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Original Paper

Beneficial Effects of Bushen Formula Combined with Enticavir on Chronic Hepatitis B Patients with Suboptimal Response to Enticavir by Regulating B-Cell Differentiation

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Key Words
Chronic hepatitis B • Bushen Formula • Enticavir • Humoral immunity • B-cell • B cell-activating factor

Abstract
Background/Aims: To investigate the clinical effects of the combination therapy with Bushen Formula (BSF) plus enticavir (ETV) on chronic hepatitis B (CHB) patients with suboptimal response to ETV and explore the regulatory mechanisms of BSF on B cells-mediated humoral immunity. ***Methods:*** Sixty-four HBeAg-positive CHB patients with suboptimal response to ETV were enrolled, and were randomly assigned into control group (C-Group, placebo combined with ETV for 12 months) or treatment group (T-Group, BSF combined with ETV for 12 months). Serum samples from 57 treatment-naïve CHB patients and 15 healthy controls were collected. Serum HBV DNA levels were evaluated by real-time PCR. Characteristics of peripheral blood B-cell subtypes were analyzed by flow cytometry. Serum HBV markers and B cell-activating factor (BAFF) levels were detected by ELISA. Chinese medicine symptom complex score was evaluated and recorded. ***Results:*** After treatment, the rates of patients with a reduction of HBsAg >0.5 $\log_{10}$ IU/ml or 1.0 $\log_{10}$ IU/ml and the rates of HBeAg clearance in T-Group were all higher than those in C-group, with no significant intergroup difference. Only in T-Group, Chinese medicine symptom complex score and the frequency of total B cells were significantly decreased, and the frequencies of Bm1, $CD24^{+}CD27^{-}$switched B cells and plasma cells were markedly increased after treatment compared with those before treatment. Compared with healthy controls, serum BAFF levels in treatment-naïve CHB patients were increased, and

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there was a significant positive correlation between serum BAFF and HBsAg levels. However, serum BAFF levels did not differ after treatment in T-Group and C-Group. ***Conclusions:*** The combination therapy with BSF plus ETV promotes the reduction of HBsAg level and the clearance of HBeAg in CHB patients with partial response to ETV through regulating the differentiation of B-cell subsets.

Introduction

Hepatitis B virus (HBV) infection is a global health problem that caused over 400 million people suffering chronic HBV infection worldwide [1-3]. Chronic hepatitis B (CHB) can progress to cirrhosis, hepatocellular carcinoma and ultimately liver-related death [4, 5], and some 887, 000 people died from HBV-related liver disease in 2015. For CHB, the World Health Organization recommends treating the individual with an antiviral medication. Nucleos(t)ide analogs including lamivudine, adefovir dipivoxil, entecavir (ETV), telbivudine and tenofovir were approved by the US FDA for CHB treatment. These drugs can suppress HBV replication and prevent the progression of liver diseases [6]. ETV was one of the most effective therapy for Chinese CHB [7] and was recommended as the first-line therapies for CHB patients [8]. ETV effectively maintained virological and biochemical responses in long-term follow-up of CHB patients with/without cirrhosis [9]. In addition, it was an effective treatment with histological improvement [10]. However, the oral antivirals also led to obvious dose-dependent adverse reactions and drug resistance [11]. In addition, the serological response rate in oral antivirals for HBV was not ideal. There are limited data on how to effectively treat CHB patients with suboptimal response to ETV. Alternative and complementary medicines(ACM) can be considered for improving the treatment for these patients.

