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Dual TCR-α Expression on Mucosal-Associated Invariant T Cells as a Potential Confounder of TCR Interpretation

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Mucosal-associated invariant T (MAIT) cells are innate-like T cells that are highly abundant in human blood and tissues. Most MAIT cells have an invariant TCRα-chain that uses T cell receptor α-variable 1-2 (TRAV1-2) joined to TRAJ33/20/12 and recognizes metabolites from bacterial riboflavin synthesis bound to the Ag-presenting molecule MHC class I related (MR1). Our attempts to identify alternative MR1-presented Ags led to the discovery of rare MR1-restricted T cells with non–TRAV1-2 TCRs. Because altered Ag specificity likely alters affinity for the most potent known Ag, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), we performed bulk TCRα- and TCRβ-chain sequencing and single-cell–based paired TCR sequencing on T cells that bound the MR1-5-OP-RU tetramer with differing intensities. Bulk sequencing showed that use of V genes other than TRAV1-2 was enriched among MR1-5-OP-RU tetramer^{low} cells. Although we initially interpreted these as diverse MR1-restricted TCRs, single-cell TCR sequencing revealed that cells expressing atypical TCRα-chains also coexpressed an invariant MAIT TCRα-chain. Transfection of each non–TRAV1-2 TCRα-chain with the TCRβ-chain from the same cell demonstrated that the non–TRAV1-2 TCR did not bind the MR1-5-OP-RU tetramer. Thus, dual TCRα-chain expression in human T cells and competition for the endogenous β-chain explains the existence of some MR1-5-OP-RU tetramer^{low} T cells. The discovery of simultaneous expression of canonical and noncanonical TCRs on the same T cell means that claims of roles for non–TRAV1-2 TCR in MR1 response must be validated by TCR transfer-based confirmation of Ag specificity. *The Journal of Immunology*, **2022, 208: 1–7.**

Adaptive cellular immunity relies on recombination of the TCRβ (TRB), TCRγ (TRG), TCRα (TRA), and TCR-δ (TRD) genomic loci during T cell development in the thymus (1). Remarkable TCR diversity is achieved by combinatorial use of genome-encoded variable (V), diversity (D), and joining (J) genes and addition of intervening nontemplated (N) nucleotides (2). Many T cells recognize peptide Ags in the context of highly polymorphic HLA molecules (3). In parallel, some T cells bind nonpeptide Ags presented by non–MHC-encoded Ag-presenting molecules, including the MHC-related protein 1 (MR1) and CD1 proteins (reviewed in 4, 5). Unlike MHC, CD1 and MR1 proteins are almost monomorphic (6), and consequently CD1- and MR1-reactive T cells tend to express characteristic TCR motifs, shared by many individuals regardless of their HLA haplotypes (7). These invariant TCR motifs (7) recognize unique Ag classes, including pathogen-derived mycobacterial lipids for CD1b (8), α-galactosyl ceramides for CD1d (9), and metabolites from active bacterial biosynthetic enzymes for MR1 (10). These invariant TCRs are thought to have coevolved with cognate nonclassical Ag-presenting molecules in different species (11).

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Abbreviations used in this article: CDR3α, complementarity-determining region 3α; DURT, donor-unrestricted T cell; 6-FP, 6-formylpterin; HEK293, human embryonic kidney 293 cell; MAIT, mucosal-associated invariant T cell; MR1, MHC class I related; 5-OP-RU, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil; TB, tuberculosis; TRA, TCRα; TRAC, TCRα chain; TRAV1-2, T cell receptor α-variable 1-2; TRB, TCRβ; TRD, TCR-δ; TRG, TCRγ.

