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Notch 2 receptor expression and reduced cytotoxicity in MAIT cells of active pulmonary TB patients

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Abstract

Background: Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*M.tb*), resulting in significant increase in mortality rate worldwide. Immunity against *M.tb* consists of both innate and adaptive immunity, with emerging evidence of the role of the unconventional mucosal-associated invariant T (MAIT) cells. MAIT cells are MR1-restricted T cells that possess anti-bacterial activity, including activity in response to *M.tb*. To date, human studies regarding peripheral blood MAIT cell frequency and MAIT cell activity in TB patients are still controversial and most studies are cross-sectional.

Objective: To evaluate MAIT cell function and TCR repertoire over the course of anti-TB drug treatment of active pulmonary TB patients.

Methods: MAIT cells isolated from blood of active pulmonary TB patients at various anti-TB drug treatment time points were stimulated with anti-CD3/CD28 or PMA/Ionomycin and evaluated for their cytokine and cytotoxic molecule profile. In parallel, Notch signaling was determined. Clonal analysis of MAIT cells at various anti-TB drug treatment time points was also performed in one individual.

Results: We found that there was reduced perforin and granzyme B production on MAIT cells upon stimulation with PMA/Ionomycin along with decreased cell surface expression of Notch 2 receptor. Notch 2 regulates granzyme B expression in T cells, and this reduction may indicate a similar role of Notch 2 in regulating MAIT cell function. Lastly, MAIT cell diversity with increased non-canonical TRBV usage was highest and observed at 2 months into treatment.

Conclusions: Our study provides preliminary data in understanding MAIT cell function, Notch signaling and TCR repertoire expression in active pulmonary TB patients.

Key words: MAIT cell phenotype, Notch signaling pathway, MAIT TCR repertoire, Active pulmonary TB patients, Anti-TB drug treatment

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Abbreviations:

TB	Tuberculosis
CD	Cluster of differentiation
GNLY	Granulysin
GrzB	Granzyme B
IFNγ	Interferon gamma
IGRAs	Interferon gamma release assay
M.tb	*Mycoplasma tuberculosis*
MAIT	Mucosal-associated invariant T cell
MR1	Major histocompatibility complex class I-related gene
PBMC	Peripheral blood mononuclear cell
PMA	Phobal-12-myristate-13-acetate
TCR	T cell receptor
TNFα	Tumor necrosis factor
TRAC	T cell receptor alpha constant region
TRAV	T cell receptor alpha variable region
TRBC	T cell receptor beta constant region
TRBV	T cell receptor beta variable region
TSTs	Tuberculosis skin tests

Introduction

Pulmonary tuberculosis (TB) is an infectious disease caused by the microorganism *Mycobacterium tuberculosis* (*M.tb*).[1] TB is the second leading cause of death worldwide and is responsible for 1.3 million deaths among HIV-negative individuals in 2016.[2] An untreated TB individual is capable of transferring a latent infection to up to 10-15 individuals in the community.[1] Subsequently, those 10-15 latently-infected TB individuals have a further possibility of developing active TB. Thus, the epidemiological burden of TB infection is high. In addition, there is also the burden of treatment where multiple drugs and a period of six months are required to treat these active TB individuals.[1]

Immunity to tuberculosis involves both the innate and adaptive immune system.[3] *M.tb* enters the lower respiratory tract and is internalized by alveolar macrophages, where it causes infected macrophages to disrupt due to cell death.[3] Then, *M.tb* is delivered to pulmonary lymph nodes by *M.tb*-infected dendritic cells or monocytes for antigen presentation to T cells. There is much evidence that T cells play a role in protective immunity against TB. In HIV-infected individuals, where $CD4^+$ T cells are dysfunctional, there is an increased incidence in *M.tb* infection.[4] *M.tb* also carries non-peptidic antigens that can stimulate unconventional T cells. More recently, emerging evidence of the unconventional MHC I-related molecule (MR1)-Mucosal-associated invariant T (MAIT) cell axis have been shown to have protective anti-TB response,[5,6] but their role is still unclear.

MAIT cells are innate-like MR1-restricted T cells[7,8] that respond to bacteria and yeasts that possess the vitamin B2 synthesizing pathway, including *M.tb*.[9-11] Many studies investigating MAIT cells and TB show controversial results regarding MAIT cell frequency in peripheral blood and MAIT cell activity.[12-18] Some studies also proposed migration of MAIT cells to local sites of infection in TB,[19] further suggesting a role for MAIT cells in *M.tb* infection.

Treatment of drug-sensitive *M.tb* infection requires an induction phase of treatment for two months followed by a consolidation phase of four months.[20] Over the course of treatment, the medication plays a role in killing the bacteria in a biphasic manner.[21] Thus, the period covering the treatment course duration provides an opportunity to observe the dynamics of MAIT cells in *M.tb* infection. Therefore, we recruited active pulmonary TB patients infected with *M.tb* and investigated MAIT cell characteristics at initial diagnosis (prior to treatment), at 2 months and 6 months into anti-TB drug treatment.

In addition, T cell subset differentiation is governed by cytokine signals and cell surface molecules, such as the Notch signaling pathway.[22] Notch receptors and Notch ligands of the signaling pathway interact with one another, causing proteolytic cleavage of the transmembrane portion of Notch receptors.[22] Subsequently, assembly of a transcription factor complex occurs and this complex binds to gene promoters within the cell nucleus,[22] inducing lineage-specific genes in T cells. Studies of Notch signaling in MAIT cells have not been reported yet. Therefore, in this study we have investigated Notch signaling pathways in an attempt to explain the functional profile of MAIT cells observed in active pulmonary TB patients.

Materials and Methods

Patient recruitment and sample collection

Twenty-two active pulmonary tuberculosis patients were recruited into the study. Seventeen patients were included in the MAIT cell functional analysis and T cell receptor studies. The remaining five patients were included in the MAIT cell Notch signaling studies. A diagnosis of TB was given based on diagnostic microbiological assays, of which include PCR for *M.tb* in sputum, sputum examination for acid-fast bacilli and/or *M.tb* culture for confirmation.[23-25] Tuberculin skin tests (TSTs) and IFNγ-release assays (IGRAs) were not included in the criteria due the endemic widespread of TB in Thailand and BCG vaccination during childhood excludes the reliability of TSTs to be used in the diagnosis of *M.tb* infection.[23] All patients were given initial regimen treatment for drug-sensitive TB infection. This included Isoniazid (H), Rifampicin (R), Ethambutol (E) and Pyrazinamide (Z) or Streptomycin (S).[21,26] Blood samples were collected from patients at the time of diagnosis (prior to treatment initiation) and at 2 months and 6 months (including 2 weeks and 4 months for MAIT TCR repertoire analysis study) into anti-TB drug treatment. Informed consent was obtained from all patients prior to obtaining clinical samples. Our procedures were approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No. 445/57 and IRB No. 207/58) in accordance with the Declaration of Helsinki. Eight healthy controls were randomly collected from individuals undergoing blood donation at The Thai Red Cross Society and another eleven healthy controls were recruited into the study as volunteers from King Chulalongkorn Memorial Hospital. All individuals had no clinical symptoms of active pulmonary TB and have not been tested for TSTs or IGRAs.

