The Effect of Entecavir Therapy on Immune Status in Chronic Hepatitis B Patients

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ABSTRACT

Background: Entecavir (ETV) is an antiviral medication effective in suppressing hepatitis B virus (HBV) replication and improving liver function. However, the relationship between antiviral effect and immune modulation after ETV therapy is not clearly understood. **Objective:** The objective of this study is to investigate the immunoregulatory effect of ETV treatment in patients with chronic hepatitis B (CHB). **Methods:** The frequencies of immune cells, including IFN-γ-producing CD4+ and CD8+ T cells, Th9 cells, regulatory T (Treg) cells, and myeloid-derived suppressor cells (MDSC) were determined in the peripheral blood from treatment-naïve and ETV-treated CHB patients. The plasma levels of IL-10, TGF-β, IL-9, TNF-α, IFN-γ, and Arg-1 were measured using enzyme-linked immunosorbent assay. **Results:** The results showed that ETV treatment significantly reduced the levels of liver function indices as well as HBV DNA loads in CHB patients. However, no significant difference in the immune cells percentage was found between the treatment-naïve and ETV-treated patients. Additionally, ETV treatment did not influence the production of TGF-β, IL-9, Arg-1, IFN-γ, and TNF-α. In contrast, the level of IL-10 was remarkably reduced after ETV therapy. **Conclusion:** IL-10 was a more sensitive effector to ETV-induced inhibition of HBV replication in chronic HBV patients.

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Keywords: Chronic Hepatitis B, Cytokine, Entecavir, Immune Cells

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INTRODUCTION

Infection with the hepatitis B virus (HBV) affects large numbers of individuals worldwide with a high risk of developing severe liver disease (1). Accumulated evidence has demonstrated that HBV is not directly cytopathic; rather, the main cause of the development of hepatitis B is an inappropriate host immune response to virus-infected hepatocytes (2,3). Therefore, the improvement in immune status is closely associated with the outcome of hepatitis B patients.

Currently, nucleoside/nucleotide analogs (NAs) have been widely provided for clinical use to treat chronic HBV infection. NAs are highly effective inhibitors of viral replication, which can selectively suppress the activity of HBV DNA polymerase (4). Of these NAs, Entecavir (ETV) is a relatively new agent that is recommended as first-line therapy by clinical practice guidelines (5). The treatment efficacy of ETV is excellent in suppressing HBV DNA and delaying disease progression (6). However, it rarely cures HBV infection because ETV alone cannot completely eliminate intracellular virus (7). It is well known that the complete eradication of HBV depends on effective anti-viral immune response, including multiple immune cells and their related cytokines (8,9). Therefore, understanding the immunomodulatory effect of ETV may provide useful information about the development of a novel immunotherapeutic approach. Despite several studies have conducted in this research field, the results are inconsistent (10-12). Moreover, several new types of immune cells, such as myeloid-derived suppressor cells (MDSC) and Th9 cells, have been recently discovered. Overall, limited information is available about the effects of ETV on these cells.

In the present study, the potential immunoregulatory properties of ETV were assessed with a high emphasis on the effects on IFN-γ-producing T cells, MDSC, regulatory T (Treg) and Th9 cells, and their related cytokines. We focused on evaluating the expression of immune cells and cytokines in peripheral blood. Although analyzing their levels in the liver could better reflect the host immune status, it is difficult to develop and implement in clinical practice due to the relative inaccessibility of liver tissue. Recently, increasing evidence has suggested the impact of peripheral immune responses on disease progression in the chronic HBV infection (13). Our results will help better understand the inadequate capacity of ETV in improving immune function and provide new ideas for researching how to further promote the therapeutic effect.

MATERIALS AND METHODS

Patients. Between August 2015 and October 2016, 38 CHB patients were recruited from Shijiazhuang Fifth Hospital, China. Among the patients, 22 were NAs-naïve patients who had never received NAs and interferons treatment and 16 were ETV-treated patients who had been treated with ETV (0.5 mg/d) for 24 weeks. The mean age of the patients was 41.05 ± 13.85 years. Of the total study population, 77.27% (17) were male in the NAs-naïve group, whereas the mean age was 42.87 ± 10.43 years, and 62.5% (10) were male in the ETV-treated group. All patients were diagnosed according to the Guideline of Prevention and Treatment for Chronic Hepatitis B in China (2010 version). Patients coinfected with hepatitis virus A, C, D, E, human immunodeficiency virus (HIV), acute hepatitis B, alcoholic, or drug-induced liver injury were excluded. The study was approved by the Shijiazhuang Fifth Hospital Ethics Committee.

