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ORIGINAL ARTICLE

Role of IL-10-Producing Regulatory B Cells in Chronic Hepatitis B Virus Infection

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Abstract

Background A subset of interleukin (IL)-10-producing regulatory B (Breg) cells that suppress T-cell-mediated immunity was recently identified; however, their role in chronic hepatitis B (CHB) remains elusive.

Aim To explore the possible role of Breg in the interaction with Th cells and consequent pathogenesis of CHB.

Methods The prevalence of Breg as well as 3 major effector T-cell subsets—CD4$^+$CD25^{high}Foxp3$^+$ regulatory T (Treg) cells, T helper 1 cells (Th1), and T helper 2 cells (Th2)—was assessed in the peripheral blood of 31 patients with CHB, 28 patients with acute hepatitis B (AHB), and 25 healthy controls (HC).

Results Compared to patients with AHB and HC, the prevalence of Breg and Treg cells and the concentration of IL-10 in the supernatant of cultured peripheral blood mononuclear cells (PBMCs) were greatly increased in patients with CHB. A significantly decreased proportion of Th1 cells was also observed in patients with CHB and was demonstrated to have a negative correlation with the prevalence of Breg. Furthermore, depletion of Treg cells in the PBMCs of patients with CHB did not alter the frequency of Breg cells or their ability to produce IL-10, indicating little, if any, impact of Treg cells on the generation and maintenance of Breg cells.

Conclusions Our data indicate that increased Breg cells might be a major source of elevated IL-10 in CHB and represent a critical and independent regulatory force in the development of impaired anti-HBV immunity, consequently contributing to the pathogenesis of CHB.

Keywords Regulatory B cells · Chronic hepatitis B · Regulatory T cells · Th1 cells · IL-10

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Introduction

Chronic hepatitis B virus (HBV) infection, which affects more than 200 million patients worldwide, has become a serious health problem and a major risk factor for cirrhosis and hepatocellular carcinoma [1]. Cellular immunity is vital in HBV clearance; however, once the chronicity of HBV infection is established, HBV-specific T-cell responses become blunted due to mechanisms that are not yet fully understood [2–4]. Increasing evidence has demonstrated that immune-suppressor cells such as CD4$^+$CD25^{high} FoxP3$^+$ regulatory T (Treg) cells exert multiple, negative regulatory functions on the effector immune cells and thus play a key role in the impairment of HBV clearance [5–7].

Recently, a new population of immune-suppressor B cells that produces large amounts of interleukin (IL)-10 and modulates the immune response through multiple mechanisms was identified and termed as regulatory B (Breg) cells [8, 9].

Table 1 Clinical and virological characteristics of subjects enrolled in the study

Parameters	AHB ($n = 28$)	CHB ($n = 31$)	HCs ($n = 25$)	p value
Age (years), $\pm S$	34 ± 7	32 ± 5	33 ± 6	>0.05
Sex (M/F)	12:9	17:8	3:1	>0.05
AST, median (range)	325 (45–1,012)*	117 (32–324)**	30 (22–39)	0.015*; 0.022**
ALT, median (range)	851 (44–1,314)*	87 (69–521)**	27 (21–38)	0.02*; <0.001**
DNA, median (range) Log 10 (copes/ml)	3.2 (2.1–5.7)*	5.2 (4.3–7.2)	–	0.011*

* AHB versus CHB

** CHB versus HCs

First, Breg cells are able to suppress the proliferation and cytokine production of effector T cells through either cell–cell contact or IL-10 release [8, 10]. Second, Breg cells can negatively regulate the ability of dendritic cells (DC) to present antigens and produce pro-inflammatory cytokines [8, 11]. Third, Breg cells are able to skew the differentiation of naive CD4$^+$T cell toward T helper type 2 (Th2) in several infectious diseases [8, 12]. Finally, Breg cells may provide synergistic effects that facilitate the generation and maintenance of Treg cells [13, 14].

