

Activated but impaired IFN-γ production of mucosal-associated invariant T cells in patients with hepatocellular carcinoma

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ABSTRACT

Objective Mucosal-associated invariant T (MAIT) cells are innate T cells with immunoregulatory activity and were recently found to be associated with various tumor types. The role of intrasinusoidal MAIT cells in hepatocellular carcinoma (HCC) has not been fully characterized. **Design** Peripheral blood samples were obtained from patients with HCC and healthy controls. Liver-associated mononuclear cells (LMCs) were collected from liver perfusions of donors and patients with HCC undergoing liver transplantation. Blood and liver perfusates from patients with HCC were analyzed by flow cytometry for CD3 +CD161+V α 7.2+MAIT cell frequency, phenotype, and function.

Results There were fewer MAIT cells in the peripheral blood and liver of patients with HCC than in the healthy controls. Interferon- γ (IFN- γ) production by these cells was also reduced. Peripheral MAIT cells showed upregulation of HLA-DR (Human Leukocyte Antigen DR) and the inhibitory molecule PD-1 (Programmed Cell Death Protein 1), but no significant differences in upregulation were found in intrasinusoidal MAIT cells. MAIT cells were significantly enriched in the liver relative to that in the peripheral blood of patients with HCC. High levels of activation markers and exhaustion markers including HLA-DR, CD69, and PD-1 were observed in LMCs of patients with HCC but not in the peripheral blood. Single-cell RNA sequencing revealed that intrasinusoidal MAIT cells exhibited distinct features in patients with HCC and the controls.

Conclusion Our study showed that alterations in MAIT cells are associated with HCC. The distinct activity and function of MAIT cells in the peripheral blood and liver of patients with HCC might suggest a potential role of these cells in disease pathogenesis.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of liver cancer. Although patients with HCC can be managed by surgical resection and liver transplantation, the overall 5-year survival rate remains poor.¹ HCC develops slowly during chronic inflammation triggered by exposure to infectious pathogens such as hepatitis B virus (HBV) and hepatitis C virus (HCV), and the risk of developing HCC increases with the severity of inflammation and fibrosis.^{2 3} HBV infection is a major cause of hepatitis, cirrhosis, and HCC,⁴ accounting for approximately 50% of HCC cases worldwide.⁵

Mucosal-associated invariant T (MAIT) cells in humans have innate-like characteristics and express a unique semi-invariant T cell receptor (TCR), including an invariant V α 7.2 and a limited array of V β 2 or V β 13 chains.⁶ In contrast to conventional T cells, MAIT cells are mostly CD8+, expressing high levels of CD161 on their surface.⁷⁸ MAIT cells can be activated directly by MR1-presented bacterial ligands or indirectly via interleukin (IL)-12 and IL-18.9 Once activated, MAIT cells secrete proinflammatory cytokines such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α). A reduction in frequency and impaired function of MAIT cells was observed in the peripheral blood of patients with chronic hepatitis B.¹⁰ Most HCCs are usually associated with chronic HBV infection. It has been reported that the frequency of circulating MAIT cells is decreased in patients with colorectal cancer,¹¹ HCC,¹² and lung cancer.¹³ Additionally, MAIT cell frequency is diminished within liver metastases of colorectal carcinoma, and the function of hepatic tumor-infiltrating MAIT cells is impaired.¹⁴ MAIT cells are abundant in healthy adult human livers and account for approximately 20%-50% of all intrahepatic T cells. The liver serves as an immunological organ where MAIT cells are enriched and the role of MAIT cells in HCC deserves further investigation.

We aimed to analyze the phenotype and function of MAIT cells in the peripheral blood and liver of patients with HCC. Our study provides evidence that MAIT cells are

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Professor Xiaomin Shi; shixm5@sysu.edu.cn associated with HCC and indicates a distinct function for peripheral blood MAIT cells and intrasinusoidal MAIT cells in patients with HCC. Our single-cell RNA sequencing (RNA-Seq) results showed that intrasinusoidal MAIT cells in patients with HCC belong to a population of cells with a high expression of LAG3.

