Hepatitis B envelope antigen increases Tregs by converting CD4+CD25- T cells into CD4+CD25+Foxp3+ Tregs

RUI TANG*, ZHIGANG LEI*, XINPENG WANG*, QIANQIAN QI, JINGJING HE, DAN LIU,
XIAOXIAN WANG, XIAOJUN CHEN, JIFENG ZHU, YALIN LI, SHA ZHOU and CHUAN SU

State Key Laboratory of Reproductive Medicine, Department of Pathogen Biology and Immunology,
Jiangsu Key Laboratory of Pathogen Biology, Center for Global Health, Nanjing
Medical University, Nanjing, Jiangsu 211166, P.R. China

Abstract. Hepatitis B virus (HBV) can establish a lifelong chronic infection in humans, leading to liver cirrhosis, liver failure and hepatocellular carcinoma. Patients with chronic hepatitis B (CHB) exhibit a weak virus-specific immune response. Regulatory T cells (Tregs) play a key role in regulating the immune response in patients with CHB. Patients with hepatitis B envelope antigen (HBeAg)-positive CHB harbored a higher percentage of Tregs in their peripheral blood than those with HBeAg-negative CHB. However, whether and how HBeAg manipulates the host immune system to increase the population of Tregs remains to be elucidated. The present manuscript describes a preliminary immunological study of HBeAg in a mouse model. Multiple potential CD4+ T cell epitopes in HBeAg were identified using Immune Epitope Database consensus binding prediction. It was demonstrated that HBeAg treatment increased the numbers of Tregs in mouse spleens *in vitro* and *in vivo*. Furthermore, it was indicated that the HBeAg-mediated increase in Tregs occurred through the conversion of CD4+CD25- T cells into CD4+CD25+Foxp3+ Tregs. Additionally, *in vitro* study illustrated that HBeAg stimulated murine spleen cells to produce increased transforming growth factor-β, which is required to enable HBeAg to convert T cells into Tregs. The results of the present study may provide further evidence of the effect of HBeAg on Tregs and aid in the development of novel HBeAg-based immunotherapy for CHB.

Correspondence to: Professor Chuan Su or Professor Sha Zhou, State Key Laboratory of Reproductive Medicine, Department of Pathogen Biology and Immunology, Jiangsu Key Laboratory of Pathogen Biology, Center for Global Health, Nanjing Medical University, 101 Longmian Avenue, Jiangning, Nanjing, Jiangsu 211166, P.R. China.
E-mail: chuansu@njmu.edu.cn
E-mail: shazhou@njmu.edu.cn

*Contributed equally

Introduction

Hepatitis B virus (HBV) is a noncytopathic virus that can establish a lifelong chronic infection in humans. Currently, 3.5% of the global population suffers from chronic HBV infection, and therefore have a high risk of developing liver fibrosis, cirrhosis and hepatocellular carcinoma (HCC), which cause nearly 1 million annual deaths (1,2). Patients with chronic hepatitis B (CHB) exhibit a suppression of virus-specific immune responses that is closely associated with viral persistence and reduced responsiveness to interferon-based therapies (3-5). However, to the best of our knowledge, the underlying mechanisms for this antiviral immune hypo-responsiveness are not yet well defined.

HBV typically encodes four antigens, hepatitis B core antigen (HBcAg), hepatitis B envelope antigen (HBeAg), hepatitis B X antigen (HBxAg) and hepatitis B surface antigen (HBsAg). Patients with CHB are known to have persistently high quantities of the viral antigens HBsAg and HBeAg in their peripheral blood (6,7). HBeAg, a secretory form of the nucleocapsid antigen, is not required for viral infection or replication (8-10). However, HBeAg clearance or seroconversion in CHB patients is closely related to the restoration of antiviral T cell function following effective treatment (11,12). Secreted HBeAg has been shown to suppress the HBV-specific immune response and promote HBV persistence (12,13).

Diverse suppressive pathways have been implicated in the dysfunctional antiviral responses in CHB (6). Among the proposed mechanisms, regulatory T cell (Treg) activation in CHB is a major concern. Tregs, an immunoregulatory T cell subpopulation, are characterized by high expression of CD25 and transcription factor forkhead box P3 (Foxp3) compared to conventional CD4+ T cells and capable of suppressing the immune functions of numerous cell types, including CD4+ and CD8+ T cells, B cells, natural killer cells, natural killer T cells and dendritic cells (14). Patients with CHB harbor an increased percentage of Tregs in their peripheral blood that is positively correlated with serum HBV DNA load, the reduction of antiviral and treatment responses and increased risk of HCC (15-19). Furthermore, patients with HBeAg-positive CHB exhibited a higher percentage of Tregs in peripheral blood than those with HBeAg-negative CHB (15,19,20). However, to the

best of our knowledge, whether and how HBeAg manipulates the host immune system to induce the conversion of T cells to Tregs remains to be elucidated.

