulatory B cell is to secrete IL-10, so it is also known as B10 cell (5). B10 cells act as an negative regulator and inhibit excessive immune response and maintain homeostasis especially in autoimmune diseases (6). In last two decades, the immunomodulatory effect of B10 on inflammation, autoimmune diseases, and cancer have attracted much attention (7–9). Previous studies were mostly limited to chronic hepatitis B infection (10, 11). However, it is clear that acute hepatitis B virus infection has a more intense dynamics of virological and immunological process. Here, a cohort of patients with acute hepatitis B who made eventually favorable recovery was analyzed and we attempted to elucidate the relationship between B10 cells and the clinical course.

MATERIALS AND METHODS

Study Objects

We conducted a retrospective observational single-center study. The acute hepatitis B (AHB) patients were initially screened according to the Chinese Management Scheme of Diagnostic and Therapy Criteria of Viral Hepatitis criteria (12): Patients needed to meet the following criteria: no history of hepatitis B virus infection (HBsAg negative), presence of clinical symptoms and signs of AHB, significant increase in serum transaminase with or without elevated bilirubin, positive for HBsAg and/or HBeAg and/or HBV DNA, and immunoglobulin M antibody to hepatitis B core antigen positive (IgM anti-HBc). Exclusion criteria were HCV, HDV, HEV, or HIV co-infection. This case series has been described in our previous study (13). Among these cases, we chose the patients with antiviral treatment intervention-free. Overall, there were 48 patients in this study. The blood samples collection was performed between -2 and 24 weeks after the acute hepatitis B diagnosis was confirmed. 24 patients with chronic hepatitis B (CHB) and 10 healthy people (normal controls, NC) as a comparison group. All fresh blood samples were tested immediately or gathering tested within 2 weeks.

This study was guided by the Declaration of Helsinki and the ethical principles developed by the American Human Research Protection Program (AHRPP), and approved by the Ethical Committee of Beijing You An Hospital, Capital Medical University. All patients provided written informed consent.

HBV Markers

Serologic markers for HBV infection were analyzed by Roche e601 automatic electrochemical luminescence instrument Roche kits (lower limit of detection of HBsAg was 0.05 IU/mL, range from 0.05 to 52,000 IU/mL), according to the manufacturer's instructions. Quantitation of HBV DNA was performed by polymerase chain reaction using the Roche Cobas/Taqman Real-Time PCR 2.0 System(Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions (lower limit of detection was 20 IU/mL).

B10 Confirming

Peripheral blood mononuclear cells (PBMCs) were isolated using lymphocyte separation medium (Ficoll-Hypaque density gradient, Axis-shield, Germany) from 10 ml fresh heparinized blood sample. And then PBMCs were incubated with 1 μM CpG-B ODN2006 (*In vivo*Gen, San Diego, CA, USA) for 96 h at 37°C. PMA (3 ng/ml) and ionomycin (100 ng/ml) were added during the last 4h in the presence of 10 μg/ml brefeldin A (all from Sigma, St Louis, MO, USA). Cells were then surface stained for the markers CD19 Pacific Blue-A, and stained intracellularly with anti-IL-10 APC. So we could assess the ability and the frequency of IL-10 production from purified B cells (14). All antibodies including CD19-PE and IL-10-APC used in flow cytometry were ordered from BD Pharmingen (BD Pharmingen, San Diego, CA, USA). PBMCs were acquired on a FACS Canton flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). FlowJo 7.6.1 (Tree Star Inc., Ashland, OR, USA) was used as the flow analysis software.

Statistical Analysis

All data were analyzed using SPSS 20.0 (IBM Inc, ArMonk, NY, USA) and the GraphPad Prism 7 software (San Diego, CA, USA). Continuous variables were expressed in mean ± SD or median (interquartile range) as appropriate. Qualitative and quantitative differences between subgroups were analyzed by chi-squared or Fisher's exact tests for categorical parameters and Student's t-test or Mann–Whitney test for continuous parameters, paired or unpaired, as appropriate. The Pearson correlation was used to analyze the linear correlation of normally distributed data, otherwise the Spearman rank correction test was used. $P < 0.05$ was considered statistically significant.

RESULTS

Patient Demography and Clinical Outcomes

In total, 48 adult hospitalized patients with AHB were enrolled, which ages ranging from 20 to 71 years (y) The most common chief complaints were dark urine (47/48, 97.9%) scleral or skin jaundice (45/48, 93.8%) and fatigue (42/48, 87.5%). All of the patients displayed significant abnormalities in liver biochemistry enzyme level. The clinical characteristics of all enrolled patients are listed in **Table 1** and the details in **Supplementary Table 1** (**Supplementary 1**). After routine symptomatic treatment and liver protection treatment (but antiviral therapy-free), all 48 patients recovered smoothly. That is, abnormal liver function returned to normal, accompanied by undetectable HBV DNA, and clearance of both HBsAg and HBeAg. At the end of the 48-week follow-up, only 2 patients (4.17%) still did not produce effective Anti-HBs. None of the 48 cases turned chronic.

TABLE 1 | Baseline Clinical and Virological Characteristics.

Characteristic	n = 48
Sex (M/F)	30/18
Age (y), mean±SD	39.6 ± 13.8
ALT (U/L), median (range)	1043 (64.1-6367)
AST (U/L), median (range)	367 (35.5-3129)
TBil (μmol/L), median (range)	83.50 (14.1-379.4)
HBV DNA (log10 IU/mL) mean±SD	3.59±1.77
HBsAg (IU/mL), median (range)	934.7 (0.05-52000)
HBeAg (S/CO), median (range)	6.21 (0.08-1193)
Transmission Route	
sexual	15 (31.3%)
tattooing/dental/body piercing	6 (12.5%)
healthcare exposure	2 (4.17%)
uncertain	25 (52.1%)
Incubation period* (days), median (range)	37.0 (21-102)
Average onset day†(days), mean±SD	11.27±6.45

M, male; F, female; y, year; SD, standard deviation; ALT, alanine transaminase; AST, aspartate transaminase; TBIL, total bilirubin.
**Incubation period, depends on data or transmission route available.*
†Onset day also means the first day of collecting blood sample.

Analysis of the Correlation of B10 and HBV Markers

In this study, we used CD19 as the marker of B cells and the percentage of IL-10 in Breg or regulatory B cells as the level of B10 (**Figure 1A**). In order to analyze the relationship between B10 and prognosis of acute hepatitis B, we first analyzed the relationship between B10 and HBV DNA, HBsAg, Anti-HBs and HBeAg. We found that B10 was positively correlated with HBsAg (r = 0.245, P = 0.015; **Figure 1B**) and HBeAg (r = 0.181, P = 0.045; **Figure 1D**), and negatively correlated with anti-HBs (r = -0.408, P = 0.007; **Figure 1C**). There was no correlation between B10 and HBV DNA (r = -0.153, P = 0.298; **Figure 1E**).

Analysis of B10 Level Before and After HBsAg Seroconversion

In order to further analyze the temporal relationship between B10 and the clearance of acute hepatitis B surface antigen. The level of B10 within one and two weeks before HBsAg turned negative and one to two weeks after HBsAg negative were estimated. The level of B10 in one week before HBsAg turning negative was significantly lower than before and after one to two weeks of that (**Figure 2A**). Compared with the chronic hepatitis B (CHB) group and the healthy normal control (NC) group, the CHB group showed the highest B10 level, while the NC group showed the lowest expression level. There were statistical differences among the three groups (**Figure 2B**).

Correlation Analysis Between Initial B10 Level and Length of Hospital Stay

We also analyzed the relationship between B10 level at the admission day (i.e., before the HBsAg clearance) and length of hospital stay. The B10 level of patients with hospitalization time more than 4 weeks was higher than that of patients within 4 weeks (**Figure 3A**). Baseline TBIL levels also show significance between

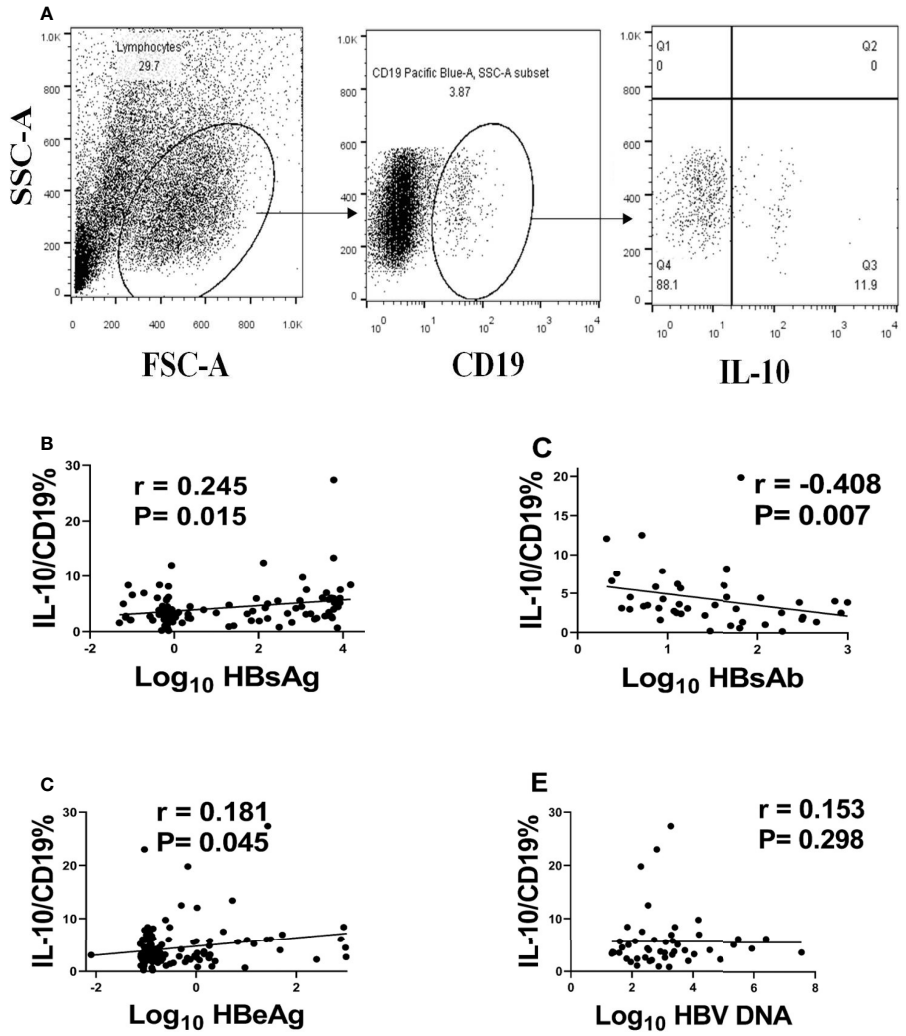

FIGURE 1 | B10 positively associated with HBsAg and HBeAg and negatively associated with anti-HBs. We defined B10 as the level of IL-10 in CD19+ cells **(A)** and analyzed the relationship of B10 with HBsAg **(B)**, anti-HBs **(C)**, HBeAg **(D)** and HBV DNA **(E)**.

