

Original Article

Toll-like receptor 2 promotes T helper 17 cells response in hepatitis B virus infection

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Abstract: Purpose: Innate and adaptive immune responses play vital roles in initiating and maintaining the immunological homeostasis in both physiological and pathological processes. However, the expression and function of the important cells and molecules as well as their interaction in hepatitis B virus (HBV) infection has not been well elucidated. The aim of the current study was to determine the pattern of Toll-like receptor 2 (TLR2) in T cells in HBV infection and the function of TLR2 in regulation of T helper 17 (Th17) cells response. Methods: Thirty-four patients with HBV infection (ten acute and twenty-four chronic) were enrolled. HBV-specific and -nonspecific Th17 cells and TLR2 expression in T cells were analyzed by flow cytometry. The function of TLR2 agonist for induction of IL-17 production was also determined. Results: HBV-specific and -nonspecific IL-17 secretion in CD4⁺ (Th17 cells) and CD8⁺ T cells was significantly elevated in chronic HBV infection. Viral-specific TLR2 expression in CD4⁺, CD8⁺, and Th17 cells was also remarkably increased in patients with chronic hepatitis B. Moreover, TLR2 agonist Pam3Csk4 directly activated Th17 cells response without antigen stimulation in HBV infection. Conclusion: TLR2, which traditionally associated with innate immunity, might also promote Th17 cells response in HBV infection. The function of TLRs in regulation of adaptive immune response in HBV infection, which might play an important role in persistent HBV infection.

Keywords: Hepatitis B virus, Toll-like receptor, T helper 17 cells, immunoregulation

Introduction

Hepatitis B virus (HBV) infection is a potentially life-threatening infectious diseases of the liver, which is a major global health problem [1]. Hepatitis B has a complex natural history and causes a wide spectrum of disease. Acute hepatitis B (AHB) runs a self-limiting course with a complete resolution in majority of the patients. However, chronic hepatitis B (CHB) often results in liver cirrhosis and hepatocellular carcinoma, leading to millions of death each year worldwide from HBV-associated end-stage liver diseases [2, 3]. The precise mechanism associated with different clinical outcomes is still not fully understood. Increasing evidence has suggested that HBV is not directly cytopathic to infected hepatocytes, and the clinical consequences of HBV infection results from complicated interactions between the virus and the host immune response [4, 5].

CD4⁺ T cells secreting of interleukin-17 (IL-17) are a newly discovered T helper cell subsets 17

(Th17), which differ from Th1 and Th2 cells. They primarily secrete proinflammatory cytokines IL-17 and IL-22, which are closely related to the host anti-microbial immunity and inflammation [6, 7]. According to recently published data, Th17 cells and secreting cytokines contribute to exacerbate liver damage during chronic HBV infection and HBV-associated liver fibrosis, leading to the severity of disease progression [8-11]. However, the mechanism for modulation of Th17 cells functions during HBV infection remains obscure. Toll-like receptors (TLRs), which play an important role in early host defense and innate immune response, induce the expression of various cytokines and chemokines to control the activation of adaptive immune response [12, 13]. Peripheral and liver-resident TLRs also contribute to different acute and chronic liver diseases by triggering of antigen-presenting cells and costimulation of T cells [14]. Moreover, TLRs are involved in T cell development and differentiation [15], and TLR-induced cytokines (IL-23 and IL-1 β) promote Th17 cell response to promptly produce IL-17

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Table 1. Clinical characteristic of enrolled subjects

	NC	AHB	CHB
Case (n)	15	10	24
Sex (Male: Female)	6:9	7:3	19:5
Age (year)	30.2 ± 8.2	27.4 ± 9.7	29.6 ± 7.4
ALT (U/L)	N.D.	1147 (306-1538)	213 (91-1289)
HBsAg positive	0	10	24
HBeAg positive	0	5	17
Anti-HBc IgM positive	0	10	0
HBV DNA positive	0	10	24

Virological and Biochemical assessments

Semi-quantification of HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc was performed by electrochemiluminescence (Architect, Abbott Laboratories, and Abbott Park, IL, USA). Serum HBV DNA was quantified by real-time polymerase chain reaction (RT-PCR) kit (Da'an Gene Co. Ltd, Guangzhou, China) with detection limit thresh-

old of 500 copies/mL. Serum biochemical parameters (hepatic and renal functions) were measured using an automatic analyzer (Hitachi 7170A, Hitachi Ltd, Tokyo, Japan) in Department of Clinical Laboratory of Tangdu Hospital.

