

# TCR $\gamma\delta^+$ CD4 $^-$ CD8 $^-$ T Cells Suppress the CD8 $^+$ T-Cell Response to Hepatitis B Virus Peptides, and Are Associated with Viral Control in Chronic Hepatitis B

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## Abstract

The immune mechanisms underlying failure to achieve hepatitis B e antigen (HBeAg) seroconversion associated with viral control in chronic hepatitis B (CHB) remain unclear. Here we investigated the role of CD4 $^-$ CD8 $^-$  T (double-negative T; DNT) cells including TCR $\alpha\beta^+$  DNT ( $\alpha\beta$  DNT) and TCR $\gamma\delta^+$  DNT ( $\gamma\delta$  DNT) cells. Frequencies of circulating DNT cell subsets were measured by flow cytometry in a retrospective cohort of 51 telbivudine-treated HBeAg-positive CHB patients, 25 immune tolerant carriers (IT), 33 inactive carriers (IC), and 37 healthy controls (HC). We found that  $\gamma\delta$  DNT cell frequencies did not significantly change during treatment, being lower at baseline ( $P=0.019$ ) in patients with HBeAg seroconversion after 52 weeks of antiviral therapy ( $n=20$ ) than in those without ( $n=31$ ), and higher in the total CHB and IT than IC and HC groups ( $P<0.001$ ).  $\alpha\beta$  DNT cell frequencies were similar for all groups. In vitro,  $\gamma\delta$  DNT cells suppressed HBV core peptide-stimulated interferon- $\gamma$  and tumor necrosis factor- $\alpha$  production in TCR $\alpha\beta^+$ CD8 $^+$  T cells, which may require cell-cell contact, and could be partially reversed by anti-NKG2A. These findings suggest that  $\gamma\delta$  DNT cells limit CD8 $^+$  T cell response to HBV, and may impede HBeAg seroconversion in CHB.

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## Introduction

Worldwide, more than 350 million people have chronic hepatitis B virus (HBV) infection. The serious sequelae of this disease include liver cirrhosis, liver failure, and hepatocellular carcinoma [1]. Hepatitis B e antigen (HBeAg) seroconversion, defined as the loss of serum HBeAg with development of anti-HBe, is a stage in the evolution of chronic HBV infection, which can occur either spontaneously or as a result of antiviral therapy. For HBeAg-positive patients with chronic hepatitis B (CHB), HBeAg seroconversion is one of the primary goals of antiviral treatment, as it is associated with control of viral load, cessation of liver inflammation, decreased risk for serious sequelae, and increased survival [2]. Unfortunately, most patients do not achieve HBeAg seroconversion on treatment, for reasons that are poorly understood.

Evidence that HBeAg seroconversion is the result of a host immune response to HBV has recently been summarized [3]. Genetic polymorphisms in IL-10 and IL-12 loci [4], and differences in serum IL-12 [5] and serum IL-21 [6] concentrations may predict HBeAg seroconversion. However, there is little information on the cellular immune mechanisms that might be responsible for these associations. These mechanisms

need to be identified to allow optimizing treatment strategies in individual patients and developing novel immunotherapies to increase seroconversion rates. We focused on the CD4 $^-$ CD8 $^-$  subset of regulatory T cells, also known as double-negative T (DNT) cells.

DNT cells are classified into  $\alpha\beta$  DNT and  $\gamma\delta$  DNT subsets, depending on whether their TCR is comprised of  $\alpha\beta$  or  $\gamma\delta$  chains.  $\alpha\beta$  DNT cells can regulate antigen-specific T-cell responses in both mice [7] and humans [8,9], and HBeAg-activated  $\alpha\beta$  DNT cells can inhibit the murine HBeAg-specific effector T-cell response [10].  $\gamma\delta$  T cells, which are usually CD4- and CD8-negative, have a role in immunoregulation, including maintenance of oral tolerance [11], ocular tolerance [12], and hyporesponsiveness to tumors [13] in mice. Human  $\gamma\delta$  T cells also possess immune-suppressive capabilities [14,15,16,17,18]. Thus, DNT cells may involve in suppression of any cellular immune response that promotes HBeAg seroconversion. To investigate this possibility, we conducted a longitudinal study comparing DNT cell frequencies in patients with CHB who either did or did not undergo HBeAg seroconversion with antiviral therapy, and a cross-sectional comparison of DNT cell frequencies in subjects who were either HBeAg-positive or HBeAg-negative.

## Materials and Methods

### Human Subjects

This study was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the ethics committee of Nanfang Hospital. All subjects provided written informed consent.

HBeAg-positive patients with CHB for the longitudinal study ( $n = 51$ ) were participating in either a phase IV, multi-center, open-label clinical trial of telbivudine (LdT 600 mg/d,  $n = 21$ , trial number CLDT600ACN07T) or the Efficacy Optimization Research of Telbivudine Therapy trial (EFFORT,  $n = 30$ , trial number NCT00962533). From each patient, 20 mL of heparinized blood was taken at baseline and at 12, 24, and 52 weeks of LdT treatment. Patients were classified as responders ( $n = 20$ ) if they achieved HBeAg seroconversion by treatment week 52, and as non-responders ( $n = 31$ ) if they did not. Baseline characteristics of the patients in these two groups are compared in Table 1. Sixteen responders who received LdT consolidation therapy and maintained a durable response (HBeAg-negative, anti-HBe-positive, HBV DNA  $< 300$  copies/mL, and normal alanine aminotransferase (ALT)) for 48 weeks were followed up for another 52 weeks of treatment. Virologic relapse was defined as an increase of DNA level to  $> 10,000$  copies/mL [19]. The subjects for cross-sectional study included IT ( $n = 25$ ), CHB ( $n = 51$  from the above CHB), IC ( $n = 33$ ) and HC ( $n = 37$ ), who were recruited according to definition in AASLD criteria (see Methods S1) [2]. Their characteristics are summarized in Table 2.

### Synthetic Peptides

A panel of 26 18-mer peptides overlapping by 8 or 10 residues and covering the full HBV core open reading frame (Table S1 and S2) and HBV core18–27 (FLPSDFPSPV) were obtained (Sigma-Aldrich; St. Louis, MO, USA). 18-mer Peptides were dissolved in dimethyl sulfoxide (DMSO), pooled, and named the core peptides pool. An 18-mer HIV gag peptide (VDRFYKTLRAEQAS-QEVK) dissolved in DMSO was used as the negative control, while medium only as the blank control.

### Cell Isolation and Flow-cytometry Analysis

Isolation of peripheral blood mononuclear cells (PBMC) [6] and liver-infiltrating lymphocytes (LIL) [20,21] was performed (see

Methods S2). For analysis of the DNT cell subset frequency, cells were stained with anti-CD3-APC (UC7H1), anti-TCR $\gamma\delta$ -FITC (11F2), anti-CD4-PE, anti-CD8-PE (all BD Biosciences, Pharmingen, San Jose, CA, USA) and anti-TCR $\alpha\beta$ -PE-Cy7 (IP26; BioLegend, San Diego, CA, USA). Flow-cytometry analysis was performed using a FACS CanTo II flow cytometer and BD FACSDiva software (BD Biosciences).

