Received: 19 August 2019 | Revised: 29 February 2020 | Accepted: 3 March 2020

DOI: 10.1111/liv.14438

Liver INTERNATIONAL WILEY

ORIGINAL ARTICLE

HBV promotes the recruitment of IL-17 secreting T cells via chemokines CCL22 and CCL17

Ka Zhang[1] | Yanqiong Liu[2] | Xiaoan Yang[1] | Haixia Sun[1] | Xin Shu[1] | Yeqiong Zhang[1] | Hong Cao[1] | Mengdi Wu[3] | Nan Liu[3] | Yong Zou[4] | Qihuan Xu[1] | Gang Li[1]

[1]Department of Infectious Diseases, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, P. R. China

[2]Department of Infectious Diseases, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, P. R. China

[3]General Practice Center, Nanhai Hospital, Southern Medical University, Foshan, P. R. China

[4]Department of Blood Transfusion, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, P. R. China

Correspondence
Qihuan Xu, Department of Infectious Diseases, The third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, P. R. China.
Email: xqh032014@163.com

Yong Zou, Department of Blood Transfusion, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, P. R. China.
Email: zouyong@mail.sysu.edu.cn

Funding information
National Natural Science Foundation of China, Grant/Award Number: 81500462 and 81872584; Natural Science Foundation of Guangdong, Grant/Award Number: 2016A030313357; Science and Technology Planning Project of Guangdong, Grant/Award Number: 2016A020215215

Handling Editor: Gabriele Missale

Abstract

Background and Aims: Little is known about the mechanisms of IL-17 secreting T cells accumulation in HBV-transfected livers. Here, we investigated the role of the chemokines CCL17, CCL20 and CCL22 in this process.

Methods: Peripheral blood and liver tissues were obtained from 30 chronic hepatitis B (CHB) patients and 15 healthy volunteers and were evaluated by flow cytometric analysis and immunohistochemistry. Chemokine production by monocyte-derived dendritic cells (MoDCs) cocultured with HBV-transfected or untransfected Huh7 cells was measured by quantitative real-time PCR and enzyme-linked immunosorbent assay. The chemotactic activity of the culture supernatants was also tested.

Results: The proportions of IL-17 secreting CD4 (Th17) and CD8 (Tc17) T cells were both increased in liver and peripheral blood mononuclear cells of CHB patients compared to those in HVs. CHB patients showed higher intrahepatic levels of CCL17 mRNA, CCL22 mRNA, CCR6 mRNA and CCR4 mRNA than HVs. The expression of CCR6 and CCR4 on the surface of Th17 and Tc17 cells in CHB patients was also significantly higher than that in HVs. Significant correlations existed between the CCR4/CCR6 levels and both the alanine transaminase levels and HBV DNA loads. Contact between MoDCs and pBlue-HBV-transfected Huh7 cells induced the expression of CCL17 and CCL22 dependent on the dose of HBV DNA. However, CCL20 expression was lower in CHB patients than in HVs. Transwell experiments showed that upregulation of CCL17 and CCL22 enhanced the migration of IL-17 secreting T cells.

Conclusions: Contact of HBV-transfected cells with MoDCs induces CCL17 and CCL22 chemokine production, which may favour the recruitment of Th17 and Tc17 cells to liver tissue in CHB. Our results reveal the mechanism of IL-17 secreting T cells recruitment to liver tissue and thus provide new immunotherapy targets for CHB patients.

Abbreviations: ALT, alanine aminotransferase; CHB, chronic hepatitis B; HBV, hepatitis B virus; IFN, interferon; MoDCs, monocyte-derived dendritic cells; PBMC, peripheral blood mononuclear cells; qPCR, quantitative real-time-PCR; RT-PCR, reverse transcription-polymerase chain reaction; Tc17, interleukin-17 secreting CD8 T helper cells; Th17, interleukin-17 secreting CD4 T helper cells.

Ka Zhang and Yanqiong Liu shared first authorship.

KEYWORDS
chemokine, chronic hepatitis B, hepatitis B virus, interleukin-17, T helper cell

1 | INTRODUCTION

Chronic hepatitis B virus (CHB) infection is still considered a serious health problem. It is estimated that more than 275 million people are chronically transfected with HBV worldwide, and HBV is one of the major causes of liver disease.[1] CHB infection leads to severe sequelae, including CHB, liver failure, cirrhosis and hepatocellular carcinoma.[2,3] Nucleoside analogs (NAs) and interferon (IFN) are commonly used and effective drugs for the treatment of patients with chronic HBV (CHB) infection, and they show potent efficacy in inhibiting HBV replication, improving the histology and prognosis of CHB patients.

To date, the exact mechanism of the development of HBV-related hepatitis remains unclear. Adaptive and innate immunity play an important role in the pathogenesis of acute and chronic HBV infection.[4] Th17 cells initiate immune-mediated pathogenesis and play a key role in the process of HBV-related diseases.[5] A previous study reported that the proportion of Th17 cells in peripheral blood mononuclear cells (PBMCs) of patients transfected with HBV was significantly higher than that in healthy controls, which suggests that Th17 cells influence the regulation of inflammation after HBV infection.[6] Our previous study also confirmed that the proportion of Th17 cells was significantly increased in patients with HBV infection and acute-on-chronic liver failure (HBV-ACLF).[7] We also found that the increased levels of Th17 cells and regulatory T cells were upregulated by hepatic stellate cells (HSCs) and that HSCs, Th17 cells and regulatory T cells played a key role in the persistence of fibrosis and the development of hepatocellular carcinoma following cirrhosis.[8]

However, the role played by HBV in the exact mechanisms of Th17 cells and CHB remains unknown. The proportion of Th17 cells and the level of IL-17 in patients treated with entecavir were lower than those before treatment with entecavir.[9] A longitudinal observational study found that the frequency of Th17 cells in the peripheral blood of CHB patients was significantly increased compared with that in healthy controls and that the Th17 cell frequency showed a reverse 'V'-type change during telbivudine treatment.[10] Parallel to the reduction in HBV DNA and the normalization of serum alanine transaminase (ALT), a significant reduction in Th17 cells was observed during antiviral therapy.[1,11] All these studies indicated that HBV may be associated with the expression or aggregation of Th17 cells in liver tissue. However, how Th17 cells infiltrate the liver tissue remains unclear.

It was found that the chemokine CCL20 could facilitate CCR6-expressing Th17 cell infiltration into inflamed joints in the pathogenesis of rheumatoid arthritis.[12] It was reported that CCL20 aggravates neuroinflammation following spinal cord injury via regulating Th17 cell recruitment and IL-17A levels.[13] It was also demonstrated that Th17 cells in peripheral blood were induced by CCL22 and CCL20 in malignant pleural effusion.[14] It was also shown that hepatitis C virus (HCV) induces the expression of CCL17 and CCL22, which attract regulatory T cells to the infection site.[15] Herein, we hypothesized that Huh7 cells transfected with HBV strongly stimulate the expression of the chemokines CCL17/CCL20/CCL22, which act as chemotactic factors for the recruitment of Th17 cells to liver tissue.

