Persistent reduction of mucosal-associated invariant T cells in primary biliary cholangitis

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Abstract

Background & Aim: Mucosal-associated invariant T (MAIT) cells constitute a novel subset of innate-like T-lymphocytes characterized by a semi-invariant T-cell receptor repertoire capable of recognizing bacterial products. Considering the abundance of MAIT cells in the liver and the possible association between bacterial infections and primary biliary cholangitis (PBC), we aimed to analyze the involvement of MAIT cells in the immunopathogenesis of PBC.

Methods: Peripheral blood and liver biopsy specimens were collected from 25 patients with PBC and 19 patients with chronic viral hepatitis. Surgically removed liver tissues distant from tumors in patients with metastatic liver tumors were used as controls. Mononuclear cells were separated using Ficoll-gradient, and the expression of various markers was investigated by flow cytometry. Cytokine production was investigated using blood MAIT cells after stimulation by anti-CD3/CD28-coupled beads with/without interleukin-7 (IL-7).

Results: MAIT cells were significantly reduced in both the blood and liver of PBC patients compared with those in controls. MAIT cells in the blood of PBC patients expressed significantly lower levels of activation markers and IL-7 receptor. Moreover, MAIT cells in the blood of PBC patients showed impaired production of cytokines, especially tumor necrosis factor-α, after *in vitro* stimulation with IL-7. Interestingly, even after biochemical responses were achieved by ursodeoxycholic acid (UDCA) treatment, the frequencies of MAIT cells did not fully recover to normal levels.

Conclusions : These findings suggested that MAIT cells were activated, exhausted and persistently depleted in PBC patients even after UDCA treatment, possibly as a consequence of persistent liver inflammation.

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Key words: mucosal-associated invariant T cells, primary biliary cholangitis, interleukin-7, exhaustion, ursodeoxycholic acid

Introduction

Primary biliary cholangitis (PBC) is a progressive chronic cholestatic liver disease of unknown etiology characterized by autoimmune-mediated destruction of small- and medium-sized intrahepatic bile ducts.[1,2] Although its etiology remains to be determined, several infectious and chemical candidates have been proposed to trigger the disease via molecular mimicry.[1,3] Recently, in addition to adaptive immunity, contributions of the innate immune systems, such as natural killer cells and natural killer T cells, have been highlighted in PBC.[4] Indeed, recent genome-wide case-control association studies in PBC have identified a significant association of PBC risk with genes of the innate immune system.[5-8] However, the role of innate immune responses in the pathogenesis of PBC has not yet been fully elucidated.

Mucosal-associated invariant T (MAIT) cells are a novel subset of innate-like T cells that are characterized by the expression of a semi-invariant Vα7.2-Jα33 chain of the T-cell receptor (TCR). This invariant TCR restricts MAIT cells to the non-polymorphic major histocompatibility complex (MHC)

class I related protein1 (MR1).[9] Vitamin B2 (riboflavin) metabolites produced by bacteria and yeasts have been reported to activate MAIT cells in an MR-1-dependent manner.[10] Moreover, MAIT cells can also be activated in a MR-1-independent manner and can immediately produce various pro-inflammatory cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin (IL)-17.[11] Several studies have reported a contribution of MAIT cells to the protection against mycobacterial and enterobacterial infections.[12-15]

In humans, MAIT cells acquire a memory phenotype and expand dramatically after birth; these cells ultimately comprise up to ~5% of the total number of TCR-$\alpha\beta^+$ T-cells in the blood.[16] MAIT cells can also be found in peripheral tissues, such as the mucosa of the intestine and lung, and they are particularly abundant in the liver.[17] Although MAIT cells have been reported to play an important role in several autoimmune diseases, such as inflammatory bowel diseases (IBD), systemic lupus erythematosus (SLE) and multiple sclerosis,[18-21] the involvement of MAIT cells in the immunopathology of autoimmune liver diseases remains unclear. Considering the importance of innate immune responses in PBC and the abundance of MAIT cells in the liver, we aimed to analyze the involvement of MAIT cells in the immunopathogenesis of PBC.