Currently, few studies have investigated the role of Breg cells in chronic hepatitis B (CHB). A pioneering study performed by Abhishek et al. [15] reported increased Breg cells in the PBMCs of patients with CHB, which was associated with disease severity in these patients as well as suppression of HBV-specific CD8$^+$ T cell responses in an IL-10-dependent manner, indicating a role of Breg cells in regulation of T-cell immunity in CHB. However, it remains unclear whether Breg cells interact with CD4$^+$ Th cell subsets (i.e., Th1, Th2, and Treg) and the role their interaction plays in the pathogenesis of CHB. In this study, we carried out a series of in vitro experiments to explore the possible interaction of Breg cells with Th cells and consequent pathogenesis of CHB.

## Materials and Methods

Study Subjects

This study was approved by the Institutional Review Board of Jiangsu University, and written informed consent was obtained from all subjects. Blood samples were collected from 31 patients with chronic HBV infection (CHB), 28 patients with acute HBV infection (AHB), and 25 healthy controls (HC). The diagnosis of CHB or AHB was made according to the criteria of the Chinese Society of Hepatology [16]. None of the enrolled patients had received antiviral or immunomodulatory therapy before sampling. Exclusion criteria included the concurrence of hepatitis C

virus (HCV), hepatitis E virus (HEV), HIV, and/or autoimmune liver disease. Clinical characteristics of all enrolled subjects are listed in Table 1.

The Prevalence of Breg, Treg, Th1, and Th2 Cells

Breg, Treg, Th1, and Th2 cells were defined as CD19$^+$IL-10$^+$, CD4$^+$CD25^{high}Foxp3$^+$, CD4$^+$IFN-γ$^+$, and CD4$^+$IL-4$^+$, respectively, and were detected by flow cytometry with surface and intracellular stainings. All staining antibodies were purchased from BD Biosciences. PBMCs were isolated from fresh heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation and were resuspended in RPMI-1640 complete medium supplemented with 10 % fetal calf serum, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (all from Invitrogen, Carlsbad, CA, US). For Breg detection, PBMCs were first incubated with 1 μM CpG-B ODN 2006 (InvivoGen, San Diego, CA, USA) for 96 h at 37° and then PMA (3 ng/ml) and ionomycin (100 ng/ml) were added during the last 4 h in the presence of 10 μg/ml brefeldin A (all from Sigma, St Louis, MO, USA). Cells were then surface-stained with anti-CD19-APC, fixed, permeabilized, and stained intracellularly with anti-IL-10-PE. For Treg detection, isolated PBMCs were first surface-stained with anti-CD25-PE and anti-CD4-FITC, then fixed, and permeabilized before intracellular staining with anti-Foxp3-APC. For Th1 and Th2 cell detection, isolated PBMCs were first stimulated for 5 h with PMA (50 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (10 μg/ml). Upon collection, cells were surface-stained with either PerCP- or APC-conjugated anti-CD4 for 20 min, fixed, permeabilized, and intracellularly stained with anti-IFN-γ-FITC and anti-IL-4-PE. All antibodies used in flow cytometry were from BD Pharmingen, San Diego, CA, USA. Cells were acquired on a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and analyzed by FlowJo software (TreeStar, San Carlos, CA, USA).