Traditional Chinese medicine(TCM), a type of ACM, originated in ancient China and has developed over thousands of years [12]. CHB has been treated for centuries with TCM [13]. With the development of TCM, CHB was named as stagnancy of liver-qi and blood or hot liver. TCM syndrome categorization (also defined as Zheng differentiation) and treatment were considered as the basis thought of TCM theory. TCM syndrome, a summary of symptoms and signs as a series of clinical phenotypes, played an important role in understanding the human homeostasis and directing the applications of Chinese herbs and acupuncture. Heat, cold, excess and deficiency are the four basic syndromes of maladjustment nature in TCM [14]. Damp heat stasis syndrome and liver and kidney Yin deficiency syndrome, classified as excess syndrome and deficiency syndrome, respectively were the common syndromes in CHB patients [15]. In this study, 64 HBeAg-positive CHB patients with suboptimal response to ETV were enrolled, and they belonged to liver and kidney Yin deficiency and damp-heat syndrome. The Bushen formula (BSF) is one empirical TCM formula for CHB patients, and our previous studies showed that the BSF had positive effects on CHB patients with mildly elevated ALT (1-2 times ULN) by reducing serum ALT and HBV-DNA levels [16]. In another Multicenter, Randomized, Double-Blind, Placebo-Controlled Trial study, it was shown that after the combination treatment of BSF and ETV, the serum HBV-DNA levels decreased and the HBeAg negative conversion rate increased in HBeAg-positive CHB patients with mildly elevated ALT without any serious adverse events [17].

The host immune response, especially the T-cell-mediated immune response, was usually considered as a central factor in regulating the outcome of HBV infection. Many researchers showed that dysfunctional T-cell immune responses may promote the procession of chronic HBV infection [18-21]. However, B cell-mediated humoral immune response in CHB patients was still unknown. In fact, HBV could be detected in B lymphocytes [22] and reversal of B-cell hyperactivation and functional impairment was associated with HBsAg seroconversion in CHB patients [23]. Therefore, in this study, the clinical effects of the combination treatment of BSF with ETV on CHB patients with partial response to ETV will be observed and the regulatory mechanisms of BSF on B cells-mediated humoral immunity will be explored.

Materials and Methods

Patients and control groups

Sixty-four CHB patients with liver and kidney Yin deficiency and damp-heat syndrome were included in this study (Table 1). All patients were chosen according to the diagnostic criteria published [24](HBsAg positive for at least 6 months, HBeAg and anti HBeAb (positive or negative) and HBV DNA positive). All patients, once treated by ETV from 6 months to 3 years, were normal serum ALT/AST levels and negative for HBV DNA, but were positive for HBeAg. TCM syndromes of patients were diagnosed according to Criteria of Diagnosis and Therapeutic Effect of Diseases and Syndromes in TCM. The patients were randomly divided into the control group(C-Group) and the treatment group(T-Group), with 32 patients in each group. Serum samples from 57 treatment-naïve CHB patients and 15 healthy controls (HC) were collected to examine serum BAFF levels (Table 2).

Treatments

All patients were treated with ETV 0.5mg daily and were randomly assigned into T-Group or C-Group to receive the BSF or placebo (4.5g twice daily, oral administration) for 12 months. The concentrated granules of BSF and the placebo were prepared as our previous paper [17]. In summary, the concentrated granules of the BSF and the placebos were prepared and provided by Jiang Yin Tianjiang Pharmaceutical Company Limited. Weights of the six herb concentrated granules in the BSF (4.5 g) are Longspur epimedium 0.375 g, Astragalus mongholicus 1.125 g, Rhizoma Picrorhizae 0.75g, ClawVine 0.75g, Fructus Ligustri Lucidi 0.75g and Pericarpium citri reticulatae viride 0.75g. The placebos were composed of food coloring (tartrazine, sunset yellow, and caramel color), bittering agent (sucrose octaacetate), and pharmaceutical excipient (Lactose). Food coloring, bittering agent, and pharmaceutical excipient were conducted in accordance with the Chinese regulation on the management of pharmaceutical excipients and Chinese hygienic standards for uses of food additives. The placebo granules shared the same package and label as the herb granules. The appearance, taste and solubility of placebos were similar to the herb granules. They were taken the same way as the BSF. This study was conducted in accordance with the ethics principles of the Declaration of Helsinki and regulation of clinical trial. This study was approved by the IRB of Shuguang Hospital affiliated with Shanghai University of TCM. All patients were given informed consent.