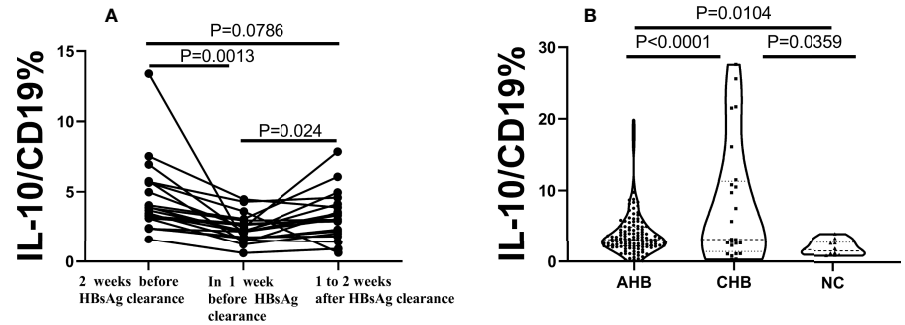

FIGURE 2 | B10 significantly decreased before HBsAg serum clearance in AHB, show significance among the 3 groups. AHB, acute hepatitis **(B)** CHB, chronic hepatitis **(B)** NC, normal control. **(A)**. The dynamics of B10 in each patient with two weeks before HBsAg clearance, one week and within 1-2 weeks after HBsAg clearance. **(B)**. The average of B10 show significance among the 3 groups. the CHB group (n=24) was the highest, followed by the AHB group (n=48), and the lowest was the NC group (n=10).

the 2 groups (**Figure 3D**), but not ALT, AST, HBsAg, HBeAg, and HBV DNA (**Figures 3B, C, E–G**). The hospitalization time of acute hepatitis B patients are not only related to the time of HBsAg clearance, but also related to the time of liver function recovery. We also analyzed the relationship between B10 and ALT. The levels of ALT (**Figure 4A**) and B10 (**Figure 4B**) gradually decreased with a smooth recovery from the disease. B10 was positively correlated with ALT (r = 0.293, P = 0.001; **Figure 4C**).

DISCUSSION

Regulatory B cells are a subgroup of B cells. Regulatory B cells are determined by their secretion of negative cytokine IL-10, so the B cells secreting IL-10 are also known as B10. Very little was found in the literature on the question of the role of B10 cells in acute HBV infection. In this study, we found that B10 was closely related to the prognosis of acute HBV by series of samples from 48 cases of acute HBV patients.

The role of B lymphocytes in the pathogenesis of hepatitis B may have been underestimated (15). It is thought that the robust specific T lymphocyte response largely determines the clearance or persistence of HBV infection. Related research also focuses on the depletion mechanisms of HBV-specific T lymphocyte responses (16, 17). B lymphocytes comprise a major part of adaptive immunity, and their main functions in resisting viral infection include a series of events, including complex antigen identification and antigen presentation, as well as the ultimate production of viral antigen-specific antibodies (18). Mizoguchi et al. first coined the term regulatory B cell to describe B cells that suppress disease in a mouse inflammatory bowel disease model (19, 20). B lymphocytes can perform duplex immunomodulation. A prominent example is that B lymphocytes are highly capable of antigen processing and presentation and are necessary for activating CD4+ T lymphocytes. In recent years, attention has been paid to the potential of B lymphocytes in negative immunomodulation, manifested by a number of studies on autoimmune diseases and inflammatory diseases (21–26).

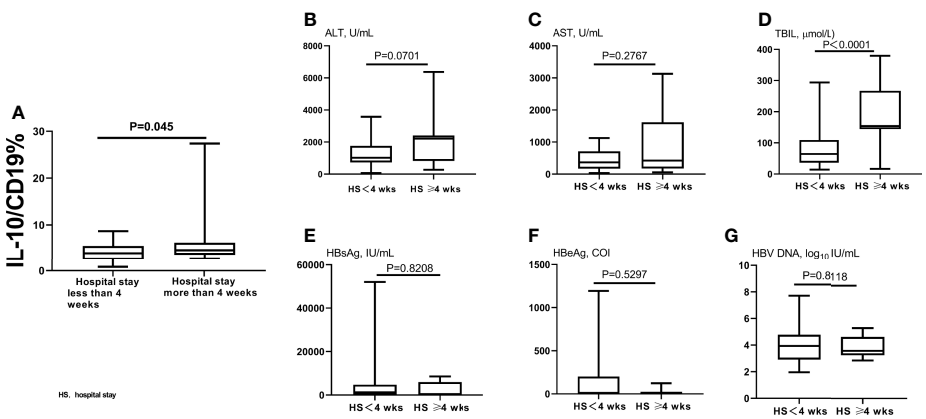

FIGURE 3 | Compared B10 levels and other liver function index between different hospitalization stay time patients. The admitting day's B10 levels was significant lower in patients within 4 weeks of hospitalization stay than those of more than 4 weeks **(A)**. TBIL levels also show significance between the 2 groups **(D)**, but not ALT **(B)**, AST **(C)**, HBsAg **(E)**, HBeAg **(F)**, and HBV DNA **(G)**.

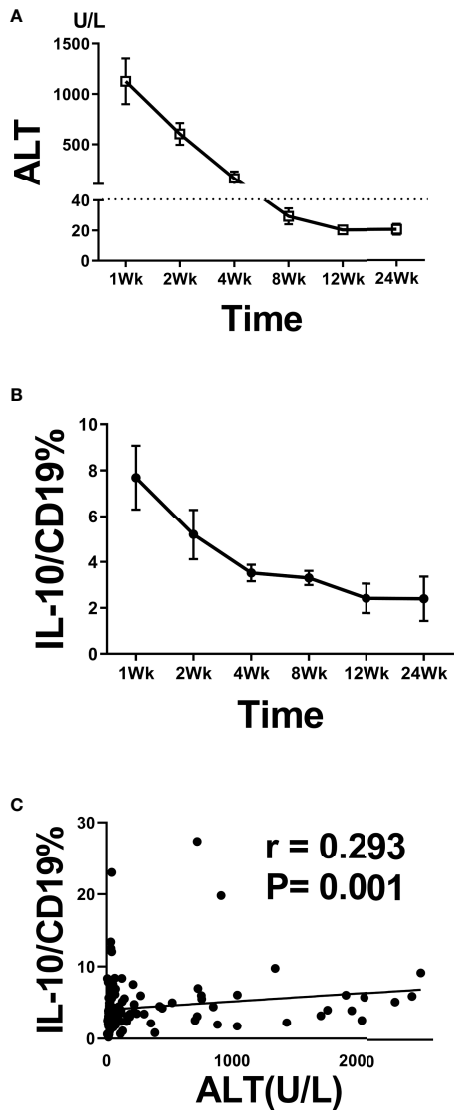

FIGURE 4 | B10 temporal positively associated with ALT during acute HBV infection. Dynamic of ALT **(A)** and B10 **(B)** during hospitalization stay time. The positive correlation between B10 and ALT during hospitalization stay time **(C)**.

IL-10 is an important inhibitory cytokine in the human body, with a low absolute concentration in peripheral blood. In various microenvironments, it inhibits pro-inflammatory cytokines, Th1 cell responses, and T lymphocyte proliferation, performing multiple negative regulations. In a certain sense, the number of IL-10-producing Breg cells and the IL-10 production level reflect the changes in the body's inflammation level and immune status. According to a pioneer study by Dr. Maini and her colleagues in 2012, IL-10 levels in plasma and liver inflammation levels of chronic hepatitis B patients were positively correlated, suggesting that the elevation of IL-10 levels is a protective mechanism (10). In terms of the disease course, compared to chronic hepatitis B, acute hepatitis B is characterized by the clinical features of acute onset and self-limiting viral clearance. In acute hepatitis B, inflammation changes rapidly but can eventually recover in a relatively short time. This study revealed the obvious correlation between the B10 frequency and the changes in liver inflammation: the B10 frequency increased significantly when liver inflammation intensified and decreased rapidly to normal during disease recovery. This also fully demonstrates the potential leading role B10 cell plays in regard to inflammation and immunity.

As stated above, because the secretion of IL-10 is not limited to a particular cell or cell subset, B10 cells may also comprise different cell subsets and may have certain heterogeneities. Indeed, it significantly increased at the peak of inflammatory activities and decreased when inflammation was eased, suggesting that it can inhibit inflammation. The inflammation levels of acute hepatitis B can be self-limiting, synchronically related to the levels of B10 frequency, indicating the latter's function in inhibiting overdevelopment of inflammation to prevent spread. In the AHB recovery phase, B10 drop to a low level and prevent the disease from turning into a chronic persistent infection. Even if the P values have statistical significance, the absolute values of Pearson correlation coefficient (r) between B10 cell and HBsAg, HBeAg and ALT are not strong enough. A larger sample size to verify the correlation is expected. It is no doubt that regulatory T cells can perform negative regulation by means of IL-10, and this has been confirmed for chronic hepatitis B. B10 cells exerted dysregulation in T cell function through IL-35 dependent mechanism and depend on cell-to-cell contact style (27, 28).

With AHB standard management procedure and no antiviral intervention, we could conclude that the length of hospital stay is directly related to severity of illness. In this study, the frequency of B10 cell was higher in patients hospitalized for longer than 4 weeks than in patients hospitalized for less than 4 weeks. These results further suggest that B10 is positively correlated with the course and prognosis of acute liver inflammatory disease. Before and after HBsAg clearance, we also clearly observed the critical time point which the expression of B10 frequency is the lowest. Accompanied by HBsAg clearance and anti-HBs appearance gradually, B10 return to the previous normal level. These regulatory cells are also regulated by regulators and may be related to IL-35 or IL-21 (8), which needs to be confirmed by further studies.

Previous study revealed that the depletion of Treg cells in the PBMCs of patients with chronic hepatitis B did not alter the frequency of B10 cells or their ability to produce IL-10 (11), indicating regulatory T or B lymphocyte have their own unique pathway to influence immune response. Also, there are other IL-10-producing immune cells such as T cell, monocytes and NK cells, not involved in this assay with regret (29–31). We speculate that these immune cells focus on secreting IL-10, forming a complex and interacting regulatory network, and exert inhibitory effects organically. Furthermore, the lack of acute fulminant hepatitis and liver specimens is the deficiency of this study. Additionally, the development about HBsAg-specific and/or HBcAg-specific B cells maybe novel targets for antiviral strategies toward functional cure of chronic HBV infection attracted more attentions (32–34). The failure to explore the

phenotype features of those HBV-specific B cells in the environment of acute hepatitis B is also one of the shortcomings of this study.

In conclusion, we found that B10 is closely related to the clinical prognosis of acute HBV infection. Whether B10 is one of the pathogenic mechanisms of acute fulminant hepatitis and whether intervention of B10 cells is helpful to the elimination of chronic HBV deserves further study.

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 Ethical Committee of Beijing YouAn Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL, XC, and JZ conceived and designed the experiments; YL and XD performed the experiments; YL and HB analyzed the data; XD, LM, JL, and YJ contributed patients recruiting and management. YL and JZ wrote the paper. All authors contributed to the article and approved the submitted version.

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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.2022.906650/full#supplementary-material

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Study on the Retreatment, Outcome, and Potential Predictors of Recurrence in Patients With Recurrence of Hepatitis B After Functional Cure

Xiao Lin[1†], Aixin Song[1†], Junfeng Lu[1], Sujun Zheng[1], Zhongjie Hu[1], Lina Ma[1], Zhenhuan Cao[2], Hong Li[2], Yanhong Zheng[1], Shan Ren[1] and Xinyue Chen[1*]*

[1] First Department of Liver Disease Center, Beijing Youan Hospital, Capital Medical University, Beijing, China, [2] Third Department of Liver Disease Center, Beijing Youan Hospital, Capital Medical University, Beijing, China

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Edited by:
Sheikh Mohammad Akbar, Ehime University, Japan

Reviewed by:
Joel V. Chua, University of Maryland, United States
Mamun Al Mahtab, Bangabandhu Sheikh Mujib Medical University (BSMMU), Bangladesh

***Correspondence:**
Shan Ren
13811361553@163.com
Xinyue Chen
chenxydoc@ccmu.edu.cn

[†]These authors have contributed equally to this work

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Background: Studies about the retreatment and predictors for patients with hepatitis B recurrence after functional cure are rare. This study aimed to evaluate the effect of retreatment, outcome, and potential predictors of recurrence in patients with recurrence after functional cure.

Methods: A long-term follow-up was conducted with 32 cumulatively obtained patients who relapsed after cessation of pegylated interferon (Peg-IFN)-based antiviral treatment. The decision of whether to treatment or which therapeutic method to use [Peg-IFN or nucleos(t)ide analogs (NAs)] was based on the patient's preferences and wishes. The rate of achieving functional cure and the clinical outcomes of different therapeutic methods were analyzed. Hepatitis B surface antibody (anti-HBs) and hepatitis B core antibody (anti-HBc) levels were detected in patients with blood samples during follow-up to evaluate the predictive ability of recurrence.