Peripheral blood mononuclear cells (PBMCs) isolation and stimulation

PBMCs were isolated using density gradient centrifugation by Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO, USA). The isolated PBMCs were cryopreserved at 5×10^6 /mL in 10% dimethyl sulfoxide (DMSO) and 90% fetal bovine serum (FBS; Invitrogen GIBCO, Grand Island, NY, USA) and thawed prior to analysis.

The PBMCs were thawed once day before the experiments, and cell viability was over 90% by trypan blue exclusion. The concentration of cells (10^6 /mL) was adjusted using RPMI 1640 (Hyclone, Logan, Utah, USA) supplemented with 10% FBS, and cells were incubated in 5% CO₂ incubator. PBMCs were stimulated with PMA (50 ng/mL) and ionomycin (1 µg/mL), supplemented with Brefeldin A (BFA, 10 µg/mL) for 6 hours for measurement of non-specific Th17 cells. Otherwise, cells were incubated with HBV envelope peptide pool (15 amino acids of each peptide with 5 amino acids overlapping, final concentration 2.5 µg/mL), with BFA (10 µg/mL) for 12 hours for detection of HBV-specific Th17 cells. In some experiments, PBMCs were stimulated Pam3Csk4 (InvivoGen, San Diego, CA, USA; final concentration 3 µg/mL) for 24 hours to analysis the influence of TLR2 in Th17 cells responses.

Flow cytometry

The monoclonal antibodies (mAb) CD3-FITC (eBioscience, San Diego, CA, USA), CD4-PE (eBioscience) and TLR2-PE-Cy7 (eBioscience)

and IL-22 [16]. Our previous study revealed that overexpression of TLR2/4 on monocytes modulates the activity of regulatory T cells (Tregs) in patients with HBV infection [17]. Furthermore, Reynolds *et al.* [18] demonstrated that T cell expression of TLR2 directly regulated Th17 cell proliferation and Th17 cytokine production in experimental autoimmune encephalomyelitis (EAE). Thus, we hypothesized that TLR2 regulates Th17 cells response to contribute to the establishment of chronic HBV infection. To test this possibility, we therefore examined the frequencies of Th17 cells and TLR2⁺CD4⁺ T cells in response to either HBV peptides or TLR2 agonist.

Materials and methods

Subjects

A total of 34 patients with HBV infection, including 10 AHB patients and 24 CHB patients were enrolled in this study. The standards of diagnosis conformed to the diagnostic standard of Chinese National Program for Prevention and Treatment of Viral Hepatitis. All patients were followed up in Tangdu Hospital from July 2012 to July 2013. For normal controls, fifteen healthy age- and sex-matched individuals were selected in the present study. All enrolled subjects were confirm without co-infection of other viral hepatitis, HIV, or concurrently had immune-compromised diseases or autoimmune disorders. Patients who received nucleotide analogues or interferon therapy during the preceding year were excluded. The clinical data obtained for the enrolled subjects are listed in **Table 1**. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and written informed consent was obtained from each participant. The study protocol was approved by the Ethics Committee of Tangdu Hospital.

