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ORIGINAL ARTICLE

Effects of antiviral therapy with Telbivudine on peripheral iNKT cells in HBeAg(+) chronic hepatitis B patients

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Abstract iNKT cells are relatively abundant in the liver, which suggests that they may play important roles in the clearance of invading foreign pathogens such as hepatitis B virus. In this study, we investigated the frequencies, functions and PD-1 expression of peripheral iNKT cells from HBeAg-positive chronic hepatitis B (CHB) patients during antiviral treatment with Telbivudine. Results demonstrated that as compared with the healthy donors, the peripheral iNKT cells from these patients existed at lower frequencies, displayed impaired capabilities to produce IFN-γ and expressed higher PD-1. Antiviral treatment with Telbivudine significantly increased IFN-γ production by iNKT cells, which may be associated with the decreased PD-1 expression as manifested by blocking experiments. Our study suggested that iNKT cells played an important role in the chronicity of HBV infection and antiviral therapy with Telbivudine could restore at least in part the impaired host immune response in the HBeAg-positive CHB patients.

Keywords Hepatitis · Invariant NKT · PD-1 expression · Hepatitis B virus (HBV) · Telbivudine

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Introduction

As a distinct subtype of lymphocytes, natural killer T (NKT) cells share some common features and functions of both classical T cells and natural killer cells [1]. Upon recognition of glycolipid antigens presented by CD1d molecules, NKT cells rapidly become activated and secrete large amounts of interferon-γ and interleukin-4 [2], playing important roles in infectious diseases, tumor surveillance, autoimmune diseases and establishing peripheral tolerance [3]. Generally, NKT cells can be divided into three categories based on their TCR repertoire, CD1d dependence, α-Galcer reactivity and NK1.1 expression [1]. Here, we focused on the widely studied NKT subset named invariant NKT (iNKT) or classical NKT cells, which express a semi-invariant TCR, consisting of the homologous invariant Vα24-JαQ and Vα14-Jα18 rearrangement in human and mice, respectively, paired with junctionally diverse Vβ11, Vβ8.2, Vβ7 and Vβ2 TCR chains in human and mice, respectively [4]. iNKT cells are restricted by the non-classical MHC classI-like molecule CD1d and can react with glycolipid α-Galcer binding CD1d [5].

As a major worldwide public health problem, hepatitis B virus (HBV) infection is also highly endemic in China, with about 100 million asymptomatic carriers and more than 20 million patients suffering from chronic HBV infection. Substantial advances have been made in the treatment of chronic hepatitis B in the past decades. Approved antiviral agents including nucleoside/nucleotide analogues lamivudine, adefovir dipivoxil, Telbivudine and entecavir, as well as the immune modulator interferon are now clinically prevalent in China. Telbivudine is a synthetic thymidine nucleoside analogue, which has been widely used in the treatment of HBV infection in China during recent 4 years. Large clinical trials have shown

potent suppression of viral replication with Telbivudine, along with a good safety profile and superiority over the other nucleoside/nucleotide analogues [6, 7].

iNKT cells are relatively abundant in the liver, representing approximately 25–40% or 12% of intrahepatic lymphocytes in mice and human, respectively [8, 9]. This characteristic of distribution suggests that iNKT cells may play a critical role in defense against foreign pathogens such as HBV. It has been reported that intrahepatic NKT cells activated by α-Galcer inhibited HBV replication noncytopathically in the liver of transgenic mice, and this effect was mediated by antiviral cytokines such as IFN-γ directly produced by activated NKT cells and/or by other cytokine-producing inflammatory cells that were recruited into the liver [10]. It is universally acknowledged that chronicity of HBV infection is characterized by a weak immune response, especially an impaired CTL response to the virus. Furthermore, according to de Lalla et al. [11], iNKT cells increased in chronically infected livers and underwent a substantial modification in their effects or functions, consisting in the production of the type 2 profibrotic IL-4 and IL-13 cytokines, which characterizes the progression of hepatic fibrosis to cirrhosis. Yet, little research has been done so far on the frequency and function of iNKT cells in chronic hepatitis B patients during the course of antiviral therapy.

