

Roles of the programmed cell death 1, T cell immunoglobulin mucin-3, and cluster of differentiation 28 pathways in the low reactivity of invariant natural killer T cells after chronic hepatitis B virus infection

Zhixin Yang¹ · Yu Lei¹ · Chunbo Chen¹ · Hong Ren¹ · Tongdong Shi¹

Received: 27 January 2015 / Accepted: 17 July 2015 / Published online: 29 July 2015
© Springer-Verlag Wien 2015

Abstract One of the main responses of invariant natural killer T (iNKT) cells to antigen stimulation is the rapid production of interleukin (IL)-4 and interferon (IFN)- γ cytokines. There is a decline in the function of iNKT cells in chronic hepatitis B (CHB) patients. In this study, we explored the impact of programmed cell death 1 (PD-1), T cell immunoglobulin mucin-3 (Tim-3), and cluster of differentiation 28 (CD28) expression on iNKT cell functions in CHB patients. Flow cytometry was used to test iNKT frequencies and levels of PD-1, Tim-3, CD28, IL-4, and IFN- γ secreted by iNKT cells. An enzyme-linked immunosorbent assay (ELISA) was used to measure IL-4 and IFN- γ secretion upon α -galactosylceramide (α -GalCer) activation *ex vivo*. We found that the levels of expression of PD-1 and Tim-3 from iNKT cells in CHB patients were significantly higher than in healthy donors ($p < 0.05$), but there was lower expression of CD28 ($p < 0.05$) and an impaired capability to produce IL-4 and IFN- γ ($p < 0.05$). *In vitro* α -GalCer stimulation upregulated the expression of PD-1⁺ iNKT cells ($p < 0.05$), Tim-3⁺ iNKT cells ($p < 0.05$), and CD28⁺ iNKT cells ($p < 0.05$). In response to combination therapies consisting of α -GalCer and anti-PDL1 monoclonal antibody (mAb) and/or anti-Tim-3 mAbs and/or anti-CD80/anti-CD28 mAbs, IL-4⁺ and IFN- γ ⁺ iNKT cells demonstrated different degrees of growth ($p < 0.05$). The functional decline of iNKT cells was closely related to the decrease in CD28 expression and the

increases of Tim-3 and PD-1. In addition, clinical antiviral treatment with lamivudine could partially restore the immune function of iNKT cells in CHB patients.

Introduction

Hepatitis B virus (HBV) is a noncytopathic enveloped virus that causes acute and chronic necroinflammatory liver diseases that affect more than 350 million people worldwide [1, 2]. Approximately 1 million people die annually from HBV-induced liver diseases [1, 2]. Antiviral-therapy-induced host immune responses against HBV play a crucial role in viral clearance [3, 4]; however, the pathogenesis and immunologic mechanisms of these responses remain unclear.

Invariant natural killer T (iNKT) cells express an invariant α -chain T-cell receptor (TCR; V α 24-J α 18 chain in humans and V α 14-J α 18 chain in mice), which is paired with one of three different β chains [5–7]. CD1d-restricted T cells are less diverse. Many use the same TCR α chain, but they also express natural killer (NK) cell receptors. The CD1d-restricted population is called iNKT cells, and one recognized ligand for CD1d molecules is α -galactosylceramide (α -GalCer) [5–8]. iNKT cells rapidly produce large quantities of both type 1 and type 2 helper-biased cytokines, including interleukin (IL)-4 and interferon (IFN)- γ [9]. It is thought that these cells primarily have a regulatory function, which enhances the function of NK cells, dendritic cells, and B cells, as well as that of conventional CD4⁺ and CD8⁺ T cells [9].

Research on chronic hepatitis B (CHB) infections has mainly focused on CD8⁺ T cells [10–12], but the role of iNKT cells has been neglected. Recently, iNKT cells were found to inhibit HBV replication *in vivo* directly and not

✉ Tongdong Shi
1452381090@qq.com; shitongdong@cqmu.edu.cn

¹ Key Laboratory of Molecular Biology for Infectious Diseases, Ministry of Education, Institute of Viral Hepatitis, Chongqing Medical University the Second Affiliated Hospital, 74 Linjiang Road, Chongqing 400010, China

rely on conventional CD4⁺ T and CD8⁺ T cells [13, 14]. In chronic HBV infections, iNKT cells exhibit functional decline. Unfortunately, the mechanism underlying the malfunction of iNKT cells in chronic HBV infections is not well understood. This functional decline could be related to the upregulation of surface programmed cell death 1 (PD-1) and T cell immunoglobulin mucin-3 (Tim-3) molecules [15–22]. PD-1 is a inhibitory receptor related to CD28, which was found to be upregulated on the surface of exhausted T cells in human immunodeficiency virus (HIV) [23–25], chronic lymphocytic choriomeningitis virus (LCMV), hepatitis C virus (HCV), and chronic hepatitis B virus infections [15, 24, 25]. PD-1 can bind to the B7-family ligands PDL1 (B7-H1) and PDL2 (B7-DC). Binding of PD-1 to PDL1 has been studied in detail and has been shown to transduce strong inhibitory signals to the iNKT cell [20]. The Tim-3 protein is specifically expressed on type 1 helper T (TH1) cells and negatively regulates TH1 cell responses. Some reports have suggested that Tim-3 is upregulated on effector CD8⁺ T cells in chronic virus infections [26]. Tim-3-expressing T cells have an inhibitory ability and dysfunctional cytokine responses, and blockade of Tim-3 improves the proliferative ability of HIV-specific T cells [27].

The aim of this project was to study the frequencies, functions, and surface expression levels of the co-stimulatory factors CD28, PD-1, and Tim-3 on iNKT cells in chronically HBV-infected patients. The low activities of iNKT cells by blocking the *in vitro* PD-1 and Tim-3 signaling pathways and/or the activation of CD28 signals were also investigated, which may improve the immune responses mediated by α -GalCer-activated iNKT cells in chronic HBV infection. The results indicated that the functional decline of iNKT cells was closely related to the decrease in CD28 expression and the increase of Tim-3 and PD-1 expression. In addition, clinical antiviral treatment with lamivudine could partially restore the immune function of iNKT cells in CHB patients.

Materials and methods

Subjects

Twenty-eight patients with CHB were enrolled in the study. Peripheral blood was taken from each patient, and none of the patients received interferon or nucleoside analogue drug treatment before sampling.

Fifteen patients who were pathologically confirmed to have chronic hepatitis B and stage I or II hepatocellular carcinoma (HCC) were enrolled. The clinical stage of tumor progression was determined according to the tumor-nodes-metastasis (TNM) classification system of the

International Union Against Cancer (Edition 6). Of these 15 cases, five cases had received lamivudine orally as an anti-HBV treatment (LAM 100 mg/day) for 2–3 years, and the remaining 10 cases received no interferon or nucleoside analogue drug treatment before surgery and sampling. None of these patients received anticancer therapy before surgery and sampling. Fresh nontumor hepatic tissues, with approximate dimensions of 1.5 × 1.5 cm each, were obtained from the patients undergoing surgical resection from a location at least 3 cm distant from the tumor site.

Control fresh hepatic tissues were obtained from 10 patients diagnosed with hepatolithiasis or hepatic angiomas who did not have an HBV infection. Control peripheral blood samples were obtained from 16 healthy donors. Individuals with concurrent autoimmune disease or HIV, hepatitis C virus, or syphilis infections were excluded (Table 1).

