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HBcAg induces PD-1 upregulation on CD4$^+$T cells through activation of JNK, ERK and PI3K/AKT pathways in chronic hepatitis-B-infected patients

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Hyper-expression of programmed death-1 (PD-1) is a hallmark of exhausted T cells. In chronic hepatitis-B virus (HBV)-infected patients, PD-1 upregulation on T cells was often observed. The mechanism of it has not been fully understood. In this study, we examined the dynamic changes of PD-1 expression on T cells during the natural history of chronic HBV infection and explored the signaling pathway of PD-1 upregulation by the hepatitis-B core antigen (HBcAg). Sixty-seven chronic HBV-infected patients were categorized into an immune tolerance group, an immune clearance group and an inactive virus carrier group, and 20 healthy volunteers were chosen as normal control group. Peripheral blood mononuclear cells from patients and healthy volunteers, and T lymphocytes from healthy volunteers were separated. Results showed that the PD-1 expression level on CD4$^+$T cells in every phase of chronic HBV infection was significantly higher than that in healthy volunteers, whereas such effects were not observed on CD8$^+$T cells. In the immune clearance phase, a positive correlation was found between serum HBV DNA level and the PD-1 expression level on CD4$^+$T cells. In all phases, no correlation was shown between serum alanine amino transferase (ALT) level and PD-1 expression level. Phosphorylation of JNK, ERK and AKT was induced by HBcAg, and inhibitors of JNK, ERK and PI3K/AKT significantly decreased the HBcAg-induced PD-1 upregulation on CD4$^+$T cells. In conclusion, the PD-1 expression level on CD4$^+$T cells was upregulated in every phase of chronic HBV infection, which was induced by HBcAg through JNK, ERK and PI3K/AKT signaling pathways.

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Hepatitis-B virus (HBV) poses a great threat to humans, with serious consequences including hepatocirrhosis, hepatocellular carcinoma (HCC) and polyarteritis nodosa.[1] This infection is prevalent in Asia, Africa, Southern Europe and Latin America.[2] An individual can develop HBV infection that is acute and achieve complete immune clearance of the virus, yielding lifelong immunity; however, an alternate fate of the host is development of chronic hepatitis-B (CHB). Chronic infection may persist for life and cause varying grades of chronic liver injury, which may lead to hepatocirrhosis and/or HCC.[3,4] During chronic HBV infection, a dynamic balance between viral replication and host immune response is pivotal to the pathogenesis of liver disease. According to immune characteristics, chronic HBV infection is clinically categorized into three periods, namely an immune tolerance phase, an immune clearance phase, and an immune stable phase or inactive virus carrier phase.[5–7]

The interaction between positive and negative co-stimulatory molecules expressed on T cells and antigen-presenting cells is essential for the development of T-cell responses. Programmed cell death-1 (PD-1), a member of the CD28 family, which is expressed on activated T and B cells,[8] was first isolated from T-cell hybridoma by subtractive hybridization in 1992.[9] PD-1 has a significant role in some autoimmune diseases[10–12] and viral infectious diseases, especially in chronic infectious diseases caused by HIV,[13–15]

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hepatitis-C virus (HCV)[16,17] and HBV.[18–20] PD-1 has been found to have a role in immunity regulation as an inhibitory co-stimulation molecule,[21] and has a crucial role in initiating and maintaining peripheral tolerance.[22,23] PD-1-deficient mice (PDCD1−/−) develop various spontaneous autoimmune diseases depending on the genetic background.[24]

Moreover, blockade of the PD-1 pathway was shown to enhance antiviral and antitumoral immunity.[24–28]

Recently, Peng *et al*[20] reported that the PD-1 expression level was elevated on HBV-specific T cells in chronic HBV-infected patients. PD-1$^+$ tumor-infiltrating lymphocytes correlate with portal vein thrombosis and might serve as a potential prognostic marker of and a novel therapeutic target for HBV-related HCC.[29] One recent report showed that PD-1 polymorphism was related with HBV infection, which suggested that the PD-1 gene may be one of the genes predisposing to chronic HBV infection and disease progression.[30] Here, we focused on the dynamic changes of PD-1 expression on T cells from peripheral blood in the natural history of chronic HBV infection. Understanding such changes in PD-1 expression in each phase in the natural history of chronic HBV infection is crucial to the management of HBV carriers. Furthermore, the mechanism of PD-1 upregulation in chronic HBV-infected patients is still unknown. A better understanding of the mechanism of PD-1 upregulation is important to the study of blocking the PD-1/programmed death ligand-1 (PD-L1) pathway. Hepatitis-B core antigen (HBcAg) is a hepatitis-B viral protein;[31,32] it is produced by the HBV DNA and is an indicator of active viral replication. Therefore, we analyzed PD-1 expression levels on T-cell surface in chronic HBV-infected patients in different infectious phases, as well as the correlation between PD-1 expression level and serum HBV DNA level and serum alanine amino transferase (ALT) level. Further investigations of PD-1 expression signaling pathways induced by HBcAg were also performed.

Our results suggested that the PD-1 expression levels on CD4$^+$T cells in peripheral blood in chronic HBV-infected patients with different infectious phases were all elevated compared with normal control. In the inactive virus carrier phase, the PD-1 expression level on CD4$^+$T cells tended to be the highest, but there was no significant difference as compared with other two phases. In the immune clearance phase, the PD-1 expression level on CD4$^+$T cells was positively correlated with serum HBV DNA level. There was no correlation between PD-1 expression level and serum ALT level. More importantly, PD-1 expression on CD4$^+$T cells can be upregulated by HBcAg *in vitro*, which was related with signaling molecules, including JNK, ERK and PI3K/AKT.

