M1 macrophages may be effective adjuvants for promoting Th-17 differentiation in HBeAg positive hepatitis patients with ALT ≤2ULN

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Abstract. Hepatitis B virus (HBV) infection can activate macrophages to accelerate liver disease progression, including inflammation and fibrosis. However, the exact mechanism remains undetermined. The present study assessed the effects of macrophage polarization and the related cytokines on Th-17 differentiation in HBeAg positive individuals with a HBV infection, and also evaluated the potential association of Th-17 cell frequency with the severity of liver injury. A cross-sectional study design was used to collect the clinical parameters, blood samples and liver tissue samples of patients with alanine transaminase £2x upper limit of normal and confirmed hepatitis B who underwent liver puncture in Qishan Hospital between January 2019-December 2021. Macrophage and Th-17 cell related factors were assayed using ELISA. The expression and quantification of cell surface antigen and intracellular markers in cells were assessed using flow cytometry. Pathological staining, including hematoxylin and eosin, reticular fiber staining and immunohistochemical staining were used to assess inflammation and fibrosis in the liver tissue. In the peripheral blood of patients with HBV infection, the number of $CD14^+$ macrophages was significantly increased compared with the healthy control, especially in the hepatitis B e antigen (HBeAg) positive group. $CD14^+$ macrophages were predominantly of the M1 type based on the assessment of the phenotype using flow cytometry and cytokine secretion. Furthermore, the percentage of M1 phenotype and related cytokines were positively correlated with Th-17 differentiation. IL-17A secreted by Th-17 was positively correlated with the degree of liver inflammation and fibrosis, as well as with the severity of liver disease, which indicated that the differentiation of Th-17 may be involved in the progression of liver disease. HBeAg may promote Th-17 differentiation and IL-17A production by M1 macrophages to accelerate the pathogenesis of liver inflammation and fibrosis in CHB patients.

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Abbreviations: ULN, upper limit of normal; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; AIH, autoimmune hepatitis; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; ALT, alanine transaminase; Th17, T helper cell 17; IL-17A, interleukin 17A; IL-6, interleukin 6; IL-10, interleukin 10; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor-α; ELISA, enzyme linked immunosorbent assay; CD4, cluster of differentiation 4; CD3, cluster of differentiation 3; CD86, cluster of differentiation 86; CD163, cluster of differentiation 163; CD14, cluster of differentiation 14; OD, optical density; RLU, relative light units; S/CO, ratio of sample RLU to control cutoff RLU; ANOVA, analysis of variance; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, glutamyltranspeptidase

Key words: HBeAg, macrophages, Th-17, IL-17A, liver fibrosis

Introduction

Hepatitis B virus (HBV) infection is a major public health problem worldwide (1). In 2016, the 194 member states of the World Health Organization committed to eliminating viral hepatitis as a public health threat by 2030, with a particular focus on hepatitis B virus and hepatitis C virus infection (2). China is the country with the highest burden of HBV infection in the world and will be a major contributor to the global elimination of hepatitis B disease by 2030 (3).

However, there are still large gaps in achieving the goals of reducing mortality and improving coverage of diagnosis and treatment, which shows that China still faces enormous challenges in the elimination of HBV infection by 2030 (3). High coverage of hepatitis B diagnosis and treatment has become one of the most difficult goals to achieve globally. A report in 2016 estimated that only 16.1 million people (19% of the estimated total) with chronic HBV infection were diagnosed

in China (compared to a target of 90% by 2030) (3,4), and only 2.8 million people (10-11% of those with chronic HBV infection treated in China in 2016) with chronic HBV infection were currently receiving the necessary treatment (compared to a target of 80% by 2030) (3,5). Diagnosis and treatment of HBV infection need to be improved urgently; however, the pathogenesis of chronic hepatitis B (CHB) is complicated and has not previously been elucidated. One of the crucial concepts, which is generally accepted, is that HBV does not directly kill liver cells, and it is the immune response induced after HBV infection, which is the main cause of liver cell injury and inflammation (6). To address this knowledge gap, the present study evaluated the immune mechanism of the pathogenesis of hepatitis B, especially the mechanism of hepatitis B e antigen (HBeAg)-induced immune injury, in order to support the development of better diagnosis and treatment methods, to support the World Hepatitis 2030 goal.

HBeAg has strong immunogenicity and serves an important role in the early stage of HBV infection (7). The natural history of chronic HBV infection still lacks accurate indicators. Given the importance of HBeAg in the pathogenesis of CHB, recent European Association for the Study of the Liver guidelines (8) suggested that based on the status of HBeAg, chronic HBV infection could be divided into 5 stages: HBeAg (+) chronic infection stage, HBeAg (+) chronic hepatitis stage, HBeAg (-) chronic infection stage, HBeAg (-) chronic hepatitis stage and hepatitis B surface antigen (HBsAg) (-) stage. It can therefore be concluded that HBeAg serves an important role in the progression of HBV infection.

Our previous studies have reported the immunopathogenesis of HBV-related antigens in liver disease after HBV infection. Preliminary results showed that HBeAg, but not hepatitis B core antigen or HBsAg, serves a key role in the activation of macrophages, ultimately aggravating the liver injury by stimulating the production of macrophage inflammatory factors (9-11). Therefore, HBeAg levels have been advocated as a useful biomarker for HBeAg positive patients.

Macrophages, one of the most important innate immune cells, are known for their powerful phagocytosis and high plasticity. Liver macrophages, also known as Kupffer cells, are essential for liver tissue homeostasis and promote the progression of liver disease (12). Certain previous studies have focused on the inflammatory diseases caused by macrophages since the discovery of the two subtypes of macrophages (M1 and M2) (12,13). M1 macrophages are pro-inflammatory cells that are responsible for inflammatory signaling, whereas M2 macrophages are anti-inflammatory cells that participate in the resolution of the inflammatory process through the production of anti-inflammatory cytokines that contribute to tissue healing (14). Specific environmental signals further determine the polarization and function of hepatic macrophages (14,15). After HBV infection, HBeAg induces microRNA (miR)-155 expression and promotes liver injury by increasing inflammatory cytokine production in macrophages (9,11). Therefore, it can be hypothesized that HBeAg could improve M1 activation and promote the production of inflammatory cytokines.

