Activated and Exhausted MAIT Cells Foster Disease Progression and Indicate Poor Outcome in Hepatocellular Carcinoma

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Abstract

Purpose: Innate immunity is an indispensable arm of tumor immune surveillance, and the liver is an organ with a predominance of innate immunity, where mucosal-associated invariant T (MAIT) cells are enriched. However, little is known about the phenotype, functions, and immunomodulatory role of MAIT cells in hepatocellular carcinoma (HCC).

Experimental Design: The distribution, phenotype, and function of MAIT cells in patients with HCC were evaluated by both flow cytometry (FCM) and in vitro bioassays. Transcriptomic analysis of MAIT cells was also performed. Prognostic significance of tumor-infiltrating MAIT cells was validated in four independent cohorts of patients with HCC.

Results: Despite their fewer densities in HCC tumor than normal liver, MAIT cells were significantly enriched in the HCC microenvironment compared with other mucosa-associated organs. Tumor-derived MAIT cells displayed a typical CCR7⁺CD45RA⁻CD45RO⁺CD95⁺ effector memory phenotype with lower costimulatory and effector capabilities. Tumor-educated MAIT cells significantly upregulated inhibitory molecules like PD-1, CTLA-4, TIM-3, secreted significantly less IFNγ and IL-17, and produced minimal granzyme B and perforin while shifting to produce tumor-promoting cytokines like IL8. Transcriptome sequencing confirmed that tumor-derived MAIT cells were reprogrammed toward a tumor-promoting direction by downregulating genes enriched in pathways of cytokine secretion and cytolytic effector function like NFKB1 and STAT3B and by upregulating genes like IL8, CXCL12, and HAVCR2 (TIM-3). High infiltration of MAIT cells in HCC significantly correlated with an unfavorable clinical outcome, revealed by FCM, qRT-PCR, and multiplex IHC analyses, respectively.

Conclusions: HCC-infiltrating MAIT cells were functionally impaired and even reprogrammed to shift away from antitumor immunity and toward a tumor-promoting direction. See related commentary by Carbone, p. 3199

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh among women in the world (1). HCC represents a typical inflammation/immune-related tumor that usually develops in an inflamed fibrotic or cirrhotic liver (2). The recent breakthrough in HCC immunotherapy targeting immune checkpoint PD-1/PD-L1 has substantially improved the patient survival (3, 4). A deeper understanding of interactions between immune cells and cancer cells within the HCC microenvironment may reveal diverse therapeutic approaches for HCC (5, 6). In addition to adaptive immune system specialized in recognizing tumor antigen, innate immunity is another indispensable arm of immune system, directly or indirectly participating in tumor control (7, 8). Indeed, the liver is an organ with a predominance of diverse range of innate lymphocytes, among which mucosal-associated invariant T (MAIT) cells are of paramount importance.

MAIT cells are a specialized innate-like T-cell subset, accounting for 10% of circulating CD4⁻ T cells in adults, featured with the expression of the semiinvariant T-cell antigen receptor (TCR, Vα7.2-Jα33) that detects microbial vitamin B metabolites presented by major histocompatibility complex class I–related protein 1 (MR1; refs. 9, 10). Both the TCR expressed by MAIT cells and the antigen-presenting molecule MR1 are evolutionarily conserved among mammals, indicating a strong selective pressure to maintain...
Immunity mediated by MAIT cells (11). Several subsets of MAIT cells have been defined, most of which, if not all, are CD161<sup>hi</sup>I<sup>17</sup>-secreting CD8<sup>+</sup> T-cell subset (9, 12). After being activated by microbial antigen bound with MR1, MAIT cells are licensed to kill targets by secreting IFN<sub>γ</sub>, granzyme, and perforin (13).

Intriguingly, contrary to the name, MAIT cells are most enriched in normal human livers, comprising up to 50% of all intrahepatic T cells (14). It has been reported that hepatic MAIT cells are highly activated within the liver and may be protective against a range of bacteria, fungi, and viruses along with the large phagocytic Kupffer cell population (8). Overall, a reduction and dysfunction in MAIT cells are observed in blood and livers from patients with HCC and healthy donors. A high density of tumor-infiltrating MAIT cells display a typical effector memory phenotype, their effector functions and cytotoxic capability are significantly impaired. Upregulation of PD-1, CTLA-4, and TIM-3, a common feature of T-cell exhaustion, is evidenced in HCC-derived MAIT cells. MAIT cells are found to be activated within tumor milieu and reprogrammed to produce a significant amount of proinflammatory cytokines. A high density of tumor-infiltrating MAIT cells significantly and independently correlated with dismal clinical outcomes in patients with HCC. Thus, strategies to modulate the functional activities of MAIT cells may provide a new avenue for antitumor therapy in HCC. Extended understanding of interactions between innate immunity and the HCC microenvironment may further provide new clues for more effective immune therapy.