Isolation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated by ficoll density gradient centrifugation. Briefly, peripheral blood was collected into EDTA-coated tubes and mixed with the same volume of PBS. Diluted blood was carefully added to human lymphocyte separation medium (Solarbio Science & Technology, Beijing, China). After centrifugation, the interphase containing mononuclear cells was obtained. The cells were washed 3 times with RPMI 1640 and resuspended in culture medium.

Flow cytometric analysis. For MDSC examination, 200 μL of whole blood sample was stained with the following monoclonal antibodies: CD11b-APC, CD33-PE, and HLA-DR-PE/Cy7 (all from BioLegend, San Diego, CA, USA). After incubation for 15 min, each sample was treated with 1 mL of lysing solution (Beckman Coulter, Miami, FL, USA) for 10 min. To stain intracellular cytokines, PBMCs were stimulated with phorbol-12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μg/mL). Meanwhile, GolgiStop (Becton Dickinson, San Diego, CA, USA) was added to each well and treated for 4 h at 37℃. After harvesting, the cells were stained with CD4-FITC and CD8a-PerCP/Cy5.5 (all from BioLegend, San Diego, CA, USA), fixed and permeabilized with Fixation and Permeabilization solution (Becton Dickinson, San Diego, CA, USA), and then were intracellularly stained with IFN-γ-PE/Cy7 and IL-9-PE (BioLegend, San Diego, CA, USA). For Treg cell examination, PBMCs were surface stained with CD4-FITC and CD25-APC (all from BioLegend, San Diego, CA, USA) for 20 min, and then fixed and permeabilized with Fixation and Permeabilization Solution (eBioscience, San Diego, CA, USA), followed by staining with Foxp3-PE (BioLegend, San Diego, CA, USA). All samples were detected by FACS CantoII flow cytometer and analyzed by FACS Diva software.

Enzyme-linked immunosorbent assay (ELISA). Plasma samples were collected, and the levels of IL-10, TGF-β, TNF-α, IL-9, IFN-γ, and Arg-1 were measured using human ELISA assay kits (Multi Sciences, Hangzhou, Zhejiang, China) according to the manufacturer's instructions. The optical density (OD) at 450nm was measured by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Biochemical and virological assessments. The biochemical indices, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), total bilirubin (TBIL), and direct bilirubin (DBIL), were measured using an automated analyzer with standard techniques. Serum HBV DNA load was detected by real-time PCR using an ABI7500 quantitative PCR instrument (Applied Biosystems, Foster City, CA, the USA), where the lowest detection limit was 500 copies/mL.

Statistical analysis. All statistical analyses were performed using SPSS 19.0 software (SPSS Inc, Chicago, IL, USA). Data were expressed as mean ± SD or median with range. The comparison of differences between each group was analyzed using Student's t-test, Mann-Whitney U test, or Chi-square test. Pearson correlation tests were done for correlation analysis. A two-sided P value of <0.05 was considered to be statistically significant.

RESULTS

Antivirus efficacy of ETV treatment. After treatment with ETV for 24 weeks, the levels of ALT and AST were significantly decreased ($P<0.05$). The proportion of ALT

and AST normalization was 81.25% and 87.50%, respectively. Additionally, treatment with ETV led to a significant decline in HBV-DNA loads (P<0.05) (Table 1).

Table 1. Demographic and clinical characteristics of study subjects.