MATERIALS AND METHODS

Subjects

Sixty-seven chronic HBV-infected patients (50 males and 17 females), enrolled in the study (Table 1), were positive for HBV surface antigen (HBsAg) and anti-HBc, but negative for Abs to HCV, hepatitis-D virus, HIV-1 and HIV-2, and had no other symptoms of chronic liver damage. The patients were observed for more than 72 weeks during which liver function and serum DNA level were tested every 12 weeks. No patients were treated for chronic HBV infection or received any other medication 72 weeks prior to blood sampling. Among the 67 patients, 19 were in the immune tolerance phase with the presence of the hepatitis-B e-antigen (HBeAg), a high serum HBV DNA level and a normal ALT level; 28 were in the immune clearance phase with a persistent elevated serum ALT level and positive serum HBV DNA level; and 20 were in the inactive virus carrier phase, being negative for HBeAg and positive for Abs to HBeAg, with an undetectable or low HBV DNA level. The normal control group, matched for age, sex and race, comprised 20 healthy volunteers who had no evidence of exposure to HBV (HBsAg-negative).

All patients and normal controls were Chinese. Our study was approved by the local ethics committee and all patients provided written informed consent according to a protocol reviewed and approved by the institutional review board of Shanghai Shuguang Hospital.

Serum Viral Load, HBV Serum Markers and ALT Determination

Serum HBV DNA load in chronic HBV-infected patients was measured by real-time PCR using a LightCycler PCR system (FQD-33A) with a lower limit of approximately 1000 viral

Table 1 Characteristics of HBV-infected patients and normal controls

	Sex		Age (year)[a]	ALT (U/l)[a]	eAg(+)	HBV DNA(copies/ml)				
	Male	Female				NA	$<10^3$	10^3–10^5	10^5–10^7	$>10^7$
NC	14	6	29.0 (24.0–35.0)	20.0 (7.0–38.0)	—	20				
Immune tolerance	13	6	30.0 (19.0–55.0)	38.0 (20.0–55.0)	19		0	0	3	16
Immune clearance	25	3	32.0 (21.0–49.0)	90.0 (65.2–232.0)	23		0	0	9	19
Inactive carrier	12	8	34.5 (16.0–65.0)	25.5 (14.0–58.5)	0		4	16	0	0

Abbreviations: ALT, alanine aminotransferase; HBV, hepatitis-B virus; NA, not applicable.
[a]Values are expressed as median (range).

genome copies per milliliter. The handling procedures were performed in strict accordance with the instructions in the reagent kit (Shenzhen PG Biotech Co. Ltd.). Results were considered abnormal when HBV DNA load was >1000 copies/ml. HBsAg; HBeAg; anti-HBs; anti-HBc; anti-HBe; and Abs against HCV, hepatitis-D Virus, HIV-1 and HIV-2 were detected by ELISA using commercially available kits (Sino-American Biotechnology Company).

Serum ALT levels were assayed by using the DXC 800 Fully-auto Bio-Chemistry Analyzer, at the Department of Clinical Laboratory, Shuguang Hospital, Shanghai University of Traditional Chinese Medicine, China.

Isolation of PBMCs and T Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by standard density-gradient centrifugation using Lympholyte-H (Cedarlane) according to the manufacturer's protocol. T cells were isolated from PBMCs using the Dynabeads Untouched Human T Cells kit (Invitrogen Dynal AS) according to the manufacturer's protocol. $CD4^+$ T cells were isolated from PBMCs using the Dynabeads Untouched Human $CD4^+$ T cells kit (Invitrogen Dynal AS). After isolation, T cells and $CD4^+$ T cells were resuspended in the respective supplemented RPMI-1640 medium (Gibco). Subsequently, cells were cryopreserved in a medium containing 75% FBS, 15% RPMI-1640 and 10% DMSO. The cells were thawed by a step-by-step, gradual dilution method. Cell viability was over 90%, as assessed by Trypan blue exclusion.

Real-Time RT-PCR

RNA was treated with RNase-free DNase-I (Takara Bio Inc.) and reverse-transcribed using avian myeloblastosis virus reverse transcriptase (Promega). Real-time PCR was performed using the Mastercycler ep realplex 4 real-time PCR system (Eppendorf) with an SYBR Green qPCR Master Mix (Fermentas), according to the manufacturer's protocol. The sequences of primers for human PD-1 (NM_005018.2) were 5′-ATCAAAGAGAGCCTGCGGG-3′ (forward) and 5′-GGTG GGCTGTGGGCACT-3′ (reverse).[33] The primers for human GAPDH (NM_002046.3) were 5′-TGCACCACCAACTGCTT AGC-3′ (forward) and 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse). The amplification conditions were as follows: 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 64 °C for 30 s and 72 °C 20 s. The amount of PD-1 transcripts of individual samples was normalized to GAPDH.

Flow Cytometry

Cells were stained with Peridinin–Chlorophyll Protein Complex (PerCP)-, FITC-, phycoerythrin (PE)- and allophycocyanin (APC)-labeled mAbs, respectively, according to instructions of the respective manufacturers. FCM was performed using FACSAria (Becton Dickinson, San Jose, CA, USA). PerCP-anti-CD3, FITC-anti-CD4, PE-anti-PD-1 and APC-anti-CD8 mAbs were obtained from BD PharMingen (BD Biosciences, San Jose, CA, USA).

Gene Expression after *In Vitro* Stimulation and Inhibitor Experiment

In order to examine PD-1 expression on T cells, thawed T cells or $CD4^+$ T cells were cultured overnight in culture medium (RPMI-1640, 10% FBS). Cells (1×10^6) were further diluted with 200 μl of RPMI-1640 medium in the presence of HBcAg (10 μg/ml) and IL-2 (20 U/ml). IL-2 (20 U/ml) was added to the culture medium to prevent the anergic state of T cells.[34,35] HBcAg (HBV-232) was purchased from Prospec-Tany TechnoGene Ltd. IL-2 (200-02) was purchased from PeproTech. HBcAg (10 μg/ml) was added to culture medium of T lymphocytes to examine its effects on the expression of PD-1 in every group. T cells were respectively cultured for 0, 12, 24, 48 and 72 h in 96-well cell culture plates at 37 °C under 5% CO_2. Then the T cells were collected and PD-1 expression was examined by real-time RT-PCR and FCM.