MATERIALS AND METHODS Human samples

Blood samples were obtained from patients with HCC (n=34) and age-matched controls (aged 22–73 years; n=52). The phenotype, immune activity, and immunosenescence of intrasinusoidal MAIT cells were assessed in patients with HCC. Liver-associated mononuclear cells (LMCs) were collected from donors (n=16) after portal flush, using cold (0°C–4°C) preservation solution following the removal of the donor livers and from the explanted livers (n=18) of patients with HCC undergoing liver surgery. The collection was performed according to standard protocols prior to liver transplantation.¹⁴ Subsequently, LMCs and peripheral blood mononuclear cells (PBMCs) were isolated using density-gradient centrifugation with Ficoll-Paque Plus (GE Health-care, Uppsala, Sweden).

Flow cytometry

PBMCs were stained with the following antibodies: anti-TCR-Va7.2 FITC (REA179, Miltenyi Biotec, GmbH), peridinin anti-CD3 PerCP-Cy5.5 (HIT3a), anti-CD4 BV510 (OKT4), anti-CD161 APC (4AHP-3G10, 4A Biotech Co. Ltd.), anti-CD8 PE-Cy7 (SK1), anti-IFN-γ APC-Cy7 (4S. B3), anti-TNF-α BV421 (Mab11), anti-HLA-DR APC-Cy7 (L243), and anti-CD279 (PD-1) BV421 (EH12.2H7). All antibodies were from BioLegend, unless otherwise specified. The samples were stained at 4°C for 30 min and then processed and analyzed on a FACSCantoII machine using FACSDiva software. Flow cytometric analysis was performed using FlowJo V.10.0 for Windows.

Cell sorting strategy

MAIT cells were sorted based on CD3 +CD161+TCR Va7.2+ expression using magnetic cell separation (Miltenyi Biotec, GmbH). MAIT cells were defined as CD161 +TCR-Va7.2+CD3+ T cells (figure 1A). More than 88% purity and 90% viability were obtained after sorting by positive selection. A minimum of 4×10^4 cells/sample was sorted from 10^7 perfusates or PBMC samples.

MAIT cell activation and intracellular staining

MAIT cells were subjected to intracellular staining for IFN- γ and TNF- α . Cells were stimulated with 100 ng/mL PMA (Sigma-Aldrich, USA), 1 µg/mL ionomycin (EMD Millipore, USA), 50 ng/mL IL-12 (PEPROTECH, USA), and 50 ng/mL IL-18 (BioLegend, USA) at 37°C with 5% CO₂ for 6 hours prior to immunostaining. Brefeldin A (10 µg/mL) was added during the final 4 hours of stimulation. The immunostained samples were washed twice prior to acquisition on a FACSCantoII Flow Cytometry system (BD Biosciences).

Cytokine multiplex bead-based assay and Luminex

Cytokine concentrations in cell culture supernatants were assessed using ProcartaPlex, a multiplex bead-based immunoassay (Invitrogen), according to the manufacturer's instructions (Luminex, FLEXMAP 3D). IL-12/IL-18 and PMA (Phorbol 12-Myristate 13-Acetate) /ionomycin were used to stimulate sorted MAIT cells (10,000 MAIT cells per patient sample) and PBMCs/LMCs across the clinical cohorts and controls for 24 hours. The supernatants were then collected and analyzed with a cytokine human 18-plex (IL-27, TNF- α , IL-1 β , IL-4, IL-6, IL-12p70, GM-CSF, IFN- γ , IL-13, IL-9, IL-17A, IL-10, IL-5, IL-21, and IL-22) assay on the Luminex system.

Single-cell RNA-Seq analysis

The cells were sorted and combined from the perfusates of patients with HCC and healthy controls. Only cells expressing SLC4A10 and IL-18RA were designated as MAIT cells and used for further analysis based on the RNA-Seq results. Briefly, trypan blue staining was used to assess the viability of sorted cells, and the samples (cell viability >90%) were prepared using a 10× Genomics Single Cell 3' v2 Reagent Kit according to the manufacturer's instructions. Single-cell libraries were prepared as per the protocol and sequenced on an Illumina HiSeq X Ten system (Illumina). Samples were filtered for lowquality reads and unmapping sequences by importing them into CellRanger and aligned to human reference genomes (hg19, GRCh37). The unique molecular identifiers per gene were counted for each cell. Differentially expressed genes were selected based on a normalized value. Principle component analysis was performed and tSNE (t-Distributed Stochastic Neighbor Embedding) was used for dimensionality reduction. Cells were represented in two dimensions, and clusters were identified and annotated according to marker gene composition. A marker gene was defined as having an expression level >0.25 logfold higher than the mean expression value of the other subclusters and detectable expression in >25% of the cells from the corresponding subcluster.