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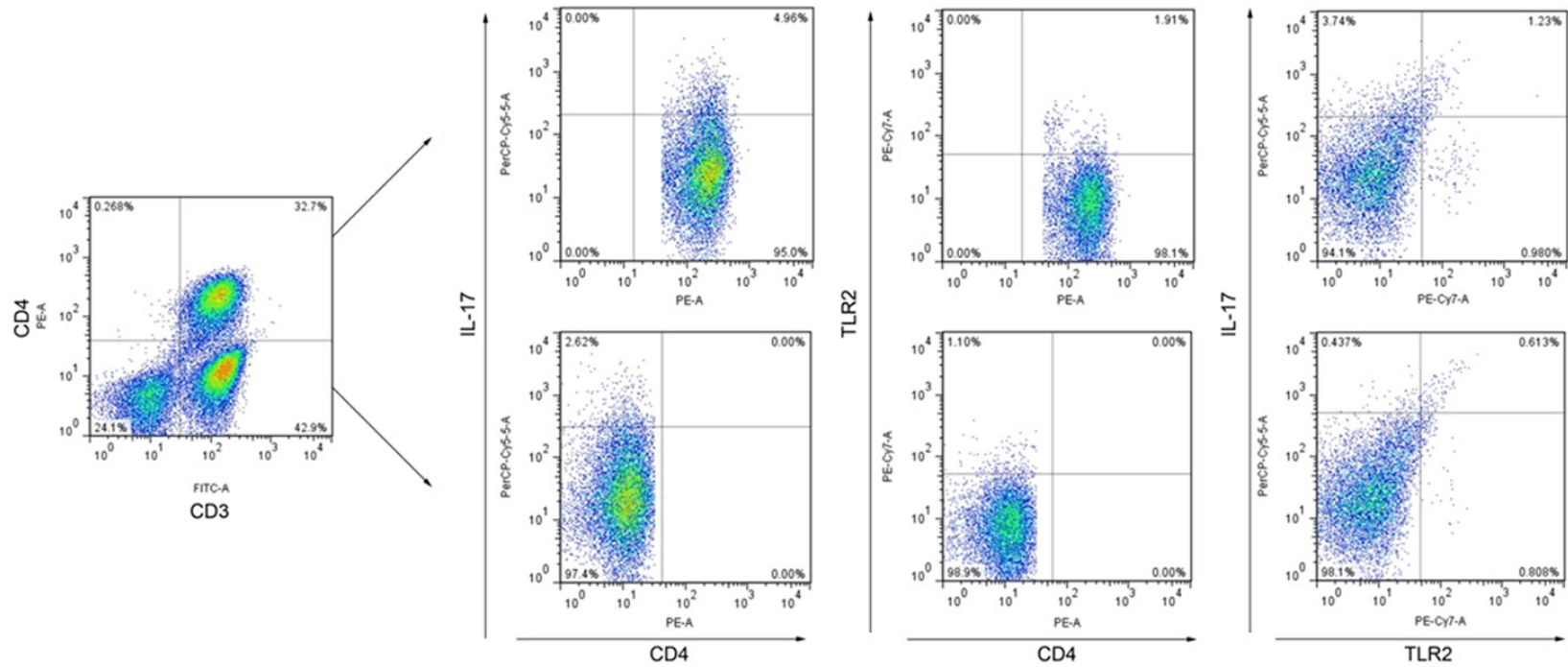


Figure 1. Typical IL-17 and TLR2 profile in peripheral blood lymphocytes from enrolled subjects. Isolated PBMCs from a representative patients with chronic hepatitis B were separated using the gates shown by flow cytometry after antigen stimulation. IL-17 production and TLR2 expression in CD3⁺CD4⁺ (upper panels) and CD3⁺CD4⁻ (mostly CD8⁺, lower panels) subsets was shown.