Due to their potential to elicit generalizable population-level immune responses, donor-unrestricted T cells (DURTs) and the Ags they recognize are attractive targets of vaccination against microbes such as *Mycobacterium tuberculosis* (12). In particular, mucosal-associated invariant T (MAIT) cells, which recognize Ags presented by MR1, are attractive candidates due to their abundance in the blood (13), their high reactivity against several bacterial infections (14–17), and their documented roles in vaccination (18, 19). MR1 tetramers bind directly to TCRs and allow unequivocal identification of MAIT cells and more diverse MR1-restricted αβ (20) and γδ (21) T cells, and they provide a unique opportunity to identify novel TCR rearrangements and Ag specificities (22). Human MAIT TCRα chains display a characteristic complementarity-determining region 3α (CDR3α) formed by a rearrangement between T cell receptor α-variable 1-2 (TRAV1-2) and TRAJ33, or sometimes TRAJ12 or TRAJ20, with few nontemplate encoded (N)-nucleotides (22–24) and a biased preference for some TRB genes (23, 25, 26). Diversity in TRB gene use in MAIT cells is potentially associated with recognition of different microbes (25, 27–29) or different ligands (30). These canonical MAIT cells have a preferred specificity for 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) over 6-formylpterin (6-FP) (10, 31, 32). Although TCR conservation, especially "canonical" TRAV1-2 use, has been considered a key defining feature of human MAIT cells for decades, a new direction in the field has resulted from identification of "noncanonical" TRAV1-2–negative (TRAV1-2$^-$) and γδ T cells (21) that recognize MR1 and that are suggested to have unique Ag specificities (20, 33–37). MAIT cells have broadly reported roles in infection (17), cancer (38), and autoimmunity (39). Hence, defining MAIT TCR motifs can be used to infer pathogenic and protective TCR clonotypes relevant to immunodiagnosis or vaccination.

Several new technologies and algorithms for high-dimensional TCR sequencing analysis have successfully identified clonally expanded populations of Ag-specific T cells and their TCR motifs among large numbers of blood- and tissue-derived T cells (40–43). These sequencing technologies derive TCR sequences either from single cells, which identify paired TCRα and TCRβ (44, 45), or from bulk genomic (46) or transcriptomic sequencing data (41, 47). In this study, we sought to use MR1 tetramers and high-throughput TCR sequencing to identify noncanonical TCR patterns. We observed MAIT cell populations with differing binding intensities to the 5-OP-RU–loaded MR1 tetramers. We hypothesized that MAIT cells with lower MR1 tetramer binding intensities would reveal unique TCR motifs consistent with lower preferential binding to the 5-OP-RU/MR1 Ag complex. Consistently, we detected an enrichment of TRAV1-2$^-$ TCRs in MR1 tetramer$^+$ MAIT cells, especially those with lower MR1 tetramer intensity. However, detailed TCR gene transfer studies revealed that the lower tetramer binding was explained by dual expression of canonical and noncanonical TCRα chains in the same TRAV1-2$^+$ clonally expanded MAIT cells, as opposed to a single noncanonical TCR with lower affinity for MR1-5-OP-RU. Dual TCR expression was previously observed in HLA-restricted (48) and CD1d-reactive T cells (49), but it takes on special importance in the MAIT cell system because it can confound the assignment of noncanonical TCRs for MR1 specificity. These data establish the need to validate the Ag specificity of newly described TCR motifs from large-dimensional sequencing platforms by TCR gene transfer and other alternative techniques (50).

Materials and Methods

Human participants

Lima, Peru. We recruited Peruvian participants with active tuberculosis (TB) disease or asymptomatic household contacts of TB cases with positive or negative QuantiFERON TB Gold In-tube test results from Lima, Peru, as described previously (51, 52). The institutional review board of the Harvard Faculty of Medicine and Partners HealthCare (protocol IRB16-1173) and the institutional committee of ethics in research of the Peruvian Institutes of Health approved this study protocol. All adult study participants and parents and/or legal guardians of minors provided informed consent, whereas minors provided assent. The protocol is approved by the institutional review board of Harvard Faculty of Medicine and Partners HealthCare and the institutional committee of ethics in research of the Peruvian Institutes of Health.

