Expression of programmed cell death1 in T follicular helper cells is regulated by prostaglandin E2 secreted by HBV-infected HepG2.2.1.5 cells

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Abstract. The present study aimed to investigate the distribution of T follicular helper (Tfh)-cell subsets in patients with hepatitis B virus (HBV) and determine the underlying mechanism of HBV regulation of Tfh cells. The frequency of peripheral blood Tfh subsets was analyzed using flow cytometry. The expression level of programmed cell death-1 (PD-1) and prostaglandin E2 (PGE2) was quantified using reverse transcription-quantitative polymerase chain reaction and western blotting. The PGE2 level in culture supernatant was detected using enzyme-linked immunosorbent assay. A Transwell chamber was used to co-culture Tfh cells with HepG2 and HepG2.2.1.5. The percentage of inducible T-cell costimulator (ICOS)+ and total Tfh cells was high at the immune activation (IA) group; however, it was reduced in the immune tolerance (IT), responders with HBsAg seroconversion (RP) and healthy control (HC) groups. The percentage of PD-1+ Tfh cells was significantly higher in IA and IT compared with RP and HC. The ratio of PD-1+/total Tfh cells was positively correlated with the load of HBV DNA; therefore, this ratio may act as an indicator for HBV replication. The expression level of PD-1 in Tfh cells was higher in the HepG2.2.1.5 co-cultured group compared with the HepG2 group, this may be due to the high PGE2 expression level in HBV-infected HepG2.2.1.5 cells. The findings of the present study revealed an imbalanced distribution of PD-1+ Tfh cells in patients with HBV at different immune phases. Additionally, HBV may upregulate the expression of PD-1 in Tfh cells by promoting HepG2.2.1.5 to secret PGE2. Identifying the effect of HBV on Tfh-cell subsets is crucial for improving immuno-based therapy for HBV.

Introduction

It is estimated that 317,000 people worldwide with human hepatitis B virus (HBV) developed liver cirrhosis, and 300,000 patients with HBV developed hepatocellular carcinoma (HCC) in 2013 (1,2). The pathogenic process of HBV has been determined to be closely associated with deficient host immune responses, involving the dysregulation of HBV-specific T cells, B cells, infiltrating neutrophils, natural killer cells and other lymphocytes (3-5). Clearance of hepatitis B infection is directly performed by B cells by secretion of specific antibodies, this function is primarily mediated by T follicular helper (Tfh) cells. The dysfunction of Tfh and B cells has been associated with the pathogenic process of various autoimmune diseases and tumors (5-7).

Tfh cells have been established as the primary helpers to promote the proliferation, differentiation, maturation and antibody class switching of B cells in germinal centers (GCs) or outside GCs. They are positive for certain surface markers, including CD4, C-X-C motif chemokine receptor 5 (CXCR5), B-cell CLL/lymphoma 6 (Bcl-6), interleukin (IL)-21, inducible T-cell costimulator (ICOS) and programmed cell death-1 (PD-1). Previous studies have suggested that as the two key molecules expressed on Tfh cells, ICOS and PD-1 perform differential immune functions (8-11). ICOS has always been regarded as a marker for activity, whereas PD-1 and PD-ligand lare usually highly expressed on lymphocytes and tumor cells in an immune tolerance microenvironment (12-14). Although the effect of PD-1 on inducing immunosuppression has been previously investigated, the features of PD-1+ Tfh cells during HBV infection remain to be elucidated.

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The activation of the intrahepatic cytokine network has an important role in the pathogenesis of acute and chronic liver inflammation. Expression levels of tumor necrosis factors (15,16), interferons and interleukins (17) were increased in response to HBV infection and may lead to the development of HBV into HCC. Prostaglandin E2 (PGE2) is one of the most abundant prostaglandins in the body and an important causative cytokine of inflammation that results from tissue damage or infection (18). PGE2 is usually produced by epithelial cells and is highly expressed in lung cancer and other malignancies (19). Additionally, it may upregulate the activity of T-regulatory cells, which primarily mediate the suppression of immune responses (20). However, whether PGE2 is capable of direct regulation of PD-1+ Tfh cells in patients with HBV remains to be elucidated.

