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Potential role of soluble B7-H3 in liver immunopathogenesis during chronic HBV infection

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SUMMARY. Immune-mediated mechanisms have been implicated in liver pathogenesis and subsequent progression in hepatitis B virus (HBV) infection. Costimulatory molecules, the important regulators of immune responses, participate in the regulation of liver pathology in HBV infection. However, the role of B7-H3 (CD276, a new member of B7 family) in this process has not been investigated. In this study, we detected abundant soluble B7-H3 (sB7-H3) in the plasma of patients with chronic HBV infections. The increase of the plasma B7-H3 was associated with the progression of liver cirrhosis and accompanied by decreased expression of B7-H3 on hepatocytes. The identification analysis suggests that the plasma B7-H3 might be derived from the membrane-bound B7-H3 on hepatocytes. A functional study showed that immobilized (4Ig) B7-H3Ig fusion protein could inhibit TCR-induced proliferation and IFN-γ secretion of T cells, which could be partially blocked by soluble B7-H3flag fusion protein. These results suggest that the reduced expression of B7-H3 in the livers might temper the inhibition of T-cell responses mediated by B7-H3 expressed on hepatocytes and thus promote the hepatic inflammation and hepatitis progression in the chronic HBV-infected patients.

Keywords: B7-H3, chronic hepatitis B, HBV, liver cirrhosis, T-cell immunity.

INTRODUCTION

More than 350 million people worldwide suffer from persistent infection with hepatitis B virus (HBV), and are at risk for developing liver cirrhosis (LC) and hepatocellular carcinoma (HCC) [1]. A large body of evidence indicates that the outcome of HBV infections and the pathogenesis of the attendant liver diseases are determined by immune-mediated host–virus interactions. Control of HBV infection is correlated with efficient induction of HBV-specific $CD8^+$ cytotoxic T-cell responses [2,3]. The marked quantitative and qualitative defects in HBV-specific $CD8^+$ T-cell response have been described in patients with chronic hepatitis B (CHB) [4–6]. Patients with uncontrolled infection are distinguished from healthy HBV carriers by the presence of a large lymphocytic infiltrate in their livers, containing a high proportion of non-antigen-specific $CD8^+$ T cells [2,7]. Other inflammatory cells including macrophages and neutrophils are also recruited by IFN-γ and its inducible chemokines [8]. The continuous recruitment of inflammatory cells to the liver ultimately results in liver injury, fibrosis, cirrhosis and possible carcinoma. However, the underlying mechanisms have not been well characterized.

Costimulatory molecules play pivotal roles in regulating immune responses and are engaged in the pathogenesis of many diseases especially in chronic virus infection [9]. In chronic HBV infection, high levels of programmed death-1 (PD-1) expression can lead to T-cell exhaustion and favour virus persistence [10–13], while the increased expression of PD-1 or its ligands (B7-H1 and B7-DC) may also

Abbreviations: (4Ig) B7-H3, B7-H3 4Ig isoform; B7-H3flag, 4Ig B7-H3 with Flag tag; B7-H3Ig, 4Ig human B7-H3 and human Ig Fc fusion protein; B7-H3mIg, 2Ig B7-H3 and mouse Ig Fc fusion protein; CHB, chronic hepatitis B; CIV, collagen type IV; HA, hyaluronic acid; hB7-H3, human B7-H3; HBV, hepatitis B virus; HC, healthy control; HCC, hepatocellular carcinoma; HPF, high power field; LC, liver cirrhosis; MMPs, matrix metalloproteinases; PBMCs, peripheral blood mononuclear cells; PD-1, programmed death-1; PIIINP, N-terminal procollagen III peptide; sB7-H3, soluble B7-H3; T.Bili., total billirubin; TIMP, tissue inhibitors of metalloproteinase.

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potentially be a mechanism to reduce liver damage [14,15]. During acute HBV infection, the delayed PD-1 expression may be associated with liver failure [16]. These data indicate that costimulatory molecules may actively participate in the regulation of liver pathogenesis in HBV infection.

B7-H3 (CD276) was originally identified as a costimulatory molecule inducing T-cell proliferation and IFN-γ production *in vitro* [17]. Unlike the other members of B7 family, B7-H3 has two forms. Mouse B7-H3 has extracellular IgV–IgC domains, whereas human B7-H3 (hB7-H3) contains tandemly duplicated IgV–IgC–IgV–IgC domains because of exon duplication, known as (4Ig) B7-H3 [18]. B7-H3 can be weakly induced on T cells, NK cells and antigen-presenting cells such as dendritic cells and macrophages. In addition, B7-H3 mRNA has also been identified in human liver, lung, bladder, testis, prostate, breast and placenta [17]. Although the functional roles of B7-H3 on T-cell responses are controversial [17,19], the clinical studies suggest that B7-H3 is exploited by tumours as an immune evasion pathway. The B7-H3 up-regulation can impair T-cell-mediated anti-tumour immunity and is markedly associated with disease severity in human malignant tumours [20–23]. Up to now, little information about the roles of B7-H3 is available in HBV infection.