Methods

Subjects

Heparinized peripheral blood and liver biopsy specimens were consecutively collected from 25 patients with PBC and 19 patients with chronic viral hepatitis (CVH). Blood samples were also collected from 13 healthy individuals who had no history of any liver disease. Surgically removed liver tissues obtained from 13 patients with metastatic liver tumors were used as control livers (Control). Those liver tissues were distant from the tumors, and histology findings confirmed that the patient had no chronic liver disease. The diagnosis of PBC was based on established international and Japanese criteria and excluded any autoimmune hepatitis (AIH)-PBC overlap. The demographic and clinical characteristics of these subjects are indicated in Table 1. This work was conducted in accordance with the Declaration of Helsinki. Written informed consent under institutional review board-approved protocols (approval no. 1474) at Niigata University Medical and Dental Hospital was appropriately obtained from all the individuals enrolled in the study.

Cell isolation

Liver specimens were pressed through 200-gauge stainless steel mesh and suspended in Eagle's MEM medium (GIBCO/Life Technologies, Grand Island, NY, USA) supplemented with 5 mM HEPES and 10% heat-inactivated fetal calf serum (FCS). Liver mononuclear cells and peripheral blood mononuclear cells (PBMCs) were then isolated by density-gradient centrifugation using Ficoll-Paque Plus (GE Healthcare UK Ltd, Little Chalfont, UK). Obtained liver and

blood mononuclear cells were resuspended in phosphate-buffered saline (PBS) (GIBCO) containing 2% heat-inactivated FCS, and counted in a hemocytometer; the viability of cells was determined using trypan blue exclusion.

Reagents

Monoclonal antibodies (mAb) used for immunofluorescence assay were anti-CD161-FITC (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany), anit-TCR PAN γ/δ-PC5 (Beckman Coulter Brea, CA, USA), anti-CD3-PerCP, anti-CD8α-PerCP/Cy5.5, anti-IL-7R-PE (Becton Dickinson, San Jose, CA, USA), anti-TCR-Vα7.2-APC, anti-TNF-α-PerCP/Cy5.5 (BioLegend, San Diego, CA, USA), anti-CD69-PE, anti-IFN-γ-PE (BD Biosciences, San Diego, CA, USA), anti-NKG2D-PE, anti-IL-17-PerCP (R&D systems Inc., Minneapolis, MN, USA).

Flow cytometry

Cells (10^5) were labeled with several mAbs at 4°C for 30 min in darkness for the surface antigens, and then washed with PBS 2 times and acquired by FACSCalibur flow cytometer (Becton Dickinson). Data were analyzed using Flow-Jo software (v7.7.2, Tree Star Inc., Ashland, OR, USA). The percentage of MAIT cells was calculated as follows: % MAIT cells (number of $CD3^+TCR\text{-}\gamma\delta^-CD161^{high}TCR\text{-}V\alpha7.2^+$ cells) / (number of $CD3^+TCR\text{-}\gamma\delta^-$ cells).

In vitro cytokine production and apoptosis assay

PBMCs were suspended in RPMI1640 medium (GIBCO/Life Technologies)

supplemented with 10% FCS and cultured in the presence or absence of IL-7 (10ng/ml) (R&D Systems Inc.) for 2 days as described previously.[22] Subsequently, cells were stimulated with anti-CD3/28-coupled beads (1:1 bead/cell ratio) (Invitrogen, Carlsbad, CA, USA) overnight. Monensin (Golgi Stop, BD Biosciences) was added for the last 3 hours of incubation. Cells were then stained for several surface molecules. For intracellular staining, BD Cytofix/Cytoperm Fixation/permeabilization Kit (BD Biosciences) was used according to the manufacturer's specification. Apoptosis assays were performed by Annexin V Apoptosis Detection Kit (BD Biosciences). The percentages of cytokine-positive cells among MAIT cells ($CD3^+CD161^{high}$TCR-$V\alpha7.2^+$ cells) were examined as in a previous study.[23]

Statistical analysis

The nonparametric Mann-Whitney *U* test was used to determine the statistical significance of differences. Relationships of surface markers in MAIT cells were examined using Spearman's rank correlation test. Wilcoxon signed rank test was used for the comparison of MAIT cell levels before and after UDCA treatments. Statistical analyses were performed by Prism software ver.7.02 (GraphPad Software, San Diego, CA, USA). The level of significance was set at P value <0.05.

