Circulating and Liver Resident CD4$^+$CD25$^+$ Regulatory T Cells Actively Influence the Antiviral Immune Response and Disease Progression in Patients with Hepatitis B[1]

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CD4$^+$CD25$^+$ regulatory T cells (Treg) have been shown to maintain immune tolerance against self and foreign Ags, but their role in persistent viral infection has not been well-defined. In this study, we investigated whether and where CD4$^+$CD25$^+$ Treg contribute to the development of chronic hepatitis B (CHB). One hundred twenty-one patients were enrolled, including 16 patients with acute hepatitis B, 76 with CHB, and 29 with chronic severe hepatitis B. We demonstrated that in chronic severe hepatitis B patients, the frequencies of CD4$^+$CD25$^+$ Treg in both PBMC and liver-infiltrating lymphocytes were significantly increased and there was a dramatic increase of FoxP3$^+$-cell and inflammatory cell infiltration in the liver compared with healthy controls. In CHB patients, circulating CD4$^+$CD25$^+$ Treg frequency significantly correlates with serum viral load. In acute hepatitis B patients, circulating CD4$^+$CD25$^+$ Treg frequency was initially low and with time, the profile reversed to exhibit an increased number of circulating Treg in the convalescent phase and restored to normal levels upon resolution. In PBMC taken from infected patients, depletion of CD4$^+$CD25$^+$ Treg led to an increase of IFN-γ production by HBV-Ag-stimulated PBMC. In addition, CD4$^+$CD25$^+$ Treg were capable of suppressing proliferation of autologous PBMC mediated by HBV Ags, which probably reflects the generation of HBV-Ag-specific Treg in circulation and in the liver of HBV-infected patients. Together, our findings suggest that CD4$^+$CD25$^+$ Treg play an active role not only in modulating effectors of immune response to HBV infection, but also in influencing the disease prognosis in patients with hepatitis B. *The Journal of Immunology,* **2006, 177: 739–747.**

Hepatitis B virus (HBV)[4] is a noncytopathic, hepatotropic DNA virus that infects ~350 million people worldwide. Ten percent of adults and 90% of children become persistent HBV carriers after the infection, and 1–2 million people died annually as the consequence of infection with the virus, such as liver cirrhosis and hepatocellular carcinoma (1, 2). As the most affected area by HBV infection, China alone has 130 million carriers of HBV and 23 million patients with chronic hepatitis B (CHB) (3). It has been demonstrated that HBV-specific CTL and CD4$^+$ Th lymphocytes are essential for the control of HBV infection. Persistent carriers of HBV, however, fail to generate sufficient cellular immunity against the virus (4–6). The mechanisms responsible for the T cell tolerance in chronic HBV infection are not completely understood. It is possible that negative selection, immunological ignorance, peripheral anergy, and dysregulation of lymphokine production can all contribute to hyporesponsiveness in hosts which are continuously exposed to viral Ags (7). In addition, defective Ag presentation cell function in chronic HBV infection has been proposed (8, 9), though it is still a controversial issue (10). Knowing the extent to which each of these factors contributes to the hyporesponsiveness will have a profound influence attempting to break the immunological tolerance and thereby terminate the persistent viral infection.

It has been revealed that CD4$^+$CD25$^+$ regulatory T cells (Treg) play an important role in the maintenance of immunologic tolerance to both self and foreign Ags by suppressing aggressive T cell response (11–13). In human, this regulatory CD4$^+$ T cell population is identified by high expression of IL-2Rα chain (CD25), these cells represent 2–4% of peripheral blood CD4 T cells (14). Though the expression of CD45RO and CTLA-4/CD152 are also characteristics of CD4$^+$CD25$^+$ Treg (15), the forkhead/winged helix transcription factor (FoxP3) has been demonstrated to be a unique marker restricted to the Treg (16). CD4$^+$CD25$^+$ Treg are implicated in a range of disease states including cancer (17–20), allograft rejection (21, 22), allergy (23), autoimmune diseases (24, 25), and infectious diseases (26–28). Emerging evidence support the hypothesis that pathogenesis of persistent virus infections may be directly related to the levels of circulating CD4$^+$CD25$^+$ Treg or to the balance of the Treg vs effector T cells. In hepatitis C virus

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[4] Abbreviations used in this paper: HBV, hepatitis B virus; CHB, chronic hepatitis B; Treg, regulatory T cell; HCV, hepatitis C virus; AHB, acute hepatitis B; CSHB: chronic severe hepatitis B; TBIL, total bilirubin; PTA, prothrombin time activity; LIL, liver-infiltrating lymphocyte; HDV, hepatitis D virus; HGV, hepatitis G virus; Ct, cycle threshold; rh, recombinant human; hpf, high-powered field; lpf, low-powered field; HBsAg, hepatitis B surface Ag; HBcAg, hepatitis B core Ag; HBeAg, hepatitis B e Ag; ALT, alanine aminotransferase.

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(HCV) and HIV-infected subjects, $CD4^+CD25^+$ Treg may contribute to the persistence of the infections by down-regulating HCV- or HIV-specific T cell response (29–33). Recent data from two independent groups indicated that $CD4^+CD25^+$ Treg were linked to the chronicity of the disease in patients with CHB (34, 35). However, it is still controversial whether circulating $CD4^+CD25^+$ Treg frequency is increased in CHB patients and whether the frequency is correlated with HBV replication due to discrepant studies. More importantly, $CD4^+CD25^+$ Treg profiles in other states of HBV infection such as acute hepatitis B (AHB) and chronic severe hepatitis B (CSHB) remain unknown, and there is a paucity of data showing Treg profiles in the liver in these disease states.

In this study, we investigated the association between circulating $CD4^+CD25^+$ Treg and different disease states for HBV-infected patients. We found that the circulating Treg frequency was significantly increased in CSHB patients. Treg frequency was positively associated with serum HBV DNA load in CHB patients, and in AHB patients, the Treg frequency was initially at relatively low levels, then increased in the convalescent phase and restored to normal levels upon resolution. Treg were also found to be capable of inhibiting HBV-specific T cell responses in infected patients. Furthermore, we also observed the accumulation of Treg infiltrating in the liver of the CSHB and CHB patients. The results suggest that $CD4^+CD25^+$ Treg may play an active role not only in modulating the effectors of the cellular immune response to HBV infection in peripheral blood as well as in the liver, but also can influence the disease progression of hepatitis B in humans. The correlation between the $CD4^+CD25^+$ Treg profile and HBV replication or liver inflammation in chronic HBV infection suggests that the level of $CD4^+CD25^+$ Treg could be a potential prognostic factor and the liver may be a critical site for the specific inhibition of immune response by these cells.

