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Mucosal-Associated Invariant T Cells in Tuberculosis Pleurisy

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Abstract

Mucosal-Associated Invariant T (MAIT) cells, which is a prevalent and unique innate T-cell population that expresses an evolutionarily conserved invariant T cell receptor TCRVα7.2, are present at high frequencies at mucosal tissue sites and have an intrinsic capacity to respond to microbial infections. However, the local immune responses of MAIT cells at the site of *M.tb* infection is unclear. We compared the PFMCs from TB (n = 57) with the PBMCs from TB (n = 57) and HD (n = 50), and characterized those T-cell phenotypes and functions. Our direct ex vivo analysis demonstrated that the frequencies of MAIT cells in PFMCs were much higher than those in PBMCs from TBP patients ($P<0.001$), however, lower than those in PBMCs from HD ($P<0.01$). Those infiltrating MAIT cells expressed high levels of tissue-tropism chemokine receptors ($CXCR3^{hi}CXCR4^{hi}CXCR6^{hi}CCR6^{hi}CXCR5^{hi}CCR5^{hi}$) and displayed an effector memory phenotype ($CD45RO^{+}CCR7^{-}CD62L^{-}$), which indicated preferential accumulating these cells into infected lung lesions. Further, the majority of MAIT cells in PFMCs expressed CD69, a marker for tissue resident memory T cells, which suggested that specialization of these T cells into unique tissue-resident subsets given the host enhanced regional immunity. In addition, MAIT cells from PFMCs produced IFN-γ and TNF-α, and exhibited cytotoxic activity molecules CD107a/b, suggesting that poly functional *M.tb*-reactive MAIT cells played an significant role against *M.tb* infection in the local lesions. This study addressed that the *M.tb*-reactive MAIT cells exerted unique innate functions in immune responses to *M.tb* at local infection sites.

Keywords: Cellular immune response; MAIT; PFMCs; TB

Introduction

Invariant Natural Killer T (iNKT) cells and Mucosal-Associated Invariant T (MAIT) cells represent peculiar T-lymphocyte subpopulations with innate-like properties that differ from conventional T cells. CD1d-restricted iNKT cells and MR1-restricted MAIT cells are defined by invariant or semi-invariant repertoires [1-3]. These invariant lymphocyte subsets follow specific onto genic pathways, home to particular tissues and have larger clonal sizes than do conventional lymphocytes [4,5] MAIT cell is an abundant population of innate-like T-cell subset in humans, which is characterized by expression of an evolutionarily conserved invariant T Cell Receptor (TCR) carrying the canonical Vα7.2-Jα33 TCR rearrangement and activated by an antigen bound to the major Histocompatibility Complex (MHC) class I-like molecule MR1 [6-8].

In humans, contrary to NKT cells, MAIT cells display a naive phenotype in the thymus as well as in cord blood where they are in low numbers. However, after birth, MAIT cells have a memory phenotype early in life and are abundant in human blood (1-8% of T cells versus 0.01-1% for natural killer T cells), which suggests that MAIT cell populations expand after birth and acquire their memory phenotype in the presence of commensal flora [9, 10]. It is shown that the development of MAIT cells are dependent upon the expression of MR1 on bone marrow-derived cells, and are selected by MR1 in the thymus on a non-B non-T hematopoietic cells [11]. However, they acquire a memory phenotype and expand in the

periphery in a process dependent both upon B cells and bacterial flora [12]. In support of that hypothesis, MAIT cells are not detectable in germ-free mice and/or B-cell-deficient patients and mice, but can be induced to expand after microbial colonization, which is a complex relationship between microbes and MAIT cells [13]. MAIT cells are abundant within the CD8αα$^+$, an intermediate level of CD8αβ and scarce in the CD4$^+$ T cell subset, which account for up to ~15% of the CD8$^+$ T-cell population in the blood of healthy individuals [14]. Meanwhile, MAIT cells gradually obtain some other functional surface makers in the process of development. The specific anti-Va7.2 antibody allows the human MAIT cell population to be characterized in detail. This marker, together with the high expression of CD161 identifies MAIT cells in peripheral blood and other tissues [15]. CD161, is a c-type lectin family member, which is expressed on a significant proportion of tissue-infiltrating T cells, such as the majority of NKT cells, MAIT cells and TCRγδ T cells, mediates cytokines and cytotoxicity after immune stimulation [16,17].

Tuberculosis (TB), caused by the highly infectious intracellular pathogen *Mycobacterium tuberculosis* (*M.tb*), remains a highly leading cause of infectious rates and mortality worldwide [18]. It has shown that the frequencies of MAIT cells are severely reduced in circulation but high levels in the lungs of patients with active TB, suggesting that MAIT cells contribute to protection against *M.tb* infection in humans [19,20]. Although these T cell clusters are important for the persistence of MAIT cells resident in lungs, it is not clear whether these MAIT cells in pleurisy effusion are long term resident and what proportion of MAIT cells in pleurisy effusion are resident versus recirculating. What's more, much less is known about what phenotypes and functions of these MAIT cells and how these cells provide protection. The role of MAIT cells at the local sites of *M.tb* infection is currently unknown. In the current study, we examined the frequencies, phenotypes and functions of MAIT cells in a relatively large number of patients with active tuberculosis pleuritis and healthy donors.

Materials and Methods

Human Subjects

Fifty-seven patients with diagnosed Tuberculosis Pleurisy (TBP) were enrolled in this study from the Chest Hospital of Guangzhou, China (Table 1). The diagnosis of TBP has been described in our previous publication [21]. The peripheral blood and pleural fluid samples were obtained from the patients with TBP during therapeutic thoracentesis operated by Dr. Suihua Lao according to the strict medical operation rules and methods. All participating patients had received less than one week of anti-tuberculosis therapy. Patients who had a history of autoimmune diseases or co-infected HIV, HBV or HCV were excluded from the study. Fifty Healthy Donors (HD) were recruited from the blood center of Guangzhou, China (Table 1). The peripheral blood samples were obtained from volunteers. The written consents were obtained from all the subjects and this study was approved by the ethics committee of the Zhongshan School of Medicine, Sun Yat-sen University and the Chest Hospital of Guangzhou, China.