Boston, MA. We obtained de-identified leukoreduction filter samples (leukopaks) from healthy blood bank donors through the Brigham and Women's Hospital Specimen Bank, as approved by the institutional review board of Partners HealthCare.

Memphis, TN. PBMC samples were obtained from healthy children and adult and elderly donors from St. Jude Children's Research Hospital (XPD12-089 IIBANK and 1545216.1).

Melbourne, Australia. Spleen lymphoid tissues were collected from deceased donors whose deaths were caused by conditions other than influenza (DonateLife, Canberra, Australia) after written informed consent was provided by the donors' next of kin (53). The University of Melbourne Human Ethics Committee approved experiments (identification numbers 1443389.4, 1955465, and 1545216.1).

Flow cytometric analysis

The protocol and primary analysis of Peruvian samples by flow cytometry were reported previously (51). MR1 monomers were obtained from the University of Melbourne, Australia (10, 22), and were used to generate tetramers in Boston as previously described (51). For HEK293T cell validation experiments, we used MR1 tetramers obtained from the National Institutes of Health Tetramer Core facility.

Genomic bulk TCR sequencing

For TCR sequencing from genomic templates, 3900 MR1 tetramer^{hi} and 4500 MR1 tetramer^{int} cells were doubly sorted from PBMC samples from Peruvian donor 58-1 after 14 d of polyclonal T cell expansion. For expansion, 10^6 cells were cultured with 25×10^6 irradiated allogeneic PBMCs, 5×10^6 irradiated allogeneic Epstein-Barr virus–transformed B cells, 30 ng/ml anti-CD3 mAb (clone OKT3) for 14–16 d, in the presence of 1 ng/ml IL-2 (52). PBMC samples from healthy Boston blood bank donors LP1 and CO2 were not expanded before double cell sorting. Cell numbers obtained from the sorted tetramer^{hi}, tetramer^{int}, and tetramer^{low} populations were 2000, 5800, and 3100, respectively, for LP1 and 1100, 4000, and 2300, respectively, for CO2. High-throughput TCR sequencing and assignment of V and J genes was performed for the TCRβ locus and the TCRαδ locus (Adaptive Biotechnologies, Seattle, WA) using a multiplex PCR approach on genomic DNA isolated from sorted T cells using the Qiagen QIAamp DNA Mini Kit, followed by Illumina high-throughput sequencing (46).

Sorted single-cell paired TCR sequencing

Single-cell TCR sequencing was adapted from a previously published protocol (41). Briefly, single MR1 tetramer binding cells from Peruvian participant 7-3 and blood bank donors 702A and 703A were sorted into a 96-well plate coated with Vapor-Lock (Qiagen) containing iScript cDNA synthesis mixture (Bio-Rad Laboratories) and 0.1% Triton X-100 for direct cell lysis. Reverse transcription was performed in a thermocycler (25°C for 5 min, 42°C for 30 min, 80°C for 5 min). Subsequently, cDNA samples were amplified in a nested PCR using Denville Choice Taq Polymerase (Thomas Scientific) and previously described primers (41). Briefly, the first external reaction contained a mixture of all TCRα and TCRβ forward primers, combined at 1 μM each, and reverse TRA chain (TRAC) and TRB chain primers at 10 μM each: 95°C for 2 min, 35 cycles of (95°C for 20 s, 50°C for 20 s, 72°C for 45 s), and 72°C for 7 min. A second internal PCR used a mix of TCRα forward primers at 1 μM each with a reverse internal TRAC primer at 10 μM or a mix of TCRβ forward primers and reverse TRB chain primer, separately at cycling conditions: 95°C for 2 min, 35 cycles of (95°C for 20 s, 56°C for 20 s, 72°C for 45 s), and 72°C for 7 min using previously described primers (41). Amplicons were analyzed on an agarose gel, and bands were excised using a UV lamp and purified using the QIAquick Gel Extraction Kit (Qiagen), then sent for Sanger sequencing (GENEWIZ). Sequences were reverse complemented and analyzed using 4Peaks software and mapped to the reference sequences for the genome-encoded V and J segments for both the TCRα and TCRβ genes on the ImMunoGeneTics information system database. The unmapped sequences were considered N-nucleotides and/or Dβ segments for TCRβ to determine the CDR3. CDR3α and CDR3β amino acid sequences were predicted by in silico translation,