Evaluation of Serum viral load, ALT/AST levels and HBV serum markers

Serum HBV DNA levels in CHB patients were tested with real-time PCR using a lightcycler PCR system (FQD-33A), and the lower limit was about 1000 viral genome copies/mL by using the reagent kit (Shenzhen PG Biotech Co Ltd.). The serum ALT / AST levels were assayed by DXC 800 Fully-auto Bio- Chemistry Analyzer, at the Department of Clinical Laboratory, Shuguang Hospital. The results were considered abnormal if ALT /AST was >40 U/L. HBsAg, HBeAg, anti-HBs, anti-HBc, anti-HBe, and Abs to HCV, Hepatitis D Virus, HIV-1, and HIV-2 were detected with ELISA kits (Sino-American Biotechnology Company).

Table 1. Clinical characteristics of enrolled HBeAg positive CHB patients with suboptimal response to ETV. CHB, chronic hepatitis B; ETV, enticavir; HBeAg, HBV envelope antigen; HBsAg, HBV surface antigen; ALT, valanine transaminase; AST, aspartate transaminase; T-Group, treatment group; C-Group, control group

Group	Case	Sex (male/female)	Age (years)	HBsAg (log10)	ALT (U/L)	AST (U/L)	HBVDNA (100IU/ml)
T-Group	32	22/10	34 (16-65)	3.84 (2.13-5.09)	18 (7-35)	20 (10-36)	LDL
C-Group	32	20/12	36 (15-65)	3.74 (1.92-4.85)	26 (13-37)	29 (17-35)	LDL

Table 2. Clinical characteristics of treatment-naïve CHB patients and healthy controls in this study. CHB, chronic hepatitis B; HBeAg, HBV envelope antigen; HBsAg, HBV surface antigen; ALT, valanine transaminase; AST, aspartate transaminase

Group	Case	Sex (male/ female)	Age (years)	HBsAg (log10)	HBeAg (+/-)	ALT (U/L)	AST (U/L)	HBVDNA (log10)
CHB patients	57	40/17	32 (18-68)	3.18 (0.52-4.41)	28/29	37 (9-189)	29 (15-120)	6.01 (3.54-7.64)
Healthy Controls	15	10/5	29 (21-45)	ND	ND	ND	ND	ND

Isolation of peripheral blood mononuclear cells (PBMCs)

Blood samples were collected from patients. PBMCs were isolated from heparinized blood by standard density gradient centrifugation with Lympholyte-H (Cedarlane) according to the manufacturer's protocol. The cell viability was over 90%, as assessed by trypan blue exclusion.

Flow cytometry analysis and cell sorting of blood B-cell subsets in CHB patients

PBMCs were stained with BV421-CD45, APC-CD24, PE-Cy5-CD3, PE-CF594-CD20, FITC-IgD, APC-Cy7-IgM, PE-CD38, PE-Cy7-CD27 monoclonal antibodies respectively, according to the instructions of the respective manufacturers. The above monoclonal antibodies were obtained from Biolegend (San Diego, CA). Flow cytometry was performed using BD LSRFortessa in Institut Pasteur of Shanghai Chinese Academy of Science.

ELISA

Serum BAFF levels were tested by ELISA (Multi Sciences), and the experiment was carried out according to the manufactures.

Symptom scores

According our previous paper [17], symptoms in patients were recorded before and after treatment including hypodynamia, shortness of breath, sweating, palpitations, poor appetite, insomnia, characteristics of urine and stools, appearance of tongue, and characteristics of pulse. Then Chinese medicine symptom complex score was calculated by recording the severity of the symptoms: none, light, moderate and severe were scored as 0, 1, 2 and 3, respectively.

Statistical Analysis.

Statistical analyses were performed using SPSS statistics software (version 21.0, IBM Inc., NY, USA). The measurement data and numeration data were statistically analyzed with t test and Fisher's exact test respectively. All statistical tests were two-sided test, $P < 0.05$, the difference was statistically significant.