Results

MAIT cells are abundant in the liver, but significantly decreased in PBC

MAIT cells were defined as $CD3^+$TCR-$\gamma\delta^-CD161^{high}$TCR-$V\alpha7.2^+$ lymphocytes (Fig. 1A).[13,16] MAIT cells were significantly increased in the liver of controls,

accounting for up to 35% of αβ T-cells compared with the blood (10.57 ± 8.83% vs. 1.89 ± 2.05%, P <0.001) (mean ± SD) (Fig. 1B). In PBC patients, the frequencies of MAIT cells were significantly decreased in both the blood and liver compared with those in controls (0.66 ± 0.77% vs. 1.89 ± 2.05%, P = 0.038, and 2.23 ± 2.80% vs. 13.01 ± 9.42%, P = 0.002, respectively) (Fig. 1B). Moreover, we also found that the absolute numbers of MAIT cells in the blood was significantly decreased in PBC patients compared with those in controls (Table 2). However, we did not find any association between the frequencies of MAIT cells in both the blood and liver with the liver histology of PBC patients (data not shown). Because the expression of CD161 was down-regulated after *in vitro* stimulation of MAIT cells,[12,24] we also analyzed the frequencies of $CD161^{low}$TCR-Vα7.2^{+} T-cell subset (Fig. 1A). Although the $CD161^{low}$TCR-Vα7.2^{+} T-cell subset was relatively increased in the blood compared with the liver, there were no significant differences in the frequencies of $CD161^{low}$TCR-Vα7.2^{+} T-cell subsets among the groups (Fig. 1C).

Expression of functional markers on MAIT cells in PBC

MAIT cells in the liver expressed significantly higher levels of activation marker CD69 than those in the blood of controls, patients with PBC and CVH (all P values <0.01) (Fig. 2A). Expression of CD69 in MAIT cells in the blood was significantly decreased in PBC patients compared with controls (13.11 ± 9.89% vs. 22.76 ± 9.10%, P = 0.02) (Fig. 2A). However, there was no significant difference in CD69 expression in MAIT cells in the liver among the groups. Although MAIT cells in the blood have been reported to display high expression levels of IL-7 receptor (IL-7R), IL-18 receptor (IL-18R) and natural

killer group 2, member D (NKG2D),[12,13,17,22,24,25] MAIT cells in the liver also expressed similar levels of those surface markers compared with MAIT cells in the blood (Fig. 2B-D). Among those markers, the expression levels of IL-7R and IL-18R in MAIT cells were significantly decreased in the blood of PBC patients compared with those in controls (79.33 ± 24.20% vs. 92.81 ± 10.97%, P <0.01, and 73.40 ± 23.52% vs. 82.25 ± 19.67%, P = 0.03, respectively) (Fig. 2BC). Interestingly, the expression levels of CD69 in MAIT cells in the blood of PBC patients were positively associated with IL-7R (r = 0.52, P = 0.02) and IL-18R (r = 0.48, P = 0.03) expression levels (Fig. 2EF). We also analyzed the expression levels of natural killer group 2, member A (NKG2A), C-C chemokine receptor type 5 (CCR5) (data not shown) and CCR6 (Supplementary Fig. 1), but we detected no significant differences.

Clinical relevance of MAIT cells in PBC

We then analyzed the clinical relevance of MAIT cells in PBC patients. Although we did not find any association between the frequencies of MAIT cells (data not shown) or $CD69^+$ MAIT cells with serum alkaline phosphatase (ALP) or gamma-glutamyl transpeptidase (GGT) levels (Fig. 3A), MAIT cells in the blood of PBC patients with elevated alanine aminotransferase (ALT) levels (n = 10) exhibited significantly higher expression levels of CD69 compared with those in the blood of PBC patients with normal ALT levels (20.20 ± 16.61% vs. 10.38 ± 7.45%, P = 0.04) (Fig. 3B). NKG2D expression was also significantly increased in MAIT cells in the blood of PBC patients with elevated ALT levels compared with PBC patients with normal ALT levels (64.04 ± 20.77% vs. 25.70 ± 14.96%, P = 0.02) (Fig. 3B). We further analyzed

the effects of ursodeoxycholic acid (UDCA) treatment on the frequency of MAIT cells in PBC patients by comparing the frequencies of MAIT cells in the blood before and after UDCA treatment (n = 7). All the patients were treated with UDCA alone for at least 6 months, and we found that serum levels of ALT, ALP and GGT normalized after the treatment. The frequencies of MAIT cells were significantly increased after UDCA treatment (0.42 ± 0.28% vs. 0.27 ± 0.26%, P = 0.02), and we confirmed that the absolute numbers of MAIT cells were also increased after treatment (Fig. 3C). However, the frequency and absolute number of MAIT cells remained lower than those of controls after UDCA treatment (Fig. 1B, Table 2). In addition, the expression levels of CD69, IL-7R and IL-18R in MAIT cells did not recover after UDCA treatment (Supplementary Fig. 2).