showing productive in-frame rearrangements, using the online ExPASy translate tool (https://web.expasy.org/translate/).

For Australian samples, single MR1-5-OP-RU-tetramer$^+$TRAV1-2$^+$ PBMCs from healthy donors and spleen tissues were sorted into 96-well plates using a FACSAria cell sorter (BD Biosciences). Paired CDR3αβ regions were determined using multiplex-nested RT-PCR before sequencing of TCRα and TCRβ products, as previously described (41, 54) and reported (55). For paired TCRαβ analyses, sequences were parsed into the IMGT/HighV-QUEST web-based tool using TCRBlast1 (kindly provided by Paul Thomas and Matthew Caverley) to determine V(D)J regions.

TCR transfection assay

Synthetic TCRα and TCRβ sequences (GENEWIZ) from MR1 tetramer binding sorted single T cells, separated by self-cleaving picornavirus 2A-linker sequence (5′-GGATCCGGCGCCACCAATTTCTCGCTGCTTAAG-CAGGCCGGCGACGTCGAAGAGAACCCCGGGCCC**ATG**-3′), were cloned into a GFP-containing pMIG vector using standard restriction digestion and cloning procedures. Human embryonic kidney (HEK293T) cells were cultured overnight on a 6-well plate containing 4 ml of DMEM-10 media supplemented with 10% FBS and penicillin-streptomycin at 37°C, and they were subsequently cotransfected with the pMIG-TCR and pMIG-CD3 plasmid (56) using FuGENE HD transfection reagent (Promega). Transfected HEK293T cells were analyzed for tetramer binding by flow cytometry 48–72 h after transfection. Abs used to stain transfected 293T cells were Brilliant Violet 421–conjugated anti-human CD3 Ab (BioLegend) and PE-conjugated anti-human TCRαβ Ab (BD Biosciences).

Results

During a quantitative study of MAIT cells in a Peruvian TB cohort (51), we observed MAIT cell populations with variable staining intensities for the 5-OP-RU–loaded MR1 tetramer (Fig. 1A). This phenomenon was observed in participants with and without evidence for *M. tuberculosis* infection and did not seem to be correlated with TB disease. Although canonical MAIT TCRs typically show high affinity for MR1-5-OP-RU, we hypothesized that MAIT cells with lower tetramer staining intensity may reflect different and variable TCR motifs, consistent with their lower affinities to the MR1-5-OP-RU complex. To define TCR gene use in high, intermediate, and low staining populations, we sorted MAIT cell populations with different MR1 tetramer staining intensities and performed bulk TCRα and TCRβ sequencing from genomic DNA and subsequent V- and J-gene assignment of rearranged genes. Subsequently, we sorted MAIT cell populations from one Peruvian sample (participant number 58-1) after polyclonal T cell expansion and from two random Boston blood bank donors (LP1 and CO2) without expansion (Fig. 1B). The populations were sorted on the basis of MR1 tetramer fluorescence intensities and resorted before sequencing to ensure the purity and preservation of MR1 tetramer binding levels (Supplemental Fig. 1). Regardless of the source of PBMCs, we saw similar patterns with TRAV1-2 TCRs in brightly staining cells, and TCRα V-genes other than Vα7.2 (TRAV1-2) were enriched in sorted MAIT populations with low and intermediate MR1 tetramer staining (Fig. 1B and 1C). This pattern of atypical TRAV gene use in MAIT cells with lower MR1 tetramer binding relative to MAIT cells with high MR1 tetramer staining was observed even after discarding unproductive TCRα-chains (Supplemental Table I). Frequencies of TRAV1-2$^-$ MAIT cells in blood did not differ by TB status in Peruvian samples (Kruskal-Wallis test $p = 0.75$; Fig. 1D). TRAV1-2$^-$ MAIT cells in these samples (Fig. 1B and 1C) were similar to frequencies previously reported in other populations (20) representing a minority of T cells (0.6–40%), but they were potentially biologically significant because TCRα diversity diverges from the conventional understanding of MAIT cell function.