Materials and Methods

Subjects

Blood samples were collected from 121 HBV-infected patients, including 16 patients with AHB, 76 with CHB, and 29 with CSHB. The diagnoses were complied with the diagnostic criteria of the 2000 Xi'an Viral Hepatitis Management Scheme issued by the Chinese Society of Infectious Diseases and Parasitology, and the Chinese Society of Hepatology, of the Chinese Medical Association (36). The standards for diagnoses of AHB, CHB, and CSHB have been described in detail previously (37, 38). Briefly, AHB patients are defined as those who displayed hepatitis B surface Ag (HBsAg)-negative conversion within 6 mo after the initial onset of symptoms due to HBV infection. A HBV carrier with chronic hepatitis required a clinical course of hepatitis B for >6 mo and may have exhibited symptoms or signs of hepatitis and abnormal hepatic function on this occasion, or with a histological confirmation. The diagnostic standard for CSHB mainly includes a history of CHB or liver cirrhosis with serum HBsAg positivity >6 mo, serum total bilirubin (TBIL) >10 times of normal level (> 171 μM/L), and prothrombin time activity (PTA) <40%. All patients were hospitalized or followed-up in our unit, Beijing 302 Hospital, from August 2004 to August 2005. No patients received anti-HBV agent or steroid 6 mo before sampling. The fresh blood samples from 42 healthy individuals were taken as controls. The liver tissues from 9 of the CSHB patients receiving liver transplantation and 5 of the healthy controls were collected for isolation of liver-infiltrating lymphocytes (LIL) and immunohistochemical analysis. In addition, biopsy liver specimens from 13 CHB patients were also collected for immunohistochemical analysis. Clinical characteristics of enrolled subjects were listed in Table I. Concurrence of HCV, hepatitis D virus (HDV), hepatitis G virus (HGV), HIV infections and autoimmune liver disease was excluded for all enrolled individuals. The study protocol was approved by the ethics committee of our unit, and written informed consent was obtained from each subject.

Isolation of PBMC and LIL

PBMC were isolated by Ficoll-Hypaque density gradient centrifugation either from 10 ml of heparinized blood or 1 ml of leukapheresis-derived PBMC-enriched sample. LIL were isolated based on the method we established previously (39) with minor modifications. In brief, liver tissues were carefully washed with Hank's solution containing 2% FCS and 1% EDTA to remove peripheral blood, whittled into small pieces, homogenized and pressed between two semifrosted microscopic slides. Dissociated cell suspension were mixed and sustained in ice for 15 min. The upper part of suspension were carefully recovered, passed through a 70-μm cell strainer (BD Labware), and underlaid onto Ficoll-Hypaque separation solution. LIL were then isolated by density gradient centrifugation. The viability of isolated cells was determined by trypan blue exclusive staining. In general, $>1 \times 10^6$ LIL could be obtained from 1 g of liver tissue and viable LIL were >95%.

T cell isolation

$CD4^+CD25^+$ Treg were isolated from PBMC or LIL by CD4-negative selection followed by CD25-positive selection, using $CD4^+CD25^+$-T cell isolation kit (Miltenyi Biotec), with a MidiMACS separator unit, according to the manufacturer's instructions. The purity of $CD4^+CD25^+$ Treg was ≥90% (see Fig. 1*a*) and the depletion efficiency of the Treg in $CD4^+CD25^-$ cell population was ≥85%, as determined by CD4/CD25 flow cytometric analysis. PBMC-Treg population was prepared by mixing CD4-depleted PBMC, obtained by negative $CD4^+$ cell selection from whole PBMC, and the $CD4^+CD25^-$ fraction, obtained by depleting the negatively selected $CD4^+$ cell fraction of $CD25^+$ cells, using positive selection beads.

Flow cytometric analysis

The Abs for FoxP3 staining was purchased from eBiosciences. Other Abs and isotypes were all purchased from BD Pharmingen. For staining of $CD4^+CD25^+$ Treg, PerCP-anti-CD3, FITC-anti-CD4, and PE-anti-CD25 were used. Only $CD4^+$ T cells expressing high level of CD25 were taken

Table I. *Clinical characteristics of studied subjects*

Group	AHB[a]	CHB	CSHB	Healthy Controls
Case	16	76	29	42
Sex (male)	15 (93.8%)	55 (72.4%)	13 (44.8%)	31 (73.8%)
Age (years)	34.3 ± 12.9	28.6 ± 7.9	44.9 ± 8.6	33.8 ± 9.6
ALT (U/L)[b]	>400 (1084 ± 470)	41–400 (98 ± 68)	>40 (64 ± 83)	<40 (21 ± 9)
TBIL (μM/L)	>17.1 (32 ± 27)	<85 (45 ± 3)	>171 (353 ± 176)	<17.1 (8 ± 4)
PTA[b]	>40% (77 ± 23%)	>65% (84 ± 17%)	<40% (20 ± 10%)	>65% ND
HBsAg positive	16	76	29	0
HBeAg positive	5 (41.7%)	76	9 (31.0%)	0
HBeAb positive	4 (33.3%)	0	4 (17.4%)	0
HBcAb positive	16	76	29	0
HBcAb IgM positive	16	0	0	0
HBV DNA positive	12 (75.0%)	76	12 (41.4%)	0
Recovery	<6 mo	>6 mo	>6 mo	

[a] Parameters at week 1 after the onset of illness.
[b] Median ± SD.