In present study, we found that the soluble form of B7-H3 (sB7-H3) was abundantly presented in the plasma of the CHB patients, and was closely correlated with LC. Moreover, the elevated plasma sB7-H3 was accompanied by the decreased expression of B7-H3 on the hepatocytes, which might enhance the responses of T cells to promote the inflammatory diseases in liver. Our studies might suggest a new pathogenic mechanism of CHB.

MATERIALS AND METHODS

Study objects

Plasma samples were obtained from 136 HBV infected patients (80 male, 56 female; median age, 37.2 years) hospitalized in Beijing 302 Hospital and 259 gender- and age-matched healthy donors. According to the imaging and biopsy examination of liver, the patients were assigned into 59 CHB patients with LC and 77 CHB patients without LC. The characteristics of the patients are shown in Table 1. None of the patients received anti-HBV agents or steroids in 6 months before sampling. Concurrence of HCC, HCV and HIV infections, autoimmune or alcoholic liver disease was excluded for all enrolled individuals. Liver specimens of healthy donors were collected during liver transplantations, and those of patients are collected in biopsy. This study was carried out in accordance with the World Medical Association Helsinki Declaration and was approved by the Ethics Committee of Beijing 302 Hospital and the Institutional Review Board of Institute of Biophysics, Chinese Academy of Sciences. Informed consent was obtained from all patients.

Preparation of fusion proteins and monoclonal antibodies

The fusion proteins of 4Ig human B7-H3 and human Ig Fc (B7-H3Ig), 2Ig B7-H3 and mouse Ig Fc (B7-H3mIg), and 4Ig B7-H3 and Flag tag (B7-H3flag) were prepared as described previously [17]. Anti-B7-H3 mAbs were produced by immunizing BALB/c mice with B7-H3mIg fusion protein by standard methods. Three clones of hB7-H3.1M, hB7-H3.3M, hB7-H3.5M were detected to recognize both 2Ig and 4Ig hB7-H3.

Table 1 Characteristics of the populations enrolled in the study

Variable	Healthy control	Patients	
		CHB & non-LC	CHB & LC
Cases	259	77	59
Age (years)	32 (21–58)	33 (20–53)	48 (24–68)
Gender (male/female)	133/126	43/34	37/22
Plasma HBV DNA (copies/mL)	U.D.	7.76×10^6 (200–9.26×10^8)	1.45×10^5 (730–2.83×10^7)
Plasma alanine transaminase (U/L)	13 (2–25)	45 (10–598)	50 (6–165)
HBsAg, +/−	0/259	77/0	59/0
HBsAb, +/−	234/25	0/77	0/59
HBeAg, +/−	0/259	42/35	15/44
HBeAb, +/−	0/259	30/47	26/33
HBcAb, +/−	0/259	77/0	59/0

Data are shown as median and range.
U.D., undetected; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBsAb, hepatitis B surface antibody; HBeAg, hepatitis B e antigen; HBeAb, hepatitis B e antibody; HBcAb, hepatitis B core antibody; LC, liver cirrhosis; CHB, chronic hepatitis B.

Detection of sB7-H3

To detect sB7-H3, a double-antibody sandwich ELISA was established with clone hB7-H3.3M (5 μg/mL) as capture antibody and biotinylated clone hB7-H3.5M (2 μg/mL) as detection antibody. For the competition ELISA assay, B7-H3Ig fusion protein was diluted to 2.5 ng/mL in the plasma from LC patients or healthy controls (HC), or foetal bovine serum as control, and added into the plates coated with clone hB7-H3.3M in triplicate. After incubation at 37 °C for 2 h, the plates were incubated with HRP-conjugated anti-human IgG (Sigma-Aldrich, St. Louis, MO, USA).

Immunohistochemistry

Paraffin-embedded, formalin-fixed liver tissues were cut into 5-μm sections and treated according to our previously described protocols [24]. For B7-H3 staining, sections were incubated with mouse anti-B7-H3 mAb (hB7-H3.1M) at 1 μg/mL, then incubated with biotinylated secondary antibody followed by streptavidin-peroxidase (Zhongshan Goldenbridge Biotech, Beijing, China). The peroxidase activity was detected with 3-amino-9-ethyl-carbazole (AEC; red colour) and the sections were counterstained with haematoxylin. The immunohistochemical blocking studies were carried out by pre-exposing anti-B7-H3 mAb (hB7-H3.1M) with B7-H3Ig at 37 °C for 30 min before immunohistochemical staining.

