RESEARCH ARTICLE

Enhanced Functions of Peripheral γδ T Cells in Chronic Hepatitis B Infection during Interferon α Treatment In Vivo and In Vitro

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Abstract

Background
γδ T cells play an important role in infectious, autoimmune, or neoplastic diseases. Here, a study was conducted to investigate the dynamic changes in phenotype and function of peripheral γδ T cells in patients with chronic hepatitis B (CHB) during pegylated-interferon (pegIFN)-α treatment, and to explore their roles in IFN-α therapy.

Methods
Total 15 CHB patients with pegIFN-α therapy and 6 healthy controls (HC) were enrolled in this study. Flow cytometry was used for the study of frequency of peripheral γδ T cells, subtypes, effector or memory γδ T cells, and also the IFN-γ+, TNF-α+, CD107a+ or Granzyme B+ γδ T cells in 10 patients at week 0, 4, 8, 12, 24, 36 and 48 of treatment. Another 5 CHB patients and 6 HC were recruited for the γδ T cell isolation, and gene expression in γδ T cells was evaluated before or after IFN-α treatment in vitro.

Results
Although γδT cells decreased in CHB patients during pegIFN-α therapy, their capacities to produce TNF-α and to express CD107a were enhanced. More effector γδT cells (CD27-CD45RA+) were found in the response group than in non-response group. Furthermore, IFN-α boosted the expression of Mx2 and cytokine genes in γδT cells from CHB patients in vitro.

Conclusion
IFN-α could enhance the cytokine production or cytotoxicity potential of γδT cells in vivo and in vitro. The enhanced function of γδT cells might contribute to the effect of IFN-α treatment.
Introduction

Chronic hepatitis B (CHB) is a serious public health problem in Eastern Asia. The disease is caused by persistent infection with hepatitis B virus (HBV), and has many complex clinical manifestations [1,2]. To date, interferon (IFN)-α is one of the main antiviral drugs against HBV, which has potential advantages compared to the nucleoside analogs including a lack of drug resistance, a finite and defined treatment course, and a higher likelihood of hepatitis B surface antigen (HBsAg) clearance. After about 10 years of pegylated-interferon (pegIFN)-α-based therapy for hepatitis B, pegIFN-α has attracted increased interest due to its more effective antiviral activity than standard IFN-α [3,4].

It is well established IFN-α induces anti-viral proteins and mediates the immune response. First, after interacting with the cell surface receptors, IFN-α activates the JAK-STAT signaling pathway. This results in transcription of interferon-stimulated genes (ISGs) and expression of proteins (protein kinase R, 2′-5A oligosynthetase/RNAse L system, or the Mx system), which induce antiviral activity. Second, and perhaps more importantly, IFN-α directly or indirectly modulates the immune system including activation of nature killer (NK) cells, B cells, CD8+T cells, or production of some inflammatory cytokines[5,6].

In recent years, an increasing number of studies have shown that innate immune cells, especially NK cells, play an important role in the IFN-α treatment of viral hepatitis. Those studies have revealed that during IFN-α treatment, the specific T-cell response is suppressed but NK cells proliferate and are activated. Activation of NK cells in the early stage of IFN-α treatment is associated with the control of hepatitis C virus (HCV) infection [7,8].

γδ T cells, one type of innate immune cells, share many aspects of phenotype and function with NK cells. Similar to NK cells, γδ T cells express NKG2D, CD56 or other NK markers, exert strong cytotoxicity, and secrete different types of cytokines. As reported recently, γδ T cells have multifaceted immunological functions and might be a good immunotherapeutic tool for infectious diseases and tumors [9]. These cells display their activity either through directly affecting microbial growth, destroying the infected or transformed cells, or by modulating other immune functions, such as T helper (Th)1 immune response polarization, B cell activation, or dendritic cell maturation [10,11]. And Cimini’s study has shown that IFN-α improves phosphoantigen-induced Vγ9Vδ2 T cell production of IFN-γ during chronic HCV infection [12].

In our previous study, we found that the proportion of total γδ T cells increased in the blood of CHB patients, but the frequency of Vδ2 T cells decreased [13,14]. In this study, we further investigated the change in γδ T cells in CHB patients treated with IFN-α, and explored the possible effect of γδ T cells in the treatment of CHB with IFN-α.

