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Original Article

Change of Cytokines in Chronic Hepatitis B Patients and HBeAg are Positively Correlated with HBV RNA, Based on Real-world Study

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Abstract

Background and Aims: The natural course of chronic hepatitis B virus (HBV) infection is widely studied; however, follow-up studies of the same patients are scanty. Here, we studied the dynamic changes of serum HBV RNA and cytokines in hepatitis B virus e antigen (HBeAg)-positive patients treated with entecavir (ETV) to explore the relationship between the HBV serum viral nucleic acids and host immunity. ***Methods:*** Thirty-three chronic hepatitis B patients who are HBeAg-positive, with high virus load (HBV DNA >20,000 IU/mL), and received standard nucleos(t)ide analogue (NA) antiviral therapy (ETV) for more than 48 weeks were included. The serum levels of HBV nucleic acids and selected cytokines were measured at 0, 12, 24, and 48 weeks respectively. ***Results:*** Serum HBV RNA could still be detected while serum HBV DNA had fallen below the detection limit in patients treated with ETV. There was a strong positive correlation between HBV RNA and HBeAg, with a concomitant decrease in the secretion of cytokines from type 1 helper T (Th1)/type 2 helper T (Th2)/interleukin (IL)-17 producing T (Th17) cells. IL-4 and IL-10 were the main cytokines negatively associated with serum HBV RNA. ***Conclusions:*** HBeAg can be used to reflect the load of HBV RNA indirectly, because serum HBV RNA has not been widely used in clinical practice. Meanwhile, serum IL-4 and IL-10 might be explored in combination with HBV RNA in guiding future clinical antiviral therapy.

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Introduction

Globally, chronic infection with the hepatitis B virus (HBV) affects more than 250 million individuals, and can lead to the development of liver cirrhosis, liver failure, and hepatocellular carcinoma, despite the availability of effective HBV vaccines.[1] As a highly prevalent virus, hepatitis B infection is considered to be a non-cellular disease, with its pathogenesis mediated by innate immunity and adaptive immune response. Although HBV may initially escape the innate immune defense, T cells have dual antiviral effects on HBV infection, both cytopathic and non-cytopathic.[2] In addition, effective B cell and neutralizing antibody responses are lacking.[3] There was evidence published showing that the virus could be cleared by activation of immunity. HBV control is correlated with the expansion of intrahepatic HBV-specific $CD8^+$ T cells and peripheral cytotoxic T cells with the enhanced production of cytokines.[4]

Serum HBV DNA load is the only biomarker for clinical monitoring of the number of circulating viral particles in peripheral blood.[1] In addition, serum HBV load can be used as an independent factor for the prediction of the patient response to antiviral treatment.[5,6] However, HBV DNA was undetected in most patients treated with nucleos(t)ide analogues (NAs). Of note, there is no reliable indicator to reflect the replication of HBV in the liver. In fact, the disappearance of HBV DNA in serum only indicates an effective inhibition of the HBV reverse transcription but not the transcriptional

Keywords: HBV RNA; Cytokines; HBeAg positive; Entecavir; Chronic hepatitis B.
Abbreviations: ALT, alanine aminotransferase; CHB, chronic hepatitis B; cccDNA, covalently closed circular DNA; ETV, entecavir; HBV, hepatitis B virus; HBeAg, hepatitis B virus e antigen; HBeAg(+), HBeAg-positive; anti-HBe, anti hepatitis B virus antigen; anti-HBs, anti hepatitis B surface antigen; anti-HBc, anti hepatitis B core antigen; IFN-γ, interferon-gamma; IL, interleukin; IL-12p70, IL-12 p70 heterodimer; MIP-1β, macrophage inflammatory protein-1-beta; NAs, nucleos(t)ide analogues; NSC, non-HBeAg seroconversion; pgRNA, pregenomic RNA; SC, HBeAg seroconversion; TBIL, total bilirubin; Th1, type 1 helper T cells; Th2, type 2 helper T cells; Th17, interleukin-17 producing T; TNF-α, tumor necrosis factor-alpha.

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Table 1. Baseline characteristics of CHB patients

Characteristics	All
Participants	33
Participants with HBeAg positivity	33
Participants with HBeAg negativity	0
Age in years	47.4±1.9
Male/Female	25/8
HBV DNA in log_{10}IU/mL	6.81±0.64
ALT in U/L	191.98±37.54
TBIL in μmol/L	37.20±10.30

CHB, chronic hepatitis B; HBeAg, hepatitis B virus e antigen; ALT, alanine aminotransferase; TBIL, total bilirubin.