The present manuscript describes a preliminary study to investigate the involvement of HBeAg in the activation of Tregs using mouse model. *In vitro* and *in vivo* studies indicated that HBeAg may convert naive T cells into Tregs, potentially due to increased transforming growth factor (TGF)-β production induced by HBeAg. The results of the present study may provide further evidence of the effect of HBeAg on Tregs and of the benefit of the development of novel HBeAg-based immunotherapy for CHB.

Materials and methods

Animal studies. Animal experiments were conducted in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (date issued, November 14th, 1988; http://en.pkulaw.cn). All efforts were made to minimize suffering, and all procedures for the use of laboratory animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University (Permit Number: IACUC-1601123).

Specific pathogen-free 7-8-week-old female BALB/c mice (22~25 g body weight) were purchased from the Laboratory Animal Center of Nanjing Medical University (Nanjing, China) and bred in a specific pathogen-free animal facility. At the end of the experiments, mice were sacrificed by cervical dislocation under isoflurane anesthesia (5% in oxygen).

MHC class II binding prediction. The Immune Epitope Database (IEDB 2.22; http://tools.iedb.org/mhcii/) was used to predict human and mouse MHC class II binding peptides in HBeAg (GenBank accession no. AUR80753.1) from the HLA-DRB1*03:01 and H2-IAb/H2-IAd MHC class II alleles. In the prediction, percentile ranks ranged from 0-100, and low percentile ranks were good MHC II binders. As predicted by IEDB, MHC class II epitopes with a percentile rank below 10 were considered good binders.

Cytokine assays. The amounts of total and active TGF-β in the supernatants of spleen cells were determined with commercial ELISA kits (cat. nos. 437707 and 436707; BioLegend, Inc.) according to the manufacturer's instructions. The cytokine concentration in each supernatant was extrapolated from a standard curve.

Cell isolation. Spleens from 7-8-week-old female BALB/c mice were pressed through nylon nets to prepare single-cell suspensions. Cells were washed with PBS containing 1% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 0.2 M EDTA, counted after lysing the red blood cells, and then used to isolate CD4+CD25- T cells by using a mouse regulatory T cell isolation kit (Miltenyi Biotec GmbH) and a magnetic activated cell sorter (MACS; Miltenyi Biotec GmbH) according to the manufacturer's instructions, achieving ~90% purity as determined by flow cytometry (FCM).

Antigen-presenting cells (APCs) were prepared from spleen cell suspensions by negative selection using CD90.2 magnetic microbeads (Miltenyi Biotec GmbH) to deplete T cells. Isolated APCs were treated with mitomycin-C (Sigma-Aldrich; Merck KGaA) at 50 μg/ml for 30 min and then washed thoroughly before incorporation into cocultures.

FCM analysis. To analyze CD4+CD25+Foxp3+ Tregs, the Mouse Regulatory T Cell Staining kit (eBioscience, Thermo Fisher Scientific, Inc.) was used according to the manufacturer's instructions. In brief, cells from mice spleens or cell cultures were surface stained with anti-CD3-PerCP-Cy5.5 (clone 145-2C11), anti-CD4-FITC (clone RM4-5) and anti-CD25-APC (clone PC61.5) and subsequently permeabilized with cold Fix/Perm Buffer. The Fc receptors were then blocked with anti-CD16/32 for. A PE-labeled anti-Foxp3 (clone FJK16s) was then added. Following staining, the cells were examined using a FACS Verse flow cytometer (BD Biosciences) and data were analyzed using FlowJo (v10.0.7; FlowJo LLC).

In vitro cell cultures. To determine the *in vitro* activation of Tregs by HBeAg, spleen cells from normal BALB/c mice were cultured in 96-well round-bottom plates at a density of 5x10⁵ cells/well in complete RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc.). Corresponding concentrations of HBeAg (0.2, 1, 5 or 10 μg/ml; Shanghai Yuan Mu Biotechnology Co., Ltd) or Grade VII chicken egg ovalbumin (OVA; 1 μg/ml; Sigma-Aldrich; Merck KGaA) were added to each culture in four wells per condition. An inhibitor of transforming growth factor-β type 1 receptor (TGF-βRI signaling; 20 μM; SB-431542; Sigma-Aldrich; Merck KGaA) was added to a proportion of the wells. After 3 days, cells were harvested from cultures and washed twice by centrifugation (300xg, 10 min, 4℃) in PBS. After counting, cells were stained for FCM analysis, and supernatants were collected for cytokine detection by ELISA.

To investigate the Treg conversion of naive T cells induced by HBeAg *in vitro*, MACS-isolated CD4+CD25- T cells (2x10⁵ cells/well) were cocultured with mitomycin-C-treated APCs (1x10⁵ cells/well) in triplicate in 96-well round-bottom culture plates and stimulated with HBeAg (1 μg/ml) or OVA (1 μg/ml). After 3 days, cells were collected for FCM analysis.

