Preserved Function of Circulating Invariant Natural Killer T Cells in Patients With Chronic Hepatitis B Virus Infection

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Abstract: To date, the role of invariant natural killer T (iNKT) cells in chronic hepatitis B virus (HBV) infection is not fully understood. In previous reports, iNKT cells were identified by indirect methods. However, discrepancies regarding the prevalence and function of iNKT cells during HBV infection were observed.

In this study, we have devised a direct, highly specific CD1d tetramer-based methodology to test whether patients with HBV infection have associated iNKT-cell defects. In our study, a total of 93 chronic HBV-infected patients and 30 healthy individuals (as control) were enrolled. The prevalence of iNKT cells, their cytokine producing capacity, and in vitro expansion were determined by flow cytometric analysis with CD1d tetramer staining.

Our observation demonstrated that there was no significant difference in circulating CD1d-tetramer positive iNKT cell numbers between HBV-infected patients and healthy controls. The capacity of iNKT cells to produce IFN-γ or IL-4 as well as their in vitro expansion was also comparable between these 2 groups. However, among chronic HBV-infected patients, a decrease in iNKT cell-number was observed in chronic hepatitis B (CHB) and cirrhosis patients in comparison to that in immune tolerant (IT) patients.

These results indicated that patients with chronic HBV infection may have normal prevalence and preserved function of circulating iNKT cells. And antiviral therapy with nucleotide analogue does not alter the frequency and function of circulating iNKT cells in chronic Hepatitis B patients.

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Abbreviations: α-GalCer = alpha-galactosylceramide, ALT = alanine aminotransferase, APC = antigen presenting cells, CHB = chronic hepatitis B, CTL = cytotoxic lymphocyte, DC = dendritic cell, HBeAg = hepatitis B e antigen, HBsAg = hepatitis B surface antigen, HBV = hepatitis B virus, IC = immune carrier, IL = interleukin, iNKT = invariant natural killer T, IR = immune reactive, IT = immune tolerant, PBMC = peripheral blood mononuclear cell.

INTRODUCTION

Despite the introduction of effective hepatitis B vaccine, more than 360 million people are chronically infected with hepatitis B virus (HBV) worldwide. Most adults can mount an effective immune response to eliminate HBV after infection. However, HBV infection tends to become chronic when infection takes place during childhood or infancy and can lead to liver cirrhosis and cancer. Generally the prognosis of HBV infection depends on the intensity of the host adaptive immunity. An abundant HBV specific polyclonal cytotoxic lymphocyte (CTL) response can effectively control HBV infection, while a weak monoclonal CTL response results in chronicity. The effectiveness of CTL responses to HBV infection is sometimes dictated by the microenvironment in the liver which is largely regulated by the innate immune response.

Invariant natural killer T (iNKT) cells are a subset of T lymphocytes recognizing lipid-based antigens in context with the MHC-like molecule CD1d. Therefore, iNKT cells can link the innate and adaptive immune responses. The T cell receptors expressed by iNKT cells are highly conserved. These T cell receptors are composed of Vα24-Jα18 segments paired with Vβ11 in humans and nonhuman primates and Vα14-Jα18 segments paired with one of Vβ8.2, Vβ7, or Vβ2 in mice. Upon activation, iNKT cells initiate immune responses through their unique ability to activate antigen presenting cells (APC) (eg, dendritic cells [DCs]), natural killer cells, and CD8+ T cells through cytokines produced by activated iNKT cells or direct cell-to-cell contact. Therefore, the function of iNKT cells affects early immune responses to many diseases including viral infection.

Although iNKT cells are important for immune-responses against viral infections,11,12 their role in responses against HBV viral infection is controversial. In an HBV transgenic animal model, it has been found that iNKT cells control HBV replication through induction of hepatic IFN-α/β/γ and natural killer cell activation. And activation of iNKT cells by α-galactosylceramide (α-GalCer) can enhance HBV-specific CTL responses following hepatitis B surface antigen (HBsAg)-immunization.14 iNKT cells have been reported to decrease in hepatitis B e antigen (HBeAg) positive chronic hepatitis B (CHB) patients, and the decreased iNKT cell numbers were not associated with viral load.15,16 However, de Lalla et al17 showed that the numbers of iNKT cells in chronic HBV-infected patients with high viral load were comparable to those in
healthy controls (HCs). Therefore, the role of iNKT cells in the immunological pathogenesis of chronic HBV infection has not been clarified so far.