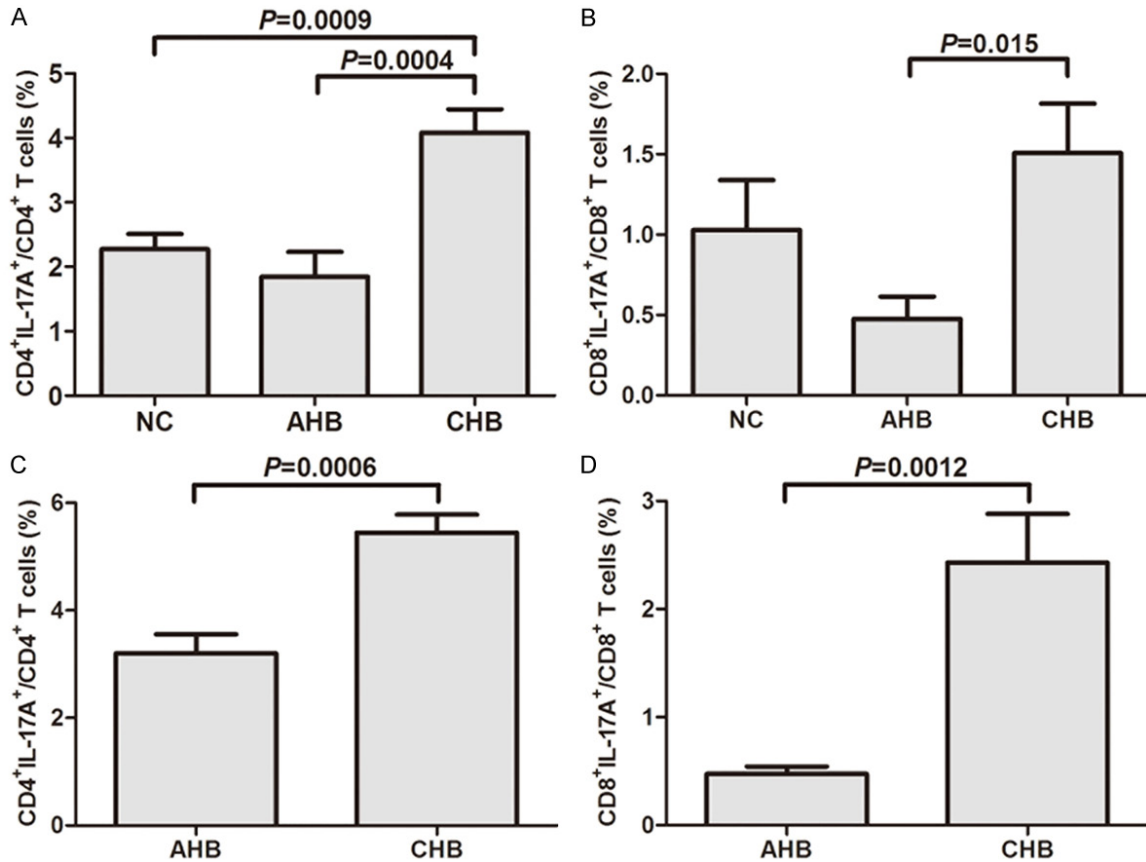


Figure 2. HBV-nonspecific and -specific IL-17 production in CD4⁺ and CD8⁺ T cells was significantly elevated in chronic HBV infection. Viral-nonspecific IL-17 production in CD4⁺ T cells (A) and CD8⁺ T cells (B) was analyzed in normal controls (NC), acute hepatitis B (AHB), and chronic hepatitis B (CHB). Viral-specific IL-17 production in CD4⁺ T cells (C) and CD8⁺ T cells (D) was analyzed in AHB and CHB patients.

were added and incubated at 4°C in the dark for 30 min. After staining, the cells were fixed by adding 100 µL of Fixation & Permeabilization Medium A (Caltag Laboratories, Invitrogen, Carlsbad, CA, USA), then incubated in the dark at room temperature for 15 minutes. Cells were resuspended in 100 µL of Fixation & Permeabilization Medium B (Caltag Laboratories) containing mAb IL-17A-PerCP-Cy5.5 (eBioscience) for 20 min incubation. Isotype control antibodies were used to separate positive and negative cells in the PerCP, FITC, PE, and PE-Cy7 fluorescence channels. Cell samples were analyzed with a four-color FACS Calibur analyzer (BD Biosciences Immunocytometry Systems, San Jose, CA, USA). Acquisitions were performed with CellQuest Pro software (BD Biosciences Immunocytometry Systems) and analyses were performed with FlowJo version 8.7.2 for Windows (Tree Star Inc., Ashland, OR, USA).

Statistical analysis

Data were analyzed using SPSS version 13.0 for Windows (SPSS, Chicago, IL, USA). The Kruskal-Wallis H test and Dunn's Multiple Comparison test were used for comparison between groups. A value of $P < 0.05$ was considered to indicate a significant difference.

Results

Elevation of HBV-specific and non-specific Th17 cells in CHB patients

For each tested PBMCs, 10,000 events were acquired in a stored live lymphocyte gate. Typical flow cytometry determination of IL-17A secretion in response to either PMA+ ionomycin or HBV envelope peptides pool stimulation were shown in **Figure 1**. On the basis of intracellular cytokine staining (ICS) analyses, little IL-17A production can be found in either CD4⁺ or CD8⁺ (gated as CD3⁺CD4⁻) T cells without

