Dissection of a Circulating and Intrahepatic CD4⁺Foxp3⁺ T-Cell Subpopulation in Chronic Hepatitis B Virus (HBV) Infection: A Highly Informative Strategy for Distinguishing Chronic HBV Infection States

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Background. The definition of CD4⁺Foxp3⁺ regulatory T cells (Tregs) is challenging as it relates to chronic hepatitis B virus (HBV) infection. Recently, the heterogeneity of human CD4⁺Foxp3⁺ T cells has been confirmed.

Methods. Three circulating CD4⁺Foxp3⁺ T-cell subpopulations in chronic HBV patients were identified, and their frequencies associated with clinical parameters were analyzed. Antigen specificity of Tregs was further studied.

Results. We found that circulating and intrahepatic CD4⁺CD45RA⁻Foxp3hi–activated Tregs (aTregs) were selectively increased in patients with chronic active hepatitis B and acute-on-chronic liver failure (ACLF) but not in asymptomatic carriers. The aTreg frequency was strongly correlated with HBV DNA load but not liver damage. In both peripheral blood mononuclear cells and livers, ACLF patients showed a dramatically elevated frequency of interleukin 17A–secreting CD45RA⁻Foxp3lo nonsuppressive T cells (non-Tregs), which were shown to be associated with severe liver damage. Interestingly, an HBV core antigen (HBcAg)–derived peptide could preferentially expand CD4⁺CD25⁺Foxp3⁺T cells and aTregs in HLA-DR9⁺ chronic active hepatitis B patients, and these Tregs required ligand-specific reactivation for suppressor function.

Conclusions. The delineation of a CD4⁺Foxp3⁺ T-cell subpopulation is a highly informative strategy for distinguishing different chronic HBV infection states. HBcAg-derived peptides may be responsible for activation of Tregs that, in turn, specifically inhibit anti-HBV immune response but not liver inflammation.

Hepatitis B virus (HBV) infection is a global health problem. Worldwide, 400 million people suffer from chronic HBV infection and approximately 1 million people die annually from HBV-related diseases [1]. It has been demonstrated that chronic HBV pathogenesis is largely attributed to the host immune system, in particular, the cellular immune response [2]. However, the exact cellular mechanism(s) is not yet completely understood.

Recently, CD4⁺Foxp3⁺ regulatory T cells (Tregs) have become a research focus in diseases that involve the immune mechanism. CD4⁺Foxp3⁺ Tregs have been linked to chronicity of HBV infection [3–6] and were inducible from human peripheral blood mononuclear cells (PBMCs) exposed to HBV antigen [7, 8]. Unfortunately, several studies have been based on Tregs identified by CD25high and/or Foxp3 expression, which has been identified as an insufficient marker of Tregs because both can be induced by general T-cell receptor stimulation in conventional human T cells [9]. Thus, CD4⁺CD25high or CD4⁺CD25⁺Foxp3⁺ T cells represent a heterogeneous population of Tregs and recently activated...
Figure 1. Separation of circulating CD4\(^+\)Foxp3\(^+\) T-cell subpopulations in chronic hepatitis B virus patients by phenotype, proliferative state, and cytokine production. A, Surface CD4 and CD45RA and intracellular Foxp3 expression. Representative fluorescence-activated cell sorting (FACS) histogram
effect T cells, especially in samples obtained from patients with persistent viral antigen exposure.

Recently, Miyara et al [10] showed that human CD4\(^+\)Foxp3\(^+\) T cells comprised 3 phenotypically and functionally distinct subpopulations: CD45RA\(^+\)Foxp3\(^{lo}\) resting Tregs (rTregs), CD45RA\(^-\)Foxp3\(^{hi}\) activated Tregs (aTregs), and cytokine-secreting CD45RA\(^-\)Foxp3\(^{lo}\) nonsuppressive T cells (non-Tregs). It was suggested that the delineation of Treg cell subpopulations would prove highly informative when assessing the dynamics of Treg differentiation under distinct physiological and disease conditions. Hence, it is necessary to examine the role of rTregs, aTregs, and non-Tregs subpopulations in chronic HBV infection and their respective proportions.