Peripheral blood mononuclear cell (PBMC) isolation

Collected whole blood was layered on Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation at 1500 rpm for 20 minutes at room temperature (no deceleration force). Cells were washed twice with RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco, Life Technologies Corporation, NY, USA). Isolated cells were cryopreserved in 10% DMSO (PanReac AppliChem, Darmstadt, Germany) in FBS for further experiments.

T cell functional analysis

10^5 cells of PBMCs were cultured in the presence or absence of 5 ng/ml PMA (Sigma-Aldrich, MO, USA) and 1 μg/ml ionomycin (Thermo Fisher Scientific, OR, USA), or anti-CD3/anti-CD28 beads (Invitrogen, CA, USA), with 20 μg/ml brefeldin A (BFA, (Invitrogen, CA, USA)) at 37°C for 5 hours. Cells were labeled with anti-CD3-PE/Cy7, anti-CD4-PE/Cy5, anti-CD8-APC, anti-CD161-APC/Cy7 and anti-Vα7.2-FITC at 4°C for 30 minutes, fixed with 1% formaldehyde for 30 minutes and permeabilized with 0.3% (w/v)

saponin (Sigma-Aldrich, MO, USA). Intracellular cytokines were labelled with anti-IL2-PerCp/Cy5.5, anti-IFNγ-AF700, anti-TNFα-AF700, and anti-IL17-PE for 1 hour at 4°C in the dark. For cytotoxic molecule determination, intracellular cytotoxic molecules were labeled with anti-CD107a-PE/Cy7, anti-granulysin-PE, anti-granzyme B-FITC and anti-perforin-PerCP/Cy5.5. All antibodies were purchased from Biolegend (CA, USA). After washing twice with PBS containing 2% FBS, cells were then acquired on the BD LSRII flow cytometer (BD Biosciences, CA, USA) and data was analyzed using the Flowjo software (Tree star, Inc., OR, USA).

Determination of cell surface Notch expression

10^5 cells of PBMCs were stimulated for 24 hours with or without anti-CD3/CD28 beads (1:1 bead-to-cell ratio). Then cells were labelled with anti-CD3-PE/Cy7, anti-CD4-PE/Cy5, anti-CD8-PerCP/Cy5.5, anti-CD161-APC/Cy7, anti-Vα7.2-FITC, anti-Notch1-APC and anti-Notch2-PE, at 4°C for 30 minutes. Antibodies were purchased from Biolegend (CA, USA) and BD Biosciences (Franklin Lakes, NJ, USA). Cells were washed 3 times with PBS containing 2% FBS and acquired on the BD LSRII flow cytometer. Data was analyzed using the Flowjo software (Tree star, Inc., Ashland, OR, USA).

MAIT T cell receptor repertoire analysis

Lymphocytes isolated from blood of patients were labeled with anti-CD3-PE/Cy7, anti-CD161-APC and anti-Vα7.2-FITC (Biolegend, CA, USA). Live cells were screened by labelling with propidium iodide (Biolegend, CA, USA). Single cells of $CD3^+Vα7.2^+CD161^+$ population were sorted into a 96-V well PCR plate using a BD FACS Aria II-cell sorter. RNA extraction and cDNA synthesis were performed using SuperScript VILO cDNA Synthesis Kit (Invitrogen, CA, USA) according to the manufacturer's instructions. Transcripts of T cell receptor (TCR) were then amplified by multiplex nested PCR using specific primers. The first-round PCR, the cDNA template was added to PCR reaction mixes (1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 0.75 U of Taq DNA polymerase) containing 2.5 pmol each of the external sense (TRBV and TRAV) and external antisense (TRBC and TRAC) primers.[27] In the second-round, PCR products were used as templates to amplify TCR transcripts using internal sense TRAV primers and internal antisense TRAC primer[27] for alpha chain and using internal sense TRBV primers and internal antisense TRBC primer[27] for beta chain. The PCR reaction mixes were incubated at 95°C for 2 min, following 35 cycles of 95°C for 20 sec, 52°C for 20 sec, 72°C for 45 sec, and 1 cycle of 72°C for 7 min. After cleaning with Illustra ExoProStar 1-Step Kit (GE Healthcare, Uppsala, Sweden), PCR products were detected by 2% agarose gel electrophoresis and sequenced. TCR was analyzed using the ImMunoGene Tics database.[28]

Statistical analysis

All data were analyzed using GraphPad Instat version 5.0 software (San Diego, CA, USA) and the SPSS software package (SPSS Inc, Chicago, IL, USA). Descriptive statistical analysis was used to analyze the continuous data and were expressed as frequency and percentage (n (%)). Comparison of MAIT cell population and production of cytokines and cytotoxic molecules from stimulated MAIT cells or conventional T cells at various treatment time points were compared using one-way ANOVA with post-hoc Tukey HSD (Honestly Significant Difference) test. Two-way ANOVA was used to analyze significant expression levels of Notch receptors and Notch ligands among three cell populations at various treatment time points. Data was shown as mean ± SEM and statistical significance was defined as $p \leq 0.0001$ (****), $p \leq 0.001$ (***), $p \leq 0.01$ (**) and $p \leq 0.05$ (*).