In the inhibitor experiment, T cells or $CD4^+$ T cells were incubated with LY294002 (PI3K/AKT inhibitor, 25 μM), U0126 (ERK inhibitor, 150 nM), SB203580 (p38 inhibitor, 1 μM) and SP600125 (JNK inhibitor, 100 nM) for 1 h and then HBcAg (10 μg/ml) was added to culture medium of every group. After 48 h, T cells were collected and PD-1 expression was examined by FCM and western blotting. LY294002 (V1201), U0126 (V1121) and SB203580 (V1161) were purchased from Promega Corporation. SP600125 (s5567) was purchased from Sigma. PD-1 monoclonal antibody was purchased from Abcam.

Western Blotting

Cells were washed twice with ice-cold PBS and prepared with RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate and 0.1% SDS) containing protease inhibitor mixture (Roche). The samples were separated by SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Millipore) using SemiDry Transfer Cell (Bio-Rad). The polyvinylidene difluoride membrane was blocked with 5% non-fat milk and incubated with the first Ab at 4 °C overnight. The blots were incubated with an HRP-conjugated secondary Ab (Santa Cruz Biotechnology) for 1 h at room temperature. The Abs against PD-1 were purchased from Abcam Inc. and the Abs against AKT/p-AKT, ERK/p-ERK, JNK/p-JNK and p38/p-p38 were purchased from Santa Cruz Biotechnology.

Statistical Analysis

One-way ANOVA was used to compare multiple groups and two groups using the SPSS software. Pearson correlation analysis of PD-1 expression and HBV viral titers, and ALT level, was performed. Differences were considered statistically significant at $P<0.05$. The test of significance was two-sided.

RESULTS

PD-1 Expression Level was Elevated on CD4$^+$T Cells in Peripheral Blood in Chronic HBV-Infected Patients

Chronic HBV infection develops when the host's immune response fails to eradicate the primary infection. Among the many co-stimulatory molecules, PD-1, which is expressed on T cells, is an important molecule that regulates and fine-tunes immune responses.[36–38] In this study, the PD-1 expression levels on CD4$^+$T cells and CD8$^+$T cells in all 67 chronic HBV-infected patients were analyzed by FCM. Representative FCM histograms of circulating PD-1-positive CD4$^+$T cells and PD-1-positive CD8$^+$T cells in one chronic HBV-infected patient and one normal control people are presented in Figure 1.

Both the percentage of PD-1-positive T cells and the mean fluorescence intensity (MFI) of PD-1 expression were used to evaluate the PD-1 expression level. Our results showed that the percentage of PD-1-positive CD4$^+$T cells in chronic HBV-infected patients was more than that in normal controls (Figure 2a). Also, the MFI of PD-1 expression in chronic HBV-infected patients was significantly increased as compared with that in normal controls (Figure 2b). By contrast, both the percentage of PD-1-positive CD8$^+$T cells and the MFI of PD-1 expression on CD8$^+$T cells in the patients showed no significant difference between the two groups (Figures 2c and d). These results indicated that the PD-1 expression level on CD4$^+$T cells was elevated in chronic HBV-infected patients compared with that in normal controls.

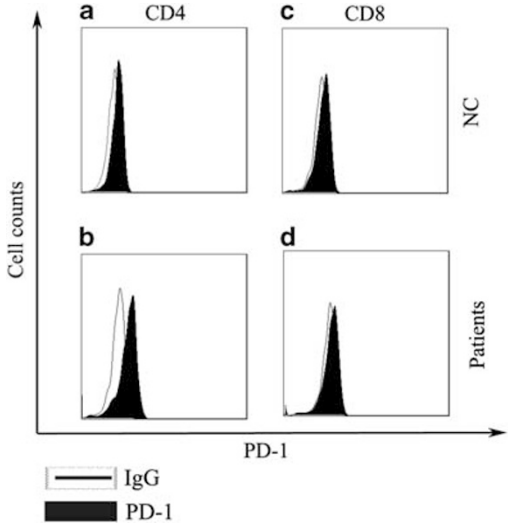

Figure 1 Representative histograms of PD-1 expression on CD4$^+$T cells and CD8$^+$T cells. (**a**) Representative histogram illustrating PD-1 expression on CD4$^+$T cells in a normal control people. (**b**) Representative histogram illustrating PD-1 expression on CD4$^+$T cells in a chronic HBV-infected patient. Increased PD-1 expression on CD4$^+$T cells is illustrated in this patient. (**c**) Representative histogram illustrating PD-1 expression on CD8$^+$T cells in a normal control patient. (**d**) Representative histogram illustrating PD-1 expression on CD8$^+$T cells in a chronic HBV-infected patient. In panels **a** and **b**, the histogram is gated on CD3$^+$ PBMCs and co-stained for CD4 and PD-1 to identify PD-1$^+$CD4$^+$T cells. In panels **c** and **d**, the histogram is gated on CD3$^+$ PBMCs and co-stained for CD8 and PD-1 to identify PD-1$^+$CD8$^+$T cells.