Materials and Methods

Patients

Total fifteen treatment-naive patients with CHB were selected from the Department of Infectious Diseases, Second Affiliated Hospital of Chongqing Medical University, China, during August 2011 to May 2013. All enrolled patients met the criteria for treatment with IFN-α, including serum alanine aminotransferase (ALT) level 80–400 U/L (2~10-fold the upper limit of normal), total bilirubin (TB) normal, HBV-DNA load 10⁵–10⁸ copies/mL, and positive HBsAg and hepatitis B e antigen (HBeAg) for at least six months. Patients were excluded if they were co-infected with other hepatitis viruses or had other liver diseases, such as hepatocellular carcinoma, autoimmune disease, or alcoholic hepatitis. Six healthy age-matched controls (HCs) were also recruited. In these 15 CHB patients, ten of them were recruited for the dynamic observation of peripheral γδ T cells during IFN-α treatment for 48 weeks. The other five
patients before IFN-α treatment and six HCs were blood sampled for γδ T cell sorting and IFN-α treatment in vitro. The characteristics of all these subjects are shown in Tables 1 and 2. Peg-IFN-α-2a (Pegasys; Hoffman-La Roche, Shanghai, China) was administered subcutaneously at a dose of 180μg once weekly for 48 weeks. At the treatment endpoint, the patients who had normal ALT level, loss of HBeAg, and >3 log10 decrease in HBV-DNA were considered to be responders.

The trial was carried out with approval of the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University, and written informed consent was obtained from all participants.

Clinical evaluation and HBV-DNA quantification

HBsAg and HBeAg were measured by the Roche electrochemiluminescence method. HBsAg and HBeAg values were evaluated by a cut-off index (COI): a COI of over 1.0 indicated a positive response. Hepatitis B surface antibody, hepatitis B e antibody and hepatitis B core antibody were detected with a commercial enzyme immunoassay kit. Serum HBV-DNA levels were

Table 1. Clinical parameters of the 10 CHB patients enrolled for the dynamic observation of γδ T cells during pegIFN-α treatment for 48 weeks.

| Sample | Sex | Age | ALT (IU/L) | HBsAg (COI) | HBeAg (COI) | HBV-DNA (log10 copies/mL) | ALT (IU/L) | HBsAg (COI) | HBeAg (COI) | HBV-DNA (log10 copies/mL) | Group |
|--------|-----|-----|------------|--------------|-------------|----------------------------|------------|--------------|-------------|----------------------------|--------|
| ID     | (M/F) | (Year) |            |              |             |                            |            |              |             |                            |        |
| 1      | M   | 26  | 104       | 6866         | 951.4       | 7.12                       | 134        | 6584         | 2.11         | 6.09                       | N      |
| 2      | M   | 38  | 107       | 793.7        | 1005        | 8.91                       | 225        | 6637         | 0.59         | 7.42                       | N      |
| 3      | M   | 28  | 395       | 1700         | 1075        | 8.81                       | 295        | 3350         | 1.27         | 7.54                       | N      |
| 4      | M   | 22  | 106       | 6098         | 185.5       | 7.81                       | 33         | 6999         | 0.29         | <3                         | R      |
| 5      | M   | 28  | 129       | 6844         | 60.74       | 6.39                       | 48         | 6530         | 0.31         | <3                         | R      |
| 6      | M   | 25  | 106       | 3469         | 1355        | 8.24                       | 46         | 7230         | 690.9        | 5.63                       | N      |
| 7      | M   | 26  | 143       | 5365         | 650.5       | 7.99                       | 34         | 17.59        | 1.00         | <3                         | R      |
| 8      | M   | 28  | 211       | 2057         | 1114        | 7.88                       | 32         | 1632         | 0.23         | <3                         | R      |
| 9      | M   | 28  | 123       | 2411         | 1076        | 8.65                       | 43         | 545.3        | 64.7         | <3                         | N      |
| 10     | M   | 21  | 413       | 2179         | 784.9       | 8.22                       | 42         | 4062         | 0.19         | 4.68                       | R      |

Abbreviations: CHB, chronic hepatitis B; pegIFN-α, pegylated-interferon α; ALT, alanine aminotransferase; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; COI, cut-off index.