For analysing the B7-H3 positive percentage on hepatocytes, five high-power fields (HPF, 400×) centred on lobules or regenerative nodules of hepatocyte were selected for each sample at relative random. The B7-H3$^+$ and B7-H3$^-$ hepatocytes per HPF were counted and percentage of B7-H3$^+$ hepatocyte were calculated. The values for the different fields were then averaged to obtain the final values of each sample for statistical analysis.

Immunoprecipitation and Western blotting

Forty microlitres Streptavidin-Sepharose Beads (GE Healthcare, Piscataway, NJ, USA) were labelled with 5 μg biotinylated clone hB7-H3.3M at 4 °C for 2 h and washed in PBS, and incubated with plasma samples at 4 °C for 24 h. After precipitation, the beads were resuspended in SDS sample buffer for SDS-PAGE. Western blotting was performed under standard conditions by using clone hB7-H3.1M at 2 μg/mL as primary antibody and HRP-conjugated goat-anti-mouse IgG (Sigma-Aldrich) as secondary antibody.

Cytokine and serum biochemical analysis

The levels of cytokines in the serum or the culture supernatant were detected with human Th1/Th2 cytokine kit and inflammatory cytokine kit (BD Biosciences, San Diego, CA, USA). Serum level of hyaluronic acid (HA), N-terminal procollagen III peptide (PIIINP) and collagen type IV (CIV) were measured by radioimmunoassay (RIA-HA and CIV kit, Shanghai, China; RIA-PCIII kit, Chongqing, China). Total billirubin (T.Bili.) was measured using T.Bili. kit (Biosino Biothec, Beijing, China).

T-cell preparation and proliferation assay

Peripheral blood mononuclear cells (PBMCs) were separated from healthy human peripheral blood (Beijing Central Blood Bank, China) by Ficoll–Hypaque gradient centrifugation. CD4$^+$ or CD8$^+$ T cells were positively isolated using anti-CD4-PE or anti-CD8-PE followed by anti-PE-beads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to standard protocol. Flat-bottom 96-well plates were first coated with anti-CD3 overnight at 4 °C and subsequently coated with B7-H3Ig or control Ig at indicated concentrations at 37 °C for 2 h. PBMCs or purified CD4$^+$ and CD8$^+$ T cells (2×10^5) were cultured in the plates coated with anti-CD3 and B7-H3Ig proteins, or cultured with soluble B7-H3flag fusion protein in the plate only coated with anti-CD3. For the blocking experiments with sB7-H3flag fusion protein, PBMCs (2×10^5) were cultured with sB7-H3flag in the plates coated with anti-CD3 and B7-H3Ig proteins. Proliferation of T cells was determined by incorporation of 1 μCi per well ^{3}H-TdR during the last 12 h of the 72 h culture. ^{3}H-TdR incorporation was counted on a MicroBeta Trilix liquid scintillation counter (Wallac, Turku, Finland). To detect cytokines, supernatants were collected at the indicated time points of the cultures.

Statistical analysis

Statistical analyses were performed using Graphpad prism 4 software (Graphpad software Inc., San Diego, CA, USA). Comparison between various individuals was performed using the Mann–Whitney *U* test. Correlation analysis was evaluated by the Spearman rank correlation test, other comparisons were performed using two-sample *t*-test. For all tests, two-sided $P < 0.05$ was considered statistically significant.

RESULTS

The elevated plasma sB7-H3 correlates with liver diseases in the CHB patients

To detect sB7-H3 in plasma, we set up a double sandwich ELISA system using two clones of anti-B7-H3 mAbs (hB7-H3.3M and hB7-H3.5M). sB7-H3 was detected in the plasma of 77 CHB patients without LC (CHB & non-LC), 59 patients with LC (CHB & LC) and 259 HCs. As shown in Fig. 1a, the level of sB7-H3 in plasma of the CHB patients was significantly higher than that in the HCs, and moreover, the CHB patients with LC had higher sB7-H3 concentration in the