Until now, staining with CD1d tetramer loaded with lipid antigen has been a sensitive and accurate method for the identification of iNKT cells. To investigate the role of iNKT cells in the development of chronic HBV infection, we analyzed iNKT cells and their function in chronic HBV infected patients with tetramer staining. The results revealed that the fraction of iNKT cells among peripheral blood mononuclear cells (PBMCs) in chronic HBV-infected patients was not statistically different from that in healthy donors. However, among chronic patients, a decrease in iNKT cell-number was observed in patients with CHB and cirrhosis in comparison to that in immune tolerant (IT) patients. Reduction of viral load by tenofovir (TDF) antiviral treatment did not rescue iNKT cell numbers. The functions of iNKT cells were comparable between HBV-infected patients and HCs. Our results indicate that iNKT cells are mainly influenced by the inflammatory circumstance caused by HBV infection rather than HBV itself.

MATERIALS AND METHODS

Patient Populations

A total of 75 treatment naïve patients with chronic HBV infection (in various infectious phases) and 18 cirrhosis patients participated in this study between 2010 and 2012. Thirty age and gender-matched healthy individuals were recruited as HCs. The ethics committee at Huashan Hospital, Fudan University granted approval for all aspects of this study. Blood samples were obtained with informed written consent from healthy individuals and HBV-infected patients. The inclusion criterion for chronic HBV infection was defined by the presence of detectable HBsAg for at least 6 months. Individuals with concurrent HCV, HDV, HIV, autoimmune liver disease, or alcoholic liver disease were excluded.

Based on the European Association for the Study of the Liver clinical practice guidelines (2009), patients were classified into 4 groups according to the natural phases of chronic HBV infection as follows: The IT phase was defined by HBeAg positive, high viral load (usually >10^7 IU/mL), normal or low level of alanine aminotransferase (ALT), mild or no liver necro-inflammation, and no or slow progression of fibrosis. The immune reactive (IR) phase was defined by: HBeAg positive, elevated viral load, increased or fluctuating levels of ALT, moderate or severe liver necro-inflammation, and more rapid progression of fibrosis. The inactive carrier (IC) state was defined by HBeAg negative and anti-HBeAg antibody positive, very low or undetectable serum HBV DNA levels and normal ALT. The HBeAg-negative CHB (HBeAg-negative hepatitis) phase was defined by: HBeAg negative with fluctuating levels of serum HBV DNA and ALT. Cirrhosis was diagnosed by imaging, biochemical, and histological examination. In this study, both IR and HBeAg-negative hepatitis were classified into the CHB group and the cut-off value for normal ALT was 50 U/L. Patient characteristics are summarized in Table 1. A flow chart for subject enrollment and study design is shown in Figure 1.

Isolation and In Vitro Stimulation of PBMCs

PBMCs were isolated from heparinized whole blood by Ficoll density gradient centrifugation on Lymphoprep (AXIS-SHILD, Oslo, Norway). Cells were resuspended in RPMI 1640 medium (GIBCO, Carlsbad, CA) with 10% fetal bovine serum and 1% penicillin–streptomycin at a concentration of 10^6 cells/mL. Then these cells were cultured with 50 ng/mL phorbol myristate acetate (PMA) (Sigma, St. Louis, MO) and 200 ng/mL ionomycin (Sigma) at 37°C for 5 hours in the presence of 0.7 μL/mL monensin during the last 1 hours.