Results

The combination therapy with BSF plus ETV promoted HBsAg decline and the clearance of HBeAg in CHB patients

Yellow Emperor's Internal Classic indicates that TCM remedies have been used to treat chronic liver disease in China at least Since 475 BCE. The BSF is one empirical Chinese medicine formula and our previous papers confirmed that BSF decreased ALT levels and promoted HBeAg clearance in CHB patients [16, 17] . The ultimate goal of CHB treatment is HBsAg clearance and/ or even seroconversion. In this study, our results showed that after 12-month combination treatment, the rate of the reduction of HBsAg >0.5 $\log_{10}$ IU/ml and the rate of the reduction of HBsAg >1.0 $\log_{10}$ IU/ml were 40.63% and 9.38% respectively in T-Group, while in C-Group, the rates were 31.25% and 0% respectively. In addition, the rate of HBeAg clearance (9.38%) in T-Group were higher than those (0%)in C-group, with no significant intergroup difference (Fig. 1). These results suggested that the combination therapy with BSF plus ETV had better effects in HBsAg reduction and HBeAg clearance in CHB patients with partial response to ETV in long-term treatment.

The combination therapy with BSF plus ETV improved Chinese medicine symptoms in CHB patients

Nowadays, TCM treatment has been commonly used to treat CHB patients in Asian countries. TCM is featured by such treatment based in pathogenesis obtained through differentiation of symptoms and signs as its essence. Chinese medicine symptom complex score is the traditional method for evaluating the change of Chinese medicine symptom. Damp heat stasis syndrome and liver and kidney Yin deficiency syndrome, classified as excess syndrome and deficiency syndrome, respectively are the common syndromes in CHB patients [15] . The

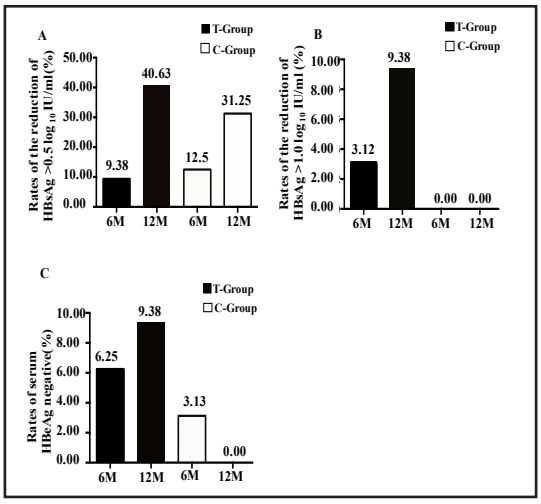

Fig. 1. Beneficial effects of the combination therapy with BSF plus ETV on CHB patients with partial response to ETV. Serum HBsAg and HBeAg levels were evaluated before and after treatment both in T-Group and C-Group. A. Rate of the reduction of HBsAg >0.5 $\log_{10}$ IU/ml after treatment for 12 months in T-Group was higher than that in C-Group, with no significant intergroup difference. B. Rates of the reduction of HBsAg >1.0 $\log_{10}$ IU/ml after treatment for 6 months and 12 months, in T-Group were higher than those in C-Group, with no significant intergroup difference. C. Rates of serum HBeAg negative after treatment for 6 months and 12 months in T-Group were higher than those in C-Group.

standard for Chinese medicine symptom complex score is set based on Guiding principles for clinical research of new drugs of traditional Chinese medicine. In this study, our results showed that the combination therapy of BSF and ETV decreased Chinese medicine symptom complex score in CHB patients from 3.94±1.865 to 1.97±1.356 after treatment for 12 months, as shown in Table 3. The above results suggested that Chinese medicine symptoms in CHB patients were substantially improved after the combination therapy with BSF plus ETV.

Table 3. Changes of Chinese medicine symptom complex score in CHB patients after the combination therapy with BSF plus ETV. CHB, chronic hepatitis B; BSF, Bushen Formula; ETV, enticavir; T-Group, treatment group; C-Group, control group. *P<0.05

Group	Case	Time		
		0 month	6 months	12 months
T-Group	32	3.94±1.87	3.28±1.42	1.97±1.36*
C-Group	32	3.59±1.24	3.69±1.615	3.50±1.34

Serum BAFF levels were positively correlated with HBsAg levels in treatment-naïve CHB patients