Immunization protocol. In each experiment, 10 normal BALB/c mice were randomly divided into two groups consisting of 5 mice per group. Mice were subcutaneously (inguinal region) injected with 100 μl of a 1:1 (v/v) mixture of antigen (25 μg of HBeAg or PBS alone) and incomplete Freund's adjuvant (Sigma-Aldrich; Merck KGaA). Each mouse was injected twice with a 14-d internal injection. Immunized mice were sacrificed under isoflurane anesthesia 10 days after the last injection and their spleens were collected for analysis.

Statistical methods. All statistical calculations were performed by using the SPSS program (v26 for Windows; IBM Corp.). The comparisons between more than two groups were analyzed with one-way analysis of variance (ANOVA) followed by an LSD post hoc test (n<4 groups) or a Tukey's post hoc test (n≥4 groups). P<0.05 was considered to indicate a statistically significant difference.

Results

Prediction of potential CD4+ T cell epitopes in the HBeAg. The IEDB consensus binding prediction platform was employed to

Table I. Prediction of human MHC class II epitopes for HBeAg.

MHC class II Epitopes	Peptides	Percentile rank[a]
1	SYVNVNMGLKIRQLL	1
2	VNVNMGLKIRQLLWF	1.04
3	YVNVNMGLKIRQLLW	1.07
4	NVNMGLKIRQLLWFHI	1.19
5	PASRELVVSYVNVNM	1.94
6	SRELVVSYVNVNMGL	1.94
7	ASRELVVSYVNVNMG	2.07
8	DPASRELVVSYVNVN	2.6
9	IRDLLDTASALYREA	3.26
10	RDLLDTASALYREAL	3.55
11	PSDFFPSIRDLLDTA	3.64
12	QLFHLCLIISCSCPTVQAS	3.93
13	SIRDLLDTASALYRE	3.97
14	TTVVRRRGRSPRRRT	4.1
15	TVVRRRGRSPRRRTP	4.14
16	ETTVVRRRGRSPRRR	4.2
17	PETTVVRRRGRSPRR	4.26
18	LPSDFFPSIRDLLDT	4.45
19	LPETTVVRRRGRSPR	4.54
20	SCLTFGRETVLEYLV	5.73
21	PSIRDLLDTASALYR	5.76
22	SCPTVQASKLCLGWL	5.85
23	CLTFGRETVLEYLVS	5.86
24	CLIISCSCPTVQASK	5.87
25	CPTVQASKLCLGWLW	5.92
26	SDFFPSIRDLLDTAS	5.94
27	SCSCPTVQASKLCLG	6.04
28	CSCPTVQASKLCLGW	6.24
29	LIISCSCPTVQASKL	6.33
30	FLPSDFFPSIRDLLD	6.37
31	ISCLTFGRETVLEYL	6.56
32	LLSFLPSDFFPSIRDLL	7.64
33	LSFLPSDFFPSIRDL	7.71
34	HISCLTFGRETVLEY	8.48
35	MNLATWVGSNLEDPA	8.5
36	NLATWVGSNLEDPASRELV	8.84
37	DFFPSIRDLLDTASA	9.53
38	LTFGRETVLEYLVSF	9.83

[a]In the prediction method of IEDB, percentile ranks range from 0 to 100, and low percentile ranks are good MHC II binders. As predicted by IEDB, MHC class II epitopes with a percentile rank below 10 were considered good binders. HBeAg, hepatitis B envelope antigen; IEDB, Immune Epitope Database; MHC, major histocompatibility complex.

Table II. Prediction of mouse MHC class II epitopes for HBeAg.

MHC class II Epitopes	Peptides	Percentile rank[a]
1	RTPPAYRPPNAPILST	0.53
2	PPAYRPPNAPILSTLP	0.65
3	IRTPPAYRPPNAPIL	0.81
4	AYRPPNAPILSTLPET	2.1
5	CSPHHTALRQAILCW	4.33
6	WIRTPPAYRPPNAPI	4.76
7	SPHHTALRQAILCWGE	4.89
8	HCSPHHTALRQAILC	5.09
9	VWIRTPPAYRPPNAP	5.65
10	SFGVWIRTPPAYRPP	7.02
11	IDPYKEFGASVELLS	7.32
12	HHTALRQAILCWGEL	7.36
13	GVWIRTPPAYRPPNA	7.46
14	VSFGVWIRTPPAYRP	7.57
15	FGVWIRTPPAYRPPN	7.98
16	FGRETVLEYLVSFGV	8.16
17	DPYKEFGASVELLSF	8.39
18	MDIDPYKEFGASVELL	9.02
19	LTFGRETVLEYLVSFG	9.32
20	CLTFGRETVLEYLVS	10

[a]In the prediction method of IEDB, percentile ranks range from 0 to 100, and low percentile ranks are good MHC II binders. As predicted by IEDB, MHC class II epitopes with a percentile rank below 10 were considered good binders. HBeAg, hepatitis B envelope antigen; IEDB, Immune Epitope Database; MHC, major histocompatibility complex.

suggest that HBeAg has multiple high-affinity agonist peptides and may potentially induce CD4^{+} T cell activation.