Results

Demographic characteristics of recruited active TB patient population

Twenty-two active pulmonary TB patients were recruited from King Chulalongkorn Memorial Hospital. Due to the limited number of cells obtained from patients, we categorized our patient population in this study into two groups. Seventeen patients were used to test for MAIT cell function. Five patients were used in the Notch signaling studies. Patient demographic characteristics were shown in **Table 1**. Due to Thailand being in an endemic area of tuberculosis infection, we defined "healthy individuals" in this study as individuals that did not have clinical symptoms of active or subclinical pulmonary TB regardless of their *M.tb* infection status.[3,29]

Table 1. Demographic characteristics of TB patient population

	Number of TB patient (N = 17)	Number of TB patient (for Notch expression study) (N = 5)
Age (years)		
< 35	8 (47.08%)	5 (100%)
35-60	5 (29.40%)	-
> 60	4 (23.52%)	-
Gender		
Male	12 (70.59%)	2 (40%)
Female	5 (29.41%)	3 (66.67%)
Laboratory investigation		
PCR (+)	7 (41.12%)	3 (66.67%)
M.tb culture (+)	16 (94.12%)	5 (100%)
Sputum examination (+)	14 (82.35%)	2 (40%)
Susceptibility testing		
Drugs (H, R, S, E)* response	12 (70.59%)	5 (100%)
Multidrug resistance	2 (11.76%)	-
Mono-drug resistance	3 (17.65%)	-

Table 1. (Continued)

	Number of TB patient (N = 17)	Number of TB patient (for Notch expression study) (N = 5)
Treatment drug		
Start first line drug regimen		
2HRZE/4HR*	16 (94.12%)	5 (100%)
2HRSE/4HR*	1 (5.88)	-
Change to MDR regimen		
Km, Eto, CS, MXF*	1 (5.88%)	-
Km, Eto, Cs, IV*	1 (5.88%)	-
RZE* (for H-resistance)	3 (17.65%)	-

The demographic data are presented as number of TB patients (% of total patients).
*H = Isoniazid, R = Rifampicin, S = Streptomycin, E = Ethambutol, Z = Pyrazinamide, CS = Cycloserine, MXF = Moxifloxacin, Km = Kanamycin, IV= Levofloxacin, Eto = Ethionamide

For active pulmonary TB patient data, analysis was performed with samples collected at the time of diagnosis (prior to treatment), at 2 months and 6 months into treatment.

Perforin and granzyme B production from MAIT cells in active pulmonary TB patients was decreased upon stimulation

We firstly identified MAIT cell population in peripheral blood of TB patients. MAIT cells were defined as Vα7.2^{+}CD161^{hi} T cells, which is equivalent to MR1-5-OP-RU tetramer purified cells, and conventional T cells were defined as CD3^{+}Vα7.2^{-} T cells (**Figure 1**). We evaluated MAIT cell activity by stimulating MAIT cells from active pulmonary TB patients and healthy individuals with anti-CD3/anti-CD28 beads or PMA/Ionomycin and evaluated for their cytokine (TNFα, IFNγ, IL-2 and IL-17) and cytotoxic molecule production (granulysin, granzyme B and perforin). Our results showed that there were not significant differences in cytokine production upon stimulation in MAIT cells between healthy individuals and active pulmonary TB patients regardless of stimulating with anti-CD3/anti-CD28 beads or PMA/Ionomycin (**Figure 2A**). No significant changes were observed with CD107a nor granulysin expression in both stimuli conditions (**Figure 2B**). However, in active pulmonary TB patients, there was a significant reduction in granzyme B and perforin expression in MAIT cells when activated with PMA/Ionomycin, but not anti-CD3/anti-CD28 bead stimulation, from active pulmonary TB patients at 2 and 6 months into treatment; and prior to treatment, respectively (**Figure 2B**). From these results, only granzyme B and perforin expression upon stimulation were affected in active pulmonary TB patients.

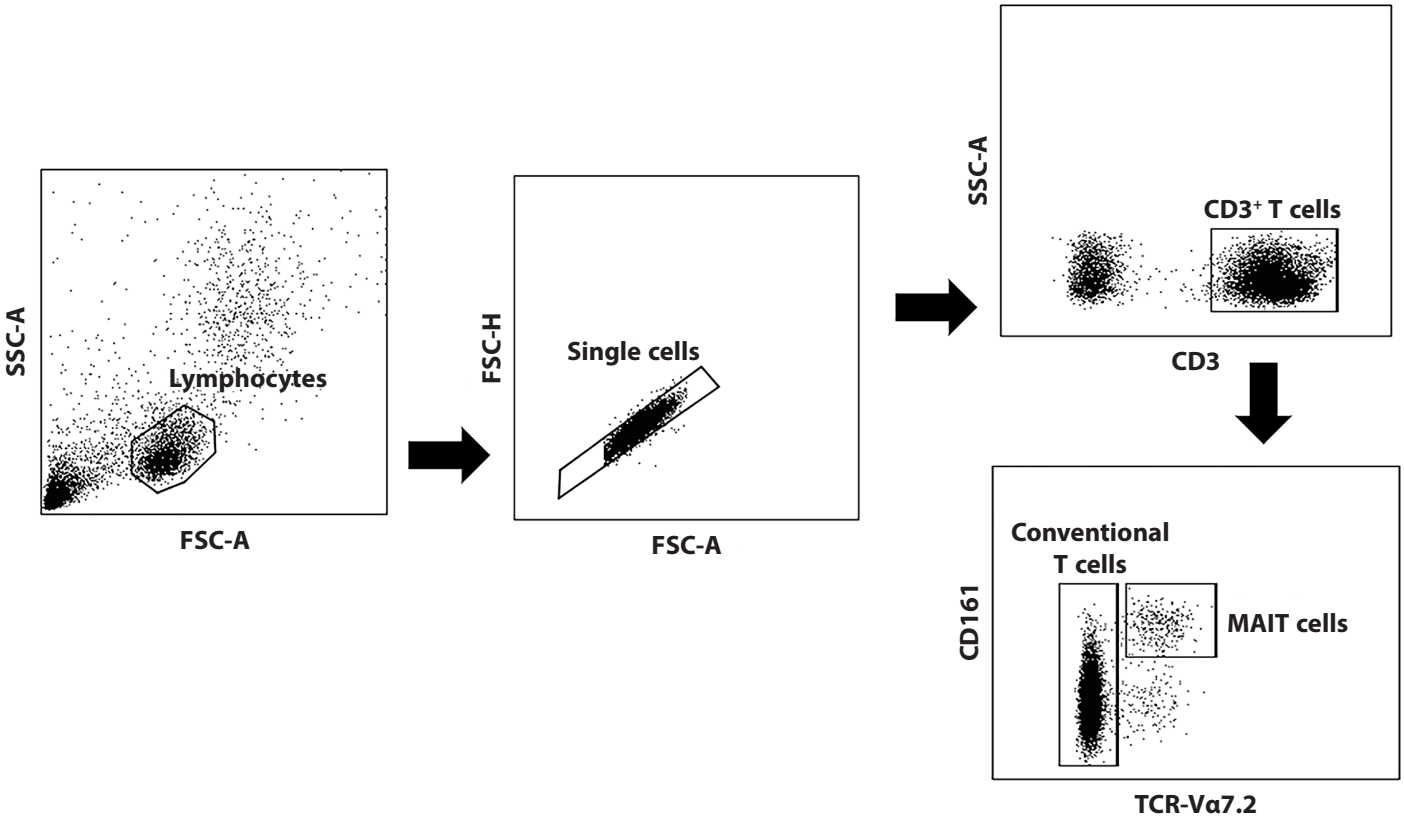

Figure 1. Gating strategies of MAIT cells and conventional T cells.
Isolated PBMCs from peripheral blood of active pulmonary TB patients and healthy individuals were labeled with anti-CD3-PE/Cy7, anti-CD161-APC/Cy7 and anti-Vα7.2-FITC and were detected by flow cytometry. MAIT cell population was gated on CD3^{+} T cells which expressed both TCR-Vα7.2 and CD161 high, and conventional T cell population was defined as CD3^{+}Vα7.2^{-} T cells.