All patients were from the Department of Infectious Diseases and the Department of Hepatobiliary Surgery of the First and the Second Affiliated Hospitals of Chongqing Medical University. The patients were enrolled between January 1, 2012, and December 31, 2013. This study received ethical approval from the Medical Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University, and written informed consent was obtained from all participants.

Reagents

Allophycocyanin (APC), α -GalCer/human CD1d dimer, was purchased from Proimmune (USA). Anti-human V α 24-J α 18 TCR fluorescein isothiocyanate (FITC), anti-human CD279 (PD-1) FITC, phycoerythrin (PE)-conjugated anti-CD28 monoclonal antibody (mAb), anti-human IL-4 PE-Cy7, anti-human IFN- γ PerCP-Cy5.5, anti-human CD274 (PD-1; functional grade, purified), anti-human CD28 (functional grade, purified), and anti-human CD80 (B7-1; functional grade, purified) were purchased from eBioscience (San Diego, CA, USA). PE anti-human Tim-3 and anti-human Tim-3 (functional grade, purified) were purchased from Biolegend. The human IFN- γ enzyme-linked immunosorbent assay (ELISA) and human IL-4 ELISA were from R&D Systems (USA).

Cell isolation

Venous blood was sampled from healthy controls and patients. The venous blood was separated to obtain peripheral blood mononuclear cells (PBMCs) by Ficoll density gradient centrifugation. Hepatic mononuclear cells (MNCs) were isolated from the fresh nontumor hepatic tissues (at least 3 cm distant from the tumor site) from the patients who underwent surgical

Table 1 Baseline characteristics of CHB patients (n = 53) enrolled in the study, including the HBeAg (+) and HBeAb (+) patients

Index	Male/female	ALT (IU/ml)	AST (IU/ml)	HBV-DNA (log10)	TBil (mmol/l)
Patients	31/22	313.64 ± 74.29	278.62 ± 62.14	6.34 ± 0.45	20.1 ± 7.2

Data are presented as the mean ± SD. The upper limit of the normal AST and ALT levels is 40 IU/ml. HBV-DNA was quantified by RT-PCR, and the lower limit of quantification is 52 IU/ml (<10³ cp/ml). The normal range of serum TBil levels is 1.7–17.1 mmol/L
 ALT, alanine aminotransferase; AST, aspartate transaminase; TBil (TB), total bilirubin

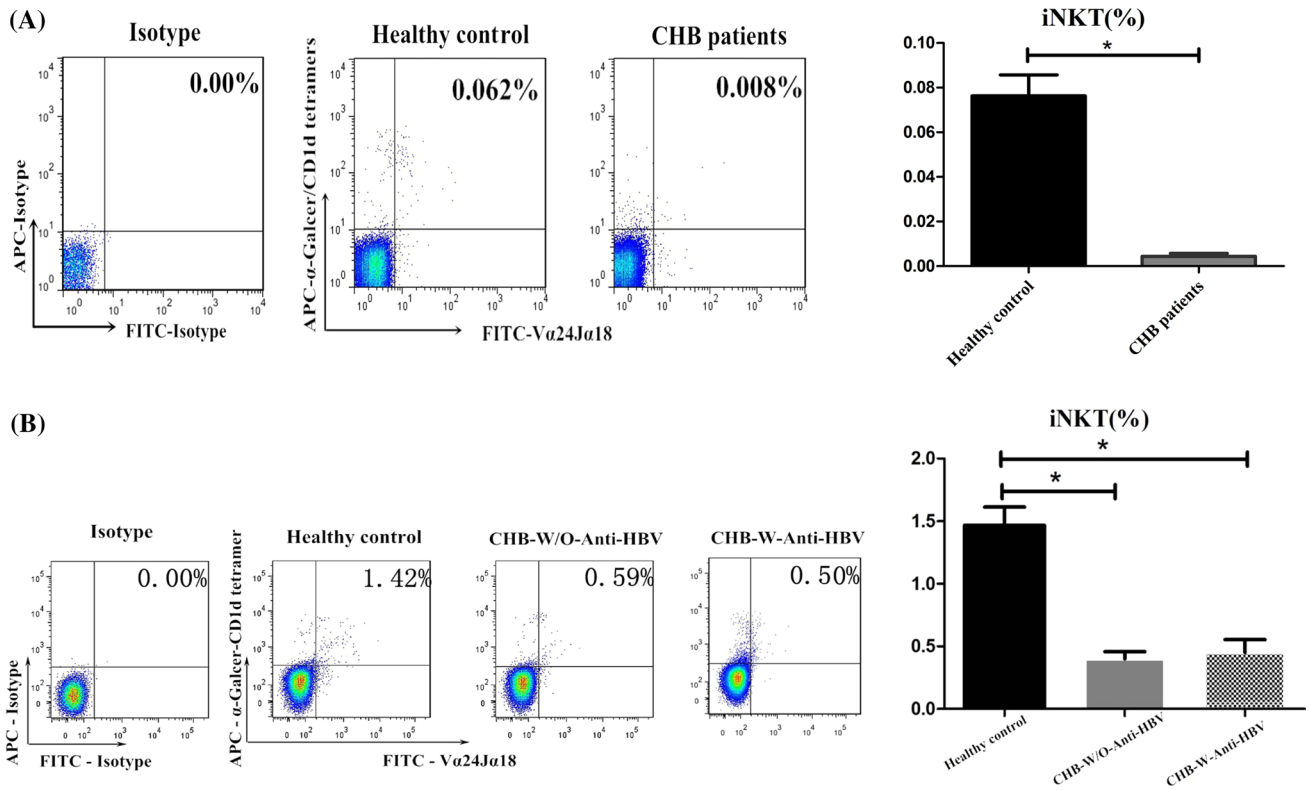


Fig. 1 iNKT cell frequencies in MNCs from different groups, as analyzed by flow cytometry. iNKT cells (shown in the right upper quadrant) were defined as APC α-GalCer/CD1d tetramers and Vα24-Jα18 TCR FITC T cells. (A) The numbers indicate the percentage of iNKT cells in the isolated PBMC population. (B) Graph comparing

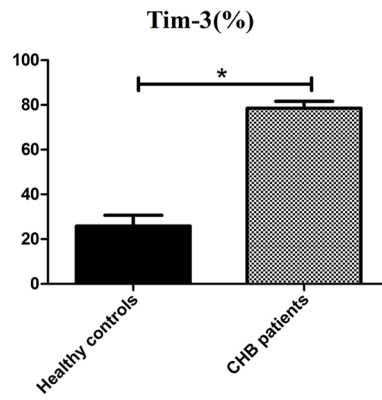
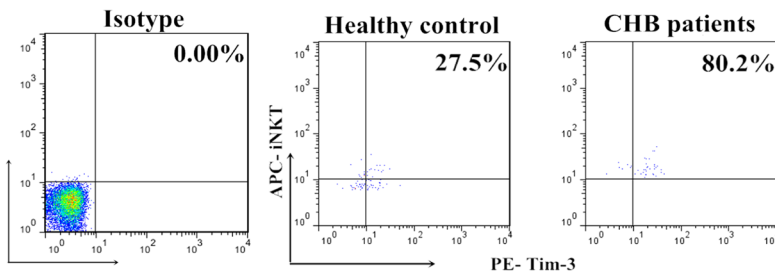
the frequency of iNKT cells from liver MNCs between CHB with (w) anti-HBV treatment, CHB without (w/o) anti-HBV treatment and normal controls. *, *p* < 0.05, as determined by ANOVA. MNCs, mononuclear cells; PBMCs, peripheral blood mononuclear cells

resection. Freshly obtained tissues were washed three times in sterile phosphate-buffered saline (PBS) before being cut into small pieces with scissors. The specimens were then homogenized in RPMI 1640 medium containing 10 % fetal calf serum (FCS) using a gentleMACSTM dissociator (Miltenyi Biotec, Germany). Dissociated cells were filtered through a 70-μm mesh and separated by Ficoll centrifugation. The fresh hepatic MNCs were washed two times in sterile PBS and resuspended in RPMI 1640 medium containing 10 % FCS and 1 % penicillin/streptomycin solution (penicillin, 10,000 units/ml; streptomycin, 10 mg/ml).