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Table 2. The clinical parameters of 5 CHB patients with and 6 HCs enrolled for the peripheral γδ T cells isolation and IFN-α stimulation in vitro (Mean±SD).

| Group | CHB | HC |
|-------|-----|----|
| N     | 5   | 6  |
| Sex(M/F) | 3/2 | 3/3 |
| Age(year) | 32.8±5.1 | 27.5±2.6 |
| ALT(IU/L) | 109.4±34.6 | 15.8±9.1 |
| AST(IU/L) | 73.4±20.5 | 22.3±5.3 |
| TB(μmol/L) | 14.5±3.3 | 11.6±2.3 |
| HBV-DNA(copies/mL) | 7.7±0.6 | 0 |

Abbreviations: CHB, chronic hepatitis B; HC, healthy control; ALT, alanine aminotransferase; AST, Aspartate Aminotransferase; TB, total bilirubin.

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determined by Roche real-time fluorescent quantitative polymerase chain reaction (PCR) (Lightcycler; Hoffman-La Roche, Swiss). The detection limit of this HBV-DNA assay was 1,000 copies/mL. All liver function parameters were evaluated using an automatic biochemical analyzer. The normal level of ALT, AST or TB was 6–48 IU/L, 10–45 IU/L, or 3–21 μmol/L.

Detection of γδ T cell phenotype by fluorescence activated cell sorter (FACS)

Blood samples from 10 CHB patients were collected for determination of the phenotype and immune function of γδ T cells before and during IFN-α treatment at 4, 8, 12, 24, 36 and 48 weeks. The phenotypes T cell receptor (TCR) γδ, Vδ1, Vδ2, CD45RA or CD27 were detected according to the manufacturers’ instructions (BD Biosciences, La Jolla, CA, USA). The following fluorochrome-conjugated monoclonal antibodies (mAbs) were used: Peridinin chlorophyll (PerCP)-conjugated anti-CD3 mAb (clone SK7), allophycocyanin (APC)-conjugated anti-TCR γδ mAb (clone B1), phycoerythrin (PE)-conjugated anti-Vδ2 mAb (clone B6), PE-conjugated anti-CD45RA mAb, and fluorescein isothiocyanate (FITC)-conjugated anti-CD27 mAb, and were purchased from BD Biosciences (La Jolla, CA, USA). FITC-conjugated anti-Vδ1 mAb was purchased from Thermo Fisher Scientific (clone TS8.2; Rockford, IL, USA). The appropriate volume of antibody was added to 100 μL fresh peripheral anticoagulated blood and incubated for 30 minutes in the dark at 4°C. Erythrocytes were lysed using BD FACS lysing solution, and cells were washed in PBS supplemented with 1% fetal calf serum (FCS). Stained cells were immediately analyzed using the FACS Canto II flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA). Data were analyzed using FACSDiva 2.0 software (BD Immunocytometry Systems).

Intracellular cytokine staining (ICS)

At the same time as phenotype detection, expression of intracellular IFN-γ, tumor necrosis factor (TNF)-α, CD107a or Granzyme B was determined by ICS. Total leukocytes were obtained from whole blood by lysis of erythrocytes using PBS containing 0.85% NH₄Cl. The cells were adjusted to ~5×10⁶/mL in RPMI 1640 culture medium supplemented with 10% FCS and stimulated with 100 ng/mL phorbol myristate acetate (PMA) plus 1 μg/mL ionomycin for 4 hours, in the presence of the secretion inhibitor monensin (BD Biosciences). Cells were stained with APC-conjugated anti-TCR γδ mAb, followed by washing with PBS, and fixation in 4% paraformaldehyde. Stained cells were permeabilized using 0.1% saponin (Sigma, St. Louis, MO, USA). Cells were incubated with FITC-conjugated anti-IFN-γ (clone 4S.B3; eBioscience, San Diego, CA, USA), PE-conjugated anti-TNF-α (clone Mab11; BD Biosciences), PE-conjugated anti-CD107a (clone H4A3; BD Biosciences), or FITC-conjugated anti-Granzyme B (clone GB11; BD Biosciences) for 30 minutes at 4°C. Finally, cells were washed with PBS, and analyzed using the FACS Canto II flow cytometer (BD Immunocytometry Systems). Data were analyzed using FACSDiva 2.0 software (BD Immunocytometry Systems).