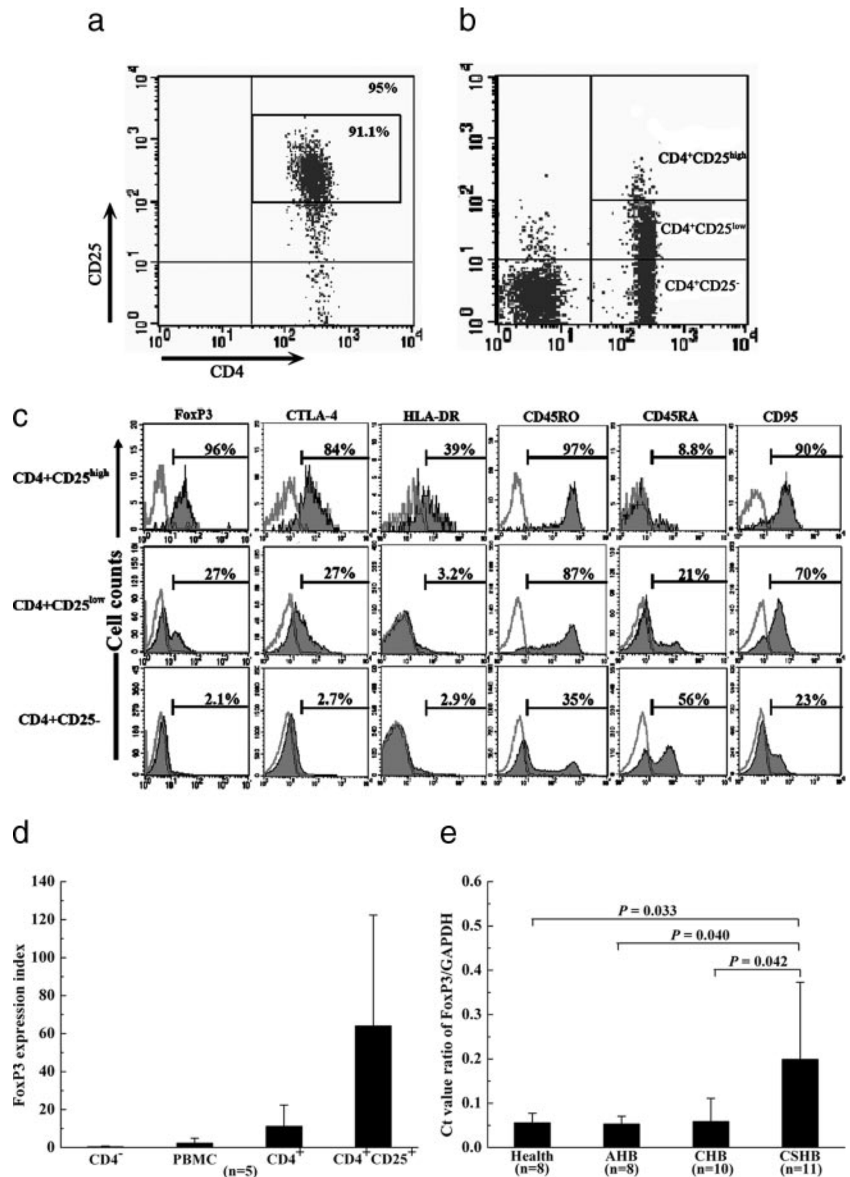

FIGURE 1. Characterization of $CD4^+CD25^+$ Treg. $CD4^+CD25^+$ Treg were isolated from PBMC by CD4-negative selection followed by CD25-positive selection, using magnetic beads (*a*). $CD4^+CD25^+$ Treg were gated from the $CD4^+CD25^{high}$ subset of $CD3^+$ T cells, defined by obtained with isotypic control Ab (the fluorescence intensity of CD25 $\geq 10^2$) (*b*). Analysis of a representative phenotypic profile of CTLA-4, HLA-DR, CD45RO, CD45RA, CD95, and FoxP3 from gated $CD4^+CD25^{high}$, $CD4^+CD25^{low}$, and $CD4^+CD25^-$ T cell subsets, respectively (*c*). Relative FoxP3 mRNA expression level was determined by real-time RT-PCR, with the GAPDH as an internal control. FoxP3 mRNA levels in total $CD4^-$, PBMC, total $CD4^+$, and $CD4^+CD25^+$ cell populations were presented as the expression index calculated by taking the Ct value ratio of FoxP3/GAPDH of CD4 $^+CD25^-$ T cell population as 1 (*d*). FoxP3 mRNA levels in various subjects were presented as the Ct value ratio of FoxP3/GAPDH from $CD4^+$ cells in patients with various statuses of hepatitis B and healthy controls. Value of $p = 0.029$ for multiple comparisons by Kruskal-Wallis H nonparametric test (*e*). Data are expressed as mean ± SD.

into count of $CD4^+CD25^+$ Treg (see Fig. 1*b*), as $CD4^+CD25^{high}$ cell population rather than $CD4^+CD25^{low}$ population exhibited high regulatory activity (14, 40). Other fluorochrome-conjugated Abs specific for the surface markers included PerCP-anti-HLA-DR, FITC-anti-CD95, FITC-anti-CD45RA, and allophycocyanin-anti-CD45RO. For intracellular staining of CTLA-4, the cells were first stained with FITC-anti-CD4 and PE-anti-CD25 Abs, then permeabilized and fixed using Cytofix/Cytoperm (BD Pharmingen) according to the manufacturer's instructions. PE-Cy5-anti-CTLA-4 was added during permeabilization. Similarly, intracellular staining of FoxP3 was conducted using PerCP-anti-CD3, allophycocyanin-anti-CD4, and PE-anti-CD25 for surface marker staining, followed by FITC-anti-FoxP3 staining after permeabilization. After staining, the cells were fixed in 1% of paraformaldehyde and three- or four-color flow cytometric analyses were performed using FACSCalibur and CellQuest software (BD Biosciences).

FoxP3 mRNA quantification

Cell RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The FoxP3 mRNA levels were determined in a real-time RT-PCR with the TaqMan (ABI Prism 7900 sequence detector; Applied Biosystems), using GAPDH as an internal control. The probe and primers used for FoxP3 were as follows: FAM-5′-CAC AGA TGA AGC CTT GGT CAG TGC CA-3′-TAMRA; 5′-GAG AAG CTG AGT GCC ATG CA-3′; and 5′-AGG AGC CCT TGT CGG ATG AT-3′. The probe and primers used for GAPDH were as follows: FAM-5′-AAG GTG AAG GTC GGA GTC AAC GGA TTT G-3′-TAMRA; 5′-CCA CAT CGC TCA GAC ACC AT-3′; and 5′-CCA GGC GCC CAA TAC G-3′ (41). The one-step RT-PCR kit (Qiagen) was used for RT-PCR amplification. The FoxP3 and GAPDH were amplified in two independent tubes with identical amount of template, the probe, the primers, and reaction buffer. Thermal cycling consisted of 55°C for 30 min, 95°C for 15 min, 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. Relative expression level of FoxP3 mRNA was described as the cycle threshold (Ct) value ratio of FoxP3/GAPDH. A RNA sample in aliquot from a healthy control was used in each RT-PCR as quality control.

IFN-γ detection and cellular proliferation assays

The effects of $CD4^+CD25^+$ Treg on IFN-γ production and cellular proliferation were tested with three sets of PBMC: (1) PBMC; (2) PBMC-Treg; and (3) PBMC-Treg + Treg at a ratio of 3:1, if sufficient PBMC sample could be obtained. A total of 3×10^5 effector cells were suspended in RPMI 1640 medium containing 10% FCS and seeded in a U-bottom 96-well plate in triplicate. To form the set of PBMC-Treg + Treg, 1×10^5 $CD4^+CD25^+$ Treg and 3×10^5 PBMC-Treg were mixed. The cells were stimulated either with 1 μg/ml anti-CD3 or 10 μg/ml HBV mixture Ags (containing 5 μg/ml purified homogenous HBsAg and 5 μg/ml recombinant hepatitis B core Ag (HBcAg)). Negative control was created by adding HCV mixture Ags (containing 5 μg/ml core Ag and 5 μg/ml envelope Ag; Invitrogen Life Technologies) or omitting stimulator. Each well was supplemented with the medium to a 200-μl volume. For IFN-γ detection, the plate was cultured at 37°C in 5% CO_2 atmosphere for 2 days before the harvest of supernatant. IFN-γ in the supernatant was measured with a commercial available ELISA kit (BioSource International) according to the

manufacturer's instructions. For proliferation test, the plate was cultured at the same condition for 5 days, with replenishment of fresh medium containing 20 U/ml recombinant human (rh) IL-2 (EuroCetus) at day 3. For the last 18 h the cultures were pulsed with 0.5 μCi/well [^{3}H]thymidine and harvested using a multichannel harvester. The amount of incorporated [^{3}H]thymidine was determined by liquid scintillation spectroscopy. Percentage suppression of cellular proliferation by depletion of $CD4^+CD25^+$ Treg from PBMC or by adding of $CD4^+CD25^+$ Treg to PBMC-Treg was calculated using the formula: $1 -$ (cpm in the presence of $CD4^+CD25^+$ Treg/(cpm in the absence of $CD4^+CD25^+$ Treg) $\times$ 100%.