Intracellular cytokine staining (ICS) was performed as previously described [6,22,23]. Briefly,  $4 \times 10^5$  PBMC were incubated with either the core peptide pool (each peptide at a final concentration of  $1 \mu\text{g/mL}$ ) plus anti-CD49d ( $1 \mu\text{g/mL}$ ) and anti-CD28 ( $1 \mu\text{g/mL}$ ), or with anti-CD3 (OKT,  $5 \mu\text{g/mL}$ ) plus anti-CD28 ( $1 \mu\text{g/mL}$ , BD Biosciences), or with phorbol-12-myristate-13-acetate (PMA,  $50 \text{ ng/mL}$ ) plus calcium ionomycin ( $1 \text{ nmol/mL}$ ; Sigma-Aldrich) for 1 hour in  $200 \mu\text{L}$  R10 (RPMI 1640 containing  $2 \text{ nmol/mL}$  glutamine, 10% FBS,  $100 \text{ U/mL}$  penicillin, and  $100 \mu\text{g/mL}$  streptomycin) in a round-bottomed 96-well plate, followed by  $1 \mu\text{g/mL}$  brefeldin A (BD Biosciences) for an additional 5 hours. The cells were harvested, stained with cell surface markers, fixed, permeabilized, and stained with either anti-interferon (IFN)- $\gamma$ -PerCP Cy5.5, anti-tumor necrosis factor (TNF)- $\alpha$ -PE or isotype controls (BD Biosciences) for flow-cytometry analysis.

The frequency of a positive CD8<sup>+</sup> T-cell response to core peptides in patients with CHB is usually  $< 30\%$  [23,24,25]. In this study, positive response was defined as greater production of either IFN- $\gamma$  or TNF- $\alpha$  by core peptide-stimulated CD8<sup>+</sup> T cells compared with either culture medium-stimulated or HIV gag peptide-stimulated CD8<sup>+</sup> T cells. Patients with a positive CD8<sup>+</sup> T-cell response were chosen for the T-cell suppression assays, transwell assays, and blocking assays.

### T-cell Suppression Assays

Because the proportion of CD4<sup>+</sup>  $\gamma\delta$  T cells in the  $\gamma\delta$  T-cell pool is extremely low ( $< 1\%$ ), the isolation of  $\gamma\delta$  DNT was carried out using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) to remove CD8<sup>+</sup> cells from PBMC, followed by positive selection of  $\gamma\delta$  T cells.  $\gamma\delta$  T(-) PBMC were obtained by direct removal of  $\gamma\delta$  T cells. The  $\gamma\delta$  DNT :  $\gamma\delta$  T(-) PBMC co-cultures were set up at a ratio of 1:2 with a total of  $4.5 \times 10^5$  cells/well. After overnight incubation, both the  $\gamma\delta$  DNT :  $\gamma\delta$  T(-) PBMC and  $\gamma\delta$  T(-) PBMC were cultured with the core peptide pool, and the production of IFN- $\gamma$  in TCR $\alpha\beta$ <sup>+</sup>CD8<sup>+</sup> T (CD8<sup>+</sup>  $\alpha\beta$  T) cells was detected by ICS. Negative or blank controls were  $4.5 \times 10^5$   $\gamma\delta$  T(-) PBMC cultured with or without the HIV gag peptide, respectively.

### Transwell Assays

Transwell assays were performed using cell-culture inserts with a  $1 \mu\text{m}$  polycarbonate membrane to avoid direct cell contact between the top and bottom chambers in 96-well plates (Transwell; Corning Inc., Corning, NY, USA). The  $\gamma\delta$  T(-) PBMC ( $4.5 \times 10^5$ ) were added to the bottom chamber, while  $\gamma\delta$  DNT cells ( $1.5 \times 10^5$ ) were added to either the top or the bottom chamber. Production of IFN- $\gamma$  and TNF- $\alpha$  in core peptide-stimulated CD8<sup>+</sup> $\alpha\beta$  T cells in the bottom chamber was detected by ICS.

### Blocking Assays

For more sensitive detection, blocking assays were performed as previously described [24,26,27], with minor modifications. Briefly,  $1 \times 10^5$   $\gamma\delta$  DNT cells were respectively incubated with anti-NKG2A (Z199,  $10 \mu\text{g/mL}$ ; Beckman Coulter Inc., Brea, CA, USA), anti-HLA-E(3D12,  $10 \mu\text{g/mL}$ ), or mouse IgG1 isotype

**Table 1.** Clinical characteristics at baseline of subjects in the cohort receiving telbivudine therapy.

Groups	Responders	Non-responders	P values
Cases	20	31	
Gender, M/F	14/6	26/5	0.408 <sup>b)</sup>
Age, years <sup>a)</sup>	28 (25–35)	27 (24–29)	0.250 <sup>c)</sup>
ALT, IU/L <sup>a)</sup>	166 (98–252)	116 (82–244)	0.370 <sup>c)</sup>
AST, IU/L <sup>a)</sup>	104 (53–129)	88 (57–146)	0.946 <sup>c)</sup>
HBV DNA, log <sub>10</sub> copies/mL <sup>a)</sup>	8.16 (7.32–8.72)	8.73 (8.59–9.03)	0.002 <sup>c)</sup>
Genotype B/C	16/4	16/15	0.080 <sup>b)</sup>

ALT, alanine aminotransferase; AST, Aspartate aminotransferase; HBV, hepatitis B virus; M/F: Male/female.

<sup>a)</sup>Data are median (interquartile range).

<sup>b)</sup>Compared by  $\chi^2$  test (continuity correction).

<sup>c)</sup>Compared by Mann-Whitney U-test.

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**Table 2.** Clinical characteristics of subjects in cross-sectional study.

Groups	IT	CHB	IC	HC
Cases	25	51	33	37
Gender, M/F	11/14	40/11	10/23	15/22
Age, years <sup>a)</sup>	26 (23–30)	27 (24–33)	29 (24–38)	24 (23–26)
ALT, IU/L <sup>a)</sup>	27 (17–32)	127 (88–244)	19 (16.5–25)	13(8–22)
AST, IU/L <sup>a)</sup>	25 (22–27)	99 (57–137)	24 (21–28)	20 (16.5–24)
HBV DNA, log <sub>10</sub> copies/mL <sup>a)</sup>	7.80 (7.49–8.30)	8.62 (8.00–8.90)	<3.00(<3.00)	NA
HBeAg/anti-HBe	25/0	51/0	0/33	0/0

ALT, alanine aminotransferase; AST, Aspartate aminotransferase; HBeAg, HBV e antigen; HC, healthy controls; IC, inactive carriers; IT, immune tolerant carriers; M/F: Male/female; NA, not applicable.

<sup>a)</sup>Data are median (interquartile range).

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(10 µg/mL) (both Biologend) for 45 min at 37°C, and then cocultured with  $3 \times 10^5$   $\gamma\delta$  T(-)PBMC in the presence of the core peptides pool, anti-CD49d, and anti-CD28 (1 µg/mL). The gag peptide was the negative control. IL-2 (25 U/mL) was added on day 4. After 10 days, cells were resuspended, and stimulated again with peptides for 1 h, then Brefeldin A was added for a further 5 h. Production of IFN- $\gamma$  and TNF- $\alpha$  in CD8<sup>+</sup> $\alpha\beta$ T cells was measured by flow-cytometry analysis.

PBMC from some patients were stained with anti-HLA-A2-PE (BD). The HLA-A2 positive patients were chose for the detection using PE-labeled pentamer complexed with HBV core 18–27 (Proimmune, Oxford, UK). With the method of blocking assays, cells were expanded and re-stimulated with HBV core18–27 (5 µg/mL), and stained with anti-CD3-FITC, anti-CD8-APC-Cy7, pentamer-PE, anti-IFN- $\gamma$ -PerCP-Cy5.5 and anti-TNF- $\alpha$ -PE-Cy7.

