

Original Article

Characteristics of peripheral and intrahepatic regulatory B cells in HBV-related liver cirrhosis

Yali Xiong¹, Hongyan Wu², Yang Li³, Rui Huang³, Yong Liu⁴, Yuxin Chen⁴, Xiangan Zhao³, Haiyan Chang³, Jun Chen², Chao Wu^{1,3}

¹Department of Infectious Diseases, Nanjing Drum Tower Hospital, Clinical College of Traditional Chinese and Western Medicine, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China; Departments of ²Pathology, ³Infectious Diseases, ⁴Laboratory Medicine, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, Jiangsu, China

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Abstract: Regulatory B (Breg) cells have been demonstrated to play a suppressive role in chronic hepatitis B virus infection. However, the role of Breg in HBV-related liver cirrhosis has not been determined. Patients with chronic hepatitis B virus infection and HBV-related liver cirrhosis were enrolled. Frequencies of peripheral B cell subsets were assessed by flow cytometry. Intrahepatic B cells, IL-10 positive cells and Breg cells were determined by immunohistochemistry. We found that frequency of peripheral Breg cells was significantly higher in cirrhotic patients compared with that in healthy volunteers. Further, the amount of intrahepatic Breg cells was elevated in patients with HBV-related liver cirrhosis. We identified a positive correlation between intrahepatic Breg cells and histological fibrosis stages. Our results suggested that Breg cells may play a role in HBV-related liver cirrhosis, but the function of Breg cells in cirrhosis remains to be further elucidated.

Keywords: Regulatory B cells, IL-10, liver cirrhosis

Introduction

Liver cirrhosis is a serious global health problem, and results in 1.03 million deaths per year worldwide [1]. The most common cause of liver cirrhosis in China is chronic hepatitis B virus infection. Liver fibrosis is an immune-driving process, during which natural killer cells, T cells, dendritic cells and macrophages are involved in regulating the liver fibrosis and extracellular matrix production [2-4].

B lymphocytes can modulate immune responses by presenting antigens and secreting cytokines during infection, inflammation, and autoimmunity [5]. In recent years, B cells were thought to contribute to liver injury. In a mouse liver fibrosis model, B cells have been proven to promote liver fibrosis in an antibody-independent manner [6]. And in chronic hepatitis B (CHB) patients, intrahepatic CD20 expression was associated with stage of fibrosis [7]. In our previous research, we also found that intrahepatic B cells may play a pathologic role in inflammation in chronic liver disease patients [8].

B lymphocytes are subdivided into effector B cells (Be) and regulatory B cells (Breg) based on cytokine secretion [9]. Breg cells inhibit the function of other immune cells mainly by IL-10 secretion [10]. Some studies have suggested that Breg cells are enriched in CHB patients, which suppress HBV-specific T cell responses, enhance regulatory T cells function in an IL-10-dependent manner [11-13]. However, the role of Breg cells in HBV-related liver cirrhosis remains unclear. To explore the role of Breg cells in HBV-related liver cirrhosis, we investigated the characteristics of Breg cells in blood and liver tissues of patients with HBV-related liver cirrhosis by flow cytometry and immunohistochemistry.

Materials and methods

Patients and samples

Fresh heparinized peripheral blood samples from 32 antiviral treatment-naïve chronic hepatitis B patients, 16 HBV-related liver cirrhosis patients hospitalized to Nanjing Drum Tower Hospital during 2014-2017 and 10 healthy vol-

Table 1. Clinical profile of enrolled subjects

Group	HC (n=10)	CHB (n=32)	HBV-LC (n=16)
Age (years)	31.25 ± 8.54	39.09 ± 9.48	48.66 ± 10.49
Gender (M/F)	5/5	18/14	11/5
ALT (U/L)	17.62 ± 8.30	52.82 ± 51.19	36.93 ± 32.71
AST (U/L)	16.76 ± 5.15	34.12 ± 19.44	36.00 ± 23.26
ALB (g/L)	44.35 ± 2.52	41.87 ± 3.81	36.49 ± 6.87
GLB (g/L)	20.51 ± 2.77	22.59 ± 3.22	25.43 ± 5.56
A/G	2.71 ± 0.18	1.88 ± 0.26	1.50 ± 0.47
TBIL (μmol/L)	7.93 ± 1.85	14.22 ± 5.85	19.33 ± 11.58
WBC (×10 ⁹ /L)	7.43 ± 0.86	5.66 ± 1.68	3.88 ± 2.20
PLT (×10 ⁹ /L)	181.66 ± 18.53	160.80 ± 55.37	102.20 ± 91.87
CRP (mg/L)	1.66 ± 0.72	2.26 ± 1.97	2.55 ± 1.91
Child-Pugh score	NA	NA	5.91 ± 1.56