Impaired cytokine production by MAIT cells in PBC

Previous studies established that MAIT cells can be activated and produce various cytokines upon TCR signaling.[12,26] Moreover, without TCR stimulation, MAIT cells can also be activated by cytokines, including IL-7.[11,18,22] Therefore, we investigated cytokine production by MAIT cells in the blood of control individuals (n = 9) and PBC patients (n = 19) after *in vitro* stimulation of the TCR by using CD3/28-coupled beads or IL-7 alone either without TCR stimulation or with TCR stimulation in the presence of IL-7 (Fig. 4A). MAIT cells were activated using CD3/28-coupled beads or IL-7 alone and were found to produce TNF-α, IFN-γ and IL-17 (Fig. 4B). Moreover, MAIT cells were strongly activated and produced increased amounts of these cytokines upon stimulation with CD3/28-coupled beads and IL-7 (Fig. 4B). TNF-α production

by MAIT cells in PBC patients was significantly reduced after IL-7 stimulation compared with that in controls (36.74 ± 23.43% vs. 59.02 ± 17.69%, $P = 0.04$). Moreover, MAIT cells in PBC patients produced lower amounts of IFN-γ and IL-17 after stimulation with CD3/28-coupled beads and IL-7 than did controls, although the difference was not statistically significant (Fig. 4B). The frequencies of MAIT cells were significantly decreased by these stimulations, and the reduction in the frequencies of MAIT cells was likely caused by apoptosis because the frequencies of MAIT cells that expressed Annexin V were significantly increased upon those stimulations (Fig. 4C).

Discussion

Several reports have described associations between MAIT cells and autoimmune diseases, such as SLE, rheumatoid arthritis (RA), multiple sclerosis and IBD.[18,19,21] MAIT cells are decreased in the blood of patients with these autoimmune diseases. In contrast, in patients with multiple sclerosis or RA, MAIT cells accumulate in the inflamed foci of diseases.[21] We also reported that MAIT cells accumulated in the inflamed colonic mucosa of IBD patients, although MAIT cells were significantly decreased in the blood of those patients.[27] However, the present study revealed that MAIT cells were significantly decreased in the liver of PBC patients in addition to being reduced in the blood. Consistent with our findings, Jeffery *et al.* recently reported a decreased frequency of MAIT cells in explanted liver tissues of end-stage PBC patients.[23] These researchers observed that intrahepatic MAIT cells expressed the biliary tropic chemokine receptors CCR6 and CXCR6,[23] while we observed that circulating MAIT cells expressed CCR6. Although the reason for the differences between PBC and other autoimmune diseases, such as RA and IBD, remains to be elucidated, we speculate that an alteration of cell trafficking may influence the frequency of MAIT cells in the livers of PBC patients. Moreover, the reduction of circulating MAIT cells may simply result in the decrease of trafficking of MAIT cells to the liver.

The reduction of circulating MAIT cells was also reported in patients with chronic viral infections, including human immunodeficiency virus and hepatitis C virus (HCV),[28-30] although we could not identify this reduction of MAIT cells in the blood of patients with CVH. We could not determine the

reason for the discrepancy between previous studies and our study; however, the median value of ALT in the patients with CVH in our study seems to be lower than that in patients with HCV infection in the previous studies.[28,29] We speculate that our study included patients with less active liver inflammation and that MAIT cells might be less activated. Therefore, in our study, the frequencies of MAIT cells might not be as strongly decreased in patients with CVH.

In addition to the reduction of MAIT cells, we observed that the expression levels of CD69, IL-7R and IL-18R in MAIT cells were significantly reduced in the blood of PBC. Interestingly, IL-7R has been identified as a susceptibility locus for PBC patients in a genome-wide association study conducted in Japan.[5] Its ligand, IL-7, is primarily produced in the liver, especially in an injured liver, and influences homeostasis throughout the whole body.[31] Moreover, it has been reported that MAIT cells can be "licensed" by IL-7 stimulation and produce abundant pro-inflammatory cytokines in an MR-1-independent manner.[22] Yamano *et al.* also reported that serum levels of IL-18 were increased in patients with PBC.[32] In the normal liver, Jeffery *et al.* observed that intrahepatic MAIT cells were predominantly localized to bile ducts in the portal tracts and that MAIT cells may protect the biliary epithelium, playing an important role in immune surveillance and homeostasis of the biliary mucosal barrier.[23] These reports may indicate the importance of MAIT cells in PBC.