Generation of α-GalCer Loaded Dendritic Cells for In Vitro iNKT Stimulation

α-GalCer loaded DCs were produced as described previously. Briefly, CD14+ monocytes were separated from PBMCs with anti-CD14 microbeads (Mitenyi, Bergisch Gladbach, Germany) and cultured in Iscove’s Modified Dubecco’s Medium (IMDM) (GIBCO, Carlsbad, CA) with 2% human serum. Granulocyte macrophage colony-stimulating factor (800 U/mL) (Peprotech, Rocky Hill, NJ) and interleukin (IL)-4 (1000 U/mL) (Peprotech) were added, and cells were incubated at 37°C for 5 days. At day 5, α-GalCer (100 ng/mL) was added. At day 6 of culture, tumor necrosis factor-α (10 ng/mL) (Peprotech) was added to trigger the maturation of DCs. DCs were harvested at day 8 of culture to be used for the stimulation of iNKT cells in vitro.

In Vitro Expansion of iNKT Cells

PBMCs from chronic HBV-infected patients were mixed with α-GalCer pulsed DCs at the ratio of 10:1, then co-cultured in AIM-V medium (GIBCO, Carlsbad, CA) with 10% human serum and IL-15 (10 ng/mL) (Peprotech). IL-2 (100 U/mL) (Peprotech) was added on the second day after co-culture. After 14 days of co-culture, iNKT cells were restimulated by

| TABLE 1. Clinical Characteristics of Subjects Enrolled in This Study |
|---------------------------------------------------------------|
| Characteristic | HC | IT | CHB | IC | Cirrhosis |
|----------------|----|----|-----|----|-----------|
| Number         | 30 | 18 | 38  | 19 | 18        |
| Gender (M/F)   | 18/12 | 12/6 | 23/15 | 11/8 | 13/5 |
| Age (y)        | 27 (23–57) | 25 (13–33) | 33 (19–63) | 41 (20–57) | 54 (37–67) |
| ALT (U/L)      | 18 (8–30) | 30.5 (11–45) | 127.5 (33–847) | 27 (19–40) | 38 (21–123) |
| HBV-DNA (log_{10}copies/mL) | ND | 7.89 (6.76–8.51) | 7.85 (4.11–10.26) | <3–3.66 | <3–5.99 |
| HBsAg (log_{10}U/mL)  | ND | 4.63 (4.34–4.87) | 3.5 (2.07–5.28) | 3.16 (0.19–4.04) | 2.65 (0.87–3.63) |
| HBeAg (+/−)   | 0/30 | 18/0 | 22/16 | 0/19 | 6/12 |
| HBeAb (+/−)   | 0/30 | 0/18 | 16/22 | 19/0 | 12/6 |

Data were shown as median (range). CHB = chronic hepatitis B, HC = healthy control, IC = inactive carrier, IT = immune tolerant, ND = not determined.
aGalCer-pulsed DCs.19 Expanded iNKT cells were quantified by staining with aGalCer-CD1d tetramer (kindly provided by the NIH Tetramer Facility), anti-CD3, and anti-CD4 antibodies (eBioscience, San Diego, CA).

Flow-Cytometric Analysis
PBMCs or expanded iNKT cells were harvested, washed with PBS, and stained with APC-PBS57-CD1d tetramer at 37°C in the dark for 20 minutes. Cells were then incubated with near-IR fluorescent reactive dye (Invitrogen, Darmstadt, Germany), PE-Cy7-anti-CD3 and PerCP-Cy5.5-anti-CD4 at 4°C for 20 minutes. After incubation, cells were extensively washed, fixed, and permeabilized using a BD Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA), according to the manufacturer’s instructions. These cells were finally stained with either PE-anti-IFN-γ or PE-anti-IL-4 (eBioscience) and analyzed on a BD Canto II flow cytometry.