Previous studies have reported that M1-type inflammatory macrophages are not only conventionally associated with T helper cell 1 response, but also T helper cell 17 (Th-17) responses, including promotion of interleukin 17A (IL-17A) production and Th-17 differentiation (16,17). IL-17A secreted by Th-17 is a proinflammatory cytokine with dual effects in immune responses, including the early beneficial responses against infection and the detrimental effects associated with autoimmunity and inflammatory diseases. During HBV infection, Th-17 cells may contribute to disease progression and the pathogenesis of liver injury (18). Whether HBeAg, as a strong immune mediator, can promote the differentiation of Th-17, thus leading to liver inflammation and fibrosis has been rarely reported.

The present study evaluated the relationship between M1 macrophage and related cytokines, and Th-17 differentiation in CHB infection and assessed the hypothesis that the HBeAg-M1 macrophage-Th-17-IL-17A axis may be another immune pathway by which HBeAg positive patients develop inflammation and fibrosis.

Materials and methods

Patients and healthy controls. A total of 42 participants, including 15 healthy controls, 15 patients who were HBeAg positive and 12 patients who were HBeAg negative, were recruited. The 27 patients with CHB were divided into three groups according to their pathological results as follows: i) light-moderate CHB (n=12), ii) severe CHB (n=8), and iii) hepatitis B cirrhosis (n=7). All participants were negative for antibodies to hepatitis A virus, hepatitis C virus (HCV), hepatitis D virus, hepatitis E virus and human immunodeficiency virus (HIV), and other causes of chronic liver damage, including alcoholic hepatitis, nonalcoholic hepatitis, drug-induced liver injury, autoimmune liver disease or Wilson disease, liver cirrhosis, decompensated liver disease, hepatocellular carcinoma and other kinds of malignancies were excluded. The CHB patients with alanine transaminase (ALT) £2x upper limit of normal (ULN; ULN, 40 U/l) (8) were hospitalized or followed up in Yantai Qishan Hospital from January 2019-December 2021 and all underwent liver biopsies. The liver tissues of healthy volunteers with normal liver functions were obtained during surgery following liver trauma. None of the patients received anti-HBV agents or steroids in the 6 months prior to sampling. The diagnostic criteria for CHB were based on the Guidelines for the Prevention and Treatment of Chronic Hepatitis B (2019) issued by The Chinese Society of Infectious Diseases (Chinese Medical Association) (6) and the degree of pathological injury of liver tissue (6). There were no significant differences in age and gender between patients and healthy controls. Clinical characteristics of the enrolled participants were summarized in Table I. Peripheral blood samples were collected from the patients and the healthy control after obtaining written informed consent. The present study received ethical approval from Yantai Qishan Hospital Research and Ethics Committee.

Clinical parameters. All patients systematically underwent a complete biochemical examination, ultrasonography and liver biopsy within 3 days. Blood samples for use in the present study were collected before liver biopsy. Biochemical tests were performed using commercial assays in the Yantai Qishan hospital laboratory according to the manufacturer's protocols. The ULN for ALT was set as 40 U/l (8). HBeAg

Table I. Clinical and laboratory characteristics of enrolled subjects.

Characteristics	Healthy control (n=15)	HBeAg positive (n=15)	HBeAg negative (n=12)
Age, median ± SD (years)	49.93±14.25	49.87±12.05	53.50±13.79
Sex			
Male, n	9	5	7
Female, n	6	10	5
ALT, median ± SD (U/l)	28.20±9.06	50.34±20.25	34.75±16.47
AST, median ± SD (U/l)	31.27±8.71	63.35±37.96	36.73±20.45
GGT, median ± SD (U/l)	28.67±10.40	78.06±77.66	54.83±42.67
ALP, median ± SD (U/l)	56.47±11.08	124.00±80.34	96.00±38.00
logHBVDNA	-	4.09±1.85	3.17±1.34

SD, standard deviation; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; GGT, glutamyltranspeptidase; HBVDNA, Hepatitis B virus DNA.

levels were assessed using a microparticle-based enzyme immunoassay in a commercially available HBeAg Regent Kit (cat. no. 07P6474, Abbott Pharmaceutical Co. Ltd.), in which there was a direct relationship between the HBeAg level in the sample and the intensity of the resulting chemiluminescent reaction, measured as relative light units (RLU). HBeAg levels were based on the ratio of sample RLU to a control cutoff RLU (S/CO), which thereby produced semi-quantitative results that were proportional to the HBeAg level. HBeAg titers ≥1.0 S/CO were scored as positive, whereas HBeAg titers <1.0 S/CO were scored as negative.

Liver biopsy. Liver tissue was obtained by ultrasound-guided percutaneous liver biopsy using a 16-guage biopsy needle. These specimens were fixed using 4% paraformaldehyde at room temperature for 72 h, paraffin-embedded, cut into 4mm thick sections and stained using hematoxylin and eosin (H&E; as detailed below) and Gomori reticular fiber staining (adtailed below). A minimum of 1.5 cm of liver tissue with at least 6 portal tracts was required for diagnosis. According to Scheuer's classification, the degree of inflammatory activity and fibrosis were divided into 0-4 grades (G) and 0-4 stages (S) (19) by two pathologists blinded to the clinical data. G1-2, S0-2 were defined as mild-moderate CHB; G3, S1-3 were defined as severe CHB, G4, S2-4 were defined as cirrhosis. When the opinions of the two experts were inconsistent, the higher G score was used (19).

H&E staining. After heating at 65˚C for 30 min, liver tissue sections were dewaxed at room temperature (soaked in xylene, anhydrous ethanol and 95% ethanol for 5, 1 and 1 min respectively, and washed with tap water for 2 min), hydrated (washed with distilled water for 2 min), stained with hematoxylin for 5 min, differentiated using hydrochloric acid alcohol (pH 3, 75% alcohol) for several seconds, soaked in ammonia for 1 min and stained with eosin for 2 min, all at room temperature. Sections were then washed with distilled water, dehydrated using an increasing alcohol gradient (95% alcohol for 1 min and anhydrous alcohol for 1 min), washed with xylene and sealed with neutral resin. The stained section were assessed and imaged using a light microscope.