Fig. 1 Prevalence of IL-10$^+$ CD19$^+$ regulatory B (Breg) cells in patients with CHB. **a** Flow cytometry analysis showed that the prevalence of Breg cells in patients with CHB (13.9 ± 0.66 %) was significantly higher than that of patients with AHB (10.4 ± 0.77 %, $p < 0.05$) and HC (9.9 ± 0.79 %, $p < 0.05$). **b** Three representative analyses are shown
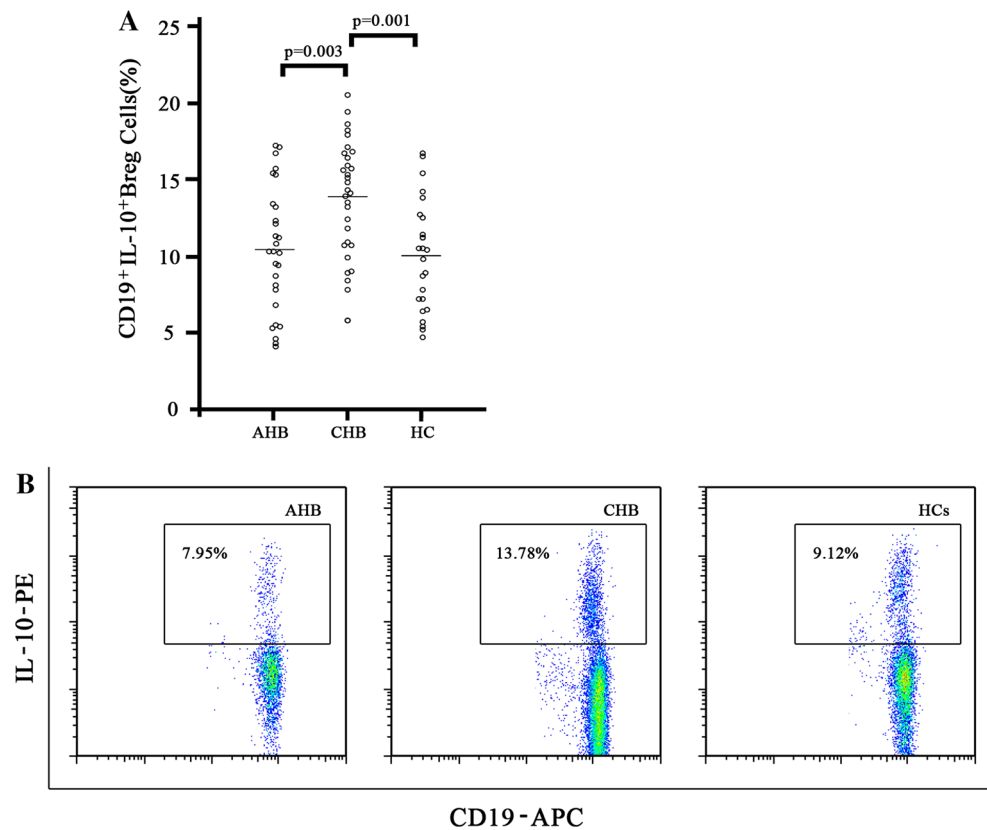
IL-10 Measurement

Concentration of IL-10 in the culture supernatant was assessed using an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instruction.

Depletion of Treg Cells

Treg cells were depleted as previously described [17]. Isolated PBMCs were cultured for 18–20 h in RPMI-1640 complete medium with denileukin diftitox (2–4 nM; Eisai, Woodcliff Lake, NJ, USA), washed, and replated in complete media for 1 day to allow recovery before being subjected to the analysis of Breg cell prevalence and IL-10 production by flow cytometry and ELISA, respectively, as described above. Flow cytometric analysis demonstrated that treatment with denileukin diftitox concentrations ≥2 nM successfully depleted more than 90 % of Treg cells in treated PBMCs (data not shown).

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 (SPSS, Chicago, IL, USA). Differences between groups were analyzed using one-way ANOVA followed by the Student–Newman–Keuls post hoc test for normally distributed data

or the Kruskal–Wallis test for non-normally distributed data. Spearman's correlation tests were performed for correlation analysis. The significance level was set as $p < 0.05$.

Results

Increased Prevalence of Breg Cells in Patients with CHB

The PBMCs from all subjects were stimulated with CpG-B ODN 2006, PMA, and ionomycin. The prevalence of Breg cells (CD19$^+$ IL-10$^+$) was then assayed by flow cytometry. As shown in Fig. 1a, the prevalence of IL-10$^+$ Breg cell in patients with CHB (13.9 ± 0.66 %) was significantly higher than that of patients with AHB (10.4 ± 0.77 %, $p < 0.05$) and HC (9.9 ± 0.79 %, $p < 0.05$). No difference was found in Breg cell frequency between the AHB group and the HC group.

Increased Prevalence of CD4$^+$CD25^{high}Foxp3$^+$ Treg Cells in Patients with CHB

We further examined the prevalence of CD4$^+$CD25^{high}Foxp3$^+$ Treg cells in all subjects. As shown in Fig. 2a, the prevalence of Treg cells in patients with CHB (6.8 ± 1.1 %) was