Elevated PD-1 Expression on CD4$^+$T Cells in Different Phases in the Natural History of Chronic HBV Infection

To study the correlation between PD-1 expression and progress of chronic HBV infection, the PD-1 expression profile in three phases in the natural history of HBV infection was analyzed. Patients were classified into respective phases of chronic HBV infection in the following manner: immune tolerance phase, immune clearance phase and inactive virus carrier phase.[5–7] Accordingly, all patients enrolled in the study were categorized into three phases in order to analyze the correlation between the immune phase and PD-1 expression. We found that the percentages of PD-1-positive CD4$^+$T cells in immune tolerance phase, immune clearance phase and inactive virus carrier phase were all higher than those in normal controls (Figure 3a). The percentage of PD-1-positive CD4$^+$T cells increased gradually from the immune tolerance phase, the immune clearance phase to the inactive virus carrier phase; however, no significant difference was found between these groups (Figure 3a). Similar results were obtained with respect to the MFI of PD-1 expression on CD4$^+$T cells (Figure 3b). By contrast, there were no significant differences between the percentages of PD-1-positive CD8$^+$T cells in all phases in the natural history of chronic HBV infection and that in normal controls (Figure 3c), and the MFI of PD-1 expression on CD8$^+$T cells in patients with the three phases showed no difference from that in normal controls (Figure 3d). Our results suggested that, in the three phases in the natural history of chronic HBV infection, the PD-1 expression levels on CD4$^+$T cells in patients were all elevated.

PD-1 Expression Level on CD4$^+$T Cells was not Correlated with Serum ALT Level in Chronic HBV Infection

Studies showed that the intra-hepatic interaction between PD-1 and PD-L1 might have an important role in balancing the immune response to HBV and immune-mediated liver damage in chronic HBV infection.[39] In this study, we analyzed the correlation between PD-1 expression level on CD4$^+$T cells in peripheral blood and serum ALT level in the different phases of chronic HBV infection. Results showed that both the percentage of PD-1-positive CD4$^+$T cells and the MFI of PD-1 expression on CD4$^+$T cells were not correlated with serum ALT level in each phase of chronic HBV infection (Figure 4).

PD-1 Expression Level was Correlated with Serum HBV DNA Level in the Immune Clearance Phase of Chronic HBV Infection

One recent report showed that PD-1 expression on CD4$^+$T cells may inhibit the cellular immune response against HBV and affect viral clearance, leading to persistence of chronic

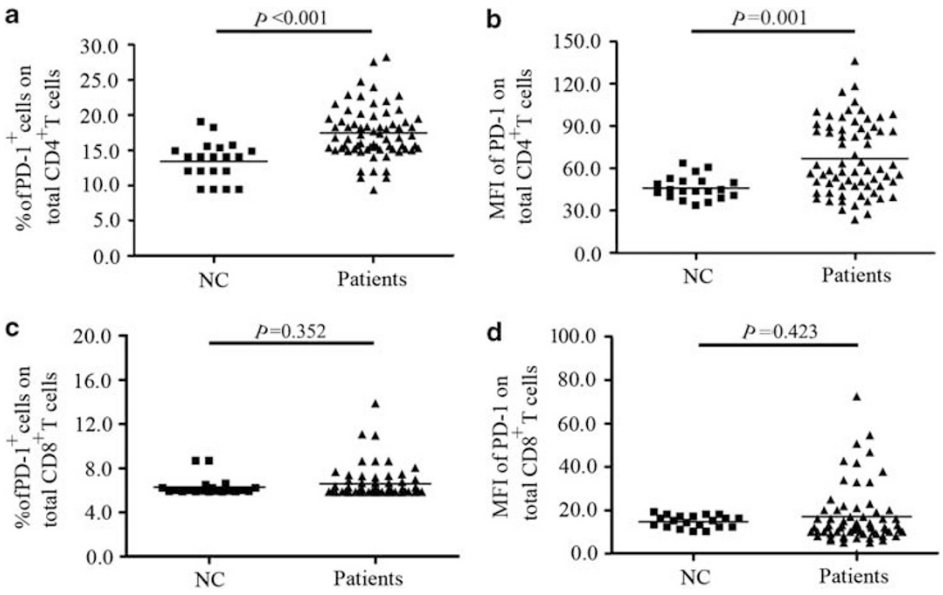

Figure 2 PD-1 expression on CD4$^+$T cells and CD8$^+$T cells. (**a**) The dot plots show the percentage of PD-1-positive CD4$^+$T cells from chronic HBV-infected patients and normal controls, and the horizontal bars indicate the median percentage of PD-1-positive on CD4$^+$T cells. (**b**) The dot plots show the MFI of PD-1 expression on CD4$^+$T cells and the horizontal bars indicate the median MFI of PD-1 expression on CD4$^+$T cells. (**c**) The dot plots show the percentage of PD-1-positive CD8$^+$ T cells from chronic HBV-infected patients and normal controls, and the horizontal bars indicate the median percentage of Pd-1-positive on CD8$^+$ T cells. (**d**) The dot plots show the MFI of PD-1 expression on CD8$^+$ T cells and the horizontal bars indicate the median MFI of PD-1 expression on CD8$^+$ T cells.