Gomori reticular fiber staining. The staining was performed according to the manufacturer's protocol. Ammoniacal silver solution was prepared using 0.4 ml of 10% sodium hydroxide solution and 4 ml of 10% aqueous silver nitrate solution (Merck KGgA), with the precipitate dissolved by the gradual addition of with concentrated ammonia (28%) while shaking the container continuously until the solution was clear. The working solution was 1 ml ammoniac silver solution diluted to 10 ml with distilled water. Staining was performed as previously described (20). Gomori-stained sections were imaged at x200 magnification using an Olympus BX51 light microscope. Reticular fibers were identified by black staining.

Flow cytometry analysis. Peripheral blood monocytes were prepared using Human Lymphocyte Separation liquid (cat. no. 7121011; Dakewe Biotech Co., Ltd.), according to the manufacturer's protocols. Th-17 cells were prepared and activated as follows, the monocytes were stimulated using Cell Activation Cocktail (with Brefeldin A) (cat. no. 423303; BioLegend, Inc.) stimulating solution and then blocked using Human TruStain FcX™ Fc receptor blocking solution (cat. no. 422301; BioLegend, Inc.). $CD4^+$ T cells were gated on the basis of forward and side light scatter using a FITC-conjugated anti-human CD3 antibody (1:20; cat. no. 300305; BioLegend, Inc.) incubated at 4˚C for 20 min in the dark and APC-conjugated antihuman CD4 antibody (1:20; cat. no. 357407; BioLegend, Inc.) incubated at 4˚C for 20 min in the dark. For intracellular staining, the cells were fixed and permeabilized using fixation buffer (cat. no. 420801; BioLegend, Inc.) at room temperature for 20 min and intracellular staining permeabilization wash buffer (cat. no. 421002; BioLegend, Inc.) at room temperature for 10 min, and then stained using PE-conjugated IL-17A antibodies (1;20; cat. no. 512305; BioLegend, Inc.) incubated at room temperature in the dark for 20 min. IL-17A staining was performed to label Th-17 cells. The Th-17 cell subset was defined as CD4IL-17A double positive.

M1 and M2 macrophages were prepared and activated by assessing the membrane expression levels of CD14, CD86 and CD163 in human monocytes. Macrophages were gated on the basis of forward and side light scatter and using a FITC-conjugated anti-human CD14 antibody

(1:20; cat. no. 301803; Dakewe Biotech Co., Ltd.) incubated at 4˚C for 20 min in the dark and Fixable Viability Dye (cat. no. 65-0865-14, Thermo Fisher Scientific, Inc.) at room temperature for 10 min. The following antibodies were used for staining: PE-conjugated CD86 (1:20; cat. no. 305405; Dakewe Biotech Co., Ltd.) and APC-conjugated antihuman CD163 (1:20; cat. no. 326509; Dakewe Biotech Co., Ltd.), both were incubated at 4˚C for 20 min in the dark. All flow cytometry data were acquired using a BD LSRFortessa Cell Analyzer (BD Biosciences) and analyzed using FlowJo version 10 software (FlowJo LLC).

Enzyme-linked immunosorbent assay (ELISA). Peripheral serum was separated by centrifugation at 400 x g for 40 min at room temperature and the concentration of interleukin 6 (IL-6) (cat. no. KE0007, Proteintech Group, Inc.), tumor necrosis factor-α (TNF-α) (cat. no. KE000681, Proteintech Group, Inc.), interleukin 10 (IL-10) (cat. no. 1111002, Dakewei Biological), IL-17A (cat. no. ab216167, Abcam) and interleukin 1β (IL-1β) (cat. no. 1110122, Dakewei Biological) were assessed using commercially available ELISA kits, which were used according to the manufacturer's protocols. Optical density values were quantified at 450 nm. Standard curves were established and cytokine levels were calculated.

Statistical analysis. All numerical data were presented as mean ± standard deviation or median (interquartile range). Comparison of two groups was performed using Student's t-test or Mann Whitney U test. Comparison of ≥3 groups was performed using one-way analysis of variance (ANOVA), LSD was used as the ANOVA post-hoc pairwise test when 3 group were compared and Tukey's was used as the ANOVA post-hoc pairwise test when >3 groups were included in the comparison. Spearman correlation analysis was performed between the frequency of circulating Th-17 cells and other parameters. All analyses were performed using SPSS 17.0 (SPSS, Inc.) and GraphPad Prism (GraphPad Software; Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

Results

HBV infection promotes the accumulation of CD14$^+$ cells in the peripheral blood. To evaluate changes in the macrophage subset in peripheral blood between patients with HBV infection and healthy controls, the proportion of CD14$^+$ (a surface localized indicator of macrophage maturation) cells was first assessed using flow cytometry. The number of CD14$^+$ macrophages was significantly increased after HBV infection compared with the healthy control group (3.26±1.75% vs. 8.75±4.42%; Fig. 1). Furthermore, the HBeAg positive group had a significantly higher proportion of CD14$^+$ macrophages compared with the healthy control group (10.63±4.40% vs. 3.26±1.75%). The HBeAg negative group showed an increase; however, this was not statistically significant compared with the healthy control group (6.33±3.29% vs. 3.26±1.75%) and was markedly lower compared with the HBeAg positive group (6.33±3.29% vs. 10.63±4.40%).

HBeAg promotes the polarization of macrophages towards the M1 phenotype. As previously reported (14,15,21), CD14$^+$ CD86$^+$ was used to identify M1 macrophages and CD14$^+$ CD163$^+$ was used to identify M2 macrophages for the assessment of the polarization of macrophages using flow cytometry. The results demonstrated that M1 was the main phenotype of macrophages in the peripheral blood of patients with HBV infection with significantly higher levels of M1 macrophages compared with M2 macrophages (9.32±5.19% vs. 2.54±5.49%; Fig. 2A and B). Moreover, the proportion of M1 polarization was significantly higher in the HBeAg positive group compared both with the healthy control group and HBeAg negative groups (1.79±0.77% vs. 0.24±0.17% and 1.79±0.77% vs. 0.76±0.59%, respectively; Fig. 2C and D). Furthermore, the protein expression levels of IL-6, IL-1β and TNF-α in the peripheral serum was assessed and the protein expression levels of these cytokines were all significantly higher in HBeAg positive group compared with the healthy control group (Fig. 2E-G). Moreover, IL-6 and TNF-α levels were significantly higher in the HBeAg positive group compared with those in the HBeAg negative group (Fig. 2E and G).