Data presentation: M ± SEM; NA: not applicable.

unteers were collected. Patients who had received immunotherapy or combined with infection, immune diseases and cancer were excluded. Liver biopsy specimens from 16 patients with chronic hepatitis B and 8 patients with HBV-related liver cirrhosis among these patients were included. Healthy liver specimens from 10 transplantation donors were included as controls. Clinical data from the patients were acquired from their medical records at the time of blood collection or liver biopsy before therapy. The research protocol was approved by the Human Ethics Committee of the Nanjing Drum Tower Hospital, and all participants offered written informed consent in this study.

Flow cytometry

A four-parameter flow cytometric analysis of fresh whole blood cells was performed. Fifty microliters of whole blood were directly stained with combinations of the following monoclonal antibodies: anti-CD45 conjugated to PerCP-Cy5.5 (BD Biosciences), anti-CD19 conjugated to APC (BD Pharmingen, cat # 652804), anti-CD24 conjugated to PE (BD Pharmingen, cat # 555428), and anti-CD38 conjugated to FITC (BD Pharmingen, cat # 555459). After 20 min incubation, whole blood was lysed with red blood cell lysis buffer (BD Biosciences, cat # 349202), then the cells were washed twice with PBS, further resuspended in PBS and immediately analyzed.

Histopathology

Liver tissue was fixed in paraformaldehyde for 48 h, then dehydrated, embedded with paraffin and sectioned to 3 μm-thin slices. Hematoxylin-eosin staining of liver tissue specimens was performed to evaluate the inflammation grade and fibrosis stage according to the *Batts-Ludwig* grading and staging system [14]. The sections were judged by two pathologists separately.

Immunohistochemistry

Immunohistochemical staining of liver tissue sections was processed by EnVision two-step method. Monoclonal antibodies against CD20 (DAKO, cat # M0755, 1:100, USA), IL-10 (Abcam, cat # ab134742, 1:100, USA), PAX5 [15] (Abcam, cat # ab109433, 1:100, USA) and IL-10 double-labeled were used to represent for B cells, IL-10 positive cells and IL-10 producing regulatory B cells, respectively. The dewaxing, antigen retrieval, antibody fixation, and DAB/Fast Red staining were performed according to the standard protocol. The evaluation was carried by two pathologists. Five high-power visual fields per slide were analyzed. The mean number of staining positive cells per high-power field (HPF) was counted for each slide.

Statistical analysis

All statistical analyses were used with the statistical software SPSS 22.0 (Chicago, USA) and *P* value <0.05 was considered statistically significant. ANOVA or Mann Whitney U test was used to compare data among groups. Correlation analysis was performed by Pearson or Spearman correlation coefficient.

Results

General characteristics of the study population

There were normal or mild to moderately elevated transaminase levels among 48 patients, and the range of alanine aminotransferase level was 10-235 U/L, and that of aspartate transaminase level was 13-105 U/L. The cirrhotic patients were all at decompensation

