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RESEARCH

Improved MAIT cell functions following fecal microbiota transplantation for metastatic renal cell carcinoma

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Abstract

Strategies to modify the gut microbiome in cancer patients using fecal microbiota transplantation (FMT) have gained momentum as a therapeutic intervention. However, how FMT impacts innate-like, antimicrobial T lymphocytes is unclear. In this study, we assessed peripheral blood (PB) mucosa-associated invariant T (MAIT) cell frequencies and functions in patients with metastatic renal cell carcinoma (mRCC) before and seven days after they received FMT as part of a clinical trial. We found comparable MAIT cell frequencies in healthy controls and mRCC patients. In contrast, γδ T cells exhibited a numerical decline in mRCC, which was partially reversed by FMT. We also found a significant increase in the PB $CD4^+$ MAIT cell compartment of mRCC patients with or without FMT. Paired sample analyses revealed CD69 upregulation on MAIT cells accompanied by decreased PD-1 levels post-FMT. These changes were unique to MAIT cells as non-MAIT T lymphocytes showed either no trend or a trend in the opposite direction. Importantly, FMT did not render MAIT cells exhausted as also judged by their stable expression of TIM-3, LAG-3, BTLA, CTLA-4, TIGIT and VISTA. These findings were corroborated in functional assays in which MAIT cells were stimulated with MR1 ligands or with a combination of IL-12 and IL-18 to produce inflammatory cytokines and granzyme B. Indeed, when stimulated ex vivo with IL-12 and IL-18, MAIT cells mounted a more rigorous TNF-α response post-FMT. In conclusion, FMT improves MAIT cell functions, which should serve patients well in subsequent microbial challenges in the face of cancer-elicited immunosuppression. *Trial Registration*: https://clinicaltrials.gov/Identifier: NCT04163289 (registration date: November 14, 2019).

Keywords MAIT cells · Fecal microbiota transplantation · Renal cell carcinoma · Kidney cancer · MR1 · Cytokine

Abbreviations

Ag	Antigen
BFA	Brefeldin A
BTLA	B- and T-lymphocyte attenuator
CTLA-4	Cytotoxic T-lymphocyte-associated antigen-4
CRC	Colorectal cancer
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
FMT	Fecal microbiota transplantation
FoxP3	Forkhead box P3
6-FP	6-Formylpterin
GATA-3	GATA binding protein 3

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gMFI	Geometric mean fluorescence intensity
GZM	Granzyme
HC	Healthy control
HLA	Human leukocyte antigen
ICI	Immune checkpoint inhibitor
IFN	Interferon
IL	Interleukin
*i*NKT	Invariant natural killer T [cell]
*i*TCR	Invariant T cell receptor
LAG-3	Lymphocyte activation gene 3
mAb	Monoclonal antibody
MAIT	Mucosa-associated invariant T [cell]
MHC	Major histocompatibility complex
MR1	MHC-related protein 1
mRCC	Metastatic renal cell carcinoma
5-OP-RU	5-(2-Oxopropylideneamino)-6-D-ribitylaminouracil
PB	Peripheral blood
PBMC(s)	Peripheral blood mononuclear cell(s)
PBS	Phosphate-buffered saline
PD-1	Programmed cell death-1
PMA	Phorbol 12-myristate 13-acetate
RCC	Renal cell carcinoma
RORγt	Retinoic acid receptor-related orphan receptor γt
T-bet	T-box expressed in T cells
TH2	T helper 2 [cell]
TIGIT	T cell immunoreceptor with immunoglobulin and ITIM domains
TIM-3	T cell immunoglobulin and mucin-3
TME(s)	Tumor microenvironment(s)
TNF	Tumor necrosis factor
VISTA	V-domain immunoglobulin suppressor of T cell activation

Introduction

Renal cell carcinoma (RCC), the malignant transformation of tubular epithelial cells, is the most common type of kidney cancer and the most deadly urological neoplasm. RCC is more frequent in men than in women, and accounts for more than 2% of all oncological diagnoses in the middle-aged and elderly populations worldwide [1]. A considerable proportion of RCC patients present with evidence of metastatic disease at diagnosis, which is associated with poor overall survival. Apart from partial or radical nephrectomy, the systemic treatment of RCC includes radiotherapy, targeted therapy with tyrosine kinase inhibitors, and immunotherapy. Immune checkpoint inhibitors (ICIs) have been used in patients with metastatic RCC (mRCC) and shown promise. However, ICIs may exert systemic toxicity and immune-related adverse effects, resulting in their discontinuation [2]. Also importantly, a sizable fraction of mRCC patients do not favorably respond to ICIs. Therefore, more tolerable and more effective treatment strategies for mRCC are urgently needed.

Fecal microbiota transplantation (FMT) is a therapeutic intervention in which fecal matter from a healthy donor is introduced to alter the intestinal microbiota of a recipient. FMT has been successfully used for the management of recurrent *Clostridioides difficile*-associated diarrhea [3], and may be efficacious in certain metabolic and autoimmune disorders [4–6]. The potential clinical benefits of FMT have also been proposed or recognized in oncology, with the possibility of alleviating the side effects of radiotherapy, chemotherapy and immunotherapy [7–11].

Mucosa-associated invariant T (MAIT) cells constitute a subset of innate-like T lymphocytes with prominent antimicrobial activities, effector memory-like characteristics and notable homing to mucosal barriers, including the gut [12]. They are also relatively abundant in the peripheral blood (PB) where they comprise up to 10% of circulating T cells. The invariant T cell receptor (*i*TCR) of MAIT cells detects microbial metabolites of the riboflavin biosynthesis pathway presented by the monomorphic antigen (Ag)-presenting molecule MHC-related protein 1 (MR1) [13, 14]. MAIT cells can also be activated by select inflammatory cytokines, including type I interferons (IFNs), interleukin (IL)-7, IL-12, IL-15 and IL-18, which are produced during many infections. Upon stimulation, MAIT cells swiftly secrete immunomodulatory cytokines of their own [*e.g.*, IFN-γ, tumor necrosis factor (TNF)-α, IL-4, IL-5, IL-13, IL-17A and/or IL-22] and destroy infected cells that display MR1 ligands [15]. Furthermore, there is growing appreciation for MAIT cell roles in tissue repair [16], which may contribute to the healing process after infections.

In addition to their crucial roles in anti-pathogen immunity, MAIT cells are implicated in mucosal homeostasis and sense the presence of commensal microbes [17]. In fact, commensal flora and their metabolites control MAIT cells' intrathymic development and extrathymic expansion in the gut lamina propria [17, 18]. Certain commensal species belonging to the Bacteroidetes and Proteobacteria phyla are among the most potent producers of riboflavin and the most powerful stimulators of MAIT cells [19].

Although tumor-derived MR1 ligands that may serve as MAIT cell Ags remain obscure, anticancer, pro-tumorigenic and pro-metastatic properties have been reported or envisaged for MAIT cells, largely due to their ability to modify the biological behaviors of downstream effector and regulatory cells [20, 21]. How MAIT cell responses to microbes and their products during natural infections or in therapeutic settings (*e.g.*, FMT) influence clinical outcomes is unclear. Such responses may alter the cellular landscape of tumor microenvironments (TEMs) and bodily macroenvironments

to patients' benefit or detriment. On the other hand, excessive exposure to bacterial products or inflammatory cytokines may overwhelm MAIT cells and impede their ability to face and overcome subsequent microbial challenges [22]. This is particularly important in cancer patients whose underlying malignancy and the treatments they receive may result in immunosuppression.