	NAs-naïve patients (n=22)	ETV-treated patients (n=16)	*P* value
ALT (U/L, median with range)	56.50 (17.00, 903.00)	36.00 (19.00, 55.00)	0.006
AST (U/L, median with range)	54.00 (18.00, 211.00)	27.00 (18.00, 70.00)	0.005
TBIL (mg/dl, median with range)	1.14 (0.53, 15.32)	1.05 (0.58, 2.28)	0.328
DBIL (mg/dl, median with range)	0.56 (0.23, 11.11)	0.37 (0.18, 0.82)	0.319
HBV DNA loads (log_{10}copies/mL, median with range)	6.17 (2.70, 8.22)	2.70 (2.70, 2.87)	<0.001

The effect of ETV treatment on the percentage of immune cells. We first investigated the effect of ETV treatment on two types of immunesuppressor cells, including MDSC and Foxp3+ cells. The results showed that there was no significant difference between NAs-naïve patients and ETV-treated patients (P>0.05) (Figure 1). Moreover, compared with NAs-naïve patients, ETV-treated patients had slightly lower frequencies of IFN-γ–producing CD4+ and CD8+ cells, and slightly higher percentage of IL-9-producing CD4+ cells, but without any significant difference (P>0.05) (Figure 1).

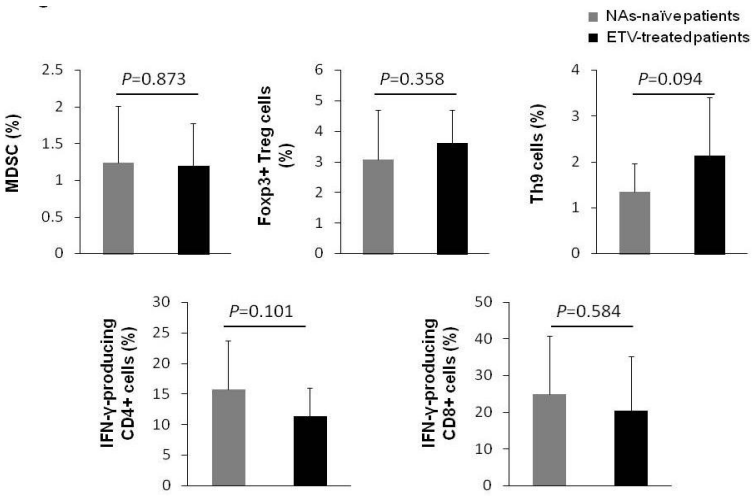

Figure 1. Effect of ETV treatment on the percentage of immune cells. Peripheral blood samples were collected from nucleoside/nucleotide analogs (NAs)-naïve patients (n=22) and entecavir (ETV)-treated patients (n=16). The percentages of MDSC, Treg, Th9, IFN-γ-producing CD4+ and CD8+ cells were determined by flow cytometry. Data are represented as the mean ± SD.

The effect of ETV treatment on cytokine production. After ETV treatment, the level of IL-10 was significantly decreased in the ETV-treated patients compared to those NAs-naïve patients (P<0.05) (Figure 2). However, there was no significant difference between the two groups in the production of other cytokines, including TGF-β, IL-9, Arg-1, IFN-γ, and TNF-α (P>0.05) (Figure 2).