Results: The follow-up time of 32 recurrence cases was 42–532 weeks after recurrence (median 226 weeks). In the 20 patients who received retreatment (15 received Peg-IFN and 5 received NAs only), the rate of functional cure was 65.0% (13/20); it was 86.7% (13/15) in the patients retreated with Peg-IFN. Three cases experienced recurrence again. Five patients received NA treatment, and no functional cure was achieved. No drug intervention was administered for 12 patients, 2 of them with hepatitis B virus (HBV) DNA spontaneous clearance, and one patient achieved spontaneous hepatitis B surface antigen (HBsAg) clearance during follow-up. Patients who relapsed after functional cure with Peg-IFN treatment did not have liver cirrhosis or hepatocellular carcinoma during the follow-up, regardless of whether they received retreatment. Anti-HBs and anti-HBc levels at the end of therapy were predictors of recurrence ($p < 0.001$, $p = 0.023$). The value of combining the above two indicators in predicting recurrence was further improved, the areas under the

receiver operating characteristic curves were 0.833, at combining predictors >-0.386, the predictive sensitivity and specificity for recurrence were 86.67% and 90.62%.

Conclusion: The functional cure rate was above 80% for patients with recurrence treated by Peg-IFN. During the follow-up, liver cirrhosis and hepatocellular carcinoma were not observed in all recurrence cases. High levels of anti-HBs and anti-HBc at the time of drug discontinuation are less likely to relapse.

Keywords: HBV, recurrence, retreatment, high clearance rate, outcome, hepatitis B core antibody, hepatitis B surface antibody

INTRODUCTION

In recent years, domestic and international chronic hepatitis B (CHB) management guidelines have recommended that the goal for CHB patients should be functional cure to improve prognosis (1–3). Recently, studies related to hepatitis B surface antigen (HBsAg) clearance have increased, but there are still fewer studies related to recurrence after HBsAg clearance. Our previous study and a meta-analysis found that the recurrence rate after functional cure was 6.19%–9.66%, including cases with HBsAg positivity and/or hepatitis B virus (HBV) DNA positivity (4, 5). Due to the few large or even sufficient sample relapse cohorts are available for analysis, the effectiveness of retreatment and clinical outcomes after relapse and the correlation between immune function and relapse are even rarer. Therefore, we conducted a long-term follow-up of CHB who achieved functional cure in the previous study cohort. And we evaluated the clinical characteristics, retreatment effect, and outcome of relapsers after functional cure, as well as the predictors of relapse.

MATERIALS AND METHODS

Study Population

During the year 2005–2021, a total of 1,326 CHB patients in our center received antiviral treatment with pegylated interferon (Peg-IFN) or Peg-IFN combined with nucleos(t)ide analogs (NAs), 358 of them achieved a functional cure. From the date of treatment cessation, the patients received regular follow-up, and a total of 32 cases of recurrence were observed. Informed consent was obtained from all subjects. The protocol and the consent form for the study were approved by the research ethics committee of the Beijing Youan Hospital, Capital Medical University, China ([2018]050).

Abbreviations: Peg-IFN, pegylated interferon; NA, nucleos(t)ide analog; anti-HBs, hepatitis B surface antibody; anti-HBc, hepatitis B core antibody; CHB, chronic hepatitis B; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; ALT, alanine aminotransferase; AFP, alpha fetoprotein; Ig, immunoglobulin; ROC, receiver operating characteristic; TDF, tenofovir disoproxil fumarate; AUROC, area under the receiver operating characteristic curve; PPV, positive predictive value; NPV, negative predictive value; PRE, predictive probability equation; ETV, entecavir.

Functional cure was defined as that after the cessation of treatment for 12 weeks or more, the virological response (HBV DNA <20 IU/ml), hepatitis B e antigen (HBeAg)-negative status, HBsAg clearance (HBsAg <0.05 IU/ml) or seroconversion [hepatitis B surface antibody (anti-HBs) ≥10 IU/ml], and normal liver biochemical indicators were maintained. The definition of recurrence is the reappearance of HBsAg, HBV DNA, or both at least two times in an interval of 4–12 weeks during the follow-up after treatment cessation. Relapse type was defined according to the HBsAg- and HBV DNA-positive statuses at the time of the first confirmation of recurrence. The patients were divided into three types: Type I: only HBsAg positive, HBV DNA negative; type II: both HBsAg and HBV DNA positive; type III: HBsAg negative and HBV DNA positive.

Follow-Up Plan and Laboratory Detection Methods

Regular follow-up was continued after treatment cessation to determine whether the patient had recurrence. After the first confirmed recurrence, the examination was repeated every 12 ± 4 weeks, and blood samples are collected for further analysis. The examination included HBV markers (HBsAg, anti-HBs, HBeAg), HBV DNA, alanine aminotransferase (ALT), alpha-fetoprotein (AFP), and abdominal ultrasound. HBV DNA sequencing was performed in some patients with a high viral load, and the hepatitis B core antibody (anti-HBc) level was tested in some patients who had retained blood samples.

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 tested using the cobas® AmpliPrep/cobas® Taqman automatic nucleic acid separation and purification and PCR analysis system (Roche Diagnostics GmbH, Germany), with a detection limit of 20 IU/ml. ALT was tested using Olympus AU5400 biochemical analyzer (Japan), and the normal value was <40 u/l. AFP was detected using Cobas E601 (Roche Diagnostics GmbH, Germany) analyzer, and the normal value was <7 ng/ml. Sequencing of the P and S regions of HBV was performed using the PCR direct sequencing method (ABI 3730XL, USA). Serum anti-HBc was quantified by using double-sandwich anti-HBc immunoglobulin (Ig)M and IgG

immunoassay (Wantai, China) and a detection range of 2–6 log10 IU/ml (6, 7).

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 data are expressed as the mean ± standard deviation or median (interquartile range), and categorical variable data are 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 recurrence. Receiver operating characteristic (ROC) curve analysis was performed to analyze the predictive value of the factors in predicting recurrence. The cutoff value corresponding to the maximum Youden index is regarded as the best cutoff (Youden index = sensitivity + specificity - 1). A p-value <0.05 was considered significant.

RESULTS

General Information and Follow-Up

A total of 358 patients with functional cure were derived from a continuation of our team's previous study cohort population (5) and followed up for 24–624 weeks after treatment cessation, with a median of 208 weeks. Cumulatively, 32 cases of recurrence were observed, with a recurrence rate of 8.94%. The average age of patients with recurrence was 39 ± 11 years, and men accounted for 59.4% (19/32). As of July 2021, the patients were followed for 42–532 weeks after recurrence, with a median of 226 weeks.

Clinical Data of the Patients at the Time of Recurrence

The recurrence time was 18–375 weeks after the end of treatment, with a median of 48 weeks. Type I recurrence accounted for 56.2% (18 cases, 4 of which had HBV DNA reversion at 100–164 weeks), 21.9% for type II recurrence (7 cases, 1 of which also had HBeAg reversion), and 21.9% for type III recurrence (7 cases, 1 of which had HBsAg reversion at 64 weeks).

The above relapsers had lower HBsAg levels at the time of HBsAg relapse, 22 patients (22/26, 84.62%) had HBsAg levels of <10 IU/ml, and 53.85% (14/26) had HBsAg levels of <1 IU/ml. The relapsed patients included 18 cases that were HBV DNA positive. Among them, HBV DNA levels fluctuated from 1.30E+1 to 6.21E+2 IU/ml in 83.3% (15/18) of the cases who experienced HBV DNA positivity. Sequencing of relevant variants and nucleotide resistance gene testing were performed in 2 patients (Nos. 27 and 29) with type III recurrence and 4 patients (Nos. 19, 20, 22, and 23) with type II recurrence among those with HBV DNA >1 E+3 IU/ml at recurrence or during follow-up. S-region variants were detected in 3 patients (Nos. 20, 23, and 29); HBV P-region drug resistance mutation was present in patient no. 22 (the nucleotide resistance gene sites detected after recurrence were identical to those detected before the conversion to HBsAg-negative status). The specific results are reported in **Table 1**.

Retreatment of Relapsed Patients

Patients' decisions about whether to take antiviral drugs need to be determined in the context of their reality. There are three main treatment measures for patients who relapse: 1) immediate treatment; 2) dynamic observation initially, followed by treatment when the condition worsens; 3) always dynamic observation (no treatment). The following describes the situation of clinical interventions in patients with recurrence.

Retreatment Immediately After Recurrence

According to the patient's wishes, 15 patients chose to start antiviral therapy immediately after recurrence. Among them, 11 patients were treated with Peg-IFN alone or combined with NAs (hereinafter referred to as Peg-IFN group), and 4 patients were treated with NAs (hereinafter referred to as NA group). The specific medication regimen, course of treatment, and outcomes are summarized in **Table 2**.

Follow-Up Treatment of Dynamic Observers After Recurrence

Seventeen patients did not receive treatment immediately after recurrence and opted to undergo dynamic observation instead. Among them, 6 patients did not receive treatment, and their HBV markers and HBV DNA during the follow-up period were not significantly different from that at the time of the first confirmed recurrence. Another eight patients showed altered HBsAg and/or HBV DNA levels. In addition, 2 patients showed spontaneous clearance of HBV DNA, and 1 patient had spontaneous clearance of HBsAg during the observation period (**Figure 1A**).

The details of the 8 patients with hepatitis B serological changes are shown in **Figure 1B**. Type I: 4 out of 5 patients had HBV DNA reversion at 100–164 weeks (mean: 128 weeks) after HBsAg reversion with normal liver function, and 2 of them received retreatment. Another one was followed for 192 weeks after recurrence: the HBsAg level increased from 0.413 IU/ml at the time of recurrence to 555.4 IU/ml, and retreatment was started. Type II: Both HBsAg and HBV DNA were significantly elevated during the observation period in 2 patients with recurrence. One patient (No. 20) was examined at 28 weeks after recurrence: HBV DNA increased from 6.21E+02 IU/ml at the time of recurrence to >1 E+08 IU/ml, HBsAg level increased from 168 IU/ml at the time of recurrence to 7,992 IU/ml (the anti-HB level was still >1,000 IU/ml), HBeAg reversion was observed, ALT was 51 U/L, and S-region mutation sequencing was positive for sI126T. This patient received retreatment. Type III: Patient no. 27 had NA drug resistance mutations (rtL180M, rtM204I) previously, but no drug resistance site was detected after recurrence. After 64 weeks of relapse, HBsAg reversion was observed (39.81 IU/ml), HBV DNA increased from 4.94E+1 IU/ml at the time of recurrence to 2.09 E+5 IU/ml, ALT was normal, and retreatment was started. The specific medication regimen, course of treatment, and outcome of the 5 patients who were retreated are shown in **Table 3**.

Ultimately, due to the significant changes in HBV markers and HBV DNA, 5 of the 17 patients who initially chose dynamic

TABLE 1 | Basic information of the 32 patients at the time of recurrence.