### Statistical Analysis

Serum HBV DNA levels were normalized by log transformation. Box and whisker plots were used to show the median, interquartile range (IQR), and range. For independent group comparisons, we used the Kruskal-Wallis *H*-test to compare all groups and the Mann-Whitney *U*-test to compare groups by pairs. For related group comparisons, the Friedman test was used to compare all groups and the Wilcoxon test to compare groups by pairs. Spearman rank order correlation was used for correlation analyses. The  $\chi^2$  test, logistic regression, and receiver-operating characteristic curves (ROC) analysis were used as appropriate, using either SPSS (version 13.0; SPSS Inc., Chicago, IL, USA), GraphPad Prism (version 5; GraphPad, San Diego, CA, USA) or SAS (SAS Inc., Cary, NC, USA). For ROC analysis, an area under the curve (AUC) of 1.0 is characteristic of an ideal test, whereas AUC <0.5 indicates a test of no diagnostic value. A (two-tailed) *P*-value of <0.05 was deemed statistically significant.

## Results

### Association between Frequencies of $\gamma\delta$ DNT Cells and HBeAg Seroconversion in Antiviral Therapy

In longitudinal study, HBeAg-positive patients treated with telbivudine (LdT) were classified as responders (*n* = 20) if they achieved HBeAg seroconversion by treatment week 52, and as non-responders (*n* = 31) if they did not. Circulating frequencies of  $\alpha\beta$  DNT cells and  $\gamma\delta$  DNT cells were examined (Figure 1A) at baseline, 12, 24, and 52 weeks. There were no significant changes in frequencies of the two DNT cell subsets during 52 weeks of

treatment, and no significant differences in frequencies of  $\alpha\beta$  DNT cells between the two groups (Figure 1B; Figure S1A). Frequencies of  $\gamma\delta$  DNT cells were higher in non-responders than in responders at baseline (median [IQR]: 10.3% [6.1–14.7%] vs 5.1% [3.6–11.2%], respectively; *P* = 0.019) and at 12 weeks (10.5% [7.0–16.5%] vs 5.8% [3.7–12.1%]; *P* = 0.041).

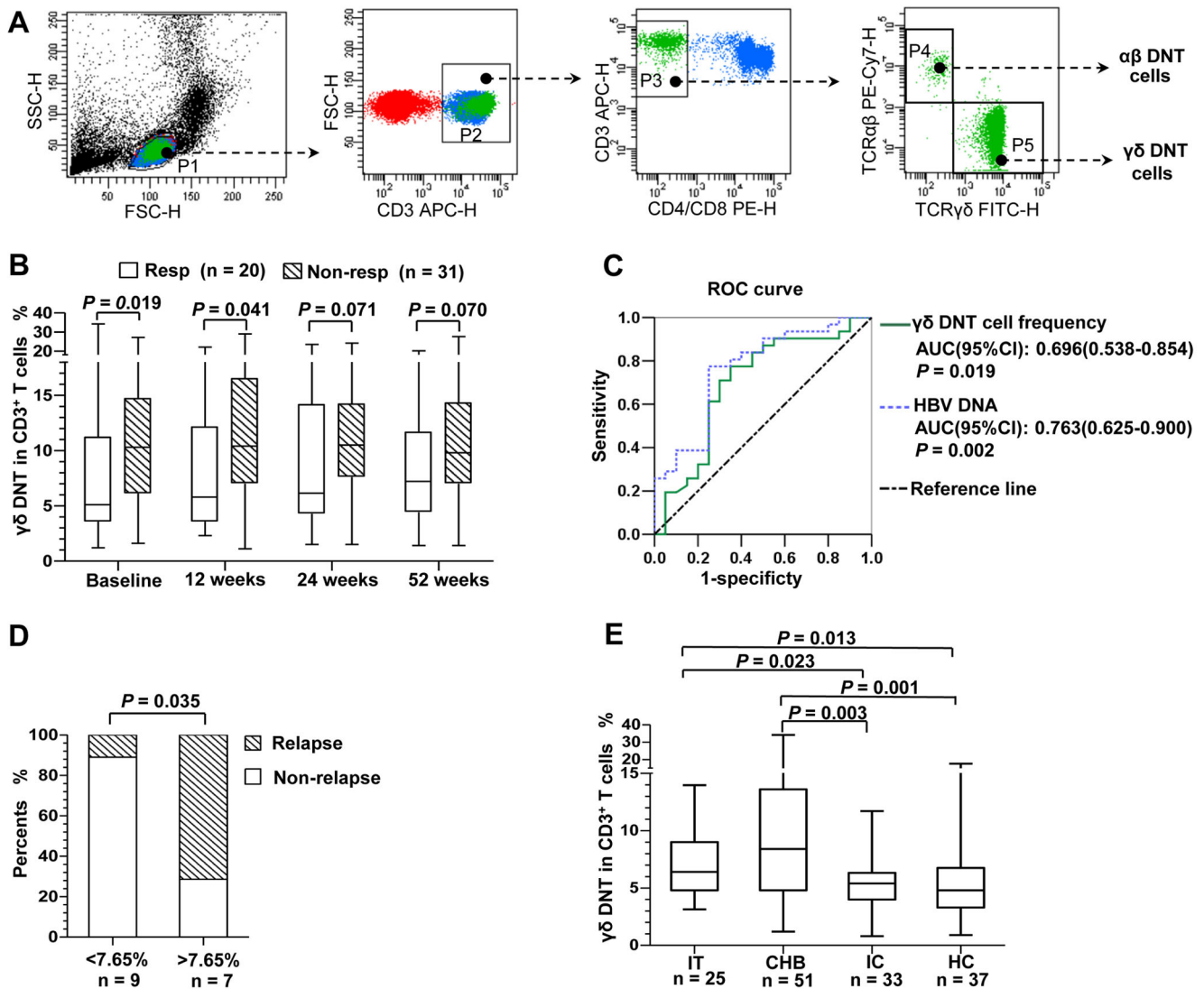
Univariate logistic regression showed that HBeAg seroconversion was associated with low baseline frequencies of both  $\gamma\delta$  DNT cells (*P* = 0.04) and HBV DNA (*P* = 0.005). Multivariate analysis identified a significant effect of HBV DNA (*P* = 0.008), and a trend towards an independent effect of  $\gamma\delta$  DNT cell frequency (*P* = 0.08). No significant interaction was found between these variables (*P* = 0.77). There were no significant correlations between frequencies of  $\gamma\delta$  DNT cells and either the serum HBV DNA levels, ALT, or aspartate aminotransferase (AST), or age of the CHB patients at baseline (Table S3).

In this cohort, ROC analysis showed that either baseline  $\gamma\delta$  DNT cell frequency or baseline serum HBV DNA level predicted the non-responders after 52 weeks of LdT therapy (Figure 1C). The optimal cut-off value for prediction was baseline  $\gamma\delta$  DNT cell frequency of 7.65% of CD3-positive PBMC, which had a sensitivity of 70.0% and specificity of 71.0%. In light of that quite a few patients who achieve HBeAg seroconversion induced by nucleotide analogues have virologic relapse off treatment [19], we observed the association between baseline  $\gamma\delta$  DNT cell frequency and viral control after 52 weeks off therapy. Sixteen patients received an average of 112 Weeks (rang 104–163 weeks) of LdT therapy before off-therapy. In the cohort of patients with baseline  $\gamma\delta$  DNT cell frequency >7.65%, five of seven patients had a virologic relapse after 52 weeks off treatment, whereas only one of nine patients with baseline  $\gamma\delta$  DNT cell frequency <7.65% had a virologic relapse (*P* = 0.035, Figure 1D). All patients (*n* = 4) who had recurrence of HBeAg had a high frequency of  $\gamma\delta$  DNT cells, with rates for the four patients being 10.4%, 11.3%, 14.2%, and 17.8%, respectively.