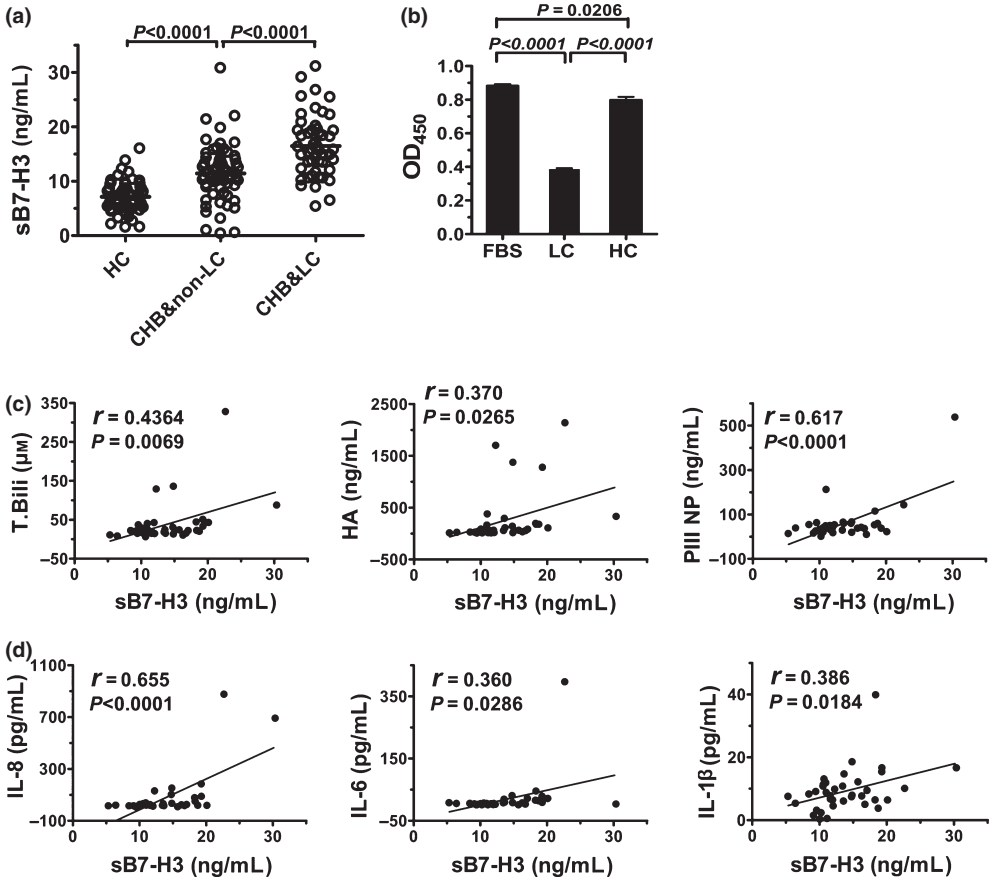

Fig. 1 The association of the plasma level of soluble B7-H3 (sB7-H3) with liver fibrosis and inflammatory cytokines in chronic hepatitis B (CHB) patients. (**a**) The plasma sB7-H3 was detected by a double-antibody sandwich ELISA from 77 CHB patients without LC (CHB & non-LC), 59 CHB patients with cirrhosis (CHB & LC) and 259 healthy controls (HC). Each dot represents one subject. Horizontal lines illustrate the median value. (**b**) Competition ELISA assay. (4Ig) B7-H3Ig fusion protein was incubated with foetal bovine serum (FBS) or the plasma of LC patients (LC) or HC in anti-B7-H3 mAb coated plate and detected by HRP-conjugated anti-human Ig antibody. (**c–d**) Correlation analysis between sB7-H3 concentration and the levels of total bilirubin (T.Bili.), hyaluronic acid (HA), procollagen III (PIIINP) (**c**) and inflammatory cytokine IL-8, IL-6, IL-1β (**d**) in the plasma of CHB patients. Solid line, linear regression trend; *r*, correlative coefficient. *P* values are shown. LC, liver cirrhosis.

plasma than the patients without LC. To confirm the specificity of the sB7-H3 assay, a competition ELISA assay was performed. The plasma from both the patients and HCs could compete with B7-H3Ig fusion protein to bind to coated anti-B7-H3 mAb, and the plasma from LC patients had much stronger competition than that from HCs, indicating the higher concentration of sB7-H3 in the plasma of LC patients (Fig. 1b).

To exploit further the potential relation of sB7-H3 with the pathogenesis of CHB, we analysed the association of sB7-H3 levels with inflammation cytokines and some hepatitis markers. Although there was no significant correlation of sB7-H3 level with serum HBV burden and alanine aminotransferase levels, sB7-H3 was found to be positively correlated with the serum levels of T.Bili., HA and PIIINP (Fig. 1c), which are the markers for the progression of LC during chronic liver diseases [25]. In addition, there were positive correlations between the plasma levels of sB7-H3 and inflammation cytokine IL-1β, IL-6 and IL-8 (Fig. 1d). No correlation was found between sB7-H3 and IL-2, IL-4, IL-5, IL-10, IL-12, TNF-α, IFN-γ and TGF-β (data not shown). These data demonstrate that the increased plasma sB7-H3 is closely associated with hepatic inflammation and fibrogenesis in CHB.