Fig. 2 Prevalence of CD4$^+$CD25^{high} Foxp3$^+$ regulatory T (Treg) cells in patients with CHB. **a** Flow cytometry analysis showed that the prevalence of Treg cells in patients with CHB (6.8 ± 1.1 %) was significantly higher than that of patients with AHB (3.9 ± 0.9 %, $p < 0.01$) and HC (4.5 ± 1.0 %, $p < 0.01$). **b** Three representative analyses are shown. **c** Positive correlation of Treg cell prevalence with Breg cell prevalence was found in patients with CHB ($r = 0.50$, $p = 0.004$)
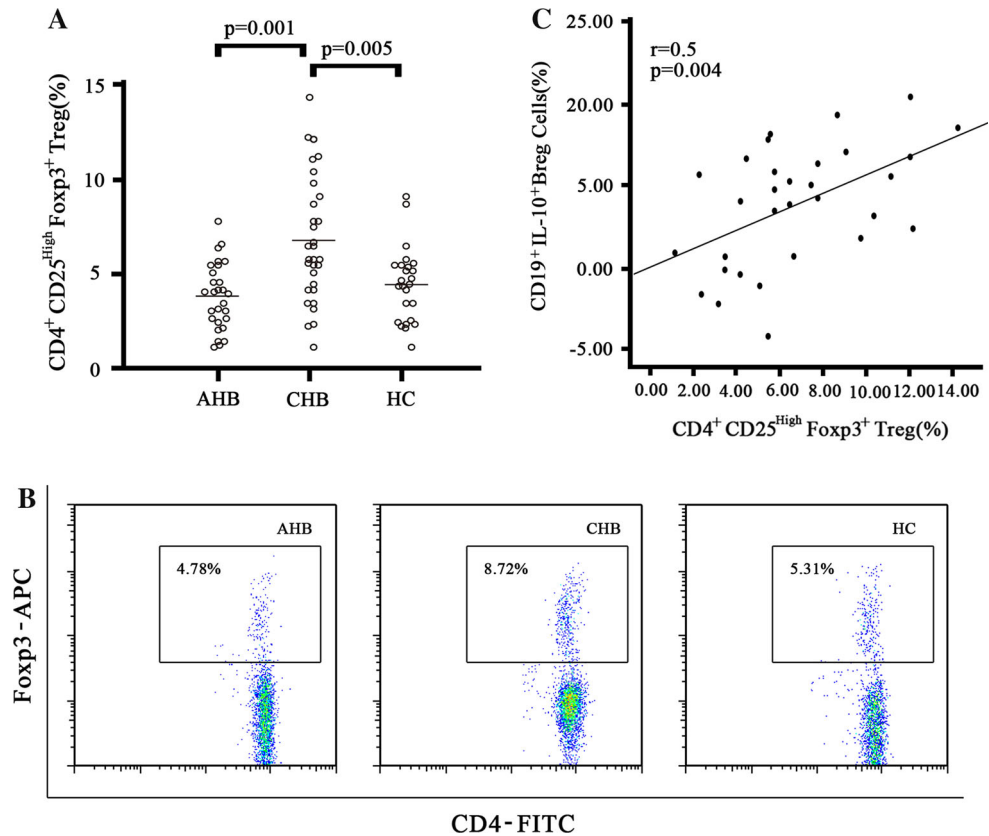
significantly increased compared to that of patients with AHB (3.9 ± 0.9 %, $p < 0.01$) and HC (4.5 ± 1.0 %, $p < 0.01$). No difference was found in Treg cell frequency between patients with AHB and HC. Furthermore, Treg cell prevalence was positively correlated with Breg cell prevalence in patients with CHB ($r = 0.50$, $p = 0.004$, Fig. 2c), but not in those with AHB or HC ($r = -0.15$, $p = 0.462$ and $r = 0.09$, $p = 0.669$, respectively).

Decreased Prevalence of Th1 Cells in Patients with CHB

Upon the stimulation of CpG and PMA, intracellular flow cytometry analysis of Th1 (CD4$^+$, IFN-γ^+) and Th2 (CD4$^+$, IL-4$^+$) was performed. As shown in Fig. 3a, the proportion of Th1 cells among the total number of CD4$^+$T cells was significantly decreased in patients with CHB (17.8 ± 3.6 %) compared to that of patients with AHB (23.4 ± 3.2 %, $p < 0.01$) and HC (24.1 ± 2.9 %, $p < 0.01$). The difference between the AHB group and the HC group was not statistically significant. The proportion of Th2 cells among the total number of CD4$^+$ T cells in patients with CHB, AHB, and HC was 3.50 ± 0.6, 4.71 ± 0.8, and 3.42 ± 0.9 %, respectively. No difference was found in the prevalence of Th2 cells between the groups of patients (data not shown). Correlation analysis showed that the prevalence of Th1 cells was negatively correlated ($r = -0.472$, $p = 0.007$, Fig. 3c)

with that of Breg cells in patients with CHB, but not in patients with AHB and HC ($r = 0.110$, $p = 0.578$ and $r = 0.234$, $p = 0.260$, respectively).