Statistical analyses

Differences between categorical variables were analyzed using a χ^2 test or Fisher's exact test, whereas continuous variables were compared using the non-parametric Kruskal-Wallis test for multiple group comparisons. Correlations between two continuous variables were analyzed based on Spearman's rank correlation. Differences were considered significant at p<0.05. GraphPad Prism V.6 software (GraphPad, La Jolla, California, USA) was used to conduct the analyses and create graphs.

RESULTS

Patient cohort characteristics

Peripheral blood samples were obtained from 34 patients with HCC and from a control cohort of 52 subjects without HCC (online supplemental table 1). Liver perfusion



Figure 1 Mucosal-associated invariant T (MAIT) cells are severely depleted in the peripheral blood and liver of patients with hepatocellular carcinoma (HCC). (A) MAIT cells were identified using flow cytometry as lymphocytes expressing CD3, CD161, and TCR-V α 7.2. The gating strategy of HLA-DR+, CD69+, CD38 +, and PD-1 +MAIT cells in this study is shown. (B) The frequency and absolute number of peripheral blood MAIT cells in patients with HCC (n=34) and healthy control groups (n=52) were determined by flow cytometry. The percentage and expression (MFI) of HLA-DR, CD69, CD38, and PD-1 in peripheral MAIT cells were determined in patients with HCC and healthy controls. The percentage and expression levels (MFI) of HLA-DR, CD69, CD38, and PD-1 in intrasinusoidal MAIT cells were determined in patients with HCC and healthy controls. The percentage and expression levels (MFI) of HLA-DR, CD69, CD38, and PD-1 in intrasinusoidal MAIT cells were determined in patients with HCC (gray) and healthy donors (black). Graphs show mean and individual data points of HLA-DR (control=29, HCC=15), CD69 (control=7, HCC=9), CD38 (control=7, HCC=9), and PD-1 (control=21, HCC=15) in peripheral MAIT cells. (C) The frequency of intrasinusoidal MAIT cells in patients with HCC (n=18) and healthy donors (n=16) was identified. Graphs show mean and individual data points of HLA-DR (control=3, HCC=3), CD69 (control=8, HCC=8), CD38 (control=8, HCC=8), and PD-1 (control=13, HCC=13) in peripheral MAIT cells. A Mann-Whitney U test was performed to detect significant differences between groups, with asterisks indicating *p<0.05, **p<0.01, ***p<0.001, and ****p<0.001.

samples were collected from 18 patients with HCC and a control group of 16 donors (online supplemental table 2). All samples were collected between November 2017 and December 2019.

MAIT cells are severely depleted in the peripheral blood and liver of patients with HCC

As previously observed,¹⁵ peripheral blood and intrasinusoidal MAIT cells were significantly depleted in patients with HCC compared with levels in the controls (figure 1B,C). We then investigated the phenotype, immune activity, and immunosenescence of peripheral and intrasinusoidal MAIT cells in patients with HCC. Although a previous study reported that MAIT cells expressed significantly higher levels of the activating markers CD38 and HLA-DR in tumors compared with those in peritumoral or normal liver tissues,¹⁴ we observed that peripheral MAIT cells from patients with HCC express significantly higher levels of HLA-DR Mean Fluorescence Intensity(MFI) than those of controls, but there were no significant differences in the levels of CD38 in peripheral MAIT cells between patients with HCC and healthy donors.¹⁵ The percentage of cells expressing activation and exhaustion markers was not significantly different between control and HCC samples (figure 1B,C, p>0.1). However, in our study, peripheral MAIT cells of patients with HCC expressed a higher level of HLA-DR (MFI) and PD-1 (MFI) than those from the controls (figure 1B), whereas the expression of HLA-DR remained unchanged for intrasinusoidal MAIT cells. There was a tendency of higher PD-1 expression on intrasinusoidal MAIT cells from patients with HCC than those from healthy controls, although it was not significant (figure 1C). Interestingly, intrasinusoidal MAIT cells of patients with HCC expressed a higher level of CD69 (MFI) than those of controls (figure 1C). These data suggest that peripheral MAIT cells are deficient in patients with HCC.