M1 macrophages may promote CD4$^+$ T lymphocyte differentiation to Th-17. As both macrophage and Th-17 cells serve important roles in the promotion of HBV-associated immune-mediated liver injury (9-11), the present study evaluated the correlation of IL-6, IL-1β, TNF-α and IL-10 protein expression levels in the serum with the percentage of Th-17 cells in the CD4$^+$ T subset (Th-17 cells/CD4$^+$T cells), in peripheral blood samples. These factors are mainly produced by macrophages and are involved in the differentiation of Th-17 in numerous cases (22-31). Significant positive correlation between IL-6 (r_s=0.709, P=0.001), IL-1β (r_s=0.542, P=0.045) and TNF-α (r_s=0.893, P=0.000) cytokines and the percentage of Th-17 cells in CD4$^+$ T subset was demonstrated in patients with CHB (including HBeAg positive and HBeAg negative patients; Fig. 3A-C. However, the data demonstrated that the protein expression level of IL-10 was significantly negatively correlated with the percentage of Th-17cells in CD4$^+$ T subset (r_s=-0.837, P=0.001, Fig. 3D). Furthermore, the percentage of Th-17 cells was significantly higher in HBeAg-positive group compared with the healthy control group (1.02±0.77% vs. 0.33±0.13%, p=0.028, Fig. 3E). These data demonstrated that the cytokines produced by M1 polarization were positively correlated with the proportion of Th-17 in CD4$^+$ cells. Moreover, the differentiation ratio of Th-17 was the highest in the peripheral blood of HBeAg positive patients. Therefore, it could be hypothesized that M1 polarization might promote Th-17 differentiation.

IL-17A is associated with disease severity and liver inflammation/fibrosis. We hypothesized that IL-17A, as a cytokine with major functions in Th-17 cells, mediated the occurrence of liver inflammation and fibrosis in patients with CHB. To evaluate this hypothesis, the relationship between IL-17A and, inflammatory/fibrosis and the severity of liver disease was assessed. In peripheral blood, the data demonstrated that, the IL-17A protein expression level was significantly positively correlated with serum ALT levels (r_s=0.737, P<0.001, Fig. 4A). To further evaluate whether IL-17A was histopathogenic, on the relationship of the level of IL-17A in the serum with inflammation and fibrosis in liver tissues was assessed. The

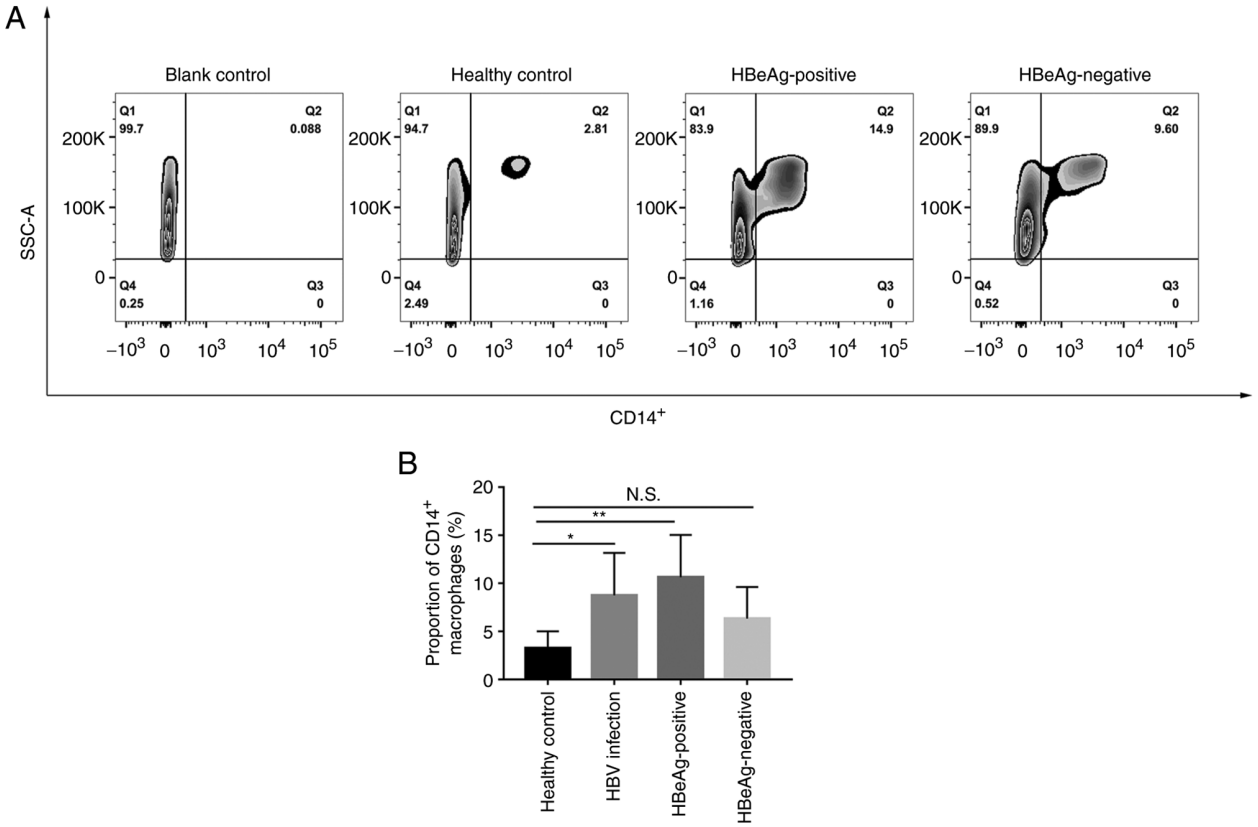

Figure 1. CD14+ macrophage ratio in patients with chronic HBV infection. (A) Flow cytometry analysis of the CD14+ macrophage ratio in healthy controls, HBeAg-positive and HBeAg-negative groups (zebra plots). Mature macrophages were defined as CD14+ subpopulations (upper right) with the purity displayed as percentage of parent population gated on forward scatter/SSC (B) CD14+ macrophage ratio in healthy controls, HBV infection (including patients who were HBeAg negative and positive), HBeAg-positive and HBeAg-negative groups. *P<0.05 and **P<0.01. SSC, side scatter; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; N.S., not significant.