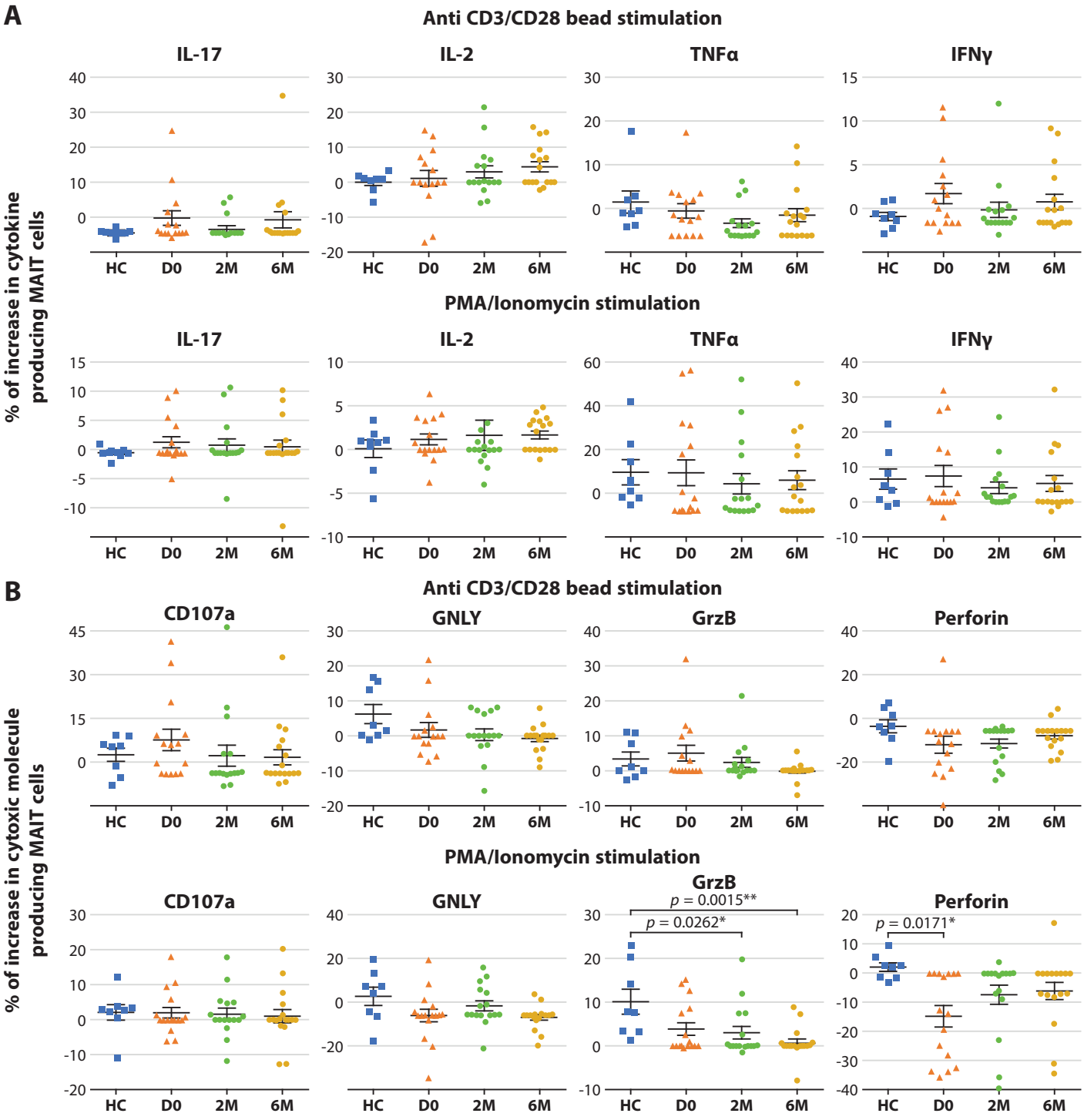

Figure 2. Cytokine and cytotoxic mediator production from MAIT cells of active pulmonary TB patients and healthy individuals.

PBMCs of active pulmonary TB patients prior to, at 2 months and at 6 months into treatment; and healthy individuals were stimulated with anti-CD3/anti-CD28 beads and PMA/Ionomycin. Cytokine and cytotoxic mediator production for each time point was determined and calculated from % of cytokine/cytotoxic mediator-producing cells in stimulated conditions subtracted by % of cytokine/cytotoxic mediator-producing cells in unstimulated conditions. (A) Percentage of increase in IL-17-, IL-2-, TNFα- and IFNγ-producing MAIT cells after stimulation with anti-CD3/anti-CD28 beads (upper panel) and PMA/Ionomycin (lower panel). (B) Percentage of increase in the degranulation marker (CD107A), granulysin, granzyme B- and perforin-producing MAIT cells after stimulation with anti-CD3/anti-CD28 beads (upper panel) and PMA/Ionomycin (lower panel). Y-axis represents the percentage increase of cytokine/cytotoxic mediator-producing cells. Each dot represents each patient. Mean values are shown with error bars.

Decreased cell surface expression of Notch 2 receptors on MAIT cells in active pulmonary TB patients

Regulation of granzyme B and perforin expression in T cells is governed by transcriptional and post-transcriptional regulators.[30-32] Among these regulators are the highly conserved membrane-bound cell surface Notch 1 and Notch 2 that mediate the Notch signaling pathway.[30,31] To investigate the potential role of Notch 1 and Notch 2 signaling pathways in MAIT cell activity in *M.tb* infection, especially perforin and granzyme B expression; cell surface expression of Notch 1 and Notch 2 on MAIT cells of active pulmonary TB patients and healthy individuals were determined. First, we determined cell surface expression of Notch 1 and Notch 2 *ex vivo* from active pulmonary TB patients and healthy individuals on MAIT cells, conventional $CD4^+$ T cells and conventional $CD8^+$ T cells (**Figure 3A**). Our data shows that Notch 2 expression on MAIT cells was significantly reduced in active pulmonary TB patients at all time points (**Figure 3B**), suggesting a consistent suppression of Notch 2 expression on MAIT cells over the course of anti-TB drug treatment. This was in contrast with conventional T cells, where a reduction of Notch 2 expression was significantly reduced in active pulmonary TB patients only at the initial time of diagnosis and no changes in Notch 2 expression were observed for conventional $CD4^+$ T cells and conventional $CD8^+$ T cells, respectively (**Figure 3B**).