MAIT cells of patients with HCC are significantly enriched in the liver

Our findings suggested that peripheral MAIT cells in patients with HCC expressed a higher level of HLA-DR and PD-1 than those of the controls, whereas the expression of these factors in intrasinusoidal MAIT cells remained unchanged. Then, we directly compared the frequency and activation status of MAIT cells in the peripheral blood and liver of control and patients with HCC, as well as their phenotypes and the expression levels of exhaustion and activation markers. We found that the frequency of MAIT cells was significantly higher in the liver than in the peripheral blood in both healthy control and patients with HCC (figure 2A). Moreover, HLA-DR, CD69, and PD-1 were expressed at a higher level in the peripheral blood than in intrasinusoidal MAIT cells in patients with HCC (figure 2B).

Production of IFN- γ by peripheral and intrasinusoidal MAIT cells is functionally impaired in patients with HCC

We showed that peripheral MAIT cells of patients with HCC expressed a higher level of the immune exhaustion marker PD-1 than those of the control subjects. Although the level of PD-1 did not change significantly in intrasinusoidal MAIT cells, it showed an overall increasing trend. Since MAIT cells of patients with HCC exhibited an exhausted phenotype, we attributed this to their function being dysregulated. We first examined the changes in soluble factors in peripheral blood and the liver environment by measuring the levels of cytokines accompanying the immune response to HCC. Several key cytokines were detected by Luminex in the supernatant of PBMCs and LMCs from patients with HCC. Interestingly, the levels

of cytokines such as IL-6 and GM-CSF increased in the liver of patients with HCC, compared with those in the controls, after stimulation with IL-12/IL-18 for 24 hours (figure 3A). An altered cytokine profile in the bulk culture could influence the cytokine profile of MAIT cells. We then sorted MAIT cells from PBMCs and LMCs obtained from patients with HCC and verified cytokine changes in their supernatants after stimulation with IL-12/IL-18 or PMA/ionomycin for 24 hours. IL-12/IL-18 stimulation induced a greater than fourfold increase in the levels of IL-4, IFN-y, IL-1b, TNF-alpha, IL-13, IL-23, IL-18, and IL-21 in intrasinusoidal MAIT cells of healthy controls, compared with levels in patients with HCC (figure 3B). To gain broader insight into the cytokine profiles of MAIT cells that were affected by the bulk environment, the levels of IFN- γ and TNF- α in MAIT cells of patients with HCC and controls were examined by flow cytometry. We found that peripheral blood MAIT cells from patients with HCC produced less IFN- γ than those from the controls when IL-12 +IL-18 was stimulated (figure 3C). However, this change was not observed when cells were stimulated with PMA/ionomycin (online supplemental figure S1). These results suggested that MAIT cells from the peripheral blood were functionally impaired in patients with HCC. IFN- γ production was also lower in MAIT cells from the liver of patients with HCC compared with control levels after stimulation with IL-12 +IL-18 (figure 3D). In agreement with the results obtained following the peripheral blood analysis, IFN-γ production by MAIT cells did not change in response to PMA/ionomycin (online supplemental figure S1), and TNF- α production was not affected by any stimulation (figure 3D). These findings indicate that the function of peripheral and intrasinusoidal MAIT cells is impaired in patients with HCC.

Single-cell RNA-Seq of intrasinusoidal MAIT cells of patients with HCC and healthy controls

To better understand the characteristics distinguishing patients with HCC from the controls, single-cell analysis was performed on sorted CD3 +CD161+innate T cells from three pooled patients with HCC and two controls. Single-cell RNA-Seq analysis revealed 854 differential genes (with 169 upregulated and 685 downregulated in HCC) between patients with HCC and healthy controls, suggesting the presence of an aberrant gene expression profile in MAIT cells of patients with HCC. In total, 15,472 and 6241 CD3 +CD161+ T cells were sequenced in the samples from controls and patients with HCC, respectively. All cells from the patients and controls expressed KLRB1 as expected. The percentage of cells expressing SLC4A10 was approximately 7% in both samples, with 12% of cells in the control samples expressing ABCB1 and only 7% in the HCC patient samples. We further filtered the MAIT cells as suggested in a previous publication,¹⁵ and post-filtering with SLC4A10/IL-18RA, 236 cells remained in the control sample (1.5%) and 141 cells remained in HCC samples (2.3%). An overview of threedimensional and two-dimensional t-SNE maps of MAIT