pathological tissues of patients with chronic hepatitis B were divided into light-moderate, severe and cirrhosis groups using the Scheuer scoring system (Fig. 4B). The IL-17A level in the serum was significantly positively correlated with the Scheuer inflammation and fibrosis scores (r_s=0.523, P=0.009 and r_s=0.434, P=0.034, respectively). IL-17A staining was used to label Th-17 cells and assess the percentage of IL-17A positive Th-17 cells in the peripheral blood using flow cytometry. These results demonstrated that the percentage of IL-17A positive cells in the peripheral blood reflected the severity of the disease. The percentage of IL-17A positive cells in the hepatitis B cirrhosis group was significantly higher compared with that in severe CHB patients (1.77±0.49% vs. 0.76±0.14%, P<0.01), light-moderate CHB patients (1.77±0.49% vs. 0.24±0.10%, P<0.01) and the healthy control group (1.77±0.49% vs. 0.39±0.21%, P<0.01); however, there was no significant difference between patients with mild-moderate CHB and the healthy control group (Fig. 4E and F). Patients with light-moderate CHB and healthy volunteers both expressed a markedly lower percentage of IL-17A positive cells compared with severe CHB patients (0.24±0.10% vs. 0.76±0.14%, P=0.004 and 0.39±0.21% vs. 0.76±0.14%, P=0.050, respectively; Fig. 4E).

Discussion

A recent study reported that people with 'normal' ALT levels can suffer from liver histology abnormalities and may require antiviral treatment (32). A study from the Netherlands (33) that included 2991 patients reported that, significant liver fibrosis occurred in 7.2% of patients with ALT <1ULN and in 25% of patients whose ALT was 1-2 times ULN. Another study (34) reported that among untreated patients with ALT <1ULN or 1-2 times ULN, the prevalence of fibrosis and cirrhosis was 35.9 and 17.9%, respectively. However, the mechanism of inflammation and fibrosis of liver tissue in patients with ALT ≤2ULN with chronic hepatitis B infection and whether there are any detectable factors to reflect the degree of liver pathological damage in patients with ALT ≤2ULN was not clear. Based on these clinical issues, the present study evaluated patients with chronic hepatitis B with ALT ≤2ULN.

Numerous articles have reported the relationship of the effect and mechanism of HBV-associated antigen and hepatitis B virus infection (9,11,35-37); however, the role of HBeAg, with strong immunogenicity, in the immune injury of liver tissue has still not been thoroughly elucidated. The present study provides new insights into HBeAgs role in the disease progression of CHB, and demonstrated a potential route for HBeAg-induced liver inflammation and fibrosis. In the present study, peripheral blood mononuclear cells from HBeAg positive and negative patients with CHB were used to analyze the frequency, cytokine production and polarization of macrophages. The results demonstrated that cytokines produced by M1 macrophages were then presented to naive T cells and promoted the differentiation of Th cells into Th-17 cells, and