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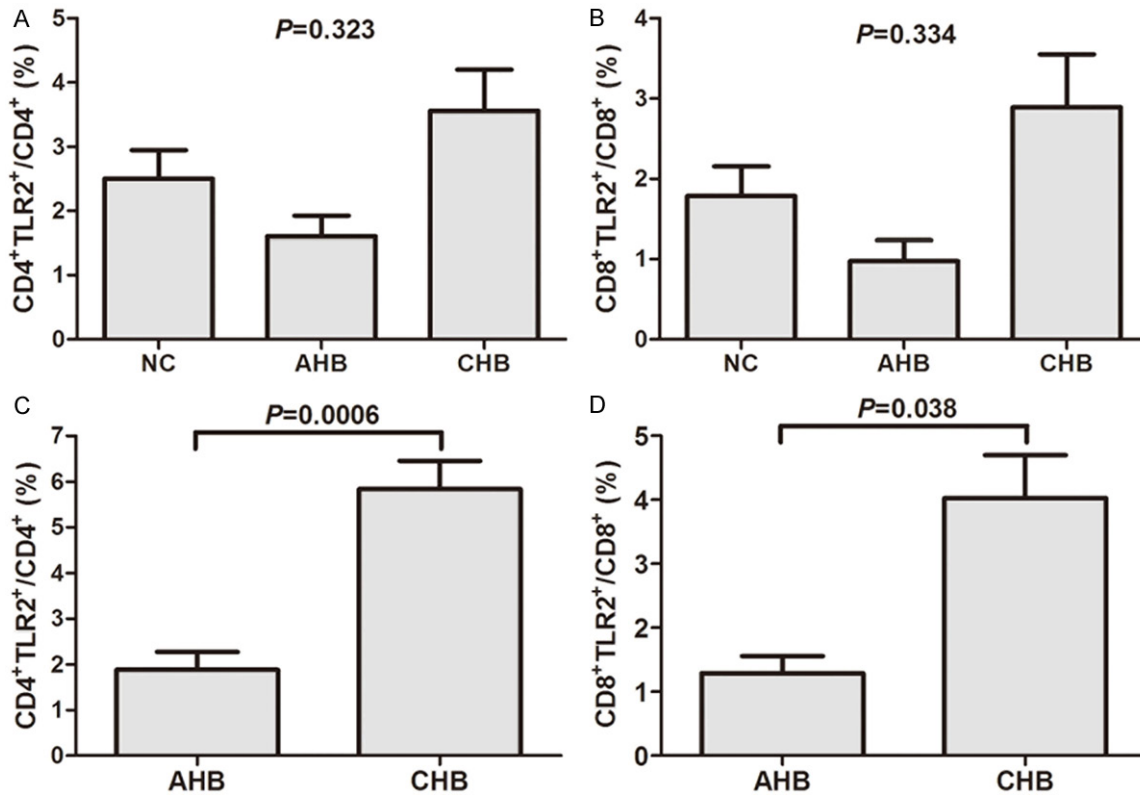


Figure 3. HBV-specific TLR2 expression in CD4⁺ and CD8⁺ T cells was significantly elevated in chronic HBV infection. Viral-nonspecific TLR2 expression in CD4⁺ T cells (A) and CD8⁺ T cells (B) was analyzed in normal controls (NC), acute hepatitis (B) (AHB), and chronic hepatitis (B) (CHB). Viral-specific TLR2 expression in CD4⁺ T cells (C) and CD8⁺ T cells (D) was analyzed in AHB and CHB patients.

stimulation. After challenge with PMA and ionomycin, the frequency of viral non-specific Th17 cells (CD3⁺CD4⁺IL-17A⁺) accounted for CD4⁺ T cells was significantly higher in patients with CHB ($4.08 \pm 1.78\%$) than either normal controls ($2.27 \pm 0.78\%$, $P = 0.0009$) or AHB patients ($1.85 \pm 1.28\%$, $P = 0.0004$, **Figure 2A**). Furthermore, increased proportion of IL-17A expression in stimulated CD8⁺ T cells in CHB patients ($1.51 \pm 1.44\%$) compared with AHB patients ($0.48 \pm 0.46\%$, $P=0.015$, **Figure 2B**). Moreover, there were consistent trends of elevated HBV-specific IL-17A productions in both CD4⁺ and CD8⁺ T cells in response to envelope peptides stimulation. Both HBV-specific Th17 cells and CD3⁺CD8⁺IL-17A⁺ cells were remarkably increased in patients with CHB ($5.45 \pm 1.61\%$ and $2.43 \pm 2.16\%$) than AHB ($3.20 \pm 1.13\%$ and $0.47 \pm 0.23\%$, $P = 0.0006$, and $P = 0.0012$ respectively, **Figure 2C** and **2D**).