Demographic and clinical characteristic of patients with TBP and HD		
	Patients with TBP	**Healthy Donors**
Total (n)	57	50
Age (year) (mean ± SD)	28.79 ± 15.41	35.24 ± 5.48
Sex (male) (n%)	37M （64.9%）	32M （64.0%）
Pulmonary TB	57	No
New pulmonary TB	54	No
Sputum smear/ culture positive	50	ND
Tuberculous pleuritis	57	No
Tuberculous pleural effusion	57	No
Antituberculosis therapy	Less than one week	ND
TB = Tuberculosis; TBP = Tuberculosis Pleuritis; M= Male; ND= Not Done.		

Table 1: The study was conducted in a total of fifty-seven patients with Tuberculosis Pleuritis (TBP) that were recruited from the Chest Hospital of Guangzhou, China. A total of fifty Healthy Donors (HD) were recruited from the blood center of Guangzhou, China. Analysis of the demographic and clinical characteristic of patients with tuberculosis pleuritis and healthy donors.

Isolation and Preparation of Pleural Fluid Mononuclear Cells (Pfmcs) And Peripheral Blood Mononuclear Cells (Pbmcs)

The PFMCs and PBMCs were isolated and prepared according to the previously described protocols [21]. Briefly, Pleural Fluid (PF) collected from TBP patients were centrifuged at 2500 rpm for 20 min, and the cell pellets were collected. The mononuclear cells were isolated and obtained through Ficoll-Hypaque (Tianjin Hao Yang Biological Manufacture, Tianjin, China) density gradient centrifugation at 2000 rpm for 20 min. PFMCs and PBMCs were collected and washed twice with Hank's balanced salt solution, then re-suspended at a final concentration of 2×10^6 cells/ml in complete RPMI 1640 medium (Life Technologies, Grand Island, USA) supplemented with 10% heated-inactivation fatal calf serum (Sijiqing, Hangzhou, China), 100 μg/

ml streptomycin, 100 U/ml penicillin, 2 mM L-glutamine and 50 μM 2-mercaptoethanol (Life Technologies, Grand Island, USA). When freezing cells, the cells were isolated and re-suspended in cell freezing medium supplemented with 90% heated-inactivation fatal calf serum (Sijiqing, Hangzhou, China) and 10% Dimethyl Sulfoxide (DMSO) (Zhanchen biological technology co., LTD, Guangzhou, China), and were cryopreserved in liquid nitrogen. When thawing cells, the microtubes were thrown into 37˚C warm water, and gently shaking them to melt as soon as possible. The cell suspension was transferred into 15 ml tube and was washed twice in the complete RPMI 1640 medium. Finally, the cells were re-suspended at a final concentration of 2×10^6 cells/ml in complete RPMI 1640 medium.

Mycobacterium tuberculosis Antigens

Mycobacterium bovis Bacillus Calmette-Guérin (BCG) was purchased from Chengdu Institute of Biological Products, Chengdu, China. *Mycobacterium tuberculosis* strain H37Rv (*M.tb*) was provided by laboratory of Baiqing Li from deparment of Immunology, research center of immunology, Bengbu Medical College, Bengbu, PR China.

Flow Cytometric Analysis

The detection of surface markers, intracellular cytokines and transcriptional factors was performed according to the references [21]. In short, the cells (2×10^6/ml) were washed twice with PBS buffer containing 0.1% BSA and 0.05% sodium azide (Sigma-Aldrich, St. Louis, MO) and incubated with the respective mAbs at 4˚ C in the dark for 30 min. The cells were fixed with 4% paraformaldehyde for 8 min followed by permeabilization in PBS buffer containing 0.1% saponin (Sigma-Aldrich, St. Louis, MO) and then incubated with the respective mAbs at 4 ˚C in the dark for 30 min. All above stained cells were and assayed by FACS Aria II (Becton Dickinson, San Jose, USA) and the data were analyzed by Flow Jo software (Tree Star, San Carlos, USA). In some experiments, at the time of stimulation, anti-CD107a/b-FITC was added, after 2 hours' incubation, 2 μmol monensin and 10 μg/ml brefeldin A (Sigma-Aldrich, USA) were added and continued overnight at 37 ˚C in 5% CO_2. The culture, differentiation, infection of THP-1 cells with *M.tb*, and the cytotoxic T lymphocyte assays according to the reference [22]. The following human antibodies/reagents for FACS: ECD conjugated-CD3 (UCHT1), PE-Cy7 conjugated-CD3 (UCHT1), PE conjugated-CD3 (UCHT1), APC-Cy7 conjugated-CD8 (SK1), FITC conjugated-CD8 (SK1), AF700 conjugated-CD8 (SK1), FITC conjugated-CD161 (DX12), PE conjugated-CD161 (DX12), PE-Cy7 conjugated-CD161 (DX12), PE conjugated-CD25 (M-A251), PE-Cy7 conjugated-CD69 (FN50), PE conjugated-CD45RO (UCHL1), AF700 conjugated-CD45RO (UCHL1), PE-Cy7 conjugated-CCR7 (3D12), PE conjugated-CCR7 (3D12), PerCP-Cy5.5 conjugated-CD62L (DREG-56), APC conjugated-CD14 (M5E2), APC conjugated-CXCR3 (1C6/CXCR3), PE-Cy7 conjugated-CCR4 (1G1), PE conjugated-CXCR4 (12G5), APC conjugated-CCR6 (11A9), PE conjugated-T-bet (O4-46), PE-CF594 conjugated-T-bet (O4-46), PerCP-Cy5.5 conjugated-IFN-γ (B27), FITC conjugated-IFN-γ (B27), PE-Cy7 conjugated-IFN-γ (B27), APC conjugated-IFN-γ (B27), PE-Cy7 conjugated-TNF-α (MA611), PE conjugated-TNF-α (MA611), APC conjugated-TNF-α (MA611), FITC conjugated- CD107a/b (H4A3/ABL-93) and PE conjugated- Granzyme B (GB11), FITC conjugated- Granzyme B (GB11) and purified anti-CD28 (CD28.2) was purchased from BD Biosciences (San Jose, CA, USA). PE conjugated-Vα7.2 (3C10) and APC conjugated-Vα7.2 (3C10) were purchased from Biolegend (San Diego, CA, USA). PE conjugated-CCR5 (2D7/CCR5), APC conjugated-CXCR5 (RF8B2), PE conjugated-CXCR6 (13B1E5) and PE conjugated-CCR10 (1B5) were purchased from eBioscience (San Diego, CA, USA). Phorbol Myristate Acetate (PMA) and ionomycin were purchased from Sigama-Aldrich (St. Louis, MO, USA).