HBeAg converts naive T cells to Tregs in spleen cells in vitro. HBeAg was further examined for its ability to induce the conversion of naive T cells into Tregs in spleen cells cultured for 3 days *in vitro*. FCM analysis suggested that there were no significant differences in the proportions of CD4^{+} T cells among all the groups (Fig. 1A and B). But notably, FCM analysis revealed that the percentage of CD25^{+}Foxp3^{+} Tregs within the CD4^{+} T cell population of HBeAg-treated spleen cells increased over the 3-day period in a dose-dependent manner (Fig. 1A and 1C). By contrast, no significant increase was observed within OVA-treated spleen cells (Fig. 1A and 1C). Different doses (0.2, 1, 5, and 10 µg/ml) were used to test the ability of HBeAg to induce spleen cell differentiation into Tregs *in vitro*. In these experiments, the medium doses (1 and 5 µg/ml) of HBeAg increased the population of Tregs, but the low dose (0.2 µg/ml) and the high dose (10 µg/ml) appeared unable to trigger this increase of Tregs (Fig. 1A and 1C).

HBeAg converts CD4^{+}CD25^{-} T cells to CD4^{+}CD25^{+}Foxp3^{+} Tregs in vitro. To determine whether the increase in Treg numbers induced by HBeAg could be due to *de novo* induction of Foxp3 expression in activated T cells, MACS-isolated CD4^{+}CD25^{-} T cells from spleens of normal BALB/c mice were cultured with HBeAg in the presence

