Study of human B7 homolog 1 expression in patients with hepatitis B virus infection

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Abstract

AIM: To further investigate the role of human B7 homolog 1 (B7-H1) in the mechanism of persistent hepatitis B virus (HBV) infection.

METHODS: Peripheral and intra-hepatic B7-H1 expression were compared by flow cytometry and immunochemical staining between two 2 distinct groups, one being chronic HBV tolerance patients (CHB-T) and the other being acute hepatitis B patients (AHB). B7-H1 mRNA expression level was also compared by real time polymerase chain reaction between CHB-T and AHB patients. The location of intra-hepatic B7-H1 and CD40 expression were analyzed by immunofluorescence. The levels of B7-H1 and CD40 expression on cultured myeloid dendritic cells (mDCs) with or without hepatitis B surface antigen (HBsAg) treatment were analyzed dynamically by flow cytometry. Intracellular interferon-γ (IFN-γ) staining and the stimulatory capacity of mDC of cultured mDC with or without HBsAg treatment were also compared by flow cytometry.

RESULTS: Peripheral B7-H1 expression on mDCs was increased significantly in AHB compared to CHB-T patients (P < 0.05). In the liver tissues from CHB-T patients, B7-H1 positive cells were almost absent despite a persistently elevated serum HBsAg load. In contrast, there were indeed increased B7-H1-positive cells in situ in the liver tissue from AHB. In vitro analysis showed the parallel upregulation of B7-H1 and CD40 on CD11c+ mDCs after the onset of stimulation. Addition of recombinant hepatitis B surface antigen (rHBsAg) significantly decreased CD40 expression (P < 0.05 at 16 h, 20 h and 24 h time points). B7-H1 expression was also inhibited by rHBsAg, and the inhibition rate of CD40 was greater than that of B7-H1. This preferential inhibition of CD40 expression on mDCs by rHBsAg resulted in the dysfunction of mDCs and T cells in the mixed leucocyte reaction (MLR) system. With rHBsAg pretreatment, in a carboxyfluorescein diacetate succinimidyl ester (CFSE) labeled MLR system at a ratio of 1:5 responder cell-stimulator cell (R/S), the CFSE dim percentage of T cells decreased from 85.1% to 25.4% and decreased from 30.3% to 12.0% at 1:10 R/S. IFN-γ production by CD8+ T cells, in the MLR system, was reduced significantly by HBsAg pretreatment. At ratios of 1:5 R/S, the percentage of IFN-γ and CD8 dual positive T cells decreased from 55.2% ± 5.3% to 15.1% ±
3.1% ($P < 0.001$), and decreased from 35.0% ± 5.1% to 7.3% ± 2.7% at ratios of 1:10 R/S ($P < 0.001$).

**CONCLUSION:** B7-H1 is not a signature of immune dysfunction, but an inflammation marker. HBsAg regulates immune response by tipping the balance between B7-H1 and CD40.

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**Key words:** Hepatitis B virus; Hepatitis B; Human B7 homolog 1; Immune tolerance; Co-stimulatory molecule

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**INTRODUCTION**

Patients with self-limited acute hepatitis B (AHB) can develop appropriate virus-specific immune responses, however these immune responses are insufficient to eliminate the virus in chronic hepatitis B patients and eventually lead to chronic hepatitis B virus (HBV) tolerance (CHB-T). To date, the mechanisms underlying this defect of the HBV specific immune response have not been fully elucidated. T-cell exhaustion, lack of CD4+ T-cell help, induction of T-cell tolerance and viral variation may contribute to HBV persistence.

HBV-DNA expression alone is not sufficient to elucidate the mechanism of this upregulation to HBV or HCV infection was determined by the lack of a significant increase in B7-H1 and PD1 in cytomegalovirus, Epstein-Barr virus and influenza A infected patients. Based on these studies, relatively high levels of B7-H1/PD-1 expression were viewed as the signature of impairments in the HBV- and HCV-specific immune response. Both HBV and HCV might exploit the B7-H1/PD1 pathway to facilitate persistent infection.

Our clinical observation revealed that chronic HBV infection was composed of 2 different components. On one hand, the CHB-T condition is characterized by active HBV-DNA replication, but shows no serum alanine aminotransferase (ALT) increase. The other component is a fluctuating inflammation condition that shows repeated inflammation flare-ups with fluctuating levels of serum ALT and HBV-DNA.