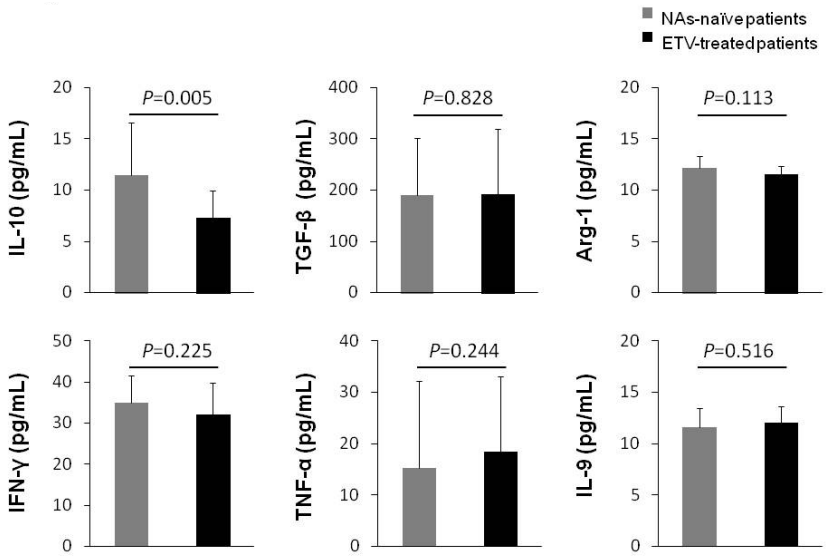

Figure 2. Effect of ETV treatment on the production of cytokines. Plasma samples were collected from nucleoside/nucleotide analogs (NAs)-naïve patients (n=22) and entecavir (ETV)-treated patients (n=16). The levels of IL-10, TGF-β, Arg-1, IFN-γ, TNF-α and IL-9 were measured by ELISA. Data are represented as the mean ± SD.

IL-10 is a critical immunoregulatory cytokine, which is known to play key roles in the pathogenesis of CHB (14,15). Moreover, it plays a major impact on the downregulation of other immune cells. Thus, the relationship of IL-10 levels with the percentage of immune cells in ETV-treated patients was analyzed. However, there was no strong correlation between the levels of IL-10 with the frequencies of MDSC, Foxp3+ cells, IFN-γ-producing CD4+, and CD8+ cells, and IL-9-producing CD4+ cells (P>0.05) (Table 2).

DISCUSSION

ETV has widely been used as a first choice drug for CHB patients because of its low rate of genotypic resistance. Several studies have shown that ETV is a highly potent agent against chronic hepatitis B (5,6). Consistent with the previous works, in this study, we found that treatment with ETV resulted in a significantly improved liver function and had a strong inhibition effect on HBV DNA. This observation supports the conclusion that ETV treatment is effective for HBV DNA suppression.

Table 2. The correlation between IL-10 and immune cells in ETV-treated patients.

	IL-10	
	r	***P* value**
MDSC (%)	0.061	0.823
Foxp3+ Treg cells (%)	-0.438	0.178
Th9 cells (%)	-0.538	0.109
IFN-γ-production $CD4^+$ cells (%)	0.207	0.566
IFN-γ-production $CD8^+$ cells (%)	0.807	0.099

Considering the impaired cellular immune response in chronic HBV infection, the effect of ETV on immune cells was observed. IFN-γ-producing CD4+ and CD8+ cells are well-known important components of HBV-specific immune response and play important roles in the control of HBV (16). In contrast, Treg cells are negative immunoregulatory cell population that suppress HBV-specific immune responses and indirectly influence the disease progression (17). Despite several previous studies have reported the effect of ETV monotherapy on IFN-γ-producing T cells and Treg cells (10-12,18), the obtained results are inconsistent. In the present study, we demonstrated that there were no significant differences in the frequencies of IFN-γ-producing CD4+ and CD8+ T cells as well as Treg cells between patients with and without ETV treatment, suggesting that ETV therapy failed to restore T cell response.

More recently, the other two immune cells (i.e., MDSC and Th9) have received increasing attention. MDSC are immunosuppressive cells that possess strong inhibitory effects toward innate and adaptive immune response. Increasing evidence has demonstrated that MDSC contributes to the persistent infection of HBV through suppressing the proliferative capacities of allogeneic T cells (19). Th9 cells are newly-discovered subset of T helper cells that are characterized by IL-9 and IL-10 secretion. Our previous study has shown that Th9 cells are negatively correlated with the development of chronic HBV infection in CHB patients (20). However, to date, the effect of antiviral therapy on MDSC and Th9 cells remains unknown. To our knowledge, the present study reports for the first time that ETV treatment did not lead to the change of the frequency of MDSC and Th9 cells. These results, together with the above data, suggest that ETV had little ability to regulate immune cells.