FMT has been employed in recent preclinical studies and clinical trials to improve the efficacy of programmed cell death-1 (PD-1)-based ICIs in cancer [8, 9, 23]. However, whether and how FMT alters MAIT cell functions is essentially unexplored. In this study, we have used paired peripheral blood mononuclear cell (PBMC) samples from RCC patients before and after FMT, along with samples from a healthy control cohort, to assess MAIT cell frequencies and responses to a panel of *i*TCR-dependent and -independent stimuli. We demonstrate, for the first time, that FMT improves certain aspects of MAIT cell functions, including their cytokine production capacities. Therefore, MAIT cells should effectively fulfill their cognate and cytokine-mediated roles in host defense or may operate more optimally post-FMT.

Materials and methods

Subjects

Ten consenting patients, two females and eight males (age range: 47–71) (Supplementary Table 1), with intermediate- or high-risk mRCC, without prior immunotherapy for their advanced disease, were enrolled. This study was approved by the Western University Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB protocol #114962). Ten closely age- and sex-matched healthy blood donors (Supplementary Table 1) were consented and recruited using HSREB protocol #5545, which was approved by the same institutional entity.

All patients had received a histologically confirmed diagnosis of advanced or metastatic (stage IV) RCC based on the American Joint Committee on Cancer staging system. Nine had clear cell RCC, and one was diagnosed with sarcomatoid RCC. Subjects younger than 18 or older than 100 were excluded. Other exclusion criteria were pregnancy, psychiatric illness, immunodeficiency, active systemic infections, active autoimmune disorders, inflammatory bowel disease, immunosuppressive therapy, radiation therapy within a four-week timeframe before the study commencement date, and the use of antibiotics within a two-week period prior to FMT. A more detailed list of inclusion and exclusion criteria can be found at ClinicalTrials.gov (Trial Identifier: NCT04163289).

To select fecal transplant donors, subjects with disorders and conditions listed in Table 1 were excluded [24, 25].

Blood processing and PBMC isolation and storage

PB from RCC patients was drawn into sodium heparin-coated tubes (BD Biosciences) before and seven days after FMT. Healthy controls (HCs) provided a single PB sample.

To isolate PBMCs, anticoagulated PB was diluted with sterile PBS and transferred into SepMate-50 tubes (STEMCELL Technologies) containing Ficoll-Paque PLUS (GE Healthcare) and subjected to density gradient centrifugation at $1200 \times g$ for 10 min. PBMCs were then washed and resuspended at 10^7/mL in a freezing medium consisting of 12.5% human serum albumin, 30 µg/mL gentamicin and 10% dimethyl sulfoxide (DMSO) in RPMI 1640. Cryovials containing PBMC aliquots ($5–10 \times 10^6$ cells/vial) were transferred into a container filled with 200 mL of isopropanol and placed inside a − 80 °C freezer to enable a gradual drop in temperature at a rate of ~ 1 °C/minute. After 24–48 h, cryovials were moved into a − 150 °C freezer where they were kept until use.

Fecal donor screening and FMT

Donors were screened for known transmissible pathogens following our established protocol [24, 25]. Each recipient was given approximately 40 oral capsules cumulatively containing 80–100 g of starting fecal material from a qualified donor (Table 1). FMT was performed seven days before recipients started dual immunotherapy with ipilimumab [an anti-cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) monoclonal antibody (mAb)] and nivolumab (an anti-PD-1 mAb) for mRCC as part of an ongoing clinical trial (Trial Identifier: NCT04163289).

Ex vivo PB MAIT cell stimulation

Escherichia coli (strain DH5α) lysate was prepared from bacterial cultures, stored at − 80 °C and used as we previously described [22]. To generate the MR1 ligand 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), equimolar concentrations of 5-amino-6-D-ribitylaminouracil, generously provided by Dr. Olivier Lantz (Institut Curie), and methylglyoxal were mixed in DMSO for 24 h at room temperature.

Frozen PBMCs were thawed at room temperature before they were washed and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, 2 mM GlutaMAX-I, 1 mM sodium pyruvate, 10 mM HEPES and 100 U/mL penicillin/streptomycin, which we will refer to as complete medium. Typically, more than 80% of thawed PBMCs

Table 1 Exclusion criteria for fecal transplant donor selection

General				
Age over 50				
BMI of under 18.5 or above 24.9				
Past/present malignancies				
Autoimmune diseases				
Hospitalization within the past 3 months				
Recent travel to a developing country and to Zika-endemic regions within the past 3 months				
Street drug use; alcohol intake of > 10 g/day in women and > 20 g/day in men				
New sexual partners (within the last 3 months); high-risk sexual behavior				
Ongoing or recent use of any prescription or over-the-counter medications				
Getting a tattoo(s) or body piercing in the past 6 months				
Known HIV or viral hepatitis B and C exposure within the past 12 months				
Metabolic disorders				
Hypertension, hyperlipidemia, diabetes, insulin resistance, atherosclerosis				
History of gastrointestinal, hepatic and/or biliary disorders				
GERD, peptic ulcer, celiac disease, ulcerative colitis, Crohn's disease, microscopic colitis, irritable bowel syndrome, gastroparesis, diverticular disease				
Previous intestinal or hepatic surgeries (except for remote appendectomy)				
Psychiatric history				
Major affective disorders, psychotic illness				
Ongoing treatment with psychotropic medications				
Family health history				
Diabetes, early-onset coronary disease, gastrointestinal or hepatic diseases, colon cancer				
Positive laboratory results for the following pathogens				
Viruses	Bacteria	Fungi	Helminths	Protozoa
Adenovirus	Campylobacter	Microsporidia	Ova	Babesia
Cytomegalovirus	*Chlamydia trachomatis*		Schistosoma	Cryptosporidium
Epstein–Barr virus	*Clostridium difficile*		*Strongyloides stercoralis*	*Entamoeba histolytica*
Hepatitis A	Enterobacteriaceae			Giardia
Hepatitis B	(CRE and ESBL-producing)			Plasmodium[a]
Hepatitis C	Enterococci (vancomycin-resistant)			*Trypanosoma cruzi*[a]
HIV types 1 and 2	*Escherichia coli* 0157H7 (Shiga toxin)			
HTLV types 1 and 2	*Helicobacter pylori*			
Norovirus	Listeria			
Rotavirus	MRSA			
SARS-CoV-2	*Neisseria gonorrhoeae*			
	Salmonella			
	Shigella			
	Treponema pallidum			
	Yersinia			

BMI body mass index; *CRE* carbapenem-resistant Enterobacteriaceae; *ESBL* extended spectrum beta lactamase; *GERD* gastroesophageal reflux disease; *GI* gastrointestinal; *HIV* human immunodeficiency virus; *HTLV* human T cell lymphotropic virus; *MRSA* methicillin-resistant *Staphylococcus aureus*; *SARS-CoV-2* severe acute respiratory syndrome coronavirus 2

[a]test conducted in case of travel history to affected areas

were viable as judged by trypan blue dye exclusion. Paired pre- and post-FMT PBMC samples were thawed, stimulated (as applicable), stained and analyzed on the same day by the same experimentalist.