Patient	Gender	Age (years)	Recurrence time‡ (weeks)	HBsAg£ (IU/ml)	anti-HBs£ (IU/L)	HBeAg£ (COI)	HBV DNA£ (IU/ml)	ALT£ (U/L)	S-region variant	Drug resistance detection
1♦/★	Female	31	47	0.287	<2	0.120	<20	12.5	/	/
2♦/★	Female	24	208	1.680	7.66	0.100	<20	11.7	/	/
3♦/★	Female	56	52	0.052	20.36	0.112	<20	22.1	/	/
4♦/★	Male	39	134	0.089	<2	0.105	<20	26.2	/	/
5♦	Female	20	147	1.750	185.40	0.102	<20	7.8	/	/
6♦	Male	27	52	0.413	23.24	0.076	<20	37.8	/	/
7♦	Male	36	48	0.621	836.40	0.112	<20	19.4	/	/
8♦	Male	45	60	0.055	<2	0.111	<20	20.0	/	/
9♦	Female	42	25	0.166	24.99	0.104	<20	20.1	/	/
10♦	Female	43	154	0.197	39.56	0.507	<20	11.2	/	/
11♦	Female	34	46	2.000	<2	0.099	<20	10.4	/	/
12♦	Female	30	20	0.082	23.25	0.086	<20	9.0	/	/
13♦	Male	49	48	1.200	38.41	0.128	<20	25.3	/	/
14♦	Female	44	48	2.770	767.20	0.116	<20	8.9	/	/
15♦	Female	31	60	0.073	32.89	0.131	<20	7.0	/	/
16♦	Female	32	21	1.540	517.90	0.139	<20	12.3	/	/
17♦	Male	49	28	0.301	205.90	0.103	<20	16.0	/	/
18♦	Male	34	18	0.052	36.94	0.091	<20	24.7	/	/
19★	Female	26	39	184.800	<2	0.112	1.66E+2 - 2.56E+3	4.8	Negative	Negative
20★	Male	35	21	168.000	>1,000	0.157	6.21E+2 - 1.70E+8	27.7	sI126T	Negative
21★	Male	33	188	2.460	<2	0.099	3.45E+1	28.4	/	/
22★	Male	55	34	12,602.000	785.50	1,703.000	3.02E+7	243.2	Negative	rtL180M, rtM204V, rtS202G
23★	Male	28	43	2.440	211.80	0.076	7.02E+3	31.5	sI126T, sT140I, sD144A	Negative
24★	Male	26	73	0.437	3.50	0.108	2.02E+1	16.8	/	/
25★	Male	26	48	0.21	<2	0.121	1.30E+1	10.0	/	/
26□	Male	51	99	<0.05	530.80	0.164	4.28E+1	20.4	/	/
27□/★	Male	38	43	<0.05	489.00	0.120	4.94E+1-2.09E+5	35.8	Negative	Negative
28□	Male	54	208	<0.05	59.44	0.095	2.14E+1	21.5	/	/
29□	Male	44	34	<0.05	88.10	0.180	1.71E+3	15.0	sD144A	Negative
30□	Female	62	156	<0.05	<2	0.107	2.47E+1	35.0	/	/
31□	Male	46	52	<0.05	>1,000	0.113	2.12E+1	27.4	/	/
32□	Male	52	375	<0.05	7.93	0.207	2.60E+1	35.0	/	/

ALT, alanine aminotransferase. Type of recurrence: ♦type I; ★type II; □type III; ♦/★ type I at the time of recurrence and then converted to type II; □/★ type III at the time of recurrence and then converted to type II; ‡ weeks from the date of treatment cessation to the recurrence; £ viral quantities and ALT at recurrence; "/" represents not tested.

observation after relapse started retreatment. The other 12 patients remained under dynamic observation.

Final Retreatment Measures in 32 Relapse Cases

Ultimately, 20 patients were retreated, including 15 who started treatment immediately after relapse and 5 who started retreatment after HBV markers and HBV DNA changes occurred during observation. A total of 15 of these patients were treated with Peg-IFN and 5 were treated with NAs only. In addition, 12 cases were always under dynamic observation.

Outcomes of Patients With Recurrence

In our study, the criteria for achieving functional cure of hepatitis B with retreatment in relapsed patients were the same as those described above. As of July 2021, 16 of the 32 relapsed patients (50.0%) achieved functional cure again, of which 13 achieved functional cure after retreatment and 3 patients achieved spontaneous clearance of HBV DNA or HBsAg without treatment. The recurrence rate was higher in the treated group (65%, 13/20) than in the untreated group (25%, 3/12), and there was a significant statistical difference between the two groups ($c^2 = 4.800$, $p = 0.028$). In one case, S-region mutation (sD144A) was detected. Under NA maintenance treatment, HBV DNA was below the lower limit of detection, HBsAg was negative, and anti-HBs was positive. However, this patient was not determined to be functionally cured.

Outcomes of Patients Who Were Retreated After Recurrence

A total of 20 relapsed patients received antiviral treatment, including Peg-IFN and NA treatment, and the overall functional cure rate was 65.0% (13/20). The functional recured patients were all from the Peg-IFN group, and 86.7% (13/15) of patients who received Peg-IFN retreatment achieved functional cure again with a shorter treatment course with a median of 28 weeks. However, none of the 5 patients who were treated with NAs achieved functional cure. The recurrence rates between the Peg-IFN and NA groups were statistically

TABLE 2 | Therapeutic method and outcomes of 15 patients who were treated immediately after recurrence.

Patient	Follow-up time (weeks)§	Therapy	Functional cure (weeks)	Consolidation therapy (weeks)	Therapy duration (weeks)	Follow-up after treatment cessation (weeks)
12◆	52	Peg-IFN	12	24	36	16
13◆	532	Peg-IFN	12	12	24	508
14◆	221	Peg-IFN	32	12	44	177
15◆	62	Peg-IFN	12	14	26	36
16◆	288	ETV+Peg-IFN	32	26	58	230
17◆	168	ETV+ Peg-IFN &	36	36	72	22
18◆	329	ETV	–	–	329	/
21★	224	ETV+Peg-IFN	12	24	36	188
22★	208	ETV+TDF+Peg-IFN &	–	–	60	/
23★	370	ETV	-	-	370	/
24★	284	TDF	–	–	284	/
25★	66	ETV+Peg-IFN	42	12	54	12
28□	444	TDF+ Peg-IFN	16	32	48	396
29□	392	TDF	-	-	392	/
32□	52	TDF+Peg-IFN	28	12	40	12

Peg-IFN, pegylated interferon; TDF, tenofovir disoproxil fumarate; ETV, entecavir. §weeks from the date of recurrence to the last follow-up; &NA treatment was administered first, then combined with Peg-IFN treatment; "/" represents the patient was still under treatment at the last follow-up.

significant (c^2 = 12.381, p < 0.001). The functional recure rates for different recurrence types were different. Based on the HBsAg and HBV DNA status at the time of retreatment, the recure rates were 87.5% (7/8) for type I recurrence, 44.4% (4/9) for type II recurrence, and 66.7% (2/3) for type III recurrence. There was no statistically significant recurrence rate among the relapse types (all p > 0.05).

Peg-IFN retreatment was not successful in 2 cases. Patient no. 22 had HBsAg level 1,703 IU/ml at the time of retreatment, and the drug resistance test was positive (rtL180M, rtM204V, rtS202G) Patient no. 20 had an S-region mutation (sI126T). None of the patients who were treated with NAs achieved functional cure. One case of type III recurrence had S-region mutations (sD144A), HBV DNA-negative conversion by treatment with NAs, and long-term NA administration for maintenance therapy. In the remaining 4 cases, only HBV DNA was inhibited and HBsAg was still positive.

After the first recurrence, a total of 13 patients reached functional cure after retreatment and discontinued drug treatment. Among them, 10 patients were followed up for 12–395 weeks, with a median of 88 weeks, and maintained functional cure. The other 3 patients (Nos. 6, 13, and 21) experienced another recurrence. The second recurrence times were 33, 244, and 52 weeks after the end of treatment. Two patients treated with Peg-IFN achieved functional cure again. The other patient was treated with tenofovir disoproxil fumarate (TDF); HBV DNA was negative but still had low-level HBsAg positivity (**Figure S1**).

Outcomes of Untreated Patients With Recurrence

Twelve patients were followed up to the present date without retreatment. The follow-up period was 42–269 weeks after recurrence, with a median of 178 weeks. Six patients had

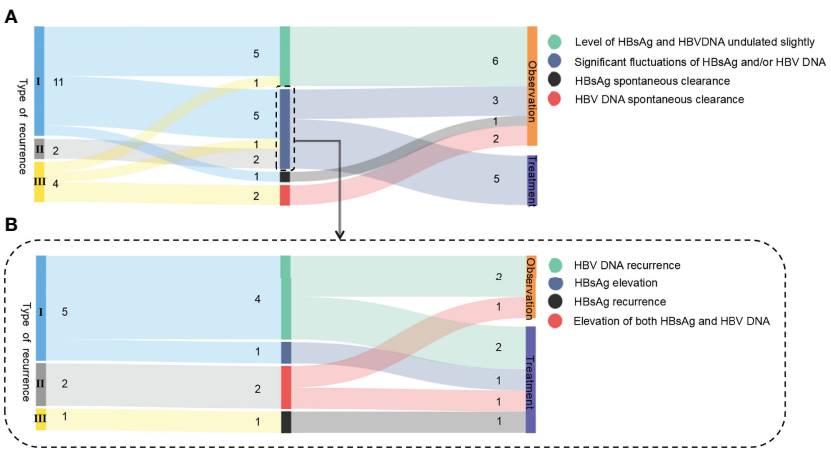


FIGURE 1 | Sankey diagram of the 8 cases of recurrence. **(A)** Follow-up retreatment of 17 relapsed patients selected for dynamic observation initially. **(B)** Follow-up retreatment of 8 relapsed patients with hepatitis B serological changes. I, II, and III in the figure represent the type of recurrence, and the Arabic numerals represent the number of cases.

TABLE 3 | Therapeutic method and outcomes of 5 patients who were under dynamic observation initially, followed by treatment.

Patient	Follow-up time (weeks)§	Therapy	Functional cure (weeks)	Consolidation therapy (weeks)	Therapy duration (weeks)	Follow-up after treatment cessation (weeks)
3♦/★	357	ETV+Peg-IFN	12	24	36	144
4♦/★	296	ETV	-	-	166	/
6♦	402	Peg-IFN	60	24	84	114
20★	227	TDF+Peg-IFN &	-	-	92	/
27□/★	258	TDF+Peg-IFN	32	20	52	141

Peg-IFN, pegylated interferon; TDF, tenofovir disoproxil fumarate; ETV, entecavir. §weeks from the date of recurrence to the last follow-up; &NA treatment was administered first, then combined with Peg-IFN treatment; "/"represents the patient was still under treatment at the last follow-up.

HBsAg and/or HBV DNA fluctuations at low levels, which did not cause liver function abnormality or disease progression. Three other patients experienced more pronounced fluctuations in hepatitis B virology (**Figure 1A**). Among the untreated patients, 2 type III recurrence patients became HBV DNA negative spontaneously, and 1 type I recurrence patient had spontaneously turned HBsAg negative during the follow-up period. These 3 patients continued to be followed up for 225, 269, and 24 weeks (**Figure S1**), and HBV DNA and HBsAg remained negative and met the functional cure criteria.

Prognosis of Relapsed Patients

Liver function and AFP tests as well as liver ultrasound and FibroScan were performed in all of the recurrence patients during the follow-up period. Two patients developed ALT abnormalities after recurrence, and both returned to normal after retreatment. AFP increased intermittently in 3 patients with recurrence, fluctuating between 7.12 and 9.97 ng/ml (normal value <7 ng/ml). No disease progression, liver cirrhosis, or liver cancer was observed in all of the patients. In general, relapsed hepatitis B patients with functional cures had a good prognosis.

Recurrence-Related Factors

The Anti-HBs Levels Between Recurrence and Non-Recurrence Groups

According to whether they relapsed or not, the 358 functionally cured patients included in this study were divided into two groups. Among them, 32 relapsed patients were defined as the recurrence group. Based on the median of 48 weeks at the point of recurrence, another 236 cases were followed up to 48 weeks after drug withdrawal and were defined as non-recurrence groups. Comparing the anti-HBs levels of the two groups at drug withdrawal and recurrence (or 48 weeks after drug withdrawal), the recurrence group had significantly lower anti-HBs levels than those in the non-recurrence group (1.87 ± 1.14 vs. 2.48 ± 0.77, $p < 0.001$; 1.48 ± 1.09 vs. 2.34 ± 0.77, $p < 0.001$, **Table S1**). Anti-HBs levels were lower in both groups at relapse and 48 weeks after discontinuation of drug therapy than those at treatment cessation. In particular, the recurrence group showed a greater decrease in anti-HBs levels (0.3897 ± 1.62 vs. 0.1389 ± 0.2844, $p = 0.032$), while anti-HBs levels of the non-relapse group remained high after drug withdrawal (**Figure 2A**).