Virological assessment

HBsAg, anti-HBs, total and IgM anti-HBc, hepatitis B e Ag (HBeAg), anti-HBe, anti-HCV, anti-HDV, anti-HGV and anti-HIV were determined by commercial enzyme immunoassay kits (Kewei Diagnostic), respectively. Serum HBV DNA was quantified by using a commercial real-time PCR kit (PG Biotech) according to the manufacturer's instruction. HBV DNA detection limit threshold is 5×10^2 copies/ml.

Immunohistochemical staining of paraffin-embedded liver tissue

Paraffin-embedded, formalin-fixed liver tissue was cut into 5-μm sections and placed on polylysine-coated slides. Slides were processed for immunohistochemistry as previously described (42). Ag retrieval was achieved via pressure cooking for 3 min in citrate buffer (pH 6.0). mAb of anti-human CD4 (Vector Laboratories), CD8 (DakoCytomation) and biotinylated goat anti-mouse Ig (Zhongshan Goldenbridge Biotech) were used for CD4 and CD8 staining, respectively. Goat-anti-human FoxP3 (Abcam) and biotinylated rabbit anti-goat Ig were used for FoxP3 staining. The slides were stained with hematoxylin following immunohistochemical staining. For the enumeration of positive lymphocytes, lymphocytes were counted in three high-powered fields (hpf; $\times$400) by two independent observers. Low-powered fields (lpf; $\times$100) were used for counting $FoxP3^+$ cells because of less abundance of the cells in the liver. For each sample, the mean percentage of positive cells was taken. Results were expressed as the median and range of all tested patents in each group.

Statistic analysis

All data were analyzed using SPSS version 13.0 for Windows software. The Kruskal-Wallis H test and Mann-Whitney nonparametric *U* test were used for comparison between groups. Bonferroni step down (Holm) correction was applied when multiple comparisons were made. Spearman correlation analysis was performed between the frequency of $CD4^+CD25^+$ Treg and other parameters. Value of $p < 0.05$ is considered as a significant difference.

Results

Phenotypic characterization of $CD4^+CD25^+$ Treg

As illustrated in Fig. 1*c*, the $CD4^+CD25^{high}$ subpopulation expressed a significantly higher level of intracellular FoxP3 and CTLA-4, surface HLA-DR, CD45RO, and CD95 but a lower level of surface CD45RA compared with $CD4^+CD25^{low}$ or $CD4^+CD25^-$ subpopulations. $CD4^+CD25^{low}$ subpopulation expressed significantly higher levels of intracellular CTLA-4 and FoxP3, surface CD45RO, and CD95 but similar levels of HLA-DR and lower levels of surface CD45RA than $CD4^+CD25^-$ subpopulation. FoxP3 expression was highly enriched in $CD4^+CD25^{high}$ subpopulation (96%) rather than subpopulations of $CD4^+CD25^{low}$ (27%) and $CD4^+CD25^-$ (2.1%) which is consistent with the current description of Treg. There is no significant difference in the phenotypic profile of $CD4^+CD25^+$ Treg among patients with AHB, CHB, CSHB, and healthy controls (data not shown).

FoxP3 mRNA is selectively expressed in $CD4^+CD25^+$ Treg

Because FoxP3 was reported as a unique marker primarily restricted to $CD4^+CD25^+$ Treg (16), we measured FoxP3 mRNA levels in HBV-infected patients with different disease statuses. To determine the fractional abundance of FoxP3 mRNA in various cell subsets, RNA extracted from PBMC and isolated $CD4^-$, $CD4^+$, $CD4^+CD25^-$, and $CD4^+CD25^+$ cell subsets were individually subjected to real-time PCR to quantify FoxP3 expression. The results showed the expression indexes of FoxP3 mRNA were 2.3, 0.4, 11.2, and 64.0 for PBMC, $CD4^-$, $CD4^+$, and $CD4^+CD25^+$ subpopulations, respectively (Fig. 1*d*). FoxP3 mRNA level in $CD4^+CD25^+$ Treg was 63-fold higher than the $CD4^+CD25^-$ cell subset, suggesting FoxP3 mRNA expression was highly enriched in $CD4^+CD25^+$ Treg. Considering the limitation of obtaining blood specimen in clinic, we measured FoxP3 mRNA expression from total $CD4^+$ T cells rather than the isolated $CD4^+CD25^+$ Treg. The results showed that the mean relative FoxP3 mRNA levels of $CD4^+$ T cells in healthy controls, AHB patients, CHB patients, and CSHB patients were 0.056 ± 0.021, 0.053 ± 0.017, 0.059 ± 0.053, and 0.199 ± 0.174, respectively (Fig. 1*e*). Therefore, FoxP3 mRNA is selectively expressed in $CD4^+CD25^+$ Treg and CSHB patients have higher levels of FoxP3 mRNA.

Increased circulating $CD4^+CD25^+$ Treg frequencies in chronic severe HBV-infected patients

Studies with humans have shown that the majority of $CD4^+$ T cells with regulatory function are segregated to the CD25 ^{high} subset (40), and the $CD4^+CD25^{high}$ subset exhibits more homogeneous characteristics of Treg than $CD4^+CD25^{low}$ subset (14). The $CD4^+CD25^{high}$ subset has been counted to represent the $CD4^+CD25^+$ Treg frequency in HBV and HIV-infected patients (35, 43). We thus determined the frequency of $CD4^+CD25^+$ Treg by measuring $CD4^+CD25^{high}$ subset (Fig. 2). Circulating $CD4^+CD25^+$ Treg frequency in CSHB patients has not been described previously. Our study showed that the frequency of the circulating Treg in these patients was significantly higher than healthy controls (CSHB patients vs healthy controls, median 6.59%, mean 6.65%, SD, 2.01 vs 3.57%, $3.60 \pm 0.75\%$, $p < 0.01$), CHB patients (vs 3.74%, $3.90 \pm 1.41\%$, $p < 0.01$), and AHB patients in the early acute phase (week 1) of their illness (vs 3.02%, $3.10 \pm 0.87\%$, $p < 0.01$). CHB patients had a higher percentage of circulating $CD4^+CD25^+$ Treg than early acute phase AHB patients ($p = 0.017$), but had no significant difference compared with healthy controls. The comparison of circulating $CD4^+CD25^+$ Treg frequencies between early acute phase AHB patients and healthy controls exhibited no significant difference (Fig. 2). We have also noticed that increased numbers of higher Treg was clinically associated with more severe illness of CSHB.