Interestingly, we observed that the frequency of MAIT cells in the blood of PBC patients was significantly increased after UDCA treatment, but it had not fully recovered to normal levels. In addition, the expression levels

of CD69, IL-7R and IL-18R in MAIT cells did not recover after UDCA treatment. These data suggested that immune responses that involved MAIT cells might continue in the liver, even after biochemical responses had been achieved by UDCA treatment. Indeed, Poupon *et al.* reported that by analyzing paired liver biopsy samples, two years of UDCA treatment only improved periportal necroinflammation and ductular proliferation in 33% and 23% of the treated PBC patients, respectively.[33] UDCA has also been shown to improve inflammation in the liver in only 10% of PBC patients treated with UDCA for 3 years based on an analysis of paired liver biopsy samples.[34] Therefore, we believe that the persistent reduction in circulating MAIT cells after UDCA treatment was a robust finding, and we speculate that such persistent reduction might be associated with the continuous inflammation in the liver of PBC patients. Moreover, we observed that activated $CD69^+$ MAIT cells were significantly increased in PBC patients who exhibited elevated ALT levels relative to the levels in PBC patients with normal ALT levels. These findings also suggest that activated MAIT cells may be associated with the severity of inflammation in the livers of PBC patients.

Impaired cytokine production by MAIT cells after *in vitro* TCR stimulation has been reported to occur in patients with type 2 diabetes and HIV infection.[24,26] The MAIT cells in those patients displayed decreased expression levels of activation markers and increased expression levels of CD38 and programmed cell death-1 (PD-1), which suggested the exhaustion of MAIT cells in a chronically inflamed environment.[24,26] In the present study, we observed that circulating MAIT cells in PBC patients showed significantly decreased expression levels of CD69 and impaired production of TNF-α upon

IL-7 stimulation. The production of IFN-γ and IL-17 also tended to decrease. Such decreases of CD69 expression and cytokine production after stimulation with IL-7 might be associated with the decreased expression of IL-7R in circulating MAIT cells in PBC patients. Moreover, after *in vitro* stimulation with CD3/28-coupled beads and/or IL-7, apoptosis was significantly induced in MAIT cells. Although we did not analyze the expression levels of CD38 and PD-1, our findings suggested that MAIT cells in PBC patients might be persistently activated and exhausted.

We recognize that the present study has several limitations. First, the relatively small sample size might have provided inadequate statistical power to detect definitive differences among patients with PBC, CVH and healthy controls and to identify associations between MAIT cells and clinical parameters in PBC patients. Second, the limited number of liver samples from PBC patients might also explain the low statistical power of our data on MAIT cells in the liver. Third, we could not investigate cytokine production by MAIT cells in the liver after *in vitro* stimulation because the sample volumes from the liver were limited, while MAIT cells in the liver were significantly depleted in PBC patients. Moreover, because of the difficulty in repeatedly obtaining liver samples from the same patients or stable patients after treatment, we could not analyze MAIT cells in the liver after UDCA treatment. Therefore, further studies will be required to clarify the role of MAIT cells in PBC.

In conclusion, our findings are consistent with a model in which MAIT cells are activated, exhausted and persistently depleted in PBC. This even occurred after biochemical responses were achieved by UDCA treatment, possibly as a result of persistent inflammation in the liver.

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Table1. Characteristics of patients and controls