Based on the above hypothesis, we determined the proportion of IL-17 secreting CD4 (Th17) and CD8 (Tc17) T cells and the mRNA levels of CCL17/CCL20/CCL22 and their associated chemokine receptors in the liver and PBMCs of CHB patients. We also investigated the CCL17/CCL20/CCL22 expression characteristics in co-cultured monocyte-derived dendritic cells/Huh7 cells to reveal the function of these chemokines in facilitating the movement of Th17 and Tc17 cells. Finally, we investigated whether HBV promoted the recruitment of Th17 and Tc17 cells via the chemokines CCL17/CCL20/CCL22. Our findings will be helpful for better understanding the role of HBV in promoting the recruitment of IL-17 secreting T cells to liver tissue and provide clues to the mechanisms of CHB and new immunotherapy targets for treating chronic HBV infection.

2 | MATERIALS AND METHODS

2.1 | Patients and healthy controls

The study protocol was approved by the ethics committee of the Third Affiliated Hospital of Sun Yat-Sen University, and all patients were required to provide written informed consent. For immunochemistry staining and quantitative real-time PCR, needle biopsy liver tissues were obtained from 12 patients with low- or moderate-grade CHB at the department of infectious disease, the Third Hospital of Sun Yat-Sen University. Five normal liver specimens were obtained from patients who had undergone liver resections because of haemangioma. The diagnostic criteria for CHB were HBsAg positivity and a detectable HBV-DNA load for at least 6 months. No patient included in this study had been treated with NAs, IFN or immunomodulatory drugs for at least 6 months. The exclusion criteria were as follows: infection with other hepatitis viruses or human immunodeficiency virus, alcoholic hepatitis, drug-induced liver disease, steatohepatitis, autoimmune diseases, Wilson's disease or carcinoma, etc. There were no differences between the patients with CHB and the HV with respect to age and sex ratio.

2.2 | Blood sample preparation

Peripheral blood samples (10 mL/tube) containing heparin were obtained from all patients and the healthy volunteers. Plasma was isolated and stored at −80°C until use in the enzyme-linked immunosorbent assay (ELISA). Peripheral blood mononuclear cells were obtained using lymphocyte separation medium (BDscience Co. Ltd)

based on the manufacturer's protocol and were used for flow cytometric analysis and real-time quantitative polymerase chain reaction (RT-PCR). The cell viability was >95% by trypan blue dye exclusion test. Specific methods and steps were performed as previously described.[7]

2.3 | Flow cytometric analysis

FACS acquisition was performed on FACSCanto II (BD company, USA) and results were analysed with CELLQUEST software (BD Biosciences). PBMCs were stained with the following mAbs: FITC anti-CD3, PE anti-CD4 cells, APC-Cy7 anti-CD8, APC anti-CD196 (CCR6), PerCP-cy5.5 anti-CD194 (CCR4), V450 anti-human IL17A, APC mouse IgG1, κ isotype control, PerCP-Cy™5.5 mouse IgG1 κ isotype control and V450 Mouse IgG1, κ isotype control which were obtained from BD Biosciences. For intracellular cytokine analysis, PBMC were stimulated with PMA/ionomycin in the presence of GolgiPlug (BD Biosciences) and intracellular cytokine staining was performed according to the manufacturers' instruction. Lymphocytes were firstly gated according to forward scatter and side scatter; then the Th17 cells ($CD3^+$ $CD4^+$ $IL17^+$) and the Tc17 cells ($CD3^+$ $CD8^+$ $IL17^+$) were gated and the percentages of Th17/Tc17 cells expressing CCR4 and CCR6 were determined individually.

2.4 | Purification of IL-17 secreting T cells

IL-17 secreting T cells were positively sorted by IL-17 secretion assay-cell enrichment and detection kit (PE) (Miltenyi Biotec) according to the manufacturer's protocol. The sorted cells were >90% pure. The viability was >95% by the trypan blue exclusion test.

2.5 | Stimulated dendritic cell preparation

Monocyte-derived dendritic cells were acquired by cultivating peripheral blood monocytes in RPMI 1640 (10% FCS) medium supplemented with GM-CSF and IL-4 (50 ng/mL) for 5 days. On the sixth day, LPS (5 μg/mL) was added for stimulation for 1 day. Specific methods and steps were performed as previously described.[16]

2.6 | Cell lines and recombinant plasmids

Human hepatocellular carcinoma cells (Huh7 cells) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 g/mL streptomycin and 100 g/mL penicillin at 37°C in a 5% CO_2 incubator. Expression plasmids and paired empty plasmids, including the full-length HBV DNA (pBlue-HBV), were kindly provided by Dr Guanxin Shen (Department of Immunology, Basic Medicine Institute, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, People's Republic of China).

2.7 | Transient transfection

Huh7 cells were plated at a density of 4×10^5 cells per 24-well plate or 6-well plate. After incubation for 24 hours, cells were transfected with pBlue-HBV. Lipofectamine 2000 (Invitrogen) was used to transfect plasmids. Specific methods and steps were similar to a procedure described previously.[17]

2.8 | RNA extraction and quantitative real-time PCR

Total RNA was extracted from fresh frozen liver specimens (obtained via needle liver biopsy or liver resection) and Huh7 cells using TRIzol (Invitrogen) according to the manufacturer's protocol. The quantitative real-time PCR reactions were performed using a SYBR green PCR kit (TOYOBO) in an ABI 7500 Sequence Detection System (Applied Biosystems, Sunnyvale, CA). For RT-PCR, primers for CCL17, CCL22, CCL20, CCR4 and CCR6 and amplification conditions were used as previous reported.[18-21] The standard curve is used to estimate the PCR amplification efficiency and efficiency within the range 0.9-1.1 was considered valid. Specificity of the PCR reaction was verified by dissociation-curve analysis and agarose gel electrophoresis.

2.9 | Quantification of CCL17, CCL20 and CCL22 protein levels

According to the manufacturer's instructions, the levels of the chemokines CCL17, CCL20 and CLL22 in coculture supernatants and serum were quantified by ELISA according to the manufacturer's protocol (Quantikine, R&D Systems, USA).

2.10 | Immunohistochemistry staining

Liver tissues were embedded in paraffin using a standard protocol. The sections (5 μm) were treated with 0.03% H_2O_2/$NaNO_3$ to block endogenous peroxidase, treated with 10% normal goat serum (30 minutes) to block non-specific staining, incubated with an antibody against IL-17 (Catalog# ab79056, Abcam, USA, 1:50) or CCL17 (Catalog# ab195044, Abcam, USA, 1:50) or CCL20 (Catalog# ab9829, Abcam, USA, 1:100) or CCL22 (Catalog# ab9847, Abcam, USA, 1:50) or CCR4 (Catalog# ab216560, Abcam, USA, 1:100) or CCR6 (Catalog# ab227036, Abcam, USA, 1:100) with rabbit serum as a negative control at 4°C overnight in a humid chamber, washed in PBS, incubated with HRP-conjugated secondary antibody (Catalog# ab205718, Abcam, USA, 1:50) for 30 minutes, and visualized after staining with 3,3,2-diaminobenzidine (DAB) solution, followed by counterstaining with a haematoxylin solution. The sections were analysed under a light microscope. Positive cells were detected using microscopy (Leica, Germany).