Materials and Methods

Patients

Twenty-nine compensated chronic hepatitis B patients (aged 20–47 years, mean 32.03 years) from an open-label, single-arm, multicenter, 1-year phase IV clinical trial of Telbivudine antiviral therapy were included in our study (Table 1). All patients were seropositive for hepatitis B surface antigen (HBsAg) and HBeAg and had elevated serum levels (2–10 times above upper limit of normal) of alanine aminotransferase (ALT) and positive serum HBV loads (above 2×10^5 IU/ml by real-time PCR). All patients were negative for markers of human immunodeficiency virus, hepatitis C virus and hepatitis D virus infections. Telbivudine (Sebivo, Novartis) was orally administered at a dose of 600 mg per day, and heparinized venous blood was taken at four study visits: baseline and treatment weeks 12, 24 and 52. As controls, 17 healthy donors (aged 18–45 years, mean 28.17 years) were included in this study. The study protocols were approved by the Ethical Committee of Chongqing Medical University the Second Affiliated Hospital, and a written informed consent was obtained from all participants.

Agents and antibodies

Anti-TCR Vα24-FITC and anti-TCR Vβ11-PE were purchased from BECKMAN COULTER. Anti-PD-1(CD279)-APC, anti-IFN-γ-PE-Cy7, anti-IL-4-APC, mAbs against human PD-L1 and PD-L2, FACS lysing solution and α-galactosylceramide (α-Galcer) were purchased from eBioscience. Anti-IL-13-APC and Fixation/Permeabilization Solution Kit with BD GolgiStop were purchased from BD Pharmingen. Phorbol 12-myristate 13-acetate (PMA) and Ionomycin calcium salt were purchased from Alexis.

Flow cytometry

Anti-TCR Vα24 and Vβ11 were used to identify the iNKT cell population. For determination of iNKT frequency in peripheral blood, 100-μl heparinized venous blood was incubated at room temperature (RT) with anti-TCR Vα24 and Vβ11 for 30 min in the dark, followed by adding 2–3 ml FACS lysing solution and incubated at RT for another 15 min. The samples were then washed with PBS and suspended in 1% paraformaldehyde prior to FCM analysis. For determination of PD-1 expression on iNKT cells, anti-PD-1 was added to the blood samples. For analysis of intracellular cytokines produced by iNKT cells, 2×10^6 PBMCs were suspended in 1 ml RMPI 1640 with 10%FBS, then activated with 20 μl PMA and 20 μl Ionomycin in the presence of 0.7 μl Monensin at 37°C in a 5% CO_2 incubator for 6 h. The final concentrations of PMA and Ionomycin were 20 ng/ml and 1 μg/ml, respectively. The harvested cells were stained with anti-TCR Vα24 and Vβ11 to identify NKT cells and then were fixed, permeabilized and incubated with anti-IFN-γ, IL-4 and IL-13 for intracellular staining. After being washed and resuspended in 500 μl PBS/2% paraformaldehyde solutions, cells were

Table 1 Baseline characteristics of HBeAg(+) chronic hepatitis B patients ($n = 29$) enrolled in the current study ($\bar{x} \pm s$)

Index	Male/female	ALT (IU/ml)	HBV-DNA ($\log_{10}$IU/ml)	TBil (mmol/l)	ALB (g/l)
Patients	18/11	158.45 ± 66.30	6.85 ± 0.99	18.1 ± 5.6	44.9 ± 2.3

The upper limit of normal ALT level is 40 IU/ml; Serum HBV loads were quantified by a sensitive real-time–polymerase chain reaction (RT–PCR) technique with a lower limit of quantification of 52 IU/ml; The normal range of serum TBil and ALB levels are 1.7–17.1 mmol/L and 40–55 g/L, respectively. *ALT* alanine aminotransferase, *TBil* total bilirubin, *ALB* albumin

analyzed by flow cytometry on a FACS Calibur flow cytometer (BD Biosciences).

Blockade of the PD-1/PD-L pathway

To block the PD-1/PD-L pathway, freshly isolated PBMCs were plated in round bottomed 96-well plates at 2×10^6 cells per well in complete RPMI-1640 culture medium containing 10%FCS, penicillin–streptomycin (100 U/ml), 10 mM HEPES buffer solution, 0.1 mM MEM non-essential amino acids, 1 mM sodium pyruvate and 5.5 mM 2-mercaptoethanol, in the presence of α-Galcer (100 ng/ml) or vehicle. To block the interaction between PD-1 and its ligands, mAbs against human PD-L1 (final concentration 5 μg/ml) and PD-L2 (final concentration 5 μg/ml) were added to the cultures. After in vitro culture for 48 h, supernatants were collected and analyzed for intracellular cytokines production of iNKT cells by flow cytometry.

Statistical analysis

All data are shown as mean ± SD. Statistical significance between multiple groups was determined by application of ANOVA. All statistical analyses were performed using GraphPad Prism 5 software.