activity of covalently closed circular DNA (cccDNA).[7] Wang *et al*.[8] revealed that HBV RNA is transcribed from the active cccDNA in infected liver cells. Such RNA is pregenomic RNA (pgRNA) that has not been reverse-transcribed and exists in the nucleocapsid of mature virus particles. Since the transcription from cccDNA to pgRNA is one of the important steps in HBV replication, pgRNA is regarded as a template for reverse transcription and synthesis of the HBV genome. Therefore, serum HBV pgRNA is considered as an important new biomarker, which reflects the sustainability of HBV infection as well as the transcriptional activity of cccDNA in the liver. Furthermore, it is also regarded as a predictor of the efficacy of antiviral therapy and the development of early resistance in patients, or a biomarker for prompting the discontinuation of NAs therapy.[9,10]

The serum HBV pgRNA load has been shown to be associated with the development of the cytotoxic lymphocyte (CTL) immunodeficiency in chronic hepatitis B (CHB) patients, as well as the induction of HBV-related immune-mediated inflammatory liver injury.[11,12] Therefore, it is necessary to understand the relationship between HBV pgRNA and host immunity in CHB patients receiving antiviral treatment. In this work, we conducted a study on the dynamic changes of serum HBV RNA levels and representative cytokines released by type 1 helper T (Th1)/type 2 helper T (Th2)/interleukin (IL)-17 producing T (Th17) cells with entecavir (ETV) treatment. We found that serum hepatitis B virus e antigen (HBeAg) levels could be used to predict serum HBV RNA levels in HBeAg-positive (+) CHB patients treated with ETV. In addition, serum IL-4 and IL-10 were the main cytokines negatively associated with HBV RNA. When combined with HBV RNA, these two cytokines could be useful in guiding clinical antiviral therapy.

Methods

Patients

We evaluated 33 HBeAg(+) individuals who had received at least 48 weeks of ETV treatment from December 2015 to July 2019 (Table 1). In short, the criteria for inclusion were: (1) HBsAg-positive for at least 6 months; (2) HBeAg-positive, anti-HBeAg (anti-HBe)-negative; (3) HBV DNA >20,000 IU/mL; and (4) patients who were naïve to NAs treatment before recruitment, but who received monotherapy with ETV. The informed consent was obtained for all patients, according to the ethical standards of the Declaration of Helsinki. The criteria for exclusion were: (1) patients who had drug-induced hepatitis, alcoholic hepatitis, nonalcoholic steatohepatitis, autoimmune hepatitis, liver fibrosis >F4 or hepatocellular carcinoma, and FibroScan was used to evaluate the degree of liver fibrosis (F4 >17.5);[13] or (2) patients receiving combination therapy with other NAs. Moreover, this study was carried out by following the guidelines set out in European Association Expert Consensus published in *J Hepatol* in 2017,[14] and the East Asian Expert Consensus published in *Aliment Pharmacol Ther* in 2020.[15]

The patients received ETV treatment continuously for 48 weeks, and were followed up every 12 weeks. Patients naïve to ETV treatment were defined as the baseline. The clinical indicators include: serum HBV DNA load, anti-HBsAg (anti-HBs), HBeAg, anti-HBe, anti-hepatitis B core antigen (anti-HBc), lymphocyte count and serum alanine aminotransferase (ALT) levels. The work was approved by the Ethics Committee of Dalian Sixth People's Hospital (approval number: 2018-026-002).

Clinical and serological parameters

The reagent kits of HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc were purchased from Abbott Laboratories in Chicago, IL, USA (HBsAg: Cat# 6C36, Lot No. 24052FN01; anti-HBs: Cat# 7C18, Lot No. 23294FN00; HBeAg: Cat# 6C32, Lot No. 23193BE01; anti-HBe: Cat# 6C34, Lot No. 24005BE01; anti-HBc: Cat# 6C33, Lot No. 24324BE01). Serum HBV DNA level was quantified using the HBV PCR Fluorescence Quantitative Detection Kit from Tianlong Bio-technology in Suzhou, China (Cat# 6C34, Lot No. P1372104001), with 500 IU/mL as the lower limit of detection. ALT was quantified using the Alanine Aminotransferase Test Kit from Ruiyuan Bio-technology in Ningbo, China (Cat# 1040-717, Lot No. 2020100902). CELLPACK DCL from Beckman-Coulter Experimental System in Suzhou, China (Cat# 628020, Lot No. 2010141) was used to test blood cell analysis, including the absolute value of lymphocytes, absolute values of monocytes and absolute values of neutrophils.

Extraction and quantification of HBV pgRNA

The levels of HBV pgRNA in serum were detected using the HBV-pgRNA assay kit from Hotgen in Beijing, China (Lot No. 20191001) following the manufacturer's protocol with the 7500 Real-time PCR Instrument from Applied Biosystems in Marsiling, Singapore. The specific primers for pgRNA were 5'-CACCGCCTCTGCTCTGTATCG-3' (forward) and 5'-TAGGGGCATTTGGTGGTCTGTAAG-3' (reverse). The lower limit of HBV RNA was calculated to be 150 copies /mL.