**Materials and Methods**

**Patients and samples**

For flow cytometry (FCM) analysis, fresh paired tumor/peritumor tissues and peripheral blood samples were obtained from a cohort of 50 patients with HCC who underwent surgical resection between 2014 and 2015. For analyzing TCR<sub>v</sub>v<sub>7</sub>-<i>j</i>33 mRNA expression, fresh frozen tumor/peritumor tissues were obtained from another cohort of 207 patients with HCC who received curative operation between 2009 and 2013. For tissue microarrays (TMA), archival tissues were obtained from two independent cohorts of 224 and 360 patients with HCC who received curative surgery in 2007 and in 2006, respectively. Patient information and clinicopathologic features of all the cohorts are summarized in Supplementary Table S1. Informed consent was obtained from each patient prior to receive the sample. This study was conducted in accordance with ethical principles that have their origin in the Declaration of Helsinki, and the ethical standards of the Research Ethics Committee of Zhongshan Hospital (Shanghai, China).

**Translational Relevance**

Hepatocellular carcinoma (HCC) represents a typical inflammation and immune-related cancer. The mucosal-associated invariant T (MAIT) cell, an innate immune cell subset, is enriched in the liver where innate immunity is dominant. Here, we demonstrate that although tumor-infiltrating MAIT cells display a typical effector memory phenotype, their effector functions and cytotoxic capability are significantly impaired. Upregulation of PD-1, CTLA-4, and TIM-3, a common feature of T-cell exhaustion, is evidenced in HCC-derived MAIT cells. MAIT cells are found to be activated within tumor milieu and reprogrammed to produce a significant amount of proinflammatory cytokines. A high density of tumor-infiltrating MAIT cells significantly and independently correlated with dismal clinical outcomes in patients with HCC. Thus, strategies to modulate the functional activities of MAIT cells may provide a new avenue for antitumor therapy in HCC. Extended understanding of interactions between innate immunity and the HCC microenvironment may further provide new clues for more effective immune therapy.

Mononuclear cell isolation and FCM

Mononuclear cells from freshly resected liver tissues and peripheral blood were isolated and stained for FCM as described previously (ref. 21; details in Supplementary Materials and Methods and Supplementary Table S2).

**Immunofluorescence**

Immunofluorescence on frozen section was performed according to a two-step way method and scanned by TCS SP5 (Leica Biosystems). Multiplex IHC on paraffin-embedded TMAs was performed using Opal Tyramide Signal Amplification (TSA)-based staining regents (PerkinElmer). Detailed information is provided in Supplementary Materials and Methods.

**MAIT cell coculture and cytokine detection**

Information of MAIT cell activation, coculture, and cytokine detection are described in Supplementary Materials and Methods.

**RNA isolation, RT-PCR, and RNA sequencing**

Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. The TCR<sub>v</sub>v<sub>7</sub>-<i>j</i>33 mRNA levels were determined by real-time RT-PCR as described previously (22). RNA sequencing (RNA-seq) was carried out as described by Picelli and colleagues with minor modifications (23). Detailed information is described in Supplementary Materials and Methods.

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism 7.03 (GraphPad Software). The experimental data were shown as mean ± SD. Cutoff values for patient grouping in all the cohorts were defined by lowest tertile (33rd percentile) of MAIT cell’s frequency/density. A two-tailed P < 0.05 was considered significant. Detailed information is provided in Supplementary Materials and Methods.

**Results**

MAIT cell infiltration is significantly decreased in HCC

We first used FCM analysis to determine MAIT cell distribution in blood and tissue of patients with HCC and healthy donors. Consistent with the previous report (9), we confirmed that CD4<sup>+</sup> cells constituted >95% of total MAIT cells, irrespective of specimens from normal liver, HCC tissues, or peripheral blood (Fig. 1A and B; Supplementary Fig. S1A).