Increase of viral-specific TLR2 expression on T cells in CHB patients

Our previous study has demonstrated the over-expression of TLR2 on monocytes in HBV-

infected patients [17]. Thus, we tried to analysis the expression of TLR2 on CD4⁺ and CD8⁺ T cells. The typical flow cytometry analyses of TLR2 expression on CD4⁺ and CD8⁺ T cells were shown in **Figure 1**. The proportion of viral non-specific TLR2 expression on CD4⁺ T cells was $2.50 \pm 1.61\%$ (NC), $1.61 \pm 0.96\%$ (AHB), $3.56 \pm 2.87\%$ (CHB) (**Figure 3A**, $P = 0.323$), and on CD8⁺ T cells was $1.78 \pm 1.38\%$ (NC), $0.97 \pm 0.82\%$ (AHB), $2.89 \pm 2.88\%$ (CHB), respectively (**Figure 3B**, $P = 0.334$). No significant differences showed of non-specific TLR2 population on T cells. In contrast, HBV envelope peptides induced notably higher percentage of viral specific TLR2 expression on CD4⁺ and CD8⁺ T cells in patients with CHB ($5.84 \pm 2.85\%$ and $1.88 \pm 1.30\%$) when compared with AHB ($1.88 \pm 1.30\%$ and $1.29 \pm 0.85\%$) ($P = 0.0006$, **Figure 3C**, and $P = 0.038$, **Figure 3D**, respectively).

TLR2 enhances Th17 cells response in CHB patients

To investigate the effect of TLR2 on Th17 cells, we firstly determine the expression of TLR2 on

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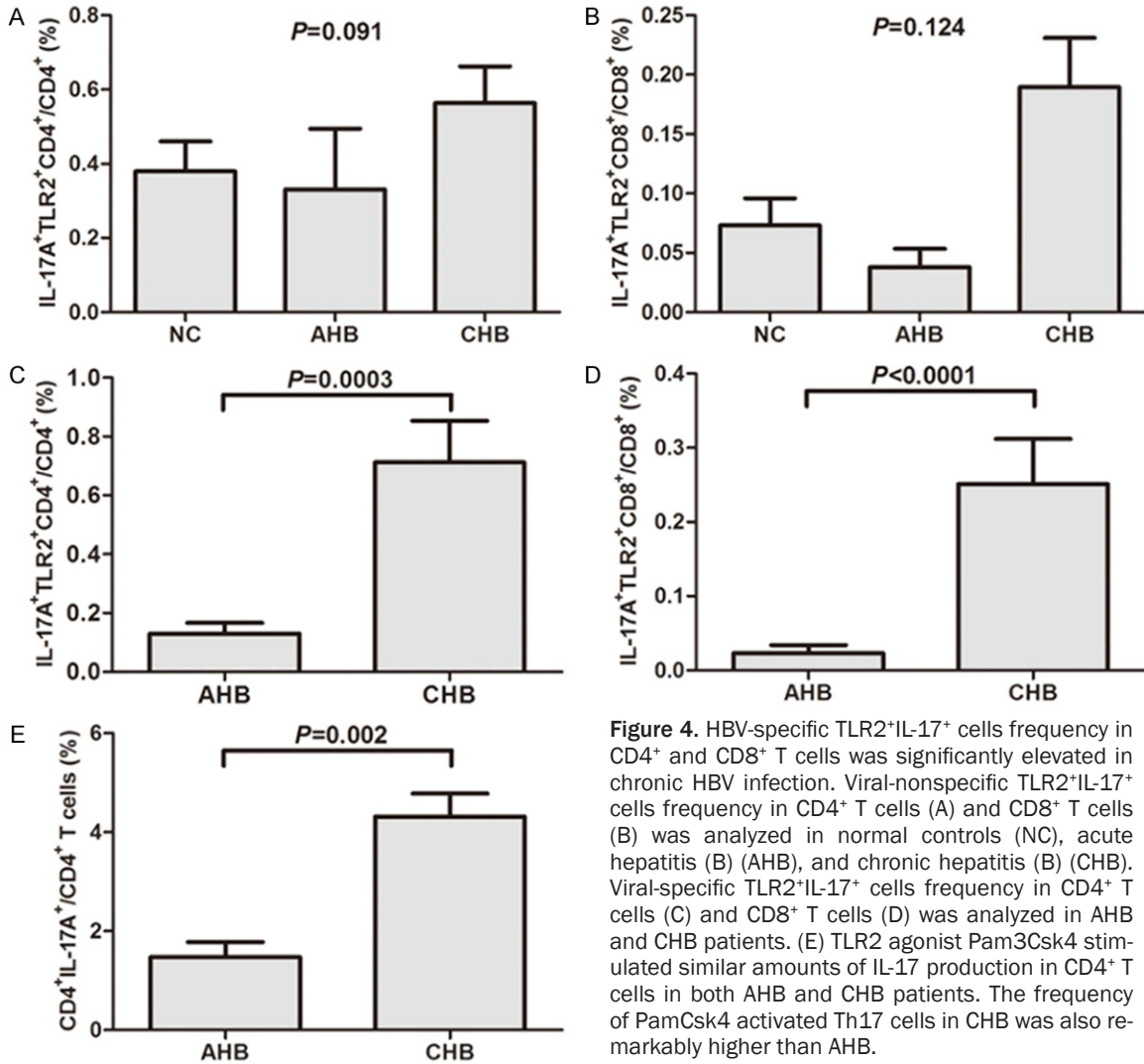