2.11 | Migration assay

Migration was assayed with a Boyden chamber. Seventy microliters of prediluted Matrigel (5 μg/mL) was added to the polycarbonate membrane (8 μm pore) in the upper chamber and the Matrigel was allowed to solidify (37°C, 30 min). Then, 100 μL of fresh IL-17 secreting T cells (Th17 and Tc17) suspension (1×10^5 cells/mL) was added to the upper chamber. A total of 600 μL of cell supernatant was added to the lower chamber and cultured for 24 h after plasmid transfection. The chamber was incubated for 3 h at 37°C in an incubator with 5% CO_2. Cells on the upper surface of the membranes were wiped off with cotton swabs; the membranes were washed with 1 mL PBS, fixed with 4% polyformaldehyde, stained with DAPI, and cells were counted under inverted phase contrast biomicroscopy.

2.12 | Statistical analysis

The Shapiro Wilk W test was used as the normality test. Descriptive data for continuous variables are reported as the means ± standard deviations. Student's *t*-test and two-way analysis of variance (ANOVA) were used for parametric data; the Kruskal-Wallis test and Mann-Whitney *U* test were used for non-parametric data. Correlation coefficients were evaluated by Pearson or Spearman correlation. All *P* values were two-tailed values, and values <.05 were considered significant. The SPSS 21.0 Windows software package was employed for statistical analysis.

3 | RESULTS

3.1 | Characteristics of patients

The characteristics of the patients with CHB and the healthy volunteers are presented in Table 1.

TABLE 1 Patient demographics and baseline characteristics

Variable	CHB	HV
Sex (M/F)	18/12	10/5
Age (years)	31.5 ± 8.6	30.2 ± 7.8
ALT (U/L)	324.3 ± 157.6	17.3 ± 11.2
TBil (μmol/L)	28.3 ± 18.9	15.8 ± 10.1
HBV DNA (log_{10} IU/mL)	5.4 ± 3.8	ND
Liver inflammation Grade (0-1, 2, 3, 4)	1/12/14/3	12/1/2/0
Liver fibrosis stage (0-1,2,3,4)	3/16/10/1	13/1/1/0

Abbreviations: ALT, alanine aminotransferase; CHB, chronic hepatitis B; HBV, hepatitis B virus; HV, healthy volunteers; ND, not determined; TBil, total bilirubin.

3.2 | Increased percentage of IL-17 secreting T cells in liver tissues and PBMCs of CHB patients

To ascertain the role of Th17 cells in CHB, we compared the percentages of hepatic and circulating IL-17 secreting T cells between CHB patients and HV. We found that the number of hepatic IL-17 secreting T cells in liver tissues was higher in CHB patients than in HV. As shown by arrow in Figure 1B, more IL-17 secreting cells were found accumulated in the portal areas of livers in CHB patients. Similarly, we compared the percentages of IL-17 secreting CD4 (Th17) and CD8 (Tc17) T cells between patients with CHB and HV by flow cytometry. The gating strategy and diversity are shown in Figure 2A. The proportion of IL-17 secreting T cells was higher in the circulation of CHB patients than in healthy volunteers. The percentage of Th17 cells was higher in CHB patients than in healthy volunteers (3.21 ± 2.26% vs 1.87 ± 0.97%, *P* = .002) (Figure 2B,D). The percentage of Tc17 cells was also higher in CHB patients than in healthy volunteers (0.92 ± 1.02% vs 0.23 ± 0.5%, *P* = .002) (Figure 2C,E).

3.3 | Upregulation of CCR6 and CCR4 on Th17 and Tc17 cells among PBMCs in CHB patients

CCR6 and CCR4 are the receptors for CCL20 and CCL17/CCL22 respectively. We found that the expression of CCR6 and CCR4 on the surface of Th17 cells was significantly higher in CHB patients than in healthy volunteers (CCR6: 12.06 ± 9.65% vs 5.23 ± 4.38%, *P* = .003; CCR4: 24.76 ± 14.78% vs 7.38 ± 6.00%, *P* = .006) (Figure 2B,D). The expression of the abovementioned two receptors on the surface of Tc17 cells in CHB patients was also significantly higher than that in healthy volunteers (CCR6: 17.34 ± 13.52% vs 5.28 ± 4.33%, *P* = .003; CCR4: 26.34 ± 24.21% vs 6.04 ± 13.42%, *P* = .006) (Figure 2C,E). We analysed the correlation between CCR6/CCR4 levels and serum ALT levels, serum total bilirubin levels and serum HBV DNA loads in CHB patients. Significant correlations existed between CCR6 and both the serum ALT level (*r* = .58, *P* = .018) and serum HBV DNA load (*r* = .56, *P* = .020). Furthermore, CCR4 levels were positively correlated with serum ALT levels (*r* = .49, *P* = .030) and serum HBV DNA loads (*r* = .53, *P* = .025). However, no significant correlation was found between total serum bilirubin levels and either CCR6 levels (*r* = .079, *P* = .370) or CCR4 levels (*r* = .076, *P* = .362) (Table 2).

3.4 | Upregulated mRNA and protein levels of CCL17, CCL22, CCR4 and CCR6 in the liver of CHB patients

Previous studies reported that HCV induces the expression of the CCL17 and CCL22 chemokines, which attract regulatory T cells to the site of infection.[15] We found that the mRNA and protein levels of CCL17, CCL22, CCR4 and CCR6 were significantly