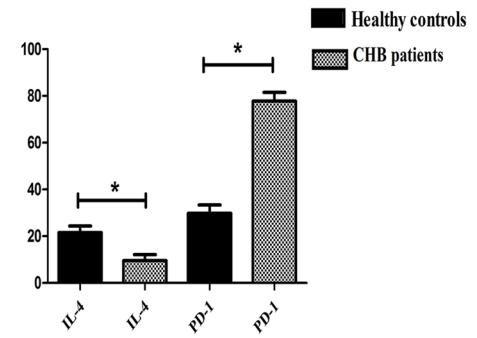
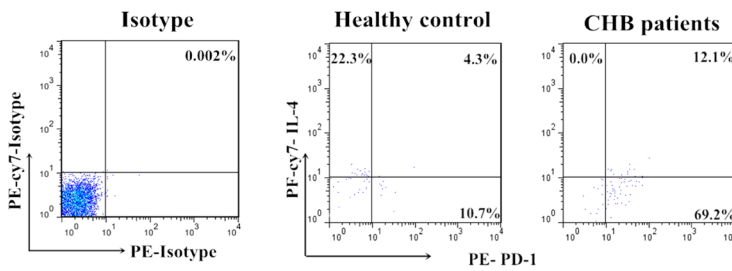
Flow cytometric analysis

The PD-1, Tim-3, and CD28 levels on iNKT cells were analyzed by flow cytometry (BD Bioscience). iNKT cells were assessed by Vα24 and α-galactosylceramide/CD1d tetramer markers. For intracellular cytokine detection in MNCs, cells were stained with mAbs against Vα24 and α-GalCer/CD1d tetramer markers, fixed with 4 % perfluoroalkoxy (PFA), and permeabilized with 0.1 % saponin/PBS. The fixed cells were then stained with PerCP-Cy5.5-anti-human IFN-γ and PE-Cy7-anti-human IL-4 and analyzed by flow cytometry as described above.

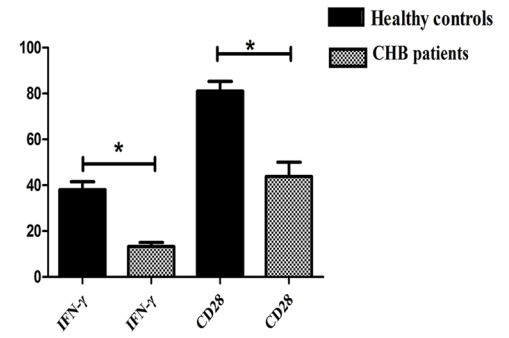
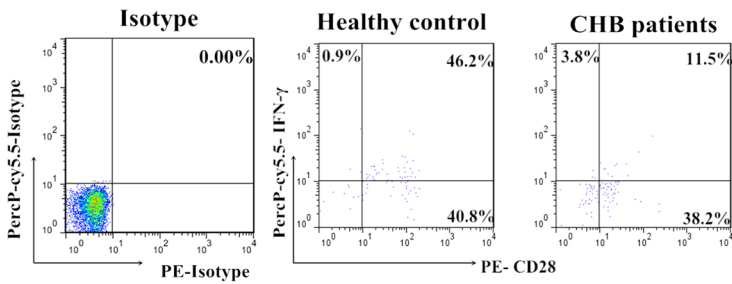
(A)



(B)



(C)



(D)

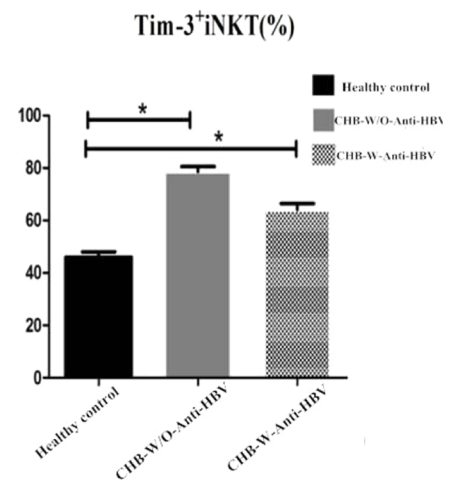
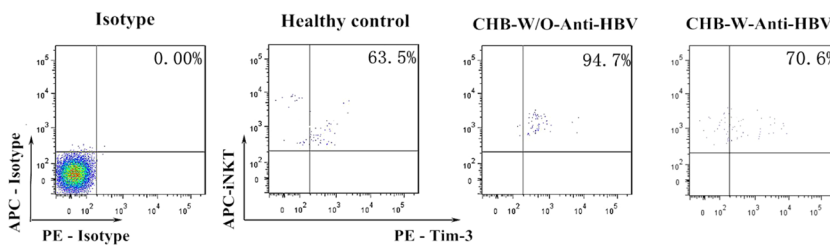


Fig. 2 Functional and phenotypic analysis of iNKT cells before cultivation. lymphocyte gating based on side-scatter (SSC) and forward-scatter (FSC) properties. Graph comparing the frequencies of (A/D) Tim-3⁺iNKT, (B/E) IL-4/PD-1⁺iNKT, and (C/F) IFN- γ /CD28⁺iNKT between CHB patients and normal controls. Panels A, B, and C show data from PBMCs, and panels D, E, and F show data from liver MNCs. Data are shown as the mean \pm SD. *, $p < 0.05$

Cell culture

MNCs were isolated as described above. Suspended enriched MNCs were cultured in RPMI-1640 medium (containing 15 % FCS, 100 U of penicillin–streptomycin per mL, 0.1 mM minimum essential medium (MEM) with nonessential amino acids, 1 mM sodium pyruvate, 5.5 mM 2-mercaptoethanol [2-ME], and 10 mM HEPES buffer) and α -GalCer (200 ng/mL) at a concentration of 1×10^6 cells/ml. The cells were cultured in flat-bottomed 96-well plates (0.2×10^6 per 96-well plate with 200 μ L/well). Briefly, these cells were divided into five groups and given different treatments. Group I was specified as the blank controls. In group II, anti-human Tim-3 (functional grade, purified; 0.5 μ g/well) was added to block the Tim-3 signaling pathway. Group III was given anti-human CD274 (B7-H1) (1 μ g/well) to block the PD-1 signaling pathways. Group IV was costimulated with anti-human CD28 (1 μ g/

well) and anti-human CD80 (B7.1) (1 μ g/well) mAb. Group V was given a mixture including anti-human Tim-3 mAb, anti-human CD274 mAb, and anti-human CD28/80 mAb. Cells were cultured in a 5 % CO₂ incubator for 72 h. The supernatant was collected and subjected to ELISA to measure the cytokines. Golgi PlugTM (1 μ L/mL) was added in the next step, and the incubation was continued for 4 h until the analysis.