Breg cells in HBV-LC

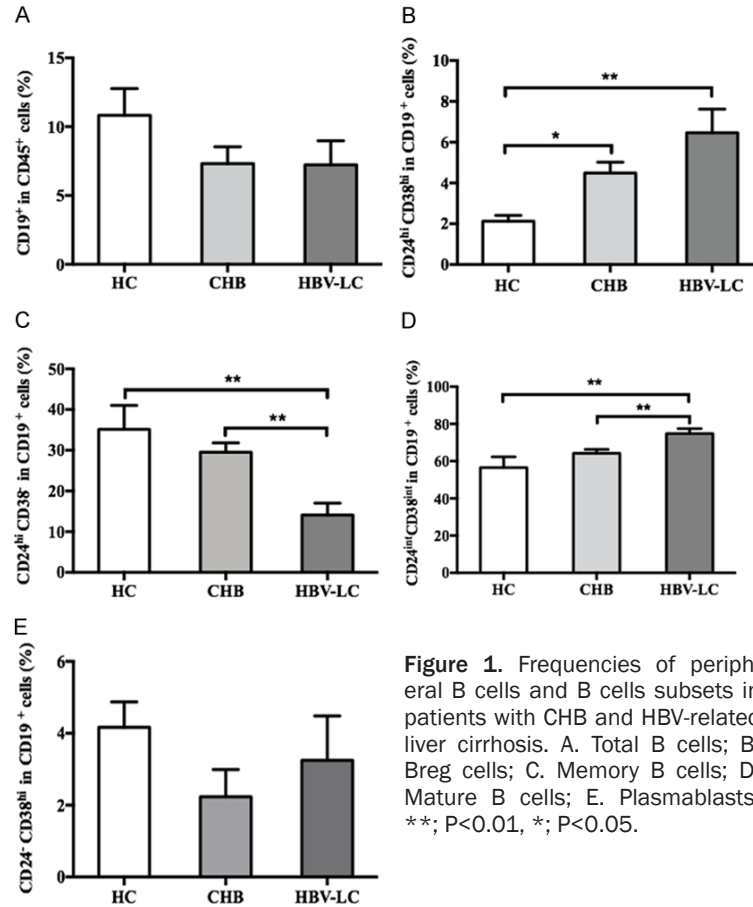


Figure 1. Frequencies of peripheral B cells and B cells subsets in patients with CHB and HBV-related liver cirrhosis. A. Total B cells; B. Breg cells; C. Memory B cells; D. Mature B cells; E. Plasmablasts. **, P<0.01, *, P<0.05.

cells, and CD19⁺CD24⁻CD38^{hi} plasmablasts. The memory B cells were significantly lower in patients with HBV-LC (14.10 ± 2.93%), compared to CHB patients (29.50 ± 2.33%) and healthy controls (35.14 ± 5.89%) (P<0.01, both) (**Figure 1C**). The mature B cells were significantly higher in patients with HBV-LC (74.80 ± 2.62%), compared to CHB (64.23 ± 2.02%) and controls (56.54 ± 5.77%) (P<0.01, both) (**Figure 1D**). There was no difference in the frequency of plasmablasts among three groups (**Figure 1E**).

Intrahepatic regulatory B cells increased in patients with HBV-related liver cirrhosis

We further investigated the intrahepatic B cells, IL-10 secreting cells and Breg cells in patients by immunohistochemistry. The intrahepatic accumulation of CD20 positive B cells in HBV-LC (44.07 ±

18.55/HPF) and CHB patients (36.49 ± 6.45/HPF) was significantly higher compared to controls (3.10 ± 0.90/HPF) (P<0.01, both) (**Figure 2A, 2B**). The intrahepatic CD20 positive B cells in patients with G2 (43.10 ± 9.58/HPF) and G3-4 (44.90 ± 16.73/HPF) were significantly increased compared to controls (3.10 ± 0.90/HPF) (P<0.01, both) (**Figure 2C**). The intrahepatic accumulation of CD20 positive B cells in patients with S1-S4 (S1: 28.86 ± 17.75/HPF, S2: 29.31 ± 9.00/HPF, S3: 47.48 ± 10.33/HPF, S4: 44.07 ± 18.55/HPF) was significantly higher compared to controls (3.10 ± 0.90/HPF) (all P<0.01) (**Figure 2D**).

The intrahepatic accumulation of IL-10 positive cells in HBV-LC patients (3.75 ± 0.16/HPF) was significantly higher compared to CHB (1.12 ± 0.20/HPF) and controls (0.05 ± 0.05/HPF) (P<0.05, P<0.01, respectively) (**Figure 3A, 3B**). The intrahepatic accumulation of IL-10 positive cells in patients with G3-4 (3.50 ± 0.28/HPF), G2 (1.70 ± 0.36/HPF) and G1 (1.66 ± 0.33/HPF) was significantly increased compared to controls (0.05 ± 0.05/HPF) (P<0.01, P<0.01,

stage, but they didn't have spontaneous peritonitis, variceal bleeding, or encephalopathy at enrollment time. The characteristics of patients were listed on **Table 1**.