Here we report that the frequency of aTregs was selectively elevated in patients with HBV-associated acute-on-chronic liver failure (ACLF) and chronic active hepatitis B (CAH) but not in asymptomatic HBV carriers (AsCs). In contrast to CAH patients and AsCs, ACLF patients showed a significantly higher frequency of interleukin 17A (IL-17A)–secreting non-Tregs, which were shown to be associated with severe liver damage. Furthermore, we found that the proportion of aTregs was strongly positively correlated with serum HBV DNA load but not with serum alanine aminotransferase (ALT) or liver histological activity index (HAI) scores. Interestingly, an HBV core antigen (HBcAg) derived peptide preferentially expanded CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells, as well as aTregs, in CAH patients carrying an HLA-DR9 allele, and these Tregs required ligand-specific activation to exert their suppressive activity. The findings provide new insights into the role and potential generation mechanism of Tregs in chronic HBV infection.

**MATERIALS AND METHODS**

**Patients and Healthy Controls**

The basic characteristics of all patients and healthy controls are listed in Supplementary Table 1. The diagnostic criteria were based on the 2000 Xi’an Viral Hepatitis Management Scheme issued by the Chinese Society of Infectious Diseases and Parasitology and the Chinese Society of Hepatology from the Chinese Medical Association [11]. Exclusion criteria included coinfection with human immunodeficiency virus or hepatitis C virus, diagnosis with autoimmune or alcoholic hepatitis, or current immunosuppressive or antiviral therapy. Heparinized peripheral blood samples were obtained from AsCs (n = 23), patients with ACLF (n = 19), patients with CAH (n = 26), and healthy controls (n = 24). Of these, 11 CAH patients and 10 ACLF patients underwent percutaneous needle liver biopsy for histological evaluation. The degree of hepatic inflammation was graded using the modified HAI described by Scheuer [12]. The liver biopsy samples of 2 AsCs patients and 3 CAH patients and the liver tissues from 1 ACLF patient and 2 healthy controls receiving liver transplantation were used to isolate liver-infiltrating lymphocytes (LLIs). HLA-DR genotyping was carried out with the Morgan HLA SSP DRB typing kit (Texas BioGene ). The study was approved by the ethics committee of the Third Military Medical University, and informed consent was obtained from all patients prior to their inclusion in this study.

**Flow Cytometry**

Surface markers and intracellular cytokines were detected as previously described [10, 13] and are detailed in the Supplementary Materials and Methods.

**Synthetic Peptides**

Peptides used in this study were synthesized by the Central Unit of Peptide Synthesis at the Shanghai Sangon Biological Engineering Technology and Services Co. (China). The purity (>95% in all cases) and identity of these peptides were determined by reverse-phase high-performance liquid chromatography and mass spectroscopic analysis, respectively.

**In Vitro HBV-Specific CD4\(^+\)Foxp3\(^+\) Treg Cultures**

PBMCs from CAH patients were stimulated for 10 days with HBV peptides (10 µg/mL) or irrelevant peptide in Roswell Park Memorial Institute 1640 medium containing 10% human AB sera, 2 mML-glutamine, 1 µg/mL anti-CD28, and 500 U/mL recombinant interleukin 2 (rIL-2; Peprotech Asia), followed by twice weekly rIL-2.

**Suppression Assay With In Vitro–Expanded Tregs**

CD4\(^+\)CD25\(^+\) (>90%) and CD4\(^+\)CD25\(^−\) (>90%) T cells were isolated from HBcAg\(_{81-105}\) or TT\(_{947-967}\) peptide-specific CD4\(^+\)Foxp3\(^+\) Treg cultures using the CD4\(^+\)CD25\(^+\) regulatory T-cell isolation kit (Miltenyi Biotec), according to the...
Figure 2. Frequencies in the 3 subpopulations of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells in peripheral blood mononuclear cell (PBMCs) and liver-infiltrating lymphocytes (LILs) of various subjects. Representative fluorescence-activated cell sorting density plots of CD45RA and Foxp3 and/or CD25 and Foxp3 in CD4<sup>+</sup>T cells from PBMCs (A) or LILs (C) in various subjects. B, Flow cytometric analysis of the frequencies of the 3 subpopulations and
manufacturer’s instructions. Suppression assay of in vitro-expanded Tregs was performed as previously described [13, 14] and is detailed in the Supplementary Materials and Methods.

Statistical Analysis
Statistical analysis was performed with SPSS software. The Mann–Whitney nonparametric U test was used to make comparisons between groups. Spearman rank correlation analysis was used to evaluate the frequencies of T-cell subpopulations and other parameters. A P value .05 was considered statistically significant.