The present study analyzed the distribution of ICOS⁺, PD-1⁺ and total Tfh cells in patients with HBV at different immune phases and investigated the possible mechanism of HBV regulation of PD-1 expression on Tfh cells. The current study determined that HBV-infected HepG2.2.1.5 may secrete more PGE2, which may contribute to the upregulation of PD-1 expression in Tfh cells. It is vital to determine the effect of HBV on the distribution of Tfh-cell subsets and the underlying regulatory mechanism in order to develop novel immunotherapy-based approaches for future HBV treatment.

**Materials and methods**

**Patients.** A total of 46 patients with HBV and 15 healthy controls (HC) were recruited from the First Hospital of Jilin University between 2014 and 2015. HBV-infected subjects were classified into three distinct groups according to their serum HBV DNA load, alanine transaminase (ALT) level and detectable hepatitis B viral protein (HBsAg). Patients with high copies of serum HBV DNA, positive HBeAg and normal ALT level were confirmed as the immune tolerance group (IT; n=13). Patients with low levels of serum HBV DNA load, positive HBeAg and increased ALT levels were defined as the immune activation group (IA; n=15); however, patients with undetectable level of HBV DNA load, negative HBeAg and normal ALT level were considered to be responders with surface antigen of HBV (HBsAg) seroconversion (RP; n=18). Therefore, HBeAg is a more specific marker indicating the immune state of HBV infection compared with HBsAg, as HBsAg may be positive in both the active and inactive phase (21-23). HBV DNA levels in serum were quantified using a qPCR HBV diagnostic kit (Roche Diagnostics, Basel, Switzerland). Serum ALT and AST levels were determined by chemiluminescent microparticle immunoassay using chemiluminescence immunoassay analyzer (Abbott Laboratories, Lake Bluff, IL, USA).

Participants suffering systemic disorders were excluded from the present study. Written informed consent was obtained from the guardians on the behalf of all participants. The present study was reviewed and approved by the Ethics Committee of the First Hospital of Jilin University (Changchun, China). The demographic and clinical characteristics of the patients enrolled in the present study are presented in Table I.

**Peripheral blood mononuclear cells (PBMCs) isolation.** PBMCs were harvested from each participant's heparinized blood sample by density-gradient centrifugation using Ficoll-Paque Plus (GE Healthcare Life Sciences, Chalfont, UK). Fluorescein isothiocyanate (FITC) conjugated-anti-CD4 (cat. no. 555346), phycoerythrin (PE) conjugated-anti-ICOS (cat. no. 557802; all at 1:100 dilution and obtained from BD Biosciences, Franklin Lakes, NJ, USA), allopurinol conjugated (APC) conjugated-anti-CXCR5 (cat. no. 356907) and peridinin chlorophyll (PerCP) conjugated-anti-PD-1 (cat. no. 329957; all at dilution 1:100 and obtained from BioLegend, CA, USA) were used to label total Tfh cells (CD4⁺CXCR5⁺), ICOS⁺ Tfh cells and PD-1⁺ Tfh cells. The frequency of ICOS⁺, PD-1⁺ and total Tfh cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences).

**Cell culture.** HepG2 and HepG2.2.1.5 liver cancer cells lines obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) were used in the present study. The HepG2.2.1.5 cell line was derived from HepG2 cells, which were transfected with a plasmid containing HBV DNA as previously described (24). Tfh cells were sorted from PBMCs using a FACSAriaII (BD Biosciences) and subsequently co-cultured (2.5x10⁴/well) in a 0.4 µm pore size Transwell system (Corning Inc., Corning, NY, USA) with HepG2 (5x10⁴/well) and HepG2.2.1.5 (5x10⁴/well) cells. All cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 1% penicillin/streptomycin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) at 37 °C with 5% CO₂. A PGE2-specific inhibitor was used to block PGE2 activity, pranoprofen (6 μM; Selleck Chemicals, Houston, TX, USA) was supplemented into the co-culture system for 6 h.