ELISA analysis

The IL-4 and IFN- γ levels in the cell supernatants were measured with human-IL-4- and IFN- γ -specific ELISA kits (RayBiotech, Inc., Norcross, GA, USA), according to the manufacturers’ instructions. Briefly, a standard curve was established by adding 100 μ l of standard-concentration solutions to the wells of the pre-coated ELISA plate. Blank wells and sample wells were prepared with 100 μ l of the appropriate samples. The reaction plate was sealed with film and allowed to incubate at 37 $^{\circ}$ C for 90 minutes. After incubation, the liquid was discarded, and the plate was washed four times with washing buffer. To each well, 100 μ l of biotinylated antibody was added, and the plate was again incubated at 37 $^{\circ}$ C for 60 minutes. The plate was washed again, as before, and 100 μ l of HRP-conjugated streptavidin was added to each well. After incubation for

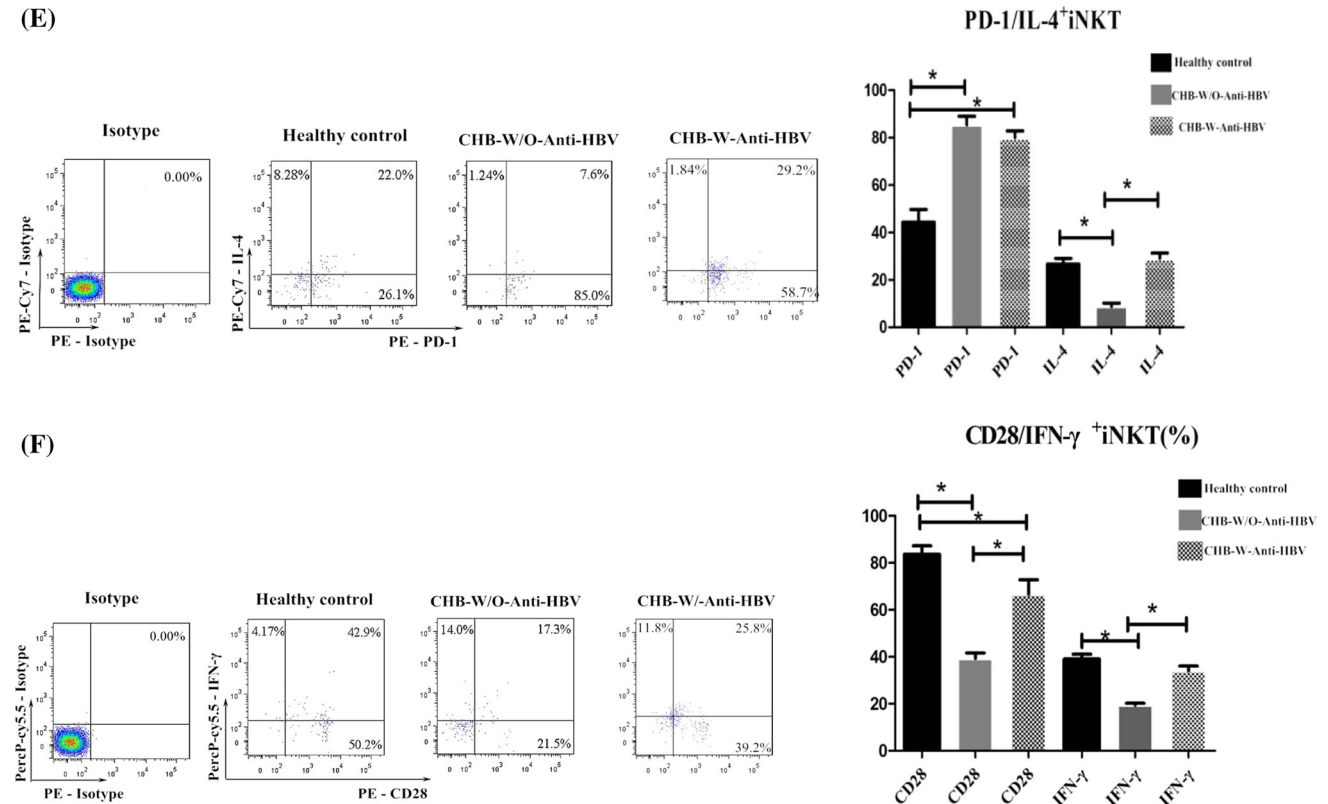


Fig. 2 continued

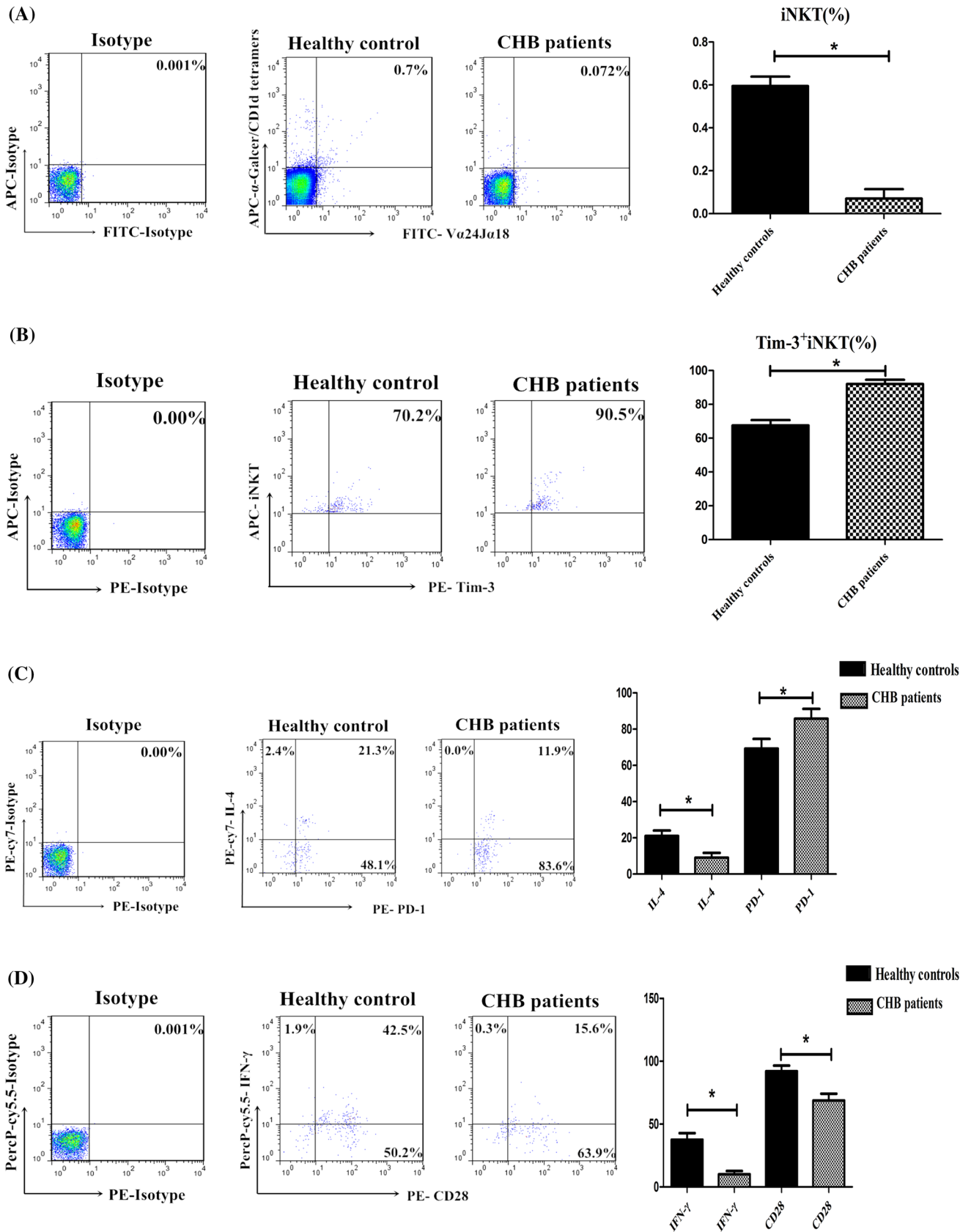


Fig. 3 *In vitro* expansion of human iNKT cells. Human PBMCs and liver MNCs were cultured in the presence of α -GalCer at a dose of 200 ng/ml for 72 h. (A/E) iNKT cells were identified by the presence of α -GalCer/CD1d tetramers with V α 24-J α 18 TCR. (B/F) Tim-3⁺iNKT, (C/G) IL-4/PD-1⁺iNKT, and (D/H) IFN- γ /CD28⁺iNKT were analyzed for their expression of the indicated cell surface markers. Panels A, B, C, and D show data from PBMCs, and panels E, F, G, and H show data from liver MNCs. Data are shown as the mean \pm SD, *, $p < 0.05$

30 minutes (37 °C), 100 μ l of TMB was added and allowed to react for 20 minutes in the dark at 37 °C. Finally, the OD₄₅₀ was determined on an ELx800NB plate reader (BioTek USA, Winooski, VT, USA). The sample concentrations were calculated using a standard curve. Data are shown as the mean \pm SD, and statistical significance was defined as $p < 0.05$.

Statistical analysis

All data are shown as the mean \pm standard deviation (SD). All statistical analysis was performed with the SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA). Multigroup comparisons were carried out by analysis of variance (ANOVA). The relationship between two quantitative indices was analyzed using Pearson's correlation coefficient. Statistical significance was set at $p < 0.05$.

Results

iNKT cells in CHB patients and healthy controls