Collectively, these data indicate that a high frequency of  $\gamma\delta$  DNT cells is associated with failure to achieve durable HBeAg seroconversion induced by LdT therapy.

### Association between Frequencies of $\gamma\delta$ DNT Cells and Serum HBeAg Status in Cross-sectional Comparison

To investigate whether frequency of  $\gamma\delta$  DNT cells is associated with spontaneous HBeAg seroconversion, we compared CHB with inactive carriers (IC), immune tolerant carriers (IT), and healthy controls (HC) at baseline. Frequencies of  $\gamma\delta$  DNT cells in the two HBeAg-positive groups (IT 6.4% [4.8–9.0%] and CHB 8.4%



**Figure 1. Comparison of the frequencies of DNT cell subsets in longitudinal and cross-sectional studies.** (A) Flow-cytometry dot plots show the strategy for identifying the  $\alpha\beta$  DNT cells and  $\gamma\delta$  DNT cells. (B) The frequencies of  $\gamma\delta$  DNT cells in peripheral blood  $CD3^+$  T cells were compared for responders (Resp,  $n = 20$ ) and non-responders (Non- resp,  $n = 31$ ) at different time points during 52 weeks of telbivudine therapy using the Mann-Whitney  $U$  test by pairs. (C) Receiver-operating characteristic curve analysis shows that either baseline  $\gamma\delta$  DNT cell frequencies or HBV DNA levels predicts the non-responders after 52 weeks of telbivudine therapy. (D) The virologic relapse rates after 52 weeks off treatment were compared between the patients with baseline  $\gamma\delta$  DNT cell frequency of  $>7.65\%$  ( $n = 7$ ) and  $<7.65\%$  ( $n = 9$ ) using  $\chi^2$  test (Fisher's exact test). (E) The frequencies of  $\gamma\delta$  DNT cells were compared in the IT, CHB, IC and HC groups using the Mann-Whitney  $U$  compared groups by pairs. (B, D) Data plots show the median, interquartile range (IQR), and range.  $\alpha\beta$  DNT cells,  $TCR\alpha\beta^+CD4^-CD8^-$  T cells;  $\gamma\delta$  DNT cells,  $TCR\gamma\delta^+CD4^-CD8^-$  T cells; AUC, area under the curve; CI, confidence interval; CHB, chronic hepatitis B; DNT cells, double-negative T cells; HC, healthy controls; IC, inactive carriers; IT, immune tolerant carriers.

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[4.8–13.6%]) were significantly higher than in either the IC (5.4% [4.0–6.3%]) or HC (4.8% [3.3–6.8%]) groups (Figure 1E). There were no significant differences in  $\gamma\delta$  DNT cell frequency between the CHB and IT groups, or between the IC and HC groups. These data indicate that a low  $\gamma\delta$  DNT cell frequency is associated with a state that is more likely to lead to spontaneous HBsAg seroconversion.

### Suppression of Anti-HBV $CD8^+$ T Cell Response by $\gamma\delta$ DNT Cells

Together, the results of the clinical studies imply that a high frequency of  $\gamma\delta$  DNT cells is not good for viral control in CHB.

Thus, we investigated whether  $\gamma\delta$  DNT cells suppress the HBV-specific  $CD8^+$  T-cell responses that are known to inhibit HBV replication [28].

In the T-cell suppression assay, the median purity of isolated  $\gamma\delta$  DNT cells within the  $\gamma\delta$  T cell compartment (Figure 2A) was 89.7% (range 86.1–96.3%,  $n = 10$ ). Addition of  $\gamma\delta$  DNT cells to the core peptide-stimulated cultures reduced the frequency of IFN- $\gamma$ -secreting  $CD8^+\alpha\beta$  T cells ( $P = 0.005$ ) (Figure 2B and C). In addition, from four patients with CHB, co-cultures of  $\gamma\delta$  T cells with a  $\gamma\delta$  DNT/ $\gamma\delta$  T frequency of  $>80\%$  and  $\gamma\delta$  T(-) PBMC at ratios of 0:16, 1:16, 1:4, 1:2, and 1:1 showed that a decreasing frequency of non-antigen specific, IFN- $\gamma$ -producing cells in the  $CD8^+$  lymphocytes cultures ( $P = 0.007$ ), and decreasing prolifera-

tion of CD8<sup>+</sup> lymphocytes were accompanied by an increasing proportion of  $\gamma\delta$  T cells, which was dose-dependent ( $P=0.003$ ) (Figure S2A–F). However, there were no similar observations from three healthy donors (Figure S2G and H). It indicated that the suppressive function of  $\gamma\delta$  T cells was associated with the condition of chronic HBV infection.

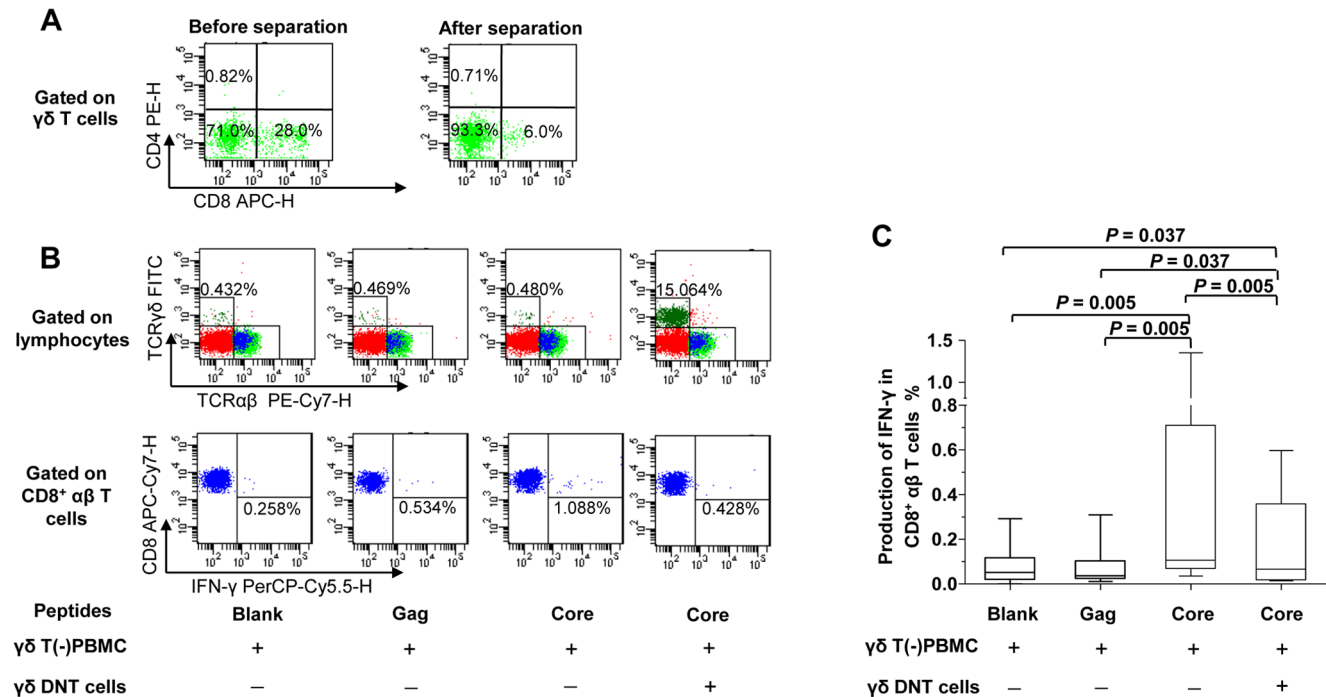
### Mechanisms Associated with the Suppression by $\gamma\delta$ DNT Cells