PBMCs were seeded at 5×10^5 cells/well in U-bottom microplates and either left untreated or stimulated with clear *E. coli* lysate (diluted 1:10 in complete medium), with human B lymphoblastoid C1R cells (pulsed with 2 nM 5-OP-RU and used at a 1:10 C1R:PBMC ratio), or with a combination of recombinant human IL-12 (rhIL-12) p70 (PeproTech) and rhIL-18 (MBL International) (5 ng/mL each). Cultures were incubated for 24 h at 37 °C and 6% CO_2. Brefeldin A (BFA) (Sigma-Aldrich) and monensin (eBioscience) were added to cultures at 10 µg/mL and 2 µM, respectively, for the final 5 h.

In several experiments, PBMCs were stimulated for 4 h with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) and 500 ng/mL ionomycin in the presence of BFA and monensin.

Cytofluorimetric enumeration and immunophenotyping of conventional and innate-like T cells

Unstimulated and stimulated PBMCs were washed and resuspended in a cold buffer (PBS containing 2% FBS and 2 mM ethylenediaminetetraacetic acid) before they were stained at room temperature with fluorochrome-conjugated tetramers, antibodies and/or control reagents (Supplementary Table 2).

Dead cells were excluded using either 7-AAD (BD Biosciences) or Fixable Viability Dye (eBioscience). After surface staining, cells were fixed and permeabilized using a Forkhead box P3 (FoxP3)/Transcription Factor Staining Buffer Set (eBioscience) to detect intracellular cytokines, transcription factors and granzyme B (GZM B) as indicated.

After dead cell and doublet exclusions, a lymphocyte gate was drawn based on forward and side scatter characteristics before MAIT cells were identified as $CD3^+$ cells that stained positively with 5-OP-RU-loaded MR1 tetramers. $CD3^+$MR1 tetramer$^-$ cells were considered non-MAIT T cells. Our gating strategy is illustrated in Supplementary Fig. 1. Invariant natural killer T (*i*NKT) cells were defined as $CD3^+$PBS-57-loaded CD1d tetramer$^+$ cells. We used 6-formylpterin (6-FP)-loaded MR1 tetramers (Supplementary Fig. 1) and empty CD1d tetramers as negative staining controls, as appropriate. All tetramer reagents were supplied by the NIH Tetramer Core Facility.

A BD FACSCanto II cytometer and FlowJo software (Tree Star) were used for data acquisition and analysis, respectively.

Statistical analyses

GraphPad Prism 8 was used for all statistical analyses. Normal distribution of data sets was assessed by the D'Agostino–Pearson test. Comparisons were made using the statistical tests identified in figure legends, and differences with $p \leq 0.05$ were considered significant. Data are presented as mean ± standard error of the mean (SEM) when applicable.

Results

Unlike γδ T cells, MAIT cells maintain their PB frequencies in RCC patients before and after FMT

Numerical declines in the PB MAIT cell compartment of cancer patients have been observed [26–29]. In addition, infections and other microbial challenges often lower PB MAIT cell frequencies, potentially due to activation-induced cell death or altered migratory patterns [30, 31]. In this study, we found comparable MAIT cell percentages between RCC patients and age/sex-matched HCs (Fig. 1). This was clear when MAIT cells were enumerated among either bulk PBMCs (Fig. 1A) or $CD3^+$ T lymphocytes (Fig. 1C). Moreover, our paired sample analyses revealed no reduction in PB MAIT cell abundance after FMT (Fig. 1B and D). In fact, we found a moderate trend toward increased, rather than decreased, MAIT cell frequencies among $CD3^+$ events post-FMT (Fig. 1D).

Most PB MAIT cells in healthy subjects express CD8, a coreceptor that binds MR1 and enhances cytokine production by MAIT cells [32]. Therefore, the functional plasticity of MAIT cell subsets may be partially due to their CD8 expression, or lack thereof. As anticipated, the vast majority of PB MAIT cells in our HC cohort expressed CD8 (Fig. 2A). However, we found significantly higher $CD4^+CD8^-$ MAIT cell levels in RCC patients than in HCs (Fig. 2A), regardless of their FMT treatment status (Fig. 2A–B). This was exclusive to MAIT cells since non-MAIT T lymphocytes from HC and RCC cohorts exhibited a similar coreceptor expression pattern (Supplementary Fig. 2A), which also remained stable after FMT in RCC patients (Supplementary Fig. 2B).

Innate-like T lymphocytes other than MAIT cells, such as *i*NKT and γδ T cells, comprise a minute fraction of non-MAIT T cells. However, they can play important roles in antimicrobial defense and in host responses to cancer [33]. Therefore, it was of interest to also enumerate these cell types in our cohorts. While circulating at low frequencies in both HCs and RCC patients, PB *i*NKT cell levels

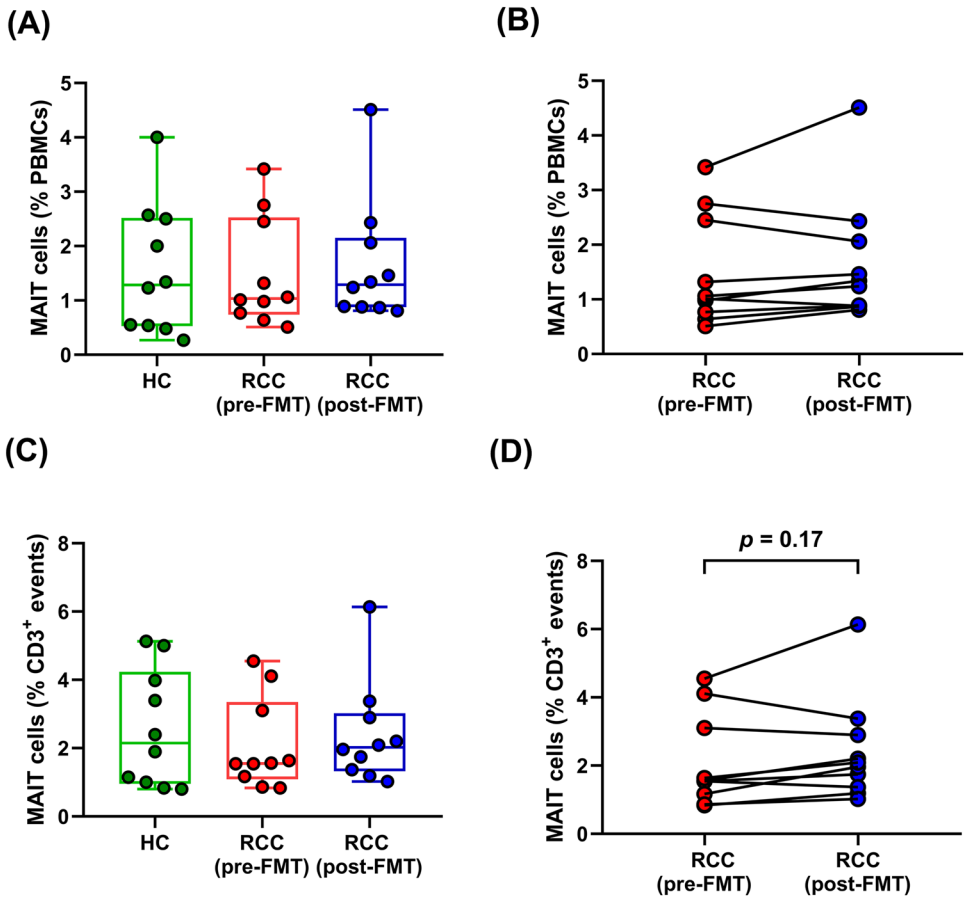

Fig. 1 PB MAIT cell frequencies remain stable following FMT for RCC. MAIT cell frequencies were determined by 5-OP-RU-loaded MR1 tetramer staining among live PBMCs (**A** and **B**) or $CD3^+$ events (**C** and **D**) in 10 healthy controls (HCs) (**A** and **C**) and 10 renal cell carcinoma (RCC) patients before and seven days after FMT (**A**–**D**). Box-and-Whisker plots (**A** and **C**) illustrate MAIT cell percentages in the three cohorts. The results of pre- and post-FMT paired sample analyses are also depicted (**B** and **D**). Each circle represents an individual sample. Kruskal–Wallis tests were followed by Dunn's post hoc comparisons (**A** and **C**), and Wilcoxon signed-rank tests were used for matched pair analyses (**B** and **D**)