Factors (Median) (range)	PBC	CVH	Control
Number of patients	PB: 25 Liver: 9	PB: 19 Liver: 14	PB: 13 Liver: 13
Age (years)	66 (29-81)	52 (30-81)	66 (44-83)
Gender (male / female)	PB: 4 / 21 Liver: 2 / 7	PB: 11 / 8 Liver: 10 / 4	PB: 8 / 5 Liver: 8 / 5
Liver histopathology			
non-cirrhosis / cirrhosis	9 / 0	11 / 3	13 / 0
Scheuer stage (I / II / III / IV)	4 / 3 / 2 / 0	-	-
Duration between initial diagnosis and present analysis (years)			
	PB: 2.2 (0-24.1) Liver: 0	-	-
Alb (g/dl)	3.8 (3.2-4.5)	3.6 (2.8-4.6)	4.0 (3.7-4.6)
AST (IU/l)	31 (20-153)	48(19-137)	20 (18-21)
ALT (IU/l)	26 (11-219)	47 (11-209)	14 (9-26)
T. Bil (mg/dl)	0.7 (0.5-1.1)	0.9 (0.6-5.4)	0.6 (0.4-0.8)
ALP (IU/l)	286 (78-1168)	257 (25-696)	209 (97-333)
GGT (IU/l)	74 (12-594)	49 (12-402)	21 (13-128)
Platelets ($x10^4/\mu l$)	19.7 (8.4-34.5)	11.7 (2.7-21)	22.2 (171.-33.1)
PT-INR	1.02 (0.91-1.25)	1.10 (0.97-1.06)	0.97 (0.86-1.07)
Child-Pugh classification (A / B / C)	PB: 25 / 0 / 0 Liver: 9 / 0 / 0	PB: 18 / 1/ 0 Liver: 13 / 1/ 0	-

Treatments (Treatment-naïve / UDCA / UDCA + BF / Steroid)			
	PB: 7 / 15 / 3 / 0	PB: 11 / 7 / 1 / 0	PB: 13 / 0 / 0 / 0
	Liver: 7 / 2 / 0 / 0	Liver: 11 / 3 / 0 / 0	Liver: 13 / 0 / 0 / 0

PBC, primary biliary cholangitis; CVH, chronic viral hepatitis; PB, peripheral blood; Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T. Bil, total bilirubin; ALP, alkaline phosphatase; GGT, gamma-glutamyl transpeptidase; PT-INR, prothrombin time-international normalized ratio; UDCA, ursodeoxycholic acid; BF, bezafibrate.

Table 2 Absolute cell numbers of MAIT cells and CD161^{-}Vα7.2^{+} cells in the blood

	n	MAIT cells (x10^4 / ml blood)	CD161^{-}Vα7.2^{+} cells (x10^4 / ml blood)
Healthy individuals	13	2.51 ± 2.90	2.34 ± 2.07
PBC	19	0.59 ± 0.56*	2.10 ± 1.46
CVH	19	1.47 ± 2.06	1.34 ± 0.76

MAIT cells, mucosal-associated invariant T cells; PBC, primary biliary cholangitis; CVH chronic viral hepatitis. *P = 0.044 (compared with healthy individuals). The mean ± SD is shown.

Fig. 1

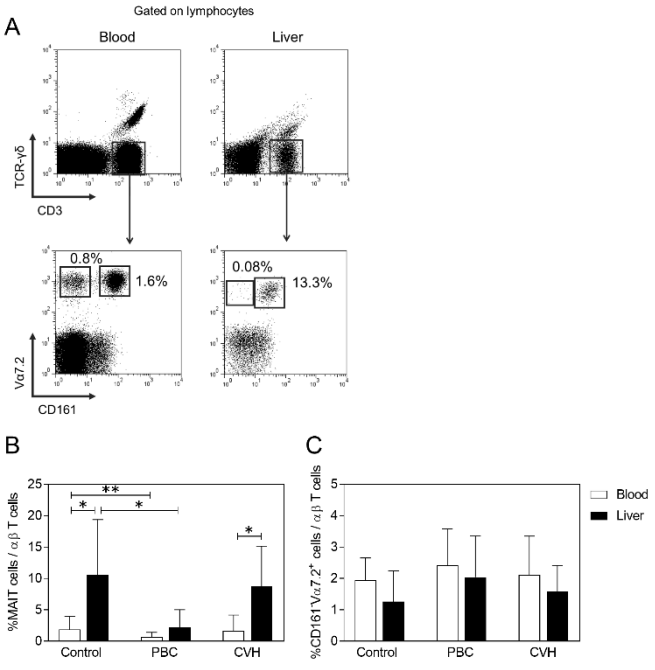

Figure 1. Frequencies of MAIT cells in the blood and liver. (A) Identification of MAIT cells in the peripheral blood and liver by flow cytometry. MAIT cells were gated as $CD3^{+}TCR\text{-}\gamma\delta^{-}CD161^{high}TCR\text{-}V\alpha7.2^{+}$ cells; $CD3^{+}TCR\text{-}\gamma\delta^{-}CD161^{low}TCR\text{-}V\alpha7.2^{+}$ cells were also analyzed. (B) The frequencies of MAIT cells in the blood and liver of patients with primary biliary cholangitis (PBC), chronic viral hepatitis (CHV) and controls (Control). MAIT cells were significantly more abundant in the liver compared with the blood. The frequencies of MAIT cells were significantly decreased both in the blood and liver of patients with PBC. (C) The frequencies of $CD3^{+}TCR\text{-}\gamma\delta^{-}CD161^{low}TCR\text{-}V\alpha7.2^{+}$ T-cells in both the blood and liver were similar between the three groups. Data are presented as the means + SD; $^{*}P$ <0.01, $^{**}P$ <0.05.