The percentage of B7-H3 positive hepatocyte decreased in CHB patients

As B7-H3 is undetectable in PBMC while its mRNA is presenting in liver [17], we hypothesized that the elevated sB7-H3 in the plasma of patients might come from the inflammatory liver. The expression of B7-H3 in liver was examined by immunohistochemical staining with anti-B7-H3 mAb (hB7-H3.1M). As shown in Fig. 2a, the hepatocytes

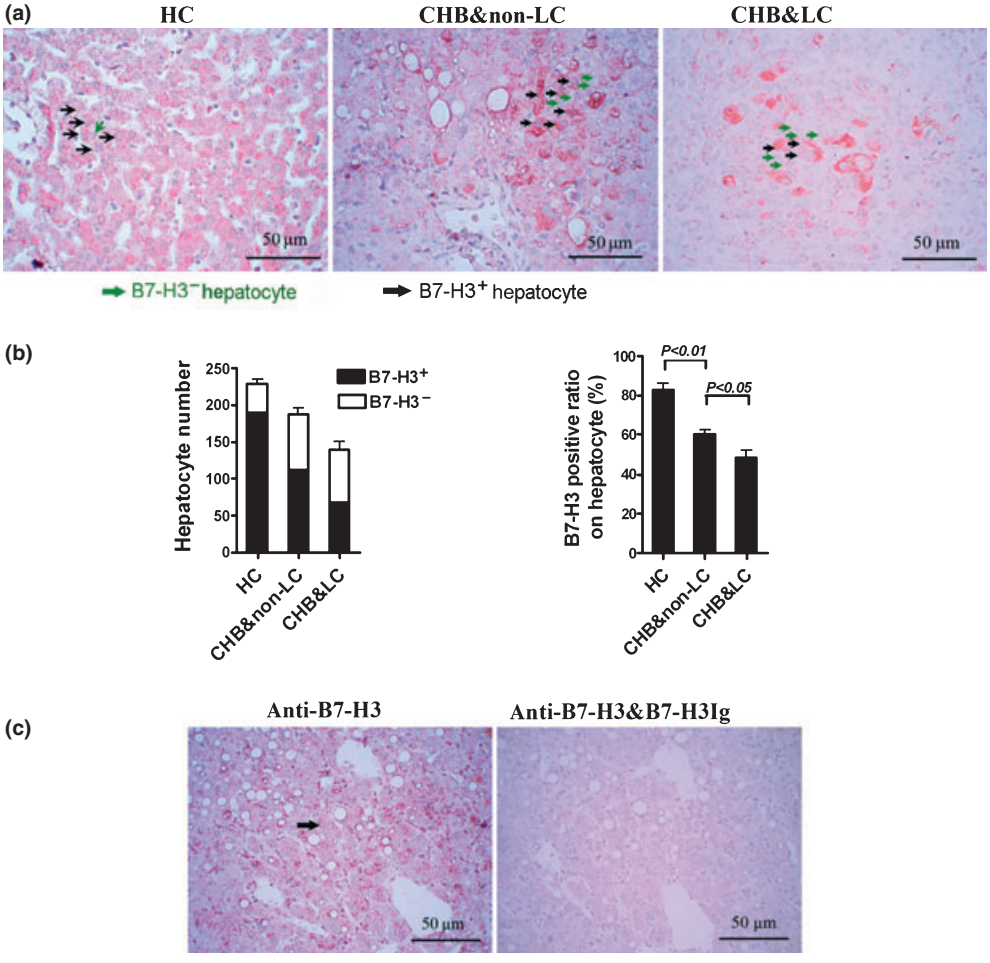

Fig. 2 Immunohistochemical detection of B7-H3 expression in liver tissues of chronic hepatitis B (CHB) patients and healthy controls. (**a**) Representative results for B7-H3 staining by B7-H3 mAb (hB7-H3.1M) in paraffin-embedded liver specimens from healthy donor (HC) and CHB patients without (CHB & non-LC) or with cirrhosis (CHB & LC). B7-H3$^+$ and B7-H3$^-$ hepatocytes were labelled representatively in each slide. (**b**) B7-H3$^+$ and B7-H3$^-$ hepatocytes number per high power field (400×, left) and statistical analysis for the B7-H3$^+$ positive ratio on hepatocytes (right) in normal and CHB liver tissue samples (n = 7–9 in each group). (**c**) B7-H3Ig fusion protein blocks anti-B7-H3 mAb staining of liver specimen of CHB patient. Paraffin-embedded liver specimen was stained with anti-B7-H3 mAb directly (left), or with anti-B7-H3 antibody preincubated with B7-H3Ig fusion protein (right). LC, liver cirrhosis.