Isolation and In Vitro Stimulation of Hepatic and Splenic Lymphocytes From HBV-Tg Mice
The male full-HBV-Tg mice with BALB/c background were purchased from Infectious Disease Center, No. 458 Hospital (China), which expresses high level of HBsAg and have detectable HBV DNA in the serum.20 The same weeks of BALB/c mice were purchased as control mice. The full-HBV-Tg mice were fed in the laboratory in accordance with Biological Safe Level 2. Animals were treated humanely, and all procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. Hepatic and splenic lymphocytes were prepared as previously described.21 The procedures of in vitro stimulation, staining and flow cytometry detection were same as mentioned before. iNKT cells were defined as CD3 and CD1d tetramer positive cells. For intracellular cytokine detection, cells were stained with APC-PBS57-CD1d tetramer and PerCP-Cy5.5-anti-CD3 and then fixed and permeabilized using BD Cytofix/Cytoperm kit. The fixed cells were then stained with PE-anti-IFN-γ or PE-anti-IL-4 and analyzed on a BD Canto II flow cytometry.

Statistical Analysis
All statistical analyses were performed using GraphPad Prism 5 software. The numbers of total or cytokine-producing iNKT cells in different groups of patients were compared by a
Kruskal-Wallis test followed by a Dunns post-test. The multiple linear regression model was used to estimate multivariate factors which influence the iNKT cell numbers, including HBsAg levels, HBV-DNA values, HBeAg status, ALT, gender, and age. Comparisons between 2 groups were conducted using Mann-Whitney test. A Friedman test followed by a Dunns post-test was used to compare iNKT cell frequency before and after antiviral therapy. A P value < 0.05 was considered to be statistically significant.

RESULTS

Circulating CD1d Tetramer-Positive iNKT Cell Counts Were Decreased in CHB and Cirrhosis Patients Compared to Those in Immune Tolerant Patients

To assess the impact of HBV on the frequency of peripheral iNKT cells, we compared the numbers of peripheral iNKT cells in 93 patients in different stages of chronic HBV infection to those in 30 HCs. Detection of iNKT cells, defined as CD1d-tetramer and CD3 double positive cells, was accomplished by flow cytometry (Figure 2A). As shown in Figure 2B, iNKT cell numbers in healthy subjects and chronic HBV infected patients were both considerably low and varied between individuals. The numbers of peripheral iNKT cells in HBV-infected patients were not significantly different from those in the IC group (P = 0.44). However, among the HBV-infected patients, the numbers of peripheral iNKT cells in IT patients (mean = 0.21%) were higher than those in CHB patients (mean = 0.14%, P < 0.05) and cirrhosis patients (mean = 0.10%, P < 0.01) (Figure 2C). The numbers of CD4⁺ iNKT and CD4⁺ iNKT cells in IT patients were also higher than those in CHB and cirrhosis patients (P < 0.05) (Figure 2D and E).

Peripheral iNKT Cells From CHB Patients Are Functionally Comparable to Those From Healthy Controls

To investigate the function of iNKT cells, we analyzed the cytokine production of iNKT cells in vitro following stimulation with PMA/ionomycin for 5 hours (Figure 3A). iNKT cells can rapidly produce a broad spectrum of cytokines such as IFN-γ and IL-4 upon activation. In our study, the major fraction of peripheral iNKT cells produced IFN-γ, irrespective of whether they were obtained from CHB patients or HCs, suggesting that IFN-γ is the dominant cytokine made by iNKT cells. The levels of cytokine IFN-γ and IL-4 production in iNKT cells from CHB patients were similar to that from HCs (Figure 3B and C).

We further analyzed the proliferative responses of iNKT cells stimulated by αGalCer-loaded DCs. Twenty cases were randomly enrolled in this study (5 each from IT, CHB, IC, and HC groups). In all subjects, iNKT cell numbers significantly increased after 14 days of culture (Figure 4A). The proliferation of iNKT cells was detected following stimulation in all groups (Figure 4A). Although the increase in iNKT cell numbers varied among individuals (2–70-fold change), we did not detect differences in fold changes of total, CD4⁺, CD4⁺ iNKT cells between CHB patients and HC (P = 0.77, 0.91, and 0.75, respectively) (Figure 4B–D).

iNKT Cell Numbers Are Not Associated With Either HBV Viral Road or HBsAg Titers

To investigate the role of iNKT cells in disease progression, we compared the numbers of peripheral iNKT cells in patients of different ages, serum ALT levels, viral copies, HBeAg status, and HBsAg titers among all 93 patients using the multiple linear regression model. As shown in Table 2, the numbers of iNKT cells were negatively correlated with age (CI = −0.0071, P = 0.003), which was consistent with the results of a previous study. And yet the levels of iNKT cells were not associated with viral load, HBsAg, ALT, HBeAg, or gender (P = 0.97, 0.81, 0.16, 0.86, and 0.22, respectively).