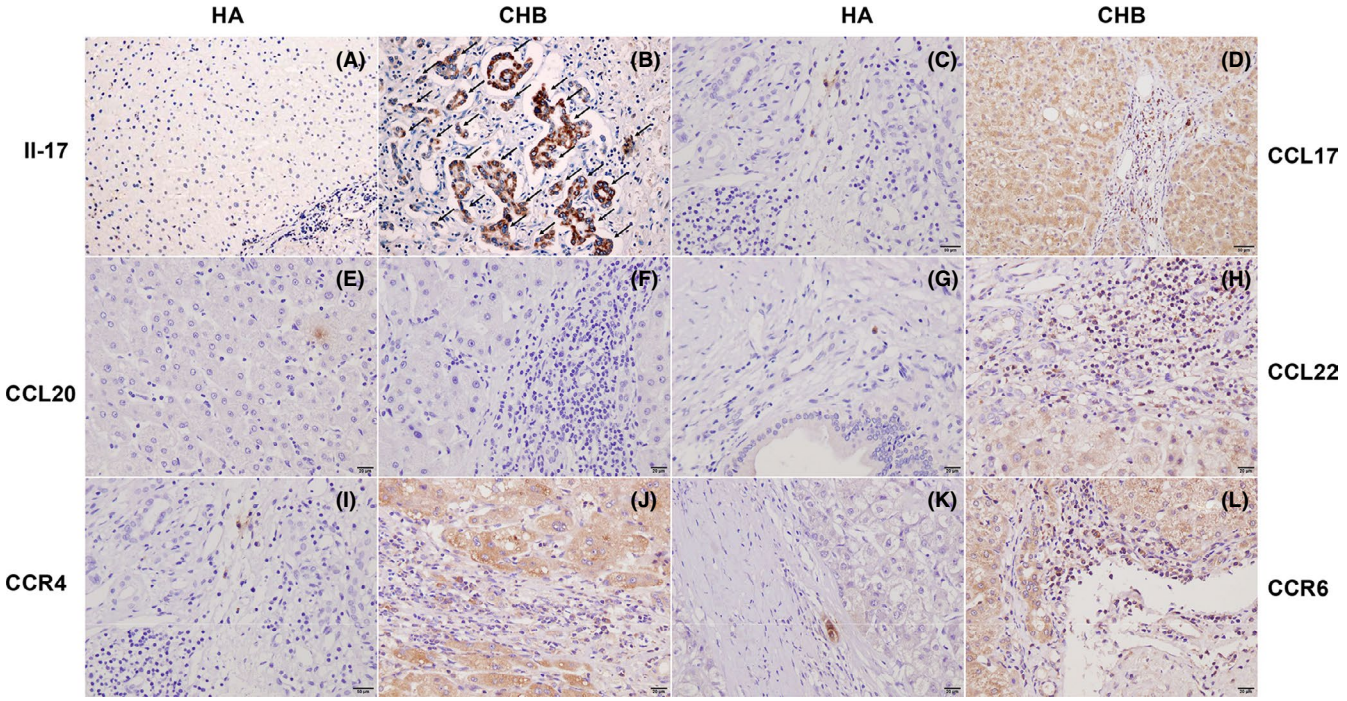


FIGURE 1 Histological expression analysis of hepatic IL-17, CCL17, CCL20, CCL22, CCR4 and CCR6 in patients with CHB. The histological expression for IL-17 (A and B), CCL17 (C and D), CCL20 (E and F), CCL22 (G and H), CCR4 (I and J) and CCR6 (K and L) in liver tissues of patients with CHB and normal liver tissues was measured by immunohistochemistry staining (original magnification 200x). As shown by arrow in Figure 1B, more IL-17 secreting cells were found accumulated in the portal areas of livers in CHB patients. CHB, chronic hepatitis B; HV, healthy volunteers

higher in liver tissue of CHB patients than in normal livers (Figures 1 and 3). However, CCL20 mRNA and protein expression levels in liver tissue in patients with CHB and HV was not significantly different (Figures 1 and 3). These results suggest that chronic HBV infection is associated with increased expression of chemokines, which are beneficial to the recruitment of Th17 cells to liver tissues.

3.5 | HBV induces CCL17/CCL22 mRNA and protein expression in a dose-dependent manner

To investigate the effect of HBV on CCL17 mRNA and CCL22 mRNA and protein expression, the plasmid pBlue-HBV was transfected into Huh7 cells. The expression of CCL17 mRNA and CCL22 mRNA was determined by real-time PCR after 24 hours. The protein levels of CCL17 and CCL22 in the culture supernatants and cell lysates were measured by ELISA after 48 hours. As the results show, the mRNA levels of CCL17 (Figure 4A) and CCL22 (Figure 5A) were significantly increased in cells transfected with the pBlue-HBV plasmid compared with those in cells transfected with empty plasmid. The protein levels of CCL17 (Figure 4B) and CCL22 (Figure 5B) were also significantly higher in the pBlue-HBV-transfected group than in the control groups. Subsequently, we investigated the dose-dependent effect of HBV on CCL17 mRNA and CCL22 mRNA and protein expression in Huh7 cells. Huh7 cells were transfected with increasing concentrations of the pBlue-HBV plasmid. The expression levels of CCL17/CCL22 mRNA and protein were measured by real-time PCR and ELISA respectively. The levels of CCL17/CCL22 mRNA and protein increased as the concentration of the pBlue-HBV plasmid increased (Figures 4C,D and 5C,D). These results indicated that the effect of HBV on the expression of CCL22 and CCL17 was concentration-dependent. This result suggested that HBV can induce CCL17 and CCL22 expression in a dose-dependent manner.

3.6 | CCL20 mRNA and protein expression inhibited by HBV in a dose-dependent manner

Similarly, the plasmid pBlue-HBV was also transfected into Huh7 cells to investigate the effect of HBV on CCL20 mRNA and protein expression. CCL20 mRNA and protein expression in culture supernatants was measured by real-time PCR and ELISA respectively. CCL20 mRNA (Figure 6A) expression was significantly decreased in cells transfected with the pBlue-HBV plasmid compared with that in cells transfected with empty plasmid, and a similar result was obtained for the protein expression of CCL20 (Figure 6B). Additionally, Huh7 cells were transfected with increasing concentrations of the pBlue-HBV plasmid to investigate the dose-dependent effect of HBV on CCL20 mRNA and CCL20 protein expression. The data revealed that the levels of CCL20 mRNA and protein decreased as the concentration of the pBlue-HBV plasmid increased (Figure 6C,D).