Increased Concentration of IL-10 in the Culture Supernatants of PBMCs in Patients with CHB

After in vitro stimulation, the supernatants of cultured PBMCs were collected for all subjects and their IL-10 concentration was assayed by ELISA. The concentration of IL-10 in the supernatants of patients with CHB (26.7 ± 3.6 pg/ml) was significantly increased compared to that of patients with AHB (5.85 ± 2.4 pg/ml, $p < 0.01$) and HC (6.4 ± 1.6 pg/ml, $p < 0.01$, Fig. 4a). A positive correlation between the amount of IL-10 and the percentage of Breg cells ($r = 0.409$, $p = 0.022$, Fig. 4b) was observed in patients with CHB, but not in patients with AHB and HC ($r = 0.296$, $p = 0.127$ and $r = 0.158$, $p = 0.450$, respectively).

Breg Cell Differentiation and Ability to Produce IL-10 Were Not Altered in the Absence of Treg Cells in Patients with CHB

Given that previous studies have shown a reciprocal interaction between Breg and Treg cells in other settings [13, 14, 18], we explored the possible role of Treg cells in

Fig. 3 Prevalence of CD4$^+$IFN-γ^+ cells (Th1) in patients with CHB. **a** Flow cytometry analysis showed that the prevalence of Th1 cells in patients with CHB (17.8 ± 3.6 %) was significantly higher than that of patients with AHB (23.4 ± 3.2 %, $p < 0.01$) and HC (24.1 ± 2.9 %, $p < 0.01$). **b** Three representative analyses are shown. **c** Negative correlation of Th1 cell prevalence with Breg cell prevalence was found in patients with CHB ($r = -0.472$, $p = 0.007$)
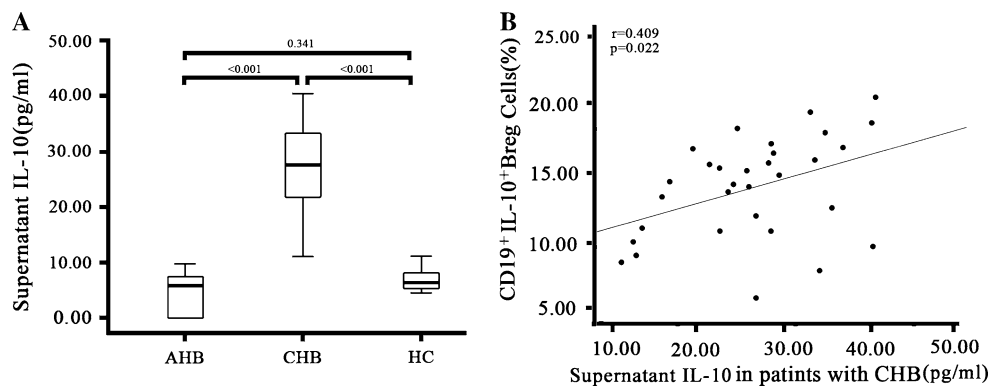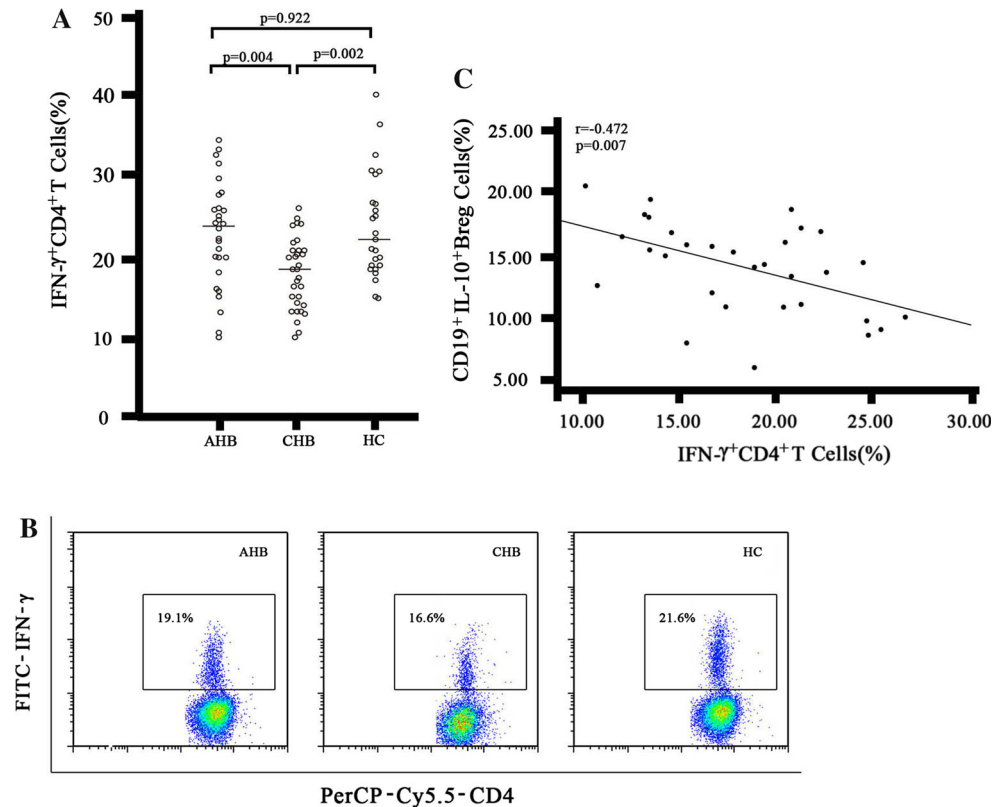