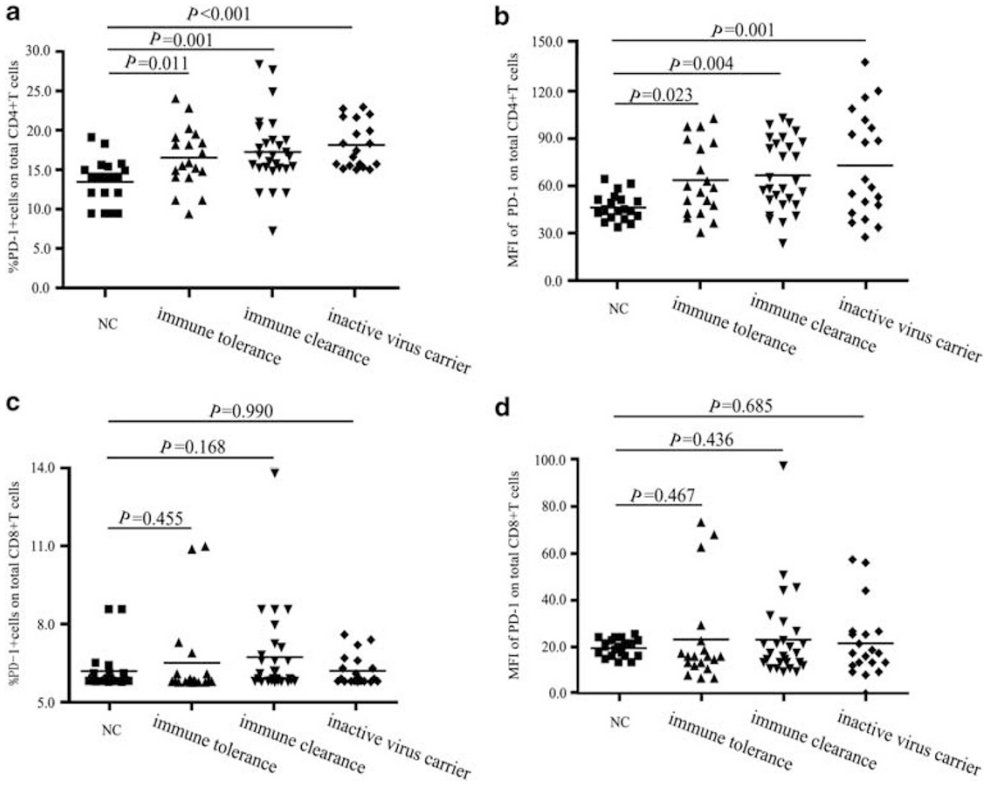

Figure 3 PD-1 expression on CD4$^+$T cells in the natural phases of chronic HBV infection. (**a**) The dot plots show the percentage of PD-1-positive CD4$^+$T cells in the different natural phases in chronic HBV-infected patients and normal controls, and the horizontal bars indicate the median percentage of PD-1-positive CD4$^+$T cells. (**b**) The dot plots show the MFI of PD-1 expression on CD4$^+$T cells and the horizontal bars indicate the median MFI of PD-1 expression on CD4$^+$T cells. (**c**) The dot plots showing the percentage of PD-1-positive CD8$^+$ T cells in different natural phases of chronic HBV-infected patients and normal controls and the horizontal bars indicating the median percentage of PD-1-positive CD8$^+$ T cells. (**d**) The dot plots showing the MFI of PD-1 expression on CD8$^-$ T cells, and the horizontal bars indicating the median MFI of PD-1 expression on CD8$^+$ T cells.

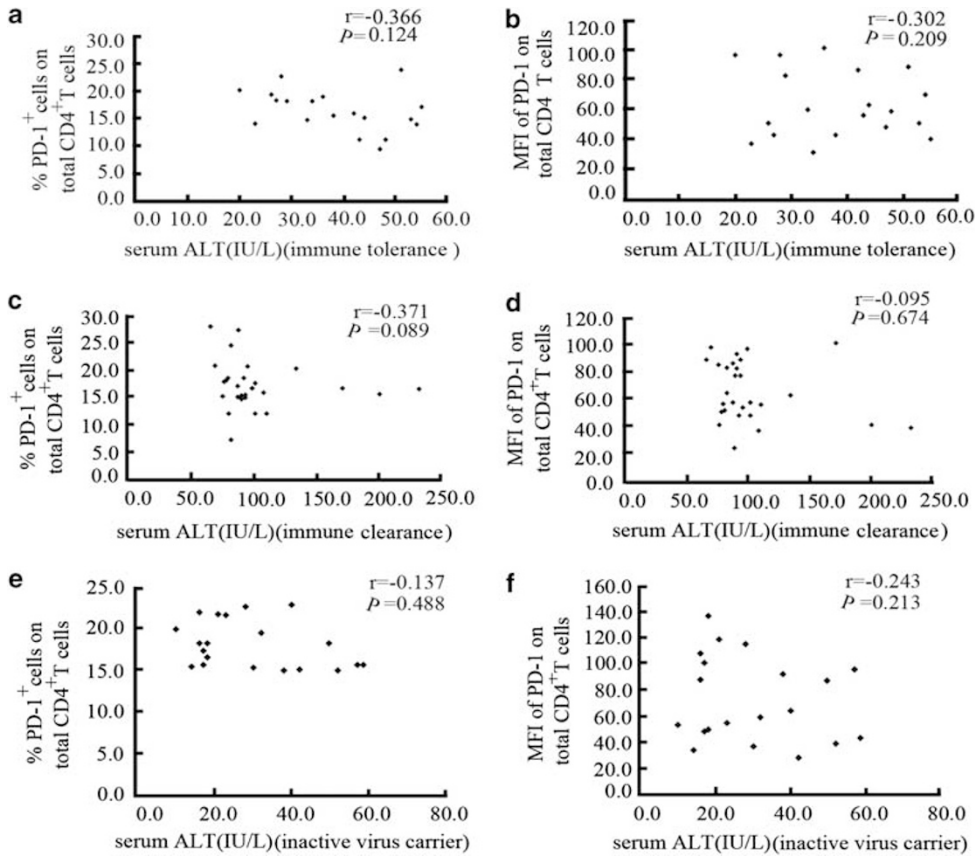

Figure 4 Correlations between PD-1 expression levels and serum ALT levels in the natural phases of chronic HBV infection. (**a**) No correlation between percentage of cells staining positive for PD-1 on $CD4^+$T cells and serum ALT level in the immune tolerance phase. (**b**) No correlation between MFI of PD-1 expression on $CD4^+$T cells and serum ALT level in the immune tolerance phase. (**c**) No correlation between percentage of cells staining positive for PD-1 on $CD4^+$T cells and serum ALT level in the immune clearance phase. (**d**) No correlation between MFI of PD-1 expression on $CD4^+$T cells and serum ALT level in the immune clearance phase. (**e**) No correlation between percentage of cells staining positive for PD-1on $CD4^+$T cells and serum ALT level in the inactive virus carrier phase. (**f**) No correlation between MFI of PD-1 expression on $CD4^+$T cells and serum ALT level in the inactive virus carrier phase.