Even in peripheral blood, the frequency of CD4<sup>+</sup> MAIT cells among total CD3<sup>+</sup> T cells was significantly decreased in patients
with HCC compared with healthy donors (mean: 0.9% vs. 2.1%, \( P < 0.01 \); Fig. 1C). Trend continues in tumor tissues, CD4^+ MAIT cell frequency within total CD3^+ T-cell population was significantly decreased in HCC tumor compared with either paired peritumor (mean: 4.2% vs. 16.5%, \( P < 0.001 \); \( n = 50 \)) or normal liver (mean: 4.2% vs. 21.6%, \( P < 0.001 \); \( n = 20 \); Fig. 1C).

Next, immunofluorescence imaging confirmed our FCM findings (Fig. 1D), reflecting a decreased density of CD161^+ TCRv\alpha7.2^+ MAIT cells in the tumor center compared with peritumor (mean: 7.2 vs. 17.4 cells/mm\(^2\), \( P = 0.014 \); \( n = 8 \); Fig. 1E). Moreover, using TCRv\alpha7.2 and IL18R as the surrogate for MAIT cell detection, the results also showed a reduction of TCRv\alpha7.2^+IL18R^+ MAIT cells in tumor as compared with peritumor (Supplementary Fig. S1B). These results indicated that either absolute or relative number of MAIT cells was significantly lower in the blood and tumor samples of patients with HCC.

We further checked the relationship between MAIT cell infiltration and clinical features and identified MAIT cell frequency was lower in patients with liver cirrhosis in FCM cohort (mean: 3.46% vs. 6.90%, \( P = 0.053 \); \( n = 50 \); Supplementary Table S1). In addition, in a larger qRT-PCR cohort (\( n = 209 \)), TCRv\alpha7.2-J33 mRNA relative expression level was also decreased in patients with liver cirrhosis (mean: 0.014 vs. 0.069, \( P = 0.057 \)), or chronic HBV infection (mean: 0.061 vs. 0.125, \( P = 0.075 \); Supplementary Table S1). The above results suggest that liver cirrhosis is a potential factor associated with the decrease of MAIT cell infiltration.

Tumor-infiltrating MAIT cells display a typical effector memory phenotype

In patients with HCC and healthy donors, we determined the phenotypic features of MAIT cells, gated on MR1-5-OP-RU^+ after CD3^+CD4^+-gated strategy, to exclude contaminations from CD161^int Va7.2^+ T CD4^+ mainstream T cells (24), by comparing with conventional T-cell–related costimulatory/memory/activation molecules. Our data indicated that most, if not all, MAIT cells displayed a CCR7^−CD45RA^− effector memory phenotype that may harbor immediate effector function (Supplementary Fig. S2A and S2B). Likewise, MAIT cells expressed high levels of CD45RO and CD95 in both patients with HCC and healthy donors, irrespective of in the liver, tumor, and blood (Fig. 2A; Supplementary Fig. S2C). Of note, the expression of

Figure 1.
MAIT cell definition, analyzing strategy, and distribution in patients with HCC by FCM and immunofluorescence imaging. A, MAIT cell staining and analyzing strategy by FCM in peripheral blood and tissues of patients with HCC and healthy donors. HD, healthy donor; PBMC, peripheral blood mononuclear cells. B, MAIT cell subset composition in tissues and peripheral blood of patients with HCC and healthy donors detected by FCM analysis (**, \( P < 0.01 \); \( P < 0.001 \) by Mann–Whitney U test or one-way ANOVA and Tukey multiple comparison tests). HD, healthy donor; PBMC, peripheral blood mononuclear cells. C, CD4^+ MAIT cell frequency in total T cells in peripheral blood and tissues of patients with HCC and healthy donors detected by FCM analysis (***, \( P < 0.01 \); \( P < 0.001 \) by Mann–Whitney U test or one-way ANOVA and Tukey multiple comparison tests). HD, healthy donor; PBMC, peripheral blood mononuclear cells. D, Representative staining for TCRv\alpha7.2 and CD161 on frozen sections, scanned by Leica SP5 under 63 x objective. E, Summary of density information of CD161^+ and TCRv\alpha7.2^+ MAIT cells in paired peritumor and tumor tissues (\( P = 0.018 \) by Mann–Whitney U test). Lines and error bars are presented as the mean ± SD.
costimulatory molecules CD28 and CD127 on MAIT cells was significantly decreased in HCC tissues compared with either peritumor or normal liver tissues (for CD28 mean: 92.6% vs. 97.5% or 98.3%, P = 0.010; for CD127 mean: 92.8% vs. 98.8% or 98.1%, P = 0.031; Fig. 2B), whereas in peripheral blood, no noticeable expression difference was found between HCC and healthy donors (Supplementary Fig. S2D). In addition, MAIT cells expressed significantly higher level of activating markers CD38 and HLA-DR in tumor compared with its counterpart in peritumor or normal liver tissues (for CD38 mean: 14.6% vs. 8.69% or 4.54%, P = 0.044; for HLA-DR mean: 15.9% vs. 6.98% or 3.44%, P = 0.014; Fig. 2C), whereas no obvious differences of these two markers were seen on peripheral MAIT cells between HCC patients and healthy donors (Supplementary Fig. S2E). Elevated HLA-DR and CD38 expression could be resulted from chronic infections that coexists and alternatively indicates a common exhausted T-cell phenotype (25, 26). Furthermore, we checked a group of ten T-cell and NK cell–related activating and inhibitory molecules (27) and found that two effector function–related molecules CD160 (mean: 39.0% vs. 72.3% or 96.3%, P < 0.001) and KLRG1 (mean: 76.5% vs. 82.7% or 95.7%, P = 0.019; Fig. 2D; Supplementary Fig. S2G) had significantly lower expression on HCC-infiltrated MAIT cells, whereas CD160 was also lower on circulating MAIT cells in patients with HCC (Supplementary Fig. S2F). Collectively, these data showed that tumor MAIT cells displayed a typical CCR7^−/CD45RA^−/CD45RO^+^CD95^+^ effector memory phenotype with activated status and potentially decreased effector capabilities.