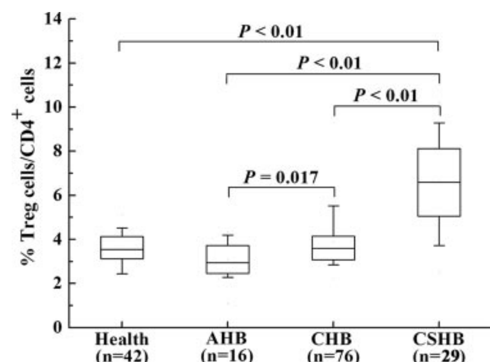

FIGURE 2. The frequency of $CD4^+CD25^+$ Treg in peripheral blood of various subjects. Data are expressed as box plots, in which the horizontal lines illustrate the 25th, 50th, and 75th percentiles of the frequencies of $CD4^+CD25^+$ Treg as measured by flow cytometry. The vertical lines represent the 10th and 90th percentiles. The *p* values for multiple comparisons were calculated by using the Kruskal-Wallis H nonparametric test. AHB patients were sampled in the early acute phase (week 1) of their illness. Value of $p < 0.001$ for multiple comparisons by Kruskal-Wallis H nonparametric test.

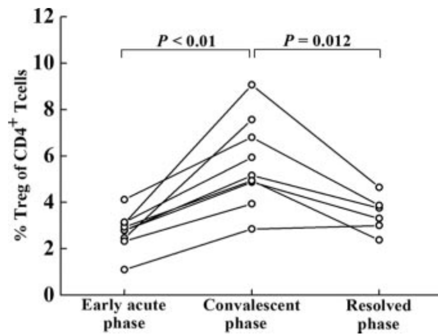

FIGURE 3. Dynamic expression of circulating CD4$^+$CD25$^+$ Treg in patients with AHB. The dynamic change of circulating CD4$^+$CD25$^+$ Treg frequency in nine AHB patients were analyzed in the various phases of illness. Value of $p = 0.002$ for multiple comparisons by Kruskal-Wallis H nonparametric test.

Thus, the results suggest that increase of the Treg is associated with poor prognosis and likelihood of CSHB.

Circulating CD4$^+$CD25$^+$ Treg is reversibly increased in AHB patients with decline of alanine aminotransferase (ALT) and HBsAg seroconversion

The level and potential role of Treg in AHB has not been well-demonstrated. We managed to collect weekly the blood samples from AHB patients to kinetically monitor the circulating Treg frequency, and analyze its association with viral and ALT parameters. In our study, nine AHB patients were followed-up with more than three time points, including six patients with more than six time points. To give a clear presentation, the sampling times were catalogued into three phases as follows: 1) early acute phase: the first week after onset of illness ($n = 9$); 2) convalescent phase: primary occurrence of both ALT level normalization and HBsAg seroconversion ($n = 9$, average week was 9.1 ± 3.2); 3) resolved phase: at least 8 wk after both ALT normalization and HBsAg-negative conversion ($n = 6$, average week was 21.8 ± 7.3). As shown in Fig. 3, the Treg frequency of the AHB patients was relatively low in the early acute phase (2.75 ± 0.84%), significantly increased in the convalescent phase (5.68 ± 1.90%), and restored to normal level in the resolved phase (3.49 ± 0.78%). The data suggest that circulating Treg is reversibly increased in AHB patients with decline of ALT and HBsAg seroconversion.

Circulating CD4$^+$CD25$^+$ Treg frequency is associated with HBV DNA load but not with ALT levels in CHB patients

CHB patients were divided into two groups based on their serum HBV DNA load or ALT level, respectively. A total of 10^7 copies/ml was taken as the cutoff value of HBV DNA was based on the report that this value could be the threshold in evaluation of HBV-specific CD8 T cell response in CHB patients (6). A total of 100 U/L was taken as the cutoff value of ALT level was due to this value close to median value (98 U/L) of ALT level of 76 CHB patients enrolled in the study. As shown in Fig. 4, *a* and *b*, patients with serum HBV DNA load $>10^7$ copies/ml had a higher mean percentage of circulating CD4$^+$CD25$^+$ Treg than patients with serum HBV DNA load $<10^7$ copies/ml (4.45 ± 1.83% vs 3.43 ± 0.63%, $p = 0.002$) and healthy controls (vs 3.60 ± 0.75%, $p = 0.033$); patients with serum ALT level >100 IU/ml and <100 IU/ml had no significant difference in circulating CD4$^+$CD25$^+$ Treg frequency. Accordingly, Spearman analysis showed that there was a positive correlation between circulating CD4$^+$CD25$^+$ Treg and serum HBV DNA load ($r = 0.32$, $p = 0.006$), and no correlation between circulating CD4$^+$CD25$^+$ Treg and serum ALT level (Fig. 4, *c* and *d*). The results suggest that increased circulating CD4$^+$CD25$^+$ Treg may be associated with a negative immune response leading to poor viral clearance in CHB patients.

Effect of CD4$^+$CD25$^+$ Treg on Ag-specific and nonspecific cellular responses

To investigate whether there is a specific suppressive effect of CD4$^+$CD25$^+$ Treg on the cellular response to HBV, we measured IFN-γ production and cellular proliferation. PBMC, PBMC-Treg (depletion of Treg), PBMC-Treg + Treg (depletion of Treg and reconstituted Treg at a ratio of 3:1) were used as effector cells with HBV-antigenic or anti-CD3 stimulation, respectively. Depletion of CD4$^+$CD25$^+$ Treg significantly enhanced the IFN-γ production of the effector cells from infected patients, while the response to anti-CD3 is relatively intact (Fig. 5*a*). The addition of CD4$^+$CD25$^+$ Treg significantly suppressed the IFN-γ production of the effector