The Numbers of Peripheral iNKT Cells Do Not Change With Inflammation or Fibrosis

Twenty-three patients including 5 IT, 14 CHB, and 4 IC patients without cirrhosis were pathologically diagnosed by liver biopsy. Based on necroinflammation grading and fibrosis staging, patients were divided into a no/mild necroinflammation and fibrosis (G0–1S0-1) group and a moderate to severe necroinflammation and fibrosis (G2–4/S2-3) group.

Nine patients were enrolled in the G0-1S0-1 subgroup and the other 14 were enrolled in the G2-4/S2-3 subgroup. As shown in Figure 5A–C, total, CD4⁺ and CD4⁺ peripheral iNKT cell numbers exhibited no significant difference between these subgroups (P = 0.33, 0.14, and 0.41, respectively). The function of iNKT cells was analyzed by measuring cytokine production following stimulation with PMA and ionomycin in vitro. The induced production of IFN-γ and IL-4 was similar between these groups (P = 0.16 and 0.26, respectively) (Figure 5D and E).

Tenofovir Therapy Does Not Alter Either Peripheral iNKT Cell Numbers or Their Function

We followed up 13 CHB patients who received tenofovir (TDF) antiviral therapy for 72 weeks to longitudinally analyze the influence of antiviral therapy on peripheral iNKT cell numbers and their function. The clinical characterization of these 13 patients was shown in Supplementary Table 1, http://links.lww.com/MD/A296. At the end of 72 weeks of treatment, HBV DNA was undetectable and ALT levels were normal in all of these patients, while no HBeAg seroconversion occurred in any patient. We compared the iNKT cell numbers and their function at baseline, 48 and 72 weeks after the initiation of antiviral therapy (Figure 6A). The numbers and function of peripheral iNKT cells were similar before and after treatment. As illustrated in Figure 6, no changes in either total, CD4⁺ or CD4⁺ iNKT cell numbers were observed in the 13 CHB patients under antiviral therapy (P = 0.74, 0.38, and 0.20, respectively) (Figure 6B–D). Likewise, IFN-γ and IL-4 producing iNKT cells were not influenced by antiviral therapy or the efficacy of therapy (Figure 6E and F) (P = 0.34 and 0.37, respectively). These results suggested that antiviral therapy with TDF had little influence on either iNKT cell numbers or their function.

The Frequency and Cytokine Producing Capacity of Hepatic iNKT Cells From HBV-Tg Mice Are Comparable to Those From WT Mice

Flow cytometric analysis revealed that there were approximately 0.5% to 1.5% iNKT cells (CD1d-tetramer⁺ CD3⁺) in the spleen and 5% to 25% iNKT cells in the liver of both HBV-Tg mice and wild type (WT) mice (Figure S1A, http://links.lww.com/MD/A296). The frequency of splenic and hepatic iNKT cells in HBV-Tg mice was comparable to that in WT mice (P = 0.28 and 0.57, respectively) (Figure S1B and D, http://links.lww.com/MD/A296), which was in accordance with previous study. Similarly, percentages of splenic or hepatic...
FIGURE 2. Numbers of peripheral iNKT cells in patients with different stages of hepatitis. (A) Representative analysis of peripheral iNKT cell numbers in patients in different stages of HBV infection compared to healthy controls. iNKT cells were identified as CD3 and CD1d tetramer double-positive cells and divided into CD4+ and CD4− subsets. (B) Comparison of iNKT cell numbers between chronic HBV-infected patients and healthy controls. (C–E) Pooled numbers of total, CD4+, and CD4− iNKT cells in IT, CHB, IC, and cirrhosis subgroups. Horizontal bars represent the median with interquartile range for each group. *P < 0.05, **P < 0.01, ***P < 0.001 using the Kruskal-Wallis nonparametric test followed by Dunn post-test. HBV = hepatitis B virus, IT = immune tolerant, CHB = chronic hepatitis B, IC = inactive carrier, HC = healthy control.
iNKT cells from HBV-infected patients have the same capacity to secrete Th1 and Th2 cytokines as those from healthy controls. (A) Representative dot plots of interferon-γ (IFN-γ) and interleukin-4 (IL-4) releasing iNKT cells in CHB patients and healthy controls. (B, C) Pooled percentages of IFN-γ and IL-4 producing iNKT cells in each group. Horizontal bars represent the median with interquartile range using the Kruskal-Wallis nonparametric test followed by Dunn post-test. IT = immune tolerant, CHB = chronic hepatitis B, IC = inactive carrier, HC = healthy control.