RESULTS
Separation of Circulating CD4⁺Foxp³⁺ T Cells From Chronic HBV Patients Into 3 Subpopulations According to Foxp3 and CD45RA Expression
As shown in Figure 1A, the 3 CD4⁺Foxp³⁺ subpopulations could be separated by detection of Foxp3 and CD45RA expression in PBMCs from chronic HBV patients, namely, CD45RA⁺Foxp³⁺ rTregs (Fr.I), CD45RA⁻Foxp³⁺ aTregs (Fr.II), and CD45RA⁻Foxp³⁻ non-Tregs (Fr.III). Non-Tregs and rTregs poorly expressed intracellular cytotoxic T-lymphocyte antigen 4 (CTLA-4), whereas aTregs expressed the highest level of HLA-DR was observed in aTregs (Figure 1C). The proliferative status of the 3 subpopulations was assessed by detecting the expression of Ki-67. Fresh rTregs expressed small amounts of Ki-67, whereas aTregs expressed the largest amount of Ki-67 (Figure 1D). Cytokine production analysis revealed that IL-17A, interferon (IFN)–γ, and IL-2 were poorly secreted by both rTregs and aTregs, whereas non-Tregs produced large amounts of these 3 cytokines (Figure 1E–G). Taken together, 3 distinct subpopulations of circulating Foxp³⁺CD4⁺ T cells can be defined in chronic HBV patients by the expression of CD45RA and Foxp3. Among them, aTregs can be considered a key effector of immune suppression in chronic HBV pathogenesis.

Three CD4⁺Foxp³⁺ T-Cell Subpopulations Reveal Distinct Distribution Pattern for Different Chronic HBV Infection States
We analyzed the proportions of the 3 subpopulations and CD4⁺CD25⁺Foxp³⁺ T cells in PBMCs from ACLF and CAH patients, AsCs, and healthy controls using flow cytometry (Figure 2A). Compared with healthy controls, all patients showed significantly increased CD4⁺CD25⁺Foxp³⁺ T-cell frequencies (Figure 2B). We found that the proportion of circulating aTregs was selectively elevated in CAH patients (mean ± SD, 1.26% ± 0.38%) and ACLF patients (mean ± SD, 1.10% ± 0.37%) but not in AsCs (mean ± SD, 0.84% ± 0.27%) compared with healthy controls (mean ± SD, 0.76% ± 0.16%). In contrast to CAH patients and AsCs, ACLF patients showed a significantly increased frequency of IL-17A–secreting non-Tregs (Figures 1F and 2B).

To determine the presence of the 3 subpopulations in patients’ livers, LILs were assessed using flow cytometry. We found that aTregs were abundantly present in the livers of CAH and ACLF patients but poorly detected in the livers of AsCs as well as healthy controls. Notably, the ACLF patients exhibited a dramatically increased frequency of non-Tregs in LILs, whereas the numbers of intrahepatic rTregs were very low in all the samples (Figure 2C). These data indicate that the 3 subpopulations of circulating and intrahepatic CD4⁺Foxp³⁺ T cells reveal a distinct distribution pattern for different chronic HBV infection states.

Association Analysis Between the Percentages of the 3 Circulating CD4⁺Foxp³⁺ T-Cell Subpopulations and the Clinical Parameters in Chronic HBV Patients
We analyzed the correlations between the frequencies of the 3 circulating CD4⁺Foxp³⁺ T-cell subpopulations and CD4⁺CD25⁺Foxp³⁺ T cells and serum HBV DNA load, ALT levels, and HAI scores in AsCs, CAH, and ACLF patients. As shown in Figure 3A, a significantly positive correlation was only found between aTreg frequency and serum HBV DNA load in all patients. Further analysis indicated that the positive association occurred only in CAH patients but not in AsCs or ACLF patients (Figure 3A and Supplementary Figure 1A). We found no statistically significant association between the percentage of CD4⁺CD25⁺Foxp³⁺ T cells, aTregs, rTregs, or non-Tregs and ALT levels in CAH and ACLF patients (Figure 3B and Supplementary Figure 1B). When CAH and ACLF patients were divided into 2 groups based on their HAI scores, no difference was observed in the proportion of aTregs, whereas we found that patients with high HAI scores (G3–G4; n = 12) had a greater proportion of non-Tregs than did patients with low HAI scores (G1–G2; n = 9; Figure 3C). Thus, these results suggest that the increase of aTregs is associated with poor anti-HBV immune response, leading to poor viral clearance in CAH patients. However, the increase is not correlated with the limitation of liver injury, whereas significantly elevated non-Tregs are associated with severe liver damage.