Next, we stimulated MAIT cells and conventional T cells with anti-CD3/anti-CD28 beads to evaluate whether the expression of Notch 1 and Notch 2 were altered. We observed that in healthy individuals, Notch 1 and 2 expressions were significantly increased on both MAIT cells and conventional T cells upon anti-CD3/anti-CD28 bead stimulation (**Figure 3C**). In addition, conventional $CD4^+$ T cells of active pulmonary TB patients had significant increased Notch 1 expression upon anti-CD3/anti-CD28 bead stimulation (**Figure 3C**). In contrast, the ability of Notch 2 receptor to increase its expression upon stimulation was significantly reduced in both conventional $CD4^+$ T cells and conventional $CD8^+$ T cells in active pulmonary TB patients when compared to healthy individuals (**Figure 3D**). In MAIT cells, there was no significant reduction in both Notch 1 and Notch 2 expression in active pulmonary TB patients when compared with healthy individuals (**Figure 3D**). However, there was an observed trend where MAIT cells seem to have increased ability to upregulate Notch 1 and Notch 2 expression upon stimulation in active pulmonary TB patients as treatment progressed (**Figure 3D**). We also observed that the ability to upregulate Notch 1 receptor upon stimulation was lowest in both conventional $CD4^+$ and conventional $CD8^+$ T cells at 2 months after treatment initiation (**Figure 3D**).

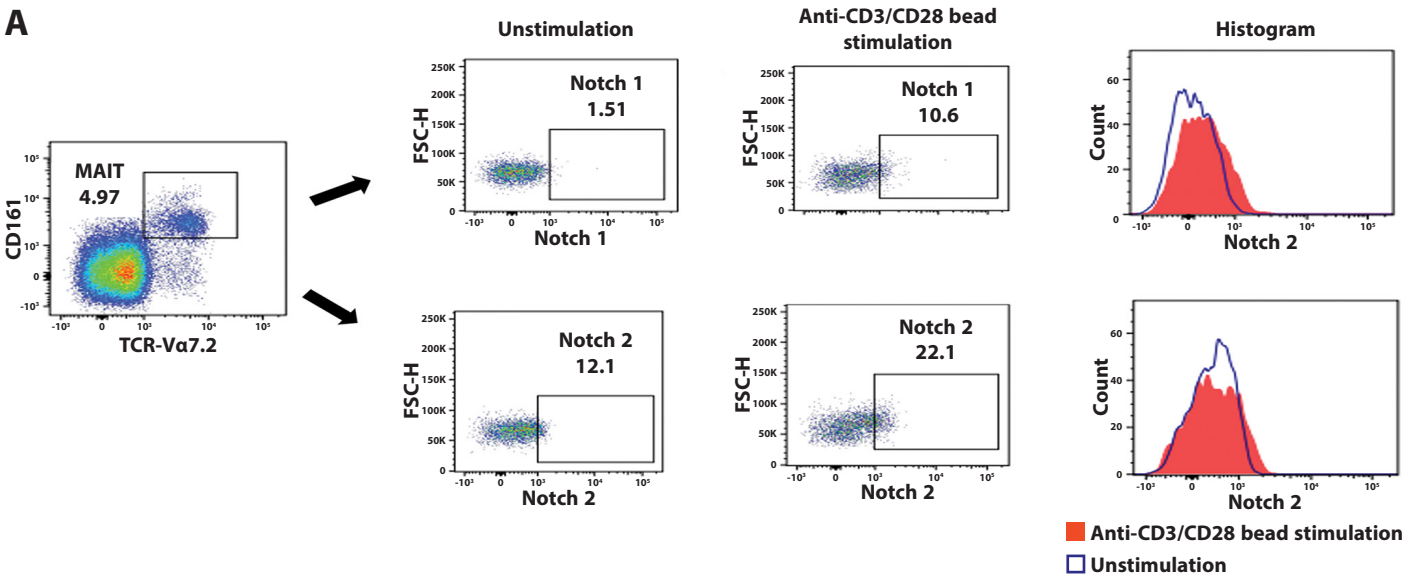

Figure 3. Cell surface expression of Notch on peripheral blood MAIT cells of active pulmonary TB patients and healthy individuals.

PBMCs of active pulmonary TB patients (N = 5) prior to, at 2 months and at 6 months into treatment; and healthy controls (N = 11) were stimulated with anti-CD3/anti-CD28 beads. Cell surface expression of Notch receptors (Notch 1 and Notch 2) were determined via flow cytometry. (A) FACS plots and histograms showing gating strategies of Notch-1 (upper panel) and Notch-2 (lower panel) expressing MAIT cells both in unstimulated (white histogram) and anti-CD3/CD28 bead stimulation (gray histogram) conditions. (B) Bar graphs showing frequency of Notch 1- and Notch 2-expressing *ex vivo* (unstimulated) MAIT cells, conventional $CD4^+$ T cells and conventional $CD8^+$ T cells. (C) Bar graphs showing frequency of Notch 1- and Notch 2-expressing MAIT cells, conventional $CD4^+$ T cells and conventional $CD8^+$ T cells; when stimulated (S) or not stimulated (U) with anti-CD3/anti-CD28 beads. (D) Bar graphs showing frequency of upregulated Notch 1- and Notch 2-expressing MAIT cells, conventional $CD4^+$ T cells and conventional $CD8^+$ T cells upon stimulation with anti-CD3/anti-CD28 bead stimulation. The percentage of upregulated Notch receptor-expressing cells was calculated by subtracting Notch receptor-expressing cells in unstimulated conditions from that of anti-CD3/anti-CD28 bead stimulation conditions. SEM values are shown with error bars.