The frequency of isolating iNKT cells was determined by flow cytometry by applying fluorescence-activated cell sorting (FACS). iNKT cells were defined by FACS analysis as cells that were positive for both α -GalCer/CD1d tetramers and V α 24-J α 18 TCR. The iNKT cell frequency was significantly lower in CHB patients than in the control groups (PBMCs, 0.055 \pm 0.012 % vs. 0.008 \pm 0.015 %; hepatic MNCs, 1.42 \pm 0.53 % vs. 0.53 \pm 0.25 % [CHB patients without clinical anti-HBV treatment], 1.42 \pm 0.53 % vs. 0.51 \pm 0.09 % [CHB patients with clinical anti-HBV treatment] $p < 0.05$). It is important to note that, due to the limited amount of some specimens, no significant difference in the percentage of iNKT cells was found between CHB W-anti-HBV and CHB W/O-anti-HBV ($p > 0.05$; Fig. 1).

Functional and phenotypic analysis of iNKT cells

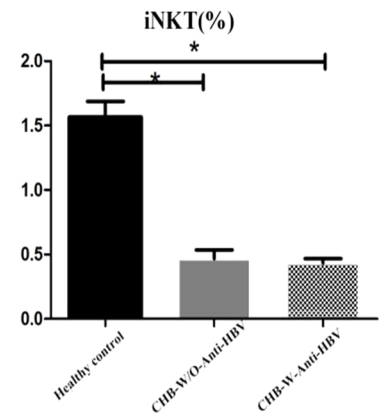
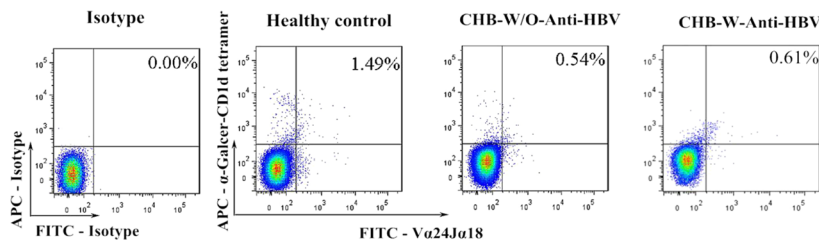
The expression levels of PD-1, Tim-3, and CD28 on iNKT cells were compared between patients with CHB and

healthy controls. In addition, comparisons were made between IL-4⁺ iNKT and IFN- γ ⁺ iNKT between the two groups. After lymphocyte gating based on forward and side scatter properties, the expression levels of Tim-3⁺ iNKT, IL-4/PD-1⁺ iNKT, and IFN- γ /CD28⁺ iNKT were analyzed for the indicated cell markers. Compared to iNKT cells from healthy controls, those from CHB patients expressed lower levels of CD28 (PBMCs, 46.2 \pm 10.6 % vs. 90.1 \pm 6.3 %; hepatic MNCs [CHB patients without anti-HBV treatment], 45.3 \pm 3.5 % vs. 87.9 \pm 9.2 %, [CHB patients with anti-HBV treatment], 67.7 \pm 7.2 % vs. 87.9 \pm 9.2 %; $p < 0.05$). In contrast, compared to iNKT cells from healthy controls, those from CHB patients expressed significantly higher levels of Tim-3 and PD-1 (PBMCs, 89.4 \pm 10.1 % vs. 23.3 \pm 12.6 % and 84.5 \pm 9.7 % vs. 35.5 \pm 5.5 %, respectively; hepatic MNCs [CHB patients without anti-HBV treatment], 89.3 \pm 8.1 % vs. 37.6 \pm 4.8 % and 89.1 \pm 7.7 % vs. 35.2 \pm 9.1 %, respectively; [CHB patients with anti-HBV treatment], 68.9 \pm 7.9 % vs. 37.6 \pm 4.8 % and 72.4 \pm 5.2 % vs. 35.2 \pm 9.1 %, respectively; $p < 0.05$). The IL-4 and IFN- γ levels and number of iNKT cells from CHB patients were much lower than those of the healthy controls ($p < 0.05$). It is noteworthy that for the CHB patients who received lamivudine as an anti-HBV treatment, the production of both IL-4 and IFN- γ by iNKT cells were increased, but the Tim-3⁺ expression was slightly reduced in comparison to patients without antiviral treatment (Fig. 2).