The Anti-HBc Levels Between Recurrence and Non-Recurrence Groups

A total of 15 relapsed patients had blood samples at the point of drug withdrawal and relapse (recurrence group). A matched control group (non-recurrence group) of 32 non-relapsed patients who had the same demographic data and the blood samples available at the time of drug withdrawal and 48 weeks after cessation of treatment (based on the median of 48 weeks at the point of recurrence). The demographic characteristics of the enrolled patients were shown in **Table S2**. Quantitative levels of anti-HBc were detected in blood samples from the above

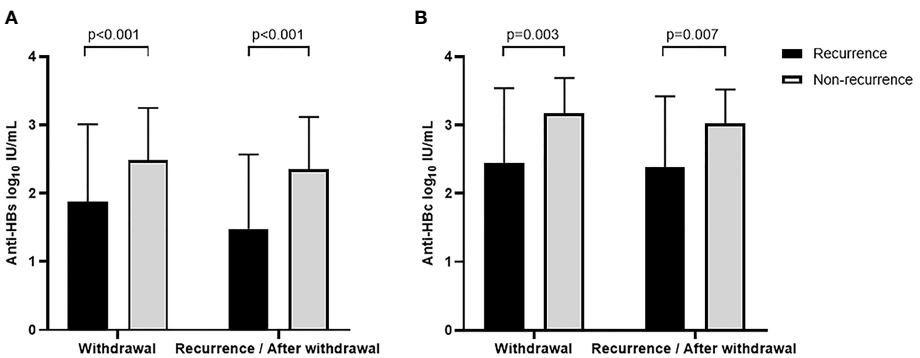


FIGURE 2 | Anti-HBs **(A)** and anti-HBc **(B)** levels at different time points between recurrence and non-recurrence groups.

patients. The results showed that the recurrence group had significantly lower anti-HBc levels at both times of drug withdrawal and relapse (or 48 weeks after discontinuation) than those of the non-recurrence group (2.44 ± 1.10 vs. 3.17 ± 0.52, p = 0.003; 2.38 ± 1.04 vs. 3.02 ± 0.50, p = 0.007, **Table S2**; **Figure 2B**). We measured anti-HBc levels in two of three patients with twice relapses (Nos. 13 and 21), all of whom had anti-HBc levels below the lower limit of detection at the first relapse.

Possibility of Anti-HBs and Anti-HBc Levels as a Predictor of Recurrence

Logistic regression analysis showed that the levels of anti-HBs [odds ratio (OR) = 0.525, p < 0.001, 95% CI: 0.369–0.746] and anti-HBc (OR = 0.226, p = 0.023, 95% CI: 0.062–0.818) at the time of drug discontinuation were both associated with recurrence. To evaluate the predictive value of anti-HBc and anti-HBs levels at drug discontinuation for relapse, areas under the ROC curve (AUROC), cutoff, specificity, sensitivity, positive predictive value (PPV), and negative predictive value (NPV) were calculated (**Figure 3**). The AUROC of anti-HBc and anti-HBs levels at drug discontinuation was 0.724 and 0.679, respectively, with low specificity and PPV, which was nsot ideal for predicting relapse.

To improve the prediction accuracy, this study established new combining predictors based on the extension of the logistic regression model in statistics and the method of the logistic model of constructing a joint predictor (8–11). Combined anti-HBs (X1) and anti-HBc (X2) at treatment cessation was expressed in a logistic regression model and predictive probability equation (PRE):

$$\text{combining predictors} = 5.1915 - 0.76996 \times X1 - 1.41559 \times X2$$

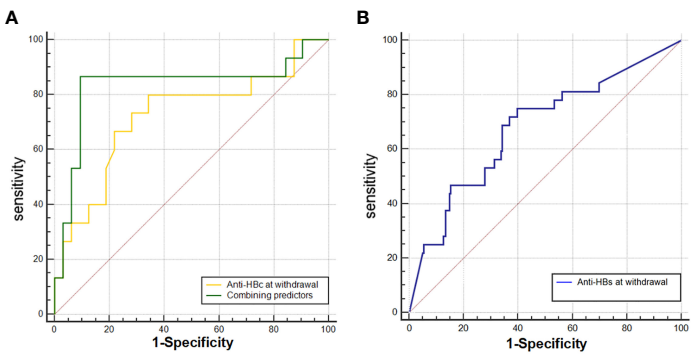
$$\text{combining predictors PRE} = \frac{1}{1 + e^{-(5.1915 - 0.76996 \times X1 - 1.41559 \times X2)}}$$

The predictive ability of the combined predictors for relapse was evaluated by logistic regression, OR = 2.718, p = 0.014, 95% CI: 1.226–6.029. The area under the curve of the combined factor was 0.833, and the sum of sensitivity (86.67%) plus specificity (90.62%) of the diagnosis was greatest when the cutoff value was equal to -0.64, with both PPV and NPV higher at 86.67% and 90.62%, respectively. The above results suggest that the combination of anti-HBs and anti-HBc at drug discontinuation was more valuable in predicting relapse.

DISCUSSION

HBsAg seroclearance or seroconversion has been considered the ideal treatment endpoint for CHB patients (1–3). Some studies showed that the long-term prognosis of patients with CHB could be improved after functional cure (4, 8, 9, 12). However, relapse still occurs in some patients, and the types of relapse are diverse. In addition, relapse cohorts are difficult to obtain. There is a lack of evidence-based medical knowledge on the effects of retreatment after recurrence and the relationship between recurrence and disease progression. Moreover, there are few studies on immunological predictors to predict relapse. Therefore, we studied the issues as mentioned above in 32 relapsed patients with functional cure observed since 2005.

In the long-term follow-up of 32 relapsed patients, 20 patients received retreatment, 13 of them achieved functional cure, and the functional cure rate was 65.0% (13/20). Of a total of 15 patients treated with Peg-IFN, 13 patients were cured, and the recovery rate of patients retreated with Peg-IFN was 86.7% (13/15). A second recurrence occurred in 3 patients who were followed up from 33 to 244 weeks after drug withdrawal. The 5 patients in the NA group did not achieve a second cure. However, among the untreated, two cases had spontaneous clearance of HBV DNA and one case had spontaneous clearance of HBsAg, resulting in functional cure of hepatitis B again. Few studies reported on the treatment outcome of



Parameter	AUROC	cut-off	Sensitivity	Specificity	PPV	NPV
Anti-HBc at drug withdrawal	0.724	2.94	80.00	65.62	52.17 (12/23)	87.50 (21/24)
Anti-HBs at drug withdrawal	0.679	2.70	75.00	60.17	20.34 (24/118)	94.66 (142/150)
Combining predictors	0.833	-0.64	86.67	90.62	81.25 (13/16)	93.55 (29/31)

FIGURE 3 | AUROC of recurrence predictors. **(A)** AUROC of anti-HBc at drug withdrawal and combining predictors; **(B)** AUROC of anti-HBs at drug withdrawal.

relapsers in clinically cured hepatitis B. Kim et al. (13) and Chu and Liaw (8) reported that HBV DNA was spontaneously cleared in patients with HBV DNA reversion, and HBsAg was spontaneously cleared in patients with HBsAg reversion. In 2020, Lok et al. (14) reported 10 relapsed patients; two of them retreated, but the treatment effect was not reported. Similar studies have been reported by Wu et al. (15), who reported a functional cure rate of 81.0% (17/21) in patients with HBsAg reversion after a median (mean) of 24 (28) weeks of Peg-IFN retreatment and observed recurrence in 2 patients. These results suggest that Peg-IFN-based retreatment can obtain higher HBsAg clearance in relapsed patients. The results of the study by Wu et al. are similar to those of our study. These results suggest that Peg-IFN-based retreatment can obtain higher HBsAg clearance.

Previous reports of relapsers after functional cure of hepatitis B were mainly HBsAg positive and both HBsAg and HBV DNA positive (16–18). In 2019, our team reported a new type of relapse, namely, "HBsAg negative, anti-HBs positive, HBV DNA positive," and sequencing results show that the mechanism may be related to the mutation of the HBV S region (5). Lok et al. (14) also observed 2 cases of recurrence with HBsAg negative but HBV DNA positive. In this study, we observed that different recurrence types had different recovery rates, and the recovery rate of patients with type I recurrence was significantly higher than that of patients with other recurrence types. In addition, in type II and III relapsers, S-region mutations were detected in 3 patients, and HBV DNA was continuously below the lower limit of detection after NA treatment, which must be maintained for the long-term according to current treatment guidelines. These patients have difficulty in obtaining functional cures. One case was resistant to NAs; despite long-term treatment with Peg-IFN combined with NAs, HBsAg conversion was not achieved. This finding, combined with the results of previous research by our team (16), suggests that NA-resistant patients are not only prone to recurrence after achieving functional cure but are also difficult to cure again. It is suggested that such patients should be discontinued with caution and closely followed after obtaining HBsAg clearance.

Twelve relapsed patients (37.5%, 12/32) did not receive drug intervention, and their follow-up period was 42–269 weeks. In 6 of 12 cases, HBsAg and HBV DNA fluctuated at low levels; and in 3 out of 12 cases, the HBsAg and/or HBV DNA fluctuations were significant (>3 log10 IU/ml), but no liver function abnormalities were observed. However, the follow-up period is limited, and the long-term outcome of untreated patients needs to be further observed.

In this study, patients who relapsed after achieving functional cure with Peg-IFN treatment did not have disease progression or the occurrence of liver cirrhosis or liver cancer during the follow-up period, regardless of whether they received retreatment. However, the follow-up period is limited, and the long-term prognosis of patients with recurrence needs to be further observed. There is no report on whether clinical cure can also improve long-term prognosis in patients with recurrence. However, several studies have shown that the incidence of hepatocellular carcinoma (HCC) in patients with CHB after obtaining HBsAg clearance ranges from 0% to 4.8% (12, 17–20). In contrast, the 5-year risk rate of HCC in patients who were HBsAg positive and without HBV DNA inhibition was 18.8% (21). The 5-year incidence of HCC in patients who achieved HBV DNA suppression and HBeAg seroconversion with NAs still ranged from 6% to 7.7% (21, 22). This indicates that functional cure for hepatitis B patients can significantly improve the prognosis. Given the low HBsAg and HBV DNA levels at the time of relapse and the high rate of functional cure in the majority of relapsers in this study, we recommend that relapsed patients should retreat if conditions permit to achieve functional cure as early as possible.

It is generally accepted that anti-HBs is a protective antibody that is produced by the body after HBV infection. Previous studies reported that anti-HBs levels at drug withdrawal were associated with recurrence; the recurrence rate in anti-HBs-positive patients was lower than that in anti-HBs-negative patients (5, 13, 23). This study compared the quantitative levels of anti-HBs between the recurrence and non-recurrence groups, and we found a significant difference between the two groups at the time of drug withdrawal and after drug discontinuation. Patients with high anti-HBs levels at drug withdrawal were less likely to relapse (OR = 0.525, $p < 0.001$). In addition, studies have shown that anti-HBc level can not only reflect the immune response (7) but also serve as a predictor of antiviral efficacy or relapse after NA withdrawal (24, 25). In this study, anti-HBc levels of the recurrence group were significantly lower than those of the non-recurrence group at both drug discontinuation and relapse (48 weeks of drug discontinuation) (all $p < 0.05$). In addition, anti-HBc levels were below the lower limit of detection in patients who relapsed again. The predictive value of anti-HBc levels at drug discontinuation for relapse after functional cure was evaluated by the ROC curve, which had an AUROC of 0.724, higher than anti-HBs levels at drug discontinuation (0.679). To more accurately predict relapse after drug withdrawal, we combined anti-HBs and anti-HBc at drug withdrawal. The combined predictors had an AUROC of 0.833, which was a better predictor of relapse than a single indicator. The combined predictors had good sensitivity (86.67%) and specificity (90.62%) for predicting relapse, which could compensate for the lower specificity of the single predictor. Humoral immunity plays an important role in the clearance of HBV infection. Anti-HBs and anti-HBc are produced by B lymphocytes specific for HBsAg and HBcAg, respectively. Therefore, the high anti-HBs and anti-HBc levels at drug discontinuation may reflect that patients have a high adaptive immune status, which may be associated with low relapse after treatment cessation. Anti-HBs and anti-HBc are noninvasive indicators, which are convenient for detection, clinical application, and observation. Therefore, they can be used as predictors of drug withdrawal and recurrence in functionally cured patients.