Figure 2.
Expression of costimulatory and activation receptors on intrahepatic and peripheral blood MAIT cells. A, Representative plots of CD45RO and CD95 expression on tissue and blood MAIT cells (gated on CD161^+^TCR^α7.2^+^MR1-tet^+^). Expression of costimulatory receptors (B), activation receptors (C), and inhibitory receptors (D) on tissue MAIT cells was also detected by FCM (gated on CD161^+^TCR^α7.2^+^MR1-tet^+^). Representative overlays for tumor, peritumor, normal liver, and isotype control and total summary data are shown (*, P < 0.05; **, P < 0.01; *** , P < 0.001 by one-way ANOVA and Tukey multiple comparison tests). Lines and error bars are presented as the mean ± SD.
Chemokine receptor expression profile in tumor-infiltrating MAIT cells

Because MAIT cells are accounting almost half of the intrahepatic T cells supposed to be mediated by CCR6 and CXCR6, and evidenced a decrease expression of gut-homing receptor CCR9 (9, 15). Herein, to further support MAIT trafficking, we screened the 18 classic chemokine receptors both in patients with HCC and healthy donors. Compared with healthy donors, the expression of all the three receptors, CCR6 (MFI mean: 568.8 vs. 876 or 817.8, \( P = 0.042 \)), CXCR6 (MFI mean: 104.9 vs. 115.1 or 177.6, \( P = 0.002 \)), and CCR9 (MFI mean: 80.4 vs. 134.9 or 168.9, \( P = 0.016 \)), on MAIT cells was significantly downregulated in the patients with HCC, particularly in the tumor center (Fig. 3A; Supplementary Fig. S3A), indicating a possible mechanism related to low infiltration of MAIT cells in HCC.

Scenario goes different while comparing CXCR3 expression with chronic liver diseases, where a positive expression of CXCR3 was detected on MAIT cells (15) and mild expression of CXCR3 was detected in patients with HCC (data not shown). CCR2 is related to IL17-secreting T cells, and is significantly upregulated in CD161\(^+\) T cells (28). In our study, there is a tendency of lower expression of CCR2 in tumor tissues compared with normal livers (Fig. 3B; Supplementary Fig. S3B). Previous study indicated heterogeneous levels of CXCR4 expression on MAIT cells (15), and here, the same phenomenon is reflected (Fig. 3B). In a normal liver, MAIT cells show a high expression of CCR5 (15), and we found that HCC-infiltrating MAIT cells maintained high expression of this marker (Fig. 3B). Taken together, these results showed a selective downregulation of certain chemokine receptors may affect the trafficking and residing capacity of tumor-infiltrating MAIT cells during the progression of hepatocarcinogenesis.