highly expressed B7-H3 in normal liver tissues. The B7-H3 expression was detected in 80% hepatocytes and localized both in the cytoplasm and on the membrane. However, the expression of B7-H3 on the hepatocytes was significantly decreased in the liver specimens of the CHB patients, which was associated with progression of the liver diseases. The liver tissues from CHB patients had less total hepatocyte number, but more B7-H3 negative hepatocyte per HPF (400×) than normal liver tissues. Moreover, the percentage of B7-H3$^+$ hepatocyte in total hepatocytes in liver tissue from CHB patients with LC was significantly less than that from the patients without LC (48% *vs* 60%) (Fig. 2b). Thus, more hepatocytes lost the expression of B7-H3 with the progression of the liver diseases. In addition, B7-H3 staining in the liver tissues of patients could be blocked by the inclusion of B7-H3Ig fusion protein (Fig. 2c), confirming the specificity of the B7-H3 staining. The associations between the elevated sB7-H3 in the plasma and decreased percentage of B7-H3-positive hepatocytes with progression of hepatitis suggested that sB7-H3 might be derived from the liver of CHB patients.

sB7-H3 comes from the extracellular domains of (4Ig) B7-H3

B7-H3 has two isoforms with 4Ig or 2Ig extracellular domains respectively in human [26,27]. To characterize the sB7-H3 in the plasma, an immunoprecipitation was carried out with the plasma from the patients with LC using anti-B7-H3 mAb (hB7-H3.3M). The precipitate and the HepG2

cell lysate were analysed by Western blot using another clone of anti-B7-H3 mAb (hB7-H3.1M). As shown in Fig. 3a, a major band at approximately 90 kDa was specifically precipitated with anti-B7-H3 mAb compared with the control antibody. The B7-H3 on HepG2 cells was shown to be around 100 kDa, which was similar to the molecular mass of surface molecule of (4Ig) B7-H3 [20]. Therefore, sB7-H3 in the plasma should be the extracellular part of membrane-bound (4Ig) B7-H3.

By searching NCBI GenBank, we found a hB7-H3 isoform (isoform c) without the transmembrane and intracellular domains, which contains 493 amino acids corresponding to the 4Ig extracellular domains of B7-H3 (GenBank: AAH62581.1) (Fig. 3b). To clarify whether the sB7-H3 comes from this isoform, a pair of primers from the common sequences of the full length B7-H3 (isoform b) and isoform c, which lie in second IgV and 3′-UTR of B7-H3 gene, were used to amplify cDNA samples from liver tissues of LC patients with high sB7-H3 (Fig. 3b). A band of 1.5 kb corresponding to isoform b, but no 0.57 kb product corresponding to isoform c, was found in the PCR products (Fig. 3c). Therefore, sB7-H3 should come from the extracellular domains of (4Ig) B7-H3, not from the alternative mRNA splicing form.

Immobilized B7-H3 protein inhibits T-cell proliferation and IFN-γ secretion

Since B7-H3 was originally identified as costimulatory molecule of T cells, we studied the costimulatory potential of sB7-H3 and immobilized B7-H3 on T-cell proliferation and cytokine production. B7-H3Flag fusion protein was prepared to mimic the plasma sB7-H3. As shown in Figs 4a,b, the immobilized B7-H3Ig fusion protein inhibited T-cell proliferation and IFN-γ production in the presence of anti-CD3, while sB7-H3 had no effects. But the sB7-H3 could partially block the inhibitory effects of immobilized B7-H3. Moreover, the inhibitory effect of immobilized B7-H3Ig was dose dependent (Fig. 4c) and showed no bias on $CD4^+$ and $CD8^+$ T cells (Fig. 4d). These data suggest that the decreased expression of B7-H3 on hepatocytes is likely to reduce the inhibitory effects of B7-H3 on T-cell responses.

DISCUSSION

In this study, we found a significant increase of circulating sB7-H3 in CHB patients compared with healthy subjects, which was positively correlated with LC. The sB7-H3 might come from the reduction of B7-H3 expression on the hepatocytes, which potentially promoted the liver inflammation by reducing the inhibitory effects of B7-H3 on T cells' responses. These findings reveal the potential role of B7-H3 in the pathogenesis of CHB. Importantly, the plasma sB7-H3 might serve as a new marker for liver fibrosis and cirrhosis in CHB patients.

B7-H3 was identified as a membrane-bound protein. It has two isoforms with 2Ig (IgV–IgC) and 4Ig (IgV–IgC–IgV–IgC) extracellular domains respectively. The (4Ig) B7-H3 is the major isoform expressed in human, which is about 100kD