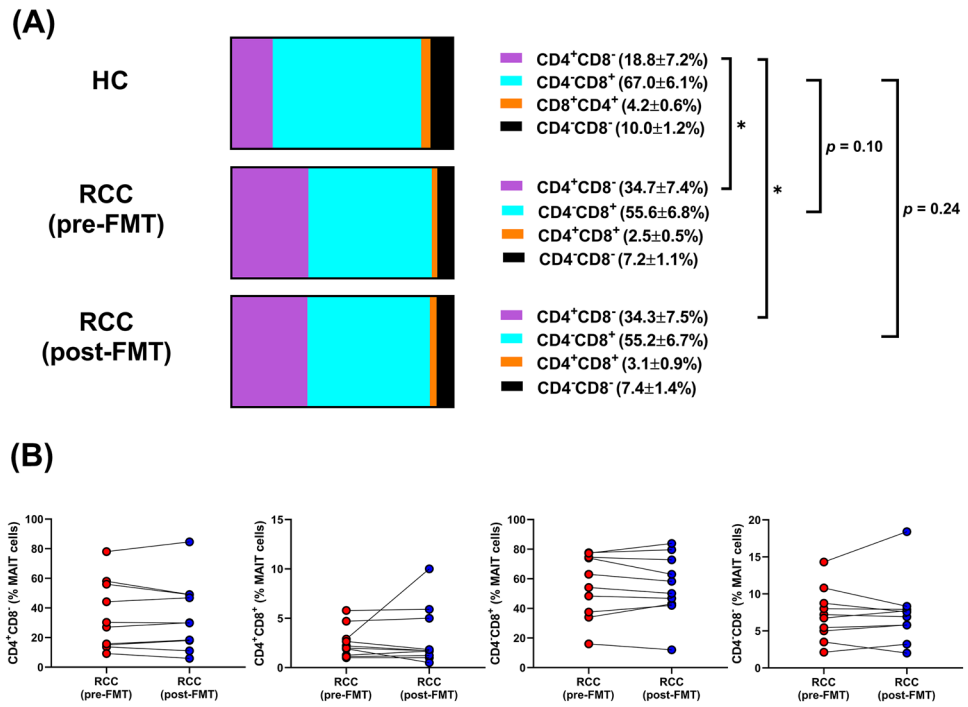

Fig. 2 $CD4^+CD8^-$ MAIT cell frequencies are increased in the PB of RCC patients. The percentages of PB $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$ and $CD4^-CD8^-$ MAIT cell subsets were determined by flow cytometry among $CD3^+$ 5-OP-RU-loaded MR1 tetramer$^+$ cells in 10 healthy controls (HCs) and 10 RCC patients before and after FMT. Horizontal slice charts (**A**) illustrate the averaged distribution of MAIT cells expressing CD4 and/or CD8, or not. The exact mean and SEM values are also shown, and * denotes a statistically significant difference with $p \leq 0.05$ using Mann–Whitney U tests (**A**). Wilcoxon signed-rank tests were employed for matched pair analyses comparing pre- and post-FMT samples (**B**)

appeared to form a larger component of $CD3^+$ T cells in patients (with or without FMT) compared with HCs (Supplementary Fig. 3A). By contrast, there was a strong trend toward reduced γδ T cell percentages among PB $CD3^+$ T cells in RCC, which were partially restored following FMT (Supplementary Fig. 3B). FMT was similarly effective in resolving γδ T cell deficits when the frequencies of these cells were determined among total PBMCs ($1.73 \pm 0.48\%$ and $2.13 \pm 0.58\%$ in pre- and post-FMT samples, respectively, $p < 0.01$).

The above results indicate that circulating MAIT cell pools are retained and γδ T cell frequencies recover after FMT for RCC.

FMT does not disrupt the MAIT1-MAIT17-MAIT1/17 balance in the PB

The immunomodulatory activities of MAIT cells are governed by the type of transcription factors that they express. MAIT cells can be generally divided into MAIT1 and MAIT17 subsets based on their ability to express T-bet (T-box expressed in T cells) or RORγT (retinoic acid receptor-related orphan receptor γt), respectively [34]. However, these transcription factors are not mutually exclusive in human MAIT cells as "MAIT1/17" cells harbor both. We found $T\text{-bet}^+ROR\gamma t^-$ (MAIT1), $T\text{-bet}^-ROR\gamma t^+$ (MAIT17), $T\text{-bet}^+ROR\gamma t^+$ (MAIT1/17) and $T\text{-bet}^-ROR\gamma t^-$ (double-negative) cell frequencies to be comparable in HCs and RCC patients (Fig. 3A and Supplementary Fig. 4). Furthermore, FMT did not tip the balance in favor of any of the above-defined subsets (Fig. 3A–B) neither did it change the geometric mean fluorescence intensity (gMFI) values of T-bet and RORγt (Supplementary Fig. 5).

Chronic stimulation of human PB MAIT cells in ex vivo settings reportedly promotes the late-onset production of T helper 2 (T_H2)-type cytokines, especially IL-13 and IL-5 and to a lesser extent IL-4 [35]. Following FMT, the presence of myriad vitamin B_2-producing bacteria within the fecal matter should result, at least in theory, in prolonged *i*TCR triggering and chronic MAIT cell stimulation. Therefore, we compared pre- and post-FMT samples for their $GATA\text{-}3^+$ MAIT cell content. As expected, only a small proportion of MAIT cells expressed GATA-3 in our cohorts (Supplementary Fig. 6A). Furthermore, $GATA\text{-}3^+$ MAIT cell frequencies within pre- and post-FMT PBMCs were statistically comparable (Supplementary Fig. 6B).