Peripheral regulatory B cells are enriched in patients with HBV-related liver cirrhosis

To determine the role of Breg cells in HBV-related liver cirrhosis, we measured the frequency of peripheral Breg cells in patients with CHB and HBV-related liver cirrhosis (HBV-LC) by flow cytometry, respectively. There was no difference in the percentage of CD19⁺B cells between CHB, HBV-LC and controls (**Figure 1A**). A significantly higher percentage of CD19⁺CD24^{hi}CD38^{hi} Breg cells was observed in both HBV-LC patients (6.46 ± 1.16%) and CHB (4.47 ± 0.52%) patients compared with controls (2.13 ± 0.28%) (P<0.01, P<0.05, respectively) (**Figure 1B**).

In addition, we determined the frequencies of other B cell subsets: CD19⁺CD24^{hi}CD38⁻ memory B cells, CD19⁺CD24^{int}CD38^{int} mature B

Breg cells in HBV-LC

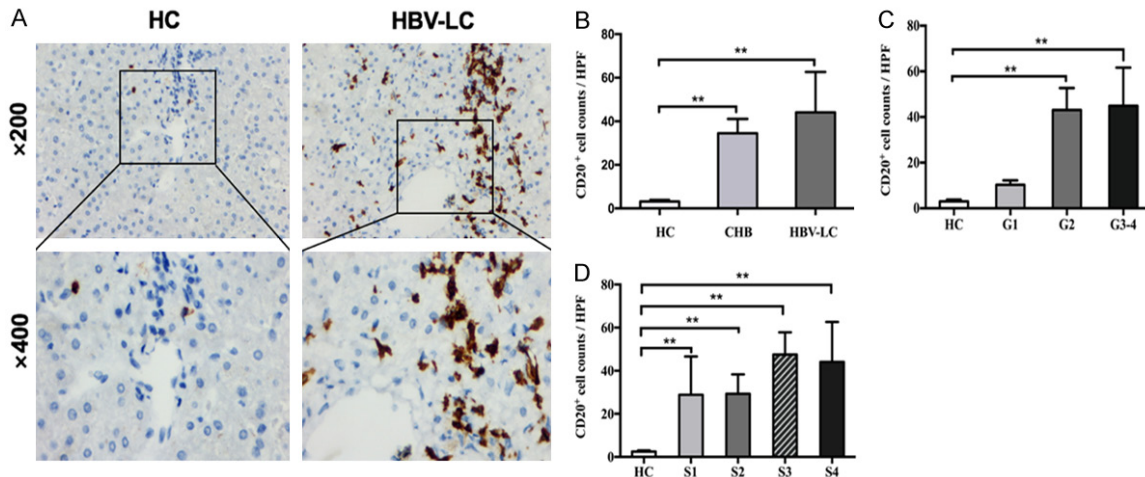


Figure 2. Intrahepatic B cells were increased in patients with HBV-related liver cirrhosis. (A) Immunohistochemical staining for CD20; (B) Comparison of intrahepatic CD20 positive B cells between healthy controls, CHB, and HBV-LC patients; Comparison of intrahepatic CD20 positive B cells in patients with different inflammatory grades (C) and fibrotic stages (D). G, inflammatory grades; S, fibrotic stages. **, P<0.01.