ELISA and ELISPOT for Cytokine Detection

The $CD3^+CD8^+V\alpha7.2^+$ T cells, $CD3^+CD8^+$ T cells, $CD3^+CD8^+V\alpha7.2^-$ T cells and total PFMCs were stimulated with or without BCG (10 μg/ml) or *M.tb* (10 μg/ml) in the presence of anti-CD28 (1 μg/ml) in a round-bottom 96-well plate, 4×10^5 cells/well, at 37 ˚C and 5%CO_2 for 72 hours. The cell-free culture supernatants were harvested and assayed for the production of IFN-γ and TNF-α by Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer's protocol (BD Biosciences, San Jose, CA, USA). The $CD3^+CD8^+V\alpha7.2^+$ T cells were stimulated with or without BCG (10 μg/ml) or *M.tb* (10 μg/ml) in the presence of anti-CD28 (1 μg/ml) in pre-coated BD™ ELISPOT plates, 1×10^5 cells/well, at 37 ˚C and 5%CO_2 for 24 hrs. The frequency of IFN-γ-producing cells in the $CD3^+CD8^+V\alpha7.2^+$ T cells was measured by Enzyme-Linked Immunosorbent Spot (ELISPOT) according to the manufacturer's protocol (BD Biosciences, San Jose, CA, USA). The levels of IP-10 (CXCL10) in pleural fluid from tuberculosis pleurisy, serum from healthy donors and pleural fluid from cancerous patients were assayed Enzyme-Linked Immunosorbent Assay (ELISA) according to the manufacturer's protocol (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Significant differences between data sets were performed with either the unpaired Student's t-test when comparing two groups, one-way ANOVA for more than two groups or two-way ANOVA for two variables (Graph Pad Software Inc, San Diego, CA, USA). $^{***}P<0.001$; $^{**}P<0.01$; $^{*}P<0.05$; and $P>0.05$, not significant, as stated in Figure legends.

Results

Identification and analysis of the expression of MAIT cells in PFMCs from TBP patients and PBMCs from TBP patients and HD

According to the publish, MAIT cells were defined by the expression of an invariant TCRVα7.2, we used anti-Vα7.2 antibodies to detect the number of cells expressing the Vα7.2 segment. We initially identified and analyzed the expression of $Vα7.2^+$ T cells in different subsets of $CD3^+$ T cells from PFMCs. We found that the expressions of Vα7.2 were mostly on the $CD3^+CD8^+CD4^-$, $CD3^+CD4^+CD8^+$ and $CD3^+CD4^-CD8^-$ T cells, scarcely on $CD3^+CD4^+CD8^-$ T cells (Figure 1A). The percentages of Vα7.2 in $CD3^+$ T cells were mostly from $CD8^+CD4^-$ (67.3% ± 1.8%) T cells, were significantly higher than those on $CD4^+CD8^+$ (12.9% ± 1.3%; P<0.001), $CD4^-CD8^-$ (7.6% ± 0.7%; P<0.001) and $CD4^+CD8^-$ (6.3% ± 0.5%; P<0.001) T cells (Figure 1B,C). To better and convenient understand the physiology of MAIT cells, we first defined MAIT cells as $CD3^+CD8^+Vα7.2^+$ T cells (Figure 1A). Next, we detected the percentages of MAIT cells in PFMCs and PBMCs from 57 patients with TBP and in PBMCs from 50 volunteers with HD (Table 1). We found that the frequencies of MAIT cells in PFMCs (TB; 10.45% ± 0.6%) were significantly higher than those in PBMCs (TB; 6.5% ± 0.3%; P<0.001), however, lower than those in PBMCs (HD; 13.76% ± 0.7%; P<0.01) (Figure 1D,E). The frequencies of MAIT cells in PBMCs (TB; 6.5% ± 0.3%) were also lower than those in PBMCs (HD; 13.76% ± 0.7%; P<0.001) (Figure 1D,E). We know that the surface marker CD161 was mostly expressed on a significant proportion of MAIT cells. To better understand the phenotype and function of tissue-infiltrating MAIT cells in pleural fluid, we detected the expression of CD161 on MAIT cells and intended to stain cells and divided MAIT cells into two subsets $CD161^+Vα7.2^+$ and $CD161^-Vα7.2^+$ T cells (Figure 1D). Interestingly, the percentages of $CD161^+Vα7.2^+$ T cells in PFMCs (TB; 3.7% ± 0.3%) were significantly higher than those in PBMCs (TB; 1.6% ± 0.2%; P<0.001), but lower than those in PBMCs (HD; 6.3% ± 0.5%; P<0.001). However, the percentages of $CD161^-Vα7.2^+$ T cells in PFMCs (TB; 6.3% ± 0.5%) were close to those in PBMCs (TB; 6.0% ± 0.4%; P>0.05) and PBMCs (HD; 5.8% ± 0.3%; P>0.05) (Figure 1F). Those data might suggest that there were high levels of MAIT cells in pleural fluid from TBP patients, and high levels of $CD161^+Vα7.2^+$ MAIT cells migrated from blood to pleural fluid and settled down being a group of tissue resident T cells.