Characterization of Antigen Specificity of Circulating Tregs in Chronic HBV Patients
We hypothesized that Tregs can be activated and expanded by HBV antigen stimulation and that, subsequently, these
HBV-specific aTregs can effectively inhibit anti-HBV immune response and facilitate HBV replication. Consequently, antigen specificity and suppressive activity of circulating Tregs in chronic HBV patients was further studied. HBCAg-specific Tregs have been described in previous studies [5, 7, 8], thus 3 promiscuous CD4+ T cell epitopes, HBCAg1-30, HBCAg81-105, and HBCAg99-109 (Supplementary Table 2) [15, 16], were synthesized and used for in vitro Treg induction in this study. OVA323-339 and a common Th epitope, TT947-967, were used as irrelevant controls. We first analyzed the expansion of CD4+CD25+Foxp3+ T cells in PBMCs of CAH patients after treatment with each peptide along with anti-CD28 stimulation by high-dose rIL-2 (previously reported as being required for Treg expansion in vitro) [13, 17]. As shown in Figure 4A, HBCAg-derived peptides that stimulated CD4+CD25+Foxp3+ T cells varied among individuals. For example, the percentage of CD4+CD25+Foxp3+ T cells was found to be consistently increased in PBMCs of CAH, CAH03, CAH08, and CAH10 patients stimulated with peptide HBCAg81-105 but not with other peptides. Also, the percentage of CD4+CD25+Foxp3+ T cells was promoted vigorously in the CAH08 patient with peptide HBCAg1-30 and in the CAH09 patient with peptide TT947-967. Notably these peptides also appeared to be able to preferentially expand CD4+CD25+Foxp3+ T cells in others (Figure 4A).

We then questioned whether certain peptides were associated with certain HLA-II alleles for Tregs recognition. Interestingly, we found that HLA-DR9 was associated with HBCAg81-105-driven CD4+CD25+Foxp3+ T-cell expansion in CAH03, CAH08, and CAH10 patients, as evidenced by HLA class II typing for each patient (data not shown). Further analysis of cytokine production showed that CD4+Foxp3+ T cells generated by HBCAg81-105 produced a large amount of interleukin 10 (IL-10) but a small amount of IFN-γ. In contrast, CD4+Foxp3+ T cells induced by TT947-967 secreted small amounts of IFN-γ but no IL-10. Furthermore, the proportion of IL-10–secreting CD4+Foxp3+ T cells decreased in the presence of anti-HLA-DR antibodies, indicating that CD4+Foxp3+ T-cell responses to HBCAg81-105 simulation were HLA-DR dependent (Figure 4B).

To further eliminate the contamination of Foxp3+ effector T cells, we examined the dynamic flux in frequency of the 3 distinct subpopulations in HLA-DR9+ PBMCs following different peptide stimulations in vitro. In DR9+ PBMC from the CAH03 patient, both the frequencies of aTregs and rTregs were increased and the frequency of non-Tregs was decreased following stimulation with HBCAg81-105. Conversely, the frequency of non-Tregs was increased after stimulation with HBCAg1-30 or TT947-967 (Figure 4C).

Finally, we examined whether HBCAg81-105 peptide–expanded CD4+CD25+ T cells in the context of HLA-DR9 required ligand-specific reactivation to exhibit their suppressive activity. As shown in Figure 4D, anti-CD3 monoclonal antibody–preactivated autologous CD4+CD25+ responder T cells alone proliferated strongly (ie, the proliferation rate was considered to be 100% for normalization purposes). When purified HBCAg81-105–generated CD4+CD25+ T cells were combined with HBCAg81-105–loaded autologous PBMCs and added at a CD25+/CD25− T-cell ratio of 1:1, the proliferation of responder T cells was significantly inhibited (mean proliferation rate, 22.1%). When either HBCAg81-105–generated CD4+CD25+ T cells along with TT947-967-pulsed autologous PBMCs or TT947-967–generated CD4+CD25+ T cells along with TT947-967–pulsed autologous PBMCs were added at a 1:1 ratio, the proliferation rate of responder T cells was only slightly decreased (respective mean proliferation rates, 79.8% and 100.8%, respectively). These results demonstrate that some HBCAg-derived peptides in the context of certain HLA class II alleles may be responsible for the peripheral activation and expansion of CD4+ Tregs in chronic HBV infection and that these Tregs require the same peptide-specific reactivation to exert their suppressive activity.