To examine the role of B7-H1 in the mechanism of HBV tolerance, we compared B7-H1 expression between AHB and CHB-T patients.

The results showed that peripheral and intra-hepatic expression of B7-H1 was more significant in AHB than in CHB-T patients. In the liver tissue of CHB-T subjects, B7-H1 was almost absent despite a persistently elevated hepatitis B surface antigen (HBsAg) load. In vitro analysis showed that CD40 and B7-H1 were upregulated synchronously after the onset of stimulation, and CD40 was preferentially inhibited by recombinant hepatitis B surface antigen (rHBsAg), which impaired the allostimulatory capacity of mDCs and interferon-γ (IFN-γ) production by CD8+ T cells in the mixed leucocyte reaction (MLR) system.

Our findings showed that the analysis of B7-H1 expression alone is not sufficient to elucidate the mechanism of HBV immune tolerance. B7-H1 is an inflammatory marker, but not an absolute indicator of HBV-specific immune tolerance. The preferential inhibition of CD40 expression by rHBsAg can be considered as part of a mechanism by which HBV impairs the immune response and results in persistent infection in CHB-T patients.

**MATERIALS AND METHODS**

**Subjects**

This study examined 27 adults with HBV infection (18
men, 9 women) from August 2008 to August 2010 that were hospitalized in our unit. None of the patients were treated with steroids before sampling. Concordance of HCV and human immunodeficiency virus infections were excluded from enrolled individuals. The study protocol was approved by the Ethics Committee of our unit, and informed consent was obtained from each subject. The patients were assigned to 2 distinct groups based on plasma HBV DNA loads and ALT levels. The first group (n = 12) was formed by CHB-T with HBV-DNA replication and HBsAg production, but normal ALT levels (normal range: 10-40 U/L). The other group (n = 15) was composed of AHB patients with active HBV replication and significantly elevated serum ALT levels. For comparison, 5 uninfected healthy controls (HC) and 3 patients with autoimmune hepatitis (AIH) were enrolled as controls. The clinical data of these patients at the time of the first physician consultation are summarized in Table 1.

### Table 1 Clinical data of these patients at the time of the first physician consultation

|          | Male/female | ALT       | HBsAg | HBV-DNA load                     |
|----------|-------------|-----------|--------|----------------------------------|
| AHB (n = 15) | 10/5        | 296 ± 110 | 404 ± 98 ng/mL | (2.7) × 10^6 (n = 4), (3.6) × 10^5 (n = 5), (1.6) × 10^6 (n = 4), (1.2) × 10^5 (n = 2) |
| CHB-T (n = 12) | 8/4        | Normal (10-40 μ/mL) | 363 ± 58 ng/mL | (3.7) × 10^6 (n = 6), (1.5) × 10^5 (n = 5), (1.1) × 10^2 (n = 1) |
| AIH (n = 3)   | 0/3        | 301 ± 79.3 | Negative | Negative                         |
| HC (n = 5)    | 3/2        | Normal (10-40 μ/mL) | Negative | Negative                         |

AHB: Acute hepatitis B; CHB: Chronic hepatitis B virus tolerance; AIH: Autoimmune hepatitis; HC: Healthy controls; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen.

### Human B7-H1 expression in circulating mDCs

B7-H1 expression on circulating mDCs was measured using 1 mL of fresh heparinized peripheral blood. Cells were lysed with fluorescence-activated cell sorter (FACS) lysing solution (BD Pharmingen) to remove red blood cells and then incubated with antibodies against B7-H1-Phycoerythrin (PE) and CD11c-fluorescein isothiocyanate (FITC) for 20 min at room temperature. After washing twice with phosphate buffered saline (PBS), the cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences).

### Preparation of mDCs and T cells

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood of healthy donors by Ficoll density centrifugation. PBMCs were then plated (5 × 10^5 cells/well) into a 6-well plate and incubated at 37 °C for 2 h. T-cell-enriched and T-cell-depleted fractions were prepared by adherence to plastic in complete RPMI 1640 medium. The immature dendritic cells (iDCs) were prepared from the T-cell-depleted fraction by culturing cells in the presence of granulocyte macrophage colony-stimulating factor (50 ng/mL) and interleukin 4 (IL-4) (50 U/mL) for 5 d.