TCR γδ T cell isolation

Blood from five CHB patients before IFN-α treatment and six HCs (clinical characters showed in Table 2) was collected to generate γδ T cells from the buffy coat, and to determine the gene expression of γδ T cells when stimulated with IFN-α in vitro. Peripheral blood mononuclear cells (PBMCs) were obtained from 30 mL fresh peripheral anticoagulated blood by Ficoll separation (Ficoll-Paque), and TCR γδ T cells were isolated from the PBMCs by positive selection using the TCR γδ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The isolations were performed according to manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach,
Germany). Purity of the sorted TCR γδ T cells was determined by flow cytometry analysis. The results showed >95% purity of γδ T cells could be obtained in the acquired fractions.

Analysis of mRNA expression of γδ T cells stimulated with IFN-α by quantitative RT-PCR

The sorted γδ T cells were divided into two parts: one for IFN-α treatment, and the other for the controls. Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/mL penicillin and 100μg/mL streptomycin in 5% CO₂ at 37°C with or without recombinant human IFN-α-2a (1,000 U/mL) (Hoffman-La Roche, Shanghai, China) for 4 hours. Cellular RNA was isolated with Zymo mini RNA isolation kit (Zymo Research Corporation, Irvine, CA, USA). About 150 ng RNA was reverse transcribed with oligo(dT) primer using the PrimeScript RT Reagent Kit with gDNA Eraser (Perfect Real Time) (Takara Bio, Japan). Real-time PCR was performed using SYBR Green quantitative PCR by SYBR Premix Ex Taq II (Tli RnaseH Plus) (Takara Bio, Japan). Primer sequences are listed in Table 3. All the samples were detected in triplicate with 7300 PCR Detection System (Applied Biosystems, Foster City, CA, USA) for 40 cycles of 5 s at 95°C followed by 31 s at 60°C. GADPH was used as an internal control. Data were analyzed using the 2−ΔΔCt method: gene expression was represented by Ct value, ΔCt = Ct(gene)-Ct(GAPDH), ΔΔCt = ΔCt(gene of HBV patients)-average ΔCt(gene of healthy controls), then one ΔΔCt would be used to produce the 2−ΔΔCt value, which was displayed as a fold change in gene expression of γδ T cells from CHB patients, with or without IFN-α treatment versus healthy controls.

Statistical analysis

All data were analyzed by SAS version 9.2 (SAS Institute, Cary, NC, USA). Repeated measures analysis of variance and simple-effects analysis were used to compare the change in proportion of total γδ T cells or the certain subtype of γδ T cells from responders and non-responders, or from the different treatment time points. A two-sided p value <0.05 was considered as

| Gene     | Gene primers          | primers     |
|----------|-----------------------|-------------|
| Ift1 (NM_001548) | forward: 5'-GCCTTGCTGAAGTGAGGAGAA-3' | reverse: 5'-ATCCAGGGCATAGCAAGACTC-3' |
| Isg15 (NM_005101) | forward: 5'-CTCTGAGATCTCTGGAGGAGA-3' | reverse: 5'-AAGGTCCAGCAGAACAGGTGGT-3' |
| Mx1 (NM_002462) | forward: 5'-GGCTGTGGATCAGGCTCGAGA-3' | reverse: 5'-CACAAAGCCTGGGAGGTTCTAC-3' |
| Mx2 (NM_002463) | forward: 5'-AAAAGCACCCCTGTGAGGAC-3' | reverse: 5'-GTGATCTTGACGGCTGAGGCT-3' |
| Cxcl10 (NM_001565) | forward: 5'-GGTGAGAGAGATGCTCTGAATCC-3' | reverse: 5'-GTCCATCTGTGAAGCACTCGA-3' |
| Ifnγ (NM_000619) | forward: 5'-GAGCTGGAGACTACAGGAAAGC-3' | reverse: 5'-TGCTTGAGGACTCTCACTG-3' |
| Tnfα (NM_000594) | forward: 5'-CTTCCGATGCTAGCTGTAC-3' | reverse: 5'-ATGGCGTACAGGCTGTCATC-3' |
| Il10 (NM_000572) | forward: 5'-TCTCCAGATGCTCAGCAGA-3' | reverse: 5'-TCAGAAGGCTGCAACAACC-3' |
| Il12a (NM_000882) | forward: 5'-TGCCCTCACCACCTCCAATAAC-3' | reverse: 5'-CAATCTCTGAGAATGCAAGG-3' |

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significant. For the comparison of gene expression *in vitro*, the gene expression from HCs was set as "1", and the significant change in gene expression from CHB patients was set as at least two fold (upregulation) or 0.5-fold (downregulation).