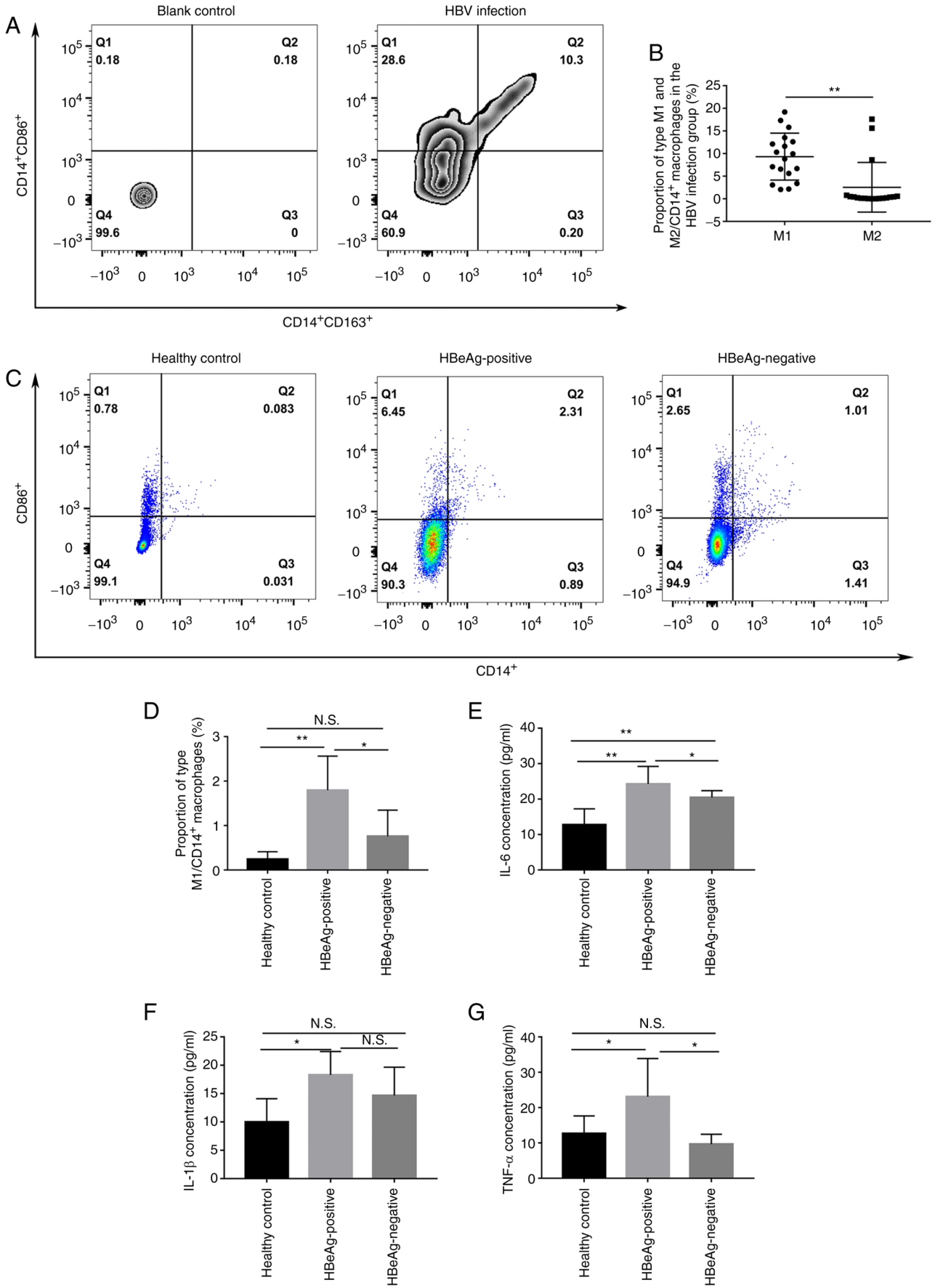

Figure 2. Macrophages from HBeAg positive patients were polarized toward M1. (A) Flow cytometry analysis of $CD14^+CD86^+$ (M1) ratio and $CD14^+CD163^+$ (M2) in blank control and HBV infection groups (zebra plots). (B) Polarization of macrophages in patients with HBV. (C) Flow cytometry analysis of $CD14^+CD86^+$ (M1) ratio (pseudo color plots). (D) Ratio of M1 macrophages. Enzyme linked immunosorbent assay analysis of (E) IL-6, (F) IL-1β and (G) TNF-α levels. $^*P<0.05$ and $^{**}P<0.01$. HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; CD, cluster of differentiation; IL, interleukin; TNF, tumor necrosis factor; N.S., not significant.

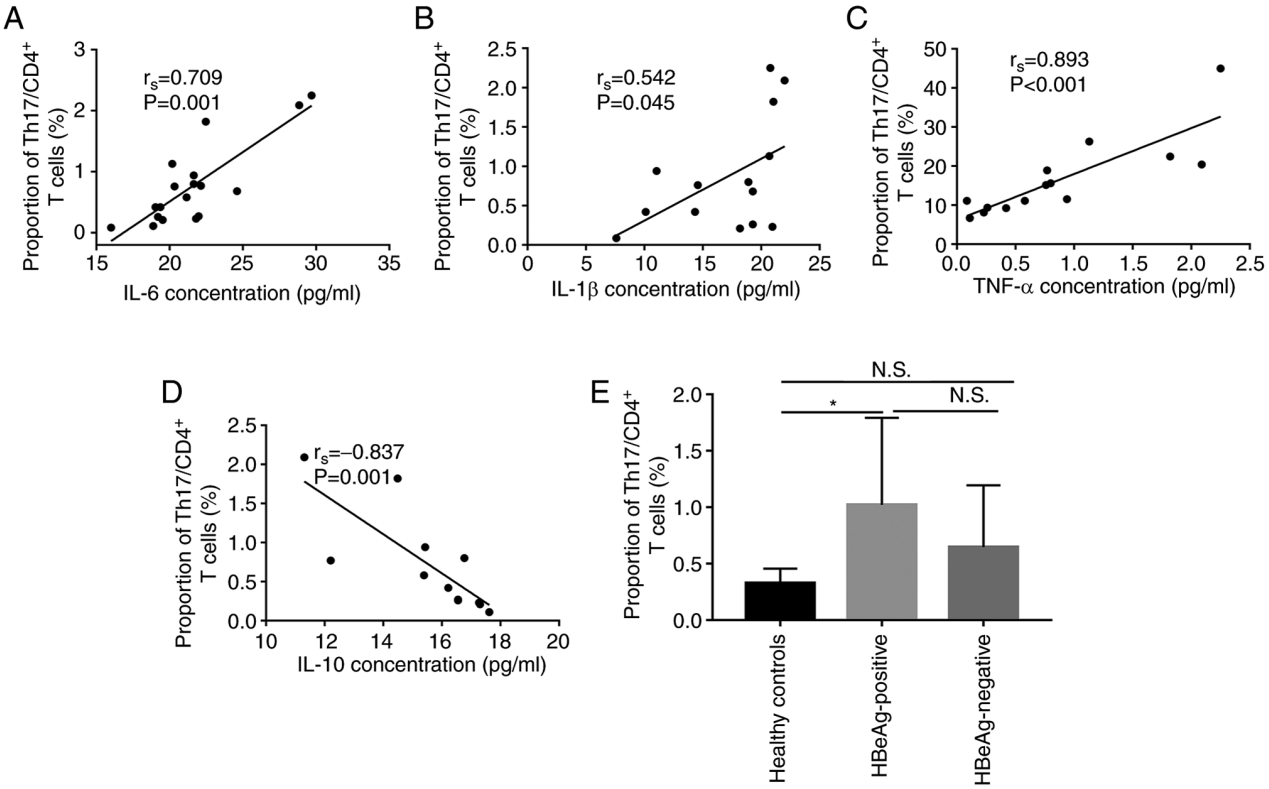

Figure 3. The percentage of Th-17 cells in the CD4+ T subset in peripheral blood and the association with serum IL-6, IL-1β, TNF-α and IL-10 levels. Correlation between the proportion of circulating Th-17 cells and serum (A) IL-6, (B) IL-1β and (C) TNF-α levels. (D) Correlation between the proportion of circulating Th-17 cells and serum IL-10 levels. (E) Comparison of Th-17 proportions. *P<0.05. Th-17, T helper cell 17; CD, cluster of differentiation; IL, interleukin; TNF, tumor necrosis factor; N.S., not significant.

then triggered over activation of Th-17 cells to produce IL-17A, which ultimately damaged the liver tissues. Circulating IL-17A levels were significantly positively correlated with the level of inflammation and fibrosis in liver histopathology, and were markedly positively correlated with the severity of the disease.