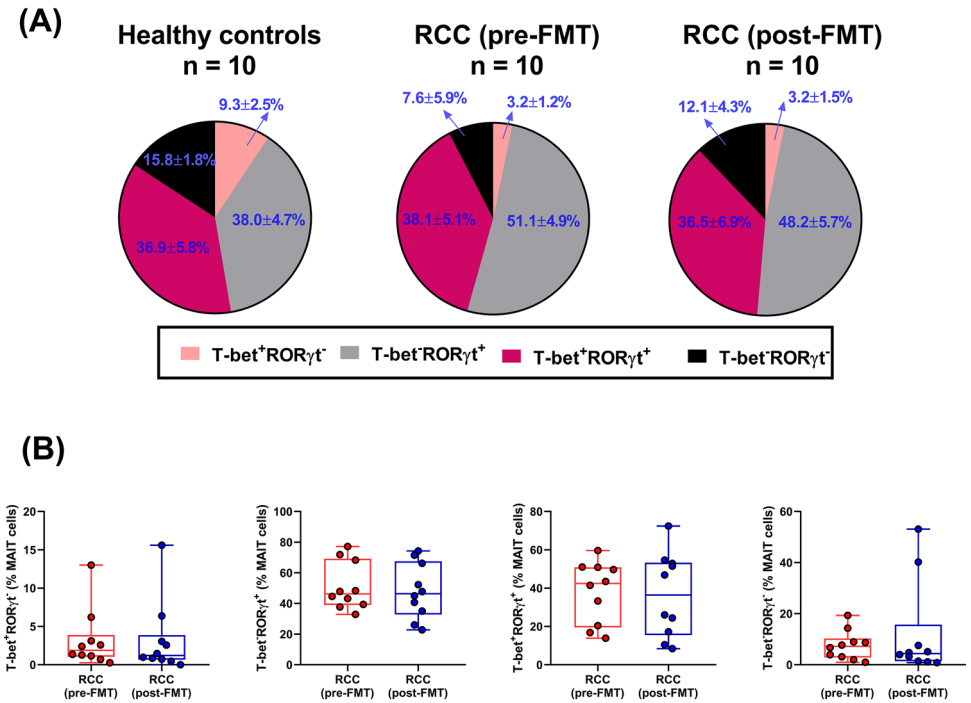

Fig. 3 PB MAIT cells maintain their MAIT1-MAIT17-MAIT1/17 balance post-FMT. PBMCs from 10 healthy controls (HCs) and 10 RCC patients (before and seven days after FMT) were analyzed by flow cytometry for indicated transcription factors within the $CD3^+$ 5-OP-RU-loaded MR1 $tetramer^+$ MAIT cell population. (**A**) Pie charts depict the frequencies of $T\text{-bet}^+ROR\gamma t^-$, $T\text{-bet}^-ROR\gamma t^+$, $T\text{-bet}^+ROR\gamma t^+$ and $T\text{-bet}^-ROR\gamma t^-$ subsets. (**B**) Box-and-Whisker plots illustrate the proportions of the above subsets in RCC patients pre- and post-FMT. Each circle represents an individual sample, and data are shown as mean ± SEM. Kruskal–Wallis tests, followed by Dunn's post hoc tests, were employed for group comparisons (**A**), and Wilcoxon signed-rank tests were used for matched pair analyses (**B**)

FMT upregulates CD69 and downregulates PD-1 on MAIT cells

Exposure to bacteria and their products activates MAIT cells, which may or may not be followed by cellular exhaustion and a failure to respond to subsequent microbial challenges [22]. Therefore, we examined the expression of activation and exhaustion markers by MAIT cells in RCC patients before and after FMT. Indeed, FMT upregulated CD69, an early activation marker, as judged both by higher $CD69^+$ MAIT cell frequencies (Fig. 4A) and by the elevated gMFI of CD69 staining, indicative of increased CD69 expression on a per-cell basis (Fig. 4B). By comparison, non-MAIT T cells downregulated their CD69 expression post-FMT (Fig. 4C–D). Comparing MAIT and non-MAIT T cells for CD38, another activation marker, revealed no changes after FMT except for a slight, but still significant, increase in the gMFI of CD38 staining in non-MAIT T cells (Supplementary Fig. 7).

Next, we assayed for the expression of surface markers associated with cellular exhaustion or co-inhibitory functions, including B- and T-lymphocyte attenuator (BTLA), CTLA-4, lymphocyte activation gene 3 (LAG-3), PD-1, T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), T cell immunoglobulin and mucin-3 (TIM-3), and V-domain immunoglobulin suppressor of T cell activation (VISTA). We detected higher levels of PD-1 and BTLA on MAIT cells in RCC patients compared with HCs (Fig. 5). Importantly, parallel sample analyses revealed a significant reduction in PD-1 expression by MAIT cells (Fig. 5). Also of note, we found increased percentages of $BTLA^+$ and $TIM\text{-}3^+$ non-MAIT T cells in RCC patients (Supplementary Fig. 8). However, FMT did not change the expression levels of any of the above markers in this compartment.

Collectively, the above data indicate that FMT results in MAIT cell activation without rendering them overwhelmed and consequently exhausted. On the contrary, FMT lowers the expression of the classic exhaustion marker PD-1.

MAIT cells remain responsive to MR1 ligands in FMT recipients

To assess the *i*TCR-coupled MAIT cell activation pathway, we stimulated PBMCs with 5-OP-RU, a potent MR1 ligand [14], which was pulsed on C1R cells to allow for stable Ag presentation to MAIT cells [36]. As illustrated in Fig. 6A–B, exposure to 5-OP-RU reduced the frequency of detectable

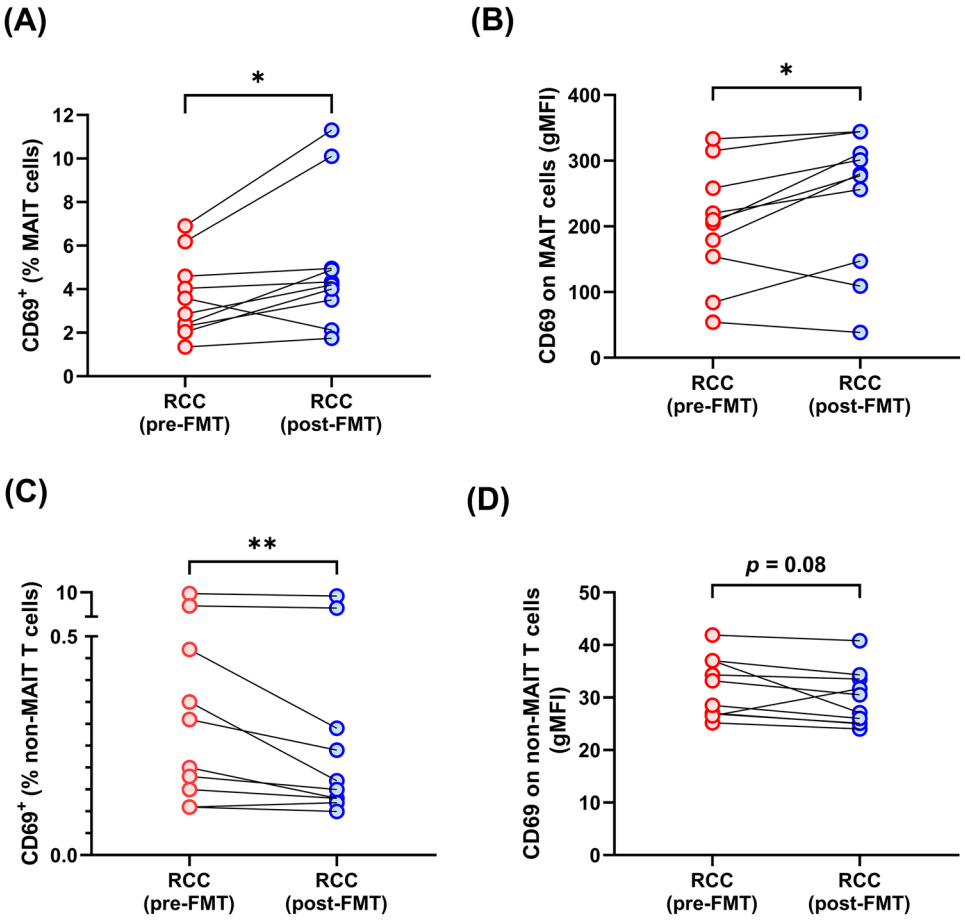

Fig. 4 FMT leads to increased CD69 expression on MAIT cells, but not on non-MAIT T lymphocytes. PBMCs from 10 RCC patients, collected before to seven days after FMT, were stained and analyzed by flow cytometry for the surface expression of CD69 on $CD3^+$ 5-OP-RU-loaded MR1 $tetramer^+$ (MAIT) cells as well as $CD3^+$ 5-OP-RU-loaded MR1 $tetramer^-$ (non-MAIT T) cells. $CD69^+$ cell frequencies (**A** and **C**) and the geometric mean fluorescence intensity (gMFI) of CD69 staining (**B** and **D**) are shown. Each circle represents an individual RCC sample. Wilcoxon signed-rank tests were used to compare paired samples, * and ** denote statistically significant differences with $p \leq 0.05$ and $p \leq 0.01$, respectively