### MDCs culture and CD40-, and human B7-H1 expression

iDCs were prepared from PBMC and incubated in a 24-well plate in RPMI 1640 medium containing 10% FCS and treated with or without rHBsAg-adr (1 μg/mL). rHBsAg adr was purified from transfected Chinese Hamster Ovary (CHO) cells. Cell lysates of CHO without HBsAg transfection served as a negative control. After 5 d, these cells were stimulated by 20 μg poly (I: C), and then collected at 4 h, 8 h, 16 h, 20 h, and 24 h after stimulation. The cells were incubated with anti-B7-H1-PE and CD40-FITC antibodies for 20 min at room temperature. After washing three times with PBS, the expression of B7-H1 and CD40 was analyzed by flowjo 7.6.

### RNA preparation and real-time polymerase chain reaction

Total RNA from liver tissues was isolated using RNeasy kits (Qiagen). Reverse transcription of RNA was performed using a SuperScript One-Cycle cDNA kit (Invitrogen). The cDNA served as a template in real-time polymerase chain reaction (PCR). The human B7-H1 primers for RT-PCR were used as follow: 5′-TTTACTGTCAGGTTCC-3′ (sense) and 5′-TGGTCTTATCCTCCATTTCC-3′ (antisense); human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, 5′-CTGCCCTCGACGTAG-3′ (sense) and 5′-TCCACGATACAAAGTTGTCATG-3′ (antisense). All reactions were performed in triplicate. The B7-H1 mRNA expression of different specimens was normalized to GAPDH. Relative mRNA levels are presented as unit values of 2^(-ΔΔCt), where Ct is the threshold cycle value defined as the fractional cycle number at which the target fluorescent signal passes a fixed threshold above baseline.

### Immunohistochemical staining

Acetone-fixed liver tissue cryosections (7 μm) were incubated with anti B7-H1 antibodies (Abcam) at 4 °C overnight. After DAB peroxidase staining, positive cells (brown color) were counted in 5 different fields by 2 independent observers. To determine B7-H1, CD40, CD68, macrophage inflammatory protein (MIP3) α, secondary lymphoid tissue chemokine (SLC), CD11c, chemokine (C-C) receptor (CCR) 7 and HBsAg expression, immunofluorescence double staining was performed. Briefly, liver tissues were incubated for 12 h at 4 °C with diluted primary Abs followed by diluted secondary Abs for 1 h at room temperature. The following primary antibodies were used for CD11c, CD40, B7-H1, CD68, SLC, MIP3α, CCR7 and HBs-Ag: mouse antihuman CD40,
CD68 and CD11c (diluted 1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States), and, goat anti-human B7-H1, MIP3α, SLC, CCR7 (diluted 1:100; Santa Cruz). As secondary antibodies, a rat anti-goat IgG PE-conjugated antibody (diluted 1:100; Santa Cruz), a rat anti-mouse IgG FITC-conjugated antibody, and a rat anti-mouse IgG rhodamine-conjugated antibody were used.

**Allogeneic mixed leukocyte reaction**

Purified mDCs with or without HBsAg treatment were matured for 24 h in a 96-well flat-bottom culture plate (at 1 × 10⁵ cells/200 μL) in culture medium containing poly (I:C) (20 μg/mL; Sigma). On the following day, mDCs were treated with mitomycin C and were added at different concentrations (1/5 and 1/10) into purified T cells from a normal healthy volunteer (1 × 10⁵ cells/200 μL) and labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE (1 mmol/mL); Molecular Probes, Eugene, OR) for 5 d. T-cells proliferation was assessed by the loss of CFSE analyzed by flow cytometry. Flow cytometric data for 5 d. T-cells proliferation was assessed by the loss of CFSE analyzed by flow cytometry. Flow cytometric data

**Intracellular IFN-γ staining**

Next, IFN-γ produced during these MLRs was measured. T cells were collected after 5 d of mixed culture, phorbol myristate acetate (1 μg/mL), ionomycin (0.1 mg/mL; T cell receptor-bypassing reagents) and 0.1 mg/mL monomycin (Sigma-Aldrich) were added into a mixed culture system 4 h before analysis. T cells were then washed in PBS, stained with anti-CD8 (FITC) mAb and then permeabilized and fixed according to the manufacturer’s instructions. After a further wash in PBS, cells were stained with anti-IFN-γ-PE mAb at room temperature for 20 min. After 2 additional washes, cells were fixed and acquired immediately on flow cytometry on a FACS Canto (BD Biosciences).