Macrophages are derived from the mononuclear phagocytic system (38,39). As precursor cells, monocytes in the blood migrate to tissues and become macrophage, especially during inflammation (40). Because it is difficult to isolate large numbers of macrophages from human organs, the present study isolated monocytes from peripheral blood instead. Compared with healthy individuals, HBV patients demonstrated a markedly higher proportion of the CD14+ macrophage subset. The aforementioned results demonstrate that HBV infection promoted the accumulation of CD14+ cells in peripheral blood.

Different stimuli activate the generation of M1 ('destroy') and M2 ('heal') macrophages (21). M1 and M2 macrophages differ in phenotype and their release of pro-and anti-inflammatory cytokines, respectively (14). M1 macrophages undergo classical activation, characterized by up-regulation of CD86 and secretion of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α. M2 polarized macrophage activation however is characterized by up-regulation of CD163 and increased IL-10 production (14). The phenotype of not only macrophages but also the cytokines were compared between CHB patients and healthy controls. These results showed markedly higher expression of CD14+CD86+ (M1 macrophages) in HBV infected patients, which was significantly higher in the HBeAg positive group. These data indicated the monocytes from HBV infected patients were predominantly M1 polarized, which was possibly related to HBeAg. Furthermore, the levels of IL-6, IL-1β and TNF-α were assessed, which were markedly higher in the HBeAg positive group compared with those in the HBeAg negative and healthy control groups. These results suggested that the polarization of M1 and M2 was unbalanced after HBV infection, and that the polarization of M1 was predominant. Cytokines produced by polarized M1 resulted in liver inflammatory damage. This phenomenon was more apparent in samples from patients with HBeAg positive HBV infection. These results indicated that, over-differentiated M1 'disruptors' led to the presence of persistent inflammation *in vivo*, which served a key role in the pathogenesis of HBeAg positive patients.

Numerous studies have reported that Th-17 cells generate a proinflammatory response (41-43). Th-17 cell subsets are differentiated from naive CD4+ T cells and mainly secrete IL-17A, IL-17F and granulocyte-macrophage colony-stimulating factor. IL-6 and IL-1β are the inducers of Th-17 differentiation and Th-17 downstream product IL-17A (43). The present study demonstrated that cytokines (IL-6, IL-1β and TNF-α) produced after M1 polarization were significantly positively correlated with the proportion of Th-17 cells, whereas the cytokine (IL-10) produced after M2 polarization demonstrated a significant negative correlation with the percentage of Th-17 cells. Th-17 cells are heterogeneous and able to display both pathogenic and non-pathogenic phenotypes depending on the microenvironment (44). IL-6 and IL-1β induce pathogenic Th-17cells to cause immune pathologies in certain tissues (45). The present study