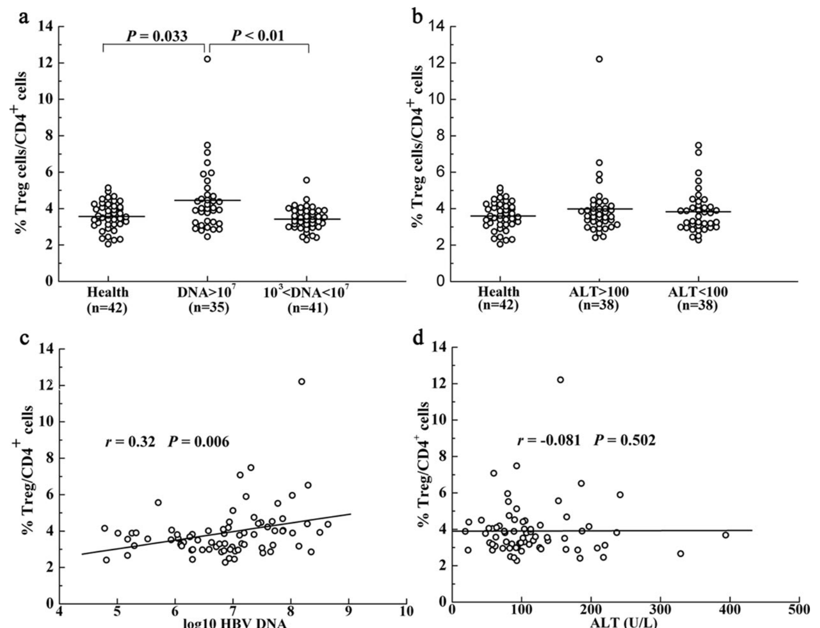

FIGURE 4. Association between circulating CD4$^+$CD25$^+$ Treg frequency and serum HBV DNA load or ALT level in patients with CHB. All patients were positive for serum HBsAg and HBeAg. Patients were divided into two groups based on serum HBV DNA load (*a*) or ALT level (*b*). The significance of difference between circulating CD4$^+$CD25$^+$ Treg frequency and serum HBV DNA or ALT level was analyzed using nonparametric Mann-Whitney *U* test. The correlation between circulating CD4$^+$CD25$^+$ Treg frequency and serum HBV DNA load (*c*) or ALT level (*d*) was analyzed using Spearman correlation analysis.

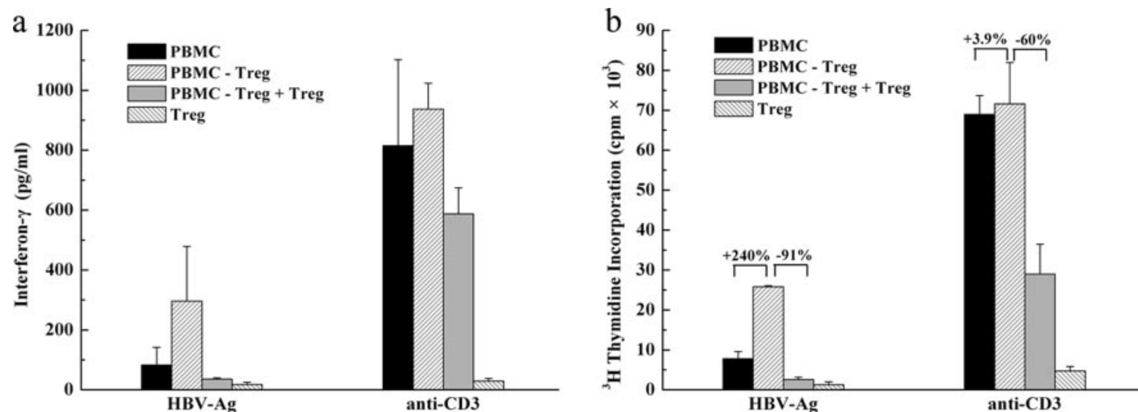

FIGURE 5. Inhibition of effector T cells against HBV by $CD4^+CD25^+$ Treg from infected patients. Blood was sampled from patients with AHB during weeks 3–5 of illness. PBMC, PBMC-Treg (depletion of Treg), PBMC-Treg + Treg (depletion of Treg and add back Treg at a ratio of 3:1) were used as effector cells, respectively. The effector cells were inoculated in 96-well plate in triplicate at 30,000 cells/well, and stimulated by 10 μg/ml HBV Ags or 1 μg/ml anti-CD3 Ab. For IFN-γ production, the cells were cultured for 48 h. IFN-γ from the supernatant was then harvested and measured by ELISA. Data were from five patients with AHB. Value of $p = 0.006$ for multiple comparisons by Kruskal-Wallis H nonparametric test (*a*). For cellular proliferation, the cells were cultured for 6 days. For the last 18 h, the cultures were pulsed with 0.5 μCi/well [^{3}H]thymidine. The results were from a representative patient with AHB. Value of $p = 0.879$ for multiple comparisons by Kruskal-Wallis H nonparametric test (*b*). Data represent the mean ± SD.

cells stimulated by HBV Ags, compared with the effector cells which were nonspecifically stimulated with anti-CD3. $CD4^+$ $CD25^+$ Treg alone only had a background level of IFN-γ under the stimulation of HBV Ags or Anti-CD3. Depletion of $CD4^+CD25^+$ Treg significantly enhanced the PBMC proliferation stimulated by HBV Ags but not by anti-CD3. Adding $CD4^+CD25^+$ Treg almost completely suppressed the PBMC proliferation stimulated by HBV Ags, and partially suppressed the PBMC proliferation stimulated by anti-CD3 (Fig. 5*b*) $CD4^+CD25^+$ Treg alone had very low levels of [^{3}H]thymidine incorporation in response to HBV Ag or Anti-CD3 stimulation. HCV Ags had no effect on stimulating IFN-γ production and cellular proliferation (data not shown). Therefore, the Ag-specific T cell response to HBV is more significantly suppressed by Treg, suggesting the possible generation of HBV-Ag-specific Treg in HBV-infected patients.

Increased Treg is associated with severity of inflammation in liver

Inhibition of primed anti-HBV T cell response by patients' $CD4^+$CD25 Treg raises the possibility that the site of inhibition may occur at liver tissues, a local inflammatory site, where the effector T cells against HBV might accumulate. To investigate whether $CD4^+CD25^+$ Treg frequency may be also accumulated in the liver for limiting local inflammation caused by increase of effector T cells against HBV, LIL were isolated from the liver tissue of nine CSHB patients receiving liver transplantation and five healthy donors as controls. CSHB patients had a dramatic increase of LIL number compared with healthy controls. The frequency of $CD4^+CD25^+$ Treg in LIL was detected by flow cytometric analysis, gated from total LIL, $CD3^+$ or $CD4^+$ T cell populations of LIL, respectively. In comparison with healthy controls, CSHB patients showed significantly higher frequencies of $CD4^+CD25^+$ Treg in the total LIL of liver tissue (patients vs healthy controls, median 0.57%, mean 0.65%, SD, 0.31 vs 0.20%, 0.24 ± 0.14%, $p = 0.004$) or $CD3^+$ T cell population of LIL (0.95%, 0.99 ± 0.43% vs 0.58%, 0.54 ± 0.15%, $p = 0.029$) (Fig. 6*a*).