**Results**

The proportion of γδ T cells in CHB patients decreased during pegIFN-α therapy

FACS gating and Figures of γδ T, Vδ1 or Vδ2 T cells were shown in Fig. 1A-D. The proportions of γδ T cells from responders or non-responders all declined consistently during treatment, from 12.9% or 11.8% before treatment to 6.6% or 7.2% at week 48 in treatment (Fig. 1E). These differences between responders and non-responders at each time point were not significant (p>0.05), but the proportion of γδ T cells at week 36 or 48 was significantly lower than that at week 0, 4, 8 and 12 (p<0.01). The proportion of Vδ1 T cells from responders was higher than in non-responders at every time point, but no significant differences were shown between the two groups (p>0.05). The proportion of Vδ1 T cells from responders was 21.1% at baseline, then it decreased slightly to 17.3% at week 36, but it increased to 22.7% at week 48. (Fig. 1F). There was no significant difference in the proportion of Vδ1 T cells between each time point in responders or non-responders (p>0.05). The proportion of Vδ2 T cells from responders decreased sharply from 75.8% before treatment to 64.0% at the end of treatment, (Fig. 1G) and the difference between week 48 and week 0, 4, 24 or 36 was significant (p<0.05). However, the differences between the responders and non-responders were not significant (p>0.05).

Responders had higher percentage of effector γδ T cells than non-responders during pegIFN-α treatment

CD45RA and CD27 molecules were used to investigate the effector or memory phenotype of γδ T cells. The γδ T cells could be divided as the naïve cells (CD45RA⁺CD27⁺), effector cells...
(CD45RA⁺CD27⁺), effector memory cells (CD45RA⁺CD27⁻), or central memory cells (CD45RA⁻CD27⁺) (FACS Fig. 2A-C). The proportion of naïve γδ T cells increased from 32.1% to 40.7% in the responders and 39.2% to 48.1% in the non-responders during 24 weeks treatment, and then declined to 39.1% in the responders and 36.6% in the non-responders at week 48 (Fig. 2D). Although the proportion of naïve γδ T cells in the non-responders was higher than in the responders, no significant difference was found between the two groups or among the time points (p > 0.05). The percentage of effector γδ T cells at the outset of treatment was 22.6% and 41.3% for non-responders and responders, respectively, and increased to 27.9% and 47.9% after 8 weeks, and finally dropped to 18.4% and 27.8% (Fig. 2E). The frequency of effector γδ T cells from responders was significantly higher than from non-responders at week 4 and 8 in treatment (p < 0.05). The non-responders patients showed a significantly higher proportion of effector memory γδ T cells (Fig. 2F) than the responders at each time point from week 0 (35.7% vs. 17.1%), week 12 (23.3% vs. 6.6%), to week 48 (41.0% vs. 23.4%) (p < 0.01). There were few central memory γδ T cells detected in the peripheral blood and the percentage

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fluctuated during treatment (Fig. 2G), and no significant differences were shown between the two groups or among the time points ($p > 0.05$).

Increased percentage of TNF-α- and CD107a-producing γδ T cells were found in CHB patients during pegIFN-α treatment

The most important properties of functional γδ T cells include cytokine production and cytotoxicity. Fig. 3A-D shows IFN-γ-, TNF-α-, Granzyme-B- and CD107a- producing γδ T cells, respectively. In the responders, the percentage of IFN-γ⁺γδ T cells declined from 70.5% at baseline to 39.2% at week 12, then increased to 66.3% at week 48 (Fig. 3E). There was a sharp decrease in the frequency of IFN-γ⁺γδ T cells in non-responders from 66.8% at 0 weeks to 27.8% at week 4, followed by an increase to 70.0% at the end of treatment. A significant difference in the percentage of IFN-γ⁺γδ T cells at week 4 was shown between responders and non-responders ($p < 0.01$). For each group, there was a significant difference between week 12 and
the other times (p<0.01). The percentage of TNF-α+γδ T cells increased from 13.6% or 13.7% at week 0 to 36.0% or 42.6% at week 48 for responders or non-responders, respectively (Fig. 3F). There were no significant differences between these two groups at each point (p>0.05), but the frequency at 48 weeks was significantly higher than at other time points for each group (p<0.01).