Determination of cytokine levels

Serum cytokine concentration, including of interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), IL-2, IL-4, IL-8, IL-10, IL-12 p70 heterodimer (IL-12p70), IL-17A and macrophage inflammatory protein-1-beta (MIP-1β), was determined using the MILLIPLEX® MAP Human High Sensitivity T Cell Magnetic Bead Panel 96-Well Plate Assay from Merck Millipore in Darmstadt, Germany (Cat# HSTCMAG-28SK, Lot No. HSTCMAG-28SK-05).

Statistical analysis

Measured concentrations of HBV nucleic acids (HBV DNA and HBV RNA) and antigen or antibody (HBeAg or ani-HBe) were logarithmically transformed (log10) for analysis. Non-

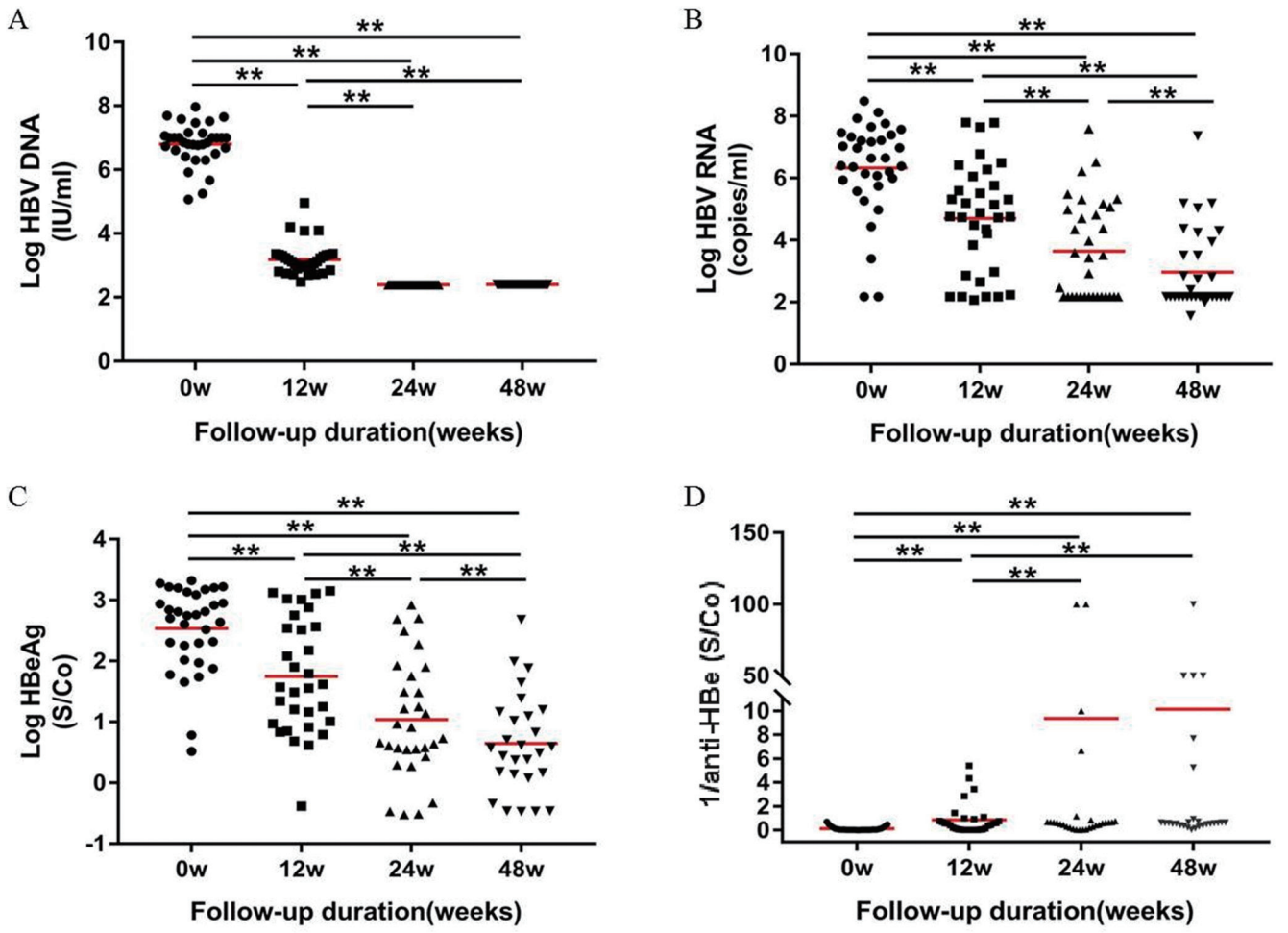

Fig. 1. Concentration and activity of HBV in HBeAg(+) CHB patients receiving ETV treatment. (A–B) The concentration of serum HBV DNA and HBV RNA during ETV treatment in HBeAg(+) CHB patients. (C–D) The level of HBeAg and anti-HBe during NAs treatment in HBeAg(+) CHB patients. Data shown are means±standard deviation. **p<0.01 compared with any two groups. HBV, hepatitis B virus; HBeAg, hepatitis B virus e antigen; anti-HBe, anti hepatitis B virus e antigen; HBeAg(+), HBeAg-positive; CHB, chronic hepatitis B; ETV, entecavir.

parametric tests, including the Mann-Whitney test, Spearman's correlation test, and multiple linear regression analysis were performed with SPSS 23.0 (IBM Corp., Armonk, NY, USA). All tests were two-tailed, and a p-value of less than 0.05 was considered to be statistically significant.