Results

Clinical outcomes

After antiviral treatment with Telbivudine for 1 year, 8 out of 29(27.6%) HBeAg-positive chronic hepatitis B patients reached HBeAg/HBeAb seroconvertion. No patient achieved HBsAg/HBsAb seroconvertion. Serum HBV loads significantly reduced in 27(93%) patients as early as at treatment week 12. By treatment week 52, all patients had undetectable serum HBV loads, and serum ALT returned to normal in all but one patient (data not shown).

iNKT cell frequencies in peripheral blood from healthy donors and HBeAg-positive chronic hepatitis B patients

We determined first the iNKT cell frequencies in peripheral blood from healthy donors and the chronic hepatitis B patients prior to antiviral treatment. iNKT cells were defined as $V\alpha24^+$ $V\beta11^+$ double-positive cells in the lymphocytes region. Our study revealed a relatively low frequency of peripheral iNKT cells, ranging from 0.01 to 0.4% of total lymphocytes in the blood, with a mean of 0.21% in healthy donors and a lower mean of 0.11% in chronic hepatitis B patients before Telbivudine treatment. A statistically significant difference ($P < 0.05$) was observed in the iNKT cell frequencies in peripheral blood between the healthy donors and the CHB patients (shown in Fig. 1).

Intracellular cytokines production by iNKT cells in healthy donors and HBeAg-positive chronic hepatitis B patients

As an important component of the innate immune system, iNKT cells can release remarkable quantities of immunomodulatory cytokines, such as IFN-γ, IL-4 and IL-17, within minutes of stimulation of their TCR via CD1d [12, 13], and are considered to play a pivotal role in communication between the innate and the acquired immunity [14]. Several studies have revealed an impaired capability of iNKT cells to proliferate and to produce IFN-γ during viral infection such as HIV infection [15]. Here, we tried to investigate the functions of iNKT cells during HBV infection. Freshly isolated PBMCs were immediately stimulated with PMA and Ionomycin in the presence of Monensin and analyzed by flow cytometry for intracellular IFN-γ, IL-4 and IL-13 production by iNKT cells. Results demonstrated that the mean percentage of IFN-γ-positive iNKT cells in healthy donors was 41.84%, while in CHB patients before antiviral treatment, the percentage significantly decreased to 23.51% or so, with a statistical difference of $P < 0.05$ between the two groups. Meanwhile, we observed low IL-4 and IL-13 productions by iNKT cells in both groups, but no statistically significant difference existed between (shown in Fig. 2).

Effects of antiviral therapy with Telbivudine on peripheral iNKT cells from HBeAg-positive chronic hepatitis B patients

In this research, 27 out of 29 HBeAg-positive CHB patients receiving antiviral treatment with Telbivudine achieved early virological and biochemical responses, which maintained to treatment week 52. Here, we tried to investigate whether these clinical outcomes resulted from Telbivudine therapy were related to the immunomodulatory effects of Telbivudine or not. Results showed that during antiviral therapy the frequencies of iNKT cells in peripheral blood increased gradually (shown in Fig. 1). A similar increase in IFN-γ production by iNKT cells after in vitro activation was also observed, with mean percentages of IFN-γ-positive iNKT cells being 28.82, 35.66 and 37.47% at treatment weeks 12, 24 and 52, respectively (shown in Fig. 2). However, no significant changes of IL-4 and IL-13 productions by iNKT cells were observed during therapy (shown in Fig. 2).