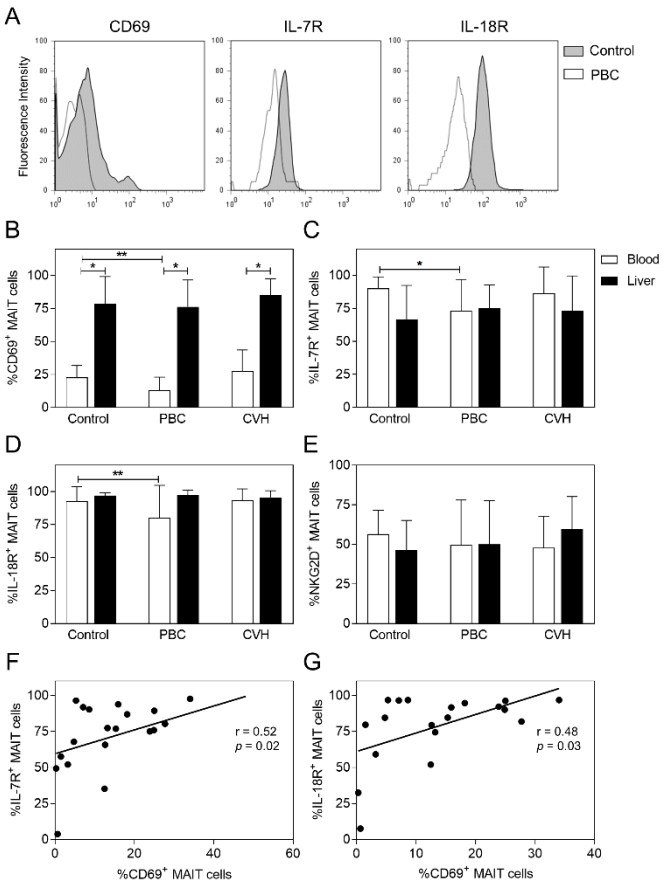

Figure 2. Expression of markers associated with the function of MAIT cells in the blood and liver. Bars indicate the percentages of indicated marker-positive cells among MAIT cells in the blood and liver of patients with primary biliary cholangitis (PBC), chronic viral hepatitis (CHV) and controls (Control). (A) Representative results of CD69, IL-7R and IL-18R expressions by flow cytometry gating on MAIT cells in the blood of PBC patients and Controls are shown. Expression levels of CD69 (B), IL-7R (C), IL-18R (D) and NKG2D (E) in MAIT cells in the blood of PBC patients were significantly decreased compared with those in controls. (F, G) Positive correlations were observed between the percentages of $CD69^+$ MAIT cells and $IL\text{-}7R^+$ or $IL\text{-}18R^+$ MAIT

cells in the blood of PBC patients by Spearman's rank correlation test. Data are presented as the means + SD; $^{*}P$ <0.01, $^{**}P$ <0.05.