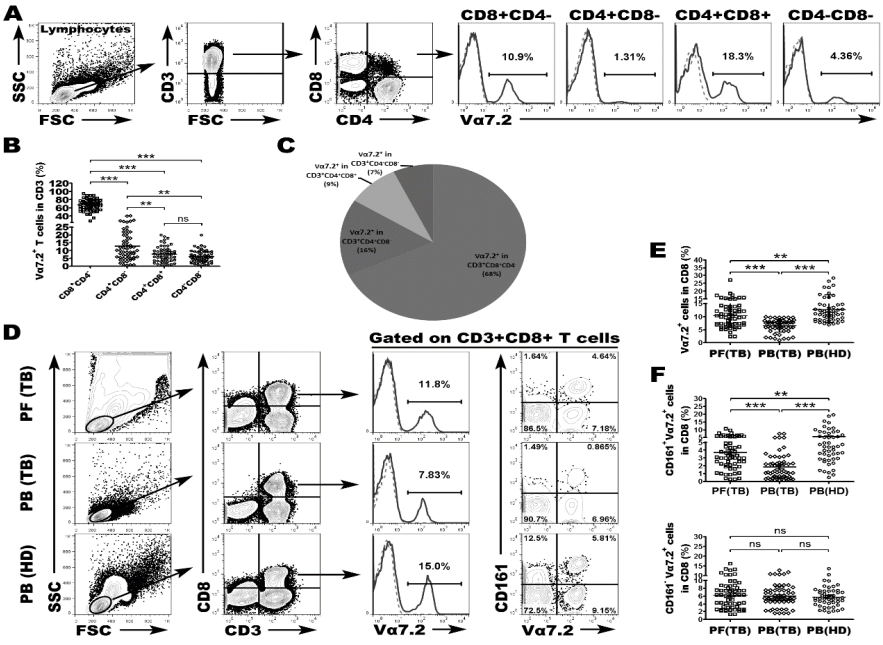

Figure 1: Identification and analysis of the expression of MAIT cells in pleural fluid from TBP patients and peripheral blood from TBP patients and HD. PFMCs from TBP (n = 57) and PBMCs from TBP (n = 57) and HD (n = 50) were harvested and stained with fluorochrome-conjugated monoclonal antibodies, and analyzed for the lineage differentiation and surface marker expression with flow cytometry. **(A):** Representative FACS gating. **(B,C):** statistical analysis showed that the expression of $Vα7.2^+$ in $CD8^+CD4^-$, $CD8^-CD4^+$, $CD8^+CD4^+$ and $CD8^-CD4^-$ of $CD3^+$ T cells from PFMCs. **(D):** Representative FACS data. **(E,F):** Statistical analysis showed that the expression of $Vα7.2^+$ cells, and two subsets $CD161^+Vα7.2^+$ and $CD161^-Vα7.2^+$ cells in $CD3^+CD8^+$ T cells from PFMCs (TB), PBMCs (TB) and PBMCs (HD). Data were shown as mean. Each dot represented one patient. ns, not significant; **P<0.01; ***P<0.001.

The tissue-tropism chemokine receptors expressed by MAIT cells in PFMCs from TBP patients and PBMCs from HD

To better understand the accumulation of MAIT cells into pleurisy effusion, we analyzed the expression of tissue-tropism chemokine receptors on MAIT cells by flow cytometry. Compared with conventional T cells, the pattern of chemokine receptor expression was, however, very specific, as MAIT cells exhibited high levels of CXCR4, CCR6 and CXCR6, heterogeneous levels of CCR4, and intermediate expression of CXCR3, CXCR5 and CCR5, but did not express CCR10. These chemokine receptors (CXCR3 and CXCR4) were involved in trafficking to local infection sites, especially the airways and lungs (Figure 2A). Altogether, these results clearly indicated that MAIT cells were circulating lymphocytes with tissue tropism, unlike other conventional T cells. Importantly, the MAIT cells in PF (TB) exhibited higher levels of CXCR3, CXCR4, CCR5, CXCR5, CCR6 and CXCR6 than those in PB (HD), but scarcely expression of CCR4 and CCR10 (Figure 2A,B). Specially, the expression of CXCR3 and CXCR4 on MAIT cells in PF (TB) were significantly higher than those in PB (HD) (Figure 2B). Next, we analyzed the indicated chemokine receptors on both subsets $CD161^{+}V\alpha7.2^{+}$ and $CD161^{-}V\alpha7.2^{+}$ T cells. It showed that the $CD161^{+}V\alpha7.2^{+}$ T cells expressed the extremely higher levels of CXCR3 and CXCR4 than counterparts in PF (TB) (Figure 2C). The chemokine IP-10 and SDF-1 for chemokine receptor CXCR3 and CXCR4 in pleural fluid from TBP patients were higher than serum from healthy donors and pleural fluid from cancerous patients (Figure 2D). It suggested that high levels of chemokine receptors on MAIT cells and chemokines in tuberculous pleural fluid were accounting for the accumulation of MAIT cells from peripheral blood into local infection sites, and to be a group of tissue resident T cells.