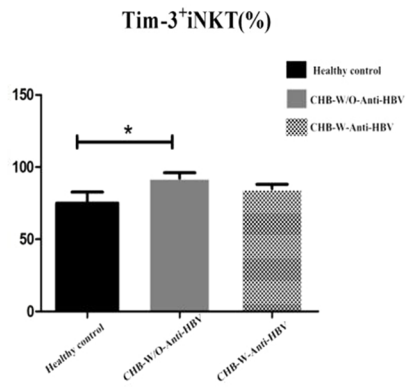
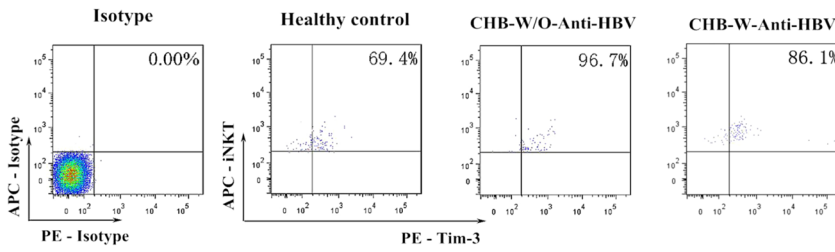
Tim-3/Tim-L3 and/or PD-1/PDL1 blockade and/or CD28/CD80 activation enhances the function of α -GalCer-activated iNKT cells *in vitro*

We designed a method for expanding iNKT cells from PBMCs and liver MNCs with α -GalCer *ex vivo*. These cells were divided into five groups and given different treatments, as described in "Materials and methods". The blank controls (group I) showed no change in the frequency of iNKT cells ($p > 0.05$), and the IL-4/IFN- γ ⁺ iNKT levels were not significantly different before and after cell culture ($p > 0.05$). The PD-1/Tim-3/CD28 expression levels of the iNKT cells after cell culture and after activation by α -GalCer were examined (Fig. 3). In the other four groups, the secretion of IL-4 and IFN- γ increased by different degrees, and IFN- γ secretion increased most obviously. It is important to note that the effect in group IV was significant (Tables 2 and 3). Finally, supernatant fluids were collected and subjected to ELISA to measure IL-4 and IFN- γ cytokines. The IL-4 and IFN- γ levels determined by ELISA and flow cytometry (FCM) technology were identical (Fig. 4)

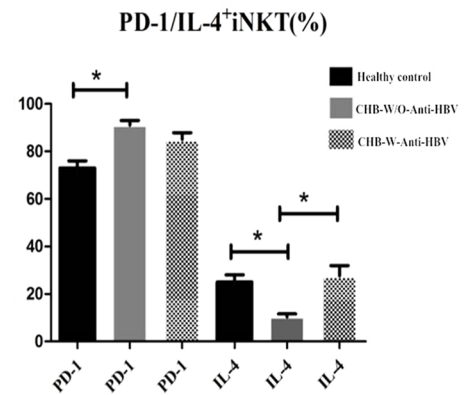
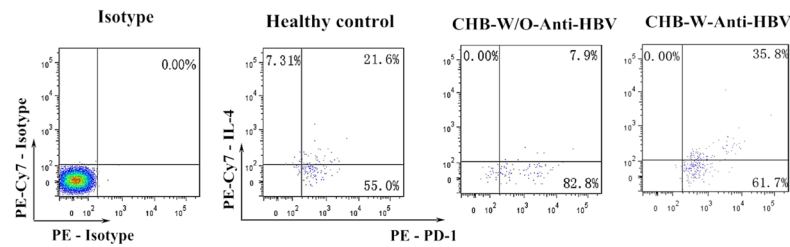
(E)



(F)



(G)



(H)

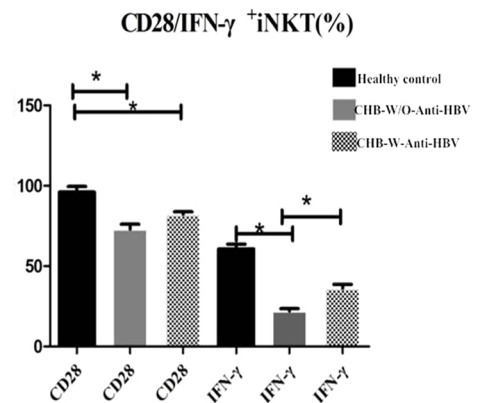
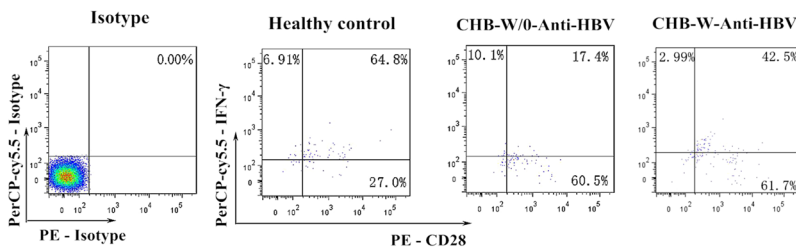


Fig. 3 continued

Table 2 PBMCs

Index	I	II	III	IV	V
IL-4 ⁺ iNKT (Healthy controls)	22.4 ± 3.8 %	42.5 ± 6.7 %	41.5 ± 7.5 %	60.1 ± 2.5 %	35.4 ± 8.2 %
IFN- γ ⁺ iNKT (Healthy controls)	40.0 ± 5.2 %	69.9 ± 8.2 %	65.4 ± 9.1 %	84.8 ± 6.3 %	56.9 ± 4.2 %
IL-4 ⁺ iNKT (CHB patients)	5.6 ± 4.7 %	20.9 ± 6.9 %	31.8 ± 2.5 %	53.7 ± 6.7 %	30.7 ± 6.3 %
IFN- γ ⁺ iNKT (CHB patients)	10.5 ± 5.7 %	39.1 ± 9.0 %	47.1 ± 7.6 %	70.5 ± 9.4 %	43.1 ± 5.8 %

I, blank controls; II, anti-Tim-3 (functional grade purified) to block the Tim-3 signaling pathway; III, anti-CD274 to block the PD-1 signaling pathways; IV, anti-CD28/CD80 McAb costimulated; V, the mixed group, including anti-Tim-3, anti-CD274, and anti-CD28/80 McAb. Cells were cultured in a 5 % CO₂ incubator for 72 h. IL-4⁺iNKT and IFN- γ ⁺iNKT were analyzed by flow cytometry for their expression of the indicated cell-surface marker

Table 3 Liver MNCs

Index	I	II	III	IV	V
IL-4 ⁺ iNKT (Healthy controls)	27.5 ± 6.5 %	38.5 ± 7.7 %	38.5 ± 8.5 %	46.1 ± 7.5 %	35.1 ± 6.2 %
IFN- γ ⁺ iNKT (Healthy controls)	45.8 ± 12.0 %	69.7 ± 13.2 %	66.5 ± 9.5 %	82.8 ± 8.3 %	54.9 ± 10.0 %
IL-4 ⁺ iNKT (CHB - W/O anti-HBV)	7.9 ± 2.1 %	21.0 ± 4.9 %	21.8 ± 4.5 %	43.5 ± 9.7 %	24.7 ± 5.7 %
IFN- γ ⁺ iNKT (CHB - W/O anti-HBV)	24.1 ± 4.6 %	59.3 ± 9.9 %	57.3 ± 7.9 %	80.5 ± 6.6 %	56.3 ± 9.1 %
IL-4 ⁺ iNKT (CHB - W anti-HBV)	33.8 ± 4.1 %	24.1 ± 6.2 %	26.5 ± 3.7 %	31.2 ± 7.2 %	23.6 ± 4.9 %
IFN- γ ⁺ iNKT (CHB - W anti-HBV)	44.5 ± 6.9 %	59.3 ± 9.9 %	57.7 ± 3.5 %	57.7 ± 3.5 %	57.7 ± 3.5 %