Many cytokines have been suggested to contribute to effective antiviral immunity and the outcome of HBV infection. The effect of ETV on the level of cytokines was also observed. The results showed that IL-10 production was significantly decreased after ETV treatment. However, the levels of other cytokines, including IFN-γ, IL-9, Arg-1, TGF-β, and TNF-α, were not significantly changed. These data suggested that IL-10 was a more sensitive effector associated with ETV treatment. This finding is consistent with that of Yu et al. (21), but different from another study in which the IL-10 level was significantly increased after ETV treatment (22). IL-10 is an important immunoregulatory cytokine involved in suppressing effective immune functions. The reduced IL-10 production after ETV therapy implies that ETV may have a certain role in restoring the impaired immune response, which is helpful for alleviating the disease

progression. To investigate the immune cells involved in IL-10 reduction, we analyzed the correlation of the level of IL-10 and the frequency of immune cells. In this study, however, no clear correlation was found between IL-10 and any immune cells in ETV-treated patients. It has been well evidenced that IL-10 is produced by various kinds of cells, such as MDSC, Th2, Th9, and Treg cells (23,24). Therefore, we speculate that the reduction of IL-10 level might be due to the decreased secretion capacity of various immune cells. Further studies are needed to investigate the major subsets of immune cells involved in the regulation of IL-10 production when HBV replication was profoundly inhibited by ETV therapy.

The major limitation of this study is that the expression of immune cells and their related cytokines was only determined from two independent cohorts at a single time point, and the dynamical change was not observed. A previous study has found that ETV therapy induces temporary increased Th17 cells and, subsequently, their level declines to the baseline level (25), suggesting that ETV may have a significant short-term and long-term effect on the immune system. The further longitudinal analysis may better clarify the change of the immune system in the process of ETV therapy.

In summary, this study demonstrated that ETV treatment displayed strong antiviral action but weak immunomodulatory property. The ETV-driven improvement of immune response seemed to be only associated with the decreased IL-10 production. Therefore, the development of the combined therapy with ETV and immune modulators may provide more clinical benefit. Currently, there has been an interest in the combined use of NAs and Peg-IFN-α, a cytokine with dual antiviral and immunomodulatory activity, to improve immune function in CHB patients (26). However, clinical use of Peg-IFN-α is limited due to the severe side effects and the contraindication in patients with decompensated liver cirrhosis or autoimmune disease. Hence, further studies should be carried out to develop new immunotherapeutic approaches to restore antiviral immunity and improve clinical efficacy in CHB patients.

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REFERENCES

1. Ott JJ, Stevens GA, Groeger J, Wiersma ST. Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. Vaccine. 2012;30:2212-2219.
2. Montuclard C, Hamza S, Rollot F, Evrard P, Faivre J, Hillon P, et al. Causes of death in people with chronic HBV infection: a population-based cohort study. J Hepatol. 2015;62:1265-1271.
3. Vyas AK, Jindal A, Hissar S, Ramakrishna G, Trehanpati N. Immune balance in hepatitis B infection: present and future therapies. Scand J Immunol. 2017;86:4-14.
4. Menendez-Arias L, Alvarez M, Pacheco B. Nucleoside/nucleotide analog inhibitors of hepatitis B virus polymerase: mechanism of action and resistance. Curr Opin Virol. 2014;8:1-9.
5. European Association for the Study of the Liver. EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection. J Hepatol. 2017;67:370-398.
6. Park JW, Kwak KM, Kim SE, Jang MK, Suk KT, Kim DJ, et al. Comparison of the long-term efficacy between entecavir and tenofovir in treatment- naïve chronic hepatitis B patients. BMC Gastroenterol. 2017;17:39.