HBV infection.[40] In this study, we analyzed the correlation between the PD-1 expression level on $CD4^+$T cells and serum HBV DNA level in each phase of chronic HBV infection. Our results showed no significant correlation between PD-1 expression level and serum HBV DNA level in the immune tolerance phase (Figures 5a and b). A strong positive correlation was found between MFI of PD-1 expression on $CD4^+$T cells and serum HBV DNA level in the immune clearance phase, but the percentage of PD-1-positive $CD4^+$T cells was not related with serum HBV DNA level (Figures 5c and d). In the inactive virus carrier phase, there were no significant correlations between the percentage of PD-1-positive $CD4^+$T cells, the MFI of PD-1 expression and serum viral level (Figures 5e and f). These results showed that elevated PD-1 expression level on $CD4^+$T cells was correlated with serum HBV DNA level only in the immune clearance phase of chronic HBV infection.

Upregulation of PD-1 on T Cells by HBcAg

To explore the involved mechanism underlying the elevated PD-1 expression levels on $CD4^+$T cells in chronic HBV infected patients, T cells from healthy volunteers were stimulated with HBcAg, which is the major component encoded by HBV DNA. T cells from healthy volunteers were sorted by the MACS method. Expression of PD-1 mRNA in T cells treated with HBcAg was examined by real-time RT-PCR. As shown in Figure 6a, the level of PD-1 mRNA in T cells was increased in response to HBcAg in a time-dependent manner. Furthermore, the expression of the PD-1 protein on T cells was examined by FCM at 0, 12, 24, 48 and 72 h after HBcAg treatment. As shown in Figures 6b and c, the percentage of PD-1-positive $CD4^+$T cells was significantly upregulated at 48 h and the MFI of PD-1 expression on $CD4^+$T cells was significantly upregulated at 24 and 48 h after HBcAg treatment. These results suggested that PD-1 expression on T cells can be upregulated by HBcAg.

JNK, ERK and PI3K/AKT Signaling Molecules were Involved in PD-1 Upregulation on T Cells by HBcAg

Studies have shown that p38 and AKT signaling molecules are involved in PD-1 expression in viral infection.[41,42] To further identify the signaling pathways underlying HBcAg-dependent

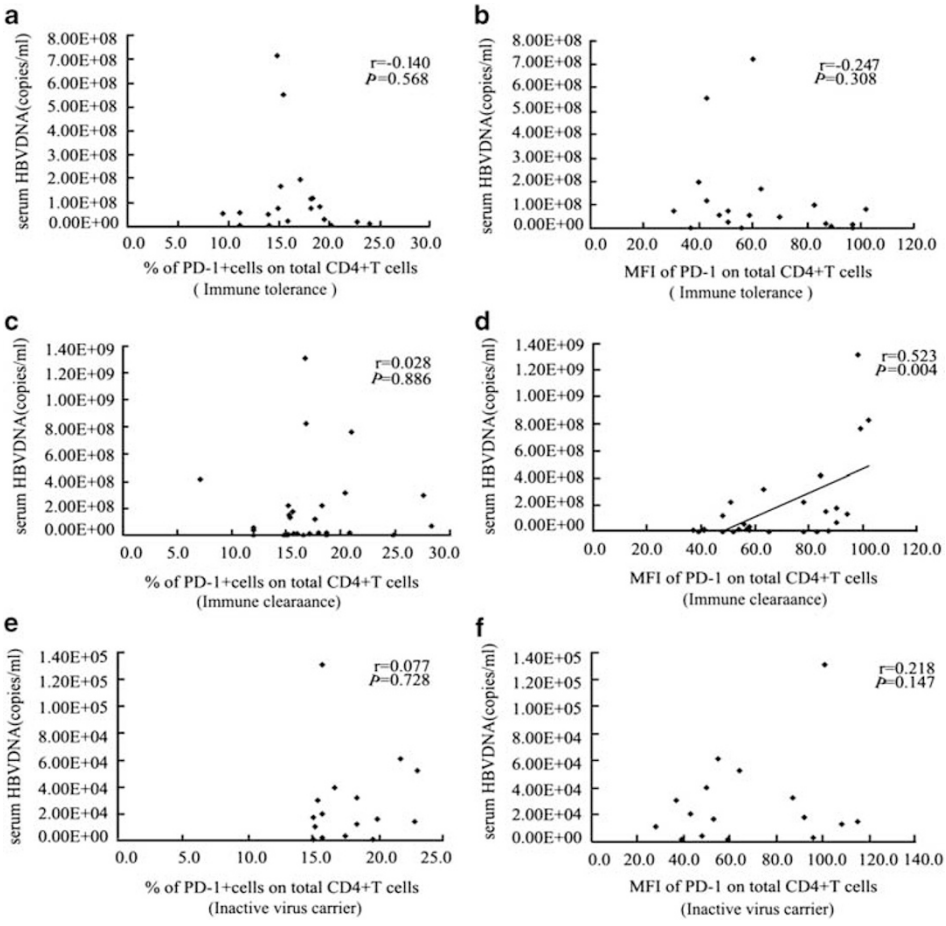

Figure 5 Correlations between PD-1 expression levels and serum HBV DNA levels in the natural phases of chronic HBV infection. (**a**) No correlation between percentage of cells staining positive for PD-1 on $CD4^+$T cells and serum HBV DNA level in the immune tolerance phase. (**b**) No correlation between MFI of PD-1 expression on $CD4^+$T cells and serum HBV DNA level in the immune tolerance phase. (**c**) No correlation between percentage of cells staining positive for PD-1 on $CD4^+$T cells and serum HBV DNA level in the immune clearance phase. (**d**) A positive correlation between MFI of PD-1 expression on $CD4^+$T cells and serum HBV DNA level in the immune clearance phase. (**e**) No correlation between percentage of cells staining positive for PD-1 on $CD4^+$T cells and serum HBV DNA level in the inactive virus carrier phase. (**f**) No correlation between MFI of PD-1 expression on $CD4^+$T cells and serum HBV DNA level in the inactive virus carrier phase.