Results

Dynamic changes of HBV RNA, HBV DNA, HBeAg and anti-HBe during ETV treatment in HBeAg(+) CHB patients

In order to understand the replication of HBV in HBeAg(+) CHB patients treated with ETV, the levels of serum HBV DNA, HBV RNA, HBeAg and anti-HBe were evaluated within 48 weeks. We found that the levels of HBV DNA, HBV RNA, ALT and HBeAg decreased (data for ALT shown in Supplementary Fig. 1). The serum concentrations of each in the ETV group (12 weeks, 24 weeks and 48 weeks) were significantly decreased, compared with the baseline group (p<0.01). At the 12th week, there were five patients with HBeAg seroconversion (SC) (5/33), and no patients achieved HBeAg seroconversion out to 48 weeks (Fig. 1).

Serum HBeAg was positively correlated with HBV RNA during ETV treatment

Serum HBV DNA, HBV RNA and HBeAg levels were found to decrease in subjects treated with ETV in this study. However, it was not clear whether the HBV nucleic acids and the HBeAg protein in the serum were interrelated. Thus, we set out to analyze the potential correlation between HBV nucleic acid and HBeAg (Fig. 2). At baseline, we found that HBV DNA was positively correlated with the level of HBV RNA (r=0.564, p<0.001) and HBeAg (r=0.464, p<0.01) respectively. However, such correlation was not observed at the 12th, 24th and 48th weeks (p>0.05). It is noteworthy that serum HBV RNA was positively correlated with HBeAg, and the correlation became stronger with the prolongation of the ETV treatment. In addition, the serum concentrations of HBsAg, anti-HBs and anti-HBc were also determined and no correlation with HBV RNA was observed (data not shown). HBeAg is not a structural component of HBV nor is it necessary for HBV DNA replication. The presence of HBeAg indicates that liver tissue is still infected with HBV continuously.