DISCUSSION

There is controversy regarding whether circulating CD4+ Tregs are increased in chronic HBV patients and whether the frequency is correlated with HBV replication or liver damage in several discrepant studies [3–7]. A possible reason for these discrepancies is the problematic definition of Tregs. Recently, heterogeneity of human CD4+Foxp3+ T cells was confirmed by Miyara [10]. Our study demonstrated that circulating CD4+Foxp3+ T cells can be dissected into 3 distinct subpopulations in chronic HBV patients. In agreement with Miyara’s study, our findings also suggest that only aTregs, not rTregs or non-Tregs, represent the major immune suppressor. Moreover, non-Tregs may possess a proinflammatory potential in chronic HBV pathogenesis.

By analyzing the 3 CD4+Foxp3+ T-cell subpopulations in PBMCs and liver tissues, we found that AsCs showed no significantly increased frequency of aTregs. However, this subpopulation was significantly increased in ACLF and CAH patients, compared with healthy controls. We propose that increased aTregs have an important effect on pathogenesis of persistent virus infections in ACLF and CAH patients. However, any other cellular mechanisms beyond Treg-mediated immune suppression may be involved in the maintenance of immune tolerance in AsCs. We also found that compared with AsCs and CAH patients, ACLF patients had dramatically increased frequencies of circulating and, in particular, intrahepatic non-Tregs, suggesting that non-Tregs are likely to be associated with the severity of immune-mediated liver injury. Thus, the distinct distribution pattern
of 3 CD4⁺Foxp3⁺ T-cell subpopulations is highly informative for distinguishing the chronic HBV infection states.

Because the proportion of aTregs was found to be significantly elevated in CAH and ACLF patients, we questioned whether these increased aTregs played an important role in the suppression of anti-HBV T-cell responses or in the downregulation of the immune responses that cause liver damage. However, we found that aTreg frequency was not correlated with a serious degree of liver injury, indicated by both serum ALT levels and liver HAI scores in these ACLF and CAH patients. Unexpectedly, we found that severe liver inflammation (indicated by HAI scores) was associated with a significantly increased frequency of non-Tregs. This finding suggests that liver damage may be a stimulus for induction of non-Tregs but not aTregs and that elevated non-Tregs may exhibit a potential to exacerbate liver damage, whereas

Figure 3. Correlation between the frequency of CD45RA⁺Foxp3⁺ activated Tregs (aTregs) or CD45RA⁻Foxp3⁻ nonsuppressive T cells (non-Tregs) and serum hepatitis B virus DNA load (A) or serum alanine aminotransferase (ALT; B) in asymptomatic HBV carriers (AsCs), chronic active hepatitis B (CAH) patients, and acute-on-chronic liver failure (ACLF) patients. C, CAH patients (n = 2) and ACLF patients (n = 10) with higher histological activity index (HAI) scores (G3–G4, n = 12) had significantly higher percentages of non-Tregs in their peripheral blood compared with CAH patients with lower HAI scores, not aTregs (G1–G2, n = 9). Abbreviation: rTregs, CD45RA⁺Foxp3⁻ resting regulatory T cells.
Figure 4. Hepatitis B virus–specific expansion of CD4+ Foxp3+ T cells in vitro from chronic active hepatitis B (CAH) patients. 

A. Antigen-specific expansion with CD25 and Foxp3 expression (gated on CD4 T cells) in peripheral blood mononuclear cells (PBMCs) following stimulation. 

B. HBCAg81–105 or TT947–967-specific CD4+ Foxp3+ T cells were generated from DR9+ PBMCs as described in the Materials and Methods section. Percentages of interleukin 10 (IL-10) or interferon (IFN)–γ production were detected by intracellular cytokine staining of CD4+ Foxp3+ and CD4+ Foxp3− subpopulations following HBcAg81–105 or TT947–967 stimulation, respectively, with or without anti-HLA-DR monoclonal antibody L243 (20 μg/mL) throughout the inoculation period.

C. Antigen-specific expansion with CD45RA and Foxp3 expression (gated on CD4 T cells) in PBMCs of the HLA-DR9+ CAH03 patient following stimulation.