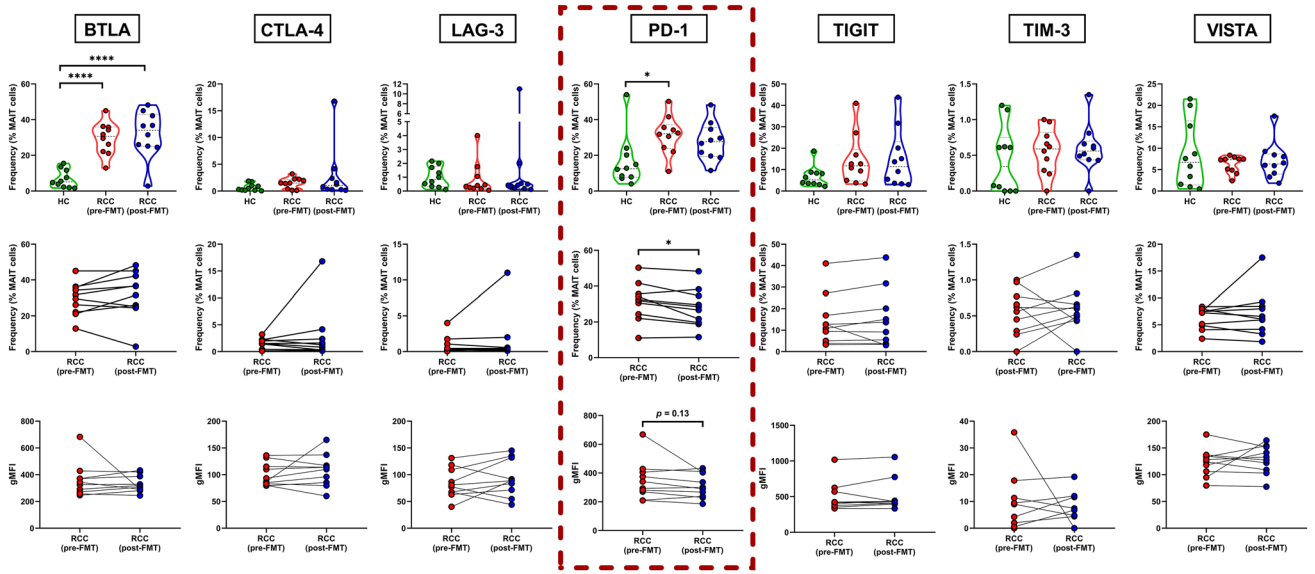

Fig. 5 FMT results in decreased PD-1 expression on MAIT cells. Pre- and post-FMT PBMC samples from 10 RCC patients along with PBMCs from 10 healthy controls (HCs) were analyzed by flow cytometry for the surface expression of BTLA, CTLA-4, LAG-3, PD-1, TIGIT, TIM-3 and VISTA by MAIT cells. The percentages of cells expressing indicated exhaustion/co-inhibitory markers are illustrated (top and middle rows), and so is the gMFI of staining for each marker (bottom row). Each circle represents an individual sample. Group comparisons (top row) were carried out by one-way ANOVA followed by the Tukey's Multiple Comparison test. For paired sample analyses (middle and bottom rows), Wilcoxon signed-rank tests were used. * and **** denote significant differences with $p \leq 0.05$ and $p \leq 0.0001$, respectively

MAIT cells in cultures containing PBMCs from HCs and RCC patients (pre- and post-FMT), due likely to activation-induced *i*TCR internalization.

To confirm MAIT cells' ability to mount *i*TCR-dependent responses, we added *E. coli* lysate, a crude source of bacterial MR1 ligands, to PBMC cultures. This stimulation mode enabled detection of a substantial number of MAIT cells with comparable levels of intracellular effector molecules, especially IFN-γ and TNF-α, across the three sample sets (Fig. 6C). Moreover, MAIT cell responses to *E. coli* were statistically similar in pre- and post-FMT cultures (Supplementary Fig. 9). Therefore, the MR1/*i*TCR-driven MAIT cell activation pathway remains operational after FMT.

MAIT cells elicit preserved or enhanced responses to TCR-independent stimuli after FMT

Many microbes, including those without an active riboflavin biosynthesis machinery that supplies MR1 ligands, can activate MAIT cells in *i*TCR-independent fashions, primarily through cytokines. To begin to evaluate MAIT cell responses under such circumstances, we stimulated PBMCs with PMA and ionomycin in short-term cultures enabling direct MAIT cell activation while avoiding or minimizing bystander responses. PMA activates protein kinase C, and ionomycin raises intracellular Ca^{++} levels, thus bypassing TCRs to induce T cell activation when used in combination. MAIT cells from RCC patients appeared more responsive than controls to PMA and ionomycin (Fig. 7A). In addition, MAIT cells in pre- and post-FMT paired samples were both able to produce IFN-γ, TNF-α, IL17A and GZM B (Fig. 7A).

Next, we stimulated PBMCs with rhIL-12 and rhIL-18, cytokines that are often released during microbial challenges and known to potently activate innate-like T lymphocytes [22, 37]. MAIT cells from HCs and RCC patients were equally capable, if not more capable, of producing cytokines and GZM B in these cultures (Fig. 7B). Of note, FMT significantly augmented the TNF-α production capacity of MAIT cells after FMT (Fig. 7B). Therefore, FMT does not compromise the functional competence of MAIT cells and may in fact improve certain aspects of their antimicrobial, and potentially anti-tumor, activities.

Discussion

The efficacy and tolerability of ICIs employed against carcinomas are influenced by the composition of patients' microbiota, which may be manipulated therapeutically, for instance, through FMT, to improve clinical outcomes and to mitigate adverse side effects [7–9, 11, 38]. However, how FMT affects immune responses at the cellular level is far from clearly understood. This is particularly important