B-cell activation is critical in the initiation of HBV infection [25]. BAFF is a cytokine critical for development and proper selection of B cells. Yang Cu et al. showed that serum BAFF levels in chronic HBV infection are elevated, correlated with the severity of CHB [26]. In this study, our results showed that serum BAFF levels in treatment-naïve CHB patients were higher than those in healthy controls, especially in HBeAg-positive patients and there was a strong positive correlation between serum BAFF and serum HBsAg levels(Fig. 2A-C). The above results suggested that the up-regulation of BAFF expression may have an adverse effect on HBsAg clearance. Therefore, we further explored the regulatory effects of BSF on serum BAFF levels in CHB patients with partial response to ETV. However, our results showed that there was no significant difference in serum BAFF levels between before and after treatment in T-Group and C-Group (Fig. 2D), which suggested that the combination therapy with BSF plus ETV cannot directly affect the expression of BAFF. In the following experiments, we wished to analyze the changes of B-cell subtypes.

Phenotypic characterization of circulating B-cell subsets in CHB patients

So far, the phenotypes of circulating B-cell subtypes in CHB patients were not well characterized. Therefore, we performed an in-depth analysis of the B cell phenotypes in CHB patients to identify the B cell subsets which may be changed after treatment.

This panel is based on surface markers CD45, CD3, CD20, IgD, IgM, CD27, CD38 and CD24 [27-29], and those markers are used to distinguish total B cells($CD45^+CD3^-CD20^+$) non-switched B cells ($CD45^+CD20^+CD3^-IgD^+IgM^+$), switched B cells($CD45^+CD20^+CD3^-IgD^-$

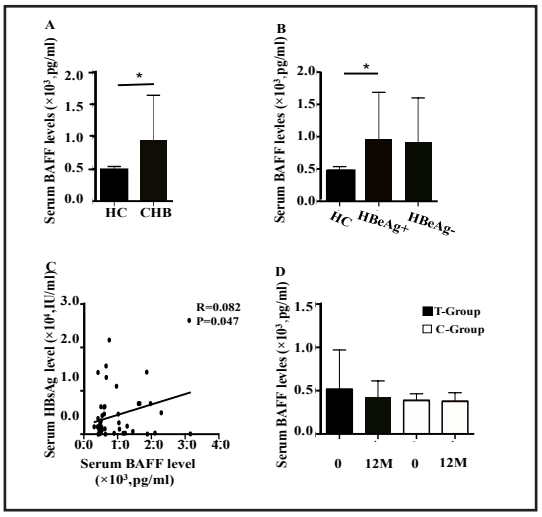

Fig. 2. Serum BAFF levels were positively correlated with HBsAg levels in treatment-naïve CHB patients. Serum BAFF and HBsAg levels were examined. A. Serum BAFF levels in treatment-naïve CHB patients were significantly higher than those in HC. B. Serum BAFF levels in HBeAg(+) and HBeAg(-)CHB patients were were significantly higher than those in HC. C. There was a positive relationship between serum BAFF levels and HBsAg levels in treatment-naïve CHB patients. D. After the treatment, serum BAFF levels in CHB patients with suboptimal response to ETV in T-Group and C-Group didn't differ much from those before the treatment. *P<0.05.