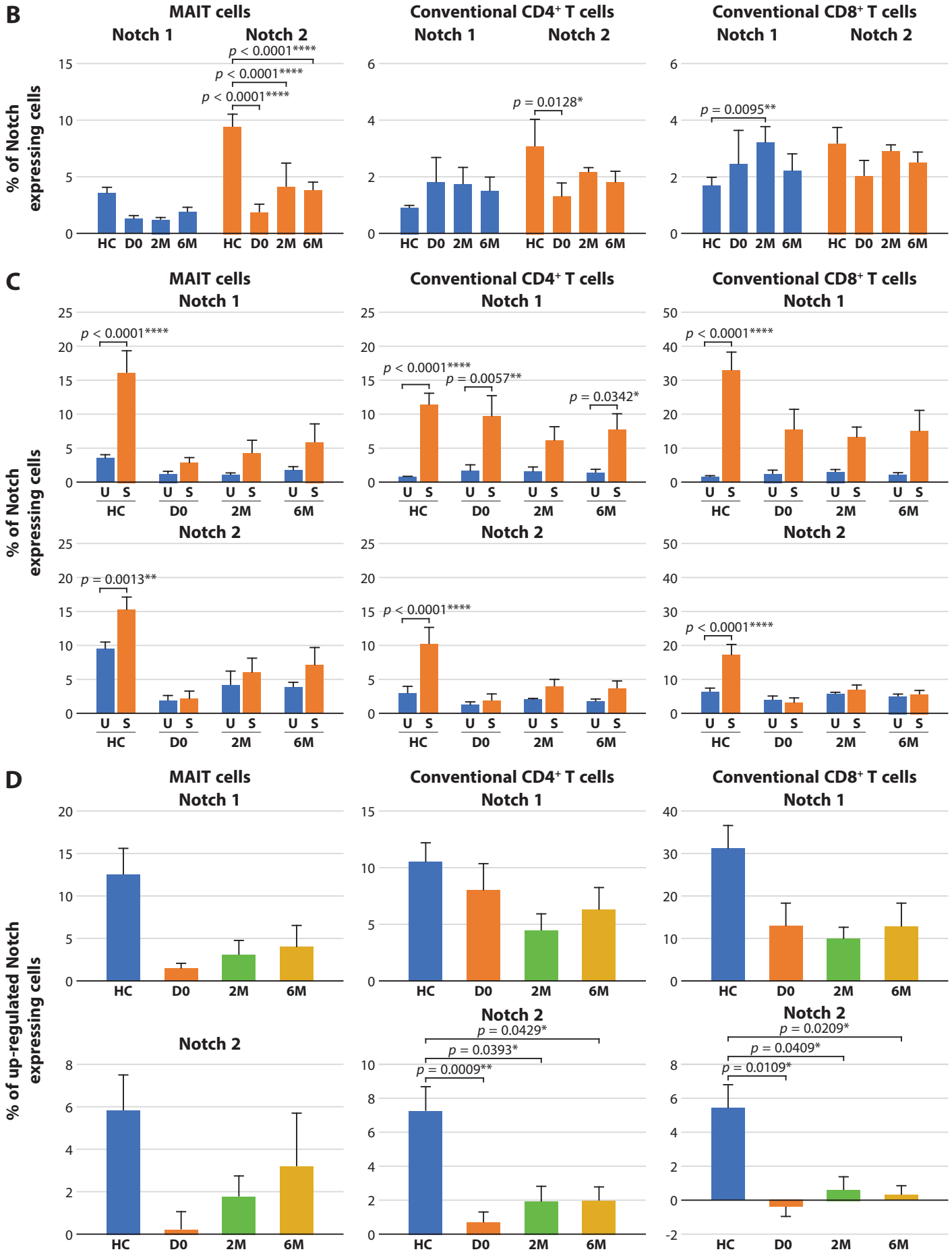

Figure 3. (Continued)