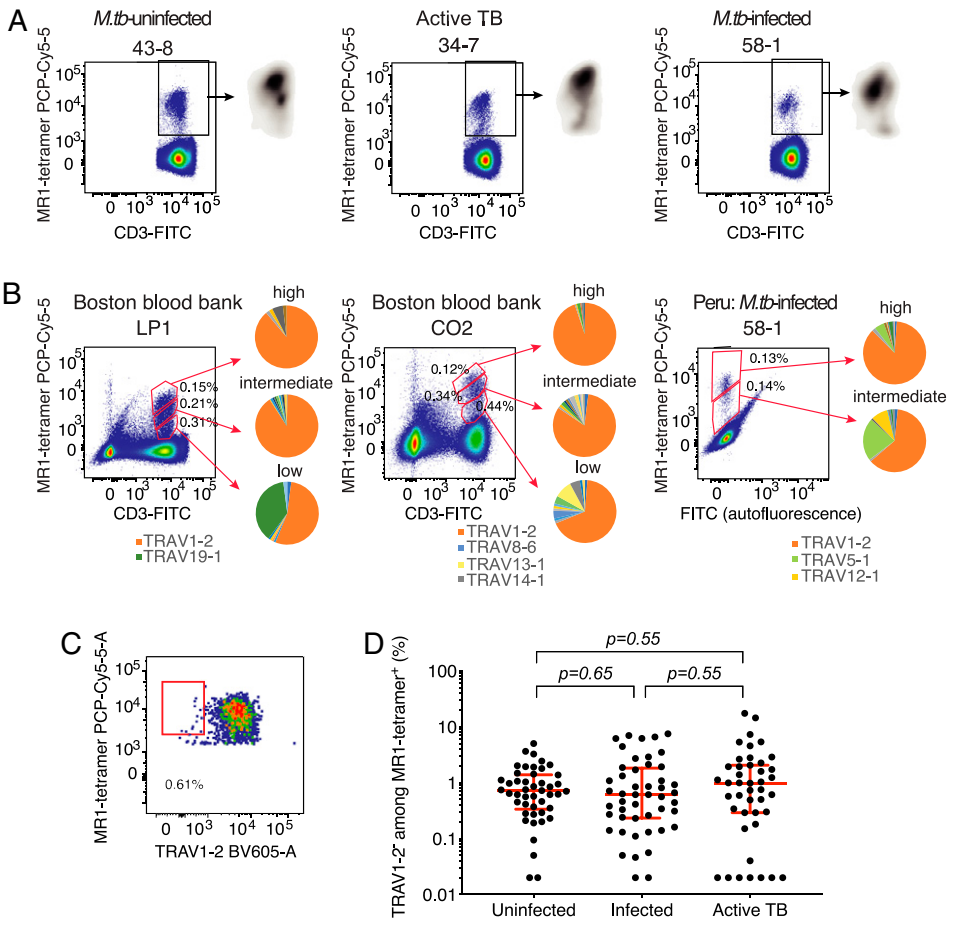

FIGURE 1. TRAV1-2$^-$ TCR sequences are enriched in MAIT cells with lower MR1 tetramer staining intensities. (**A**) Three examples of variable MR1 tetramer staining intensities by flow cytometry in pregated T lymphocytes in samples from uninfected, latent, and active TB participants. (**B**) Gating strategy for bulk-sorted MAIT cells with different 5-OP-RU–loaded MR1 tetramer staining intensities is shown. The pie charts depict the distribution of TCRα gene use from the different populations. (**C**) Gating strategy to identify TRAV1-2$^-$ MAIT cells among all MR1 tetramer binding cells is shown. (**D**) Proportions of TRAV1-2$^-$ MAIT cells among all MR1 tetramer binding cells in the Peruvian samples from healthy participants who are either uninfected or infected with *Mycobacterium tuberculosis* and patients with active TB are shown. Error bars denote medians and interquartile ranges.

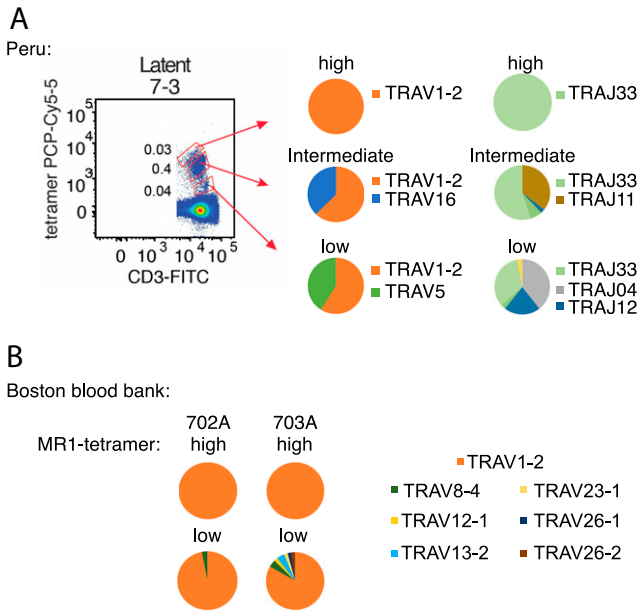

FIGURE 2. Single-cell sorted MAIT cells also show enrichment of TRAV1-2–negative TCR sequences. (**A**) Gating strategy shows single-cell sorted MAIT cells with different 5-OP-RU–loaded MR1 tetramer staining intensities in Peruvian latent sample no. 7-3. The pie charts depict the distribution of TCRα gene use from the different sorted populations. (**B**) Pie charts showing the distribution of TCRα V gene use in single-cell sorted MR1 tetramer^{high} and MR1 tetramer^{low} T cells from two additional healthy blood bank donors.