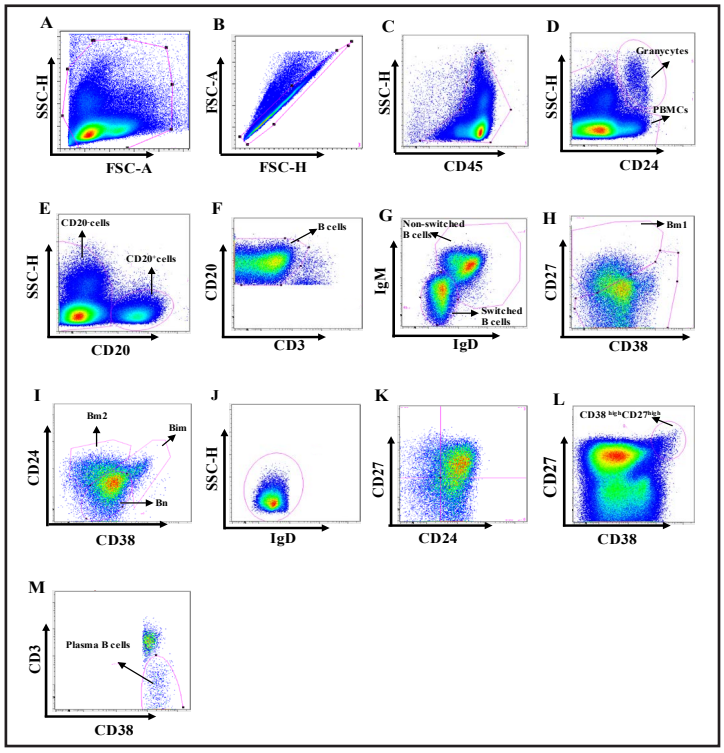

Fig. 3. Phenotypic characterization of circulating B-cell subsets in CHB patients. This panel is based on surface markers CD45, CD3, CD20, IgD, IgM, CD27, CD38 and CD24. A. All cells were gated using a FSC-H/SSC-A dot-plot. B. Single cells were gated using a FSC-H/FSC-A dot-plot. C. Total leukocytes were gated using CD45 /SSC-H dot-plot. D. Among leukocytes, PBMCs were gated using CD24/SSC-H dot-plot. E. PBMCs were categorized as $CD20^+$cells and $CD20^-$cells. F. Among $CD20^+$cells, B cells were defined as $CD20^+CD3^-$ cells. G. Among B cells, switched B cells were defined as IgD^-IgM^- cells, and non-switched B cells were defined as IgD^+IgM^+ cells. H. Among non-switched B cells, $CD27^+$ cells were Bm1. I. Among $CD27^-$ non-switched B cells, $CD38^{high}CD24^{high}$cells were Bim, and $CD24^+CD38^{\pm}$ cells were Bm2, and $CD38^{\pm}CD24^-$ cells were Bn. J and K. Among switched B cells, IgD^- cells were gated using IgD/SSH dot-plot, and then they were grouped using CD27/CD24. L and M. Among $CD20^-$ B cells, plasma cells were defined as $CD38^{high}CD27^{high}CD3^-$cells.

IgM^-), immature B cells (Bim, $CD45^+CD20^+CD3^-IgD^+IgM^+CD27^-CD24^{high}CD38^{high}$), naïve B cells (Bn, $CD45^+CD20^+CD3^-IgD^+IgM^+CD27^-CD24^-CD38^{\pm}$), non-classic B memory cell (Bm1, $CD45^+CD20^+CD3^-IgD^+IgM^+CD27^+$) , Bm2($CD45^+CD20^+CD3^-IgD^+IgM^+CD27^-CD24^+CD38^{\pm}$)and plasma cells ($CD45^+CD20^-CD3^-CD38^{high}CD27^{high}$), as shown in Fig. 3

Regulatory roles of BSF on peripheral B cellular subtypes in CHB patients with partial response to ETV

Humoral immunity is mediated by macromolecules found in extracellular fluids. Although the importance of humoral immunity mediated by B-cell to CHB has been established, the mechanisms remain to be elucidated. Our results showed that in T-Group，the frequencies of B-cell, Bm1, $CD24^+CD27^-$switched B cells and plasma cells after treatment differed from those before treatment. The frequency of B -cell was significantly decreased after treatment for 6 months (11.69±0.736% vs 9.52±0.749%, P=0.045) and 12 months (11.69±0.736%vs 8.607±0.915%, P=0.026). The frequency of Bm1 was significantly increased after treatment for 12 months (15.17±1.633 vs 21.93±3.070, P=0.049). The frequency of plasma cells was significantly increased after treatment for 12 months (0.215±0.036 vs 0.527±0.077, P=0.002). The frequency of $CD24^+CD27^-$switched B cells was significantly increased after treatment for 6 months (13.40±1.517%vs 18.92±1.679% P=0.020) and 12 months (13.40±1.517%vs 26.19±4.523%, P=0.002). However, there were no significant differences in C-Group before