Figure 2 Expression of immune activation markers and cytokines in peripheral and intrasinusoidal mucosal-associated invariant T (MAIT) cells of patients with hepatocellular carcinoma (HCC). (A) Comparison of the proportion of MAIT cells in the blood (control=52, HCC=34) and liver (control=18, HCC=16) of patients with HCC and healthy controls. (B) Comparison of the percentage of cells expressing HLA-DR, CD69, CD38, and PD-1 in peripheral (black) and liver (gray) MAIT cells of patients with HCC. (A, B) Graphs show the mean and individual data points. A Mann-Whitney U test was performed to detect significant differences between groups, with asterisks indicating *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

cell clusters from HCC and control samples is shown in figure 4A. Marker genes with considerable differences in expression between patients and controls are represented as a heatmap and listed in figure 4B. The exhaustion profile and gene activation were further analyzed in the patient and control samples (figure 4C). Interestingly, a marked increase in the level of LAG3 was also detected. LAG3 is known as a key T cell checkpoint and inhibitor of T cell responses. Together, these results suggested that the MAIT cells present in the HCC samples are activated but have an exhausted phenotype. The pathways in which the relevant key molecules are implicated are shown in figure 4D.

Intrasinusoidal MAIT cells positively correlated with plasma levels of ALT and AST

Finally, we verified whether there was any clinical correlation between MAIT cells and the clinical status of the patients. In patients with HCC, a strong correlation was found between the frequency of intrasinusoidal MAIT



Figure 3 Cytokine expression in peripheral blood and intrasinusoidal mucosal-associated invariant T (MAIT) cells in patients with hepatocellular carcinoma (HCC). (A) Expression of cytokines in the supernatant of blood and liver cells in patients with HCC after stimulation with IL-12/IL-18 for 24 hours (control=5, HCC=8). (B) Expression levels of cytokines in the supernatant of sorted MAIT cells from the blood and liver of control (n=3) and patients with HCC (n=3) after stimulation with IL-12/IL-18 for 24 hours. (C) Comparison of the percentages of IFN- γ and TNF- α in MAIT cells from the percentages of IFN- γ and TNF- α in intrasinusoidal MAIT cells of patients with HCC (n=8) and healthy controls (n=14) after stimulation with IL-12/IL-18 for 6 hours. (D) Comparison of the percentages of IFN- γ and TNF- α in intrasinusoidal MAIT cells of patients with HCC (n=9) and healthy donors (n=10) after stimulation with IL-12/IL-18 for 6 hours. (C, D) Graphs show mean and data points. The percentage of IFN- γ + and TNF- α +MAIT cells after stimulation with IL-12/IL-18 for 6 hours. (C, D) Graphs show mean and data points. The percentage of IFN- γ + and TNF- α +MAIT cells after stimulation with IL-12/IL-18 for 6 hours. (C, D) Graphs show mean and data points. The percentage of IFN- γ + and TNF- α +MAIT cells after stimulation with IL-12/IL-18 for 6 hours. (C, D) Graphs show mean and data points. The percentage of IFN- γ + and TNF- α +MAIT cells after stimulation with IL-12/IL-18 for 6 hours. (C, D) Graphs show mean and data points. The percentage of IFN- γ + and TNF- α +MAIT cells after stimulation with IL-12/IL-18 for 6 hours. (C, D) Graphs show mean and data points. The percentage of IFN- γ + and TNF- α +MAIT cells after stimulation with IL-12/IL-18 for 6 hours. (C, D) Graphs show mean and data points. The percentage of IFN- γ + and TNF- α +MAIT cells after stimulation with IL-12/IL-18 for 6 hours. (C, D) Graphs show mean and data points. The percentage of IFN- γ + and TNF- α +MAIT cells after stimulation with IL-