Fig. 4 Concentration of IL-10 in the supernatant of cultured PBMC in patients with CHB. **a** The concentration of IL-10 in the supernatant of PBMCs in patients with CHB was significantly increased compared to that of patients with AHB or HC. **b** Positive correlation of IL-10 concentration with the percentage of Breg cells was found in patients with CHB ($r = 0.409$, $p = 0.022$)

the generation and/or maintenance of Breg cells in CHB. Treg cells in the PBMCs of 10 patients with CHB were depleted by denileukin diftitox, a fusion between the active domain of diphtheria toxin and IL-2, as previously described [17]. The effectiveness of Treg cell depletion was verified by flow cytometry and was shown in Supplementary Figure 1. The resultant Treg cell-depleted PBMCs were then analyzed for the prevalence of Breg cells and IL-10 secretion. As shown in Fig. 5a, b, Treg cell depletion did not change the prevalence of Breg cells

(15.6 ± 3.3 vs. 15.7 ± 2.8 %, $p = 0.971$) nor IL-10 production (25.8 ± 8.9 vs. 25.4 ± 7.9 pg/ml, $p = 0.880$).

Discussion

Increasing evidence has demonstrated that virus-specific T cells (i.e., CD8$^+$ CTL and CD4$^+$ Th cells) play a central role in the clearance of HBV [3, 4, 19]. However, due to mechanisms that are not fully understood, the antivirus