predict the potential CD4^{+} T cell epitopes of HBeAg. Putative epitopes with a percentile rank <10% were considered MHC class II binders. As recommended by IEDB, 38 human and 20 mouse potential CD4^{+} T cell epitopes were identified in HBeAg from the HLA-DRB1[*]03:01 and H2-IAb/H2-IAd MHC class II alleles, respectively (Table I and II). These data
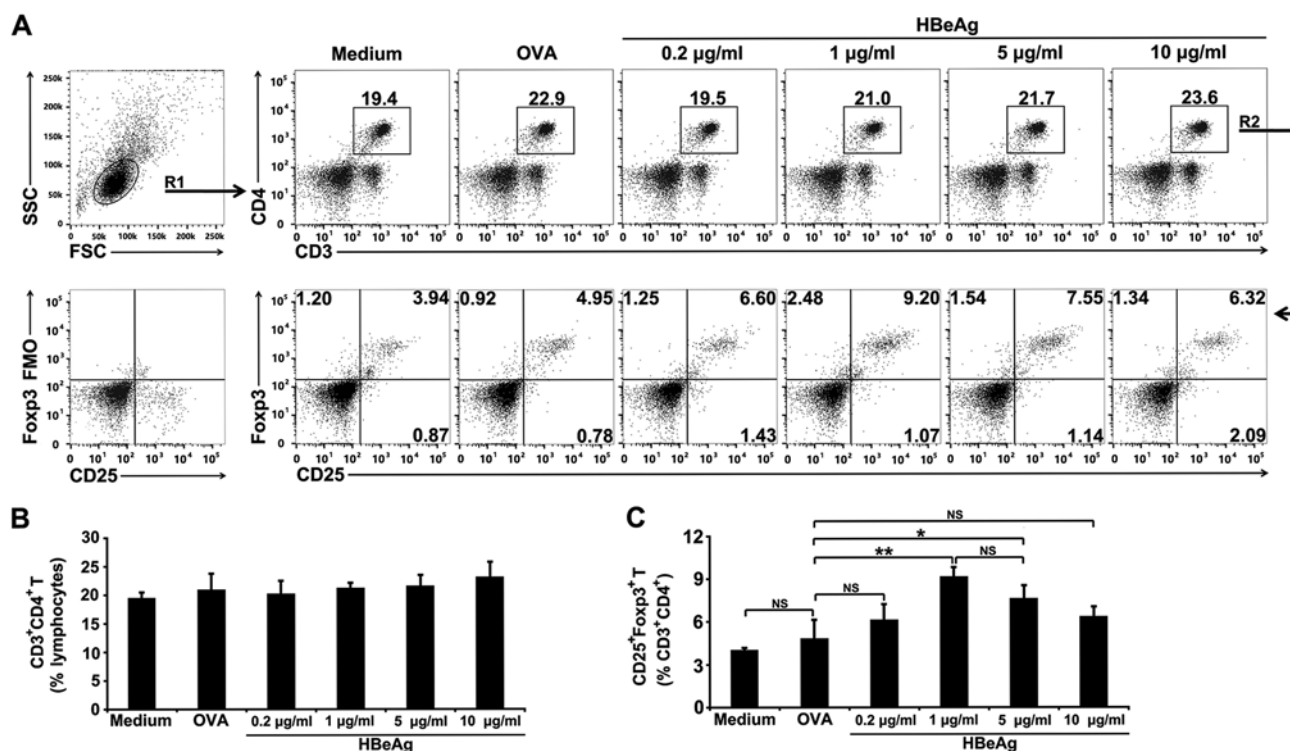
Figure 1. HBeAg treatment increases the proportion of Tregs in spleen cells *in vitro*. (A) Total spleen cells from normal mice were cultured in medium alone or in the presence of 1 µg/ml of OVA or different concentrations of HBeAg (0.2, 1, 5 or 10 µg/ml). Cells were collected, and flow cytometry was performed to identify CD3⁺CD4⁺CD25⁺Foxp3⁺ Tregs 3 days later. The gating strategy shows total lymphocytes (R1) and total CD3⁺CD4⁺ T cells (R2). Dot plots of CD3 vs. CD4 gated lymphocytes, or CD25 vs. Foxp3 expression, gated on CD3⁺CD4⁺ T cells, are representative of one of three independent experiments. Numbers on plots represent the percentage of cells for each quadrant in gated cell populations. Percentages of CD3⁺CD4⁺ T cells in (B) lymphocytes or (C) CD25⁺Foxp3⁺ Tregs in CD3⁺CD4⁺ T cells are shown as the mean ± SD of triplicate cultures and representative of three independent experiments. *P<0.05, **P<0.01. HBeAg, hepatitis B envelope antigen; Foxp3, forkhead box protein 3; NS, not significant; Tregs, T regulatory cells.
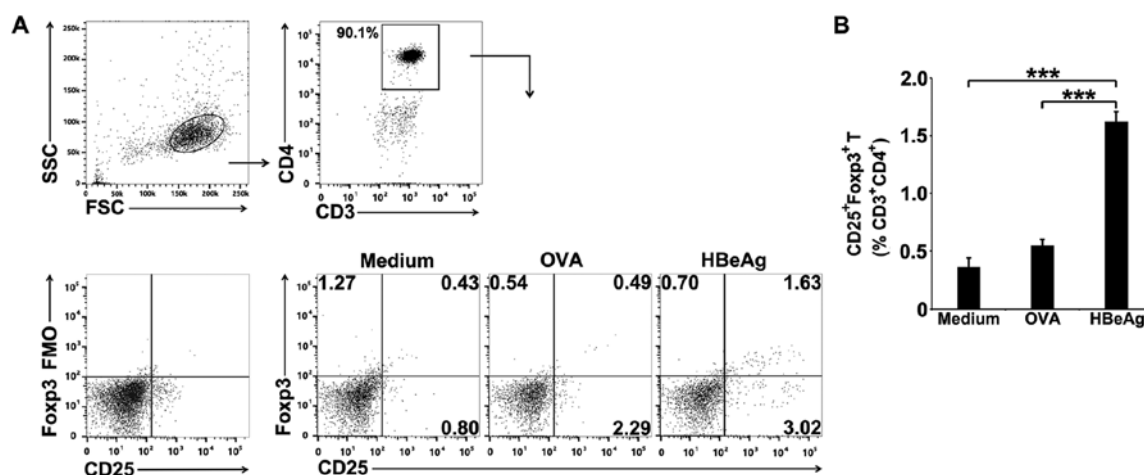

Figure 2. HBeAg converts CD4⁺CD25⁻ T cells into CD4⁺CD25⁺Foxp3⁺ Tregs in vitro. (A) MACS-isolated CD4⁺CD25⁻ T cells were cultured in medium alone or with 1 µg/ml of OVA or HBeAg in the presence of APCs at a 2:1 ratio of T cells to APC. After 72 h, percentages of CD3⁺CD4⁺CD25⁺Foxp3⁺ Tregs were determined by flow cytometry. Dot plots of CD25 vs. Foxp3 expression, gated on CD3⁺CD4⁺ T cells, are representative of one of three independent experiments. Numbers on plots represent the percentage of cells for each quadrant in gated cell populations. (B) Percentages of CD25⁺Foxp3⁺ Tregs in CD3⁺CD4⁺ T cells are shown as the mean ± SD of triplicate cultures and representative of three independent experiments. ***P<0.001. APC, antigen presenting cells; HBeAg, hepatitis B envelope antigen; Foxp3, forkhead box protein 3; OVA, ovalbumin; Tregs, T regulatory cells.

of APCs. The number of Tregs was determined 3 days later by FCM, and the results suggested that HBeAg significantly converted CD4⁺CD25⁻ T cells to CD4⁺CD25⁺Foxp3⁺ Tregs when compared to the medium or OVA control groups (Fig. 2A and 2B).