**Statistical analysis**

All experimental conditions were pair analyzed against their controls. Data on co-stimulatory molecules, and cytokine and B7-H1 mRNA expression were analyzed by the Student’s t test. The correlation between ALT levels and B7-H1 expression obtained by FACS was assessed by Pearson’s correlation analysis. Data corresponding to the expression of co-stimulatory molecules and cytokine production are expressed as mean ± SE. A P value of < 0.05 was considered statistically significant.

**RESULTS**

The expression of human B7-H1 on circulating mDCs was significantly upregulated in AHB patients and AIH

The levels of B7-H1 expression were detected on circulating CD11c+ DCs from AHB, CHB-T, and AIH patients, and HC (Figure 1A). B7-H1 expression on circulating CD11c+ DCs from enrolled patients was generally
Increased, in particular in AHB patients, who expressed higher levels of B7-H1 than CHB-T individuals ($P < 0.05$, Figure 1B). In CHB-T patients, B7-H1 expression also increased, but was not significantly different from that in HC. AHI patients exhibited the highest levels of B7-H1 expression on CD11c+ DCs among these groups (all $P < 0.05$, Figure 1B). Correlation analysis revealed that there was a significant, positive correlation between B7-H1 expression on circulating CD11c+ DCs and serum ALT levels in AHB patients ($r = 0.809$, Figure 1C).

**Intra-hepatic human B7-H1 expression is much higher in AHB than in CHB-T patients**

Immunohistochemical staining performed to analyze intrahepatic B7-H1 expression showed an increased number of B7-H1-positive cells in situ in the livers of AHB patients. In contrast, B7-H1-positive cells were almost completely absent in the livers of CHB-T subjects and healthy donors (Figure 2A). Immunofluorescence double staining revealed that almost all CD11c+ DCs, CD123+ DCs and Kupffer cells expressed B7-H1 molecules in the liver tissue from AHB patients (Figure 2B-D), while in liver tissue from CHB-T patients, B7-H1 positive cells were almost completely absent despite extensive HBsAg expression (Figure 2E). These results were in line with data obtained with circulating CD11c+ DCs. In addition, in the liver tissue from AHB patients, B7-H1 was always co-expressed with the positive co-stimulatory molecule CD40 (Figure 2F).

**Human B7-H1 mRNA expression is upregulated in liver tissue from AHB patients**

B7-H1 mRNA expression levels in liver tissues were further compared between AHB and CHB-T patients by real-time PCR. As show in Figure 3, B7-H1 mRNA expression levels were significantly higher in AHB than in CHB-T liver tissue. The highest relative B7-H1 mRNA expression level in AHB liver tissue showed a 12-fold increase over the CHB-T level. The lowest B7-H1 mRNA level showed a 2-fold increase in AHB compared to CHB-T liver tissue (Figure 3).

**Inflammatory markers showed significant expression in the liver tissue from AHB patients with significant human B7-H1 expression**

Peripheral and intrahepatic B7-H1 expression in AHB patients was far more significant than that of CH-B. These results suggest that B7-H1 is a marker of AHB. To further confirm this conclusion, serially sectioned liver tissues from AHB patients were examined for the markers of inflammatory response, including DCs, lymphocytes infiltration and chemokines expression by immunofluorescence double staining. Liver tissue with significant B7-H1 expression showed significant infiltration of CD11c+ and CD8+ positive cells. Chemokines such as MIP3α and SLC were also expressed extensively by CD11c + DCs (Figure 4A and B). Mature mDCs expressing CCR7 and activated CD8+ lymphocytes expressing PD1 were recruited to the liver lobe (Figure 4D). In contrast, in liver tissue from CHB-T patients with significant HBsAg but no B7-H1 expression, CD11c+ and CD8+ infiltration cells were predominantly localized in fibrous septa rather than in the sinusoidal area (Figure 4E). These results suggest that B7-H1 was preferentially expressed at the site of AHB.