Apoptosis is unlikely involved in the impaired infiltration of MAIT cells in HCC

In addition to aberrant chemotaxis, apoptosis could also result in less immune cells in tumor tissues. Bcl-2 family proteins are...
Moreover, PD-1high MAIT cells had simultaneously higher CTLA-4 blood counterparts (Fig. 3E; Supplementary Fig. S4A and S4B). cohort, whereas mild expression was detected in their peripheral increased in the tumor tissues than normal liver tissues in FCM cohort, whereas mild expression was detected in their peripheral blood counterparts (Fig. 3E; Supplementary Fig. S4A and S4B). Likewise, MAIT cell expression of another two immune inhibitory molecules, CTLA-4 (mean: 6.9% vs. 1.6% or 1.2%, P < 0.006 and TIM-3 (mean: 3.2% vs. 1.3% or 0.23%, P = 0.024), was also significantly increased in the tumor tissues than normal liver tissues in FCM cohort, whereas mild expression was detected in their peripheral blood counterparts (Fig. 3E; Supplementary Fig. S4A and S4B). Moreover, PD-1high MAIT cells had simultaneously higher CTLA-4 and TIM-3 expression (Fig. 3F). To validate these findings, we cocultured MAIT cells from healthy blood with HCL cell lines using Transwell system. FCM data showed that after coculture with HCC cells for 48 hours, significant upregulation of PD-1, CTLA-4, and TIM-3 on MAIT cells was observed in either contact (P = 0.001–0.014) or noncontact manners (P = 0.001–0.009; Supplementary Fig. S4C–S4F).

These results indicated that HCC-infiltrated MAIT cells were educated by tumor cells to be functionally exhausted.

HCC-infiltrated MAIT cells aberrantly produce tumor-promoting cytokines

Previous studies have indicated that MAIT cells had the ability to produce both Th1- and Th17-type cytokines after in vitro stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin or anti-CD3 and anti-CD28 (9, 15, 31). In our study, we showed that the intrinsic IFNγ- and IL17-secreting ability of tumor-derived MAIT cells was significantly inhibited compared with their counterparts in peritumor and normal liver tissues (for IFNγ mean: 47.8% vs. 83.1% or 90.2%, P < 0.001; for IL17 mean: 5.88% vs. 9.87% or 14.6%, P = 0.028; Fig. 4A and B), after stimulating with PMA and ionomycin for 5 hours. Consistently, MAIT cells from peripheral blood of patients with HCC secreted significantly less IFNγ and IL17 compared with healthy donors (for IFNγ mean: 72.9% vs. 90.2%, P = 0.013; for IL17 mean: 4.3% vs. 14.6%, P < 0.001; Supplementary Fig. S5A). Intriguingly, a significant upregulation of IL8, which has a crucial role in promoting tumor angiogenesis and progression, was detected in tumor-derived MAIT cells compared with peritumor and healthy donors for tissue mean: 0.73% vs. 0.36% or 0.02%, P = 0.017; for peripheral blood mononuclear cell (PBMC), 0.79% vs. 0.02%, P = 0.028; Supplementary Fig. S5B and S5C). By contrast, IL4, IL10, and IL22 were variable and no differences were observed among MAIT cells from different sources (Supplementary Fig. S5B–SSD). Collectively, these results indicated that HCC microenvironment inhibited the inherent cytokine-secreting potential of MAIT cells, and promoted the secretion of IL8 by MAIT cells.

HCC-derived MAIT cells produce minimal granzyme B and perforin

Besides Th1/Th17 cytokine secretion, effector function of MAIT cells depends on degranulation to kill sensitized targets (11). Previous study has indicated that resting human MAIT cells are featured by a lack of granzyme B and low perforin expression, but with high expression of granzyme A and granzyme K (13). In this study, we first determined the expression of granzymes and perforin on resting MAIT cells. After recovery from liver tissues, immune cells were stained with fluorescence dye-conjugated antibodies following a FOXP3 staining protocol. Under ex vivo condition, neither granzyme B nor perforin was detected in MAIT cells (Fig. 4C; Supplementary Fig. SSE). Different from granzyme B and perforin, the secretion of which is tightly controlled in vivo, both noncytotoxic molecules granzyme A and granzyme K had high expression in intrahepatic MAIT cells, with a relatively lower expression of granzyme K in HCC-derived MAIT cells (Fig. 4C; Supplementary Fig. SSE).