HBeAg induces conversion of T cells to Tregs in spleen cells in vitro by triggering TGF-β production. As TGF-β plays a critical role in the conversion of peripheral T cells to Tregs by promoting Foxp3 expression (21,22), whether HBeAg could induce TGF-β production in murine spleen cells *in vitro*
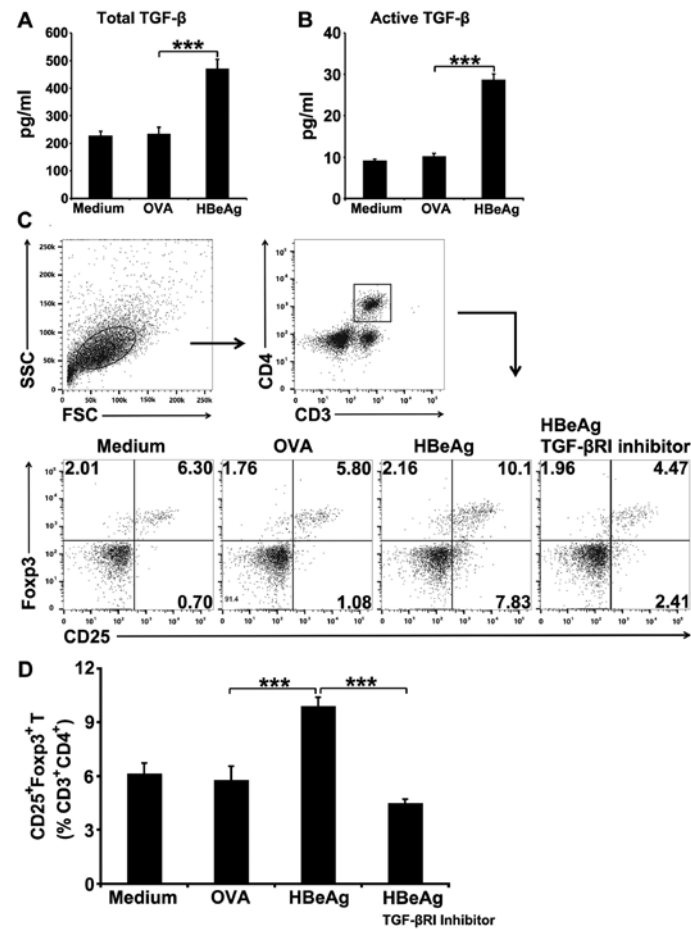
Figure 3. HBeAg triggers TGF-β production by spleen cells *in vitro*. Spleen cells from normal BALB/c mice were cultured for 3 days in medium alone or in the presence of 1 μg/ml of OVA or HBeAg. Supernatants were harvested for quantification of (A) total TGF-β and (B) active TGF-β by ELISA. Data are the mean ± SD of triplicate cultures and representative of two independent experiments. (C) Total spleen cells from normal mice were cultured in medium alone or in the presence of 1 μg/ml of OVA or HBeAg. Cultures were set up with or without an inhibitor of TGF-βRI signaling (20 μM). Cells were collected and flow cytometry was performed to identify CD3+CD4+CD25+Foxp3+ Tregs 3 days later. The gating strategy shows total lymphocytes (R1) and total CD3+CD4+ T cells (R2). Dot plots of CD25 vs. Foxp3 expression, gated on CD3+CD4+ T cells, are representative of one of two independent experiments. Numbers on plots represent the percentage of cells for each quadrant in gated cell populations. (D) Percentages of CD25+Foxp3+ Tregs in CD3+CD4+ T cells are shown as the mean ± SD of quadruplicate cultures and are representative of two independent experiments. ***P <0.001. HBeAg, hepatitis B envelope antigen; Foxp3, forkhead box protein 3; NS, not significant; OVA, ovalbumin; TGF-β, transforming growth factor-β; TGF-βR1, transforming growth factor -β receptor 1; Tregs, T regulatory cells.

was analyzed. Indeed, HBeAg induced a two-fold increase in total TGF-β production by spleen cells compared with the OVA-treated control (Fig. 3A). The presence of biologically active TGF-β was also assessed and a 3-fold increase in active TGF-β release from HBeAg-treated spleen cells was observed when compared to OVA-treated cultures (Fig. 3B). To further investigate whether TGF-β was required for HBeAg to induce conversion to Tregs, studies with TGF-βRI inhibitor in spleen cell culture were performed. It was observed that blocking of TGF-β signaling almost completely abolished the ability of HBeAg to induce conversion of T cells to Tregs (Fig. 3C and 3D). Thus, TGF-β is required to enable HBeAg to convert T cells to Tregs.