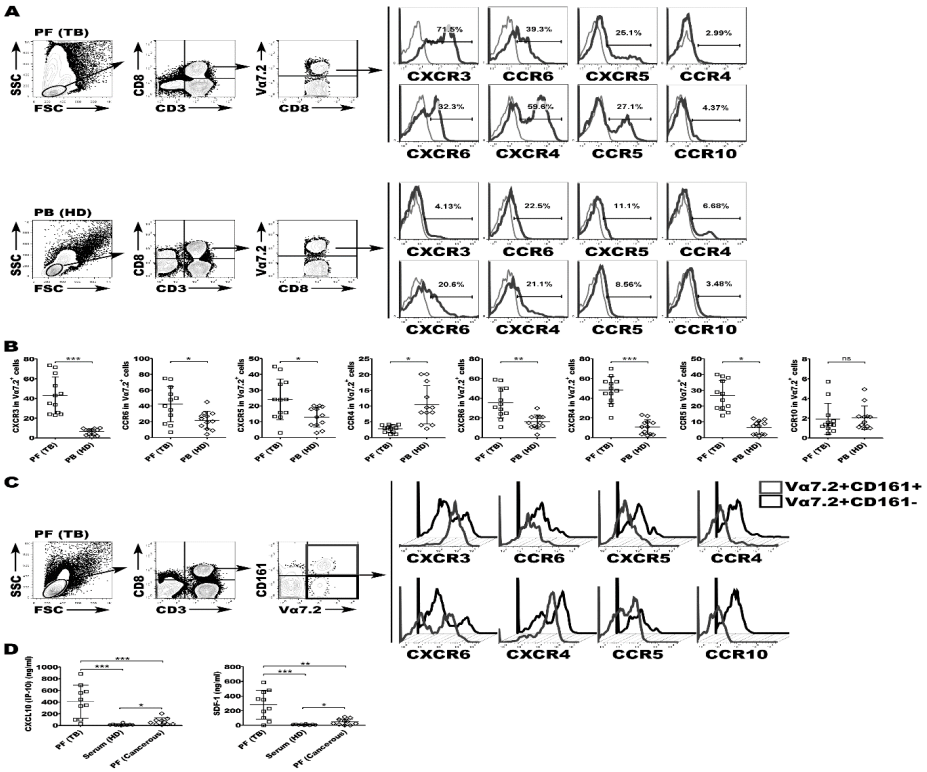

Figure 2: Analysis of the tissue-tropism chemokine receptors expressed by MAIT cells in pleural fluid from TBP patients and peripheral blood from HD. PFMCs from TBP (n = 12) and PBMCs from HD (n = 12) were harvested and stained with fluorochrome-conjugated monoclonal antibodies, and analyzed for the lineage differentiation and chemokine receptor expression with flow cytometry. **(A):** Representative FACS data. **(B):** statistical analysis showed that the expression of indicated tissue-tropism chemokine receptors CXCR3, CCR6, CXCR5, CCR4, CXCR6, CXCR4, CCR5, CCR10 in $CD3^{+}CD8^{+}V\alpha7.2^{+}$ T cells from PFMCs and PBMCs. **(C):** Representative FACS data showed that the expression of indicated tissue-tropism chemokine receptors CXCR3, CCR6, CXCR5, CCR4, CXCR6, CXCR4, CCR5, CCR10 in both $CD161^{+}V\alpha7.2^{+}$ and $CD161^{-}V\alpha7.2^{+}$ subsets of $CD3^{+}CD8^{+}V\alpha7.2^{+}$ T cells from PFMCs. **(D):** Detection of the levels of chemokines CXCL-10 (IP-10) and SDF-1 in the pleural fluid from Tuberculous Pleurisy (TB), serum from Healthy Donors (HD) and pleural fluid from cancer patients (cancerous). Data were shown as mean ± SD. Each dot represented one patient. ns, not significant; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

The memory phenotypes of MAIT cells in PFMCs from TBP patients and PBMCs from HD

To better understand the physiology of MAIT cells in tuberculous pleurisy effusion, MAIT cells were stained with memory surface markers, anti-CD45RO, anti-CCR7 and anti-CD62L, and divided into $CD45RO^+CCR7^-CD62L^-$ effector memory T cells (T_{EM}) and $CD45RO^+CCR7^+CD62L^+$ central memory T cells (T_{CM}). CCR7 is involved in homing of T cells to various secondary Lymphoid organs such as Lymph nodes. CD62L (L-selectin) also slows lymphocyte trafficking through the blood, and facilitating entry into a secondary lymphoid organ at that point. In PF (TB) and PB (HD), as conventional $CD8^+$ T cells, $CD8^+V\alpha7.2^+$ T cells expressed $CD45RO^+$ and $CD45RO^-$, however, rarely expressed CCR7 and CD62L (Figure 3A). Higher levels of MAIT cells with T_{EM} phenotypes ($CD45RO^+CCR7^-CD62L^-$) in PF (TB) were expressed compared to those in PB (HD; $P<0.001$), however, the frequencies of MAIT cells with T_{CM} phenotype in PF (TB) were similar to those in PB (HD; $P>0.05$) (Figure 3B), suggesting those MAIT cells were accumulated into pleurisy effusion and uncirculated during infection. Next, we also analyzed the T_{EM} phenotypes in both subsets $CD161^+V\alpha7.2^+$ and $CD161^-V\alpha7.2^+$ T cells. In PF (TB), the $CD161^+V\alpha7.2^+$ T cells were higher frequencies of T_{EM} phenotypes than the counterparts $CD161^-V\alpha7.2^+$ T cells ($P<0.001$). However, In PB (HD), the $CD161^+V\alpha7.2^+$ T cells with T_{EM} phenotypes were similar to the $CD161^-V\alpha7.2^+$ T cells ($P>0.05$). Meanwhile, higher frequencies of $CD161^+V\alpha7.2^+$ with T_{EM} phenotype in PF (TB) were observed compared to those in PB (HD; $P<0.001$) (Figure 3C,D). Together these data, it suggested that MAIT cells especially the $CD161^{hi}$MAIT cells in pleural fluid experienced *M.tb* infection and displayed an effector/memory potential.

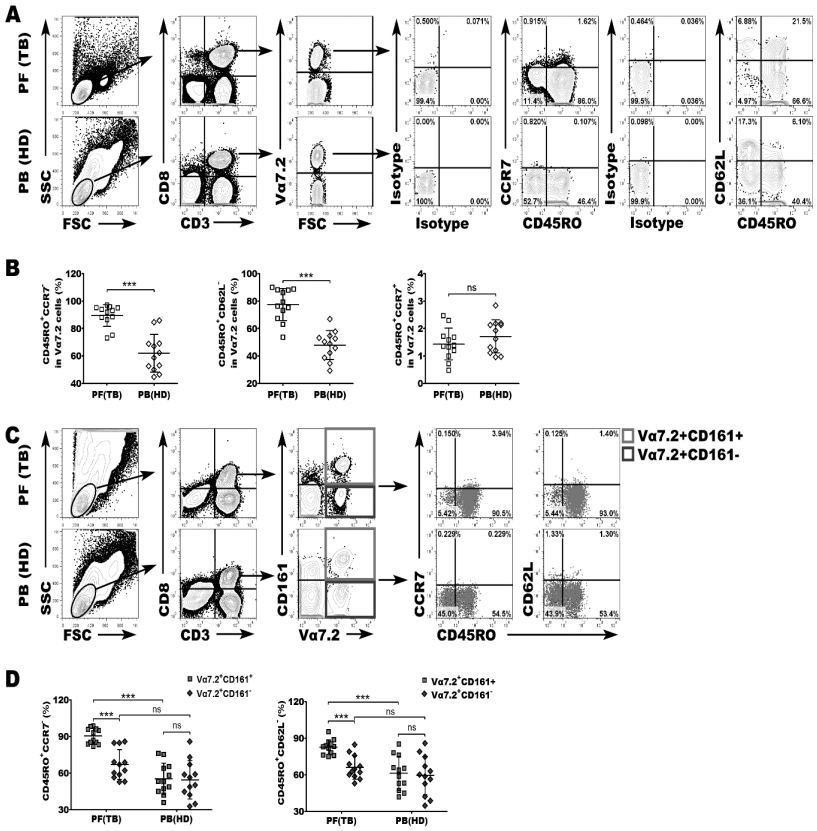

Figure 3: Detection of the memory phenotypes of MAIT cells in pleural fluid from TBP patients and peripheral blood from HD**.** PFMCs from TBP (n = 12) and PBMCs from HD (n = 12) were harvested and stained with fluorochrome-conjugated monoclonal antibodies, and analyzed for the lineage differentiation and memory phenotype expression with flow cytometry. **(A):** Representative FACS data. (**B):** Statistical analysis showed that the expression of effector memory (T_{EM}) phenotype and central memory (T_{CM}) phenotype in $CD3^+CD8^+V\alpha7.2^+$ T cells from PFMCs and PBMCs. **(C):** Representative FACS data. **(D):** Statistical analysis showed that the expression of effector memory (T_{EM}) phenotype in both $CD161^+V\alpha7.2^+$ and $CD161^-V\alpha7.2^+$ subsets of $CD3^+CD8^+V\alpha7.2^+$ T cells from PFMCs and PBMCs. Data were shown as mean ± SD. Each dot represented one patient. ns, not significant; $^{***}P<0.001$.