In the transwell assays, production of both TNF- $\alpha$  and IFN- $\gamma$  by core peptide-stimulated  $\gamma\delta$ T(-)PBMC increased when they were separated from  $\gamma\delta$  DNT cells by inserting a membrane (Figure 3A), indicating that suppression by  $\gamma\delta$  DNT cells may require cell–cell contact. In the CHB group, few  $\gamma\delta$  DNT cells expressed cell-surface CTLA-4, FasL, PD-L1, or LAG-3, and PMA-stimulated  $\gamma\delta$  DNT cells produced almost no TGF- $\beta$  or IL-10 (data not shown), implying that these molecules may not directly mediate the suppression by  $\gamma\delta$  DNT cells. More than half of  $\gamma\delta$  DNT cells expressed NKG2A, and the frequency of NKG2A expression was higher on  $\gamma\delta$  DNT cells than on  $\alpha\beta$  DNT cells, CD4<sup>+</sup>  $\alpha\beta$  T cells, NK cells, or CD8<sup>+</sup>  $\gamma\delta$  T cells. There was no significant difference between CHB and HC in the frequency of  $\gamma\delta$  DNT cells expressing NKG2A (Figure S3). Interestingly, HLA-E, the ligand of NKG2A, was expressed by all of these T-cell subsets, and the frequency of HLA-E expression on  $\gamma\delta$  DNT cells was higher than on both  $\alpha\beta$  DNT cells and CD8<sup>+</sup>  $\alpha\beta$  T cells. Moreover, frequency of HLA-E in CD8<sup>+</sup>  $\alpha\beta$  T cells was higher in CHB and IT groups compared with HC (Figure S4). To investigate whether this NKG2A-HLA-E pathway is involved in the suppression by  $\gamma\delta$  DNT cells, we used an antibody to block NKG2A or HLA-E

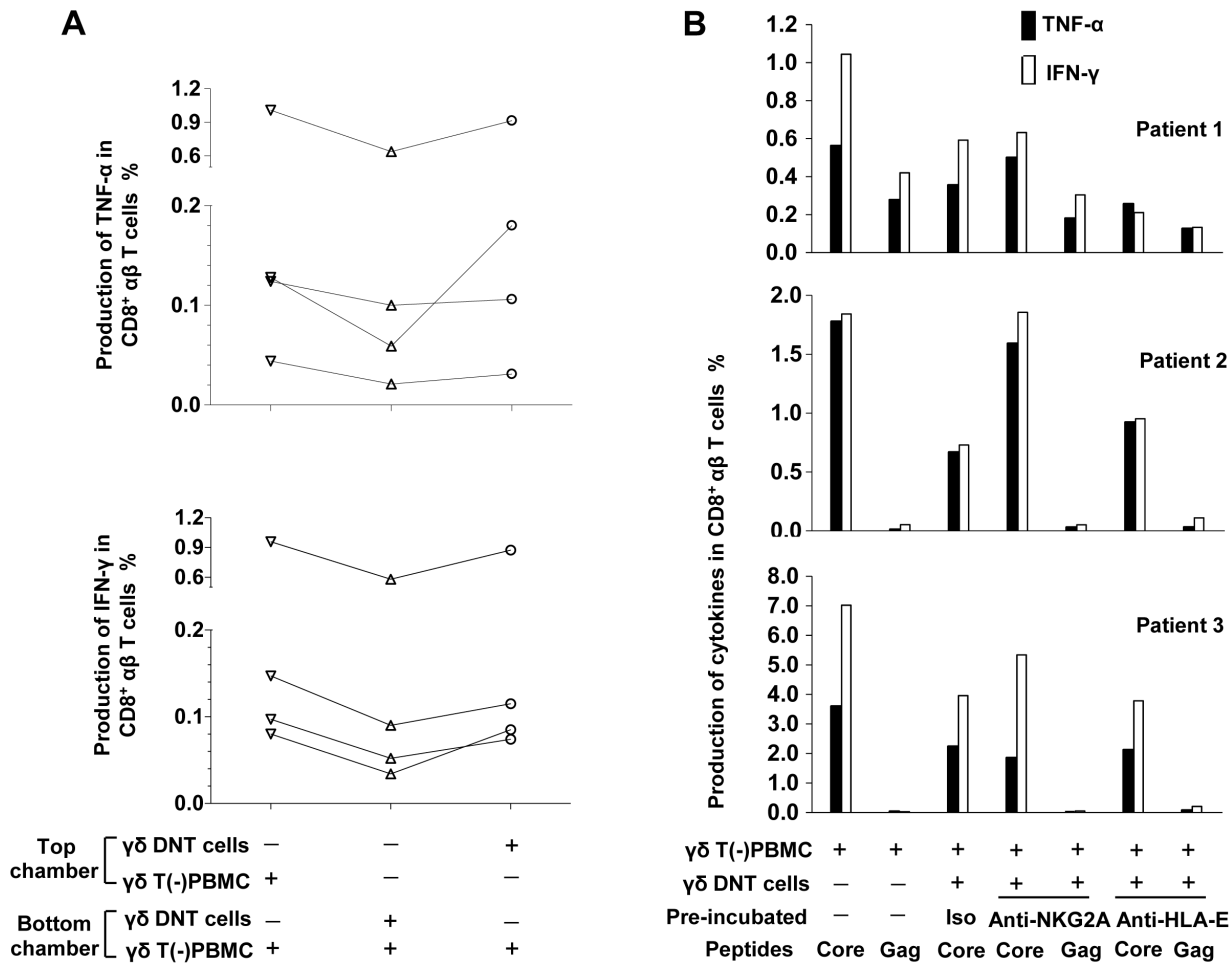
expressed on  $\gamma\delta$  DNT cells. Figure 3B (data derived from Figure S5) shows that suppression of both core peptide pool-induced TNF- $\alpha$ -secreting and IFN- $\gamma$ -secreting CD8<sup>+</sup>  $\alpha\beta$  T cells by  $\gamma\delta$  DNT cells was partly reversed in three patients after pre-incubation of  $\gamma\delta$  DNT cells with anti-NKG2A but not anti-HLA-E or IgG1 isotype. Furthermore, we investigated the influence of  $\gamma\delta$  DNT cells on the HBV-specific pentamer-positive CD8<sup>+</sup> T cells. As shown in Figure 4, the frequency of HBV-specific pentamer-positive CD8<sup>+</sup> T cells and the production of IFN- $\gamma$  or TNF- $\alpha$  in these T cells decreased when  $\gamma\delta$  DNT cells were added. And such decreases can be partially reversed when  $\gamma\delta$  DNT cells were pre-incubated with anti-NKG2A in some patients.