7. Shi M, Sun WL, Hua YY, Han B, Shi L. Effects of entecavir on hepatitis B virus covalently closed circular DNA in hepatitis B e antigen-positive patients with hepatitis B. PLoS One. 2015;10:e0117741.
8. Shih C, Chou SF, Yang CC, Huang JY, Choijilsuren G, Jhou RS. Control and Eradication Strategies of Hepatitis B Virus. Trends Microbiol. 2016;24:739-749.
9. Xia Y, Protzer U. Control of Hepatitis B Virus by Cytokines. Viruses. 2017;9:18.
10. Boni C, Laccabue D, Lampertico P, Giuberti T, Vigano M, Schivazappa S, et al. Restored function of HBV-specific T cells after long-term effective therapy with nucleos(t)ide analogues. Gastroenterology. 2012;143:963-973.
11. Zhang JY, Song CH, Shi F, Zhang Z, Fu JL, Wang FS. Decreased ratio of Treg cells to Th17 cells correlates with HBV DNA suppression in chronic hepatitis B patients undergoing entecavir treatment. PLoS One. 2010;5:e13869.
12. Kondo Y, Ueno Y, Kobayashi K, Kakazu E, Shiina M, Inoue J, et al. Hepatitis B virus replication could enhance regulatory T cell activity by producing soluble heat shock protein 60 from hepatocytes. J Infect Dis. 2010;202:202-213.
13. Conroy MJ, Mac Nicholas R, Grealy R, Taylor M, Otegbayo JA, O'Dea S, et al. Circulating CD56dim natural killer cells and CD56+ T cells that produce interferon-γ or interleukin-10 are expanded in asymptomatic, E antigen-negative patients with persistent hepatitis B virus infection. J Viral Hepat. 2015;22:335-345.
14. Das A, Ellis G, Pallant C, Lopes AR, Khanna P, Peppa D, et al. IL-10-producing regulatory B cells in the pathogenesis of chronic hepatitis B virus infection. J Immunol. 2012;189:3925-3935.
15. Shi B, Ren G, Hu Y, Wang S, Zhang Z, Yuan Z. HBsAg inhibits IFN-α production in plasmacytoid dendritic cells through TNF-α and IL-10 induction in monocytes. PloS One. 2012;7:e44900.
16. Maini MK, Boni C, Lee CK, Larrubia JR, Reignat S, Ogg GS, et al. The role of virus-specific CD8(+) cells in liver damage and viral control during persistent hepatitis B virus infection. J Exp Med. 2000;191:1269-1280.
17. Kosinska AD, Pishraft-Sabet L, Wu W, Fang Z, Lenart M, Chen J, et al. Low hepatitis B virus-specific T-cell response in males correlates with high regulatory T-cell numbers in murine models. Hepatology. 2017;66:69-83.
18. Jiang Y, Li W, Yu L, Liu J, Xin G, Yan H, et al. Enhancing the antihepatitis B virus immune response by adefovir dipivoxil and entecavir therapies. Cell Mol Immunol. 2011;8:75-82.
19. Chen S, Akbar SM, Abe M, Hiasa Y, Onji M. Immunosuppressive functions of hepatic myeloid-derived suppressor cells of normal mice and in a murine model of chronic hepatitis B virus. Clin Exp Immunol. 2011;166:134-142.
20. Cui M, Lv Y, Lu J, Zhang W, Duan Y, Huang Y, et al. Decreased frequency of circulating Th9 cells in patients with chronic hepatitis B infection. J Clin Lab Anal. 2017;32:1-9.
21. Yu XP, Guo RY, Su ML, Ming DS, Lin CZ, Deng Y, et al. Dynamic Changes of Treg and Th17 Cells and Related Cytokines Closely Correlate With the Virological and Biochemical Response in Chronic Hepatitis B Patients Undergoing Nucleos(t)ide Analogues Treatment. Hepat Mon. 2013;13:e15332.
22. Yu X, Zheng Y, Deng Y, Li J, Guo R, Su M, et al. Serum Interleukin (IL)-9 and IL-10, but not T-Helper 9 (Th9) Cells, are Associated With Survival of Patients With Acute-on-Chronic Hepatitis B Liver Failure. Medicine (Baltimore). 2016;95:e3405.
23. Mannino MH, Zhu Z, Xiao H, Bai Q, Wakefield MR, Fang Y. The paradoxical role of IL-10 in immunity and cancer. Cancer Lett. 2015;367:103-107.
24. Park MJ, Lee SH, Kim EK, Lee EJ, Baek JA, Park SH, et al. Interleukin-10 produced by myeloid-derived suppressor cells is critical for the induction of Tregs and attenuation of rheumatoid inflammation in mice. Sci Rep. 2018;8:3753.
25. Zhang JY, Song CH, Shi F, Zhang Z, Fu JL, Wang FS. Decreased Ratio of Treg Cells to Th17 Cells Correlates with HBV DNA Suppression in Chronic Hepatitis B Patients Undergoing Entecavir Treatment. PLoS One. 2010;5:e13869.
26. Konerman MA, Lok AS. Interferon treatment for hepatitis B. Clin Liver Dis. 2016;20:645-665.