The profile of tissue resident memory phenotype on MAIT cells in PFMCs from TBP patients and PBMCs from HD

The previous explanation of the expression of CD69 was that they were in an activated state, perhaps as a result of retained antigen. However, it was well known that $CD69^+$ expression was a generic characteristic of resting Tissue-Resident Memory (TRM) T cells in the infectious lungs. Thus, we next investigated the expression of tissue resident memory T cell ($CD69^+$; TRM) markers of MAIT cells in PF (TB) compared to PB (HD). We found that in PF (TB) and PB (HD), resting MAIT cells expressed higher levels of CD69 and CD25 than conventional $CD8^+$ T cells ($P<0.001$). In addition, MAIT cells in PF (TB) expressed significantly higher levels of CD69 and CD25 than those in PB (HD; $P<0.001$) (Figure 4A,B).

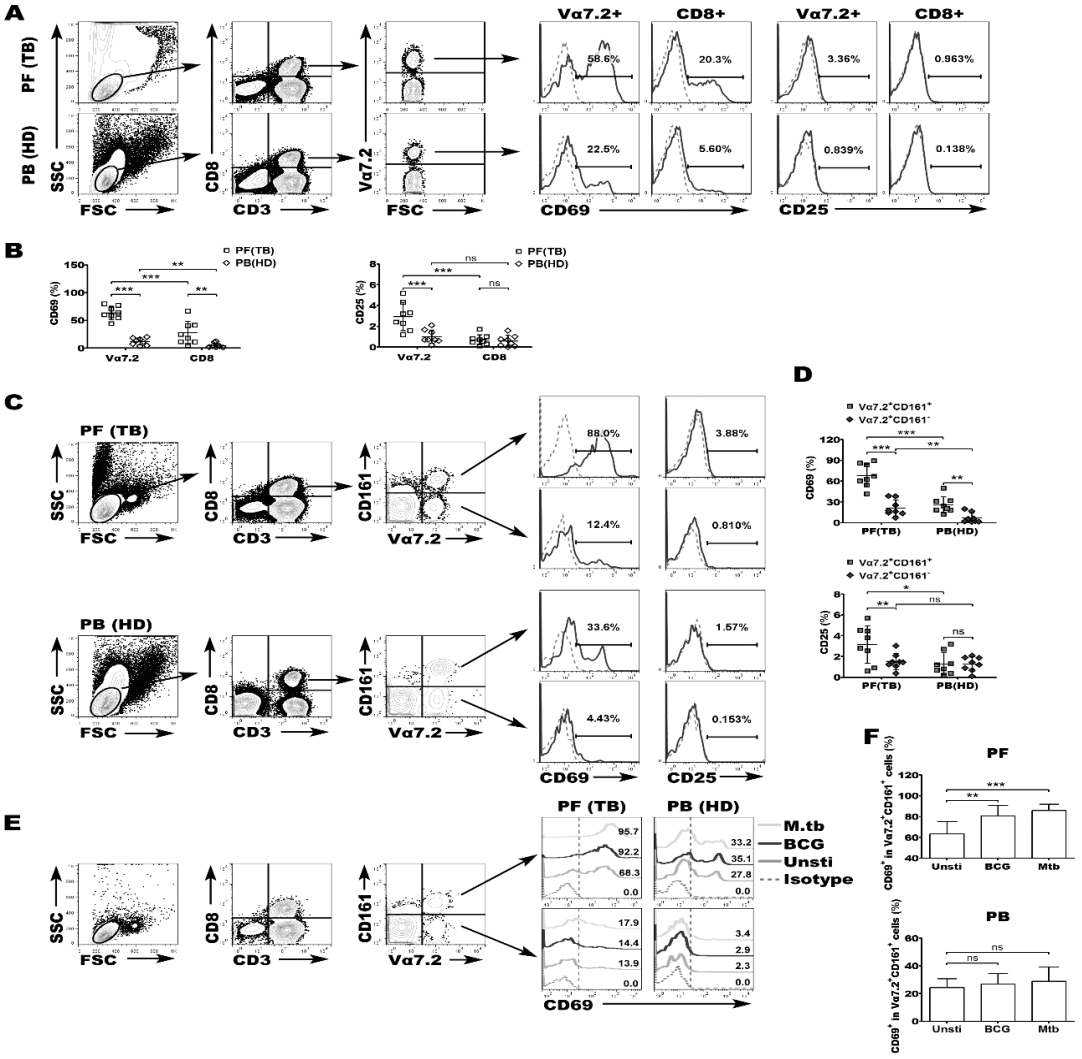

Figure 4: Detection of the profile of tissue resident memory phenotype on MAIT cells in pleural fluid from TBP patients and peripheral blood from HD. PFMCs from TBP (n = 8) and PBMCs from HD (n = 8) were harvested and stained with fluorochrome-conjugated monoclonal antibodies, and analyzed for the lineage differentiation and profile of tissue resident memory phenotype expression with flow cytometry. **(A):** Representative FACS data. **(B):** Statistical analysis showed that the expression of CD69 and CD25 on $CD3^+CD8^+V\alpha7.2^+$ T cells versus conventional $CD3^+CD8^+$ T cells from PFMCs and PBMCs. **(C):** Representative FACS data. **(D):** Statistical analysis showed that the expression of CD69 and CD25 in both $CD161^+V\alpha7.2^+$ and $CD161^-V\alpha7.2^+$ subsets of $CD3^+CD8^+V\alpha7.2^+$ T cells from PFMCs and PBMCs. **(E):** Representative FACS data. **(F):** Statistical analysis showed that the expression of CD69 in both $CD161^+V\alpha7.2^+$ and $CD161^-V\alpha7.2^+$ subsets of $CD3^+CD8^+V\alpha7.2^+$ T cells from PFMCs and PBMCs after stimulation with or without BCG or *M.tb* in the presence of anti-CD28 for 1 day, respectively. Data were shown as mean ± SD. Each dot represented one patient. ns, not significant; $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Next, we also found that the CD161^{+}Vα7.2^{+} T cells had a significantly higher expression of CD69 and CD25 in PF (TB) and PB (HD) compared to counterparts CD161^{-}Vα7.2^{+} T cells, respectively (Figure 4C,D). We found that TRM (CD69^{+}) T cells in PF (TB) were very high, however, rarely in circulation PB (HD). Clearly, almost the majority of CD161^{hi}MAIT cells expressed CD69, and being tissue resident memory T cells in local infection sites. To examine whether MAIT cells in PF (TB) could respond to TB-Ags, MAIT cells were stimulated with *M.tb* or BCG. CD161^{hi}MAIT cells in PF (TB) had higher level of expression of CD69 when responded to *M.tb* or BCG stimulation compared to un-stimulated control cells. However, in PB (HD), *M.tb* and BCG showed less effect on MAIT cells (Figure 4E,F). Together those data, it is suggesting that CD161 helped the accumulation of MAIT cells in pleural fluid after infection and being the tissue resident memory T cells in local infection site.