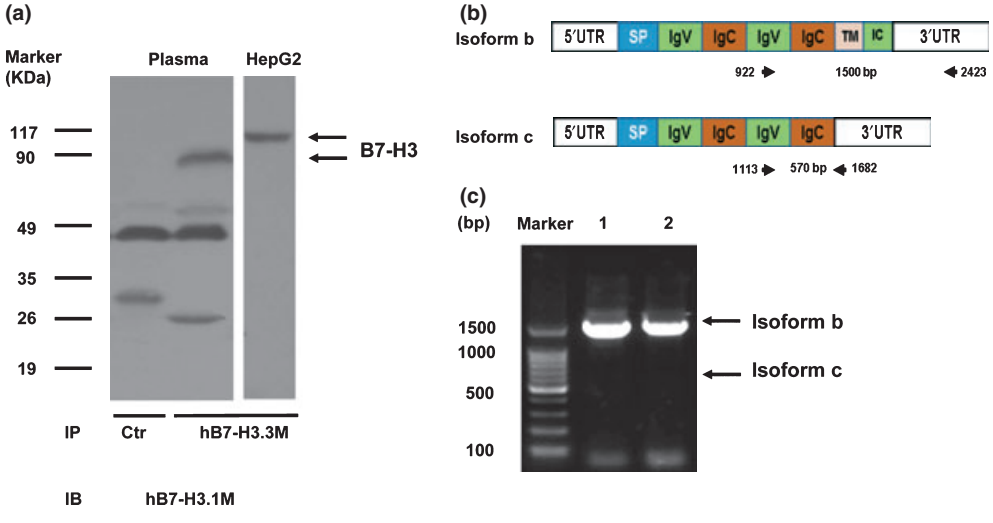

Fig. 3 Soluble B7-H3 (sB7-H3) is the extracellular domains of (4Ig) B7-H3, and not from the alternative splicing isoform. (**a**) Plasma from CHB & LC patients were immunoprecipitated with anti-B7-H3 mAb (hB7-H3.3M) or control mAb (left), HepG2 cells were boiled in SDS loading Buffer directly (right). The precipitate and HepG2 lysate were subjected to SDS-PAGE and analysed by immunoblot with anti-B7-H3 mAb (hB7-H3.1M). Molecular mass markers (kDa) and B7-H3 bands are indicated. (**b**) mRNA structures of isoform b and isoform c of (4Ig) B7-H3. The isoform b encodes full line of membrane-bound (4Ig) B7-H3 with transmembrane domain (TM) and a short intracellular tail (IC). The isoform c only encodes extracellular domains of (4Ig) B7-H3. Small arrows below indicate the positions of primers used for PCR analysis. (**c**) Representative data for RT-PCR analysis of expression of B7-H3 isoforms in liver tissues from LC patients with the primers indicated in (**b**). The theoretical size of the PCR products of isoform b and c are indicated. LC, liver cirrhosis; CHB, chronic hepatitis B.