Overall, the recovery rate of relapsed patients receiving retreatment is 65%, and the recovery rate of Peg-IFN retreatment is relatively high, reaching higher than 80%. However, the recurrence and special types of recurrence could still occur. Conversely, it is more difficult to obtain functional cure after relapse with NA retreatment and untreated patients. In this study, patients who relapsed after achieving functional cure with Peg-IFN treatment did not have disease progression or the occurrence of liver cirrhosis or liver cancer during the follow-up period, regardless of whether they received retreatment. However, the long-term

prognosis of patients with recurrence needs to be further observed. In addition, both anti-HBs and anti-HBc levels at the time of treatment cessation can be used as predictors of recurrence, and the combined predictive value of the two indicators is better. They are convenient for detection and clinical application. However, this is an exploratory study; the sample size is small and limited to one center. So, the results should be further confirmed by large, multicenter, prospective clinical trials.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary File**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

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

AUTHOR CONTRIBUTIONS

XL, AS, and XC designed the study. XL, AS, JL, LM, ZC, HL, and YZ collected the data. XL and SR tested serum samples. XL and AS analyzed the data. SR, JL, SZ, and ZH guided statistical analysis. XL, AS, and XC drafted the article. XC contributed to the interpretation of the results and critical revision of the article for important intellectual content. XC, SZ, ZH, and SR provided the financial support for the project leading to this publication. All authors read and approved the final article. All authors contributed to the article and approved the submitted version.

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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.2022.879835/full#supplementary-material

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Feng Li,
Guangzhou Eighth People's Hospital, China

REVIEWED BY
Ming Yue,
Nanjing Medical University, China
Nirupma Trehanpati,
The Institute of Liver and Biliary Sciences (ILBS), India

*CORRESPONDENCE
Junliang Fu
fjunliang@163.com
Fu-Sheng Wang
fswang302@163.com

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Quantitative anti-HBc combined with quantitative HBsAg can predict HBsAg clearance in sequential combination therapy with PEG-IFN-α in NA-suppressed chronic hepatitis B patients

Wen-Xin Wang[1,2,3], Rui Jia[2,3], Ying-Ying Gao[2], Jia-Ye Liu[2], Jun-Qing Luan[2], Fei Qiao[2], Li-Min Liu[2], Xiao-Ning Zhang[2], Fu-Sheng Wang[1,2,3]* and Junliang Fu[1,2,3]*

[1]Peking University 302 Clinical Medical School, Beijing, China, [2]Senior Department of Infectious Diseases, The Fifth Medical Center of Chinese People's Liberation Army (PLA) General Hospital, National Clinical Research Center for Infectious Diseases, Beijing, China, [3]Medical School of Chinese People's Liberation Army (PLA), Beijing, China

Background and aims: Precise predictors are lacking for hepatitis B surface antigen (HBsAg) clearance under the combination therapy of nucleos(t)ide analogs (NA) and pegylated interferon-alpha (PEG-IFN-α) in patients with chronic hepatitis B (CHB). This study aimed to determine the quantitative anti-hepatitis B core antibody (qAnti-HBc) and quantitative hepatitis B core-related antigen (qHBcrAg) as predictors for HBsAg clearance in NA-suppressed patients with CHB receiving PEG-IFN-α add-on therapy.

Methods: Seventy-four CHB patients who achieved HBV DNA suppression (HBV DNA < 20 IU/ml) and quantitative HBsAg (qHBsAg) < 1,500 IU/ml after ≥1 year of NA treatment were enrolled. Fifteen patients continued on NA monotherapy, while 59 patients received PEG-IFN-α add-on therapy. Serum qAnti-HBc and qHBcrAg levels were detected every 12 or 24 weeks for add-on and NA-alone groups, respectively.

Results: Serum qAnti-HBc but not qHBcrAg levels at baseline were negatively correlated with the duration of prior NA therapy. After 48-week treatment, both qAnti-HBc and qHBcrAg levels declined further, and 17/59 (28.81%) and 0/15 (0%) achieved HBsAg clearance in add-on and NA groups, respectively. In the add-on group, the rate of HBsAg clearance was significantly higher in patients with baseline qAnti-HBc < 0.1 IU/ml (52.63%). Logistic regression analysis identified baseline qAnti-HBc but not qHBcrAg, which was an independent predictor for HBsAg loss. Receiver operating characteristic curve analysis

showed that the combination of qAnti-HBc and qHBsAg had a better predictive value for HBsAg clearance.

Conclusions: A combination of qHBsAg and baseline qAnti-HBc levels may be a better prediction strategy for HBsAg clearance in NA-suppressed CHB patients receiving PEG-IFN-α add-on therapy.

KEYWORDS

chronic hepatitis B, anti-HBc, HBcrAg, HBsAg clearance, combined treatment, PEG-IFN-α

Introduction

Approximately 250 million individuals worldwide are chronically infected with hepatitis B virus (HBV) (1), and 10%–15% of them progress to chronic liver diseases, such as chronic hepatitis B (CHB), cirrhosis, and hepatocellular carcinoma (HCC) (2). Currently, achieving both HBV DNA undetectable and hepatitis B surface antigen (HBsAg) clearance with or without hepatitis B surface antibody (HBsAb) appearance, named functional cure, can significantly reduce the incidence of cirrhosis and HCC (3–5). However, the efficacy of available drugs, including nucleos(t)ide analogs (NA) and interferon (IFN), remains unsatisfactory when used alone. Recently developed new strategies of "add-on" or "switch-to" pegylated-interferon-alpha (PEG-IFN-α) to ongoing NA treatment have dramatically improved the chance of achieving sustained HBsAg loss/seroconversion in CHB patients (6–12). In patients with quantitative HBsAg (qHBsAg) < 1,500 IU/ml with or without early on-treatment HBsAg decline, the loss rate can reach 22.2%–58.7% after 48–96 weeks of therapy (6–10). Approximately 40%–80% of these patients remain HBsAg positive after suffering from side effects of PEG-IFN-α. Therefore, new indicators or strategies to precisely identify the patients who are most likely to achieve HBsAg clearance under PEG–IFN-α-based therapy are vital.

The quantitative anti-hepatitis B core antibody (qAnti-HBc) and quantitative hepatitis B core-related antigen (qHBcrAg) are emerging novel markers for patients with CHB. Studies have shown that the qAnti-HBc levels in CHB patients during the immune clearance and reactivation phases are significantly higher than those in the immune tolerance and low replication phases in natural history (13, 14). The qAnti-HBc levels are strongly correlated with serum ALT levels and the hepatic inflammatory degree in treatment-naïve patients (15–17) and showed a gradually decreasing trend in the course of NA therapy (18). HBcrAg levels are closely correlated with intrahepatic covalently closed circular DNA (cccDNA) transcriptional activity in CHB patients (19). Both qAnti-HBc and qHBcrAg levels could predict the clearance of hepatitis B e antigen (HBeAg) during naive treatment with PEG-IFN-α or NA therapy (16, 20–22). Moreover, the decline in qHBcrAg levels is also associated with persistent HBV DNA suppression after PEG-IFN-α-based treatment (23).

However, it remains unclear whether qAnti-HBc and qHBcrAg levels can predict HBsAg clearance in NA-suppressed CHB patients receiving combination therapy with PEG-IFN-α. This study aimed to investigate the kinetics of serum qAnti-HBc and qHBcrAg levels in CHB patients with qHBsAg < 1,500 IU/ml receiving PEG-IFN–α add-on to ongoing NA therapy, and to evaluate the role of these two biomarkers in predicting HBsAg clearance.

Abbreviations: Anti-HBs, antibody to hepatitis B surface antigen; CHB, chronic hepatitis B; ETV, entecavir; HBcrAg, hepatitis B core-related antigen; HBeAb, hepatitis B e antibody; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBsAb, hepatitis B surface antibody; HBsAg, hepatitis B surface antigen; IFN, interferon; LLD, lower limit of detection; NA, nucleos(t)ide analogs; PEG-IFN-α, pegylated-interferon-alpha; qAnti-HBc, quantitative anti-hepatitis B core antibody; qHBcrAg, quantitative hepatitis B core-related antigen; qHBsAg, quantitative HBsAg; TDF, tenofovir disoproxil fumarate.

Materials and methods

Study population and design

The subjects were enrolled from the Fifth Medical Center of Chinese PLA General Hospital. CHB patients aged 18–65 years who had received NA treatment for ≥1 year and achieved serum qHBsAg < 1,500 IU/ml and HBV DNA suppressed were eligible. HBV DNA suppressed is defined as HBV DNA < 20 IU/ml. Patient exclusion criteria were as follows: coinfection with human immunodeficiency virus, hepatitis A virus, hepatitis C virus, hepatitis D virus, or hepatitis E virus; presence of other

chronic liver disease or serious systemic diseases; and receiving IFN, glucocorticoids, or other immunomodulatory therapy within 6 months prior to enrollment. A total of 74 patients were enrolled and divided into a continuing NA monotherapy group (NA group, 15 patients) and an add-on PEG-IFN-α therapy group (add-on group, 59 patients) according to their wishes. The NA group received continuous entecavir (ETV) or tenofovir disoproxil fumarate (TDF) for 48 weeks. The add-on group received ETV or TDF combined with PEG-IFN-α-2b (180 μg once a week) for 48 weeks (**Figure 1**). The primary outcome was HBsAg loss after 48 weeks of treatment.

ETV was the main type of NA taken before enrollment (NA group: 10/66.67%; add-on group: 37/62.71%). If patients received lamivudine, adefovir dipivoxil, or telbivudine before enrollment, they were replaced with ETV or TDF. The baseline characteristics of the patients were comparable between the groups (**Table 1**). The study was approved by the Ethics Committee of the Fifth Medical Center of Chinese PLA General Hospital.

Clinical and laboratory evaluation

Twenty milliliters of peripheral blood samples were collected at study entry and every 24 weeks (NA group), or every 12 weeks (add-on group). Serological and biochemical markers were measured routinely in a central clinical laboratory. Serum HBV DNA levels were tested using the COBAS AmpliPrep/COBAS TaqMan HBV Test (Roche Molecular Systems, Inc., Branchburg, USA); the lower limit of detection (LLD) of HBV DNA level was 20 IU/ml. Serum HBsAg levels were quantified using Elecsys HBsAg II quant II (Roche Diagnostics GmbH, Mannheim, Germany); the LLD of qHBsAg was 0.05 IU/ml. Other HBV markers, including HBsAb levels, HBeAg, and HBeAb, were detected using COBAS e602 (Roche Diagnostics GmbH, Mannheim, Germany). Serum qAnti-HBc levels were measured using a double–antigen sandwich immune assay (Wantai, Beijing, China), as reported previously (24). In detail, both high-value quantitative detection (HQ) and general quantitative detection (GQ) were used in our study. The detection range of the HQ kit and the GQ kit was 100–100,000 IU/ml and 0.1–25 IU/ml, respectively. If the test result of a sample was higher than the upper limit of the GQ kit and lower than the lower limit of the HQ kit, the sample would be diluted by five times and re-tested it with GQ kit. Thus, the LLD of the qAnti-HBc level was 0.1 IU/ml. Serum qHBcrAg levels were measured with a fully automated Lumipulse analyzer (Fujirebio, Tokyo, Japan) by chemiluminescent enzyme immunoassay with the detection limit 2.0 to 7.0 $\log_{10}$ U/ml. Serum qAnti-HBc and qHBcrAg levels were measured according to the manufacturer's instructions.