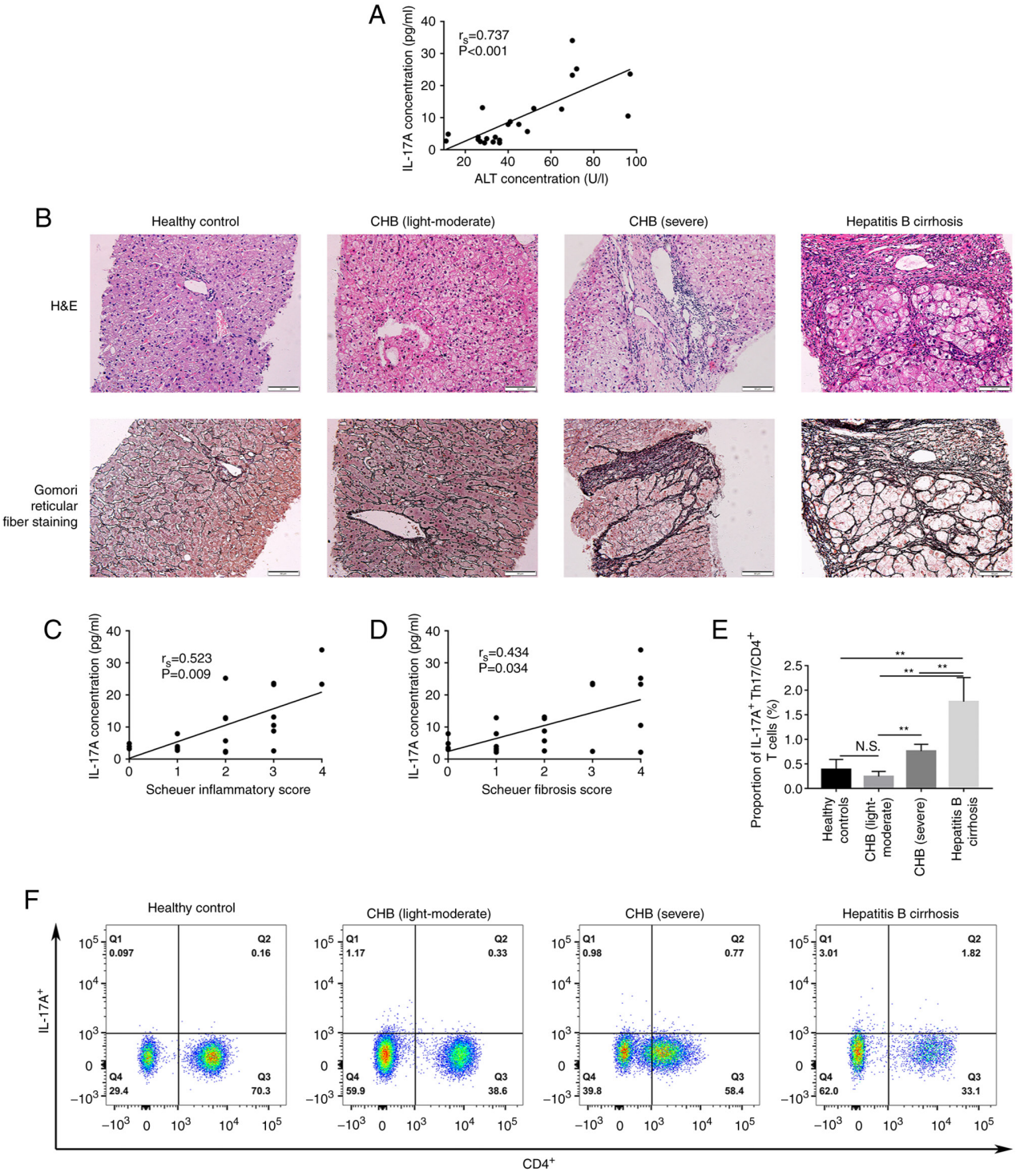

Figure 4. Correlation between IL-17A and disease severity in peripheral blood and pathological tissue. (A) Correlation between serum IL-17A level and serum ALT level in patients with CHB. (B) Representative images of the inflammation and fibrosis were scored using H&E staining and Gomori reticular fiber staining respectively (Scale bar, 50 μm). Correlation between IL-17A levels in the serum and the (C) Scheuer inflammation and (D) Scheuer fibrosis score. (E) The proportion of IL-17A$^+$ Th-17 cells in peripheral blood. (F) The proportion of IL-17A$^+$ cells were analyzed using flow cytometry. **P<0.01. ALT, alanine transaminase; CHB, chronic hepatitis B; H&E, hematoxylin and eosin; IL, interleukin; Th-17, T helper cell 17; CD, cluster of differentiation; N.S., not significant.

also demonstrated that IL-6, IL-1β and TNF-α concentrations in the HBeAg positive group were markedly higher compared with those in the HBeAg negative and control groups. Which indicated that in HBeAg positive patients, M1-released cytokines promoted the differentiation of Th-17 cells into a pathogenic phenotype rather than non-pathogenic phenotype. Furthermore, as the HBeAg positive group had a higher ratio of Th-17 differentiated cells than the HBeAg negative group and HBeAg has strong immunogenicity in the protein encoded by HBV, it could be hypothesized that HBeAg can directly promote the differentiation of pathogenic Th-17. However, further evaluation using *in vitro* cell testing is required. The participation of macrophages

in Th-17 responses has been previously proposed, though little is known about the specifics of their involvement (46) and this hypothesis requires further investigation.

To further assess the role of Th-17 in the pathogenesis of HBV-induced liver inflammation and fibrosis, the present study examined the correlation between serum IL-17A levels and, liver inflammation and fibrosis scores and disease severity. Gomori reticular fiber staining was used to evaluate the degree of liver fibrosis (47). Reticular fibers are generally located between liver cells and liver sinuses, and degradation or collapse of reticular fibers in the hepatic sinusoid can be considered a pathological feature during the initiation and/or progression of hepatic fibrosis (48). The results of the present study demonstrated that the expression of IL-17A increased with the severity of the disease. Consistent with these findings, increased IL-17A-associated tissue damage had also been reported in patients infected with HIV (49), human herpesvirus (50), respiratory syncytial virus (51), influenza virus (52) and dengue virus (53). The present study demonstrated that the level of IL-17A was positively correlated with inflammation and fibrosis. Data from numerous studies (54-57) suggest that there is a potential correlation between IL-17A-producing cells (i.e., Th-17 cells) or IL-17A levels and the development of liver fibrosis follow HBV or HCV infection. However, a previous study reported a negative correlation between the serum IL-17A concentration and the severity of inflammatory lesions in the liver tissue of patients with active autoimmune hepatitis (AIH) (58). This might be due to the different patient inclusion criteria; in the present study, patients were infected with HBV and in the aforementioned study, patients had AIH. This indicates that the mechanism by which IL-17A caused hepatitis differed depending on the infectious agent and environment. At present, the pathway that regulates the progression of the macrophage to M1 and stimulates Th-17 differentiation to produce IL-17A may be one of the pathogeneses of CHB, particularly for patients with HBeAg positive CHB.

It is important to emphasize that the relationship between HBeAg and M1 macrophages and the cytokines they secrete, such as IL-6, IL-1β and TNF-α, and Th-17 cells requires further confirmation in a cell model (using co-culture of cells) and the absence of this is a limitation of the present study. Due to the updated diagnostic and therapeutic guidelines for chronic hepatitis B in China, it is difficult to obtain liver biopsy samples from patients with chronic hepatitis B with ALT ≤2ULN (the number of liver biopsies decreased dramatically in patients with ALT ≤2ULN). Therefore, while the sample size and results of the different experimental groups in this study have reached statistical significance, the sample size was small. Moreover, the present study relied on the data from a single center and the sample size is small; hence, it is essential to confirm the findings from multiple centers or expand the sample size in future experiments to support its value in clinical practice.

The results of the present study indicated that HBeAg might be the initiator of the onset of HBeAg positive CHB by the promotion of M1 macrophage differentiation. IL-6, IL-1β and TNF-α secreted by M1-type macrophages contribute to the differentiation of naive T cells into pathogenic Th-17 cells. A potential correlation between Th-17 or IL-17A levels and a more severe disease stage of HBV progression has been suggested and mechanistic studies have reported a positive link between IL-17A production and the development of liver fibrosis/inflammation and the severity of CHB (18,42). The present study provided new evidence for a different route of pathogenesis in HBeAg positive and HBeAg negative patients with CHB. Moreover, the HBeAg-M1 macrophage-Th-17-IL-17A axis may be another immune pathway by which HBeAg positive patients can develop inflammation and fibrosis. ≤2ULN Evaluation of whether control of Th-17 functions and anti-IL-17A therapy could potentially serve as a targeted strategy for treatment of HBV infection in future studies would be of use.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the first and corresponding author on reasonable request.

Authors' contributions

JQ was responsible for conception and design. LS, NZ and YW were responsible for collection and assembly of the data. LS was responsible for the methodology and LS and JY were responsible for analysis and interpretation. LS, JY and JQ were responsible for writing the original draft. JQ provided administrative support. LS, JY and JQ confirm the authenticity of all the raw data. All authors read and approved the final draft of the manuscript.

Ethics approval and consent to participate

The research protocol and consent program were approved by Yantai Qishan Hospital Research and Ethics Committee (approval no. 201901).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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