**Human B7-H1 expression levels increased during the acute hepatitis phase and decreased with a reduction in inflammation**

The correlation between B7-H1 expression and the degree of inflammation in hepatitis was analyzed by detecting B7-H1 expression on circulating mDCs during a follow-up period ranging from 1 mo to 6 mo. In AHB patients, the suppression of HBV replication after anti-viral treatment was accompanied by a decrease in serum HBsAg and ALT levels, and followed by a gradual decrease in B7-H1 expression on circulating mDCs (Figure 5A and B). Two or three months after the ALT level returned to normal range, the percentage of B7-H1 positive cells decreased within CD11c positive cells from 21.3 ± 5.6 to 6.1 ± 1.4 (Figure 4C). B7-H1 expression levels, as assessed by mean fluorescence intensity (MFI), were also reduced (data not shown). The level of B7-H1 expression increased during the period of acute hepatitis and decreased when inflammation was reduced; supporting the conclusion that B7-H1 is an inflammatory marker. Notably, B7-H1 expression levels remained relatively high (13.2 ± 3.3) when ALT returned to normal range at 16 wk (Figure 5C). Two or three months after the return of serum ALT level to normal, B7-H1 expression further decreased to 6.1 ± 1.4, approaching healthy control levels (Figure 5B and C). Longitudinal correlation analysis revealed a positive correlation between B7-H1 expression and serum ALT levels in AHB patients (Figure 5D). In CHB-T patients, although serum HBV-DNA and HBs-Ag load remained high, peripheral B7-H1 expression levels did not increase significantly (Figure 5C).

As mentioned above, chronic HBV infection had two different components, namely the CHB-T condition and the chronic active hepatitis status, which was characterized by repeated inflammation flare ups. Detection of peripheral B7-H1 expression in 3 chronic active hepatitis B patients during the inflammatory flare up phase with increasing serum ALT and HBV-DNA level showed that B7-H1 expression on circulating CD11c positive cells increased significantly in all three patients (Figure 6).

**High HBsAg loads inhibited the upregulation of CD40 and human B7-H1 on mDCs**

The above data showed that the intra and extra-hepatic HBs-Ag load remained persistently high in CHB-T subjects, while B7-H1 and CD40 expression were nearly absent. To further explore the relationship between HBsAg load and B7-H1 and CD40 expression, the effects of HBsAg towards B7-H1 and CD40 expression were analyzed in vitro. Population of mDCs with and without HBsAg pretreatment in PBMCs was determined by flow cytorn-
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A

AHB

CHB-T

HC

B

B7-H1-PE

CD11c-FITC

B7-H1/CD11c

C

B7-H1-PE

CD123-FITC

B7-H1/CD123

D

B7-H1-PE

CD68-FITC

B7-H1/CD68

E

HBsAg-FIT

B7-H1-PE
B7-H1 and CD40 on mDCs was upregulated synchronously in response to poly (I:C) stimulation. The expression level was significantly higher in AHB than in CHB-T liver tissue (n = 5, \( P < 0.01 \)). PCR: Polymerase chain reaction.

Figure 2  Analysis of intrahepatic human B7 homolog 1 expression among acute hepatitis B and chronic hepatitis B virus tolerance patients. A: Immunohistochemical staining for intrahepatic B7 homolog 1 (B7-H1)-positive cells in acute hepatitis B (AHB), chronic hepatitis B virus tolerance (CHB-T) and healthy controls (HC). Intra-hepatic B7-H1 expression was up-regulated significantly in AHB patients, but was almost absent in both CHB-T and HC subjects. Original magnification × 200. B-D: Co-localization of B7-H1 with CD11c, CD123 and CD68 were shown by immunofluorescence double staining in liver biopsy specimens of AHB patients. B7-H1 (red) is co-localized with CD11c (green) positive mDCs (B), CD123 (green) positive pDCs (C) or CD68 (green) positive Kupffer cells (D). The 2-color merged panels were shown with co-localization visible in yellow. Original magnification × 200. E: Hepatitis B surface antigen (HBsAg) and B7-H1 expression was detected by immunofluorescence stain in the liver tissue of CHB-T patients. Original magnification × 200; F: Colocalization of B7-H1 with CD40 is showed by immunofluorescence double staining in liver biopsy specimens of AHB patients. B7-H1 (red) is colocalized with CD40 (green). The 2-color merged panels were shown with colocalization visible in yellow. Original magnification × 200. PE: Phycoerythrin; FITC: Fluorescein isothiocyanate.