Fig. 3

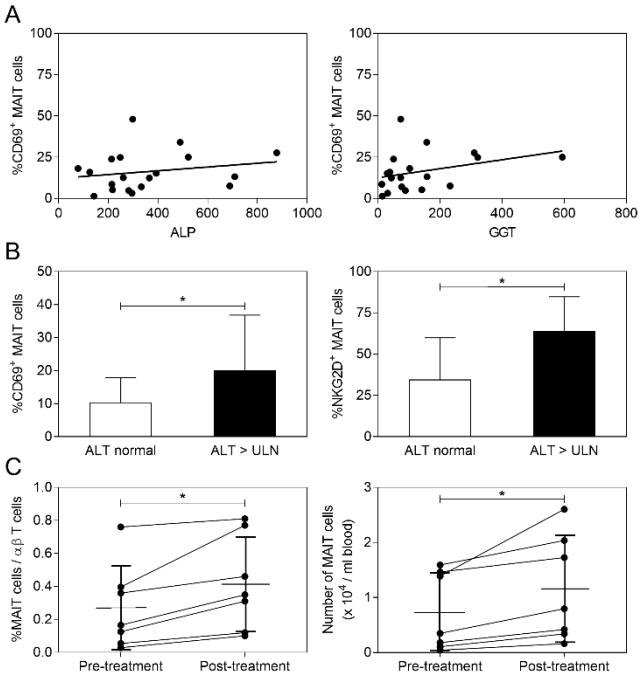

Figure 3. Clinical relevance of MAIT cells in PBC patients. (A) Associations between the frequencies of $CD69^+$ MAIT cells in the blood of PBC patients and serum levels of alkaline phosphatase (ALP) and gamma-glutamyl transpeptidase (GGT) by Spearman's rank correlation test; no significant correlation was found. (B) MAIT cells in the blood of PBC patients with elevated alanine aminotransferase (ALT) levels displayed higher expression levels of CD69 and NKG2D than PBC patients with normal ALT levels. (C) Frequencies of MAIT cells in the blood of a cohort of PBC patients before and after UDCA treatment. MAIT cells were significantly increased after biochemical responses were achieved, but this frequency remained lower

compared with controls. The individual data are presented, and bars indicate the means ± SD. Wilcoxon signed rank test was used to compare MAIT cell frequencies. $^{*}P < 0.05$.

Fig. 4

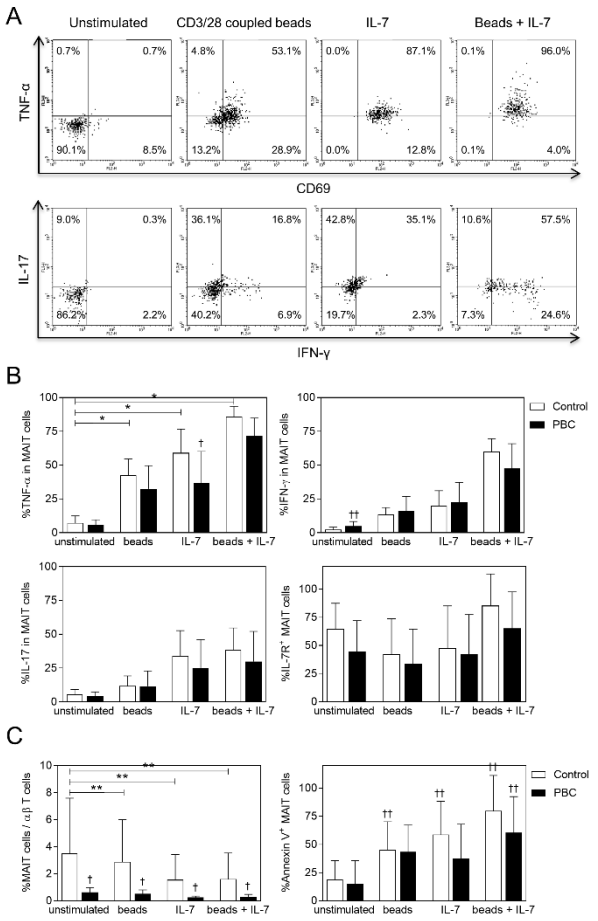

Figure 4. *In vitro* MAIT cell stimulation by CD3/28-coupled beads and/or IL-7. (A) Representative results of flow cytometry gating on MAIT cells in a healthy control after stimulations are shown. The percentages of cytokine-positive cells among MAIT cells ($CD3^+CD161^{high}$TCR-Vα7.2$^+$ cells) are indicated. MAIT cells were highly activated and produced large amounts of cytokines upon combined stimulation of anti-CD3/28-coupled beads and IL-7. (B) Frequencies of TNF-α$^+$, IFN-γ$^+$, IL-17$^+$ and IL-7R$^+$ cells among MAIT cells. The production of TNF-α from MAIT cells was significantly reduced in PBC patients after IL-7 stimulation. (C) Frequencies of MAIT cells and Annexin V$^+$ MAIT cells. Frequencies of MAIT cells significantly decreased, and apoptosis-

induced MAIT cells significantly increased after stimulations. MAIT cells in PBC patients were significantly reduced compared with those in controls before and after stimulations. The data are presented as the means + SD; $^{*}P$ <0.01, $^{**}P$ <0.05, $^{\dagger}P$ <0.01 (compared with control), $^{\dagger\dagger}P$ <0.05 (compared with unstimulated in each group).