We sought to explain the discrepancy between the low frequencies of TRAV1-2$^-$ MAIT cells as determined by flow cytometry (Fig. 1D) and the higher frequencies of TRAV1-2$^-$ TCR α-chain sequences identified in sorted MAIT cells as determined by bulk TCR sequencing (Fig. 1C). Hence, we sorted single cells from populations with different MR1 tetramer binding levels from one Peruvian participant in whom we detected three clear MR1-tetramer binding levels (MR1-tetramer^{high}, MR1-tetramer^{int}, and MR1-tetramer^{low}), and we applied a previously described nested PCR protocol to cDNA amplified from each single cell (41) to determine the sequences of paired TCRα- and TCRβ-chains (Fig. 2A). Non–TRAV1-2 TCRα gene use was enriched in populations with lower MR1 tetramer binding, with 15 (37.5%) of 40 of the MR1-tetramer^{int} cells using TRAV16 and identical CDR3α nucleotide sequences and 14 (41.2%) of 34 of the MR1-tetramer^{low} cells using TRAV5, of which 13 had identical CDR3α nucleotide sequences, suggesting clonal expansion in vivo (Fig. 2A, Supplemental Tables II, III). Similarly, we detected TRAV1-2$^-$ TCRs from single-cell sorted MR1-tetramer^{low} populations from two healthy blood bank donors: 1 (3%) of 33 and 8 (16.7%) of 48, but none in MR1-tetramer^{high} counterparts (Fig. 2B). Furthermore, the atypical TRAJ33$^-$ joining regions were seen more frequently in low MR1 tetramer staining cells. Overall, these patterns from oligoclonal T cells (Fig. 2) matched those of polyclonal T cells (Fig. 1) and demonstrated more noncanonical gene use in TCRs among low MR1 tetramer staining T cells.