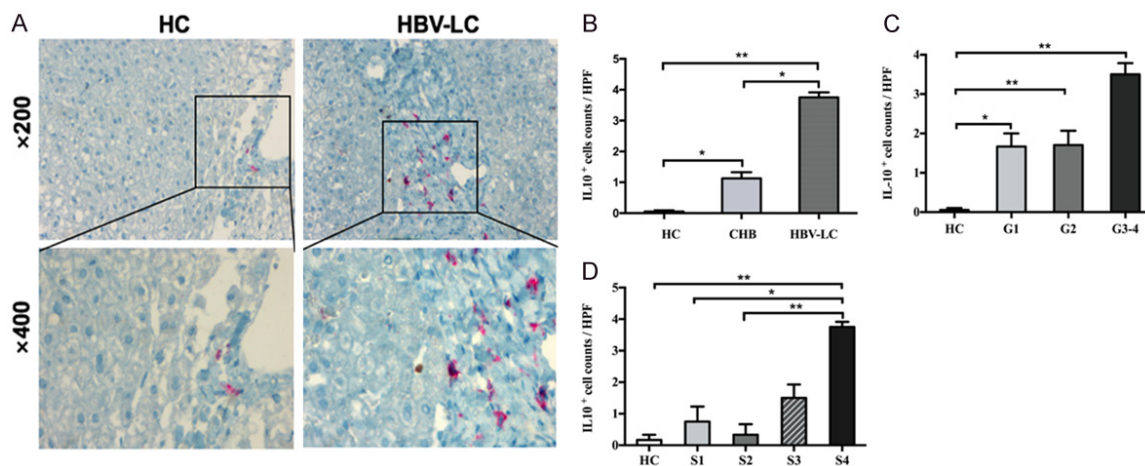


Figure 3. Intrahepatic IL10 secreting cells were increased in patients with HBV-related liver cirrhosis. (A) Immunohistochemical staining of IL-10; (B) Comparison of intrahepatic IL-10 positive cells between healthy controls, CHB, and HBV-LC patients; Comparison of intrahepatic IL-10 positive cells in patients with different inflammatory grades (C) and fibrotic stages (D). G, inflammatory grades; S, fibrotic stages. *, P<0.05, **, P<0.01.

P<0.05, respectively) (Figure 3C). There was no difference in the intrahepatic accumulation of IL-10 positive cells among other groups. The intrahepatic accumulation of IL-10 positive cells in patients with S4 (3.75 ± 0.16 /HPF) was significantly higher compared to controls (0.05 ± 0.05 /HPF), S1 (0.75 ± 0.47 /HPF) and S2 (0.33 ± 0.33 /HPF) (P<0.01, P<0.05, P<0.01, respectively) (Figure 3D).

The intrahepatic accumulation of PAX5/IL-10 double positive Breg cells in HBV-LC patients (0.87 ± 0.08 /HPF) was significantly higher com-

pared to CHB (0.11 ± 0.04 /HPF) and controls (0) (P<0.01, both) (Figure 4A and 4B). Intrahepatic accumulation of PAX5/IL-10 double positive Breg cells in patients with G3-4 (0.52 ± 0.20 /HPF) and G2 (0.38 ± 0.20 /HPF) were significantly increased compared to controls (0) (P<0.05, both) (Figure 4C).

Intrahepatic accumulation of PAX5/IL-10 double positive Breg cells in patients with S4 (0.87 ± 0.08 /HPF) was elevated compared to controls (0), S1 (0) and S2 (0.041 ± 0.041 /HPF) (P<0.01, all) (Figure 4D).

Breg cells in HBV-LC

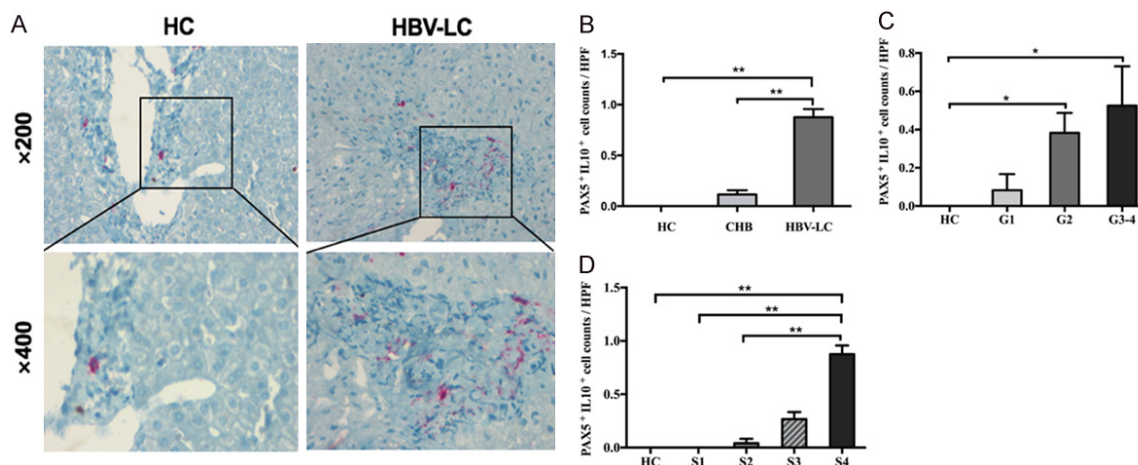


Figure 4. Intrahepatic Breg cells were increased in patients with HBV-related liver cirrhosis. (A) Immunohistochemical double-staining of PAX5/IL-10; (B) Comparison of intrahepatic PAX5/IL-10 double-positive cells between healthy controls, CHB, and HBV-LC patients; Comparison of intrahepatic PAX5/IL-10 double-positive cells in patients with different inflammatory grades (C) and fibrotic stages (D). G, inflammatory grades; S, fibrotic stages. *, $P < 0.05$, **, $P < 0.01$.