IFN-γ⁺ iNKT and IL-4⁺ iNKT cells were not different between these 2 groups (P > 0.05) (Figure S1C and E, http://links.lww.com/MD/A296).

DISCUSSION

iNKT cells are a subset of T cells which can jump start the immune activation in immune defenses and play an
important role in linking innate and adaptive immune responses. Despite their low frequency in the periphery, iNKT cells have been implicated in infectious diseases including chronic HBV infection. However, some contradictory results have been published as for the frequency and function of peripheral iNKT cells in HBV infection. Two study groups reported a significant drop in iNKT cell (6B11+CD3+Vα24+Vβ11+) frequency,15,16 while another study reported significantly increased iNKT cell (CD3+CD56+) frequency in HBeAg positive CHB patients.25 And a research in Italy revealed that the percentage of iNKT cells (CD3+Vα24+Vβ11+) in PBMCs of CHB patients was comparable with those in healthy donors.17 One of the reasons for the discrepancy between these studies may be the different identification of iNKT cells. It has been suggested that CD3+CD56+, Vα24+Vβ11+, or 6B11+CD3+ cells are not entirely iNKT cells.26 At present, a lipid antigen loaded CD1d-tetramer marker for the identification of iNKT cells is sensitive and specific, which can accurately detect 1 iNKT cell in 1 million PBMCs (0.0001%).27 Additionally, despite its binding of some non-iNKT cells,28 CD1d-tetramer can detect the entire CD1d-restricted iNKT cell population, while other antibodies only detect a subset of this population. In this study, we detected iNKT cells using CD1d tetramer staining for the first time in HBV-infected patients to accurately and comprehensively understand the role of iNKT cells in HBV infection. Our results showed that circulating iNKT cell numbers were low and variable between all individuals, including healthy subjects (range = 0.01%–0.28% in CD3+ T cells), which was similar to previous results.16 And we found no significant difference of the iNKT cell frequency between CHB patients and HCs. Despite of high viral load in IT patients, no difference were observed between this group and HCs. In addition, our study on the IT HBV-Tg mice model also revealed that the frequency of hepatic iNKT cells in HBV-Tg mice was comparable to that in WT mice. On the other hand, iNKT cell

![Figure 4](image_url)  
**FIGURE 4.** iNKT cells from CHB patients have the same capacity of in vitro expansion as healthy controls. (A) Representative dot plots of iNKT cell numbers immediately following collection and after in vitro expansion. (B–D) Pooled total, CD4+, and CD4–iNKT cell expansion fold changes in IT, CHB, IC, and HC groups. Horizontal bars represent the median, minimum, and maximum values. Comparisons between these groups were performed by Kruskal-Wallis nonparametric test followed by Dunn post test. IT = immune tolerant, CHB = chronic hepatitis B, IC = immune carrier, HC = healthy control.