The percentage of Granzyme-B+γδ T cells from the responders peaked as 63.7% at week 36 (Fig. 3H), and it was significantly higher than that from non-responders (p<0.05). However, no significant differences were found between each time point in each group (p>0.05). As shown in Fig. 3G, the frequency of CD107a+γδ T cells increased markedly from 10.1% or 32.5% at week 0 to 82.4% at week 8 or 83.3% at week 12 in responders or non-responders, respectively. The frequency at week 0 or 24 from the non-responders was significantly higher than that from the responders (p<0.05).

Mx2 expression in IFN-α-treated γδ T cells from CHB patients showed significantly higher than that from HC

We investigated changes in gene expression when γδ T cells were directly treated with IFN-α. Some IFN-α-induced genes, Ifit1, Isg15, Mx1 and Mx2, were detected (Fig. 4A-B). Among these genes, Isg15 and Mx1 expression in untreated-γδ T cells from CHB patients was higher (but not significantly) than that from HCs. When compared to IFN-α-untreated γδ T cells, expression of these four genes increased in the IFN-α-stimulated cells from HCs or CHB patients (data not shown). When compared to IFN-α-stimulated γδ T cells from HCs, Mx2 gene expression from CHB patients showed a significant increase at 2.4-fold higher than that from HCs.

Elevated mRNA expression of Ifng, Tnfa, Il10, Il12 and Cxcl10 gene in γδ T cells from CHB patients after IFN-α treatment

The expression of some cytokine or chemokine genes in γδ T cells was detected before and after IFN-α treatment (Fig. 4C-D). Before IFN-α stimulation, expression of Ifng, Tnfa, Il10 and Cxcl10 from CHB patients increased, but Il12a decreased compared to that of healthy controls. IFN-α enhanced expression of these genes in HCs and CHB patients, with a 2-4-fold increase in IFN-α-treated γδ T cells for Ifng, Tnfa, Il10 and Il12 expression, and about 300-fold increase for Cxcl10 expression. In comparison with IFN-α-stimulated γδ T cells from HCs, Il10 and Cxcl10 gene expression from CHB patients displayed a significant increase compared with that from HCs.

Discussion

CHB is a serious disease, especially in Eastern Asian countries. The immune disorder in these patients is characterized as specific immunological inadequacy to HBV and nonspecific hyper-immune reaction to hepatocytes. As a result, persistent HBV infection can be accompanied by persistent liver inflammation [15,16]. To date, IFN-α is one of the most important anti-HBV treatments, whose mechanism includes a direct influence on HBV RNA, capsid, or covalently closed circular DNA stability in hepatocytes, and more importantly, it can exert an immunoregulatory function to improve CD4+ and CD8+ T cells, NK cells or other immune cells [6-8]. However, few studies have demonstrated the changes in γδT cells during IFN-α treatment.

γδT cells are one type of innate immune cells, and their functions include cytotoxicity, cytokine secretion, antigen presentation, and crosstalk with other immune cells. In the process of infection, autoimmune reaction, or tumor development, γδT cells are activated to stimulate, regulate, or even suppress the immune response [17-19]. γδ T cells are mainly distributed in
the epithelial tissues, such as the gut, skin, lung, or liver. The percentage of total γδT cells in peripheral blood is <10% of T cells. And the major (>70%) subtype of peripheral γδT cells is Vδ2T cells. However, in skin, gut or some other epithelial tissues, the subtype of Vδ1T cells constitutes the major population.

In the present study, dynamic changes in the percentage of γδT, Vδ1T or Vδ2T cells were detected. The percentage of total γδT cells decreased during treatment with IFN- in responders and non-responders. It was particularly interesting to find that all the percentages of Vδ1T cells during treatment in the responders were higher than in the non-responders. In our previous study, increased percentages of circulating Vδ1T cells and decreased Vδ2T cells were found in patients with CHB [13]. These data imply some important role of Vδ1T in anti-HBV immunity. Vδ1T cells were reported to recognize lipids presented by CD1c/d, MICA/B, or some stress-induced self-antigens. This differs from Vδ2T cells that uniquely respond to non-peptide alkylphosphates [20,21]. Expansion of Vδ1T cells has been shown in infection with HIV-1 and cytomegalovirus, and expansion of Vδ2T cells occurs in infection with
mycobacterial species, *Listeria monocytogenes*, and Epstein-Barr virus. Certain antigens expressed on tissue cells might result in expansion of different subtypes of γδT cells [22,23].