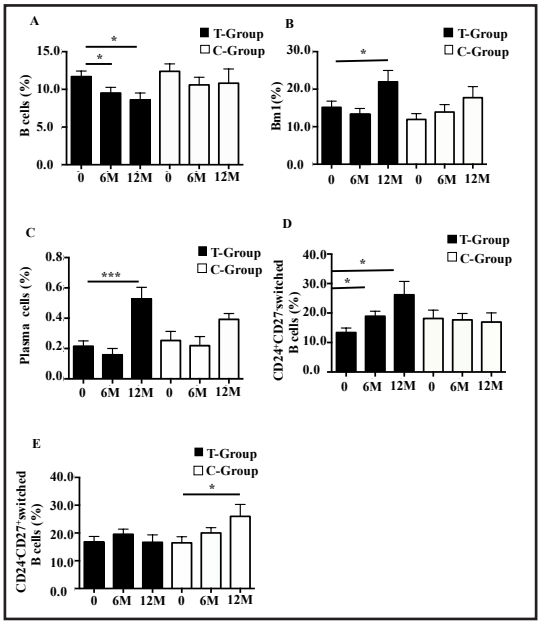

Fig. 4. Regulatory roles of BSF on frequencies of B cells, Bm1, plasma cells in CHB patients with partial response to ETV. Frequencies of peripheral B cellular subtypes in CHB patients with partial response to ETV were analyzed before and after treatment in T-Group and C-Group by flow cytometry. A. In T-Group, the frequency of B -cell was significantly decreased after treatment for 6 months and 12 months after treatment. B. In T-Group, the frequency of Bm1 was significantly increased after treatment for 12 months. C. In T-Group, the frequency of plasma cells was significantly increased after treatment for 12 months. D. In T-Group, the frequency of $CD24^+CD27^-$switched B cells was significantly increased after treatment for 6 months and 12 months. E, In C-Group, the frequency of $CD24^-CD27^+$swtiched B cells was increased after treatment for 12 months. *P<0.05; **P<0.01; ***P<0.001.

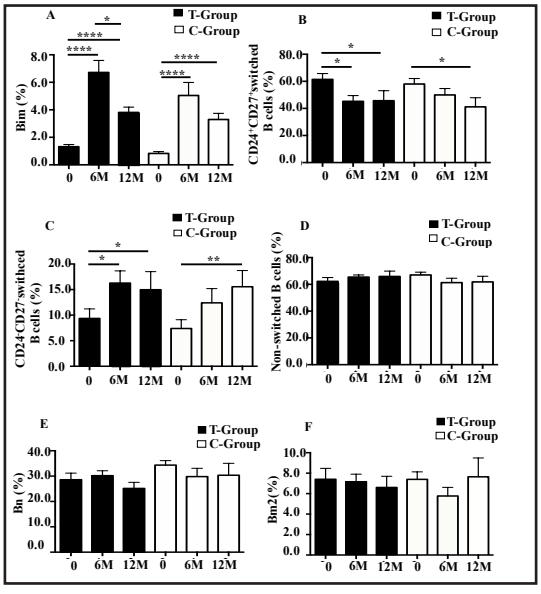

Fig. 5. Regulatory roles of BSF on frequencies of Bim, $CD24^+CD27^+$switched B cells, $CD24^-CD27^-$switched B cells, non-switched B cells, Bn and Bm2 in CHB patients with partial response to ETV. Frequencies of peripheral B cellular subtypes in CHB patients with partial response to ETV were analyzed before and after treatment in T-Group and C-Group by flow cytometry. A-C. In both groups, the frequencies of Bim and $CD24^-CD27^-$switched B cells were increased and the frequencies of $CD24^+CD27^+$switched B cells were decreased after treatment. D-F. After treatment, the frequencies of non-switched B cells, Bn and Bm2 did not much differ from those before treatment in both groups. *P<0.05; **P<0.01; ***P<0.001, ****P<0.0001.

and after treatment, as shown in Fig. 4A-D. In C-Group, the frequency of $CD24^-CD27^+$swtiched B cells was increased after treatment for 12 months (16.45±2.20 vs 25.97±4.31, P=0.04), but in T-group, there was no significant difference before and after treatment, as shown in Fig. 4E. In both groups, the frequencies of Bim and $CD24^-CD27^-$switched B cells were increased and the frequencies of $CD24^+CD27^+$switched B cells were decreased after treatment. (Fig. 5A-C). In addition, after treatment, the frequencies of non-switched B cells, Bn and Bm2 did not much differ from those before treatment in both groups (Fig. 5D-F).