Figure 4. HBV-specific TLR2⁺IL-17⁺ cells frequency in CD4⁺ and CD8⁺ T cells was significantly elevated in chronic HBV infection. Viral-nonspecific TLR2⁺IL-17⁺ cells frequency in CD4⁺ T cells (A) and CD8⁺ T cells (B) was analyzed in normal controls (NC), acute hepatitis (B) (AHB), and chronic hepatitis (B) (CHB). Viral-specific TLR2⁺IL-17⁺ cells frequency in CD4⁺ T cells (C) and CD8⁺ T cells (D) was analyzed in AHB and CHB patients. (E) TLR2 agonist Pam3Csk4 stimulated similar amounts of IL-17 production in CD4⁺ T cells in both AHB and CHB patients. The frequency of PamCsk4 activated Th17 cells in CHB was also remarkably higher than AHB.

Th17 cells. There were no notable differences of frequency in TLR2⁺IL-17A⁺ cells on CD4⁺ cells [$0.38 \pm 0.30\%$ (NC), $0.33 \pm 0.54\%$ (AHB), $0.56 \pm 0.48\%$ (CHB) (**Figure 4A**, $P = 0.091$)], or on CD8⁺ T cells [$0.07 \pm 0.08\%$ (NC), $0.04 \pm 0.05\%$ (AHB), $0.19 \pm 0.20\%$ (CHB) (**Figure 4B**, $P = 0.124$)]. Furthermore, HBV-specific TLR2/IL-17A expressions were significantly elevated in both CD4⁺ and CD8⁺ T cells of CHB patients ($0.71 \pm 0.69\%$ of CD4⁺, and $0.13 \pm 0.11\%$ of CD8⁺) than those of AHB patients ($0.13 \pm 0.11\%$ of CD4⁺, $P = 0.0003$, **Figure 4C**, and $0.13 \pm 0.11\%$ of CD8⁺, $P < 0.0001$, **Figure 4D**). More importantly, we treated the isolated PBMCs with Pam3Csk4 (TLR2 agonist) to analyze the Th17 response in HBV-infected patients. As shown in **Figure 4E**, Pam3Csk4 induced comparable level of Th17 cell in both AHB and CHB patients. The frequency of Th17 cells in CHB patients

($4.31 \pm 1.39\%$) was remarkably higher than AHB patients ($1.48 \pm 0.68\%$, $P = 0.002$).

Discussion

In the present study, we characterized IL-17 production and TLR2 expression in CD4⁺ and CD8⁺ T cells in response to antigen-specific or nonspecific stimulation during acute and chronic HBV infection. Our data showed that IL-17 secretion was significantly increased in both HBV-specific and non-specific T cells in CHB patients. We also observed that viral-specific CD4⁺ and CD8⁺ T cells from patients with chronic HBV infection expressed remarkably higher levels of TLR2 proteins compared with those from AHB patients. Moreover, TLR2 expression on HBV-specific Th17 cells (CD3⁺CD4⁺IL-17⁺ cells) was also increased in CHB patients, and

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more importantly, TLR2 agonist promoted Th17 differentiation *in vitro* in both AHB and CHB patients, which led to comparable IL-17 production in peptides stimulation manners. The current results indicated that TLR2, which play an important role in recognition and initiation of innate immune response [17], may also directly regulated adaptive immune response in chronic HBV infection.