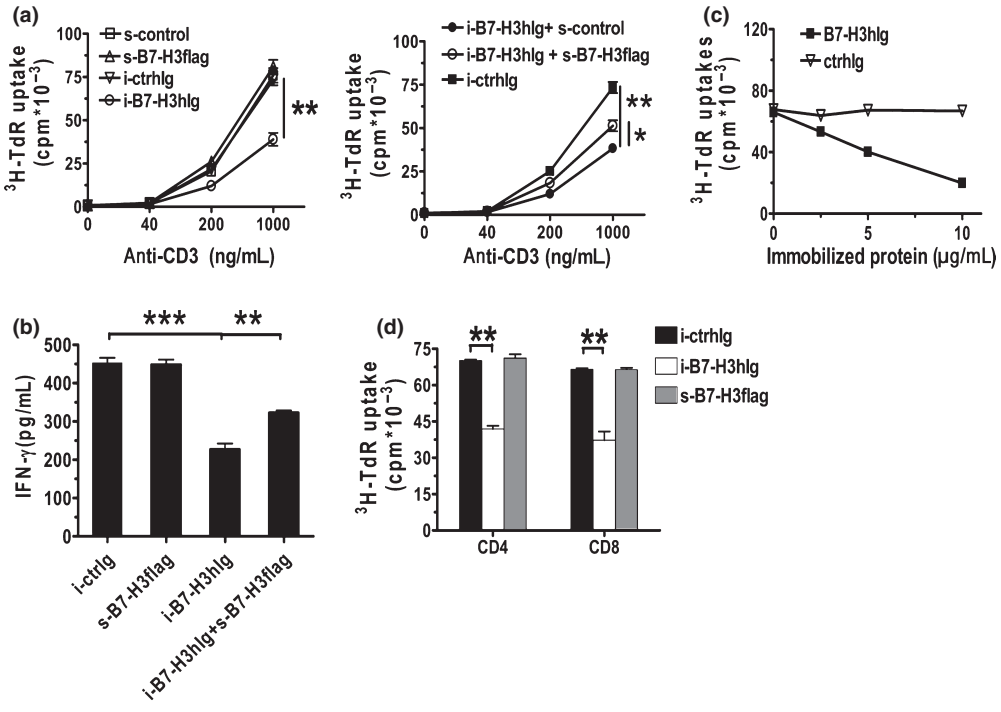

Fig. 4 Immobilized (4Ig) B7-H3Ig protein inhibits T-cell proliferation and IFN-γ secretion. (**a**) PBMC was stimulated with soluble B7-H3flag at 20 μg/mL or immobilized (4Ig) B7-H3Ig fusion proteins at 5 μg/mL in the presence of immobilized anti-CD3 mAb at indicated concentrations (left). Or, PBMC was cultured with soluble B7-H3flag at 20 μg/mL in the presence of immobilized B7-H3Ig fusion proteins at 5 μg/mL and anti-CD3 mAb at indicated concentrations (right). PBS and hIgFc protein were used respectively in soluble and immobilized forms as controls. The proliferation of T cells was determined by incorporation of 1 μCi per well of ^{3}H-TdR during the last 12 h of 72 h culture. The results are shown as the mean ± SD of triplicate wells. Data depict one representative experiment of four different donors. (**b**) Supernatants from the culture in (**a**) were collected at 48 h and the concentrations of cytokines were measured by Th1/Th2 CBA kit. Data depict the IFN-γ levels under the stimulation with 1 μg/mL of anti-CD3. (**c**) Immobilized (4Ig) B7-H3 protein inhibits PBMC proliferation in a dose dependent manner. PBMC were cultured with immobilized (4Ig) B7-H3Ig fusion protein at indicated concentrations in the presence of pre-coated anti-CD3 at 1 μg/mL. Proliferation was assayed as in (**a**). Data depict one representative experiment of three. (**d**) Purified $CD4^+$ and $CD8^+$ T cells were cultured with soluble B7-H3flag at 20 μg/mL or immobilized B7-H3Ig fusion proteins at 5 μg/mL in the presence of immobilized anti-CD3 mAb at 1 μg/mL. Data depict one representative experiment of three. *$P < 0.05$; **$P < 0.01$. s- and i- represent soluble and immobilized proteins respectively. PBMC, peripheral blood mononuclear cell.

with N-terminal glycosylations [20]. Only 2Ig form of B7-H3 is expressed in mice, which is about 50 kDa [28]. The plasma sB7-H3 was about 90 kDa, compared with the 100 kDa B7-H3 expressed on HCC cell line HepG2, indicating that it should be the 4Ig extracellular domains without transmembrane and intracellular domains. The immunohistochemistry showed that more than 80% hepatocytes in normal liver tissues were B7-H3 positive, but only 40–60% hepatocytes expressed B7-H3 in livers of CHB patients. The decreased expression of B7-H3 on hepatocytes was coincident with the elevated sB7-H3 in plasma in the patients, indicating that sB7-H3 might originate from the liver. The mRNA of the isoform encoding sB7-H3 was undetectable in the liver of CHB & LC patients, supporting that sB7-H3 might not come form the alternative splicing at mRNA level. It has been shown that the ligands of several costimulatory molecules are cleaved from the cell surface as soluble proteins by matrix metalloproteinases (MMPs) [29]. MMPs were characterized by their ability to degrade extracellular matrix components, and played roles in morphogenesis, angiogenesis and tissue remodelling. In chronic liver disease, MMPs were widely presented in liver and involved in liver diseases together with tissue inhibitors of metalloproteinases, TIMPs [30]. Therefore, it is conceivable that sB7-H3 might be shed by MMPs from hepatocytes. Further investigations are required to address this question.

The (2Ig) B7-H3Ig fusion protein was originally identified to costimulate the T-cell proliferation and cytokine production [17]. Later, the main form of B7-H3 expressed in human, including human immune cells and malignant cells, was found to be (4Ig) B7-H3 [27,28], which down-modulated human T-cell responses in the presence of strong

activating signals [31]. The up-regulated expression of B7-H3 in human malignant tumour was associated with increased disease severity, indicating its inhibitory role on anti-tumour immune responses [22,23]. In this study, we found that the immobilized (4Ig) B7-H3Ig fusion protein significantly inhibited proliferation and IFN-γ secretion of both $CD4^+$ and $CD8^+$ T cells. sB7-H3, on the contrary, had no inhibitory effects on T-cell responses, but partially blocked the inhibitory effects of immobilized B7-H3. These results suggest that reduced expression of B7-H3 in livers might temper the inhibition of membrane B7-H3 on T-cell responses and promote hepatic inflammatory responses in CHB patients.

In conclusion, we found that the plasma sB7-H3 was significantly increased and positively correlated with liver fibrosis in the CHB patients. Our study suggests that the sB7-H3 comes from the membrane-bound B7-H3 on hepatocytes, which is enhanced with the hepatitis progression. The reduction of B7-H3 expression in the livers might temper B7-H3-mediated inhibition on T-cell responses, promoting the hepatic inflammation and hepatitis progression in CHB patients.

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