Figure 3 Quantitative real-time reverse transcription-polymerase chain reaction was performed in 5 acute hepatitis B and hepatitis B virus tolerance patients. Acute hepatitis B (AHB) and chronic hepatitis B virus tolerance (CHB-T) patients were matched freely. B7 homolog 1 (B7-H1) mRNA expression level was significantly higher in AHB than in CHB-T liver tissue (n = 5, \( P < 0.01 \)), PCR: Polymerase chain reaction.

The T-cell stimulatory capacity of mDCs pretreated by HBsAg was impaired significantly

Since the inhibition rate of CD40 was greater than that of B7-H1, we further investigated whether the T-cell stimulatory capacity of mDCs pretreated by HBs-Ag was impaired. The mDC was stimulated by poly (I:C) for 20 h with or without HBs-Ag pretreatment, and co-cultured at 1:5 and 1:10 ratios with HLA-mismatched allogeneic T cells labeled with cytoplasmic dye CFSE. After 5 d of incubation, T-cell proliferation was assessed by the dilution of CFSE. In the allo-MLR system pretreated by HBsAg, with 1:5 and 1:10 responder cell-stimulator cell (R/S) ratios, approximately 25.4% or 12.0% of the responder T cells were CFSE\(^{\text{dim}}\) proliferating blasts, respectively (Figure 8A). In the allo-MLR system without HBsAg pretreatment, the percentage of CFSE\(^{\text{dim}}\) proliferating T cells was 85.1% in the 1:5 R/S ratio and 30.3% in the 1:10 R/S ratio, respectively (Figure 8B). The results showed that mDCs pretreated by HBsAg were less efficient at inducing T-cell proliferation at ratios of 1:5 and 1:10 compared with mDCs without HBsAg pretreatment.

Intracellular IFN-\(\gamma\) staining

In correlation with T-cell proliferation, the intracellular IFN-\(\gamma\) produced in these MLRs was measured after 5 d of co-culture. In the MLRs system at ratios of 1:5 and 1:10 R/S, without HBsAg pretreatment, the percentage of IFN-\(\gamma\) positive cells in CD8+ T cells was greater than 55.2% ± 5.3% and 35.0% ± 5.1% respectively. In the MLRs system, with HBsAg pretreatment, the percentage of IFN-\(\gamma\) positive cells in CD8+ T cells decreased to
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Figure 4 Localization of CD11c and programmed death 1 in liver tissue from acute hepatitis B and chronic hepatitis B virus tolerance patients. A-C: Colocalization of CD11c with SLC, MIP3α and CCR7 by immunofluorescence double staining in liver biopsy specimens of acute hepatitis B (AHB) patients. CD11c (green) is colocalsized with SLC (red) (A), MIP3α (red) (B) and CCR7 (red) (C). The 2-color merged panels were shown with colocalization visible in yellow. Original magnification × 200; D: Colocalization of CD8 with programmed death 1 (PD1) by immunofluorescence double staining in liver biopsy specimens of AHB patients. CD8 (green) is co-localized with PD1 (red). Original magnification × 200; E: Inflammatory cells, such as CD8 and CD11c positive cells, can be observed mainly in fibrous septa in the liver tissue of chronic hepatitis B virus tolerance patients. FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; SLC: Secondary lymphoid tissue chemokine; MIP3α: Macrophage inflammatory protein 3 α; CCR: Chemokine (C-C) receptor.
**Figure 5** Longitudinal analysis of serum hepatitis B surface antigen, alanine aminotransferase levels and human B7 homolog 1 expression in acute hepatitis B and chronic hepatitis B virus tolerance patients. A: In acute hepatitis B (AHB) patients, serum hepatitis B surface antigen (HBsAg) levels decreased significantly from 404 ± 98.3 ng/mL at the 1st week to less than 30 ng/mL at the 16th week. In chronic hepatitis B virus tolerance (CHB-T) patients, serum HBsAg load remained more than 231 ± 38.6 ng/mL at the 16th week; B: In AHB patients, accompanied by a decrease in serum HBsAg level, serum alanine aminotransferase (ALT) level decreased significantly from 346.5 ± 62.3 at the 1st week to 41.8 ± 82 at the 16th week; C: In AHB patients, followed by a decrease in serum HBsAg and ALT level, the percentage of B7 homolog 1 (B7-H1) positive cells decreased significantly from 25.7 ± 4.0 at the 1st week to 6.3 ± 1.37 at the 24th week. In CHB-T patients, B7-H1 expression levels were not increased significantly during the follow-up period; D: In AHB patients, positive correlation between B7-H1 expression on circulating CD11c+ DCs and serum ALT levels was revealed by longitudinal correlation analysis (r = 0.902).