TLRs were mainly considered as “first-line of defense” in the innate immune response to invading pathogens [19]. These receptors also modulated adaptive immunity indirectly through inducing the elevated expression of co-stimulatory molecules on antigen-presenting cells and maturation of dendritic cells [20]. Furthermore, TLRs have been reported to regulate the differentiations and functions of T cells. Activated CD4⁺ T cells expressed higher TLR2 level as a co-stimulatory for antigen-specific T cell development, which may directly contributed to maintenance of T cell memory [21]. M. tuberculosis and its components also directly affected cytokine production, proliferation, survival, and migration of local CD4⁺ T cells through TLR2-MyD88 signaling in patients with tuberculous pleurisy [22]. Co-stimulation with TLR2 agonist un-regulated cytotoxic antigen-activated T cells activity and proliferation, as well as increased interferon- γ and granzyme B production in both T-cell receptor transgenic CD8 OT-1 T cells line [23] and F5 TCR-transgenic mice [24], which indicating that TLR2 enhanced the effector functions of CD8⁺ T cells. Moreover, intracytosolic receptor NOD1 cooperated with TLR2 engagement in CD8⁺ T cells to enhance the T cell receptor-mediated activation [25]. Our previous study revealed that TLR2 agonist-activated CD4⁺CD25⁺ regulatory T cells (Tregs) showed increased suppression function in CHB patients, which demonstrated that overexpression TLR2 on monocytes modulated inhibitive function of Tregs and contribute to the immunotolerance for establishing persistent HBV infection [17]. Heat shock protein 60 also engaged in modulating adaptive immune response by up-regulating Tregs through TLR2 signaling [26]. Reynolds *et al.* [18] investigated TLR2 expression in CD4⁺ T cells directly enhanced Th17 differentiation, proliferation, and cytokine production in the pathogenesis of autoimmune disease. However, TLR2 expression in antigen-specific T cells remains not fully elucidated. Our

current results also showed that TLR2 was elevated expressed in both HBV-specific CD4⁺, CD8⁺, and Th17 cells in CHB patients. More importantly, TLR2 agonist solely raised IL-17 production without anti-CD3/CD28 or HBV peptides co-stimulation, indicating that TLR2 may be involved in Th17 differentiation and proliferation in HBV infection.

Th17 cells have been demonstrated to play an important role in HBV induced liver injury in many previous studies [9, 10, 27, 28]. Th17 cells and Tregs shared the same naïve CD4⁺ T cells and the developmental pathway were reciprocally interconnected [29]. Thus, the balance between Th17 and Tregs may impact the process and outcomes of autoimmune and infectious diseases. Previous studies have revealed imbalance of Tregs/Th17 ratios in HBV infection [30-32], and antiviral therapy leaded to the reduction in Tregs together with increase in Th17 cells [33, 34]. TLR2 agonist Pam3Csk4 elevated the proliferation of Tregs in the presence of antigen stimulation, thus we hypothesized that TLR2 could also regulated differentiation and proliferation of Th17 cells. We found that treatment of PBMCs with Pam3Csk4 induced similar amounts of IL-17 expression in HBV infection even without TCR stimulation. This is consistent with the previous results in EAE, which revealed that TCR activation may not be required for secretion of IL-17 in memorial Th17 cells [18]. Thus, the current results may suggested an alternative pathway for Th17 maintenance required for TLR2 stimuli in HBV infection, which may contribute to establish chronic infection.

In conclusion, the current study highlights that TLR2 expression was induced in T cells with both acute and chronic HBV infection. HBV-specific TLR2 expression was found in both CD4⁺ and CD8⁺ T cells in patients with CHB. Moreover, TLR2, which traditionally associated with innate immunity, might also promote Th17 cells response in HBV infection. These data implicated the function of TLRs in regulation of adaptive immune response in HBV infection, which might play an important role in persistent HBV infection.

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Disclosure of conflict of interest

None.

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