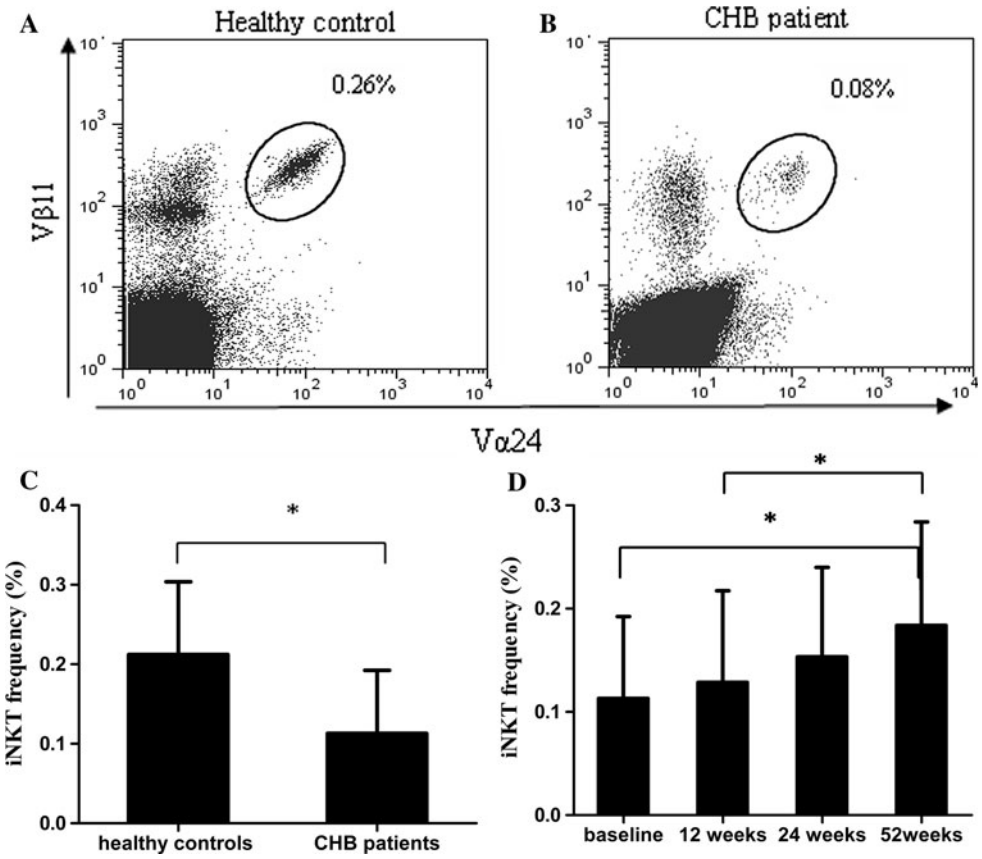

Fig. 1 Frequency of iNKT cells among PBMCs. iNKT cells were defined as Vα24$^+$ Vβ11$^+$ double-positive cells. **a** Representative *dot plot* of frequency of iNKT cells among total PBMCs from healthy controls ($n = 17$); **b** Representative *dot plot* of frequency of iNKT cells from chronic hepatitis B patients ($n = 29$); **c** *Graph* comparing the frequency of iNKT cells between healthy controls and CHB patients. **d** *Graph* comparing the frequency of iNKT cells from CHB patients among different time points during Telbivudine therapy. *Data* are shown as mean ± SD. *$P < 0.05$ as determined by ANOVA. *CHB* chronic hepatitis B patients, *PBMCs* peripheral blood mononuclear cells

PD-1 expressions on iNKT cells significantly decreased during Telbivudine therapy

Programmed death 1 (PD-1, also called CD279), a member of CD28 family of costimulatory molecules, is expressed on activated T cells, natural killer T cells, B cells and macrophages [16, 17]. Engagement of PD-1 and its ligands PD-L1 (B7-H1) or PD-L2 (B7-DC) delivers a negative signal to the activation of conventional T cells [18]. And recently, several scientists have reported a critical role of PD-1/PD-L pathway in the induction of anergy in iNKT cells [19, 20].

In this study, we found that PD-1 expression on iNKT cells was relatively lower in healthy donors, while in CHB patients before antiviral treatment, the expression of this receptor on iNKT cells was significantly elevated (shown in Fig. 3). During Telbivudine therapy, the PD-1 expression on iNKT cells decreased significantly as early as at treatment week 12 and reached almost the same level as that of healthy controls by treatment week 52 (shown in Fig. 3). We also observed that as compared to the patients remaining HBeAg positive, those acquired HBeAg seroconversion (in 8/29 patients after 1-year therapy) displayed a more evident decrease in PD-1 expression on iNKT cells (data not shown). These data indicated a positive role of antiviral therapy with Telbivudine in decreasing PD-1 expression on iNKT cells in CHB patients.

Blockade of PD-1/PD-L pathway enhanced IFN-γ production of iNKT cells from HBeAg-positive chronic hepatitis B patients

As shown in the above experiments, iNKT cells from HBeAg-positive CHB patients are characterized by impaired intracellular IFN-γ production and elevated PD-1 expression. Furthermore, antiviral therapy with Telbivudine assisted in promoting IFN-γ production by iNKT cells and suppressing PD-1 expression on iNKT cells from CHB patients. Here, we tried to investigate whether PD-1 expression level was correlated with IFN-γ production by iNKT cells. For this purpose, we tested the effects of PD-1/PD-L blockade on IFN-γ production of iNKT cells from CHB patients ($n = 5$). Results showed that when mAbs against human PD-L1 and PD-L2 were used to block their interaction with PD-1, intracellular IFN-γ production by iNKT cells was improved (shown in Fig. 4). However, application of mAbs against human PD-L2 alone, which blocks only the PD-1/PD-L2 pathway, had almost the same effects on IFN-γ production of iNKT cells as the application of mAbs against human PD-L1 alone that blocked the PD-1/PD-L1 pathway (shown in Fig. 4). This finding somewhat deviated from the result of another research [21], and perhaps, more studies are needed with larger sample size to certify this finding.