Figure 4 Comparison of gene expression patterns between patients with hepatocellular carcinoma (HCC) and healthy control subjects. (A) The cells were filtered according to SLC4A10 and IL18RA expression and the three-dimensional plot representations of tSNE analysis of mucosal-associated invariant T (MAIT) cells from controls (236 cells) and patients (141 cells). (B) Marker genes with the top differences in expression between patients and controls are presented by a heatmap. (C) The heatmap of the two-way hierarchical clustering of immune marker genes (Immune function and chemokine/cytokine profiles) from patients with HCC and controls shows distinct expression patterns of activation and exhaustion. (D) Pathways of highly expressed genes in HCC and controls. HBV, hepatitis B virus; HCV, hepatitis C virus; IFN- γ , interferon- γ ; IL, interleukin; TNF- α , tumor necrosis factor- α .

cells and the levels of ALT (alamine aminotransferase) and AST (Aspartate aminotransferase) (figure 5B). Interestingly, this correlation was not observed in peripheral MAIT cells (figure 5A). This result suggested that intrasinusoidal MAIT cells might serve an important role in the regulation of liver function.

DISCUSSION

Alterations in innate T cells are associated with various cancers.^{11–17} MAIT cells are a population of innate T cells considerably enriched in the liver,¹⁸ which suggests their potential role in HCC. In keeping with a previous study,¹² we found that the frequency of MAIT cells was reduced both in the peripheral blood and in the liver of patients with HCC. Our results further demonstrated that MAIT cells from patients with HCC exhibited an activating and

exhausted phenotype with impaired effector capability. In addition, the percentage of perfusate MAIT cells was significantly correlated with ALT and AST levels in patients with HCC, which might be indicative of a poor clinical outcome. Our findings also provided novel insights into the role of peripheral blood and intrasinusoidal MAIT cells during HCC progression. In contrast to MAIT cells in colorectalcancer (CRC), MAIT cells mainly expressed CD8 and had inflammatory functions in HCC, whereas MAIT cells comprised a subset expressing CD4 and Foxp3, suggesting a regulatory function present in the CRC environment.

The decreased frequency of circulating MAIT cells has been reported in chronic infections, such as HBV and HCV,^{19 20} as well as in colorectal¹¹ and lung cancer.¹³ Our results show that MAIT cells are decreased in the peripheral



Figure 5 Correlation between mucosal-associated invariant T (MAIT) cells and the plasma levels of ALT or AST. The correlations between the plasma levels of ALT (Alamine aminotransferase) or AST (Aspartate aminotransferase) and the frequency of peripheral (A) or liver (B) MAIT cells in patients with hepatocellular carcinoma (HCC) were analyzed using Spearman's test.

blood of patients with HCC. As HCC usually develops in the background of chronic viral infections, whether the reduction is attributable to tumor-associated factor or chronic infection conditions remains controversial. Results from our limited number of non-HBV-HCC patients showed the same MAIT cell pattern as HBV-HCC patients, suggesting the reduction might be caused by the tumor environment (data not shown). Additionally, the MAIT cells in peripheral blood highly express HLA-DR and PD-1, which could be explained by depletion by apoptosis subsequent to sustained activation.²¹ Independent of MR1 binding, MAIT cells can produce effector cytokines such as IFN-y and TNF and promote antiviral immune responses.^{22 23} However, in patients with HCC, the effector function of intrasinusoidal MAIT cells was found to be severely impaired. We found that IFN-y production was lower in HCC-sourced MAIT cells than in the controls after stimulation with IL-12/IL-18. Additionally, whereas most cytokines were not detected in sorted intrasinusoidal MAIT cells from patients with HCC, a wide range of effector cytokines was found after 24 hours of stimulation in control MAIT cells. It is possible that MAIT cells are reprogrammed within the HCC microenvironment, diminishing effector cytokine production. To characterize the cytokines secreted from the liver and in the peripheral blood of patients with HCC, we examined the cytokines present in the supernatant of PBMCs and LMCs of patients with HCC. Higher production of IL-6 and GM-CSF was observed in the supernatant of LMCs after stimulation with IL-12/IL-18 for 24 hours. The IL-6/STAT3 pathway has been reported to play a crucial role in the prevention of HCC,^{24 25} and the levels of cytokines such as IL-6 and GM-CSF are elevated in patients with HCC.^{12 26 27} A previous study suggested that the elevation in GM-CSF production might contribute to MAIT cell loss. Thus, it is possible that suppressing effector cytokine production by HCC resulted in intrasinusoidal cells.²⁸ IL-12 and IL-18 can also stimulate the monocytes in PBMCs and LMCs of patients with HCC and produce several kinds of cytokines, which might skew the cytokine profile of MAIT cells. To better characterize the cytokines produced by MAIT cells in patients with HCC, these cells were sorted from the PBMCs and LMCs. The results further suggested that in the presence of an exhausted phenotype, MAIT cells display a very limited effector function.