MAIT cells can be activated in a MR1-dependent manner or through IL12 and IL18 stimulation in a TCR-independent manner in vitro (31). MAIT cells can also be efficiently activated after cocultured with nonprofessional antigen-presenting cells (APC) pretreated with Escherichia coli stimulation, leading to substantial secretion of granzyme B and perforin (31). Following this strategy, MAIT cells were successfully activated to produce granzyme B and perforin after coculture with E. coli–pretreated THP-1 cells for 5 hours (Fig. 4D). However, the frequencies of MAIT cells expressing granzyme B (mean: 1.56% vs. 11.8% or 16.3%, P < 0.001) and perforin (mean: 36.0% vs. 46.3% or 62.7%, P = 0.002) were significantly lower in tumor compared with peritumor or normal liver, where MAIT cells from normal liver produced almost 10- and 2-fold more granzyme B and perforin than HCC tissue, respectively (Fig. 4E). Furthermore, significantly increased apoptosis and impaired proliferation of HCC cells were observed after in vitro coculture with MAIT cells derived from PBMCs of healthy donors or peritumor liver tissues, with markedly upregulated secretion of GM-CSF, TNFα, IFNγ, and MIP1α in the coculture supernatant (Supplementary Figs. S6 and S7). However, coculture supernatant from tumor-infiltrating MAIT cells and HCC cells significantly promoted proliferation and invasion, and inhibited apoptosis of HCC cells in vitro (Supplementary Fig. S7). These results indicated that normal MAIT cells could induce apoptosis of HCC cells, whereas HCC-infiltrated MAIT cells lose tumor-killing ability, promoted proliferation, and invasion of HCC cells.

Molecular characterization of intrahepatic MAIT cells by RNA-seq

Our above data revealed obvious phenotypic differences between HCC and liver-derived MAIT cells. We further determined their global gene expression differences by sorting MAIT cells from paired tumor and peritumor liver tissues in 5 patients with HCC, as well as from 5 normal liver of healthy donors, for RNA-seq. RNA-seq analysis detected more than 6,000 significantly differentially expressed genes in tumor-derived MAIT cells compared with peritumor or normal liver tissues, suggesting an...
Figure 4.
Cytokine secretion and degranulation profile of intrahepatic and peripheral MAIT cells. A, IFNγ- and IL17- secreting profiles of intrahepatic MAIT cells after PMA, ionomycin, and BFA (PIB) stimulation for 5 hours and (B) its summary data (gated on CD4^- CD161^ + TCRv7.2^ + MR1-tet^). C, Representative FCM overlay plots for perforin and granzymes secreted by peripheral and intrahepatic MAIT cells under still condition (gated on CD161^ + TCRv7.2^ + MR1-tet^). HD, healthy donor. D, Bacterial stimulation led to degranulation and changes in cytotoxic profile of MAIT cells. Sorted MAIT cells from normal liver, peritumor, and tumor tissues were cocultured with THP-1 cells, pretreated with or without E. coli, and analyzed by FCM to determine perforin and granzyme B secretion in respective tissues. E, Cumulative data showing reduced frequency of perforin- and granzyme B-expressing MAIT cells (gated on CD161^- TCRv7.2^- MR1-tet^-). GrB, granzyme B. Lines and error bars are presented as the mean ± SD (*, P < 0.05; **, P < 0.01; ***, P < 0.001 by one-way ANOVA and Tukey multiple comparison tests).
Figure 5.
Gene expression profile of intrahepatic MAIT cells based on RNA-seq data. A, Visualization of 15 tissues by first two principal components (comp) of principle component analysis computed on gene expression matrix. B, The Venn diagrams of differentially expressed genes for each pair of groups (P for peritumor, T for tumor, and HDL for normal liver tissues). DEGs were identified by negative binomial generalized linear model (nbGLM) with $|\log{2}\text{(fold-change)}| > 1$ and $P < 0.01$. C, Hierarchical clustering for all DEGs in B. D, Enrichment analysis of up- and downregulated DEGs uniquely altered in tumor MAIT cells. Dot size and color represent the number of genes and $P$ values, respectively. E, Representative expression plot of differentially expressed genes involving cytokine secretion, tumor promotion, and metabolism in MAIT cells between tumor and other tissues. $|\log{2}\text{(fold-change)}| > 1, P < 0.01$, by nbGLM.
aberrant gene expression profile in MAIT cells within HCC microenvironment (Fig. 5A–C; Supplementary Table S3).