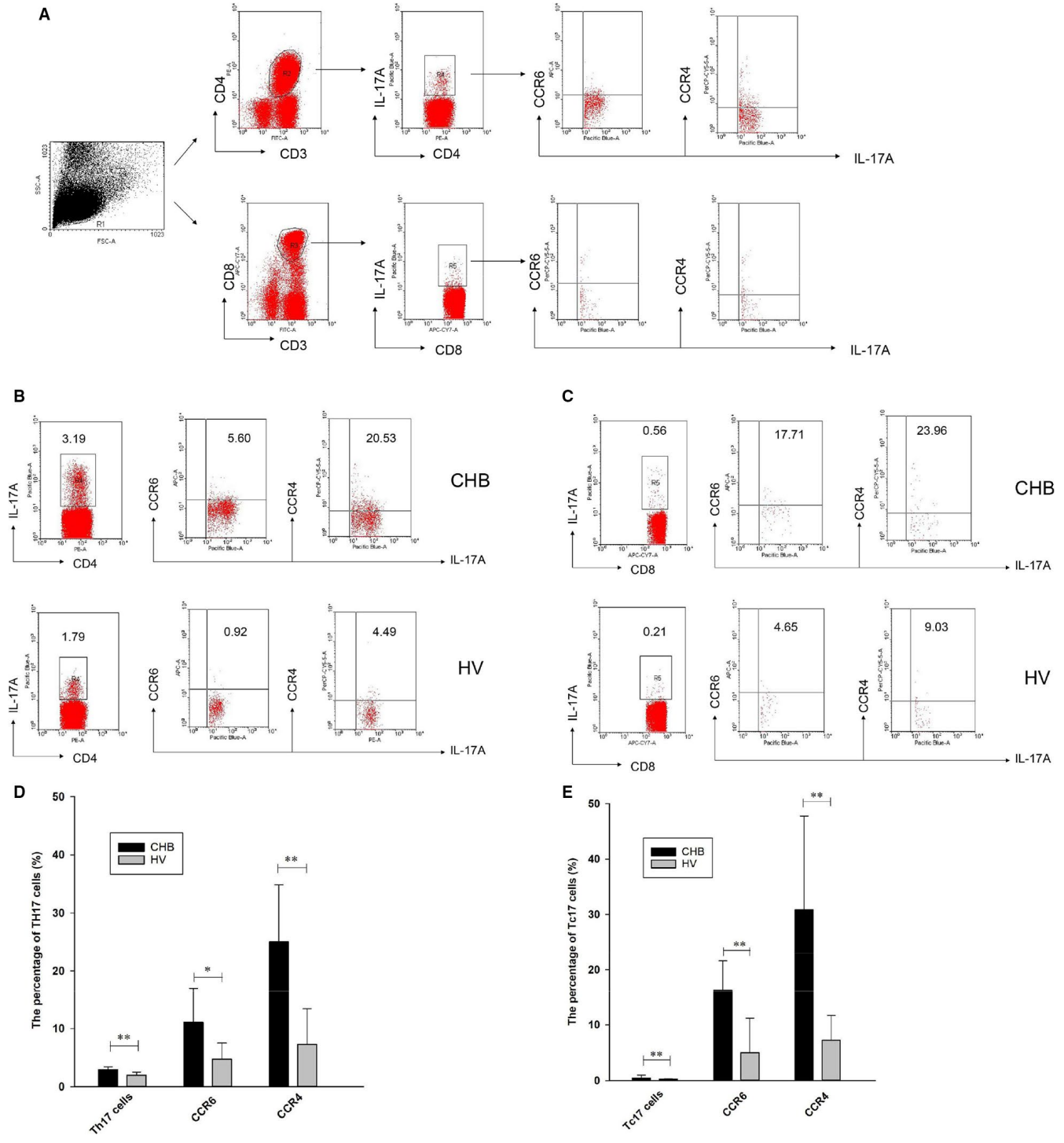


FIGURE 2 The expression of CCR4 and CCR6 on Th17 and Tc17 among PBMCs in patients with CHB (n = 30) and fifteen healthy volunteers (n = 15). (A) Gating strategy. (B and C) Representative FACS analysis of the expression of CCR4 and CCR6 on peripheral Th17 and Tc17. (D) Analysis of the percentages of CCR6 and CCR4 expression on the surface of Th17 cells. (E) Analysis of the percentages of CCR4 and CCR6 expression on the surface of Tc17 cells. CHB, chronic hepatitis B; HV, healthy volunteers; Th17, interleukin-17 secreting CD4 T helper cells; Tc17, interleukin-17 secreting CD8 T helper cells. Value represents the mean ± SD $^{*}P < .05$. $^{**}P < .01$

3.7 | Chemotactic ability of IL-17 secreting T cells reduced by blockade of CCL17 and CCL22 expression

To address whether the chemotactic ability of IL-17 secreting T cells (Th17 and Tc17) was associated with high expression of CCL17 and CCL22, the coculture supernatant including CCL17 and CCL22 was used to induce the migration of IL-17 secreting T cells as well as cells treated with isotype control antibodies, anti-CCL17, anti-CCL22 or a combination of anti-CCL17 and anti-CCL22. The chemotactic index (CI) was calculated as follows: number of IL-17 secreting T cells in the

TABLE 2 CCR4 and CCR6 were significantly correlated with the ALT level and HBV load in the CHB group

Variable	ALT (U/L) (324.3 ± 157.6)	TBil (μmol/L) (28.3 ± 18.9)	HBV DNA (log_{10} IU/mL) (5.4 ± 3.8)
CCR4	26.34 ± 24.21		
r	.49	.076	.53
P	.030	.362	.025
CCR6	12.06 ± 9.65		
r	.58	.079	.56
P	.018	.370	.020

Abbreviations: ALT, alanine aminotransferase; CHB, chronic hepatitis B; HBV, hepatitis B virus; HV, healthy volunteers; TBil, total bilirubin.

experimental group/ number of IL-17 secreting T cells in the control group. The chemotaxis assay was repeated using five different batches. A high CI indicates that a chemical acts as a strong attractant; a lower CI indicates that the chemical is a less effective attractant or does not act as an attractant. As shown in Figure 7, the CI of the experimental group was 4.47 (Figure 7B) and 4.29 (Figure 7C) before and after the addition of the isotype control antibody, respectively, relative to that of the control group (Figure 7A). The CI of the experimental group was decreased to 3.36 and 2.22 after the addition of anti-CCL17 (Figure 7D, $P < .05$) and anti-CCL22 (Figure 6E, $P < .05$), respectively, relative to that of the isotype control group. After simultaneous addition of anti-CCL17 and anti-CCL22 (Figure 7F), the CI was 1.69, and the chemotactic ability of IL-17 secreting T cells was attenuated (Figure 7G, $P < .05$).

4 | DISCUSSION

Currently, the pathogenesis of CHB is not well elucidated. Accumulating evidence suggests that Th17 cells are critically involved in the pathogenesis of liver diseases, including CHB,[22] HBV-related cirrhosis[5] and ACLF.[23] Our previous studies also confirmed that the proportion of Th17 cells is significantly increased in CHB, HBV-ACLF[7] and HBV-LF.[8] However, the mechanisms by which HBV might induce the proliferation of Th17 cells or their recruitment to sites of infection are not known. In the present work, we found that CHB patients have higher Th17 expression in liver tissues and PBMCs than healthy donors. These results are consistent with all previous data indicating the high proportion of Th17 cells among PBMCs of patients with HBV infection.[24] Furthermore, Th17 might also be linked to continuous HBV infection.[25] The Treg/Th17 balance, the Th17/IL-17 axis, IL22 and IL23, etc., have been considered the key prognostic and immunopathological elements in patients with HBV. However, little is known about the molecular basis for the recruitment and subsequent localization of these factors in the liver. Boj found that CXCR3 promotes the recruitment of Th17 cells from the blood into the liver in humans and mice; liver damage and bile localization were dependent on CCL20 secreted by bile duct cells.[26] In an HBV transgenic mouse