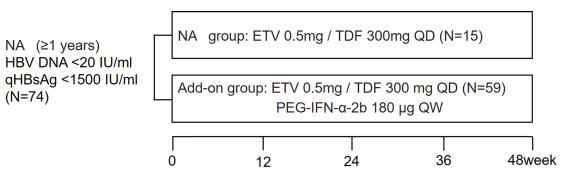


FIGURE 1
Study design. Enrolled patients were divided into NA and add-on groups according to their wishes. Blood samples were collected at study entry and every 24 weeks (NA group) or every 12 weeks (add-on group). NA, nucleos(t)ide analogs; qHBsAg: quantitative hepatitis B surface antigen; ETV: entecavir; TDF, tenofovir disoproxil fumarate; PEG-IFN-α-2b, Pegylated-interferon-alpha-2b; QD: once daily; QW: once a week.

Statistical analysis

Serum qHBsAg, qAnti-HBc, and qHBcrAg levels were log-transformed. Statistical analyses were performed using SPSS 25.0 software (IBM Corp., Armonk, NY, USA). Continuous variables were expressed as median (quartiles). The statistical significance of the difference between two groups was determined using the Mann–Whitney *U* test. The Wilcoxon signed ranks test was used for comparison of two related samples. Categorical variables were analyzed using the chi-squared test. Pearson correlation was used to determine the correlation between continuous variables. The HBsAg loss rates between patients with qAnti-HBc < 0.1 IU/ml and >0.1 IU/ml were re-evaluated with propensity score matching (PSM), adjusting for demographic characteristics and baseline indicators associated with HBV infection. A PSM with the nearest-neighbor method using 1:2 matching was performed by R version 4.1.2. Univariate and multivariate logistic regression analyses were used to identify predictors for HBsAg clearance. The discriminatory power of various predictors for HBsAg clearance was tested with the area under the receiver operating characteristic (ROC) curve. All statistical analyses were based on two-tailed hypothesis tests with a significance level of $p < 0.05$.

Results

Serum qAnti-HBc and qHBcrAg levels were decreased during treatment

The levels of baseline serum qAnti-HBc and qHBcrAg in the NA group were slightly higher than in the add-on group but without significant difference ($p > 0.05$) (**Table 1**).

In the course of treatment after enrollment, serum qAnti-HBc and qHBcrAg levels in both add-on and NA groups were further declined, and there was no difference in the reduction between the groups [qAnti-HBc: 0.19 (0.00, 0.48) vs. 0.09 (0.00, 0.36) $\log_{10}$IU/ml, $p = 0.334$; qHBcrAg: 0.23 (0.00, 0.45) vs. 0.13 (0.00, 0.31) $\log_{10}$U/ml, $p = 0.737$] (**Figures 2A, B**). Further analysis of the

TABLE 1 Characteristics of the enrolled patients.

Indicators	NA group (n = 15)	Add-on group (n = 59)	p
Male/female, n	13/2	49/10	1.000
Age (years), median (quartiles)	38.0 (34.0, 47.5)	39.0 (32.0, 47.0)	0.968
Route of transmission, maternal/paternal/others, n	1/3/11	13/9/37	0.407
Duration of prior NA treatment (months), median (quartiles)	49.0 (24.0, 70.0)	63.0 (39.0, 121.0)	0.143
Baseline qHBsAg (log_{10}IU/ml), median (quartiles)	2.73 (2.21, 3.01)	2.59 (1.97, 2.95)	0.550
Baseline HBeAg positive, n (%)	6 (40.00)	18 (30.51)	0.695
Baseline HBeAb positive, n (%)	7 (46.67)	25 (42.37)	0.764
Baseline qAnti-HBc (log_{10}IU/ml), median (quartiles)	1.81 (0.12, 2.94)	1.97 (−1.00, 2.70)	0.618
Baseline qHBcrAg (log_{10}U/ml), median (quartiles)	4.31 (3.25, 5.50)	4.22 (3.52, 5.16)	0.718
Baseline ALT (U/L), median (quartiles)	20.0 (14.0, 30.5)	22.0 (18.0, 27.0)	0.642
Baseline AST (U/L), median (quartiles)	25.0 (21.0, 29.0)	21.0 (19.0, 24.0)	0.107
HBsAg loss at week 48, n (%)	0 (0)	17 (28.81)	0.043

NA, nucleos(t)ide analogs; Add-on, nucleos(t)ide analogs combined with pegylated-interferon-alpha; HBsAg, hepatitis B surface antigen; qHBsAg, quantitative hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBeAb, hepatitis B e antibody; qAnti-HBc, quantitative anti-hepatitis B core antibody; qHBcrAg, quantitative hepatitis B core-related antigen; ALT, alanine aminotransferase; AST, aspartate amino transferase.

reduction process of these two markers showed that serum qAnti-HBc and qHBcrAg levels in the add-on group had been maintained on a downward trend at almost every time point: qAnti-HBc levels declined significantly at weeks 12, 36, and 48 compared with the previous follow-up time point ($p < 0.001$, $p = 0.012$, and $p = 0.008$, respectively) (**Figure 2A**), and qHBcrAg declined significantly at weeks 12, 24, and 36 compared with the previous follow-up time point ($p < 0.001$, $p < 0.001$, and $p = 0.031$, respectively) (**Figure 2B**). In the NA group, qAnti-HBc and qHBcrAg levels decreased significantly only in the first 24 weeks of treatment ($p = 0.047$ and $p = 0.002$, respectively) (**Figures 2A, B**).

Baseline serum qAnti-HBc levels can predict HBsAg clearance under add-on treatment

After 48-week treatment, 17/59 (28.81%) patients achieved HBsAg clearance in the add-on group, while no patients achieved HBsAg clearance in the NA group (**Table 1**). In the add-on group, the levels of baseline qAnti-HBc and qHBsAg in patients who achieved HBsAg clearance were significantly lower than those who did not, but baseline qHBcrAg levels were comparable between the two subsets of patients (**Table 2**). The rate of HBsAg clearance was significantly higher in patients with baseline qAnti-HBc < 0.1 IU/ml than in patients with baseline qAnti-HBc > 0.1 IU/ml (52.63% vs. 17.50%, $p = 0.005$) (**Figure 3A**). Meanwhile, baseline qHBsAg levels between the two groups show no significant difference (**Figure 3B**). We also compared gender, age, baseline qHBcrAg, duration of prior NA treatment before enrollment, baseline HBeAg positive rate, and baseline ALT between the two groups. We found no statistical differences ($p > 0.05$). A 1:2 PSM analysis was further conducted to avoid the influence of the above factors. After PSM, 18 patients from the baseline qAnti-HBc < 0.1 IU/ml group and 35 patients from the baseline qAnti-HBc > 0.1 IU/ml group were included in further analysis. The rate of HBsAg clearance in patients with baseline qAnti-HBc < 0.1 IU/ml and > 0.1 IU/ml was 50.00% and 20.00% ($p = 0.024$), respectively.

In those who cleared HBsAg and those who did not, qAnti-HBc levels dropped by 0.00 (0.00, 0.29) log_{10}IU/ml and 0.26 (0.00, 0.51) log_{10}IU/ml, respectively. Dynamic variation of qHBsAg during 48 weeks of add-on therapy showed that the decrease of qHBsAg at weeks 24, 36, and 48 in patients with baseline qAnti-HBc < 0.1 IU/ml was significantly greater than that in patients with baseline qAnti-HBc > 0.1 IU/ml (**Figure 4**).

Univariate logistic regression analysis showed that baseline qAnti-HBc < 0.1 IU/ml was a predictor for HBsAg loss after 48 weeks of add-on therapy (OR = 5.238 [1.554–17.653], $p = 0.008$), and other predictors included baseline HBsAg levels (<400 IU/ml) (OR = 12.187 [2.457–60.453], $p = 0.002$) and a

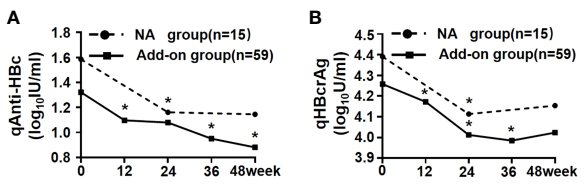


FIGURE 2
Kinetics of serum qAnti-HBc and qHBcrAg in NA and add-on groups. **(A)** Kinetics of qAnti-HBc in two groups. **(B)** Kinetics of qHBcrAg in two groups. * Comparison in biomarkers between this and last time points and $p < 0.05$. NA, Nucleos(t)ide analogs; qAnti-HBc, quantitative anti-hepatitis B core antibody; qHBcrAg, quantitative hepatitis B core-related antigen.

TABLE 2 Baseline characteristics of patients who achieved HBsAg clearance or not after 48-week add-on therapy.

Indicators	HBsAg clearance, *n* (%)		*p*
	No (*n* = 42)	Yes (*n* = 17)	
Male, *n* (%)	35 (83.33)	14 (82.35)	1.000
Age (years), median (quartiles)	39.5 (32.5, 47.0)	37.0 (31.0, 46.0)	0.592
Route of transmission, maternal/paternal/others, *n*	11/7/24	2/2/13	0.424
Duration of prior NA treatment (months), median (quartiles)	66.0 (38.0, 125.0)	51.0 (47.0, 78.0)	0.483
qHBsAg (log_{10}IU/ml), median (quartiles)	2.74 (2.46, 3.05)	1.69 (0.83, 2.05)	<0.001
HBeAg positive, *n* (%)	12 (28.57)	6 (35.29)	0.612
HBeAb positive, *n* (%)	19 (45.24)	6 (35.29)	0.484
qAnti-HBc (log_{10}IU/ml), median (quartiles)	2.11 (1.47, 2.77)	−1.00 (−1.00, 2.29)	0.031
qHBcrAg (log_{10}U/ml), median (quartiles)	4.24 (3.45, 5.18)	4.15 (3.59, 5.07)	0.958
ALT (U/L), median (quartiles)	22.0 (18.0, 27.25)	20.0 (14.0, 27.0)	0.564
AST (U/L), median (quartiles)	21.0 (19.0, 24.25)	21.0 (20.0, 24.0)	0.909

NA, nucleos(t)ide analogs; Add-on, nucleos(t)ide analogs combined with pegylated-interferon-alpha; HBsAg, hepatitis B surface antigen; qHBsAg, quantitative hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBeAb, hepatitis B e antibody; qAnti-HBc, quantitative anti-hepatitis B core antibody; qHBcrAg, quantitative hepatitis B core-related antigen; ALT, alanine aminotransferase; AST, aspartate amino transferase.

decline of qHBsAg > 1 log_{10}IU/ml at week 12 (OR = 18.571 [4.060–84.955], p < 0.001). Since previous studies found that qAnti-HBc decreased with NA treatment, and the patients had undergone NA therapy for a long time before enrollment, the duration of prior NA therapy was included in the regression analysis, while sex, age, duration of prior NA therapy, early ALT elevation, and baseline HBcrAg levels did not influence HBsAg clearance significantly (Figure 5A). Multivariate logistic regression analysis showed that baseline qAnti-HBc < 0.1 IU/ml (OR = 24.83 [2.369–260.220], p = 0.007), baseline HBsAg levels (<400 IU/ml) (OR = 11.516 [1.662–79.779], p = 0.013), and a decline of qHBsAg > 1 log_{10}IU/ml at week 12 (OR = 76.673 [5.568–1055.909], p = 0.001) remained independent predictors for HBsAg clearance after 48 weeks of add-on therapy (Figure 5A).

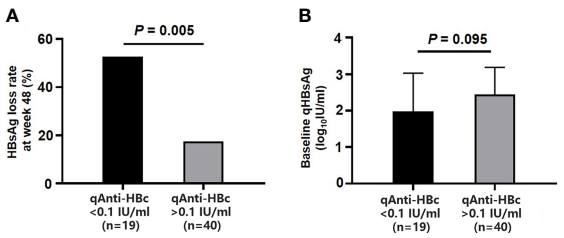


FIGURE 3
Comparison of HBsAg loss rates at week 48 and baseline qHBsAg in the add-on group with baseline qAnti-HBc <0.1 IU/ml and > 0.1 IU/ml. **(A)** Comparison of HBsAg loss rates at week 48 in two subgroups. **(B)** Comparison of baseline qHBsAg in two subgroups. HBsAg, hepatitis B surface antigen; qHBsAg, quantitative HBsAg; qAnti-HBc, quantitative anti-hepatitis B core antibody.

qAnti-HBc combined with qHBsAg can better predict HBsAg clearance under add-on treatment

Area under the ROC curve (AUROC) was used to compare the predictive effectiveness of these independent predictors for HBsAg clearance (Figure 5B). The prediction effect of the combination of baseline HBsAg and baseline qAnti-HBc levels was comparable with the combination of baseline HBsAg levels and decline of qHBsAg > 1 log_{10}IU/ml at week 12; the AUROCs were 0.830 and 0.838, respectively. Furthermore, the combination of baseline qAnti-HBc levels and decline of qHBsAg > 1 log_{10}IU/ml at week 12 had a significantly higher AUROC (0.887). When the three indexes were combined, the AUROC can reach 0.930.