Discussion

As we have known, ETV is a first-line antiviral therapy for treating CHB. However, some patients will have suboptimal response to ETV. Currently, there are limited data on how to improve the efficacy of anti-virus therapy for this group of patients. Among a large community-based real-world cohort of Asian CHB patients treated with antiviral therapy, rate of HBsAg loss was 4.58% [30]. In our study, 32 CHB patients with suboptimal response to ETV in T-Group were treated with ETV combined with BSF for 12 months, and after treatment, the rate of HBsAg reduction>0.5 $\log_{10}$ IU/ml, the rate of HBsAg reduction>1.0 $\log_{10}$ IU/ml and the rate of HBeAg clearance in T-Group were all higher than those in C-group, but no significant intergroup differences were observed because of the limited number of patients. The results suggested that the combination therapy with ETV plus BSF promoted the reduction of HBsAg and the clearance of HBeAg in patients with partial response to ETV. In addition, BSF improved Damp heat stasis syndrome and liver and kidney Yin deficiency syndrome in patients with suboptimal response to ETV. To summarize, our results provided one key tip that ETV combined with BSF was a potential effective ACM therapy for CHB patients with suboptimal response to ETV.

The clinical outcome of HBV infection depends on the interaction between virus duplication and host immune response [31] . Our previous studies showed that BSF played immunoregulatory roles in CHB paitients by regulating cellular responses including Treg cells and NKT cells [16, 32]. Humoral responses are characterized by the production of antibodies by B lymphocytes and their progeny, plasma cells. In this study, we focused on the regulatory mechanism of BSF on humoral responses. High serum levels of BAFF in treatment-naïve patients suggested that B-cell in CHB patients may be overactive compared with healthy controls. One important finding was that there was a significant positive correlation between serum BAFF and HBsAg levels, and so serum BAFF levels may be the potential marker for the forecast of clincal effects. Based on the above results, the immunoregulary roles of BSF on B celluar subtypes were explored. One recent study showed that the frequency of plasma cells correlated with anti-HBs titers in CHB patients with HBsAg seroconversion [33], which suggested that plasma cells contributed to HBsAg seroconversion. It was interesting that the frequency of B-cell was decreased and the frequency of plasma cells was increased after treatment only in T-Group, which maybe the important mechanism of HBsAg decline. Human Bm1 ($IgD^+IgM^+CD27^+$B cell) is a peculiar subset, the origin and function of Bm1 is debated. Bm1 is in charge of T-independent responses as compared with the classical B-cell involved in T-dependent responses, and most of the natural mutated IgM antibodies present in human serum are probably produced by Bm1 [34]. Our results showed that the frequency of Bm1 was significantly up-regulated after treatment only in T-Group, which showed that BSF contributed to the production of natural mutated IgM antibodies.

It has been reported that the components of BSF have a broad-spectrum immunoregulatory effect. Epimedium polysaccharide can activate peripheral T lymphocyte proliferation, and promote T cells to secrete cytokines such as IL-2, IL-4, IFN- γ, IL-12 and TNF-α [35]. Epimedium polysaccharide-propolis flavone liposome significantly promotes B lymphocyte proliferation [36]. In addition, polysaccharides from the roots of Astragalus membranaceus activate B cells via membrane Ig in a TLR4-in- dependent manner [37]. Adoptive transfer experiments have demonstrated that the hot water extract of Uncaria

tomentosa significantly prolongs lymphocyte survival in peripheral lymphoid organs [38]. In future studies, we will explore the action and material basis of BSF on the immuregulatory roles in treating CHB.

In summary, our study suggested the combination therapy with ETV plus BSF was a potential effective ACM for CHB patients with suboptimal response to ETV. Our findings demonstrated that the up-regulated expression of BAFF in treatment-naïve CHB patients was positively correlated with the persistent expression of HBsAg. The immunoregulatory mechanism of BSF was involved in decreasing the frequency of B-cell and increasing the frequencies of plasma cells and Bm1. These effects may eventually result in a significant reduction of serum HBsAg in CHB patients.

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Disclosure Statement

The authors declare to have no conflict of interests.

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