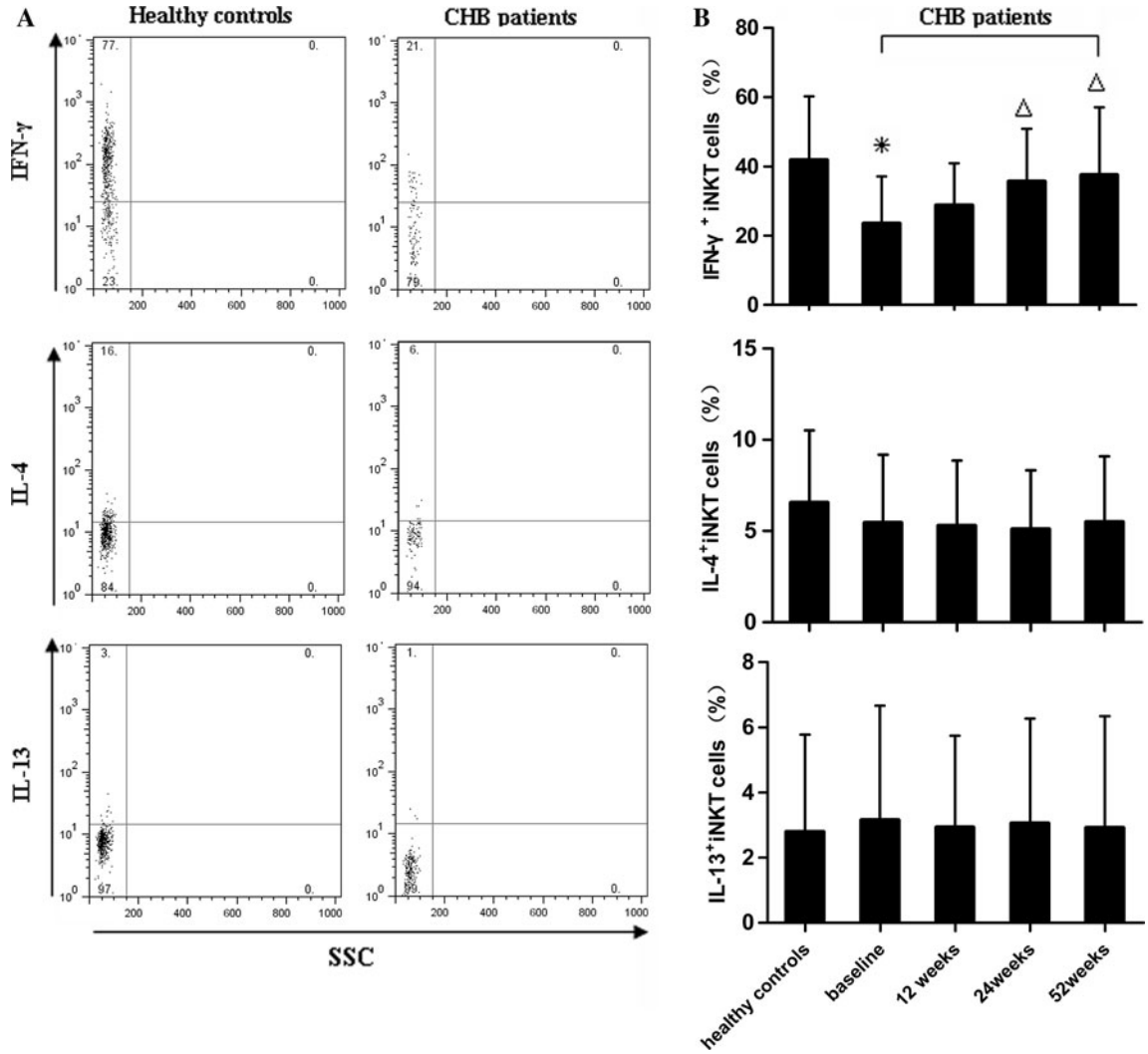

Fig. 2 Intracellular cytokines production of iNKT cells in peripheral blood from healthy controls ($n = 17$) and CHB patients ($n = 29$) during antiviral therapy with Telbivudine. **a** Representative *dot plots* of intracellular IFN-γ, IL-4 and IL-13 production of iNKT cells were determined after gating on Vα24$^+$ Vβ11$^+$ cells. **b** Graphs comparing intracellular IFN-γ, IL-4 and IL-13 production of iNKT cells from healthy controls and CHB patients during different time points (baseline, 12 weeks, 24 weeks, 52 weeks) of Telbivudine therapy. *$P < 0.05$ as compared with healthy controls, $\Delta P < 0.05$ as compared with CHB patients at baseline. No statistically significant difference of intracellular IL-4 and IL-13 production of iNKT cells was observed among healthy controls and CHB patients during the four time points of Telbivudine therapy. *CHB* chronic hepatitis B patients