To validate the MR1 reactivity of these putative MAIT TCRs, we cotransfected HEK293T cells with pMIG vectors expressing CD3 and the paired TCRα and TCRβ sequences derived from three clones with non–TRAV1-2 TCR sequences (Fig. 3), which showed clear clonal expansion in samples analyzed with bulk (Fig. 1B) or single-cell (Fig. 2A) TCR sequencing methods. Next, we measured TCR binding to the 5-OP-RU–loaded MR1 tetramer (Fig. 3). We also transfected TCRα and TCRβ from a canonical MAIT TCR (TRAV1-2-TRAJ33) identified in the bulk-sorted MR1-tetramer^{high} cells as a positive control (Fig. 3). Cotransfected HEK293T cells coexpressed CD3 and TCRαβ on the cell surface (Fig. 4, left). The 5-OP-RU–loaded MR1 tetramer, but not the MR1 tetramer loaded with the nonagonist 6-FP–loaded MR1 tetramer, stained CD3$^+$ cells from HEK293T cells transfected with the TRAV1-2$^+$ TCR, as expected. However, the MR1 tetramers, loaded with either 6-FP or 5-OP-RU, did not bind cells expressing the TRAV1-2$^-$ TCRs identified in MR1-tetramer^{low} and MR1-tetramer^{int} populations (Fig. 4), despite the original detection of these TCR sequences in MR1 tetramer binding cells (Figs. 1 and 2).

To explain the lack of binding between these TRAV1-2$^-$ TCRs and 5-OP-RU–loaded MR1, we took a closer look at the TCRβ sequences. Unexpectedly, a single TCRβ sequence consisting of TRBV24-1-TRBJ2-5 with a unique CDR3 nucleotide sequence was detected in 10 out of the 15 TRAV16$^+$ single cells (Supplemental Table III). Interestingly, the same TCRβ nucleotide sequence (TRBV24-1-TRBJ2-5) was paired with the canonical MAIT TCRα TRAV1-2-TRAJ33 in three wells (Supplemental Table III). Because the PCRs were performed in multiplex format, we hypothesized that this particular T cell clone expressed two different functional TCRα chains but that only one of the PCR products dominated the PCR. Hence, to resolve the discrepancy, we reamplified the templates that initially gave rise to TRAV16-TRAJ11 PCR products, using only the TRAV1-specific forward primer, which captures the TCRα

Tetramer level	Donor	Sort Method	TCR	V-gene	Complementarity determining region (CDR3)	J-gene
Intermediate*	Peru Latent 7-3	Single cell (PCR)	TCRα	TRAV16	CALSGRRNSGYSTLTFGKG	TRAJ11
			TCRβ	TRBV24-1	CATSDLGTDQETQYFGPG	TRBJ2-5
low	Peru Latent 7-3	Single cell (PCR)	TCRα	TRAV5	CAEAPGGYNKLIFGAG	TRAJ4
			TCRβ	TRBV6-1	CASSEAAGTGGETQYFGPG	TRBJ2-5
low	Peru Latent 58-1	Bulk (Adaptive)	TCRα	TRAV5	CAESSLDNYGQNFVF	TRAJ26
			TCRβ	TRBV15	CATSKGSERSEQYF	TRBJ2-7
high	Peru Latent 58-1	Bulk (Adaptive)	TCRα	TRAV1-2	CAVPDSNYQLIW	TRAJ33
			TCRβ	TRBV601	CASSEGPGTGHQPQHF	TRBJ1-5

FIGURE 3. TCR sequences for additional validation by HEK293T cell transfection experiments. *Templates from this reaction were reamplified using TRAV1 forward primer only with TRAC reverse primer (Fig. 4).