We analyzed the correlation between intrahepatic B cells, IL-10 positive cells, or Breg cells and liver inflammatory grade or fibrotic stage and clinical parameters. The expression of intrahepatic B cells was positively correlated with liver inflammatory grade (G). Both amounts of intrahepatic IL-10 positive cells and Breg cells were positively correlated with liver fibrotic stage (S). There was no significant correlation between the level of intrahepatic regulatory B cells and serum alanine aminotransferase, aspartate transaminase, albumin, globulin, and Child-Pugh scores.

Discussion

Regulatory B cells (Breg) have been found to suppress immune responses in autoimmunity, infection, and cancer mainly through the production of IL-10 [10]. IL-10 is an important anti-inflammatory and anti-fibrosis cytokine which can balance Th1/Th2 responses, enhance regulatory T cell function, and promote IL-4 secretion. IL-10 also can inhibit the activation, and promote the apoptosis of hepatic stellate cells, and regulate the production and degradation of extracellular matrix [16, 17]. The source of IL-10 is multicellular, as T cells, monocytes, macrophages, and hepatic stellate cells et al. are known to produce IL-10. It has been proven that B lymphocytes can produce IL-10 [18]; IL-10-producing Breg cells were enriched in CHB patients and able to suppress HBV-specific T

cell responses in an IL-10-dependent manner [11]. Breg cells could also accelerate hepatocellular carcinoma progression [19]. The role of Breg cells in fibrosis development remains elusive. An animal model had shown that adoptive transfer of Breg cells could reduce skin and pulmonary fibrosis [20], indicating that Breg cells might have a beneficial role against fibrosis.

In the present study, we determined the frequency of Breg cells in peripheral blood of HBV-related cirrhosis patients by cytometry. We found that the frequency of peripheral Breg cells significantly increased in liver cirrhosis patients without remarkable liver inflammation. Consistent with our results, Liu et al. [13] also showed that the proportion of peripheral Breg cells was positively correlated with the progression of liver fibrosis. Further, it is also shown that the dynamics of Breg cells in patients with pulmonary fibrosis. Specifically, peripheral Breg cells in patients with systemic sclerosis are reduced and functionally impaired, especially in patients with pulmonary fibrosis [21]. These studies suggested that Breg cells might play a role in the pathogenesis of fibrosis.

Previous data had indicated that the frequency of intrahepatic Breg cells was correlated with the level of peripheral Breg cells in CHB patients [13]. We also investigated the regulatory B cells in liver tissue of HBV-related cirrhosis patients by immunohistochemistry. As there is no identi-

fied surface marker for intrahepatic Breg cells, we detected the distribution of IL-10-producing B cells by IL-10 and PAX5 double labelling using immunohistochemistry. Breg cells were not detected in normal liver, but significantly increased in patients' liver even though the amount of Breg cells was still relatively low. The accumulation of intrahepatic Breg cells in patients with the G3-4 and S4 was higher than that in the other groups, and there was a positive correlation between the accumulation of intrahepatic Breg cells and fibrosis score. These results indicated that Breg cells may play a role in the pathogenesis of liver fibrosis.

In our study, elevated Breg cells were observed in the blood and liver of cirrhosis patients, suggesting they might exert regulatory effects both in the circulation and the liver. However, the mechanism and function of elevated Breg cells in liver fibrosis needs further investigation. It was thought that Breg cells could migrate from spleen to lesion location such as joints, lymph nodes, and nervous system [22, 23]. A previous study had suggested that the activation of iNKT cells in the liver could cause liver damage and raise Breg cells to enter to inhibit inflammation of the liver [24]. It was necessary to further clarify whether the proliferation of Breg cells in the liver was due to the migration of splenic Breg cells or the differentiation of liver B cells in situ under a fibrotic microenvironment. In a mouse model, B cells played a positive role in liver fibrosis through secreting cytokines such as IL-6 to promote fibrogenesis and stimulate HSC proliferation [6, 25]. But the contribution of Breg cells to liver fibrogenesis and HSC proliferation needs further investigation.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Chao Wu, Department of Infectious Diseases, Nanjing Drum Tower Hospital, 321 Zhongshan Road, Nanjing 210008, Jiangsu, China. Tel: 86-25-83105890; Fax: 86-25-83307115; E-mail: dr.wu@nju.edu.cn

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