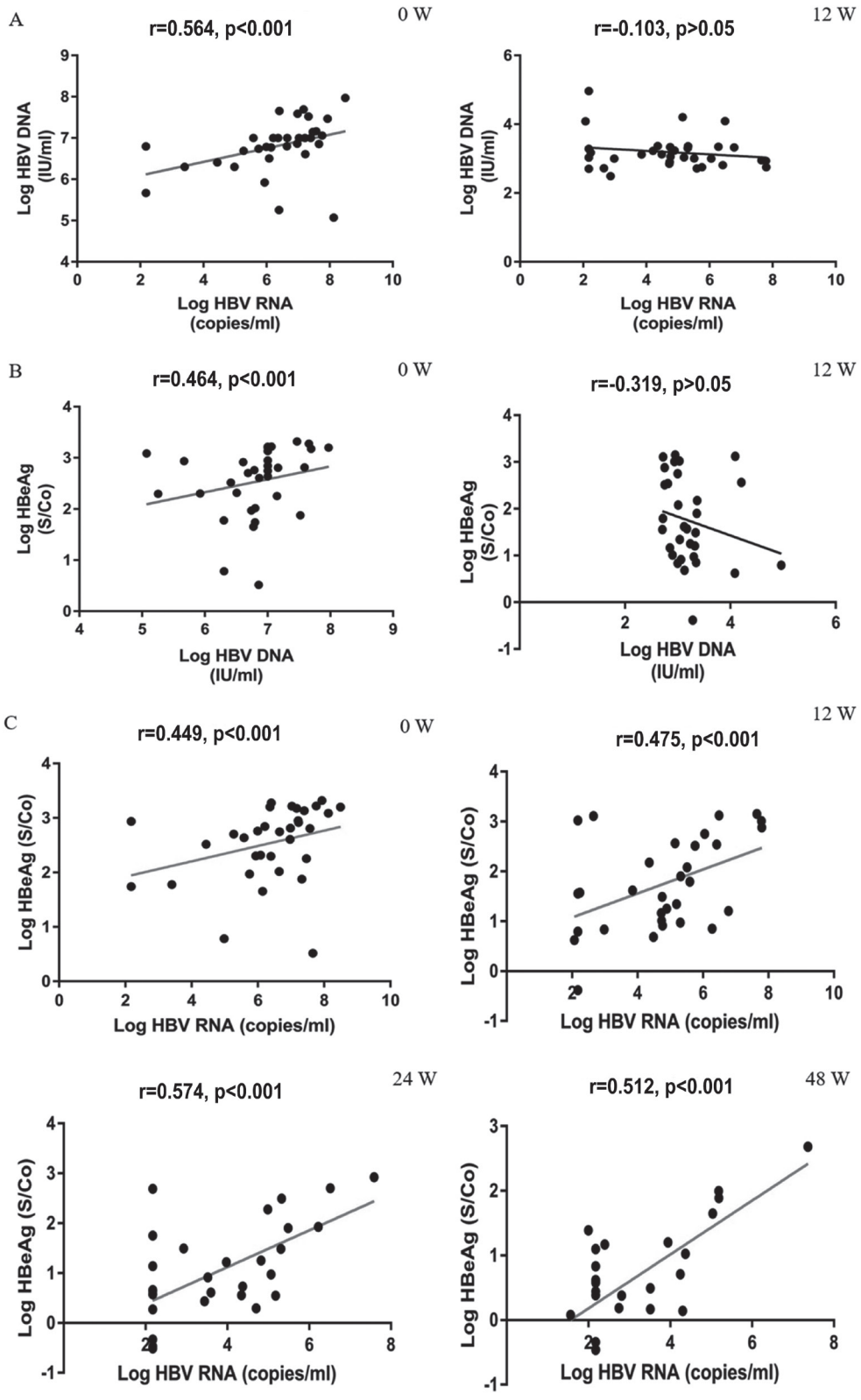

Fig. 2. Relationship between serum HBV RNA, HBV DNA and HBeAg. (A) Correlation between serum HBV DNA and HBV RNA during ETV treatment over 0–12 weeks. (B) Correlation between serum HBV DNA and HBeAg during ETV treatment over 0–12 weeks. (C) Correlation between serum HBV RNA and HBeAg during ETV treatment over 0–48 weeks. HBV, hepatitis B virus; HBeAg, hepatitis B virus e antigen; ETV, entecavir.