### Association between Intrahepatic $\gamma\delta$ DNT Cells and HBsAg Seroconversion

Because the HBV-specific CD8<sup>+</sup> T-cell response mainly occurs in the liver, which is the site of HBV replication, we investigated whether the frequency of circulating  $\gamma\delta$  DNT cells reflects the frequency in the intrahepatic compartment. LIL and PBMC were obtained from 26 patients with CHB after 104 weeks of LdT treatment (10 responders and 16 non-responders). The characteristics of these patients at 104 weeks are compared between responders and non-responders in Table S4. There was no significant difference of Knodell necroinflammatory scores or Ishak fibrosis scores between these two groups. The  $\gamma\delta$  DNT cells were identified using a modified strategy (Figure S6). There was a significant correlation between frequencies of  $\gamma\delta$  DNT cells in LIL and PBMC ( $r=0.231$ ,  $P=0.020$ , Figure 5A), both of which appeared similar ( $P=0.107$ , Figure 5B). The frequency of  $\gamma\delta$  DNT cells was higher in LIL from non-responders relative to responders



**Figure 2.  $\gamma\delta$  DNT cells suppress HBV antigen-specific CD8<sup>+</sup>  $\alpha\beta$  T-cell responses.** (A) One representative example shows the purity of  $\gamma\delta$  DNT cells in a  $\gamma\delta$  T-cell pool before and after separation. (B) One representative result shows that addition of  $\gamma\delta$  DNT cells reduced HBV core peptide-induced production of IFN- $\gamma$  in CD8<sup>+</sup>  $\alpha\beta$  T cells. A gag peptide was used as the negative control, while medium only as the blank control. (C) The pooled results from ten patients with CHB were compared using Wilcoxon test by pairs. (blank, medium only; core, HBV core peptides pool; gag, HIV gag peptide).  $\gamma\delta$  T(-)PBMC,  $\gamma\delta$  T cell-deleted-PBMC;  $\gamma\delta$  DNT cells, CD8-deleted- $\gamma\delta$ T cells; CD8<sup>+</sup>  $\alpha\beta$  T cells, TCR $\alpha\beta$ <sup>+</sup>CD8<sup>+</sup> T cells; CHB, chronic hepatitis B; DNT cells, double-negative T cells; IFN, interferon; PBMC, peripheral blood mononuclear cells. doi:10.1371/journal.pone.0088475.g002



**Figure 3. Mechanisms associated with the suppression by  $\gamma\delta$  DNT cells.** (A) The suppression of HBV core peptide-induced production of TNF- $\alpha$  or IFN- $\gamma$  in CD8<sup>+</sup>αβ T cells could be partly abrogated by inserting a membrane between the CD8<sup>+</sup> αβ T cells and the  $\gamma\delta$  DNT cells in a transwell assay of cells from four patients with CHB. Lines link the data from the same patients. (B)  $\gamma\delta$  DNT cells were isolated and pre-incubated with either anti-NKG2A, anti-HLA-E, or mouse IgG1 isotype before being co-cultured with  $\gamma\delta$  T(-)PBMC. After 10 days of stimulation with peptides, production of TNF- $\alpha$  (black bars) and IFN- $\gamma$  (blank bars) in CD8<sup>+</sup>αβ T cells was detected. Data from three patients are shown (core, HBV core peptides pool; gag, HIV gag peptide). DNT cells, double-negative T cells; IFN, interferon; PBMC, peripheral blood mononuclear cells; TNF, tumor necrosis factor. doi:10.1371/journal.pone.0088475.g003

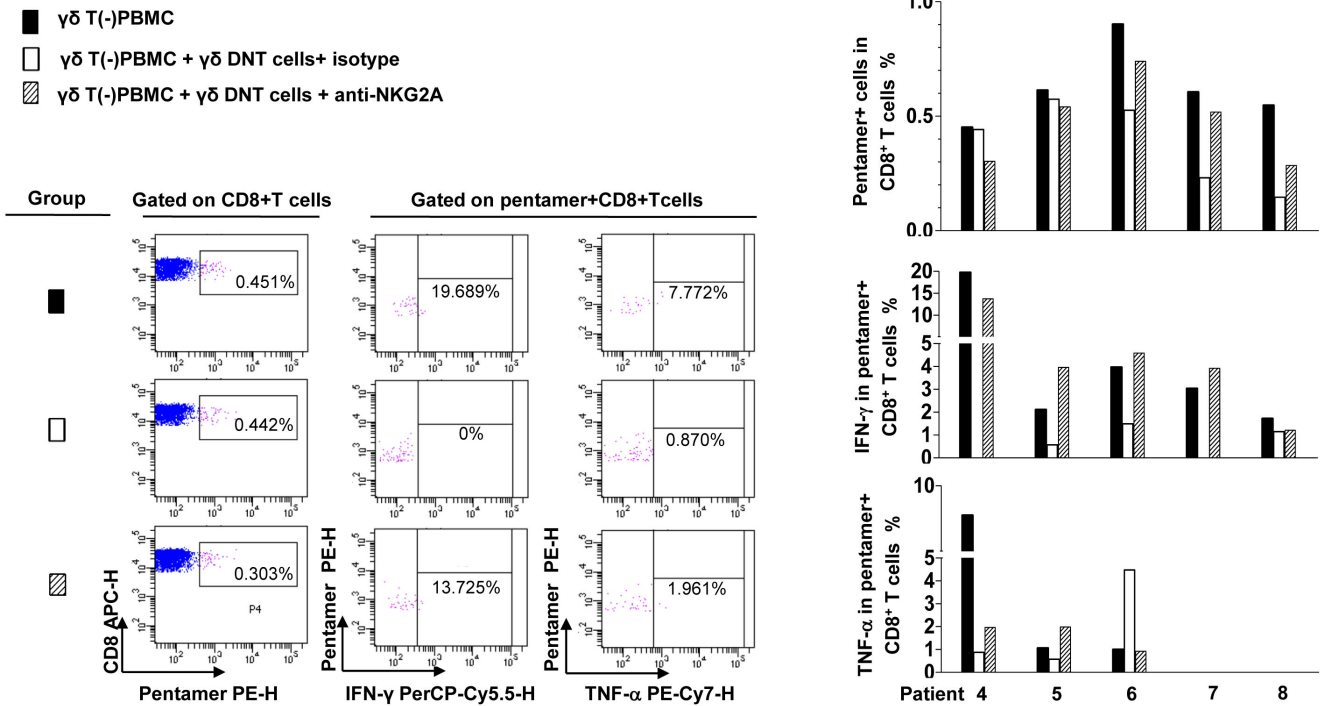
(8.5%, [7.1–14.0%] vs 5.2%, [4.6–8.5%];  $P=0.035$ ). The higher frequency of  $\gamma\delta$  DNT cells in PBMC from non-responders relative to responders approached statistical significance (6.3%, [5.2–12.7%] vs 4.8% [2.5–8.2%];  $P=0.051$ ).

**Discussion**

In this study, we tested the hypothesis that DNT cells are involved in failure to achieve HBeAg seroconversion associated with viral control, which is supported by several clinical observations. Firstly, the baseline frequency of  $\gamma\delta$  DNT cells in PBMC from the patients who seroconverted after antiviral therapy was half that of patients who did not seroconvert. Secondly, when antiviral therapy was discontinued, virologic relapse and recurrence of HBeAg tended to occur in those patients who had a high baseline frequency of  $\gamma\delta$  DNT cells. Thirdly, the frequency of intrahepatic  $\gamma\delta$  DNT cells was higher in patients who didn't achieve HBeAg seroconversion after 104 weeks of antiviral treatment. Finally, the frequency of  $\gamma\delta$  DNT cells was higher in the HBeAg-positive patients relative to IC, who are known to have a durable spontaneous HBeAg seroconversion. In the multivariate

analysis of longitudinal data, the influence of baseline  $\gamma\delta$  DNT cell frequency on HBeAg seroconversion was not statistically independent of the influence of baseline HBV DNA level, but there was no correlation between  $\gamma\delta$  DNT cell frequency and HBV DNA level. It is difficult to determine whether these two parameters are reflections of the same underlying mechanism for HBeAg seroconversion.