I, blank controls; II, anti-Tim-3 (functional grade, purified) to block the Tim-3 signaling pathway; III, anti-CD274 to block the PD-1 signaling pathways; IV, anti-CD28/CD80 McAb costimulated; V, the mixed group, including anti-Tim-3, anti-CD274, and anti-CD28/80 McAb. Cells were cultured in a 5 % CO₂ incubator for 72 h. IL-4⁺iNKT and IFN- γ ⁺iNKT were analyzed by flow cytometry for their expression of the indicated cell-surface marker

Discussion

Patients with chronic hepatitis B infections usually exhibit decreased Th1 responses and increased Th2 responses. iNKT cells have unique intermediary roles between the innate and acquired immune systems [28]. Both Tim-3 and PD-1 are important negative immune regulation factors that play essential roles in the immune response to HBV infection. However, whether the presence of Tim-3 or PD-1 molecules on iNKT cells plays a key role in HBV infection remains unclear. In this study, we demonstrated that the frequency of iNKT cells was significantly decreased in CHB patients, and high levels of Tim-3 and PD-1 inhibitory receptors were detected on these cells. However, the expression of the costimulant CD28 was much lower in CHB patients than in healthy controls. The iNKT cells had an impaired capability to secrete IL-4 and IFN- γ cytokines in CHB patients. Our study showed that the increase in Tim-3/PD-1 and the low level of CD28 were closely related to the immune impairment of iNKT cells. When the iNKT cells were activated with α -GalCer for 72 h, we found the Tim-3, PD-1, and CD28 expression levels increased, but there were no changes in IFN- γ and IL-4 production. These

experiments suggest that α -GalCer not only activates iNKT cells but also induces anergy.

Previous reports have shown that the Tim-3/galectin-9 pathway and Tim-3L, one of the ligands for Tim-3, modulate IFN- γ production. This pathway is particularly important for the immune response to virus infection [29, 30]. Research has shown that IFN- γ cytokines can inhibit HBV-DNA replication [31]. In addition, α -GalCer can induce iNKT cell anergy. In the present study, when the iNKTs from CHB patients were coactivated with α -GalCer/anti-Tim-3/anti-PDL1/anti-CD28/CD80 for 72 h, IFN- γ and IL-4 levels were significantly higher than they were before activation. In the anti-CD28/CD80 treatment group, the effect was particularly obvious. The data also showed that antiviral therapy with lamivudine resulted in a decrease in the expression of the inhibitory factors PD-1 and Tim-3, and an increase in the expression of the positive costimulatory factor CD28 on iNKTs, as compared to the levels before antiviral treatment. However, the PD-1 and Tim-3 expression levels were still higher than those of healthy controls, whereas the CD28 expression level remained relatively low. These results indicated that the Tim-3/Tim-3L, PD-1/PDL1, and CD28/CD80 pathways could regulate the functions of iNKT cells and have

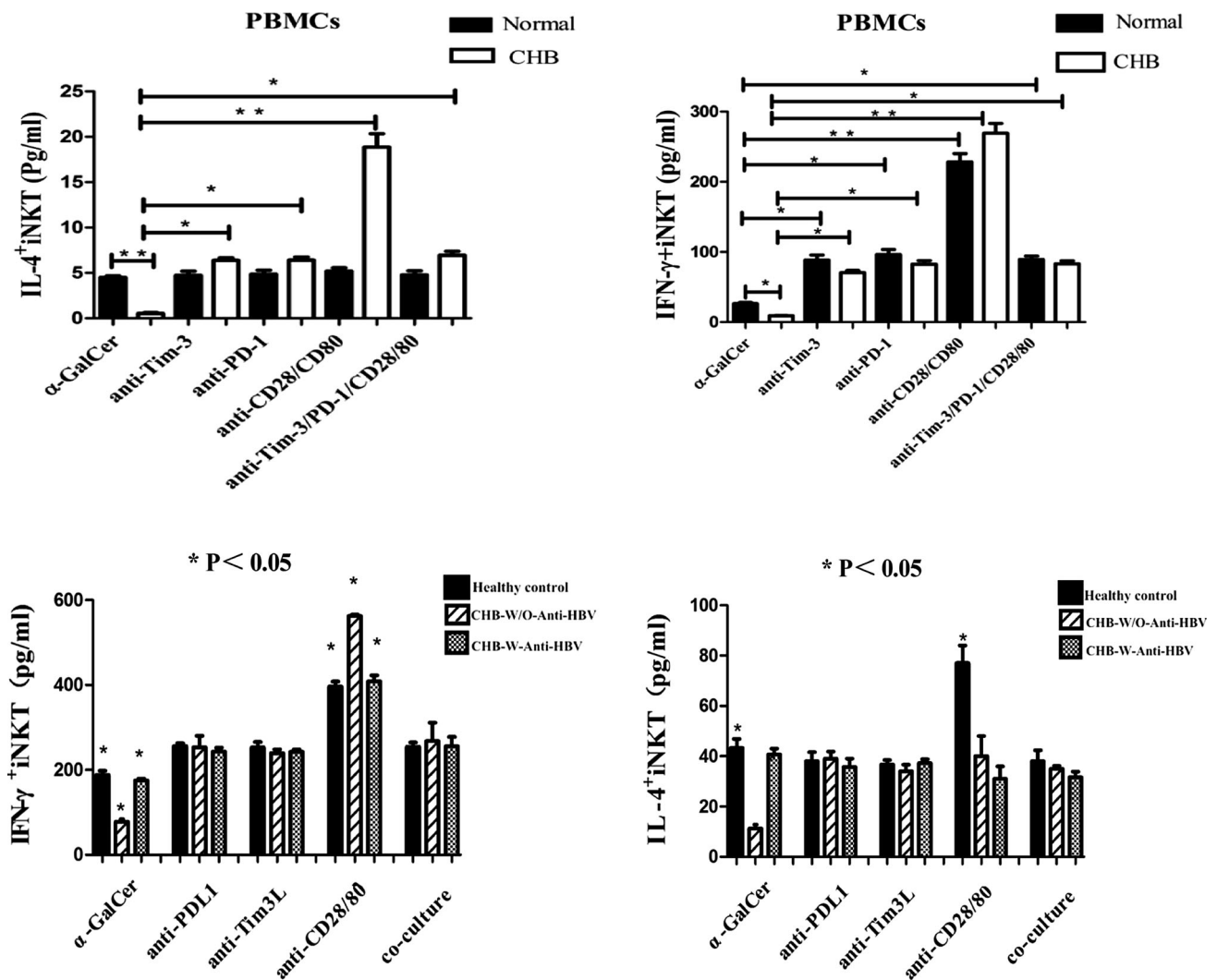


Fig. 4 Functional analysis of iNKT cells after culture. PBMCs and liver MNCs were isolated from different control groups and coactivated with α -GalCer/anti-Tim-3/anti-PDL1/anti-CD28/CD80

for 72 h. Concentrations of IL-4⁺iNKT and IFN- γ ⁺iNKT cytokines in the culture supernatants were measured by ELISA

some complementary effects. These experiments form an important theoretical basis for the clinical inhibition of HBV-DNA replication.