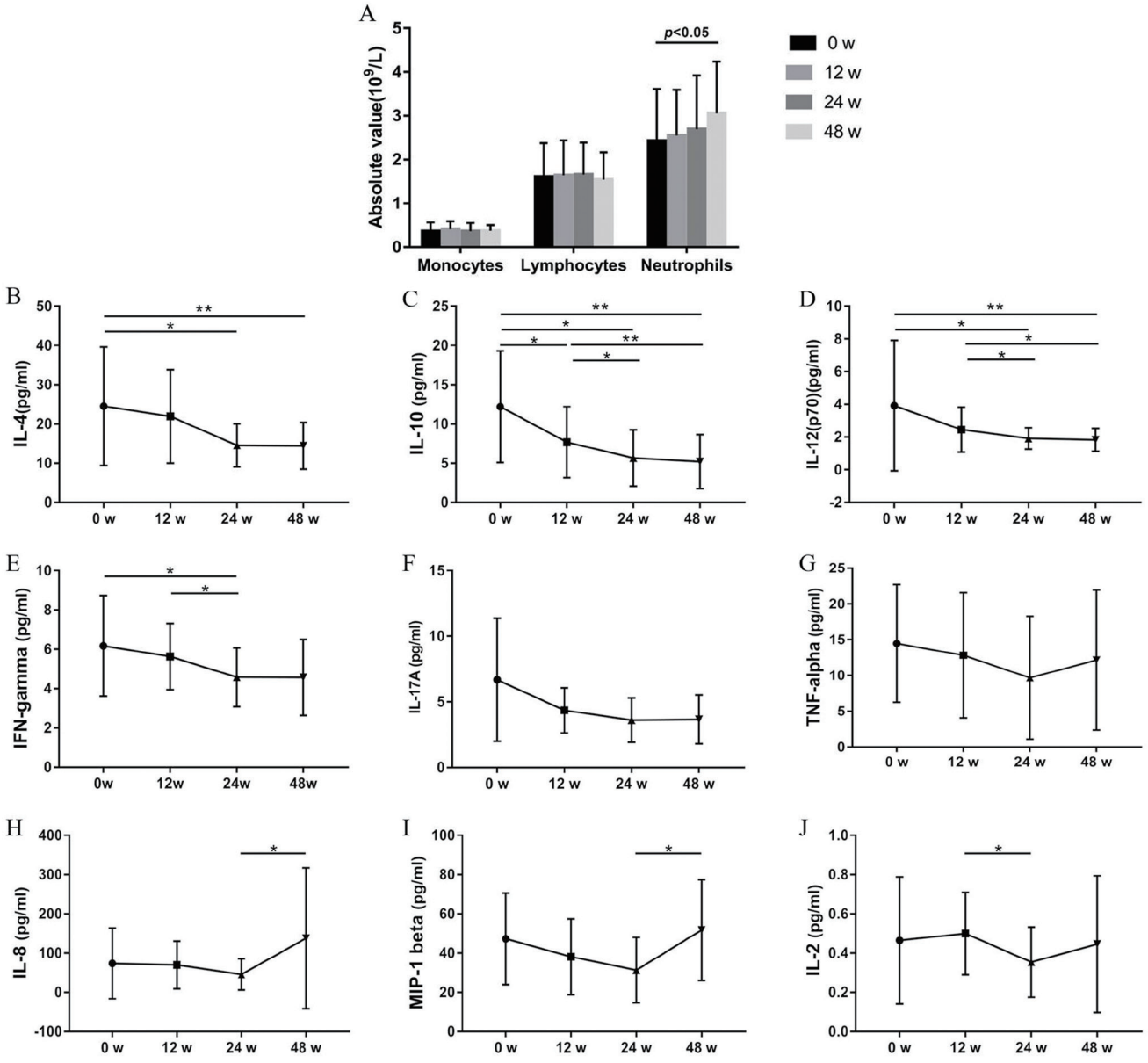

Fig. 3. Level of immune cells and serum cytokines in HBeAg(+) CHB patients treated with ETV. (A) The number of monocytes, lymphocytes and neutrophils over the course of ETV treatment. (B–J) The serum cytokines and chemokines in HBeAg(+) CHB patients treated with ETV. Data shown are means±standard deviation. $^{**}p<0.01$ compared with any two groups. $^{*}p<0.05$ compared with any two groups. IL, interleukin; IL-12p70, IL-12 p70 heterodimer; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; MIP-1β, macrophage inflammatory protein-1-beta; HBeAg(+), hepatitis B virus e antigen positive; CHB, chronic hepatitis B; ETV, entecavir.

The serum HBeAg was found to positively correlated to serum HBV RNA in this study but the underlying mechanism remains to be elucidated.

Dynamic changes of cytokines in patients with HBeAg(+) CHB treated with ETV

Chronic HBV infection leads to immune regulatory dysfunction. The balance of the ratio between Th1 and Th2 T lymphocytes is one of the main factors responsible for effective elimination of virus and virus-infected cells. Under the stimulation of HBV-specific antigens, CD4 T lymphocytes differentiate into Th1 and Th2 cells, driven by different cytokines; thus, the balance between the cytokine networks is altered. Therefore, the assessment of the relationship between HBV RNA and cytokines may provide some insight into the antiviral immune network. To this end, we first explored the number of immune cells in the peripheral blood, and found neutrophils increased significantly at 48 weeks ($p<0.05$) compared with that at the baseline, but there was no statistically significant difference in the number of lymphocytes and monocytes at various time points studied (Fig. 3A).

Interestingly, the levels of serum IL-4, IL-10, IL-12, IFN-γ and IL-17 were found to decrease following ETV treatment. Specifically, the serum levels of IL-4, IL-10, IL-12 and IFN-γ were significantly decreased at 24 weeks (Fig. 3B–E) and 48 weeks (Fig. 3B–D) compared with that at baseline. The se-

Table 2. Factors associated with HBV RNA during ETV treatment (final multivariate models)

Parameter	Baseline		12 weeks		24 weeks		48 weeks	
	Coefficient (95% CI)	*p*	Coefficient (95% CI)	*p*	Coefficient (95% CI)	*p*	Coefficient (95% CI)	*p*
IFN-γ	0.102 (−0.357, 0.560)	0.607	0.324 (−0.237, 0.885)	0.224	0.141 (−0.998, 1.279)	0.764	0.053 (−0.594, 0.699)	0.856
IL-2	−0.017 (−1.462, 1.428)	0.978	−0.415 (−1.878, 1.049)	0.538	−0.210 (−7.944, 7.524)	0.947	−0.828 (−3.189, 1.532)	0.442
IL-8	0.003 (−0.007, 0.013)	0.525	−0.007 (−0.030, 0.016)	0.521	0.000 (−0.042, 0.043)	0.985	0.002 (−0.004, 0.008)	0.488
MIP-1β	−0.002 (−0.039, 0.035)	0.906	0.036 (−0.024, 0.096)	0.211	0.038 (−0.108, 0.184)	0.533	0.004 (−0.066, 0.073)	0.908
TNF-α	−0.027 (−0.096, 0.042)	0.373	−0.075 (−0.295, 0.145)	0.462	−0.166 (−0.591, 0.260)	0.362	−0.007 (−0.073, 0.059)	0.815
IL-10	−0.010 (−0.258, 0.239)	0.928	0.057 (−0.228, 0.341)	0.663	−0.602 (−1.186, −0.017)	0.046	−0.085 (−0.502, 0.332)	0.651
IL-12p70	−0.134 (−0.507, 0.240)	0.415	−0.681 (−1.505, 0.143)	0.094	1.512 (−1.607, 4.630)	0.268	−0.990 (−3.627, 1.647)	0.412
IL-17A	−0.117 (−0.430, 0.195)	0.394	−0.070 (−0.521, 0.381)	0.732	0.135 (−0.623, 0.893)	0.666	−0.020 (−0.392, 0.351)	0.902
IL-4	−0.007 (−0.055, 0.041)	0.719	−0.055 (−0.098, −0.012)	0.017	−0.049 (−0.188, 0.091)	0.412	−0.006 (−0.019, 0.007)	0.302