To visualize the severity of inflammation and distribution of $FoxP3^+$ Treg, immunohistochemical staining was performed. As shown in Fig. 6, *b* and *c*, CSHB patients exhibited a dramatic increased infiltration of $CD8^+$, $CD4^+$ and $FoxP3^+$ LIL compared with healthy controls (patients vs healthy controls, median 84.5, mean 90.2, SD 31.1 vs 11.0, 13.7 ± 5.1/hpf for $CD8^+$ LIL; 73.0, 75.7 ± 18.5 vs 19.0, 17.1 ± 12.8/hpf for $CD4^+$ LIL; and 27.0, 32.4 ± 13.5 vs 1.0, 0.6 ± 0.5/lpf for $FoxP3^+$ LIL; P all <0.01). CHB patients had a medium increased infiltration of $CD8^+$LIL (25.0, 29.1 ± 17.7), $CD4^+$ LIL (32.0, 33.5 ± 14.3), and $FoxP3^+$ LIL (6.0, 10.0 ± 8.6) compared with healthy controls ($p < 0.01$, $p = 0.026$, $p = 0.01$, respectively), but the numbers were significantly lower compared with CSHB patients (P all <0.01). The infiltrating cells appear to accumulate near the portal tract. Together, the results confirmed the increase of Treg inside liver tissues of CHB and CSHB patients and suggested that increased Treg at the inflammatory site is associated with chronicity and severity of liver inflammation.

Discussion

HBV infection is a global health problem. The mechanisms for immune evasion of HBV to some patients leading to chronic infection are still unclear. The generation of Treg may be a normal process that occurs to prevent immunopathological damage and thereby contributes to viral persistence. In contrast, pathogens may also have evolved strategies to establish conditions favoring Treg priming, recruitment and survival (28). It is necessary to clarify whether and where Treg from infected patients inhibit HBV-specific T cells in various states of HBV infection. In this study, we compared the phenotype, frequency, and function of $CD4^+CD25^+$ Treg in peripheral blood of AHB, CHB, and CSHB patients and analyzed the correlation with disease status.

We observed that CSHB patients had a significant increase of $CD4^+CD25^+$ Treg frequency, both in peripheral blood and in LIL, compared with healthy controls. The absolute number of liver-infiltrating $CD4^+CD25^+$ Treg is much higher in CSHB patients than in healthy controls considering much greater numbers of total LIL in the patients. Immunohistochemical analysis also verified a dramatic increase of $FoxP3^+$ cells and inflammatory cells infiltrating in the liver of CSHB patients. Increased Treg with age has recently been reported (44). Therefore, we analyzed the circulating Treg frequency of 10 healthy subjects age 40–55 (47.3 ± 5.6) years old from 42 healthy controls in our study. No significant increase of the Treg frequency was observed in these people (3.90 ± 0.80%). In addition, we additionally studied eight patients with stable hepatitis B-related liver cirrhosis. Their mean age was

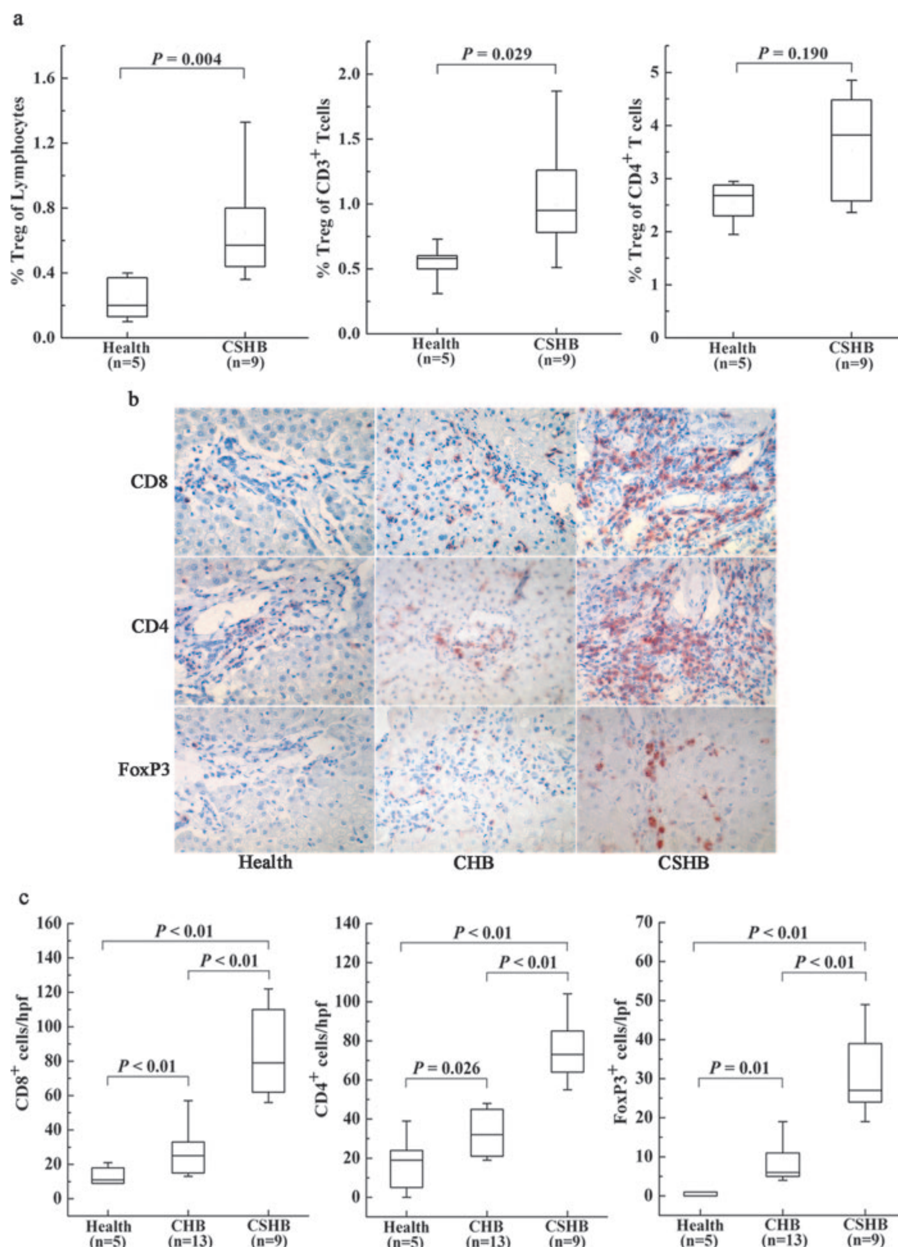

FIGURE 6. Accumulation of Treg inside liver tissue of CSHB patient. The frequency of $CD4^+CD25^+$ Treg in LIL of healthy controls and patients with CSHB were detected by flow cytometric analysis (*a*). Immunohistochemical staining for CD8, CD4, and FoxP3 within paraffin-embedded liver tissue of healthy controls, CHB, and CSHB patients (*b*). Numbers of $CD8^+$, $CD4^+$, and $FoxP3^+$ LIL in various subjects (*c*). Please note that different powered fields were used in cell counting. Data are expressed as median and range (from the 10th percentile to the 90th percentile). Value of $p < 0.001$ for multiple comparisons by Kruskal-Wallis H nonparametric test. hpf, ×400; ldf, ×100. Positive cells were stained red color.