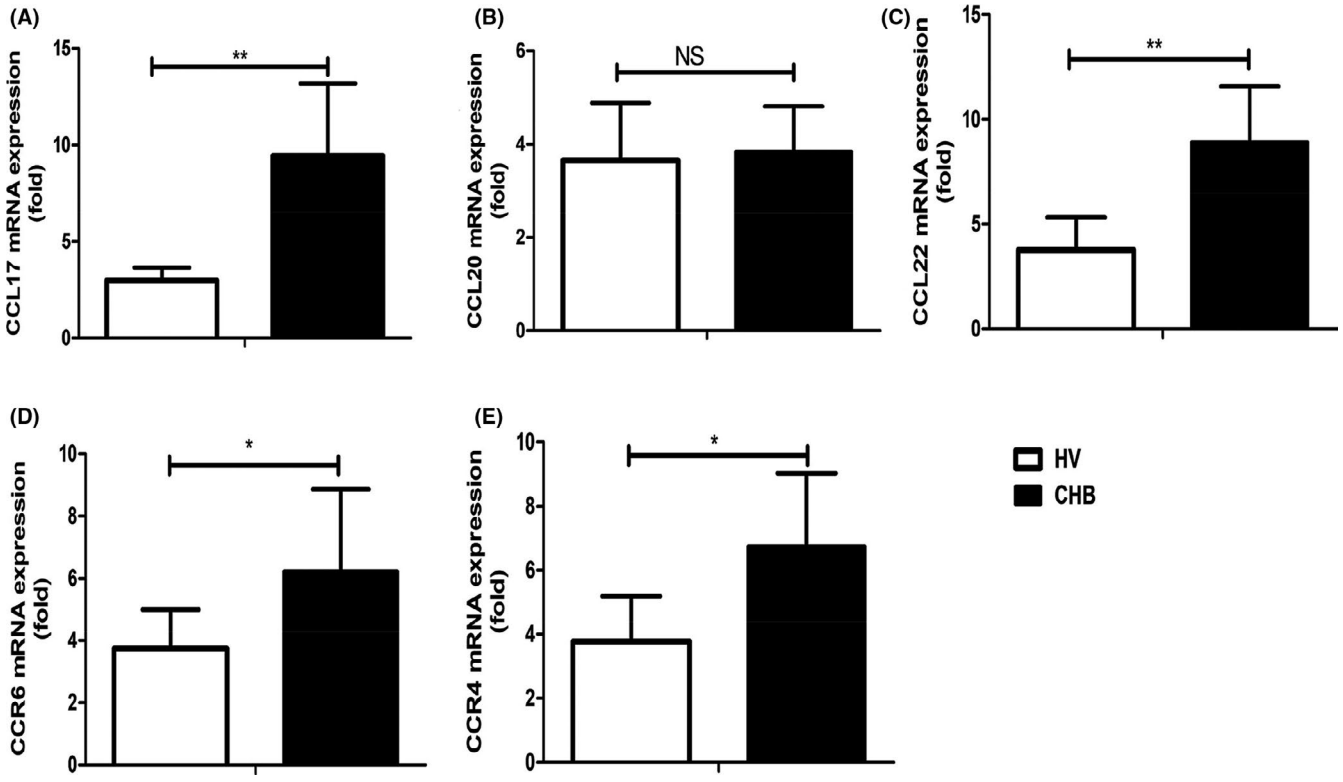


FIGURE 3 The mRNA expression of hepatic CCL17, CCL20, CCL22, CCR4 and CCR6. The mRNA expression levels for CCL17, CCL20, CCL22, CCR4 and CCR6 in liver tissues of patients with CHB was measured by qPCR. The data are representative of 12 liver specimens and expressed as mean ± SD $^{*}P < .05$. $^{**}P < .01$. CHB, chronic hepatitis B; HV, healthy volunteers

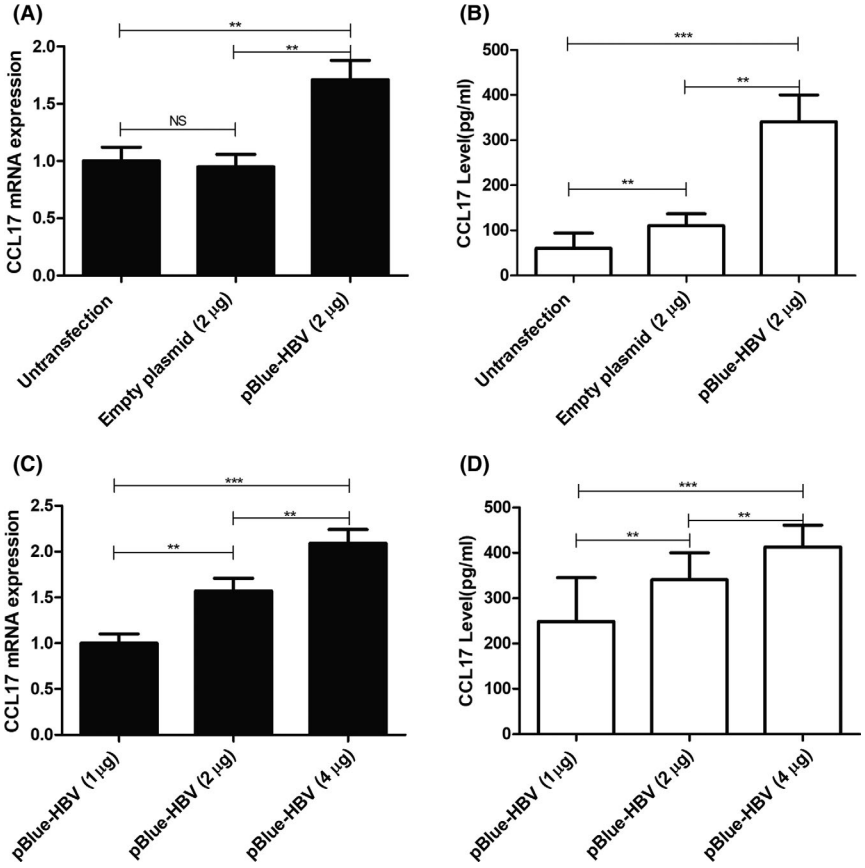


FIGURE 4 Induction of CCL17 in monocyte-derived dendritic cells after coculture with HBV-transfected or untransfected Huh7 cells. Monocyte-derived dendritic cells were co-cultured at different ratios with HBV-transfected or untransfected Huh7 cells. (A and C) mRNA expression and (B and D) protein levels for CCL17 were measured by qPCR and ELISA respectively. CHB, chronic hepatitis B; HV, healthy volunteers; Th17, T helper cell 17. Value represents the mean ± SD of three separate experiments done in triplicate. $^{*}P < .01$. $^{**}P < .001$