PD-1 expression in chronic HBV infection, the effect of pharmacological inhibitors of MAPK and PI3K/AKT on PD-1 expression was examined. T cells or $CD4^+$T cells were pre-treated with LY294002 (PI3K/AKT inhibitor), U0126 (ERK inhibitor), SB203580 (p38 inhibitor) and SP600125 (JNK inhibitor) for 1 h followed by incubation with HBcAg for 48 h. Our results showed that the elevated percentage of PD-1-positive $CD4^+$T cells induced by HBcAg can be inhibited by LY294002, U0126 and SP600125, but SB203580 had no effect on the elevated PD-1 expression (Figure 7a). Similarly, LY294002, U0126 and SP600125, but not SB203580, could inhibit the upregulated MFI of PD-1 expression by HBcAg (Figure 7b). In order to confirm PD-1 expression on $CD4^+$T cells and its mechanism by HBcAg, $CD4^+$T cells separated from PBMCs were used to perform the above inhibitor experiment through western blotting. Results showed that PD-1 expression on $CD4^+$T cells can be upregulated by HBcAg, and the upregulated PD-1 expression can be inhibited by LY294002, U0126 and SP600125 (Figure 7c). Furthermore, we found that phosphorylation of JNK, ERK and AKT on T cells was increased in response to HBcAg, and no effect on phosphorylation of p38 was found (Figures 7d–g). These data indicated that the upregulated PD-1 expression on $CD4^+$T cells by HBcAg was related to activation of ERK, JNK and AKT signaling molecules.

DISCUSSION

Chronic HBV infection is mainly related to the immune function of patients. As a negative co-stimulatory receptor, PD-1 appears particularly important in regulating T-cell tolerance. It interacts with specific ligands, PD-L1 and PD-L2, to attenuate a T-cell response. PD-L1, but not PD-L2, was also significantly upregulated on PBMCs from CHB patients.[20]

Previous reports suggested that $CD4^+$ and $CD8^+$ T cells were differentially affected by particular activation and

deactivation pathways.[43–47] The PD-1 pathway has an important role in inhibiting the function of virus-specific CD8$^+$T cells in chronic HIV,[13,14,48] HCV[17,49] and HBV infection.[18] In our study, the PD-1 expression level on CD4$^+$T cells in chronic HBV-infected patients was higher than that in normal control, but this was not observed on CD8$^+$T cells. Therefore, the mechanism of PD-1 expression on CD4$^+$T cells was not the same as that for CD8$^+$T cells.

The natural history of chronic HBV infection is characterized by three distinct phases that depend on complex

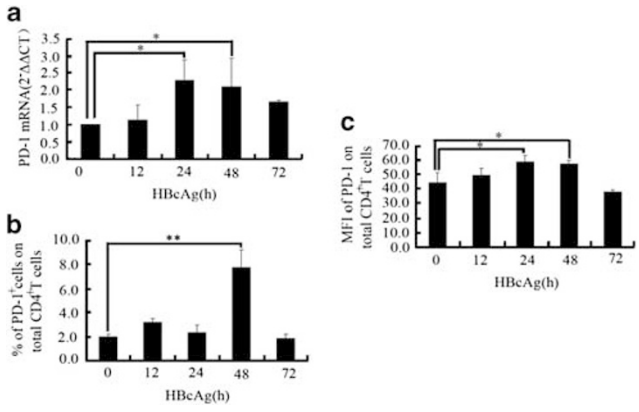

Figure 6 Upregulation of PD-1 expression on T cells was induced by HBcAg. (**a**) T cells separated from peripheral blood of healthy individuals were incubated with HBcAg for the indicated time periods, and the total RNA of T cells was extracted and mRNA expression of PD-1 was examined by real-time RT-PCR. Real-time RT-PCR results of PD-1 mRNA expression of T cells that were incubated with HBcAg for the indicated time periods have been presented. PD-1 expression was normalized using GAPDH RNA expression and the group without HBcAg was set as 1. The data shown are the mean ± s.d. ($n=3$). *$P<0.05$; **$P<0.01$. (**b**, **c**) T cells separated from peripheral blood of healthy individuals were incubated with HBcAg for the indicated time periods and PD-1 protein expression was examined by FCM. FCM results of PD-1 protein expression of T cells incubated with HBcAg for the indicated time periods have been presented. The percentage of PD-1-positive CD4$^+$T cells and the MFI of PD-1 expression on CD4$^+$T cells were analyzed. The data shown are the mean ± s.d. ($n=3$). *$P<0.05$; **$P<0.01$.