To further investigate the immune activation status of MAIT cells in the liver and peripheral blood, we measured activation and exhaustion markers such as HLA-DR, CD69, CD38, and PD-1 in these cells. The levels of HLA-DR and PD-1 were higher in the peripheral blood of patients with HCC than in the controls. This might indicate that in HCC, MAIT cells are present with an activating exhausted phenotype, which has been related to tumor promotion. In addition, high PD-1 expression in MAIT cells could suggest their impaired function in patients with HCC, which would agree with our flow cytometry results that IFN-y expression level in MAIT cells of patients with HCC is lower than in the controls. Interestingly, our single-cell analysis found that the genes involved in type I interferon signaling were expressed in control MAIT cells. Our single-cell analysis of intrasinusoidal MAIT cells from patients with HCC further demonstrated that genes required for activation and exhaustion were highly expressed in the HCC samples. Together, these results may suggest that the effector function and cytotoxic activity of MAIT cells from patients with HCC were deficient. Duan et al previously suggested that the reduced infiltration of MAIT cells in HCC is unlikely to be related to apoptosis.¹² However, our single-cell analysis suggested that genes involved in apoptotic pathways were found to be expressed in HCC-MAIT cells.

Single-cell RNA-Seq analysis provided new insights into the function of intrasinusoidal MAIT cells in patients with HCC, based on the expression of marker genes. Significant differences in functional immune markers were observed between patients with HCC and the controls, including activationassociated and exhaustion-associated genes. Most strikingly, the exhaustion marker LAG3 was found to be highly expressed in patient samples compared with levels in the controls. LAG3 is known as a key molecule involved in T cell activation, and a recent study by Wang et al suggested that the inhibitory function of LAG3 can be independent of Major Histocompatibility complex M (MHC) class II,²⁹ which raised the question of whether the LAG3 present in MAIT cells could function without interacting with MHC-II. Here, we first demonstrated that LAG3 might potentially cause MAIT cell dysfunction in HCC. Previously conducted RNA-Seq determined the global gene expression differences by sorting MAIT cells from paired tumor and peritumor liver tissues, as well as normal liver of healthy donors.¹² Their results showed that genes aberrantly upregulated in tumor-infiltrating MAIT cells included HAVCR2 (TIM-3). TIM-3 is also known as a checkpoint receptor expressed by a wide variety of immune cells.³⁰ Combined with their findings, we speculate that the dysfunction of liver MAIT cells in HCC may be due to abnormally elevated immune checkpoints in MAIT cells.

Finally, patients with a high proportion of intrasinusoidal MAIT cells exhibited a three-log elevation in the levels of ALT and AST, compared with those in patients with fewer intrasinusoidal MAIT cells. This suggests that MAIT cells, particularly intrasinusoidal MAIT cells, may be predictors of liver function in patients with HCC. Previous study in HCC results showed that patients with high MAIT cell infiltration had significantly poorer RFS (Regional Free Survial) and relatively lower OS compared with the low infiltration group.¹² We believe that the persistent presence in the liver might be associated with continuous inflammation in the liver in patients with HCC. Our findings suggest that activated MAIT cells could be associated with the severity of the intrasinusoidal liver environment in patients with HCC.

MAIT cells are increasingly known as immune protectors, particularly during infectious diseases. Here, we demonstrated that intrasinusoidal MAIT cells in the tumor environment of HCC might not favor antitumor responses owing to the presence of an exhausted phenotype and diminished effector responses. Therefore, MAIT cell reprogramming in the tumor setting might be an attractive therapeutic strategy for HCC.

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