HBeAg converts T cells to Tregs in vivo. Whether HBeAg had the capacity to convert peripheral T cells into Tregs *in vivo* was then examined. HBeAg was subcutaneously injected into normal BALB/c mice. As shown in Fig. 4A and 4B, OVA or HBeAg treatment *in vivo* did not significantly change the percentage of CD4+ T cells in total lymphocytes. HBeAg treatment *in vivo* induced a pronounced increase in the percentage

of Tregs within the CD4+ T cell population when compared to either OVA- or PBS-treated control (Fig. 4A and 4C). In addition, absolute numbers of Tregs in the spleens of HBeAg-injected mice increased consistently (Fig. 4D).

Discussion

CHB is characterized by HBeAg positivity. HBeAg has been shown to regulate the host immune response to maintain a tolerant state and promote HBV persistence in natural infection (13,23), but the mechanism by which HBeAg induces immune tolerance remains unclear. The mouse is an appropriate animal model for immunological studies (24), and the present study does not involve the investigation of HBV pathogenesis; thus, we used a murine experimental system to explore immunoregulatory function of HBeAg.

Among immunoregulatory cell populations, Tregs remain paramount in CHB patients (14). CHB patients, especially those with HBeAg positivity, exhibit a higher percentage of Tregs in their peripheral blood than those with HBeAg-negative CHB (15,19,20). Therefore, it was initially hypothesized that
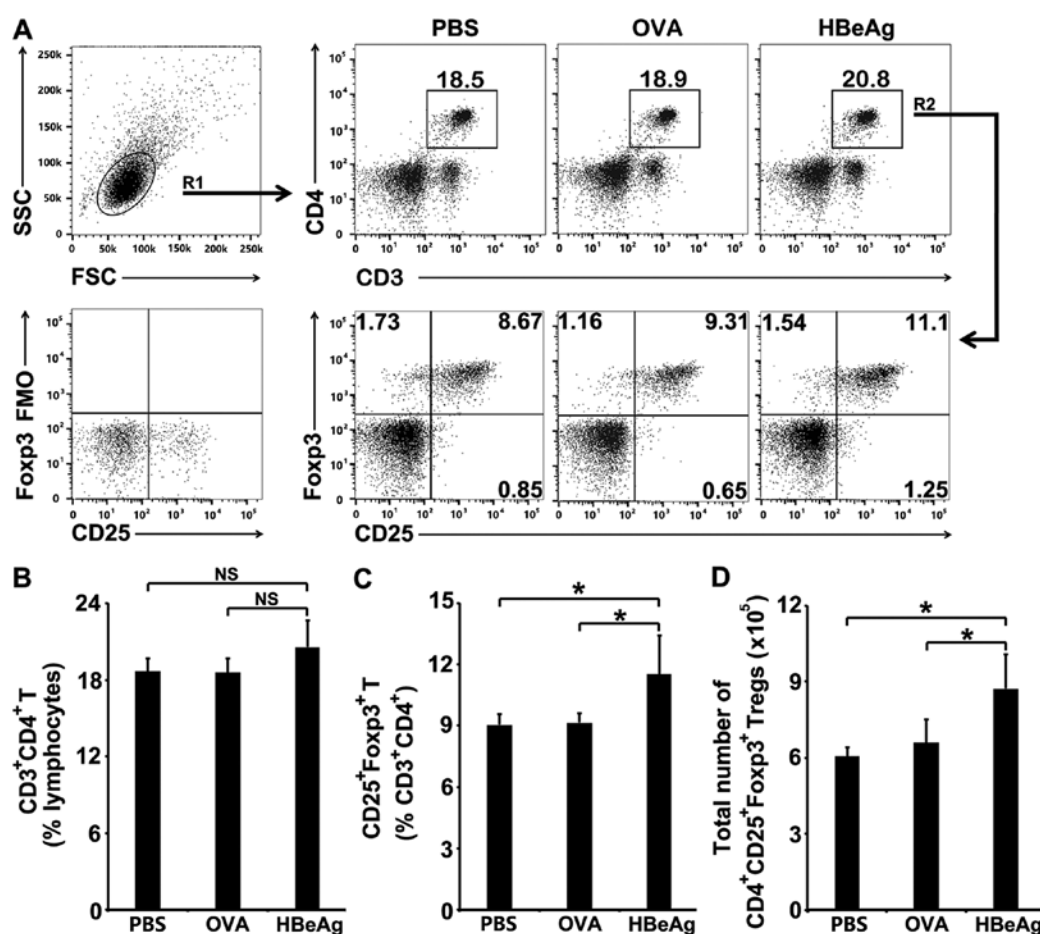
Figure 4. HBeAg induces Tregs *in vivo*. (A) Normal BALB/c mice were injected with HBeAg, OVA or PBS (control) and five mice were used in each experimental group. Flow cytometry was used to determine the numbers of CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the spleen of each mouse 7 days after the final injection. The gating strategy shows total lymphocytes (R1) and total CD3$^+$CD4$^+$ T cells (R2). Cells were gated on CD3$^+$CD4$^+$ T cells (R1). Dot plots of CD3 vs. CD4 gated on lymphocytes, or CD25 vs. Foxp3 expression are representative of one of three independent experiments. Negative gate for Foxp3 staining was set using FMO. Numbers on plots represent the percentage of cells for each quadrant in gated cell populations. Graphs indicate average percentages ± SDs of (B) CD3$^+$CD4$^+$ T cells in lymphocytes, (C) CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ Tregs and (D) absolute numbers of CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ Tregs in splenic cells from mice and are representative of two independent experiments. *P<0.05. HBeAg, hepatitis B envelope antigen; OVA, ovalbumin; Foxp3, forkhead box protein 3; NS, not significant; Tregs, T regulatory cells; FMO, fluorescence minus one.