**Plasmid construction.** The pGenesil vector, containing human U6 promoter, was obtained from GenePharma Co., Ltd. (Shanghai, China) and used to generate a series of short hairpin RNA (shRNA) expression vectors by inserting annealed oligonucleotides. The shRNA targeting the HBV preC/Csequence and HBVs sequence was designed and synthesized by GenePharma Co., Ltd. to inhibit the expression of HBeAg and HBsAg in HBV.

**Transfection.** One day prior to the transfection, HepG2.2.1.5 cells were trypsinized and ~1x10⁶ cells/well were seeded in a 6-well plate. Cells were transfected with shRNA-vectors, and a transfection efficiency was directly observed using a fluorescence microscope and an efficiency of ~75% was achieved using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. HepG2.2.1.5 cells were harvested and co-cultured with Tfh cells 72 h after the transfection in order to analyze the influence of HBeAg and HBsAg on PD-1 levels in Tfh cells.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Reverse transcription was performed using GoScript Reverse Transcription system (Promega Corporation, Madison, WI, USA) and Power SYBR-Green Master mix (Thermo Fisher Scientific, Inc.) was used for the gene amplification.
qPCR reaction conditions were as follows: 95°C for 10 min, 40 cycles of 95°C for 15 sec, 60°C for 1 min. Relative gene expression was determined using the 2^{ΔΔCq} method (25) with GAPDH used as the reference gene. The following primers were used: PD-1 forward (F) 5'-AAGGCTTATGGGGCTCGGCC-3', reverse (R) 5'-GGATCCCTCAAGAGGCC-3'; and GAPDH F: 5'-GGTGGTGTCCCTCGACTTCAAC-3', R: 5'-GTTGGTGTGGGGAATG-3'.

Western blotting. Cells were harvested 72 h after transfection and lysed in RIPA buffer (Beyotime Institute of Biotechnology, Wuhan, China). Protein concentration was tested using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). The cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Merck Millipore). Membranes were blocked with 5% non-fat dry milk for 30 min and incubated over night at 4°C with primary antibodies against PD-1 (cat. no. ab8245; 1:2,000 dilution; Abcam). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (cat. no. sc-51625; 1:1,500 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at 37°C. The protein expression levels were detected using the enhanced chemiluminescence detection system (Beyotime Institute of Biotechnology) and normalized to GAPDH. All experiments were repeated three times. Densitometry scores were determined using Quantity One software, version 4.6.9 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

ELISA. The concentration of PGE2 in the supernatant of the Tfh co-culture system with HepG2 or HepG2.2.1.5 was determined using the corresponding ELISA kit according to the manufacturers' protocol (cat. no. DPABH-29,498; Creative Diagnostics, Shirley, NY, USA).

Statistical analysis. Data are expressed as the mean ± standard deviation. Student's t-test was performed to analyze the results of the gene expression profiling assays. The comparisons between two groups were analyzed using the Mann-Whitney non-parametric test. Correlations between variables were evaluated using the Spearman's rank correlation test. All statistical analyses were performed by the SPSS version 19.0 (IBM SPSS, Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Distribution of Tfh-cell subsets in patients with HBV at different immune phases. In order to investigate the distribution of Tfh-cell subsets in patients with HBV infection the present study detected the frequencies of total Tfh, ICOS+ and PD-1+ Tfh cells in patients with HBV at different immune phases IT, IA and RP. Following the collection of PBMCs from patients and HC groups, anti-CD4-PE and anti-CXCR5-APC were used to label total Tfh cells (CD4+CXCR5+), whereas anti-ICOS-FITC and anti-PD-1-PerCP were used to distinguish ICOS+ Tfh and PD-1+Tfh cells (Fig. 1A). As presented in Fig. 1B and C, the frequency of total Tfh and ICOS+ Tfh cells was lower in the IT, RP and HC groups compared with the IA group. By contrast the percentage of PD-1+ Tfh cells was significantly higher in the IA group compared with the IT group (Fig. 1D); however, it was significantly reduced in the RP group compared with the IT and IA groups. The frequency of PD+ Tfh was higher in the HC group when compared with the RP group. Additionally, the ratio of PD-1+total Tfh cells exhibited similar changes to those observed in the PD-1+ Tfh cells, a higher frequency in IA and IT groups compared with RP and HC groups, whereas the ratio of ICOS+total Tfh cells was not changed much in HBV groups and HC (data not shown). The difference in PD-1+ Tfh cell frequency in patients at different immune stages indicated that PD-1+ Tfh cells may be closely associated with the development of HBV infection. Therefore, the potential association between PD-1+ Tfh cells and HBV-associated clinical parameters was also evaluated. It was determined that the frequency of PD-1+ Tfh cells was not associated with AST and ALT serum levels or HBV-DNA load (data not shown). Additionally, the ratio of PD-1+total Tfh cells was not associated with AST or ALT serum levels (data not shown). However, the ratio of PD-1+total Tfh cells was positively correlated with the load of HBV-DNA as presented in Fig. 2. These findings demonstrated that the ratio of PD-1+total Tfh cells may act as an indicator of HBV duplication.