As mentioned above, using coactivated iNKT cells from different treatment groups, we collected supernatant fluid and performed ELISA to measure IFN- γ and IL-4 cytokines. The results were consistent with the FACS results. These results further showed that through our different processing methods, the functions of iNKT cells could be partially restored. The data also indicated that antiviral therapy with lamivudine could partly restore the anergic T cell activities in CHB patients (although perhaps transiently) when compared to patients without antiviral therapy. However, from a therapeutic perspective, the detailed mechanism still requires further study. Unfortunately, due

to clinical and ethical constraints, few liver tissue specimens were collected after lamivudine antiviral therapy, and we did not obtain reliable statistical significance. Thus, increasing the number of samples may be necessary to obtain reliable statistical results in future studies for validation.

In conclusion, we have highlighted for the first time the role of Tim-3 on iNKT cells during HBV infection. In addition, we partly restored the function of iNKT cells by blocking the Tim-3/Tim-3L and PD-1/PDL1 pathways and/or by exciting the CD28/CD80 pathway. Our data showed that the anergic T cell activities in CHB patients were closely related to the high Tim-3 and PD-1 expression levels and low CD28 expression levels. Upregulation of the CD28/CD80 signal in α -GalCer-activated iNKT cells is

more effective in increasing IFN- γ and IL-4 secretion by iNKT cells in CHB patients than the downregulation of the Tim-3/Tim-3L and PD-1/PDL1 signals.

Acknowledgments This work was supported by Grants from the National Key Technologies Research and Development Program of China during the 11th 5-year Plan Period (No. 2008ZX10002-006), the Program for Changjiang Scholars and Innovative Research of Teamin University (No. IRT0872), and the National Science Foundation of China (No. 81172804). We also thank Medjaden Bioscience Limited for assisting in the preparation of this manuscript.

References

- Lai CL, Ratziu V, Yuen MF, Poynard T (2003) Viral hepatitis B. *Lancet* 362:2089–2094
- Jung MC, Pape GR (2002) Immunology of hepatitis B infection. *Lancet Infect Dis* 2:43–50
- Tang TJ, Kwekkeboom J, Mancham S, Binda RS, de Man RA, Schalm SW et al (2005) Intrahepatic CD8 T-lymphocyte response is important for therapy-induced viral clearance in chronic hepatitis B infection. *J Hepatol* 43:45–52
- Boin C, Penna A, Bertolotti A, Lamona V, Rapti I, Missale G et al (2003) Transient restoration of anti-viral T cell responses induced by lamivudine therapy in chronic hepatitis B. *J Hepatol* 39:595–605
- Venkataswamy MM, Porcelli SA (2010) Lipid and glycolipid antigens of CD1d-restricted natural killer T cells. *Semin Immunol* 22:68–78
- Matsuda JL, Mallevaey T, Scott-Brown J, Gapin L (2008) CD1d-restricted iNKT cells, the ‘Swiss-Army knife’ of the immune system. *Curr Opin Immunol* 20:358–368
- Taniguchi M, Harada M, Kojo S et al (2003) The regulatory role of Valpha 14 NKT cells in innate and acquired immune response. *Annu Rev Immunol* 21:483–513
- Gumperz JE, Miyake S, Yamamura T et al (2002) Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* 195:625–636
- Kronenberg M, Gapin L (2002) The unconventional lifestyle of NKT cells. *Nat Rev Immunol* 2:557–568
- Schurich A, Pallett LJ, Lubowiecki M, Singh HD, Gill US et al (2013) The third signal cytokine IL-12 rescues the anti-viral function of exhausted HBV-specific CD8 T cells. *J PLoS Pathog* 9(3):e1003208
- Bayard F, Godon O, Nalpas B, Costentin C, Zhu R, Soussan P et al (2012) T-cell responses to hepatitis B splice-generated protein of hepatitis B virus and inflammatory cytokines/chemokines in chronic hepatitis B patients. ANRS study: HB EP 02 HBSP-FIBRO. *J Viral Hepat* 12:872–880
- Grimm D, Heeg M, Thimme R (2013) Hepatitis B virus: from immunobiology to immunotherapy. *J Clin Sci Lond* 124(2):77–85
- Ito H, Ando K, Ishikawa T et al (2008) Role of Valpha14 + NKT cells in the development of Hepatitis B virus-specific CTL: activation of Valpha14 + NKT cells promotes the breakage of CTL tolerance. *Int Immunol* 20(7):869–879
- Kakimi K, Guidotti LG, Koezuka Y, Chisari FV (2000) Natural killer T cell activation inhibits hepatitis B virus replication in vivo. *J Exp Med* 192(7):921–930
- Boni C, Fisiario P, Valdatta C et al (2007) Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* 81:4215–4225
- Trautmann L, Janbazian L, Chomont N et al (2006) Upregulation of PD-1 expression on HIV-specific CD8⁺ T cells leads to reversible immune dysfunction. *Nat Med* 12(10):1198–1202
- Ha SJ, Mueller SN, Wherry EJ, Freeman GJ, Ahmed R et al (2008) Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. *J Exp Med* 205(3):543–555
- Ju Y, Hou N, Zhang XN et al (2009) Blockade of Tim-3 pathway ameliorates interferon- γ production from hepatic CD8⁺ T cells in a mouse model of hepatitis B virus infection. *Cell Mol Immunol* 6(1):35–43
- Golden-Mason L, Palmer BE, Kassam N, Rosen HR et al (2009) Negative immune regulator Tim-3 is over expressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4⁺ and CD8⁺ T cells. *J Virol* 83(18):9122–9130
- Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H et al (2002) Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* 415:536–541
- Nebbia G, Peppas D, Schurich A, Khanna P, Sing HD et al (2012) Upregulation of the Tim-3/galectin-9 pathway of T cell exhaustion in chronic hepatitis B virus infection. *PLoS ONE* 7(10):e47648. doi:10.1371/journal.pone.0047648
- Oikawa T, Kamimura Y, Akiba H et al (2006) Preferential involvement of Tim-3 in the regulation of hepatic CD8⁺ T cells in murine acute graft-versus-host disease. *J Immunol* 177:4281–4287
- Day CL, Kaufmann DE, Kiepiela P, Brown JA et al (2006) PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 443:350–354
- Barber DL, Wherry EJ, Masopust D et al (2006) Restoring function in exhausted CD8⁺ T cells during chronic viral infection. *Nature* 439:682–687
- Feuth T, Arends JE, Franssen JH, Nanlohy NM, van Erpecum KJ et al (2013) Complementary role of HCV and HIV in T-cell activation and exhaustion in HIV/HCV coinfection. *PLoS ONE* 8(3):e59302. doi:10.1371/journal.pone.0059302
- Zhang E, Zhang X, Liu J, Wang B, Tian Y et al (2011) The expression of PD-1 ligands and their involvement in regulation of T cell functions in acute and chronic woodchuck hepatitis virus infection. *PLoS ONE* 6(10):e26196. doi:10.1371/journal.pone.0026196
- Boni C, Fisiario P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, Laccabue D, Zerbini A, Cavalli A, Missale G, Bertolotti A, Ferrari C (2007) Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* 81:4215–4225
- Fujii S, Shimizu K, Smith C et al (2003) Activation of natural killer T cells by α -galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* 198(2):267–279
- Sabatos CA, Chakravarti S, Cha E et al (2003) Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nat Immunol* 4:1102–1110
- Jones RB, Ndhlovu LC, Barbour JD et al (2008) Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med* 205:2763–2779
- McClary H, Koch R, Chisari FV, Guidotti LG (2000) Relative sensitivity of hepatitis B virus and other hepatotropic viruses to the antiviral effects of cytokines. *J Virol* 74(5):2255–2264