Fig. 5 Breg cell differentiation and ability to produce IL-10 in the absence of Treg cells. **a** In vitro differentiation of Breg cells was not significantly altered with or without the presence of Treg cells in 10 patients with CHB. **b** The level of IL-10 in the culture supernatant was also not associated with the presence of Tregs
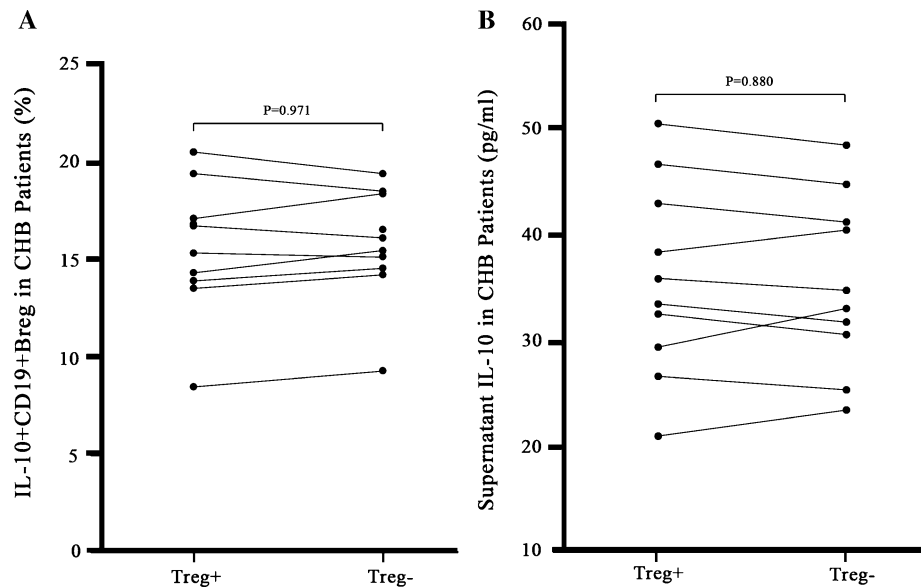
competence of HBV-specific T cells are constantly suppressed in patients with chronic HBV infection [3, 4, 19]. Consistent with previous reports [15], we found that the prevalence of IL-10-producing Breg cells was significantly increased in the peripheral blood of patients with CHB. Considering the known immune regulatory effects of Breg cells [8], our data indicate a potentially critical role of Breg cells in the impairment of HBV clearance in patients with CHB.

IL-10 was a vital determinant of persistent viral infection [20]. Elevated IL-10 has been repeatedly reported in patients with CHB and has been correlated with increased disease severity and viral load [20]. Increased IL-10 production has also been associated with suppressed T-cell responses and chronicity of HBV infection [21]. However, the cellular source of IL-10 in CHB remains elusive. In our study, Breg cells in patients with CHB but not those in patients with AHB or HC showed a potent ability to produce IL-10, suggesting that Breg cells could be a major source of the IL-10 in CHB.

Breg cells have been reported to exert regulatory functions on multiple immune effector cells, including CD4+ Th cells [8]. In order to investigate the possible interplay between Breg and Th cells in CHB, we analyzed the frequencies of Th1 and Th2 cells together with Breg cells in all subjects. Consistent with previous studies [22–24], we observed a significantly decreased percentage of Th1 cells, which was inversely associated with the prevalence of Breg cells in patients with CHB, indicating that the substantially weakened Th1 response in patients with CHB may at least be partially due to an augmented Breg cell population and its IL-10 production.

Treg cells have previously been described to play a role in the impaired immune response in chronic infectious diseases [5–7]; their production of IL-10 and interaction with Breg cells have also been reported [13, 19]. Our study demonstrated that depletion of Treg cells in the PBMCs of patients with CHB did not alter the Breg cell presence and IL-10 production. These results suggest that Breg cells might be the main source of IL-10 and that their differentiation is independent of Treg cells and Treg cell-produced IL-10 in CHB. However, the limitations of this study must be taken into account before drawing conclusions based solely on the current data. First, we only applied a "relative depletion" of Tregs using denileukin diftitox leaving a small amount in the treated PBMCs. In this circumstance, the influence of Tregs, despite much less in theory, may still exist in the system. Second, Treg cells are far more studied in CHB than Breg cells and are well known to be an important source of IL-10; thus, it is impossible to exclude the role of Treg cells in comprised anti-HBV immunity. Further and more extensive comparisons of Treg and Breg cells are warranted. Third, investigation of IL-10 production in Breg cell-depleted samples in CHB, which is a major limitation of our study, may provide further and clearer evidence on this issue.

In sum, we revealed significantly elevated IL-10-producing Breg cells in the periphery blood of patients with CHB in which a positive correlation between Breg and Treg cell prevalence and a negative correlation between Breg and Th1 cell prevalence were observed. Furthermore, increased IL-10 production from stimulated PBMCs was noted in CHB and correlated with Breg cell prevalence, and IL-10 production did not significantly change after

depletion of Treg cells as observed for Breg cells. Our data indicate Breg cells may be a major source of elevated IL-10 in CHB and, as novel immune regulatory cells, may play a pivotal role in fostering impaired anti-HBV immunity, thus contributing to the pathogenesis of CHB.

Conflict of interest   None.

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