Effect of HBV on PD-1 expression in Tfh cells. In order to investigate whether HBV may modulate PD-1+ Tfh cells, the
present study separated total Tfh cells from PBMCs using flow cytometry and subsequently co-cultured them with HepG2 and HepG2.2.1.5 cells. PD-1 mRNA and protein expression levels in Tfh cells were detected using RT-qPCR and western blotting. As presented in Fig. 3A the supernatant concentration of PGE2 was significantly higher in the HepG2.2.1.5 group compared with HepG2, it was possible that HBV may affect PD-1 expression by promoting an increase in PGE2 expression level. As presented in Fig. 4A the supernatant concentration of PGE2 was significantly higher in the HepG2.2.1.5 group compared with the HepG2 group. PGE2 protein expression was also increased in the HepG2.2.1.5 group compared with the HepG2 group (Fig. 4B). Subsequently, Tfh cells were co-cultured with HepG2.2.1.5, and a PGE2 inhibitor group, which was treated with 6 µM pranoprofen for 6 h, to evaluate the effect of PGE2 expression on PD-1 levels in Tfh cells. As presented in Fig. 4C and D, the mRNA and protein expression of PD-1 in Tfh cells was significantly reduced in the PGE2 inhibitor group. Therefore, HBV may upregulate of PD-1 expression in Tfh cells by promoting HepG2.2.1.5 to secrete PGE2.

Discussion

The initiation of HCC is closely associated with various etiological factors, including HBV and hepatitis C virus infection, carcinogen/toxin exposure and other environmental or genetic factors, such as excessive alcohol consumption, obesity and metabolic syndrome (2-4). Chronic HBV infection is a major risk factor for the development of HCC, which accounts for ~50% of all HCC cases. The pathogenesis of HBV virus-induced HCC has been previously reported to be primarily regulated by the chronic liver inflammation during the HBV infection, particularly the dysfunction of B and T cells (28,29). B cells are a major part of the lymphocyte-mediated humoral immunity and are associated with antibody production, directly contributing to the clearance of HBV. Additionally, memory
B cells may maintain the ability to quickly activate the rapid humoral response upon antigen re-encounter (30,31). The activation and functional differentiation of B cells may also be primarily regulated by one type of CD4<sup>+</sup> T cells, the Tfh cells, which are located in the GC of B cells GC, characterized by increased expression of CXCR5, ICOS, PD-1, CD40-ligand and transcription factor Bcl-6, and secretion of IL-21. Previous studies have reported that Tfh cells may promote the formation of GCs, high-affinity long-living plasma cells and memory B cells (32,33). Previous studies have shown a high frequency of ICOS and PD-1-expressing CD4<sup>+</sup>CXCR5<sup>+</sup> Tfh cells in patients with chronic HBV (34). Therefore, the disturbance of Tfh distribution may contribute to HBV development. Therefore, the present study initially analyzed the distribution of ICOS<sup>+</sup>, PD-1<sup>+</sup> and total Tfh cells in patients with HBV at different immune phases. The findings revealed that the frequencies of ICOS<sup>+</sup> and total Tfh cells were at a high level in IA; however, reduced frequency was observed in the IT, RP and HC groups. The percentage of PD-1<sup>+</sup> Tfh cells was significantly higher in the IA and IT groups compared with the RP and HC groups. The differential distribution of ICOS<sup>+</sup>, PD-1<sup>+</sup> and total Tfh cells in HBV at different immune stages indicated that the PD-1<sup>+</sup> Tfh cells maybe more sensitive to the HBV-induced immune system abnormalities. Therefore, the association between PD-1<sup>+</sup> Tfh cells and HBV-associated clinical features was investigated further. However, it was determined that the ratio of PD-1<sup>+</sup>/total Tfh cells, not the frequency of PD-1<sup>+</sup> Tfh cells was positively correlated with the load of HBV DNA in