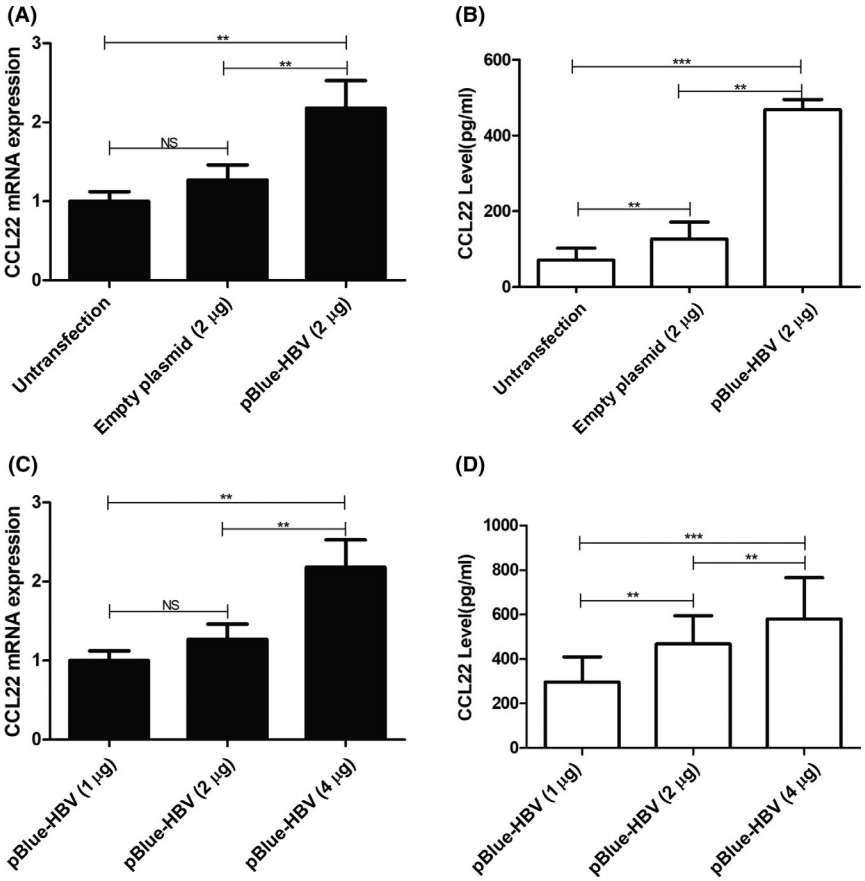


FIGURE 5 Induction of CCL22 in monocyte-derived dendritic cells after coculture with HBV-transfected or untransfected Huh7 cells. Monocyte-derived dendritic cells were cocultured at different ratios with HBV-transfected or untransfected Huh7 cells. (A and C) mRNA expression and (B and D) protein expression levels of CCL22 were measured by qPCR and ELISA respectively. CHB, chronic hepatitis B; HV, healthy volunteer; Th17, T helper cell 17. Value represents the mean ± SD of three separate experiments done in triplicate. $^{*}P < .01$. $^{**}P < .001$

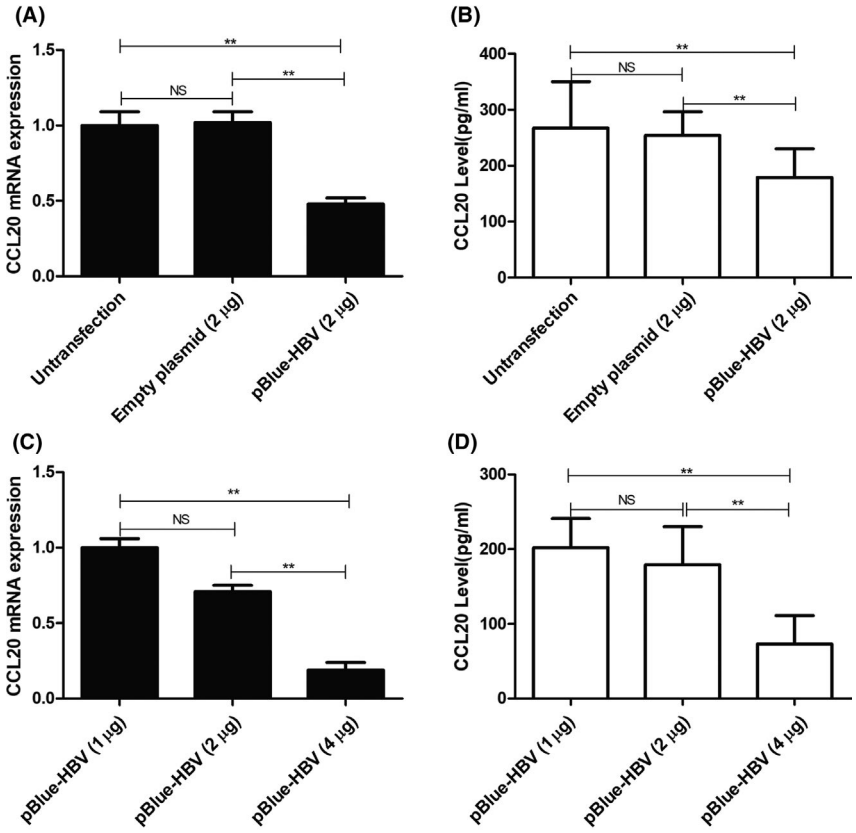
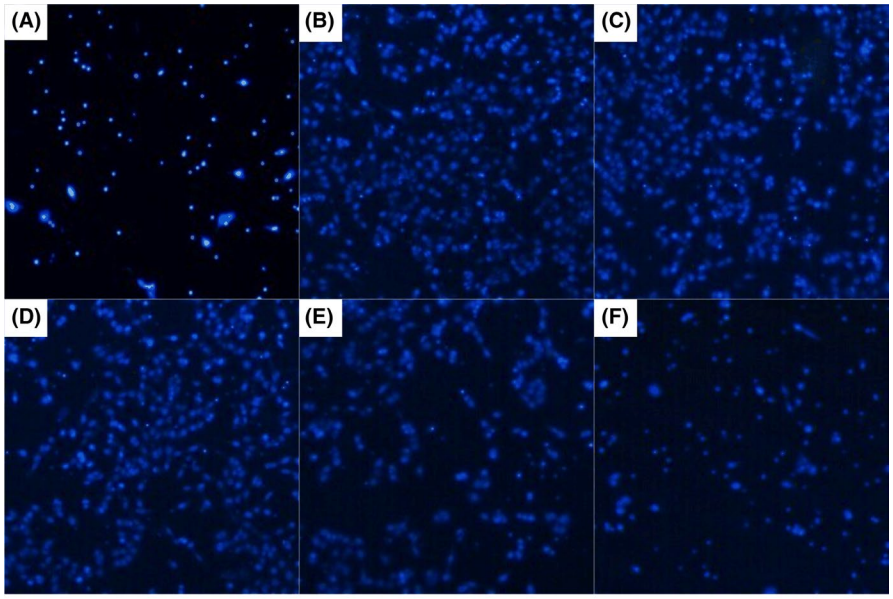


FIGURE 6 Induction of CCL20 in monocyte-derived dendritic cells after co-culture with untransfected or HBV-transfected Huh7 cells. Monocyte-derived dendritic cells were cocultured at different ratios with HBV-transfected or untransfected Huh7 cells. (A and C) mRNA expression and (B and D) protein expression levels of CCL20 were measured by qPCR and ELISA respectively. CHB, chronic hepatitis B; HV, healthy volunteers; Th17, T helper cell 17. Value represents the mean ± SD of three separate experiments done in triplicate. $^{**}P < .001$

model, blocking IL-22 attenuated the hepatic expression of CXCL20 and CCL10 and subsequently reduced the Th17 cell recruitment and liver inflammation/fibrosis progression. IL-22 may promote hepatic fibrosis progression in CHB patients by inducing intrahepatic Th17 migration.[27] HCV induces the expression of the chemokines CCL17 and CCL22, which consequently attract regulatory T cells to the site of infection.[15,28] Therefore, it is possible that HBV promotes the recruitment of Th17 cells from the peripheral blood into liver tissues by inducing the expression of CCL17, CCL20 or CCL22.