D. Percentage proliferation (relative to that for CD4+CD25− cells stimulated alone) was measured by [3H]-thymidine incorporation. CD4+CD25+ T cells isolated from HLA-DR9+ PBMCs following stimulation with peptide HBCAg81–105 were cultured with mitomycin C–treated autologous PBMCs pulsed with peptide HBCAg81–105 (△) or TT947–967 (▲). CD4+CD25+ T-cell subpopulations isolated from HLA-DR9+ PBMCs following stimulation with peptide TT947–967 were cultured with mitomycin C–treated autologous PBMCs pulsed with TT947–967 (■). Data are presented as mean of triplicate cultures and representative of 3 independent experiments.
aTregs play a poor role in liver injury control during chronic HBV infection.

It has been speculated that the activation and maintenance of CD4\(^+\) Tregs require the presence of target antigens [18]. It also has been suggested that Tregs can be induced through repetitive stimulation with high concentrations of antigen for extended periods [19]. Notably, we found that only aTreg frequency was well correlated with serum HBV DNA load, suggesting that the large amount of HBV antigen present might be responsible for the aTreg induction and that the robust levels of aTregs could likely inhibit the anti-HBV immune response efficiently, thus facilitating HBV replication.

A previous study showed that only antigen-encountering activated Tregs, and not naive Tregs, can migrate to inflamed tissue via signaling through unique chemokine receptors and exhibit more efficient and antigen-specific suppressive potency at the local site [20]. This finding was supported by our study, which showed aTregs to be abundant in the livers of CAH and ACLF patients, whereas rTregs were barely detected in inflamed livers. Because the retention of activated Tregs at the local inflamed site also seems to be antigen dependent [21], we chose to further explore the antigen specificity of such activated CD4\(^+\) Tregs in chronic HBV infection. Previous findings have suggested that the suppressive effect of Tregs present in PBMCs of chronic HBV patients are HBCAg specific [5, 7] and that a population of CD4\(^+\)Foxp3\(^+\) T cells expressing phenotypic markers of both natural and induced Tregs could be HBCAg induced from PBMCs of CAH patients [8, 15, 22]. Recent studies have demonstrated that epitope-specific CD4\(^+\) T-cell populations generated after stimulation with a MHC class II promiscuous T-cell epitope contain both CD4\(^+\) T-helper cells and Tregs [23, 24]. By using the 3 promiscuous HBCAg-derived Th epitopes, we found that these peptide-stimulated CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells varied among individuals. Interestingly, we found that within the context of HLA-DR9, which was reported to be related to susceptibility to chronic HBV infection among people in southeastern China [25], peptide HBCAg\(_{81-105}\) preferentially drove CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells expansion without being contaminated with non-Tregs. Under the same conditions of stimulation, both HBCAg\(_{61-80}\) and TT\(_{947-967}\) led to preferential expansion of a population of Foxp3\(^+\) and Foxp3\(^{lo}\) non-Tregs. These findings imply that some HBCAg-derived peptides in the context of certain HLA class II alleles might be responsible for the peripheral activation, expansion, and even local retention of CD4\(^+\) Tregs in chronic HBV infection. Another study of chronic HCV infection also suggested the existence of dominant Treg epitopes in patients with chronic HCV infection [26].

The activation of Tregs is antigen specific, which implies that the suppressive activity of Tregs is triggered in an antigen-specific manner. Although it has been demonstrated that Tregs may suppress effector T cells with different antigen specificities [27], it is believed that suppression is more effective when these Tregs and these suppressor effector T cells have the same antigen specificity [28, 29]. We demonstrated that HBCAg\(_{61-105}\)-induced Tregs required HBCAg\(_{61-105}\)-specific reactivation to exert their suppressive activity, suggesting that HBV antigenic peptides presented in the liver are very important for retention and reactivation of Tregs. We also found that these activated Tregs can come in direct contact with effector T cells that recognize the same antigen for suppressing their responses much more efficiently. This viewpoint is in agreement with the explanation that increased aTregs can effectively inhibit anti-HBV immune response but not non-HBV specific inflammatory cells, which are implied to be the major effector of mediated liver damage [30–32].

In conclusion, we present the first evidence to support the finding that the delineation of CD4\(^+\)Foxp3\(^+\) T cells into aTregs, rTregs, and non-Tregs can better distinguish chronic HBV infection states than can CD4\(^+\)CD25\(^+\)Foxp3\(^+\) T cells. Our data also indicate that HBCAg-derived peptides may be responsible for activation of Tregs that, in turn, specifically inhibit anti-HBV immune response but not liver inflammation.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

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