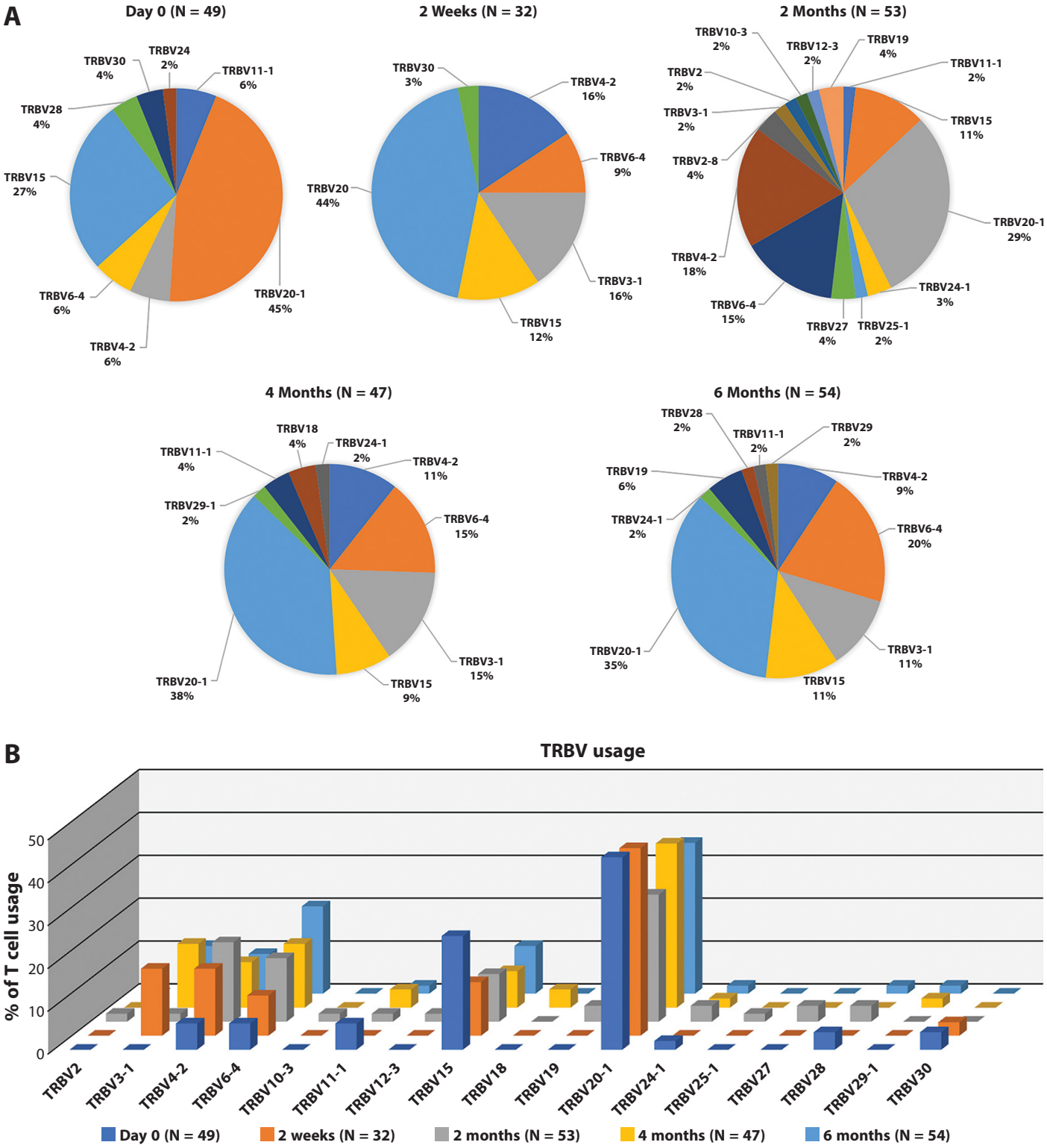

Figure 4. MAIT TCR repertoire of an individual with active pulmonary TB infection at the time of diagnosis (prior to treatment), at 2 weeks, at 2 months, at 4 months and at 6 months into treatment.

(A) Pie charts showing TRBV usage of MAIT cells of an active pulmonary TB patient prior to, at 2 weeks, at 2 months, at 4 months and at 6 months into treatment. N represents the number of clones. (B) Bar graphs comparing TRBV usage of MAIT cells of an active pulmonary TB patient prior to, at 2 weeks, at 2 months, at 4 months and at 6 months into treatment. (C) Line graphs showing the dynamic changes of each TRBV usage by MAIT cells prior to, at 2 weeks, at 2 months, at 4 months and at 6 months into treatment. Black arrows depicted represent TRBV chains that peak at the 2-month mark into treatment.

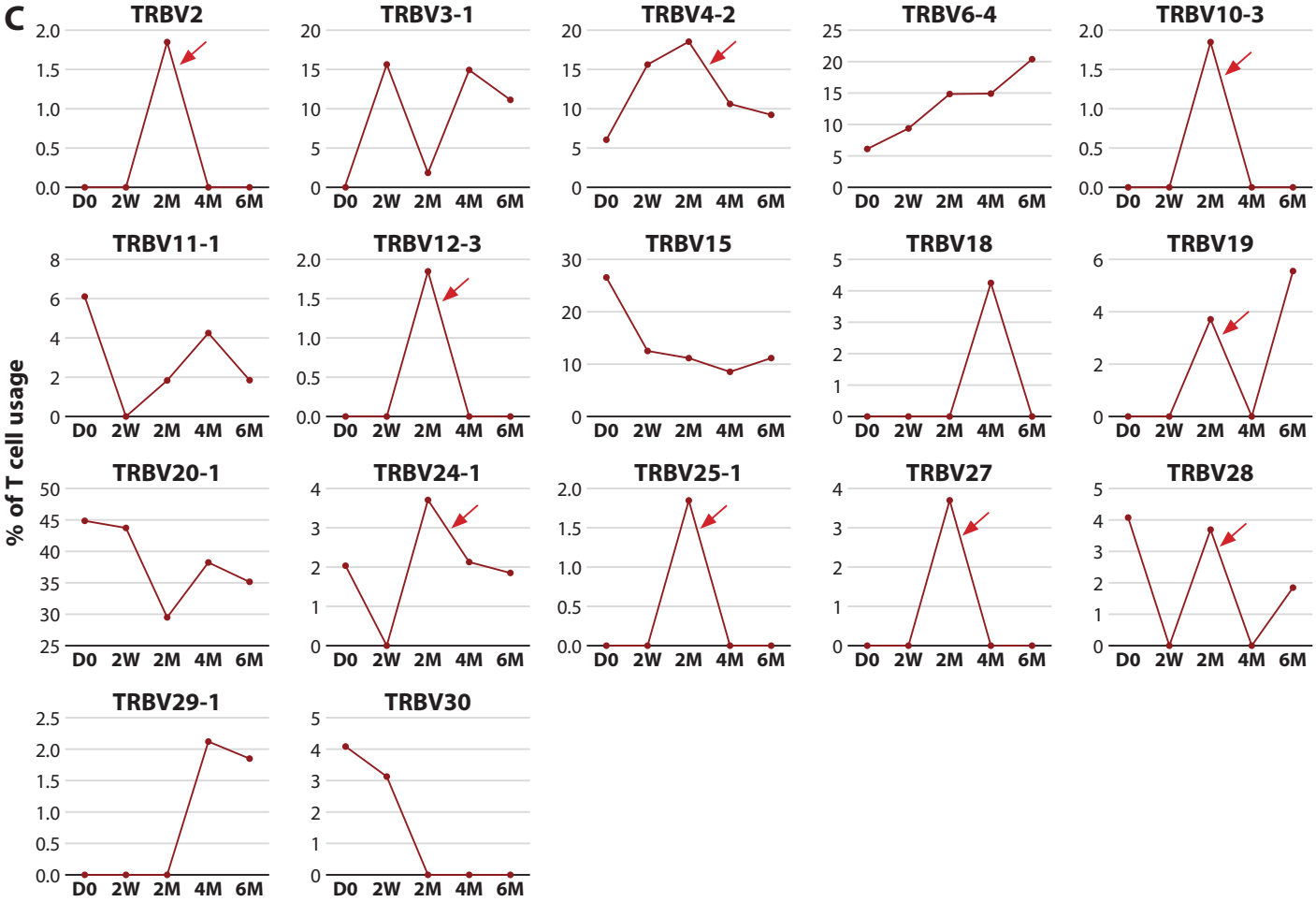

Figure 4. (Continued)

MAIT TCR repertoire of an active pulmonary TB patient reveals highest diversity and expansion of non-canonical TRBV usages at 2 months after anti-TB drug treatment initiation

T cell receptors have been shown to govern its functionality.[33,34] Therefore, we selected one active pulmonary TB patient and characterized the patient's MAIT T cell receptor (TCR) usage. We characterized the TRBV usage of MAIT cells, as defined by $CD3^{+}V\alpha7.2^{+}CD161^{hi}$ cells at the time of diagnosis; at 2 weeks, 2 months, 4 months and 6 months of anti-TB drug treatment. Our data shows that the MAIT TRBV usage was most diverse at 2 months of anti-TB drug treatment (**Figure 4A**). There were predominant TRBV chains that persisted throughout the duration of anti-TB drug treatment (**Figure 4B**). These included the TRBV20-1, TRBV15, TRBV6-4 and TRBV4-2 (**Figure 4B**). When we tracked each TRBV chain individually, we observed 8 out of 17 TRBV chains that peaked at 2 months into anti-TB drug treatment (**Figure 4C**). These included TRBV2, 4-2, 10-3, 12-3, 19, 24-1, 25-1, 27 and 28. There were also TRBV chains that increased during the treatment course (TRBV6-4) and those that decreased during the treatment course (TRBV11-1, 15, 20-1 and 30) (**Figure 4C**). These results reflect preservation of dominant TRBV usages in MAIT cells as well as demonstration of dynamic changes in non-canonical TRBV chains.