According to the surface expression of CD45RA and CD27, human γδT cells can be divided into four subsets: naïve (CD45RA⁺CD27⁺), central memory (CD45RA⁻CD27⁺), effector memory (CD45RA⁺CD27⁻), and effector cells (CD45RA⁺CD27⁻) [24–27]. In our study, the circulating γδT cells were largely composed of naïve, effector memory, and effector cells. Few central memory γδT cells could be detected in the circulating γδT cells. Our results also showed a higher percentage of late-stage effector (CD45RA⁺CD27⁻) γδT cells in the responders than non-responders. These results demonstrated that the γδT cells in the responders might have greater potential to respond to antigen and expand to the end stage of effector cells.

IFN-γ and TNF-α were the main cytokines produced in the activated γδT cells [28, 29]. We found that the production of IFN-γ decreased during the first 12 weeks of treatment and then it gradually increased, and there was an increasing trend in TNF-α production throughout the treatment course. IFN-α could enhance RNA expression of IFN-γ gene in γδT cells *in vitro*. The results revealed that interaction of γδT cells with other immune cells could influence IFN-γ production. It also implies that γδT cells are heterogeneous and different types of γδT cells have different patterns of cytokine production. So, the changing trend of IFN-γ production was not in accordance with that of TNF-α production.

CD107a (lysosomal-associated membrane protein-1) has been described as a functional marker of killer cell activity on CD8⁺ T, NK, γδT, or other immune cells [30]. During the first 12 weeks of IFN-α treatment, the percentage of CD107a⁺ γδT cells increased dramatically from 10.1% (average) to 82.4% in the responders and 32.5% to 83.3% in the non-responders. Then, it decreased to 50.7% in the responders and 65.1% in the non-responders. These results demonstrated that the γδT cells in the responders might have greater potential to respond to antigen and expand to the end stage of effector cells.

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In addition, to observing the direct effect of IFN-α on γδT, the γδT cells were isolated from CHB patients and HCs for *ex vivo* experiments. Our results show IFN-α increased expression of all the observed genes. *Isg15, Ifit1, Mx1* and *Mx2* are among the best defined ISGs in immune cells [32]. We demonstrated that after IFN-α treatment, expression of *Ifit1* and *Mx2* in CHB patients increased very much and became higher than that in HCs, especially the significant increase for *Mx2*. Although MX1 and MX2 are closely related proteins, they exert different functions in different viral infections. Goujon *et al.* have shown that MX2 suppresses HIV-1 infection, but MX1 does not. In contrast, MX1 is an inhibitor of influenza A virus, but MX2 is ineffective [33]. For HBV infection, MX1 protein inhibits viral replication by interaction with hepatitis B core antigen [34]. Our results showed that the different pattern of *Mx1* and *Mx2* RNA expression in γδT cells from CHB patients before or after IFN-α stimulation.

γδT cells have a complicated cytokine profile. When activated, they secrete Th1 type cytokines (IFN-γ and TNF-α), or Th2 type cytokines (interleukin (IL)-4 and IL-10), and they produce IL-12 or other cytokines similar to antigen presenting cells. Also, chemokines are produced in γδT cells [35]. Our results showed that before IFN-α stimulation, expression of *Ifng*, *Tnfa* and *Il10* increased, but the expression of *Il12a* and *Cxcl10* decreased. Once stimulated with IFN-α, the Th1 and Th2 cytokines were all enhanced in CHB patients. These results suggested that the γδT cells from CHB patients were heterogeneous and had different functions. This study also showed that during PegIFN-α therapy, the function of γδ T cells was enhanced, and these cells might transfer into liver tissue and exert their effects. So it appeared that decreased percentage of γδ T cells and increased function could exist simultaneously.
In conclusion, we demonstrated that, although the proportion of total γδ T cells decreased during IFN-α treatment, their functions of TNF-α production and CD107a expression were enhanced. The responders had more effector γδ T cells than the non-responders had. In vitro, IFN-α boosted many ISGs in γδ T cells. However, it was not the same in vivo, due to the complicated factors affecting the function of γδ T cells.

Author Contributions
Conceived and designed the experiments: MC HR. Performed the experiments: MC PH HP. Analyzed the data: MC YL HH. Contributed reagents/materials/analysis tools: PH NL DZ HR. Wrote the paper: MC YL.

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