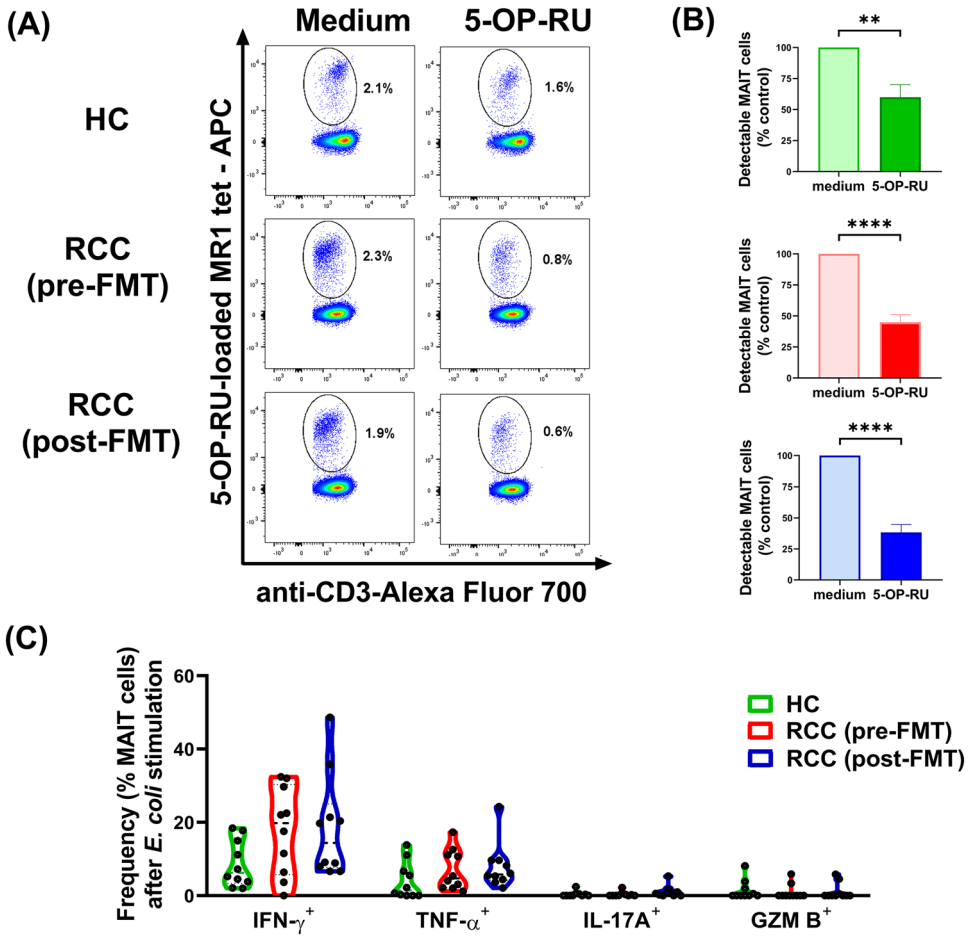

Fig. 6 MAIT cells maintain their capacity to respond to MR1/*i*TCR-dependent stimuli after FMT for RCC. Pre- and post-FMT PBMC samples from RCC patients (n = 10) and PBMCs from 10 healthy controls (HCs) were co-incubated for 24 h with C1R cells in the absence or presence of 5-OP-RU. The frequencies of detectable MAIT cells were subsequently determined by flow cytometry (**A**–**B**). Representative plots illustrating the remaining MAIT cells (**A**), and summary data depicting MAIT cell frequencies, relative to unstimulated cultures (**B**), are provided. In parallel cultures, PBMCs were stimulated for 24 h with *E. coli* lysate before MAIT cells capable of producing indicated cytokines and GZM B were enumerated (**C**). Background cytokine and GZM B levels were obtained from unstimulated cultures and subtracted for each mediator. Each circle represents an individual sample (**C**). Paired Student's *t* tests (**B**) and two-way ANOVA (followed by the Tukey's Multiple Comparison test) (**C**) were performed to compute statistically significant differences, or lack thereof. ** and *** denote $p \leq 0.01$ and $p \leq 0.0001$, respectively

in light of the robust antimicrobial properties of innate-like T cells, including MAIT cells, which also exhibit anti- and/or pro-tumor potentials [20]. MAIT cells need to stand sentinel to protect against opportunistic infections in cancer patients. While riboflavin-producing and other microbes within the transferred fecal matter should activate MAIT cells, their sheer number may also potentially cause MAIT cell exhaustion, thus making patients prone to a wide spectrum of bacterial and viral infections.

According to previous reports, PD-1$^+$ MAIT cell frequencies are elevated in the PB and bone marrow of patients with multiple myeloma [39], and PD-1$^+$TIM-3$^+$ MAIT cells accumulate in the primary tumors of patients with colon cancer [40]. In our study, RCC patients had higher PD-1$^+$ and BTLA$^+$ PB MAIT cell percentages compared with HCs irrespective of their FMT treatment status.

Importantly, we found CD69 upregulation and PD-1 downregulation by PB MAIT cells after FMT. This was not true for non-MAIT T cells, the vast majority of which are mainstream T lymphocytes that recognize peptide Ags, not riboflavin metabolites. Furthermore, MAIT cells are more sensitive than conventional T lymphocytes to cytokines like IL-12 and IL-18 [22, 37]. Finally, while MAIT cells occur in a poised effector position to respond quickly to microbial challenges [41], conventional T cells take longer to fully respond to cognate Ags. Head-to-head comparisons between innate-like and conventional T cells are not always meaningful or even possible in complex in vivo systems. However, potential differences in stimulation thresholds and response