Discussion

iNKT cells constitute the first line of immune defense against invading pathogens. The role of iNKT cells in suppressing viral replication has already been confirmed in HBV transgenic mice. During HBV infection, iNKT can be activated upon specific recognition of glycolipids and phospholipids presented by CD1d via TCR. These glycolipids and phospholipids derive from cellular membranes of HBV virions and subviral particles. Additionally, iNKT cells can be activated indirectly by virus-induced cytokines. After being activated, iNKT cells exert their antiviral effect through secreting cytokines such as IFN-γ and IFN-α/β or recruiting other lymphocytes to the liver [8].

It is now universally accepted that chronic HBV infection is characterized by a week immune response, especially impaired HBV-specific CTL responses to HBV [22]. Considering the relative abundance of iNKT cells in the liver and their important roles in suppressing HBV replication, we tried to investigate the frequencies and functions of iNKT cells from HBeAg-positive CHB patients in this study. Results demonstrated that the frequency of iNKT in peripheral blood of the HBeAg-positive CHB patients significantly decreased as compared to that in healthy controls. Possible explanations might be the down-regulation of TCRs as a result of activation, apoptosis of iNKT cells or compartmentalization of iNKT cells into the liver [23]. Meanwhile, we observed an impaired capability of

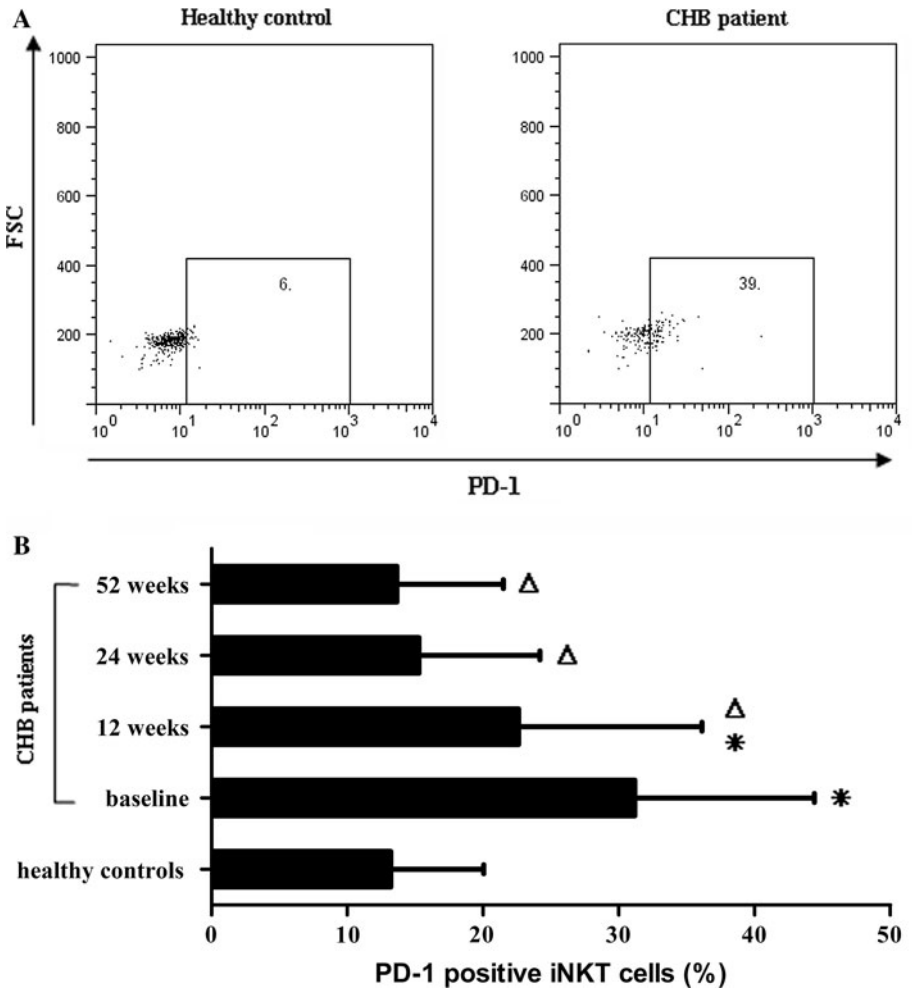

Fig. 3 PD-1 expression of iNKT cells from healthy controls ($n = 17$) and CHB patients ($n = 29$) during Telbivudine therapy. PD-1-positive iNKT cells were determined after gating on iNKT cells. **a** Representative *dot plots* of PD-1 expression of iNKT cells from healthy control and chronic hepatitis B patient; **b** *Graph* comparing PD-1 expression level of iNKT cells from healthy controls and CHB patients during different time points (baseline, 12 weeks, 24 weeks, 52 weeks) of Telbivudine therapy. $*P < 0.05$ as compared with healthy controls, $\Delta P < 0.05$ as compared with CHB patients at baseline. *PD-1* Programmed death 1, *CHB* chronic hepatitis B patients

peripheral iNKT cells to secrete IFN-γ after in vitro stimulation in CHB patients, indicating that iNKT cells in these patients may also be in a state of hyporesponsiveness, as in case of $CD8^+$ T cells.