**Figure 3. Regulation of HBV by PD-1 in Tfh cells.** Total Tfh cells were sorted from peripheral blood mononuclear cells using a FACSAria II and subsequently co-cultured with HepG2 and HepG2.2.1.5 cells for 6 h in a Transwell system. The (A) mRNA and (B) protein expression levels of PD-1 in sorted total Tfh. Tfh were co-cultured with HepG2.2.1.5 and transfected with HBeAg-shRNA or HBsAg-shRNA. PD-1 (C) mRNA and (D) protein expressions in HepG2.2.1.5 cells. Data are presented as the mean ± standard deviation. *P<0.05. HBV, hepatitis B virus; PD-1, programmed cell death 1; Tfh, T follicular helper; Con, HepG2.2.1.5 with empty shRNA vector; shRNA, short hairpin RNA; HBeAg, hepatitis B viral protein; HBsAg, surface antigen of HBV.
patients with HBV at the IA and IT stages. Therefore, this ratio may act as an indicator for HBV replication.

In order to determine the potential mechanism of HBV modulation of PD-1 expression in Tfh cells, total Tfh cells were co-cultured with HepG2 and HepG2.2.1.5 and PD-1 expression in Tfh cells was significantly increased in the co-culture group with HepG2.2.1.5 compared with the HepG2 co-culture group. In the co-culture system, HBV may influence Tfh cells primarily through the secretion of HBsAg and HBeAg; therefore, shRNA vectors targeting HBeAg and HBsAg were transfected into HepG2.2.1.5. Following the silencing of HBeAg and HBsAg, it was determined that the PD-1 expression level in Tfh cells did not change significantly. These findings implied that HBeAg and HBsAg secreted by HepG2.2.1.5 were not directly responsible for the changes in PD-1 expression in Tfh cells.

From the multiple cytokines and inflammatory factors secreted by HBV-infected cells, PGE2 may be capable of efficiently inducing immunological tolerance and maintain HBV-cell survival. The expression level of PGE2 may be modulated by inducible nitric oxide synthase, interleukins and Toll-like receptor signaling pathways and COX-2 is an upstream regulator of PGE2. Zheng et al (35) reported that HBx promoted HepG2 cell proliferation by upregulation of COX-2 expression. Therefore, the present study considered HBx may also affect PGE2 expression level. It was determined that the supernatant concentration of PGE2 was higher in the HepG2.2.1.5 group compared with the HepG2 group. Additionally, the expression of PGE2 at the transcriptional and translational level was significantly increased in the HepG2.2.1.5 group compared with the HepG2 group. Subsequently, Tfh cells were co-cultured with HepG2.2.1.5 and PD-1 expression in Tfh cells was significantly reduced in the group treated with the PGE2 inhibitor, pranoprofen. This indicated that HBV increasing PD-1 expression level in Tfh cells was primarily mediated by improving the ability of HepG2.2.1.5 to secret PGE2 as opposed to the secretion of HBeAg and HBsAg. However, whether the upregulation of PGE2 may be modulated by HBx remains to be elucidated.

In conclusion, the HBV-induced increase of PGE2 expression level in HepG2.2.1.5 contributed to the increased level of PD-1 in Tfh cells, which may lead to immune tolerance mediated by PD-1+ Tfh cells and the maintenance of chronic infection status. Understanding the regulatory mechanism of HBV in the modulation of different Tfh cell subsets is crucial for the development of novel immunotherapy-based approaches towards HBV.

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References