Next, we aimed to determine the biological pathways uniquely altered in tumor MAIT cells based on those down- and upregulated genes. The downregulated genes, like NFKB1, STAT5B, and TGFBI (32–34), were enriched in pathways of cytokine secreting and cytolytic effector function, consistent with our findings that effector function of tumor MAIT cells were severely impaired (Fig. 5D and E; Supplementary Table S3), whereas significantly upregulated genes in tumor MAIT cells, like APOE and ALDH1A2, were enriched in pathways involved in aberrant glucose and cholesterol metabolism (Fig. 5C; Supplementary Table S3). Moreover, 114 differentially expressed genes were shared when comparing either of tumor, peritumor, and normal liver tissues, and Gene Ontology analysis indicated that those genes were mainly involved in metabolism, supporting the notion that immune cells may undergo metabolic reprogramming in tumor milieu (Fig. 5B; Supplementary Fig. S8A and S8B; Supplementary Table S4; refs. 35, 36).

Genes aberrantly upregulated in tumor MAIT cells, like IL8, CXCL12, and HAVCR2 (Tim-3), foster HCC development (37, 38). Specially, IL8, an important proinflammatory and angiogenic factor (39), was one of the most upregulated genes in tumor-derived MAIT cells, which was consistent with our previous cytokine secretion result. On the basis of The Cancer Genome Atlas HCC survival data, we showed that patients with higher expression of IL8 were significantly correlated with reduced survival ($P = 0.012$, by log rank test; Supplementary Fig. S8C).

Thus, RNAseq results clearly indicated an inhibited cytolytic effector function and induced tumor-promoting potential of MAIT cells in the HCC microenvironment.

**Discussion**

Innate immunity plays an important role in antitumor immune responses, among which MAIT cells are a population of innate-type T cells preferentially enriched in human liver, indicating its pivotal role in liver immunology. Importantly, this may be the first report of such rigorous evaluation of the distribution, phenotype, function, and clinical relevance of circulating and infiltrating MAIT cells in patients with HCC. We found that overall significant decrease of MAIT cells in tumor and peripheral blood of patients with HCC signify a systemic dysregulation occurred in disease state, which was different from the conventional healthy donors. Of note, tumor-infiltrating MAIT cells displayed an activating and exhausted phenotype with impaired effector capability, and even shifted to produce tumor-promoting cytokines. Interestingly, our prime findings further reveal that the high density of tumor-infiltrating MAIT cells significantly correlated with unfavorable clinical outcomes, and we could possibly infer that MAIT cells are reprogrammed within the tumor microenvironment and may contribute to HCC development.

Reduction of circulating MAIT cell frequency has been reported in various kinds of bacterial infections and viral infections, including HBV and HCV, as well as in patients with colon cancer (16, 18–20, 42). Similar to our FCM analysis, we observed a significant decrease of circulating MAIT cell frequency in patients with HCC. This reduction may be attributed to tumor-associated factors or chronic infectious conditions, considering that majority of patients were HBV+, which couldn’t be ruled out. However, whether MAIT cell infiltration in tumor tissues was decreased or increased compared with the non tumor counterparts remains controversial. An increased MAIT cell infiltration in colon cancer and a decreased infiltration in colorectal hepatic metastases were observed, which were compared with normal colon and normal liver respectively (19, 43). Our flow cytometric, IHC, and qRT-PCR data collectively demonstrated that MAIT cell infiltration was significantly lower in HCC tumor than of peritumor tissues. Being aware of the abundance of intrahepatic MAIT cells, we wondered to compare the absolute number or frequency of infiltrating MAIT cells aided with multiplex IHC. Our results showed that the
Figure 6. Higher infiltration of MAIT cells correlates with unfavorable clinical outcomes. A, Kaplan–Meier curves for OS and RFS according to MAIT cell frequency in the FCM cohort (n = 50). B, Relative expression of TCRv7.2 in tumor and peritumor tissues (n = 209, paired t test). C, Kaplan–Meier curves for OS and RFS according to TCRv7.2 mRNA expression level in the qRT-PCR cohort. D, Representative images of MAIT cell distribution in peritumor tissues. The subset was defined as CD3 \(^+\) MDR-1 \(^+\) IL-18R \(^+\) cells using the TSA method, and immunofluorescence images were scanned at 20× on the Vectra Automated Imaging System. E, MAIT cell absolute number significantly decreased in tumor compared with peritumor (mean number: 4.1 vs. 5.8 cells/core, P < 0.001; n = 224, by paired t test). F, Kaplan–Meier curves for OS and RFS according to MAIT cell density in the TMA training cohort. Lines and error bars are presented as the mean ± SD.
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densities of MAIT cells in HCC and paired liver tissues were 7.2 versus 17.4 cells/mm², whereas MAIT cells in colon cancer and healthy colon mucosa were reported to be 6.1 versus 2.6 cells/mm² (19). Alternatively, when evaluating MAIT cell frequency among CD3⁺ T cells, similar trend was observed (4.2% and 3.2% for HCC and colon cancer, respectively; ref. 18). Obviously, MAIT cell infiltration in HCC slightly surpassed that in colon cancer. Hence, we assumed that MAIT cells were relatively accumulated in HCC although less than the peritumor liver tissues, similar to the findings that MAIT cells heavily infiltrated colorectal hepatic metastases, but to a lesser extent than the liver (19, 43). A selective downregulation of chemokine receptors in HCC-infiltrating MAIT cells observed in our study could be a possible explanation for the less infiltration.