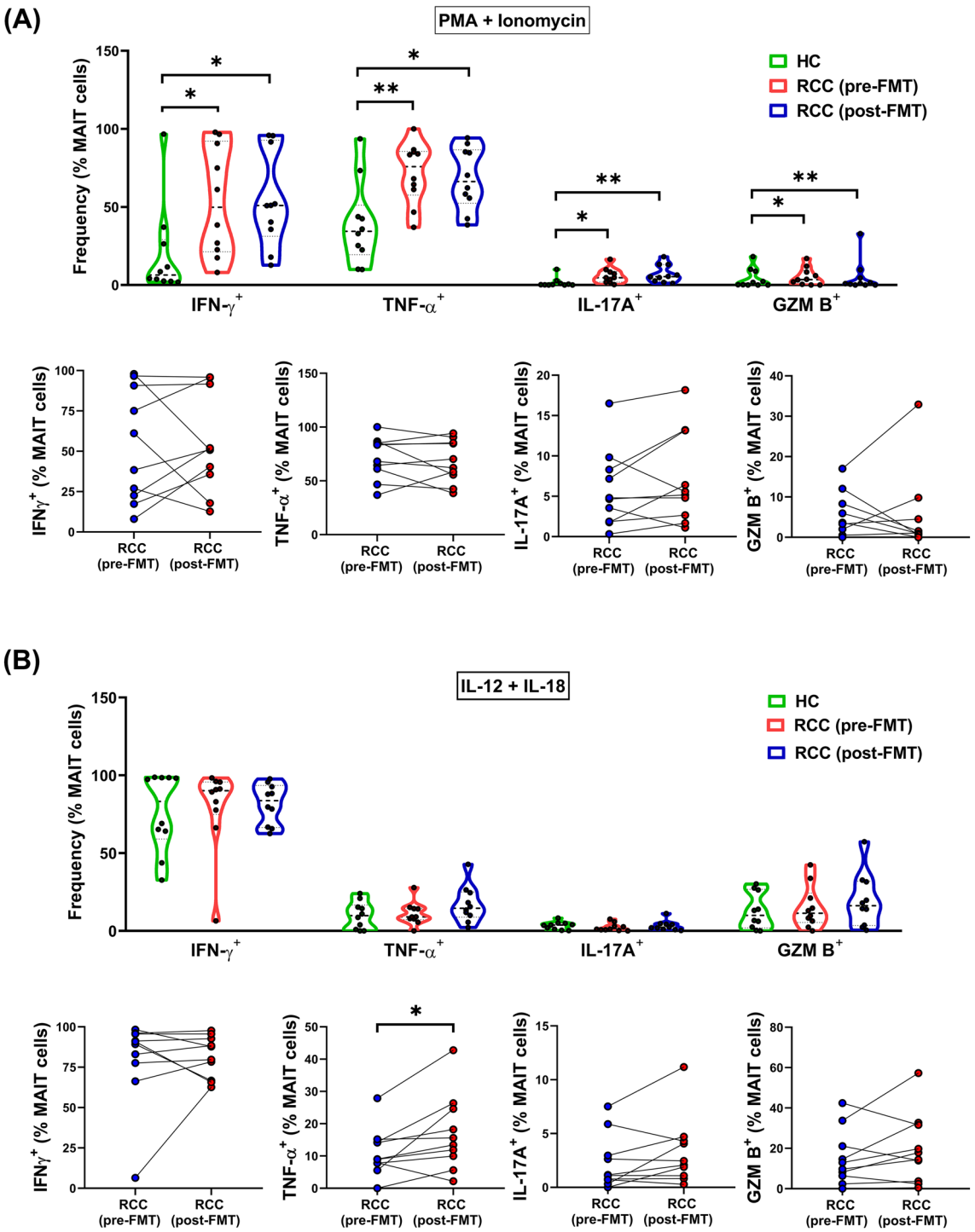

Fig. 7 MAIT cells launch equally vigorous or more vigorous responses to MR1/*i*TCR-independent stimuli after FMT for RCC. Pre- and post-FMT PBMC samples from 10 RCC patients and PBMCs from 10 healthy controls (HCs) were left untreated in medium, stimulated for 4 h with a combination of PMA and ionomycin (**A**), or stimulated for 24 h with a combination of rhIL-12 and rhIL-18 (**B**). The proportions of IFN-γ^+, TNF-α^+, IL-17A$^+$ and GZM B$^+$ MAIT cells were then determined by flow cytometry. Background cytokine and GZM B levels from unstimulated cultures were subtracted. Each circle represents an individual sample. Group comparisons were made using two-way ANOVA followed by the Tukey's Multiple Comparison test. Paired dataset comparisons were made using paired *t* tests for IFN-γ, IL-17A and TNF-α, and Wilcoxon signed-rank tests for GZM B. * and ** denote differences with $p \leq 0.05$ and $p \leq 0.01$, respectively

kinetics may dictate the divergent expression of activation and exhaustion markers by these cell types.

We found FMT not to raise the expression of BTLA, CTLA-4, LAG-3, TIM-3, TIGIT or VISTA. Our cytofluorimetric panel design also allowed us to determine the frequencies of PD-1$^+$TIGIT$^+$ and PD-1$^+$BTLA$^+$ double-expressors, which too remained stable post-FMT (data not shown).

Consistent with the above findings, MAIT cells were either equally or more capable of responding to *i*TCR/MR1-dependent and -independent stimuli. An enhanced TNF-α response to IL-12 and IL-18 was noteworthy in post-FMT PBMC cultures. A fortified IL-12/IL-18-TNF-α axis should boost immune surveillance against various pathogens, including viruses, which may cause severe infections in immunosuppressed patients.

Together, our results indicate that FMT activates MAIT cells without making them exhausted, a finding that provides additional justification for the usefulness of this therapeutic modality in human malignancies. The current study was focused on PB MAIT cells as part of a clinical trial for mRCC, and future investigations will address the impact of FMT on tissue-resident and tumor-infiltrating MAIT cells in primary sites of neoplastic transformation, including mucosal layers, and in metastatic tumor masses.

In an ex vivo lamina propria cell culture setting, Rodin et al. demonstrated stronger CD25 induction on colon tumor-infiltrating MAIT cells when pembrolizumab, a PD-1-blocking mAb, was present [40]. In our ongoing clinical trial, mRCC patients receive FMT before dual immunotherapy with ipilimumab and nivolumab, which block CTLA-4 and PD-1, respectively. We will assess the effect of this sequential treatment strategy on MAIT cell responses and their correlation with overall and progression-free survival among other clinical measures.

We found lower PB γδ T cell frequencies in our RCC cohort compared with HCs, which was partially corrected by FMT. The crosstalk between microbiota and γδ T cells controls mucosal tissue homeostasis and modulates immune responses in numerous conditions, including cancer. For instance, using the B16/F10 melanoma and Lewis lung carcinoma mouse models of pulmonary metastasis, Cheng et al. demonstrated that commensal bacteria contribute to anticancer immune surveillance through a mechanism that requires IL-17-producing γδ T cells [42]. We are currently investigating the phenotypic characteristics of γδ T cells as well as their TCR-dependent and -independent responses before and after FMT.

Another intriguing finding of this study was a drop in the CD8:CD4 MAIT cell ratio in RCC, which is reminiscent of previous reports in colorectal cancer (CRC) and melanoma cohorts [26, 29]. PB CD8$^+$ and CD4$^+$ MAIT cells from healthy donors appear to be transcriptionally distinct and likely dissimilar in terms of homeostatic, migratory, effector and regulatory functions [43]. For example, cytotoxicity-associated genes are predominantly expressed by the CD8$^+$ subset of PB MAIT cells. In a recent study, a positive correlation was found between circulating CD8$^+$ MAIT cell percentages and improved survival in patients with stage IV melanoma receiving anti-PD-1 therapy [29]. In contrast, elevated CD4$^+$ MAIT cell frequencies were correlated with poor survival. Also of note, tumor-infiltrating CD4$^+$ MAIT cells expressing FoxP3 have been previously reported in CRC patients [44]. Future studies on large patient cohorts will need to decipher the roles played by CD4$^+$ and CD8$^+$ MAIT cell subsets in various TEMs.

Innate-like T cells are not restricted by MHC and may, as such, be targeted in diverse patient populations. This is unlike mainstream T cell-based therapies tailored to patients' unique genetic makeup due to polymorphism at the MHC super-locus. MR1 and CD1d, which present a more limited array of Ags to MAIT and *i*NKT cells, respectively, are monomorphic. An added advantage of MAIT cells is their resistance to chemotherapeutic agents, owed to their high expression levels of *ABCB1* [12], which should permit intact responses to pathogens, and potentially to cancer, when/if FMT and/or immunotherapy is combined with chemotherapy. Finally, under optimal culture conditions, MAIT cells may be expanded in vitro and stored for future use as off-the-shelf therapeutics [45].

The findings of this body of work should lay the foundation for future studies in which the benefits of FMT in anti-pathogen and anti-tumor immunity are explored in the context of innate-like T cell responses.

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Authors' contributions MN designed and performed experiments, analyzed and interpreted data, and wrote the initial manuscript draft. CLS and MM recruited and obtained samples from healthy controls and participated in study design. SNP recruited fecal material donors, prepared fecal material for transplantation, administered FMT capsules and managed patient data. RF processed patient blood samples and managed patient data. JPB participated in study design. MSS participated in study design and oversaw the process of fecal material preparation and administration. RF recruited patients and participated in study design. SMV obtained funding, provided patient samples, participated in study design, analyzed data and edited the manuscript. SMMH obtained funding, conceived the idea, designed experiments, analyzed and interpreted data, and extensively edited the manuscript.

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Data availability The data generated in this study are available within the article's main body and supplementary files or may be requested from the corresponding authors. The materials we used in this investigation are available from the indicated sources or may be obtained from the corresponding authors upon reasonable request.

Declarations

Conflicts of interest The authors declare that they have no conflicts of interest. S.M. Mansour Haeryfar currently serves on the Editorial Board of Cancer Immunology, Immunotherapy.

Ethics approval Human specimens were collected and used as per study protocols 5545 and 114962 approved by the Western University Research Ethics Board for Health Sciences Research Involving Human Subjects.

Consent to participate Informed consent was obtained from all patients and healthy blood donors who participated in this study.

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