**DISCUSSION**

Recent studies have shown that functional defects in DCs may play a pivotal role in viral persistence during chronic HBV infection. However, the molecular mechanism by which the impaired mDCs induce HBV-specific T cell immune tolerance remains elusive. Previous studies implicated increased B7-H1/PD-1 signaling in DC malfunction and viral-specific T-cell exhaustion in persistent HBV or HCV infections, which were associated with disease progression. In vitro blockade of B7-H1 signaling not only enhanced the mDC mediated allostimulatory capacity, but also up-regulated IL-12 production. Based on these data, B7-H1 was viewed as a signature of impairments in HBV-specific immune response.
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A

B

C

Percentage of CD40 positive cells

Pol: (IC)  HBsAg + poly: (IC)

Percentage of B7-H1 positive cells

Pol: (IC)  HBsAg + poly: (IC)
The present data indicate that B7-H1 is not a signature of HBV immune malfunction, but rather an inflammatory marker of acute hepatitis. Firstly, AIH patients, who did not possess viral etiology, exhibited the highest levels of B7-H1 expression on mDCs among the groups studied. Secondly, B7-H1 expression at protein and mRNA levels was more significant in AHB patients, who were characterized by high levels of serum ALT, than in CHB-T subjects.

Thirdly, in CHB-T patients, despite significant upregulation of intra-hepatic B7-H1 expression, the inflammatory response in liver tissues was vigorous, while in the liver tissues from CHB-T subjects, markers of inflammation were not observed in liver lobe. Only a few inflammatory cells were located in fibrous septa, and B7-H1 molecules were mostly absent despite extensive HBsAg expression. Finally, longitudinal analysis revealed that the level of B7-H1 expression increased during the period of acute hepatitis and decreased when the inflammation was reduced.

Based on our clinical observations, chronic hepatitis B was divided into 2 different components, namely the immune tolerant HBV condition (characterized by active HBV-DNA replication, but with no serum ALT level upregulation) and the chronic active hepatitis condition (which show repeated inflammation flare ups).

In the present study, B7-H1 expression was also analyzed on circulating mDCs in chronic active hepatitis subjects during the hepatitis flare-up period, and the results showed that B7-H1 expression on circulating mDCs was up-regulated to the same extent as in AHB subjects. These data further demonstrate that B7-H1 is a marker of hepatitis.

The current longitudinal analysis revealed that B7-H1 expression on circulating mDCs remained relatively high when serum ALT returned to normal range on week 16, but decreased to near normal level after serum ALT had returned within normal range for 2 mo or 3 mo, indicating a lag phase between the decrease of serum ALT and the return of B7-H1 expression to normal levels. If the blood sample was acquired within the lag phase, B7-H1 expression was higher than in HC.

In the liver tissue of AHB patients, the majority of B7-H1-positive cells co-expressed CD40, indicating that B7-H1 is always expressed on activated APCs. These results are in line with previous findings showing that B7-H1 is significantly increased by proinflammatory cytokines, including IFN-γ and tumor necrosis factor-α.
In conclusion, the up-regulation of B7-H1 may contribute to protect the liver in preventing immune-mediated damage. This concept is in line with several studies supporting that a delicate balance between the positive and negative co-stimulatory molecules may exist in activated mDCs. The level of B7-H1 expression can serve as a marker of inflammation. On the other hand, in the liver lobes of AHB patients, with significant inflammatory characters were almost absent. Correlation analysis revealed a positive correlation between B7-H1 expression and liver damage, reflected by the levels of serum ALT, both cross-sectionally and longitudinally. Based on these data, we concluded that B7-H1 expression can be induced by inflammatory microenvironment and that the level of B7-H1 expression can serve as a marker of the degree of inflammation.