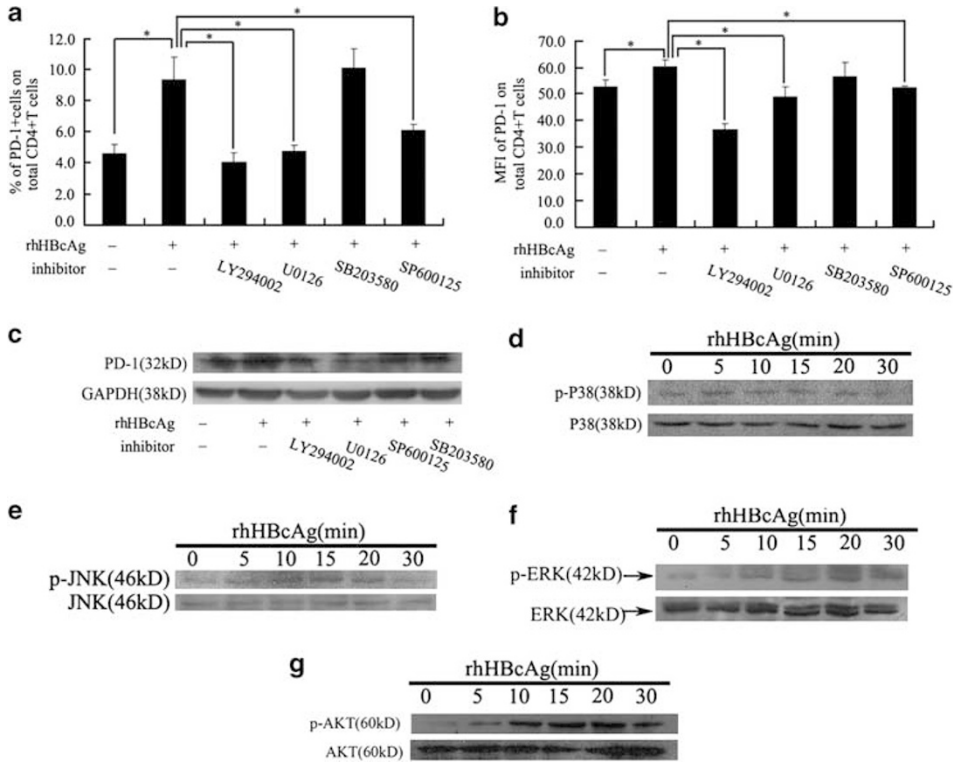

Figure 7 ERK, JNK and AKT activation was related to HBcAg-induced PD-1 expression on CD4$^+$T cells. (**a**, **b**) T cells were pretreated with U0126 (150 nM), SP600125 (100 nM), LY294002 (25 μM) and SB203580 (1 μM) for 1 h, and then incubated with HBcAg for 48 h. Normal T cells were used as negative control. The elevated percentage of PD-1-positive CD4$^+$T cells was inhibited by a PI3K/AKT inhibitor (LY294002), an ERK inhibitor (U0126) and a JNK inhibitor (SP600125). The elevated MFI of PD-1 expression on CD4$^+$T cells was inhibited with a PI3K/AKT inhibitor (LY294002), an ERK inhibitor (U0126) and a JNK inhibitor (SP600125). The data shown are the mean ± s.d. ($n=3$). *$P<0.05$. (**c**) CD4$^+$T cells were separated and pretreated with U0126 (150 nM), SP600125 (100 nM), LY294002 (25 μM) and SB203580 (1 μM) for 1 h, and then incubated with HBcAg for 48 h. Normal CD4$^+$T cells were used as negative control. The elevated PD-1 expression on CD4$^+$T cells was inhibited by a PI3K/AKT inhibitor (LY294002), an ERK inhibitor (U0126) and a JNK inhibitor (SP600125) ($n=3$). (**d**–**g**) T cells were incubated with HBcAg for the indicated time periods and phosphorylated forms of ERK, JNK, AKT and p38 were detected by western blotting. Western blot analysis of T cells after incubation with HBcAg for the indicated time periods. Phosphorylated forms of ERK, JNK, AKT and p38 were analyzed. Blots were reprobed for total ERK, JNK, AKT and p38 ($n=3$).

host, virus and environmental interactions. In each phase, it is the host's immune response that determines the outcome of infection and the severity of liver injury. In our study, we found that the PD-1 expression level on $CD4^+$T cells was dynamically changed during the natural history of chronic HBV infection. Konkel *et al*[50] reported that PD-1 was related with immune tolerance, and PD-1 signaling in $CD4^+$T cells restrained their clonal expansion to an immunogenic stimulus, but was not critically required for peptide-induced tolerance. It was an unexpected result that the highest PD-1 expression level on $CD4^+$T cells was in the inactive virus carrier phase but not in the immune tolerance phase. We supposed that during the natural history of chronic HBV infection, upregulation of PD-1 on $CD4^+$T cells maybe sustained for a long time even after clearance of HBV DNA. In addition, our study was not longitudinal, which maybe one of the reasons for this unexpected result. It deserves a more comprehensive study to well-address these questions in the future.

Among the natural phases of chronic HBV infection, the immune clearance phase is the most critical phase for the host to clear the virus. We found that the MFI of PD-1 expression on $CD4^+$T cells was positively correlated with HBV DNA level in the immune clearance phase but not in the other two phases. Activation of antiviral immunity in patients was related with the expression level of PD-1 on T cells. Elevated HBV DNA level has also been identified as a risk factor for the development of cirrhosis.[51,52]

PD-1 upregulation in the immune clearance phase may be related to the development of cirrhosis, which should be referred to in future clinical investigations.

In this study, PD-1 expression level on $CD4^+$T cells did not correlate with ALT level throughout the natural history of chronic HBV infection, which was different from a previous report.[53] As for the relationship between PD-1 expression level, serum transaminase levels and viral load, some inconsistent results were obtained in different experiments. Possibly because liver was the target organ of HBV, plasma transaminase levels did not truly reflect the status of liver damage, but it also may be an impact of PD-1 expression. Subjects in different studies have different conditions, which affects the results.

Upregulation of PD-1 expression on T cells was closely associated with occurrence of CHB. However, the mechanism of PD-1 upregulation induced by HBV is still unknown. The previous study showed that the HBV X protein induces the transcription factor AP-1 by activation of extracellular signal-regulated and c-Jun N-terminal MAPK,[54] and the HCV core protein can activate ERK, JNK and p38 MAPK.[55] However, the mechanism of PD-1 expression induced by HBcAg is still to be explored. One recent study showed Nef to be associated with HIV viral antigens to specifically increase PD-1 expression through a p38 MAPK-dependent mechanism.[41] HBcAg is the major component encoded by HBV DNA, so we focused on the correlation between HBcAg and PD-1 upregulation. In the study on lymphoma,[56] PD-L1 expression was shown to be dependent on the ERK and p38 MAPK signaling pathways. These studies provided some insight into the mechanism of how HBcAg regulated PD-1 expression on T cells. We observed that the JNK, ERK and PI3K/AKT signaling pathways were involved in PD-1 upregulation by HBcAg. These results may provide potentially immunotherapeutic strategies for chronic HBV-infected patients.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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