Although no published evidence has shown that HBeAg seroconversion is caused by the activation of CD8<sup>+</sup> T-cell responses to HBV antigens, there is a strong association between these two events [28,29,30]. Our in vitro data showed that  $\gamma\delta$  DNT cells have the ability to suppress anti-HBV-specific CD8<sup>+</sup> T-cell responses. This raises the possibility that suppression of antiviral CD8<sup>+</sup> T-cell responses by a high level of  $\gamma\delta$  DNT cells contributes to failure to control HBV replication and achieve HBeAg seroconversion. This in vitro data also adds to the list of known mechanisms that contribute to CD8<sup>+</sup> T cell hyporesponsiveness in patients with CHB, including impaired function of dendritic cells [31], increased numbers of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells [32], high antigen loads [33], high levels of expression of

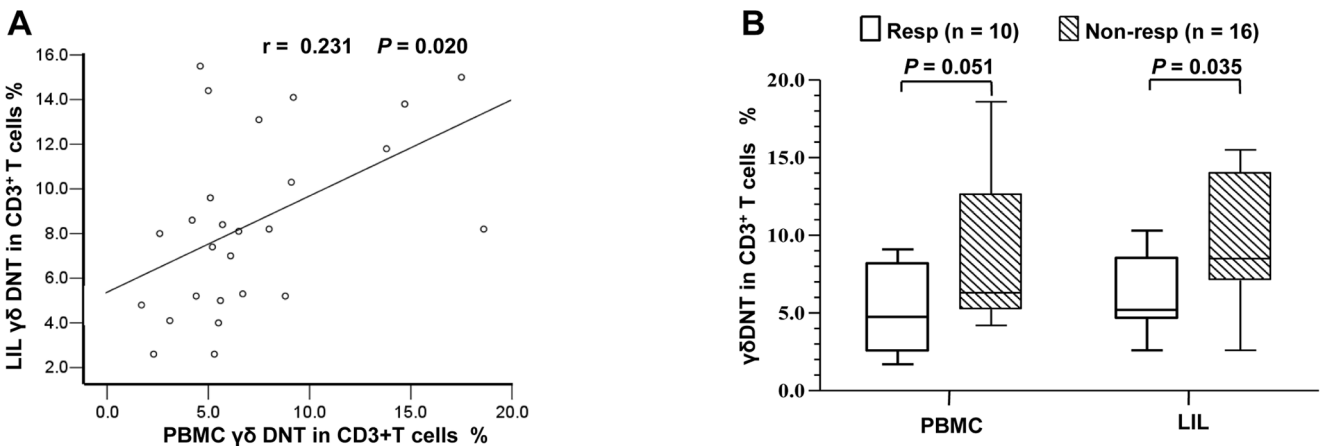


**Figure 4. Influence of  $\gamma\delta$  DNT cells on HBV-specific pentamer-positive CD8<sup>+</sup> T cells.**  $\gamma\delta$  DNT cells were isolated from HLA-A2 positive patients with CHB and pre-incubated with either anti-NKG2A (diagonal stripes bars) or mouse IgG1 isotype (blank bars) before being co-cultured with  $\gamma\delta$  T(-)PBMC. The group with  $\gamma\delta$  T(-)PBMC only (black bars) was also compared. Dot plots from one representative patient show the strategy for identifying the frequency of HBV pentamer-positive CD8<sup>+</sup> T cells, and the production of IFN- $\gamma$  and TNF- $\alpha$  in pentamer-positive CD8<sup>+</sup> T cells after 10 days of stimulation with HBV core18–27. Data from five patients are shown. doi:10.1371/journal.pone.0088475.g004

PD-1 by exhausted HBV-specific CD8<sup>+</sup> T cells [26], and deletion of HBV-specific CD8<sup>+</sup> T cells by NK cells [34].

$\gamma\delta$  T cells are known to have a wide range of functions, including immune protection against pathogens in the early  $\gamma\delta$  T-cell response and immunoregulation in some late  $\gamma\delta$  T-cell responses [35,36]. The impaired IFN- $\gamma$  production and cytotoxicity of  $\gamma\delta$  T cells in persistent HBV infection have been reported

previously [37]. Our study provides new insight into the immunoregulation of  $\gamma\delta$  T cells in CHB. The in vitro data indicates that suppression of HBV peptide-specific CD8<sup>+</sup> T-cell responses by  $\gamma\delta$  DNT cells requires cell-cell contact, and is partially mediated by the NKG2A signaling pathway. These results are in contrast to a study of human  $\gamma\delta$  T cells expanded from breast tumors [15], but are consistent with a study of



**Figure 5. Frequencies of  $\gamma\delta$  DNT cell subsets in LIL and PBMC.** LIL and PBMC were obtained from 26 patients with CHB at the same time after 104 weeks of telbivudine treatment. (A) Spearman's correlation analysis between  $\gamma\delta$  DNT cell frequencies in LIL and PBMC. (B) Comparison of the frequencies of  $\gamma\delta$  DNT cells between LIL and PBMC using a Wilcoxon test, and between responders (Resp, n = 10) and non-responders (Non-resp, n = 16) using Mann-Whitney U test. CHB, chronic hepatitis B; DNT cells, double-negative T cells; LIL, liver-infiltrating lymphocytes; PBMC, peripheral blood mononuclear cells. doi:10.1371/journal.pone.0088475.g005

intraepithelial  $\gamma\delta$  T cells in patients with celiac disease [16]. As reviewed, NKG2A, an inhibitory receptor expressed by NK cells and  $CD8^+$  T cells, plays an important role in HIV, HCV [38,39], and HBV [40] infections. NKG2A also mediates suppressive signaling in human  $\gamma\delta$  T cells [16,41]. In the current study, we found that most  $\gamma\delta$  DNT cells expressed NKG2A, and the frequency of HLA-E in  $CD8^+ \alpha\beta$  T cells was higher in patients with CHB. These findings suggest that two mechanisms may enhance  $\gamma\delta$  DNT cell-mediated suppression of anti-HBV  $CD8^+$  T-cell responses. The first is an increase in the number rather than the activity of  $\gamma\delta$  DNT cells. The second is an increase in the expression of HLA-E on  $CD8^+ \alpha\beta$  T cells from HBeAg-positive subjects, which may be similar to the findings of up-regulation of HLA-E in HCV infections [42].

Pro-inflammatory cytokine or TCR $\gamma\delta$  activation can augment  $\gamma\delta$  T-cell frequency in peripheral blood [35]. However, it seems unlikely that hepatic inflammation or viral factors are responsible for the increase in  $\gamma\delta$  DNT cell frequency seen in our study, as there were no significant changes in  $\gamma\delta$  DNT cell frequency with the decrease of ALT or serum viral components including HBV DNA, HBeAg, and hepatitis B surface antigen (HBsAg) during treatment, and there was no significant correlation between these variables at baseline. Thus, the mechanisms that regulate the circulating frequency of  $\gamma\delta$  DNT cells in CHB deserve further investigation.