HBeAg was involved in the conversion of T cells to Tregs in CHB patients. A combination of *in vitro* and *in vivo* assays appeared to validate the hypothesis that HBeAg induced a significant increase in the proportion of Tregs isolated from mouse spleens. To the best of our knowledge, the current study is the first to provide preliminary evidence for the contribution of HBeAg to Treg generation in patients with CHB. In addition, previous studies have suggested that efficient Treg induction requires low doses of antigens, which is possibly related to weak T cell receptor signaling (25-27). Consistently, in *in vitro* study, a dose of 1 µg/ml HBeAg was found to be more effective in the conversion of T cells to Tregs than that of 5 µg/ml, although the difference between the two was not statistically significant. The high dose (10 µg/ml) of HBeAg was found unable to induce the increase the number of Tregs, probably due to the induction of a strong T cell receptor stimulation.

Peripheral Treg differentiation is induced upon T cell activation with high-affinity agonist antigens (28,29). Predictions indicated multiple high-affinity potential MHC class II-binding epitopes on HBeAg. However, currently it was not possible to verify the most immunogenic epitopes that are predicted to be potentially responsible for naive CD4$^+$ T cell activation and subsequent peripheral Treg differentiation in the present study. The naive CD4$^+$ T cell population expresses a huge repertoire of receptors that are highly diverse in their epitope-binding sites (30). Only few naive CD4$^+$ T cells can be reactive to a single HBeAg epitope, so it is likely that very few CD4$^+$ T cells could be induced to differentiate into Tregs by a single epitope. HBeAg-mediated differentiation of T cells into Tregs may require a combination of multiple epitopes, in a complex process which merits further study.

Previous studies have revealed that high amounts of TGF-β are required for foreign antigen-mediated induction of Foxp3 expression in peripheral naive CD4$^+$ T cells (29,31). Considering these findings, the present data suggested that HBeAg was able to trigger TGF-β production and indeed required to enable HBeAg to induce T cell conversion into Tregs in mouse spleen. Previous research has demonstrated that patients with CHB exhibit significantly higher serum levels of TGF-β than healthy people (32) and HBeAg may

contribute to the elevated serum levels of TGF-β in patients with CHB. Diverse varieties of immune cells, such as macrophages, monocytes, NK cells and CD4^{+} T cells, are involved in the production of TGF-β during chronic HBV infection (33). However, which cells in the spleen that are primarily responsible for HBeAg-increased TGF-β production requires further investigation. The present data suggest that HBV exploits immune cells to create an TGF-β-rich microenvironment for peripheral Treg differentiation and HBV persistence, and are consistent with the notion that HBeAg can condition innate immune cells into anti-inflammatory types (34,35).

Although HBeAg is not required for HBV assembly, replication or viral infection, various studies have shown that HBeAg is capable of impairing innate immune responses or inactivating HBeAg-specific T cells by clonal deletion or anergy (34,36). The present study demonstrated the additional ability of HBeAg to convert CD4^{+}CD25^{-} T cells to CD4^{+}CD25^{+}Foxp3^{+} Tregs *in vitro* and suggest a mechanistic explanation for HBeAg, as an immune tolerogen, to modulate the host immune response and promote HBV chronicity. However, current data in mice are preliminary, and further investigation is needed to validate the T cell differentiating capabilities of HBeAg in HBV transgenic mice and to explore the underlying molecular mechanisms in future works.

In summary, the present murine experimental data indicate that HBeAg is able to convert T cells into Tregs in mouse spleen and suggest this may be due to the increased TGF-β production induced by HBeAg.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SZ and CS conceived and designed the study. RT, ZL, XW, QQ, JH, DL, XW and YL performed the experiments. SZ, XC, JZ and CS analyzed the data. SZ and CS wrote the paper. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures for the use of laboratory animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Nanjing Medical University (permit no. IACUC-1601123).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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