**TABLE 2.** Factors Independently Associated With iNKT Cell Numbers in Chronic HBV-Infected Patients by Multivariate Analysis

| Variables                | Coefficient | 95% CI          | P Value |
|--------------------------|-------------|-----------------|---------|
| Age (y)                  | −0.0071     | (−0.0118, −0.0025) | 0.003   |
| Gender (male)            | −0.0577     | (−0.1518, 0.0363) | 0.223   |
| ALT (U/L)                | −0.0002     | (−0.0005, 0.0001) | 0.157   |
| HBV-DNA (log10copies/mL) | −0.0008     | (−0.0391, 0.0376) | 0.967   |
| HBsAg (log10U/mL)        | 0.0081      | (−0.0576, 0.0737) | 0.805   |
| HBeAg (positive)         | 0.0115      | (−0.1167, 0.1396) | 0.858   |

CI = confidence interval.
frequency was not associated with either HBV viral road or HBsAg titers. And an iNKT cell stimulator α-GalCer did not affect either HBV-DNA or ALT levels despite of a strong decrease of iNKT cells when used as monotherapy for CHB infection in a phase I/II trial. On the above rationale, we assumed that iNKT cell frequency was influenced by the inflammatory circumstance caused by HBV infection rather than HBV itself.

However, discrepancy existed between subgroups of chronic HBV patients. iNKT cell numbers in CHB and cirrhosis patients were lower than those in patients in IT patients. It may be due to the migration of iNKT cells to the inflammation site in the hepatitis phase, which is consistent with a previous study that the iNKT cells were significantly enriched in chronically inflamed livers when compared with noninflamed ones. However, only cirrhosis and HCC patients were enrolled in that study and the migration and differentiation of iNKT cells in the liver of other HBV infected patients still remain unknown. In the future, in vivo imaging studies may be developed to study the migration of iNKT cells in vivo, such as using isotope-labeled α-GalCer/CD1d tetramers. On the other hand, CHB and cirrhosis patients were older than IT patients and age was an independent risk factor for iNKT cell frequency by multivariate analysis. Therefore, age might be another factor that contributes to the discrepancy between subgroups.

Upon activation, iNKT cells can produce large amount of cytokines including Th1 cytokine IFN-γ and Th2 cytokine IL-4 which may accelerate initial immune responses. Our results indicate that the production of cytokines by iNKT cells was unimpaired in all patients. Meanwhile, when stimulated by αGalCer-loaded DCs in vitro, iNKT cells from HBV-infected patients had the same proliferative responses as those from healthy subjects. Similar results were observed in the hepatic iNKT cells from full-HBV-Tg mice model. Above mentioned results indicated the preserved function of peripheral iNKT cells in chronic HBV infection.

FIGURE 5. iNKT cell frequency and IFN/IL-4 producing iNKT cells in G and S subgroups. (A–C) Pooled total, CD4⁺, and CD4⁺ iNKT cell numbers in G0-1S0-1 and G2-4/S2-3 subgroups. (D, E) Pooled percentage of IFN-γ and IL-4 producing iNKT cells in G0-1S0-1 and G2-4/S2-3 subgroups. Horizontal bars represent the median and max values. Comparisons between these 2 subgroups were performed using Mann-Whitney test.
To observe the influence of antiviral therapy on iNKT cell frequency and function, we followed up on 13 TDF treated patients for 72 weeks in this study. Despite a decline in viral load and an improvement in liver inflammation, iNKT numbers and their cytokine producing capacity did not change after 72 weeks of antiviral therapy. This result further supported our opinion that HBV itself do not influence either the iNKT cell frequency or its function.

It should be noted that our study on iNKT cells in HBV infection mainly focused on the iNKT cells in human peripheral blood. In fact, iNKT cells are more abundant in the liver and other organs. The changes in iNKT cell frequency in peripheral blood may be a result of alterations in the recruitment of iNKTs. Further studies may be needed to investigate the disease-associated function of hepatic iNKT cells.

CONCLUSION

In summary, using the direct and specific tetramer staining method for detecting iNKT cells, we demonstrated the absence of CHB-associated defect in the frequency of peripheral iNKT cells or in their ability to secrete IFN-γ and IL-4 upon primary stimulation. However, among chronic HBV-infected patients, a decrease in iNKT cell-number was observed in CHB and cirrhosis patients in comparison to that in IT patients.

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