HBV, hepatitis B virus; ETV, entecavir; IL, interleukin; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; IL-12p70, IL-12 p70 heterodimer; MIP-1β, macrophage inflammatory protein-1-beta. CI, confidence interval.

rum level of IL-10 was significantly decreased at 12 weeks compared with that at baseline (Fig. 3C). In addition, the expression of IL-10, IL-12 and IFN-γ were significantly decreased at 24 weeks (Fig. 3C–E) and 48 weeks (Fig. 3C, D) compared with that at 12 weeks. However, there was no significant difference in serum levels of IL-17A and TNF-α at the different time points during treatment (Fig. 3F, G). Moreover, the levels of IL-8 and MIP-1β decreased slowly at the time points before 24 weeks, and significantly increased until 48 weeks (Fig. 3H, I). Furthermore, the serum level of IL-2 increased at 12 weeks, and significantly decreased at follow-up treatment. At 48 weeks, expressionist serum level was similar to that at the baseline (Fig. 3J).

Correlation and multivariate analysis of the factors related with HBV RNA

In order to clarify the relationship between serum levels of HBV RNA and cytokines, we performed correlation analysis. At baseline, serum HBV RNA and IL-12p70 were negatively correlated (r=−0.509, $p<0.05$). With ETV treatment, HBV RNA was negatively correlated with IL-10 at 24 weeks (r=−0.316, $p<0.05$) and negatively correlated with IL-4 levels at 12 weeks (r=−0.476, $p<0.05$) (Supplementary Fig. 2). In addition, we explored the factors affecting HBV RNA through multiple linear regression analysis. The results suggest that serum IL-4 level is significantly associated with HBV RNA at 12 weeks (B=−0.055, $p=0.017$), while serum IL-10 level is significantly associated with HBV RNA at 24 weeks of ETV treatment (B=−0.602, $p=0.046$) (Table 2).

Discussion

HBV RNA could reflect the status of HBV cccDNA in liver cells. Thus, HBV RNA was proposed as a serum biomarker for evaluating the dynamic changes of HBV, especially when HBV DNA could not be detected with NAs treatment.[16] In this study, we found that ETV was associated with reduced serum HBV nucleic acid when the load of HBV DNA could not be detected at 24 weeks, indicating that HBV RNA could be used to monitor the level of HBV replication during NAs treatment. At the same time, not only the serum HBV nucleic acid decreased but the HBeAg protein was also significantly reduced. This might be due to the fact that pgRNA is similar in structure to HBeAg. Cornberg *et al*.[17] have found that pgRNA encodes the P protein, which is an HBcAg and HBeAg premise protein. Therefore, the dynamic changes of HBV RNA were consistent with that of serum HBeAg protein level.

Clinically, HBV RNA could be utilized to evaluate the treatment of NAs.[18,19] Furthermore, the relationship between HBV levels and HBeAg has been extensively explored.[20] In this study, we found that the HBV RNA and HBeAg were positively correlated in untreated patients. Ji *et al*.[7] found that the HBV RNA level at 12 weeks of NAs treatment could be used as an independent predictor of viral response or HBeAg seroconversion. Butler *et al*.[21] found that the correlation between HBV DNA and pgRNA levels in CHB subjects was low during NAs treatment, and the concentration of HBV RNA was usually higher than that in HBV DNA. In contrast, in patients not treated with NAs, the concentration of serum HBV RNA was positively correlated with HBV DNA. In this study, serum HBV DNA, HBV RNA and HBeAg were only positively correlated in untreated patients at the 12th week; however, the correlation disappeared during the follow-up ETV treatment. Bömmel *et al*.[22] showed that in the early stage of NAs treatment, the decrease of HBV RNA was strongly related to HBeAg seroconversion. We found only five patients whose serum anti-HBe was detectable from 12

weeks. We also analyzed the subgroups accordingly and observed that HBV RNA decreased faster and the cytokines were at a higher level in SC group compared with the that in the non-HBeAg seroconversion group (Supplementary Fig. 3). In the future, whether HBeAg seroconversion is achieved needs to be further evaluated after extending the follow-up time. Therefore, compared with HBV DNA, HBV RNA might be a better biomarker to reflect HBV replication. The detection of HBV RNA is not widely used in clinics, due to the technical complexity of the RNA assay itself. However, our results suggest that it may be more productive to use HBeAg as a surrogate to determine the virus replication in CHB patients.