Discussion

Immunity to tuberculosis requires the orchestration of innate and adaptive immune compartments for the containment of *M.tb* in immunocompetent hosts.[1,35] MAIT cells are innate-like T cells capable of killing *M.tb*-infected cells in vitro, thus, supporting a role for MAIT cells in immunity against *M.tb* infection.[10] Many studies regarding MAIT cells and TB have been published.[6,12-19,36,37] Treatment of *M.tb* infection in active pulmonary TB patients requires 2 phases: (i) an induction intensive phase to kill actively replicating bacteria and (ii) a consolidation phase to ensure persistent bacilli are targeted for a duration of 2 months and 4 months, respectively; resulting in a total of 6 months for treatment.[20] At the 2-month mark of anti-TB drug treatment, patients undergo evaluation of sputum conversion with most patients not having bacilli in their sputum to culture.[3,29] Therefore, in our study, we investigated MAIT cell characteristics at the initial diagnosis, at 2 months and at 6 months into treatment. It is worth noting that our study defined "healthy individuals" as including those without active pulmonary *M.tb* infection status, which may include uninfected and latently-infected individuals, altogether; due to the highly endemic area of TB in Thailand.

Our functional analyses revealed that there were no changes in the ability of MAIT cells to secrete IL-17, IL-2, TNFα, IFNγ and granulysin in active pulmonary TB patients. We only found detected lower ability for granzyme B and perforin production in active pulmonary TB patients upon PMA/Ionomycin stimulation, but not anti-CD3/anti-CD28 bead stimulation; at certain time points during treatment. The decrease in granzyme B production corroborated with findings from transcriptomic studies showing downregulation of the GZMB gene in MAIT cells of active TB patients.[37] However, our findings and findings of some groups have come with controversial results; some studies demonstrating increased MAIT cell activity in active TB patients[38] and others demonstrating decreased IFNγ production.[17,37] Granzyme B kills Mycobacteria-infected cells by disrupting protein synthesis of the bacteria.[39] The reduction of perforin and granzyme B production in MAIT cells may indicate an inability for the human host to eradicate *M.tb* infection.

Notch signaling plays a role in T cell development and differentiation into different T cell lineages eg. Th1, Th17 cells and Treg.[22,40] A regulator of perforin and granzyme B that was interrogated in this study was the Notch signaling pathways, especially Notch 1 and Notch 2 receptors.[30,31] Notch signaling aids in the differentiation of T cells.[22] Studies of Notch signaling in MAIT cells have not been described before. In our study, we detected a decrease in cell surface expression of Notch 1 and Notch 2 receptors on *ex vivo* MAIT cells in active pulmonary TB patients. Notch 1 was previously reported to regulate cytotoxic T lymphocyte (CTL) differentiation and function, supporting perforin and granzyme B expression.[30] Notch 2 regulates granzyme B expression via interaction with the transcription factor CREB1 and binding of the complex to the granzyme B gene promoter.[31] These observed findings suggest that MAIT cells may have reduced cytotoxicity in active pulmonary TB patients due to decreased expression of Notch 1 and Notch 2. However, due to our data being collected from different patient populations; we were not able to correlate these findings. Nonetheless, our findings provides a glimpse of a potential role of Notch signaling in defining MAIT cell function. Reduced cytotoxic function in $CD8^+$ T cells and $CD8^+$ T cell dysfunction in active TB patients have been reported.[41-43] An understanding in the regulation of Notch receptors on MAIT cells and its results in active pulmonary TB patients may offer these patients a novel approach in using synthetic Notch (SynNotch) receptors, a chimera between the core regulatory domain of the cell-cell signaling receptor Notch, an extracellular domain of a single-chain antibody structure to the cognate antigen and synthetic intracellular transcriptional domain; to target *M.tb* antigens for the treatment of *M.tb* infection.[44] MAIT cells make up the highest proportion of T cells in humans based on their antigen specificity compared to other peptide-specific T cells.[45] Thus, MAIT cells may be a potential target cell in using immunotherapy approaches. In addition, this may explain the underappreciated findings of cytotoxicity in *M.tb* infection control.

Despite MAIT cell frequency not differing between active pulmonary TB patients and healthy individuals, we further investigated qualitative changes that may occur within the MAIT TCR repertoire. An analysis of one patient with active pulmonary TB revealed at 2 months into anti-TB drug treatment had the highest MAIT TCR diversity when compared to MAIT cell diversity at other time points during the course of anti-TB treatment. The profile of TRBV usages had similar predominant to one another throughout the course of anti-TB drug treatment. Over half of all detected TRBV families peaked in their frequency at 2 months into anti-TB drug treatment and mostly were non-canonical TRBV chains. Non-canonical TRBV usages have also been described in TB patients as well.[46] MAIT cells are also able to discriminate between ligands generated from different bacterial species at a clonotypic level.[47] Anti-TB drug treatment targets *M.tb* with rapidly-killed properties (subpopulation #1) in the first 2 months of treatment and requires an extended period for the "persistent" phenotypic variant (subpopulation #2) for another 4 months.[21] The diversity of MAIT cells observed in this active pulmonary TB patient in our study may suggest expansion of MAIT cell clones that are specific to *M.tb* with unique killing properties. However, our study interrogated only one active TB patient, which is a limitation in interpretation for this dataset. Further studies that include a larger population needs to be performed to address this.

Collectively, our study provides data suggesting a potential role of Notch in MAIT cells and MAIT cell clone dynamic in active pulmonary TB patients during drug treatment in one patientt. Our data revealed MAIT cell changes in perforin and granzyme B expression, Notch 1 and Notch 2 receptors and increased MAIT TCR diversity at 2 months after initiation of anti-TB drug treatment. In-depth investigation of both Notch signaling and MAIT TCR diversity during treatment course needs to be performed for further conclusions.

Conclusion

Here, we report MAIT cell characteristics in active pulmonary TB patients and compared with those of healthy individuals. Our findings show differences in some characteristics in active TB patient in an endemic setting. The findings in this study serve as a basis for understanding the changes that occur in MAIT cells during the course of *M.tb* infection.

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Declaration of interest

None

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Supplemental material

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