Recently, many studies have reported that sites of inflammation can recruit Th17 cells to infiltrate into these sites by increasing the expression of CCL20 and CCR6 on the surface of Th17 cells.[29,30] Hirota reported that high levels of CCL20 could be measured in sites of endometrial inflammation in the synovial membrane and endometriotic tissues in mice with rheumatoid arthritis and that CCL20/CCR6 could promote the chemotaxis of Th17 cells to inflammatory tissue.[31] However, the expression of CCL20 at the transcriptional level (mRNA) and translational level (ELISA) in this study was significantly lower than that in the control group, indicating that HBV infection can inhibit the expression of the chemokine CCL20. Our result is different from those of previous reports.[13,31] The reason for this difference might be that CCL20 is widely distributed in peripheral blood lymphocytes, the appendix, lymph nodes, foetal lungs and the liver, whereas its distribution in the normal synovial cavity and endometrium is lower.[32] Different stimuli have different effects on the same chemokine. For example genipin can reduce CCL20 secretion by periodontal ligament cells,[33] while CCL20 secretion may be increased in the cells of smokers.[34] CCL20 was not the major chemokine attracting Th17 cells in chronic liver inflammation but was important in the localization of Th17 cells to cholangiocytes.[26]

CCR6 is the receptor for CCL20, and CCR4 is the receptor for CCL17 and CCL22. In our present work, the CCR6 and CCR4 levels were increased in the liver tissues and PBMCs of CHB patients, and the CCR6 and CCR4 levels were positively correlated with the serum ALT levels and serum HBV DNA loads. These results are also consistent with all the previous data showing that intrahepatic Th17 and Tc17 cells express high levels of CCR6.[24] These results indicate that CCL17, CCL20 and CCL22 may be chemotactic for Th17 cells, which migrate to the liver through the combination of CCR6 and CCR4 on their surface. Subsequently, we studied the chemokines produced by these cells in the context of HBV infection in vitro and found that transfection of the pBlue-HBV plasmid into Huh7 cells induced a strong upregulation of CCL17 and CCL22 mRNA expression and an increase in chemokine protein synthesis. Furthermore, the expression levels of the chemokines CCL17 and CCL22 were positively correlated with the HBV DNA load. This finding was also consistent with those of previous reports.[35,36] However, a previous study reported that the frequency of Th17 cells and the levels of IL-17 increased with the mitigation of liver inflammation and the decline in HBV DNA load and that the Treg/Th17 ratio decreased.[37] This experiment was carried out in vitro, and this environment is different from the complex and diverse liver inflammatory microenvironment in the human body (in vivo), which may lead to inconsistent correlation of the results. Validation in animal experiments or clinical



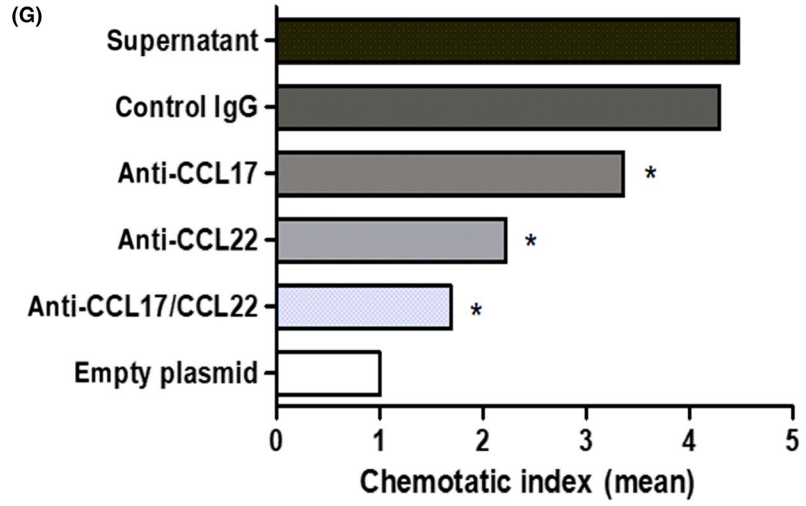


FIGURE 7 The chemotactic ability of Th17 cells was reduced by blocking the expression of CCL17 and CCL22. (A) Control group; (B) experimental group; (C) experimental group with the isotype control antibody; (D) experimental group with addition of anti-CCL17; (E) experimental group with addition of anti-CCL22; (F) experimental group with addition of anti-CCL17 and anti-CCL22. *$P < .05$

samples is required in further research. In summary, our data indicated that HBV dramatically promoted the expression of CCL17 and CCL22 in a dose-dependent manner.

In the chemotaxis experiment, the results showed that the CI of IL-17 secreting T cells (Th17 and Tc17) in the experimental group was significantly higher than that in the control group, indicating that transfection of cells with HBV significantly enhanced the recruitment of Th17 and Tc17 cells. The CI of Th17 and Tc17 cells was slightly decreased after CCL17 antibody blockade, and the chemotactic ability of Th17 and Tc17 cells was significantly decreased in the blocking experiment with anti-CCL22 antibody. These findings indicate that the chemotactic factor CCL22 plays a major role in the chemotaxis of Th17 and Tc17 cells to liver tissue in patients with CHB. Moreover, with the addition of anti-CCL17 and anti-CCL22, the chemotaxis of Th17 and Tc17 cells was significantly weakened, but a small number of Th17 and Tc17 cells migrated to the lower compartment; this migration may be related to other chemokine receptors such as CCR2, CXCR3, CCR5, CXCR6 or CXCR5 on the surface of Th17 and Tc17 cells or to interactions among different chemokines.[38]

Emerging evidences have shown that IL-17-producing $CD8^+$ T cells (Tc17) play important roles in the liver inflammation and liver injury. Increased frequency of hepatic Tc17 cells have been found in several chronic liver diseases including autoimmune hepatitis, primary biliary cirrhosis, alcoholic liver disease and non-alcoholic steatohepatitis.[39] High frequencies of Tc17 in human HCV-infected livers were correlate with control of disease progression.[40] In this study, we also observed the increased proportion of Tc17 cells and upregulated expression of CCR4 and CCR6 on Tc17 cells in the circulation of CHB patients, suggesting an involvement of Tc17 cells in the pathogenesis of CHB.

There are also some limitations in our study. First, we did not explore the relationship between HBV and CCL17, CCL20 and CCL22 expression in animal experiments. Second, we did not directly investigate CCL17, CCL20 and CCL22 expression on Th17 and Tc17 cells in the clinical samples. Further research should be performed to better understand the role of HBV in promoting the recruitment of Th17 and Tc17 cells via the chemokines CCL22 and CCL17.

5 | CONCLUSION

Our results suggest that HBV-transfected hepatocytes may induce the production of the CCL17 and CCL22 chemokines. Conversely, this effect may be beneficial for the accumulation of Th17 and Tc17 cells in the liver. Our study provides clues to better understand the mechanisms of CHB and reveals new targets for immunotherapy for chronic HBV infection.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge financial support by the National Natural Science Foundation of China (81500462 and 81872584), the Natural Science Foundation of Guangdong (2016A030313357) and the Science and Technology Planning Project of Guangdong (2016A020215215).

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.

AUTHOR CONTRIBUTIONS

KZ, NL, QX, YL, YZ and HC contributed to the experimental design, data analysis and interpretation, manuscript writing and manuscript revision. XS, MW contributed to the sample collection and processing. XY, HS GL and YZ (Yong Zou) contributed to the molecular biological experiments.

ORCID

Ka Zhang https://orcid.org/0000-0002-3673-6947
Nan Liu https://orcid.org/0000-0002-8895-3169

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How to cite this article: Zhang K, Liu Y, Yang X, et al. HBV promotes the recruitment of IL-17 secreting T cells via chemokines CCL22 and CCL17. *Liver Int*. 2020;00:1–12. https://doi.org/10.1111/liv.14438