HBV could evade recognition by the innate immune system and suppress immune response in the early stage of infection, resulting in persistence of the virus in the liver tissue.[23,24] Pathogens could activate the innate immune system by binding to pattern recognition receptors.[25] As an important part of innate immune cells, neutrophils play an indispensable role in fighting viral infections.[26] We found that the number of neutrophils increased during ETV treatment. Naumenko *et al*.[27] revealed that when the liver tissue was infected by HBV, neutrophils were rapidly recruited from the peripheral circulation to the infected site, driven by inflammatory factors and chemokines, and participated in removal of the pathogen. In this study, we found that the level of chemokines (IL-8 and MIP-1β) increased at the 48th week of ETV treatment. In addition, studies by others have shown that neutrophils regulate the maturation and activation of natural killer (NK) cells.[28] Activated neutrophils also promote the maturation of myeloid dendritic cells, the proliferation of T cells and the differentiation of Th1 and Th17, while NK cells and Th1 cells are important immune cells for virus clearance.[29] The cytokines secreted by Th1 and Th17 cells were found to decrease in their studies, which is consistent with the findings in our study. Ye *et al*.[30] also found that the balance between the effector CD4+ Th response (Th17 and Th1 response) and the regulatory response was an important part of immune regulation. Inappropriate, excessive and non-specific Th17 and Th1 effector responses might be related to the pathogenesis of HBV-associated liver inflammation and hepatocellular damage, especially the Th17 response. IL-17-mediated liver neutrophil recruitment via the induction of IL-8 might lead to liver injury in CHB patients. Neutrophils in hepatitis patients would also trigger some structural changes and dysfunctions.[31,32]

Th1/Th2 cells maintain a relatively balanced state in normal circumstances, but this balance becomes distorted with HBV infection.[33,34] At the 12th week of ETV treatment, IL-4 was the main cytokine found to be negatively correlated with serum HBV RNA level, compared to other cytokines. At the 24th week, IL-10 became the main factor to be negatively correlated with serum HBV RNA level, compared to other cytokines. It is generally believed that IL-4 promotes the differentiation of T lymphocytes into Th2 cells.[35,36] IL-4 inhibits the function of Th1 cells and limits the Th1 type response, so that the secretion of Th1 cytokines (IL-2 and IFN-γ, etc.) is reduced, leading to the reduced immune response and weakened ability to clear viruses, culminating in viral rebound.[37,38] It is also believed that the elevated serum level of IL-10 may be partly responsible for the poor clinical outcome of antiviral treatment.[39] Because IL-10 is a powerful immune and inflammatory inhibitory factor, it not only inhibits Th1 cellular immune response but also inhibits cytokines secreted from Th1 cells.[40,41] IL-10 could act on the early stage of dendritic cell maturation, inhibit the expression of costimulatory molecules, as well as reduce the expression of major histocompatibility antigen class II molecules, leading to the suppression of the maturation and differentiation of dendritic cells.[42] As a result, CD4 T lymphocytes become unresponsive to specific exogenous antigens, leading to immune tolerance to HBV and inhibiting its clearance. However, the role of IL-10 in the process of antiviral treatment of hepatitis B needs remains unclear.[43,44] In this study, the serum levels of IL-4 and IL-10 in patients treated with ETV were found to decrease along with a lowed serum HBV RNA level, suggesting that not only the virus was effectively controlled by ETV but the inflammatory response was also tamed. Therefore, it is necessary to continue antiviral treatment to prevent the virus from rebounding, because the ability for the immune system to remove the virus is concomitantly weakened.

As a key Th1 proinflammatory cytokine, IL-12 is mainly produced by antigen presenting cells.[45,46] This heterodimeric cytokine inhibits Th2 function and was originally thought to be a link between innate and adaptive immune responses.[47] In this study, serum IL-12 was found to be negatively correlated with HBV RNA at the 12th week of ETV treatment. Meanwhile, there were five patients who achieved HBeAg serological conversion. Wu *et al*.[48] have found that the higher serum levels of IL-10 and IL-12 in HBeAg-positive patients were correlated with early and spontaneous HBeAg. Such observation is consistent with the results of this study, albeit its mechanism needs to be explored in further study.

This study was of retrospective design and had a limited number of subjects. Therefore, our findings need to be verified in future prospective studies with larger sample size before they can be translated to clinical practice. Nonetheless, in summary, there seems to be a positive correlation between serum HBeAg and HBV RNA in patients treated with ETV. At the same time, antiviral therapy changed the immune response in the patients we assessed. In the early stage of ETV treatment, there was a muted immune response from the Th1/Th2/Th17 cells. At the 48th week of ETV treatment, elevated chemokines stimulated neutrophils and promoted immune responses. As serum IL-4 and IL-10 were negatively associated with the serum level of HBV RNA, these two cytokines might be used in combination with HBV RNA as new indicators of antiviral drug efficacy. Future long-term prospective studies will shed light on the relationship between HBV RNA and immunity in CHB patients.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (CP), experimental treatment (HH, CL, QW, PS), acquisition of data (QZ, FS), interpreta-

tion of data (QZ, AS, ZW and XS), drafting of the manuscript (QZ, CP), critical revision of the manuscript for important intellectual content (CP, QZ, WD, HH), and administrative, technical, or material support, study supervision (CP, YZ).

Data sharing statement

Technical appendix, statistical code, and dataset are available from the corresponding authors. Participants gave informed consent for data sharing.

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