45.6 ± 13.2 years old and mean ALT was 62 ± 35 U/L. Mean circulating $CD4^+CD25^+$ Treg frequency of these patients was 4.21 ± 1.11%, slightly higher than that of healthy controls, but significantly lower than that of CSHB patients. Immunohistochemical analysis showed that their intrahepatic $FoxP3^+$ cells and CD4, CD8 T cells were also significantly lower compared with CSHB patients (data not shown). Thus, CSHB patients have an intrinsic characteristic of $CD4^+CD25^+$ Treg expression independent of age and nondecompensated liver cirrhosis.

Our study did not show significant increase of $CD4^+CD25^+$ Treg in peripheral blood of CHB patients totally compared with healthy controls. This result is identical to the data from Bertoletti's group (34), but differs from the data from Janssen's group (35). A difference in methods, reagents, and samples used in the study might account for the discrepancies. No obvious correlation was observed between ALT and HBV DNA in these patients. Earlier studies did not find a significant association between circulating $CD4^+CD25^+$ Treg frequency and serum HBV DNA load in CHB patients (34, 35). However, we observed that CHB patients with $>10^7$ copies/ml serum HBV DNA had a significant increase of circulating $CD4^+CD25^+$ Treg compared with healthy controls. Further analysis showed that there was a positive correlation between circulating $CD4^+CD25^+$ Treg frequency and serum HBV DNA load, suggesting that the increase of the Treg correlates with the increase of HBV replication. This observation is partly supported by previous study in which an association between the presence of serum HBeAg and an increased percentage of circulating $CD4^+CD25^+$ Treg was observed in CHB patients (35). In our study, all CHB patients were HBeAg positive. We did not find association between circulating $CD4^+CD25^+$ Treg and serum ALT level, identical to previous reports (34, 35). By in situ immunohistochemical staining, we found a significantly increased infiltration of $FoxP3^+$ cells and inflammatory cells in the liver of CHB patients compared with healthy controls. These data imply that Treg are possibly involved in the mechanism of the viral persistence.

It is well-known that AHB patients are not easily observed in the clinic because 90–95% of adult patients generally have a spontaneously self-limited acute hepatitis without obvious manifestation

and often develop the convalescence period through a short-term acute phase when they start to see doctors. Our study showed a unique profile of circulating $CD4^+CD25^+$ Treg frequency in these patients, i.e., relatively low in the early acute phase, significantly increased in the convalescent phase, and restored to normal in the resolved phase. A possible interpretation is that this profile reflects compartmentalization of the Treg from periphery to the liver in the early acute phase, followed by redistribution once inflammation is resolved. This interpretation needs to be tested by the analysis of intrahepatic Treg, which is, however, prevented by difficulty in acquiring biopsy liver tissue in the clinic. Interestingly, although both CSHB and early acute phase AHB patients have strong immune responses, the $CD4^+CD25^+$ Treg profiles are quite different. CSHB patients exclusively had a high level of $CD4^+CD25^+$ Treg both in peripheral blood and liver, while AHB patients had a relatively low frequency of the circulating Treg in the early acute phase. We speculate that this difference is an important factor which influences the disease progression, and may present an important marker for patients with HBV infection.

The Ag-specific regulation of T cell immunity encompasses 3 stages: Ag presentation, Treg activation, and recognition of target cells by Treg (45). Though the effect of $CD4^+CD25^+$ Treg is generally nonspecific or in a bystander manner, preferential inhibition of Ag-specific T cell response has been observed in some cases, including in human HBV infection (35). We also found that the depletion of $CD4^+CD25^+$ Treg led to the increase of HBV Ag-stimulated IFN-γ production and cellular proliferation of PBMC from HBV-infected patients, and coculture of $CD4^+CD25^+$ Treg with effector cells significantly suppressed HBV Ag-stimulated IFN-γ production and cellular proliferation. It is notable that Ag-specific T cell response to HBV Ags is more significantly suppressed by Treg, suggesting the possible generation of HBV Ag-specific functional Treg in HBV-infected patients. This is supported by a previous study, in which depletion of $CD4^+CD25^+$ Treg enhanced cellular proliferation stimulated by HBcAg rather than by tetanus toxin (35). Thus, $CD4^+CD25^+$ Treg are capable of modulating an HBV-specific T cell response in HBV infection and may contribute to chronicity of the disease.

We showed that FoxP3 mRNA expression is relatively unique to $CD4^+CD25^+$ cell population of PBMC. This offer a practical method to measure FoxP3 mRNA expression in $CD4^+$ cell populations or even total PBMC rather than isolating $CD4^+CD25^+$ cell population to evaluate $CD4^+CD25^+$ Treg activity and predict a clinical outcome. Indeed, our results showed that FoxP3 mRNA levels of $CD4^+$ T cells corresponded to the circulating frequencies of $CD4^+CD25^+$ Treg in various subjects. $FoxP3^+$ Treg are specific for Treg (46), but their distribution inside liver has not been clarified in HBV-infected patients. Our study clearly showed an accumulation of $FoxP3^+$ cells is associated with the severity of inflammation inside the liver and located near inflammatory cells, suggesting its role in the inhibition of effector cells during inflammation. Future study is needed to further clarify the cause-and-effect relationship and underlying mechanisms between the increased Treg and changes of liver pathology in these patients.

In our study, the limited availability in collecting sufficient amount of blood specimens restricted our $CD4^+CD25^+$ Treg function analysis from CSHB patients. Another hurdle is that most CHB patients failed to generate an effective response to HBV-Ag stimulation, resulting in the difficulty of evaluating specific immunosuppression of $CD4^+CD25^+$ Treg in these patients. Though Treg-mediated suppression is generally mediated by cell-cell-dependent contact, the precise mechanism of the suppression in vivo remains to be further identified.

In conclusion, we report here the phenotype, frequency and functional property of $CD4^+CD25^+$ Treg in patients with various subgroups of HBV infection. Especially, we depict for the first time the profiles and correlation of circulating $CD4^+CD25^+$ Treg with disease progression in AHB and CSHB patients. We have also revealed accumulation of Treg inside liver tissues of CSHB and CHB patients. Such Treg can significantly inhibit HBV-specific T cell response. The results further extend our knowledge in understanding the immunomodulatory role of $CD4^+CD25^+$ Treg in various states of HBV infection and its use as a potential prognostic marker. Selective depletion or inhibition of the Treg could be developed into a new effective strategy to break immune tolerance in chronic HBV infection.

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Disclosures

The authors have no financial conflict of interest.

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