We also analyzed frequencies of  $CD4^+/CD8^+$   $\gamma\delta$  T cells both in longitudinal and cross-sectional study. There was no significant difference in  $CD4^+/CD8^+$   $\gamma\delta$  T-cell frequencies between the different groups (Figure S1B and data not shown).  $CD4^+$   $\gamma\delta$  T cells make up a small proportion of  $\gamma\delta$  T cells and most of  $CD4^+/CD8^+$   $\gamma\delta$  T cells were  $CD8^+$   $\gamma\delta$  T cells. Little is known about the different properties between human  $CD8^+$   $\gamma\delta$  T cells and  $\gamma\delta$  DNT cells [16]. A recent report showed that the absolute number of  $CD8^+$   $\gamma\delta$  T cells did not change with age, whereas  $\gamma\delta$  DNT cells decreased in healthy aged people [43]. Interestingly, in contrast, the cumulative lifetime incidence of HBeAg seroclearance increases with age [44]. Although we found higher frequencies of CD127, NKG2A, and HLA-DR, and lower frequencies of CD45 RA on  $\gamma\delta$  DNT cells compared with  $CD8^+$   $\gamma\delta$  T cells (unpublished data), the different functions of these two subsets in CHB require further investigation.

In conclusion, a high frequency of  $\gamma\delta$  DNT cells is associated with failure to control HBV. A low baseline frequency of  $\gamma\delta$  DNT cells in PBMC predicts HBeAg seroconversion during LdT therapy for CHB. In vitro data showing that  $\gamma\delta$  DNT cells suppress  $CD8^+$  T cell responses to HBV antigens raise the possibility that the NKG2A-HLA-E signaling pathway may be a target for immunotherapy.

## Supporting Information

**Figure S1 Comparison of the frequencies of  $\alpha\beta$  DNT cells and  $CD4^+/CD8^+$   $\gamma\delta$  T cells in the longitudinal study.** (TIF)

**Figure S2 Influence of different frequencies of  $\gamma\delta$  T cells on the non-specific  $CD8^+$  T-cell response.** CHB patients with a  $\gamma\delta$  DNT/ $\gamma\delta$  T-cell frequency of  $>80\%$  were recruited into this study.  $\gamma\delta$  T cells were isolated from PBMC, and  $\gamma\delta$  T :  $\gamma\delta$  T(-)PBMC overnight co-cultures were set up at ratios of 0:16, 1:16, 1:4, 1:2, and 1:1 with a total of  $4.5 \times 10^5$  cells/well, and incubated in the presence of anti-CD3 and anti-CD28 antibodies to test the production of IFN- $\gamma$  by ICS. (A,B) An individual experiment shows the influence of an increasing frequency of (A)  $\gamma\delta$  T cells (labeled “P1”,  $\gamma\delta$  DNT/ $\gamma\delta$  T = 82.9%) on (B) the production of

IFN- $\gamma$  (“P2”) in  $CD8^+$  lymphocytes stimulated with anti-CD3 and anti-CD28. In the proliferation test,  $\gamma\delta$  T(-)PBMC labeled with 1.5  $\mu\text{mol/L}$  CFSE were co-cultured with  $\gamma\delta$  T cells in the presence of anti-CD3 and anti-CD28 antibodies for 3 days, and then stained with anti-CD8-PE for analysis of the proliferation of  $CD8^+$  lymphocytes by flow cytometry. (C,D) An individual experiment shows the influence of an increasing frequency of (C)  $\gamma\delta$  T cells ( $\gamma\delta$  DNT/ $\gamma\delta$  T cells = 90.3%) on (D) the proliferation of  $CD8^+$  lymphocytes stimulated with anti-CD3 and anti-CD28. “P3” indicates the unlabeled  $\gamma\delta$  T cells, “P4” the  $\gamma\delta$  T(-)PBMC labeled with CFSE, and “P5” the proliferating  $CD8^+$  lymphocytes. (E,F) Data from four patients with CHB were analyzed by the Friedman test. (G,H) Data from three healthy donors were also shown. CHB, chronic hepatitis B; DNT cells, double-negative T cells; ICS, intracellular cytokine staining. (TIF)

**Figure S3 Expression of NKG2A on  $\gamma\delta$  DNT cells.** PBMC from CHB patients and HC were stained with anti-CD3-APC-Cy7, anti-TCR- $\gamma\delta$ -FITC, anti-CD4-PE-Cy7, anti-CD8-PerCP, anti-CD56-APC and anti-NKG2A-PE. (A) Expression of NKG2A on  $\gamma\delta$  DNT,  $CD8^+ \gamma\delta$  T,  $\alpha\beta$  DNT,  $CD4^+ \alpha\beta$  T cells, and NK cells were measured and compared between (B) different lymphocyte subsets or (C) between the CHB and HC groups. CHB, chronic hepatitis B; DNT cells, double-negative T cells; HC, healthy controls. (TIF)

**Figure S4 Expression of HLA-E on  $\gamma\delta$  DNT cells.** PBMC were stained with anti-CD3-APC-Cy7, anti-TCR $\gamma\delta$ -FITC, anti-CD4-PE-Cy7, anti-CD8-PerCP, and anti-HLA-APC. Expression of HLA-E on  $\gamma\delta$  DNT,  $CD8^+ \gamma\delta$  T cells,  $\alpha\beta$  DNT cells, and  $CD8^+ \alpha\beta$  T cells was (A) measured in HC and CHB relative to the isotype control and (B) compared in the different T-cell subsets. (C,D) Expression of HLA-E on either (C)  $\gamma\delta$  DNT or (D)  $CD8^+ \alpha\beta$  T cells was compared in the IT, CHB, and HC groups. CHB, chronic hepatitis B; DNT cells, double-negative T cells; HC, healthy controls; IT, immune tolerant carriers. (TIF)

**Figure S5  $\gamma\delta$  DNT cell-mediated suppression of cytokine production by core peptide-stimulated  $CD8^+ \alpha\beta$  T cells is partially mediated by NKG2A.** The plots were gated on  $CD8^+ \alpha\beta$  T cells. DNT cells, double-negative T cells. (TIF)

**Figure S6 Strategy for gating the  $\alpha\beta$  DNT cells and  $\gamma\delta$  DNT cells from LIL.** DNT cells, double-negative T cells; LIL, liver-infiltrating lymphocytes. (TIF)

**Table S1 The GenBank accession numbers of the sequences used to identify a panel of 26 18-mer peptides overlapping by 8 or 10 residues and covering the full HBV core open reading frame.** (DOC)

**Table S2 The amino acid sequence of core peptides.** (DOC)

**Table S3 Spearman’s correlation analyses showing associations between the frequencies of  $\gamma\delta$  DNT cells and the clinical characteristics of the CHB patients at baseline (n = 51).** (DOC)

**Table S4 Clinical characteristics of subjects in the cohort receiving telbivudine therapy at 104 Weeks.**



(DOC)

**Methods S1 Entry criteria for study subjects.**

(DOCX)

**Method S2 Isolation of peripheral blood mononuclear cells and liver-infiltrating lymphocytes.**

(DOCX)

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**Author Contributions**

Conceived and designed the experiments: QL SM JH. Performed the experiments: QL JG ZH XH XJ YL. Analyzed the data: QL MZ JS XZ WA. Contributed reagents/materials/analysis tools: QL JG ZH XH XJ YL WA. Wrote the paper: QL WA JH.

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