In vitro analysis showed that the expression of B7-H1 and CD40 on mDCs was upregulated synchronously in response to poly (I:C) stimulation. This result suggested that a delicate balance between the positive and negative co-stimulatory molecules may exist in activated mDCs. The B7-H1/PD1 interaction may be involved in weakening the positive stimulatory signal, limiting the immune response to avoid an excessive inflammatory response. This concept is in line with several studies supporting the conclusion that the B7-H1/PD1 pathway may assist the liver in protecting itself from immune-mediated destruction. Zhang et al. reported that the delayed expression of B7-H1 or PD1 lead to fulminant hepatitis. In conclusion, the up-regulation of B7-H1 may contribute to maintaining immune response under the upper limit to avoid severe immune mediated liver damage during acute HBV infection. In CHB-T patients, immune responses were not elicited and B7-H1 expression was not elevated. These phenomena are consistent with the rule of immune regulation, which states “no activation, no inhibition”.

Previous data revealed that the HBV antigen has immunoregulatory abilities. Studies have reported that exposure to higher level of HBsAg load may lead to mDC dysfunction. Op den Brou et al. reported that both HBsAg and HCsAg have the ability to suppress CD40 or HLA-DR expression and thus contribute to HBV or HCV persistence. Loirat et al. reported that recombinant HBsAg interacts with monocytes through the lipopolysaccharide (LPS) receptor CD14, resulting in diminished LPS-induced monocyte activation. HBsAg was shown to reduce LPS-induced TNF-α production through interference with the activation of...
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CD40 and B7-H1, leading to a reduction in T-cell prolif

the immune response by regulating the balance between

results suggested that HBsAg has the ability to regulate

positive in the MLR without HBsAg pretreatment.

were IFN-γ production. The specificity of this upregulation to HBV or HCV infection was deter

the HBV specific immune response have not been fully elucidated.

Several previous studies have shown the activation of DCs after HBsAg impulsion, but either uric acid or cyto-
kines were added in these investigations to active DCs.[36-38] Horii et al.[9] reported that an alum adjuvant was needed in
the HBsAg vaccine to active DCs during vaccine therapy. Proof of the low immunogenicity of HBsAg was shown in the
research by Reignat et al.[31] and Webstet et al.[31] which demonstrated that HBsAg specific CD8+ T cells are char-
acterized by an HBV-tolerant phenotype.

Whether the preferential inhibition of CD40 resulted in the impairment of T cell proliferation by mDCs re-
mains unclear. To elucidate this aspect, we compared the T cell stimulatory capacity of mDCs with or without HBs-
Ag pretreatment by MLR. The results showed that T cell proliferation at 1:5 and 1:10 (R/S) ratio was significantly

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decreased in the HBsAg pretreated MLR system. In ad-
tion to T cells proliferation, we examined whether anti-
viral ability of T cells was affected. Because IFN-γ plays a key role in the control virus infection, the intracellular
content of IFN-γ produced during these MLRs was ana-
yzed by flow cytometry. We found that in the HBsAg-
pretreated MLR system, IFN-γ production by CD8+ T
cells decreased significantly. Only 15% of CD8+ T cells were IFN-γ positive, while more than 50% were IFN-γ
positive in the MLR without HBsAg pretreatment. These results suggested that HBsAg has the ability to regulate
the immune response by regulating the balance between CD40 and B7-H1, leading to a reduction in T-cell prolif-
eration and IFN-γ production.

Our results indicated that B7-H1 is an inflammatory marker. It is induced by the inflammatory microenviron-
ment and can serve as a marker of the degree of inflammation. The increase in B7-H1 expression correlated with
the expression of positive co-stimulatory molecules to weaken the activation signal for T cells and gener-
ate a balance between co-inhibitory and co-stimulatory signal. HBsAg has the ability to tip this balance through
preferential inhibition of CD40 expression, leading to a

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reduction in T cell proliferation and IFN-γ production. In CHB-T subjects, maintenance of high HBsAg load may impair HBV immunity through the regulation of the balance between CD40 and B7-H1 expression.

Based on our results, we speculated that the outcome of hepatitis B depends on 2 factors. The first is the level of HBV antigen load and HBV replication. The second is the upper limit of the HBV specific immune response set by co-inhibitory molecules such as B7-H1. If the HBV antigen and HBV-DNA are eliminated successfully by the immune response under this upper limit, the hepata-
titis B patient should recover. The higher the upper limit, the better the chance of HBV elimination.
may exist. HBV antigens should be eliminated efficiently before the immune response reached the upper threshold level, otherwise acute HBV infection may turn into a chronic infectious state. The design of this study is reasonable; the results and conclusion of this paper are reliable, and the notion about an "upper threshold of HBV specific immune response" is somewhat attractive.

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