Viral infection is a two-way process: the host immune system, including the innate immune system and the adaptive immune system, is activated to clear invading viruses. Conversely, the viruses may develop mechanisms for evasion and modification of the host immune responses, which could result in persistent infections. According to reports [24], the PD-1/PD-L system may serve to this immune modification process. Recent studies on antigen-specific $CD8^+$ T cells from the peripheral blood of patients with HBV infection have indicated that the PD-1/PD-L pathway played an important role in the function deficits of responding T cells, and in vitro blockade of PD-1/PD-L pathway has been shown to improve the functionality of the impaired HBV-specific $CD8^+$ T cells in the peripheral blood [25–27]. In case of iNKT cells, recent researches indicated that the PD-1/PD-L pathway might play a critical role in the induction of anergy in iNKT cells [19, 20]. Research by Moll et al. [15] indicated a severely impaired capability of the remaining iNKT cells to proliferate and to produce IFN-γ in response to glycolipid antigen in chronic HIV-infected patients. Furthermore, these iNKT cells had an increased expression of the inhibitory receptor PD-1. In this study, we found that PD-1 expression on iNKT cells from HBeAg-positive CHB patients significantly increased as compared with that from healthy donors, possibly owing to persistent antigen stimulation, or a selfprotection of the host against severe injury induced by strong immune responses in light of a low PD-1 expression on HBV-specific $CD8^+$ T cells as in acute liver failure patients [23, 28]. But whether there exists a low PD-1 expression on iNKT cells in acute liver failure patients still needs to be confirmed.

Recent years have witnessed great advances in the antiviral therapy for chronic hepatitis B patients. Available antiviral drugs now include immunomodulatory agents (interferon-α) and oral nucleos(t)ide analogues (lamivudine, adefovir dipivoxil, entecavir and Telbivudine, etc.). More and more clinical evidences have shown that administration of these drugs could result in HBV-DNA reduction, liver functions normalization and hepatohistological improvements [29–31]. Telbivudine, the fourth FDA-approved oral