The expression of Tc1-type cytokines and cytotoxic molecules on MAIT cells in PFMCs from TBP patients after polyclonal stimulation

To better understand the effector functions of MAIT cells, we analyzed the secretion patterns of cytokines, cytotoxic molecules and transcription factors after polyclonal stimulation. We found that pleural fluid MAIT cells exhibited high ability of cytokine and cytotoxic molecules expression after the stimulation with PMA and ionomycin. MAIT cells expressed Tc1-type cytokines IFN-γ, TNF-α and IL-2, and also shared cytotoxic molecules CD107a/b with cytotoxic CD8^{+} T cells (Figure 5A). The majority of MAIT cells were a polyfunctional cell subset in PF (TB) when stimulated with PMA and ionomycin (Figure 5B). Next, we detected the transcription factor in regulate the expression of cytokines on MAIT cells. It showed that the transcription factor T-bet regulated the IFN-γ production of MAIT cells but less effect on the expression of TNF-α and IL-2. The T-bet^{+}MAIT cells hold higher expression of IFN-γ not TNF-α and IL-2 than T-bet^{-}MAIT cells (Figure 5C, D). Also, the CD161^{+}Vα7.2^{+} T cells expressed higher levels of Tc1-type cytokines IFN-γ and TNF-α and transcription factor T-bet than their counterparts CD161^{-}Vα7.2^{+} T cells (Figure 5E). We found that the expression of IFN-γ in MAIT cells regulated by T-bet and CD161, and CD161^{+}Vα7.2^{+} T cells expressed more IFN-γ and TNF-α compared to their counterparts CD161^{-}Vα7.2^{+} T cells. Together these data, it suggested that CD161^{+}MAIT cells exhibited stronger and more cytokines and cytotoxicity after polyclonal stimulation.

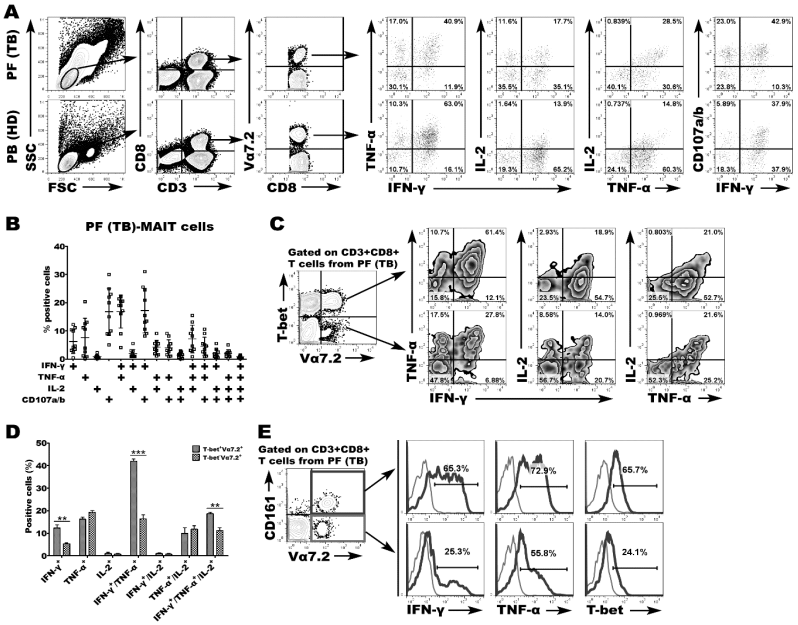

Figure 5: Detection of the Tc1-type cytokines and cytotoxic molecules by MAIT cells in pleural fluid from TBP patients after polyclonal stimulation. PFMCs from TBP (n = 8) and PBMCs from HD (n = 8) were stimulated with PMA (20 ng/ml) and Ionomycin (1 μg/ml) for 6 hours. Cells were harvested and stained with fluorochrome-conjugated monoclonal antibodies, and analyzed for the lineage differentiation, intracellular cytokine and transcription factor expression with flow cytometry. **(A):** Representative FACS data showed that the expression of IFN-γ, TNF-α, IL-2 and CD107a/b by MAIT cells after polyclonal stimulation. **(B):** The statistical analysis showed that the polyfunctional of MAIT cells in PFMCs. **(C):** Representative FACS data and **(D):** Statistical analysis showed that the expression of IFN-γ regulated by T-bet in CD3^{+}CD8^{+}Vα7.2^{+} T cells from PFMCs. **(E):** Representative FACS data showed that the expression of IFN-γ, TNF-α and T-bet in both CD161^{+}Vα7.2^{+} and CD161^{-}Vα7.2^{+} subsets of CD3^{+}CD8^{+}Vα7.2^{+} T cells from PFMCs. Data were shown as mean ± SD. Each dot represented one patient. **P<0.01; ***P<0.001.

Discussion

MAIT cells are highly conserved between biological species and are very abundant in humans [23]. MAIT cells play a critical role in the host defense against a variety of bacterial infections, including *Shigella dysenteriae*, *Klebsiella pneumonia*, *Francisella tularensis*, *M.tb bovis* BCG and *M.tb abscessus* infections, and the cytokines and cytotoxic effector molecules secreted by MAIT cells, such as IFN-γ, TNF-α and Granzyme B are probably related to the functions of anti-infection immunity [15]. The functional roles of MAIT cells during human *M.tb* infection remains to be elucidated. Those non-classically restricted *M.tb*-reactive T cells are MR1-restricted nonconventional T cells, and make up a substantial proportion of the *M.tb*-reactive non-classically restricted $CD8^+$ T-cell responses [19]. This suggested that $V\alpha7.2^+$ MR1-restricted MAIT cells could detect *M.tb*-infected cells regardless of prior exposure to the microorganisms. MAIT cell levels have been reported to be lower in blood and enriched in lung tissues in active pulmonary TB patients [24,25]. Moreover, recent studies have provided in vivo evidence demonstrating that MAIT cells could migrate into infected lung tissues during bacterial infection and that MAIT cells play a protective role against TB [26]. The observation that MAIT cells are nearly absent from the peripheral blood in those with TB, and concomitantly enriched in the lungs suggests a dynamic relationship between the presence of *M.tb* and its associated metabolites and the localization of MAIT cells.

