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

Dongping Xu,\(^2\)* Junliang Fu,\(^2\)* Lei Jin,\(*\) Hui Zhang,\(*\) Chunbao Zhou,\(*\) Zhengsheng Zou,\(*\) Jing-Min Zhao,\(*\) Bin Zhang,\(*\) Ming Shi,\(*\) Xilai Ding,\(*\) Zirong Tang,\(*\) Yang-Xin Fu,\(^3\)+ and Fu-Sheng Wang\(^3\)+\(^*\)

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-\(\gamma\) 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)\(^1\) 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 the 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-2R\(\alpha\) 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. Concordance 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 was mixed and maintained in ice for 15 min. The upper part of suspension was 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 × 10⁸ 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. 1a) 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 Abs were used. Only CD4⁺ T cells expressing high level of CD25 were taken

Table I. Clinical characteristics of studied subjects

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<tr>
<th>Group</th>
<th>AHB</th>
<th>CHB</th>
<th>CSHB</th>
<th>Healthy Controls</th>
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<tr>
<td>Sex (male)</td>
<td>15</td>
<td>55</td>
<td>13</td>
<td>31</td>
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<tr>
<td>Age (years)</td>
<td>34.3±12.9</td>
<td>28.6±7.9</td>
<td>44.9±8.6</td>
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<td>ALT (U/L)</td>
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<td>41–400 (98±68)</td>
<td>&gt;40 (64±83)</td>
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<td>TBIL (μM/L)</td>
<td>&gt;17.1 (32±27)</td>
<td>&lt;85 (45±3)</td>
<td>&gt;171 (353±176)</td>
<td>&lt;17.1 (8±4)</td>
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<td>PTA (U/L)</td>
<td>&gt;40% (77±23%)</td>
<td>&gt;65% (84±17%)</td>
<td>&lt;40% (20±10%)</td>
<td>&gt;65% ND</td>
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<td>16</td>
<td>76</td>
<td>29</td>
<td>42</td>
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<tr>
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<td>5 (41.7%)</td>
<td>76</td>
<td>9 (31.0%)</td>
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</tr>
<tr>
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<td>4 (17.4%)</td>
<td>0</td>
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<tr>
<td>HBCAb positive</td>
<td>16</td>
<td>76</td>
<td>29</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>HBV DNA positive</td>
<td>12 (75.0%)</td>
<td>76</td>
<td>12 (41.4%)</td>
<td>0</td>
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<tr>
<td>Recovery</td>
<td>&lt;6 mo</td>
<td>&gt;6 mo</td>
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* Parameters at week 1 after the onset of illness.

* 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 CD4⁺ T cells, defined by the CD4⁺CD25⁺high subpopulation, Treg were isolated from PBMC, and FoxP3 from gated CD4⁺CD25⁺high, CD4⁺CD25⁺low, and CD4⁺CD25⁻ T cell subsets, respectively (c). Relative FoxP3 mRNA expression levels were 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 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.

IN-$\gamma$ detection and cellular proliferation assays

The effects of CD4⁺CD25⁺ Treg on IN-$\gamma$ 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 $3 \times 10^5$ 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, 1 $\times 10^5$ PBMC-Treg were mixed. The cells were stimulated either with 1 $\mu$g/ml anti-CD3 or 10 $\mu$g/ml HBV mixture Ags (containing 5 $\mu$g/ml purified homogenous HBsAg and 5 $\mu$g/ml recombinant hepatitis B core Ag (HBcAg)). Negative control was created by adding HCV mixture Ags (containing 5 $\mu$g/ml core Ag and 5 $\mu$g/ml envelope Ag; Invitrogen Life Technologies) or omitting stimulator. The plate was cultured at 37°C for 2 days before the harvest of supernatant. IFN-$\gamma$ detection, IFN-$\gamma$-producing cells, and cellular proliferation assays were performed using RealTime-Glo (Promega) and CellTiter-Blue (Promega).
The results showed the expression indexes of FoxP3 mRNA were individually subjected to real-time PCR to quantify FoxP3 expression. As illustrated in Fig. 1, the CD4\(^{+}\) phenotypic profile of CD4\(^{+}\) T cells was analyzed using a commercial real-time PCR kit (PG Biotech) according to the manufacturer’s instruction. HBV DNA detection limit threshold is 5 \( \times 10^9 \) copies/ml.

**Immunohistochemical staining of paraffin-embedded liver tissue**

Paraffin-embedded, formalin-fixed liver tissue was cut into 5-\(\mu\)m 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 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 patients 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 \( t \) 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. 1c, 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\(^{+}\) low subpopulations. CD4\(^{+}\)CD25\(^{+}\) low subpopulation expressed significantly higher levels of intracellular CTLA-4 and FoxP3, surface CD45RA, 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\(^{+}\) (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. 1d). 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 \( \pm 0.021 \), 0.053 \( \pm 0.017 \), 0.059 \( \pm 0.053 \), and 0.199 \( \pm 0.174 \), respectively (Fig. 1e). 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\(^{+}\) 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.
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 10⁷ 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. 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 >10⁷ copies/ml had a higher mean percentage of circulating CD4⁺CD25⁺ Treg than patients with serum HBV DNA load <10⁷ 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. 5a). The addition of CD4⁺CD25⁺ Treg significantly suppressed the IFN-γ production of the effector...
cells were cultured for 6 days. For the last 18 h, the cultures were pulsed with 0.5 μg/ml CD4 Ags or Anti-CD3. Depletion of CD4 Treg increased infiltration of CD8 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 [3H]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.

**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 [3H]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.

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). To visualize the severity of infiltration and distribution of FoxP3+ Treg, immunohistochemical staining was performed. As shown in Fig. 6, a, b, and c, CSHB patients exhibited a dramatically increased infiltration of CD8+ 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/lpf for CD8+ LIL; 73.0, 75.7 ± 18.5 vs 19.0, 17.1 ± 12.8/lpf 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, 35.5 ± 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...
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 Bertolletti’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 offers 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 immune-suppression 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 stages 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
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References