Discussion

Many studies have demonstrated that low levels of qHBsAg, most considered less than 1,500 IU/ml, are a favorable factor for NA-treated patients receiving add-on or switching therapy of PEG-IFN-α to achieve HBsAg clearance (6–12, 25–27). However, more than half of these patients cannot achieve HBsAg clearance after 1 to 2 years of this combination treatment. More accurate and early prediction of the response to interferon-based therapy could reduce unnecessary use of drugs and avoid their associated side effects. In this study, we found that more than 50% of NA-treated patients with baseline qAnti-HBc <0.1 IU/ml obtained HBsAg clearance after 48 weeks of add-on therapy, suggesting that qAnti-HBc levels might be used to predict HBsAg clearance.

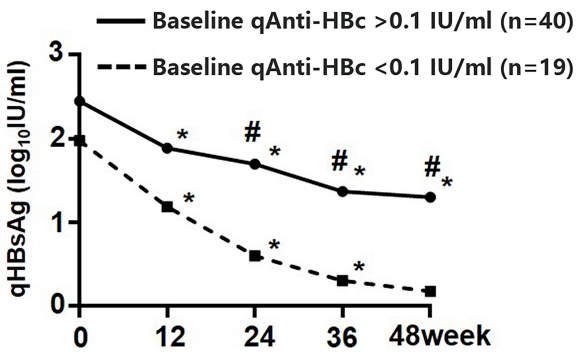
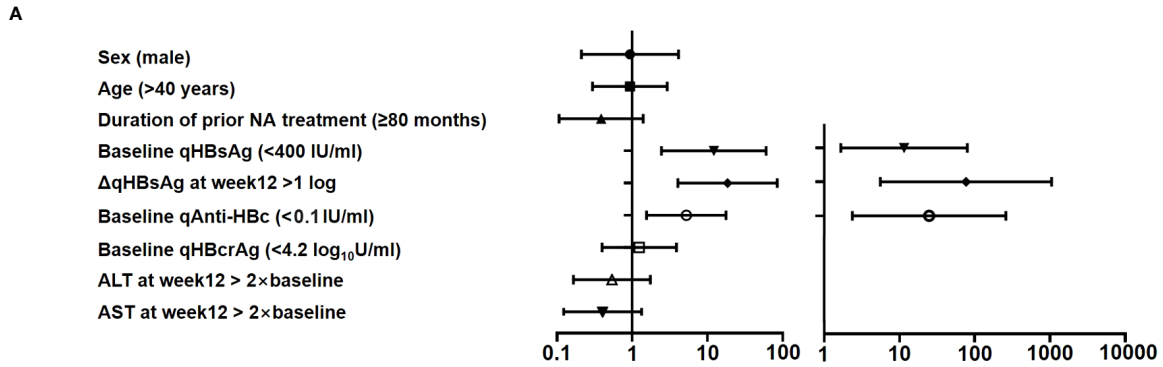
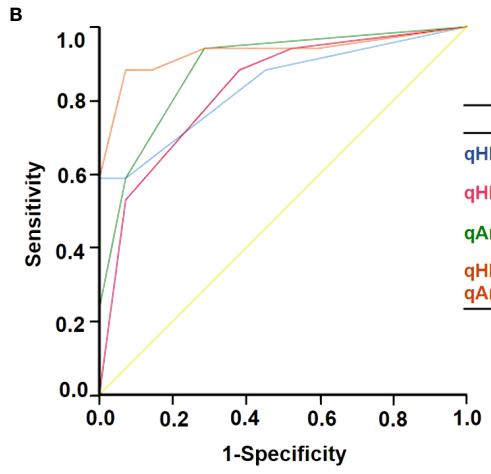


FIGURE 4
Kinetics of qHBsAg in the add-on group with baseline qAnti-HBc < 0.1 IU/ml and > 0.1 IU/ml. * Comparison in qHBsAg between this and the last time points and $p < 0.05$; # Comparison in qHBsAg between the two subgroups and $p < 0.05$. qHBsAg, quantitative hepatitis B surface antigen; qAnti-HBc, quantitative anti-hepatitis B core antibody.

Anti-HBc is a classical serological marker of HBV infection, comprising anti–HBc IgM and IgG antibodies (28). Anti-HBc IgM is positive at the phase of liver inflammation, but becomes negative in the recovery stage; therefore, it can help to distinguish acute HBV infection or acute exacerbation from quiescent CHB (29). Anti-HBc IgG is a marker of current and previous HBV infection, and may persist for a lifetime. The present study used the widely used double-antigen sandwich method to quantify anti-HBc antibodies (18, 30), and the detection results represent the sum of IgG and IgM antibodies (24). HBcrAg is a combined HBV serum biomarker that consists of three related proteins, including hepatitis B core antigen, hepatitis B e antigen, and p22cr protein encoded by the precore/core gene of HBV (31, 32). HBcrAg levels have generally been identified as closely correlated with serum HBV DNA and intrahepatic cccDNA levels in treatment-naïve CHB patients, and are considered to play a vital role in the evaluation, clinical medication, and prognosis of CHB (21, 33, 34).





Indicators	AUROC	P
qHBsAg<400 IU/ml+ΔqHBsAg at week12 >1 log	0.838(0.711-0.964)	<0.001
qHBsAg<400 IU/ml+qAnti-HBc< 0.1 IU/ml	0.830(0.714-0.945)	<0.001
qAnti-HBc< 0.1 IU/ml+ΔqHBsAg at week12 >1 log	0.887(0.792-0.981)	<0.001
qHBsAg<400 IU/ml+ΔqHBsAg at week12 >1 log+ qAnti-HBc< 0.1 IU/ml	0.930(0.836-1.000)	<0.001

FIGURE 5
Prediction of HBsAg loss at week 48 in the add-on group. **(A)** Univariate and multivariate logistics regression analysis of factors predicting HBsAg loss at week 48. **(B)** Receiver operating characteristic curve of factors predicting HBsAg loss at week 48. NA, nucleos(t)ide analogs; ALT, alanine aminotransferase; qAnti-HBc, quantitative anti-hepatitis B core antibody; HBsAg, hepatitis B surface antigen; qHBsAg, quantitative HBsAg; qHBcrAg, quantitative hepatitis B core-related antigen; AUROC, area under the receiver operating characteristic curve; Δ, decline in value.

Our follow-up study demonstrated that qAnti-HBc and qHBcrAg levels decreased persistently in the add-on group compared with the NA group after enrollment. This difference may be related to the mechanism of action of the drugs: NA inhibit HBV DNA synthesis but cannot block the activity of HBV cccDNA; therefore, their effect on the synthesis of antigen components is weak. IFN, as an innate antiviral cytokine, suppresses viral replication and spread by promoting the expression of various proteins involved in RNA degradation, translational inhibition, and cellular apoptosis (35, 36). IFN is also vital to activate innate immune cells and regulate the adaptive immune response (5, 36). Thus, IFN is able to inhibit the synthesis of various HBV components, and then lead to a further decline in antigen and antibody levels. A recent study had demonstrated that the median levels of anti-HBc IgG were significantly higher in HBV cccDNA-positive occult HBV-infected individuals than in cccDNA-negative anti-HBc-positive individuals (37). Consequently, we speculated that qAnti-HBc levels might reflect the activity of intrahepatic cccDNA transcription and mRNA translation just as qHBcrAg does and could be a predictor of HBsAg clearance. This hypothesis was tentatively confirmed in our follow-up cohort study: logistic regression identified lower baseline qAnti-HBc levels as an independent predictor for HBsAg loss after 48 weeks of add-on therapy. Long-term follow-up studies on inactive HBsAg carriers and HBeAg-negative HBV carriers also support our hypothesis to some extent, since the patients in their cohorts had similarly low levels of viral replication and inconspicuous liver inflammation to ours, and they found that patients with low qAnti-HBc levels were more likely to have spontaneous HBsAg and HBV DNA clearance (38, 39).

However, in the studies of treatment-naïve patients with CHB, the predictive role of qAnti-HBc levels seems to be opposite to the above results: Higher baseline qAnti-HBc levels were associated with a higher rate of HBeAg seroconversion or HBsAg loss in patients who were treated initially with either Peg-IFN-α or NA (16, 18, 20), and higher qAnti-HBc levels before treatment and at drug withdrawal were also related to a lower rate of viral recurrence (40). A recent study reports that a higher pretransplantation qAnti-HBc levels corresponded with sustained HBsAg loss after liver transplantation (41). Notably, the patients enrolled in these studies were mostly in the stage of hepatic inflammatory activity with markedly elevated levels of ALT and virus replication. These studies also found that qAnti-HBc levels correlated positively with ALT levels (16), and patients with ALT > two times the upper limit of normal had the highest rate of HBeAg seroconversion (17). These results suggest that a different background of liver inflammation before and after antiviral treatment might explain the completely opposite predictive effect of qAnti-HBc levels. Another possible reason is the different qAnti-HBc antibody composition in different liver inflammatory phases. The total anti-HBc antibody (IgM and IgG added together) and IgM alone levels correlated positively with ALT (38). However, high levels of anti-HBc IgM can only be detected in patients with active liver inflammation and were found at extremely low levels in inactive HBsAg carriers and in patients who achieved a complete virological response to antiviral therapy (38). Based on the above research, we speculated that anti-HBc IgM is the main component under conditions of obvious liver inflammation, whereas anti-HBc IgG is the main component in the quiescent state. Consequently, the skewed composition of qAnti-HBc might be a direct cause of its contrasting predictive effect in different types of patients, and the predictive power of IgG antibodies may be masked in certain cases. The results from a retrospective study using qualitative methods to detect anti-HBc IgG to some extent support our hypothesis and show that low levels of anti-HBc IgG prior to antiviral therapy were associated with HBsAg clearance in patients with NA-induced HBeAg seroclearance (42). Moreover, varying levels of virus replication in treated and untreated patients might also affect qAnti-HBc's predictive effect.

Consistent with Huang's findings (43), qHBcrAg decline during treatment period was observed in both patients with and without HBsAg clearance in this study (data not shown), and neither study found that baseline qHBcrAg had a predictive effect on HBsAg clearance. What is inconsistent between the two studies is that Huang's study found that qHBcrAg decline at week 24 can predict HBsAg clearance at the end of treatment. The reason for these differences may be related to the number of patients enrolled, duration of prior NA therapy, baseline qHBsAg and HBV DNA levels, and treatment course of PEG-IFN-α.

This study had limitations, such as a small sample size, short treatment time, and lack of long-term follow–up. Additionally, the proportion of IgM and IgG in qAnti-HBc was not detected. Therefore, prospective longitudinal studies with larger sample sizes are needed to further demonstrate the prediction role of qAnti-HBc, and to reveal the underlying immune mechanisms.

In summary, our study provides evidence for the first time that the baseline qAnti–HBc levels may predict HBsAg clearance in NA-suppressed patients with CHB who receive sequential combination therapy with PEG-IFN-α. However, the predictive power of qAnti-HBc alone still fails to exceed that of qHBsAg. Hence, a combination of the baseline qAnti-HBc and qHBsAg levels is a better strategy to guide clinical practice.

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 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. W-XW and J-YL performed the statistical analysis. W-XW wrote the first draft of the manuscript. RJ, F-SW, and JF wrote sections of the manuscript. 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.

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