The frequencies of MAIT cells were much lower in the peripheral blood of *M.tb* infected patients compared to healthy controls, but appeared to be better preserved in pleurisy effusion. This observation can be explained by several hypotheses, such as impaired development, promoted apoptosis stimulated by *M.tb* or the migration and recruitment of these cells into lungs [27,28], which suggested that human MAIT cells had a significant role in the anti-bacterial response in the infected lesions. The levels of MAIT cells in peripheral blood might be associated with the levels of MAIT cells in infected lung lesions. The lower frequency of MAIT cells represents a specific decrease in numbers and is not diluted by *M.tb*-reactive mainstream T cells. One possibility for the serious loss of MAIT cells in the peripheral blood of *M.tb* infected patients is that after infection, MAIT cells infiltrate into lungs and the large accumulation into the pleurisy effusion, which would directly decrease their numbers in peripheral blood. This hypothesis was tested by tissue immunofluorescence with anti-CD3 and anti-Vα7.2 (defined as MAIT cells), and we observed that MAIT cells in lung lesions from patients infected with *M.tb* [29]. Human MAIT cells seemed to migrate into infected lungs and were better preserved, which suggested that the *M.tb*-reactive MAIT cells were involvement in anti-tuberculosis defense. During the early innate phase of infections, MAIT cells were required for prompt production of pro-inflammatory cytokines and timely recruitment of activated conventional $CD4^+$ and $CD8^+$ T cells in the lungs. During later phase of infection, when conventional $CD4^+$ and $CD8^+$ T cells were activated, MAIT cells continued to infiltrate into the lungs of infected mice and produced foremost cytokines such as IFN-γ, TNF-α and cytotoxic granules [30]. Potential MAIT cell contributed to the acquisition of *M.tb*-specific T-cell responses. A wide variety of mycobacteria including *M.tb* have the capacity to infect lung epithelial cells [31]. Moreover, lung epithelial cell lines as well as primary lung epithelial cells infected with *M.tb* efficiently process and present bacterially derived antigens to both classically and non-classically restricted T cells [32]. In humans, the majority of $V\alpha7.2^+$ T cells directly isolated from lungs were pathogen reactive T cells. MR1-restricted MAIT cells have been shown to produce IFN-γ and TNF in response to *M.tb* infected lung epithelial cells [33]. Additionally, MAIT cells could induce target cell lysis of epithelial cells and contain granulizing previously shown to have anti-microbial properties [34]. Given this Th1-like cytotoxic effector phenotype, MAIT cells in the lung tissues have the potential to provide pro-inflammatory cytokines and potentially kill *M.tb*-infected cells in the infected lungs.

In Humans, MAIT cells are capable of producing IFN-γ in *M.tb*-infected lung tissues [35]. MAIT cells isolated from peripheral blood and lung lesions respond rapidly to *M.tb*-infected cells. Functionally, this response is characterized by the release of IFN-γ and TNF-α, which is critical for the control of *M.tb* infection, and also involves the granule exocytosis pathway, which results in target cell apoptosis and delivery of anti-microbial peptides [36]. In this case, activation of MAIT cells by APCs producing IL-12 would induce the secretion of cytokines such as IFN-γ, which increases the adaptive and innate immune response to the infection [37]. In infected patients, MAIT cells have an anti-bacterial function that could be attributed to the production of IFN-γ. The production of IFN-γ by MAIT cells is moderate compared with that of conventional memory $CD4^+$ and $CD8^+$ T cells stimulated with TB-Ags. In response to infected APCs, MAIT cells share functional characteristics with cytolytic $CD8^+$ effector T cells [34]. The cytotoxic capacity of MAIT cells, with a wide microbial reactivity, could have major impacts on many infectious diseases caused by microbial expressing MAIT specific ligands. The resting MAIT cells are uniquely characterized by a lack of Granzyme B and low perforin expression, key granule proteins required for efficient cytotoxic activity. However, bacterial activation of MAIT cells rapidly induced Granzyme B and perforin, licensing these cells to kill their cognate target cells. The lysis of infected target cells could supplement the immune control of microorganism infections by limiting the spread of the microorganism throughout the host.

MAIT cells kill *M.tb*-infected target cells contributing to the control of intracellular infections. In mouse models, MR1-deficient mice, after aerosol infection with *M. bovis* BCG, showed a lower ability to control the *M. bovis* BCG than wild-type mice, as

evidenced by a higher *M. bovis* BCG burden in the lung lesions [38]. In this case, MR1-dependent protection occurs within the first few days of a *M. bovis* BCG infection, consistent with the interpretation that MAIT cells are unconventional innate-like lymphocytes [39]. The capacity to react rapidly to bacterial challenge enables MAIT cells to display anti-microbial function in vivo. Recently, Hiroshi Wakao reported an Induced Pluripotent Stem Cell (IPSC)-based reprogramming approach for the expansion of functional MAIT cells in anti-mycobacterial activity [40,41]. Because of the prevalence, location, and effector functions of MAIT cells in conjunction with their ability to detect nearly all intracellularly infected cells, MAIT cells could be targeted to aid in the clearance or control of *M.tb*. To some extent, the potential use for MAIT cell targeting vaccination or therapy will depend on whether or not vaccination can elicit long-lived memory [42]. Alternately, the presence of MAIT cells in the lungs and other tissues, and their inherent effector function could suggest a role for these cells as a target of host-directed therapy. It is also possible that MAIT cell ligands could be used as adjuvants in the delivery of traditional antigens [43]. MAIT cells will be as a diagnostic or as a target of vaccination to fight against *M.tb* infection.

In the current study, we provided evidence that MAIT cells exerted unique innate functions in immune responses to *M.tb*. The results presented here have shown that MAIT cells represented an evolutionarily conserved innate-like lymphocyte population that sensed and participated in immune responses to *M.tb*. Given the abundance of this cell type in humans, their wide microbial specificity, their protective capacity and the manipulation of MAIT cells could have a considerable effect on the development of vaccines and therapeutic pathways for infectious diseases.

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