In both the patients with HCC and healthy donors, MAIT cells displayed a typical CCR7⁺CD45RA⁻CD45RO⁺CD95⁺ effector memory phenotype (9, 44). MAIT cells are known to respond against external stimuli reflecting with the release of cytokines in the surroundings and have some important implications in disease regulation. However, HCC-infiltrating MAIT cells' effector function was found to be severely impaired, and even could produce tumor-promoting cytokine like IL8. First, both IFNγ and IL17 secreted by tumor-derived MAIT cells were significantly lower than the counterparts in peritumor and healthy liver tissues. Meanwhile, our observations also detect an obvious decline of CD160 and CD127 expression in tumor-derived MAIT cells. Generally, MAIT cells are known to express higher level of CD160 and CD127 both in peripheral blood and liver tissues, which are need for Th1 and Th17 cytokine production (14, 45). Thus, it is possible that suppressed IFNγ and IL17 secretion of intratumor MAIT cells could be ascribed to CD160 and CD127 downregulation. Second, MAIT cells' effector function mainly relied on granzyme B and perforin, which are key molecules necessary for the efficient cytotoxic activity (46, 47). Our data demonstrated that HCC-infiltrating MAIT cells produced significantly less granzyme B and perforin than control, indicating the cytotoxic potential of intratumor MAIT cells was substantially inhibited, assuming a consistent local microenvironmental impact induced the transition. Third, we found that PD-1, CTLA-4, and TIM-3 expression was markedly upregulated in HCC-infiltrating MAIT cells, together with a high expression of the activating markers, CD38 and HLA-DR, which share common features of exhausted T cells, was thought to be negative immune regulator in the tumor milieu (26, 48). Finally, a significant upregulation of IL8, known for tumor-promoting factor (49), was detected in HCC-derived MAIT cells. Additional in vitro coculture experiments confirmed the tumor-promoting function mediated by intratumor MAIT cells as compared with peritumor or circulating MAIT cells. Altogether, similar to tumor-associated macrophages and neutrophils, we postulated that tumor-infiltrating MAIT cells were reprogrammed to a tumor-promoting direction. As such, coculture with HCC cells could lead to a significant upregulation of PD-1, CTLA-4, and TIM-3 on MAIT cells. RNA-seq analysis of infiltrating MAIT cells further validated that genes related to cell activation, cytokine secretion, and metabolism were rerouted to favor a tumor-promoting function in HCC.

Consistent with their tumor-promoting function, we found that high levels of tumor-infiltrating MAIT cells significantly and independently correlated with dismal clinical outcomes as established in four independent cohorts of patients with HCC. To date, the prognostic value of MAIT cells has only been reported in colon cancer, where high densities of tumor-infiltrating MAIT cells were also associated with poor survival and serum CEA level positively correlated with MAIT cell infiltration (18). Nonetheless, our results were mainly derived from patients with HBV-related HCC. It will be important to validate the prognostic value of MAIT cells among patients with HCC with other etiologies like in HCV or fatty liver.

In summary, our findings showed that HCC-infiltrating MAIT cells were skewed toward a tumor-promoting direction and were detrimental to patient prognosis. Soluble factors derived from HCC cells or direct contact with HCC cells can activate MAIT cells within the tumor milieu, markedly suppress their cytotoxic capability, and induce them to produce significant amount of protumor cytokines. These reprogrammed MAIT cells shifted away from antitumor toward tumor-suppressive and proangiogenic pathways. Strategies that modulate the function of MAIT cells may provide a new avenue for antitumor therapy in HCC.

Transcript Profiling

The RNA-seq data in this paper have been submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; accession GSE117627).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Activated and Exhausted MAIT Cells Foster Disease Progression and Indicate Poor Outcome in Hepatocellular Carcinoma

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