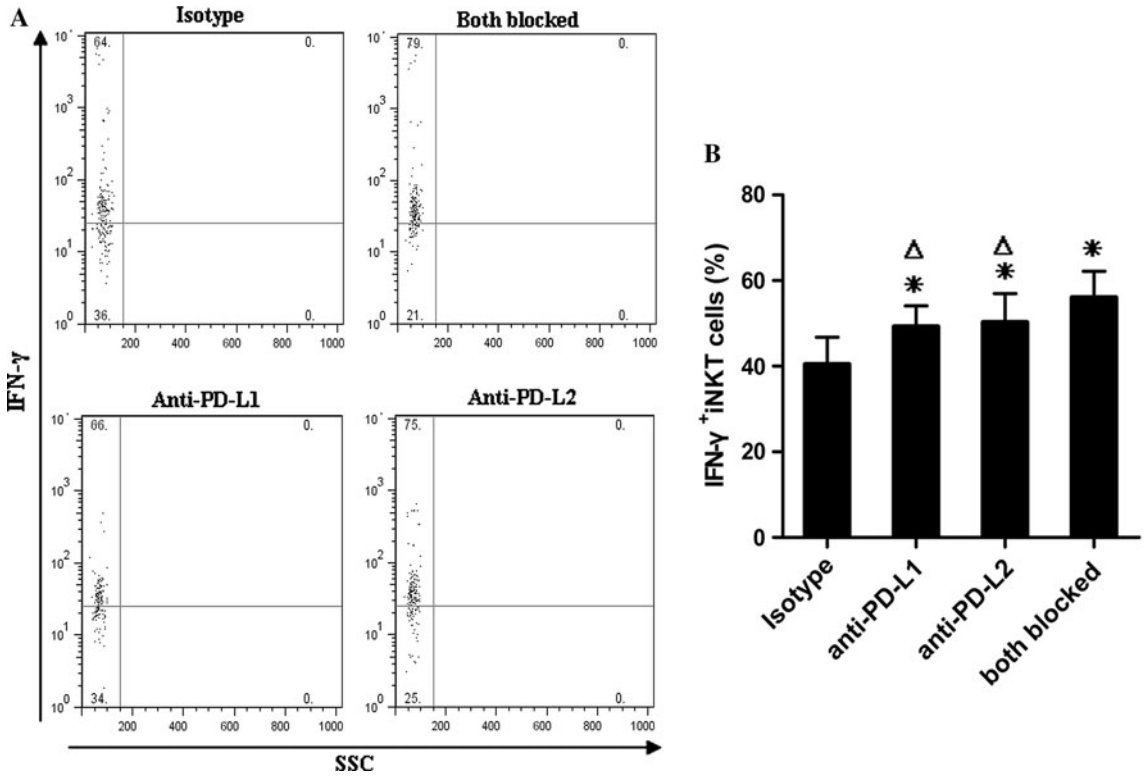

Fig. 4 Blockade of PD-1/PD-L pathway enhanced IFN-γ production of iNKT cells from CHB patients ($n = 5$). **a** Representative *dot plots* of intracellular IFN-γ production of iNKT cells from CHB patients in the presence of isotype control, anti-PD-L1 or/and anti-PD-L2 antibodies. **b** *Graph* comparing intracellular the IFN-γ production of iNKT cells from CHB patients in the presence of isotype control, anti-PD-L1 or/and anti-PD-L2 antibodies. $^*P < 0.05$ as compared with "isotype control"; $\Delta P < 0.05$ as compared with "both blocked" group

antiviral drug, is highly potent for viral suppression for HBV. And notably, Telbivudine produces higher HBeAg seroconversion rate in patients with baseline ALT ≥ 2 times upper limits (27.6% patients reach HBeAg seroconversion after 1-year therapy with Telbivudine in this study), which was thought to be related to the immunomodulatory effects of Telbivudine [32], although may be indirect. In this study, we tried to investigate the effects of antiviral treatment with Telbivudine on the iNKT cell frequency in peripheral blood and the cytokines production from the HBeAg-positive CHB patients. Our results revealed that during the four study time points of antiviral treatment, iNKT cell frequency in peripheral blood gradually increased. We gathered that the underlying causes might be as follows: (1) the redistribution of iNKT cells in the host, i.e., being transferred from the liver to peripheral blood, considering that inflammation in the liver has been controlled during antiviral treatment reflected by normalization of serum ALT levels or (2) less apoptosis occurred in iNKT cells because the PD-1 expression on iNKT cells significantly decreased during antiviral treatment. In terms of cytokines production, we observed a remarkably elevated capability to secrete IFN-γ by iNKT cells after in vitro stimulation. Furthermore, through blockade of the PD-1/PD-L pathway using mAbs against PD-L1 and PD-L2, we found that IFN-γ production by iNKT cells from CHB patients got elevated. These were different from the findings by Moll et al. [15] that antiretroviral treatment did not lead to changes in PD-1 expression and that PD-1/PD-L blockade was unable to overcome the NKT cell defect. The underlying cause remains unknown. Taken together, these results suggested that the elevated IFN-γ production by iNKT cells from HBeAg-positive CHB patients during antiviral therapy with Telbivudine may be associated with decreased PD-1 expression on iNKT cells and that in addition to the suppression of HBV replication. Nucleot(s)ide analogues such as Telbivudine may play some role in regulating the host immune responses in HBeAg-positive chronic hepatitis B patients, either directly or indirectly.

Conclusion

Our study revealed an impaired capability of cytokines production and high PD-1 expression on iNKT cells in HBeAg-positive chronic hepatitis B patients. Antiviral treatment with Telbivudine could elevate IFN-γ production and decrease PD-1 expression on iNKT cells. Blocking

experiments suggested that the elevated IFN-γ production by iNKT cells from HBeAg-positive CHB patients during antiviral therapy with Telbivudine might be associated with decreased PD-1 expression on iNKT cells. We believe that the behaviors of iNKT cells deserve further investigation to eventually achieve a comprehensive understanding of the state of the innate immune system during the course of antiviral treatment. It is also greatly interesting to conduct a long-time observation to confirm whether the effects persist after the withdrawal of antiviral treatment.

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Conflict of interest The authors have no conflict of interest to declare in relation to this manuscript.

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