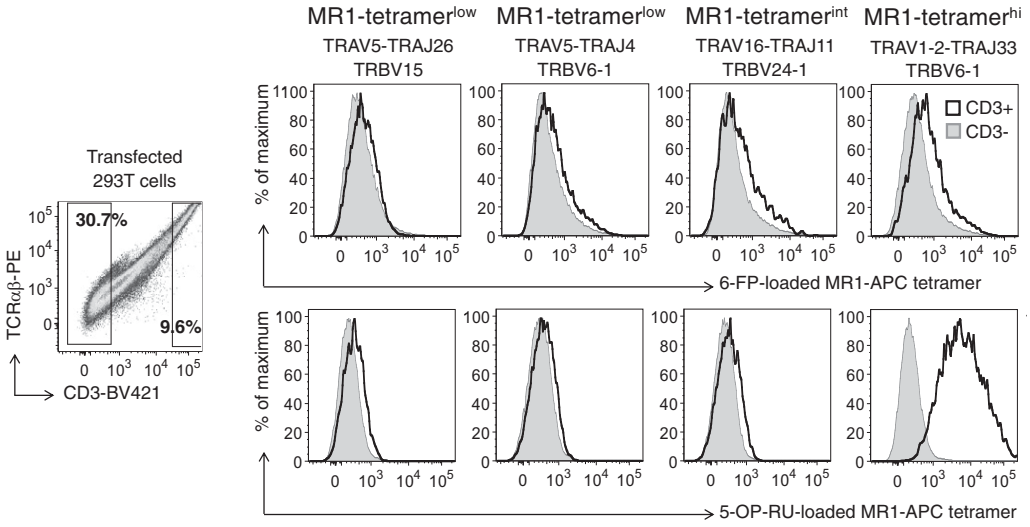

FIGURE 4. HEK293T cells transfected with non–TRAV1-2 TCRs from MR1 tetramer sorted cells do not bind MR1. The plots show flow cytometry of HEK 293T cells cotransfected with pMIG vectors expressing CD3 and paired TCRα and TCRβ sequences from TCR sequences identified in sorted MR1 tetramer binding populations with different MR1 binding intensities. The left panel shows gating of $CD3^-$ and $CD3^+$ populations used to derive the overlaid histograms are gated on $CD3^-$ (gray) and $CD3^+$ (black).

variable genes TRAV1-1 and TRAV1-2 only, as previously described (41). Using this approach, 10 out of the 15 templates initially giving rise to TRAV16-TRAJ11 sequences now gave rise to a PCR product that resulted in identical TRAV1-2-TRAJ33 sequences and paired with the same TRBV24-1-TRBJ2-5 TCRβ (Supplemental Table III). Although we initially interpreted these results as noncanonical TCRs binding to MR1, the data were more consistent with clonal expansion of a T cell coexpressing one TCR β-chain, a $TRAV1\text{-}2^+$ invariant MAIT TCR α-chain, and an additional noncanonical TCRα-chain. If only the canonical TCRα-chain binds MR1, the lower tetramer binding of these TCRs could be caused by competition of two different TCRα-chains with the same TCRβ-chain (TRBV24-1-TRBJ2-5), analogous to what has been described for NKT cells (49).

Finally, to reproduce our finding of dual TCRα expression on MAIT cells in an independent experiment, we analyzed paired TCR sequences in MR1 tetramer binding cells from different blood donors (55). Although in this experiment we sorted all MR1 tetramer binding T cells, including the MR1-$tetramer^{high}$ ones, we identified cells that coexpressed the canonical invariant $TRAV1\text{-}2^+$ TCR α-chain with a $TRAV1\text{-}2^-$ α-chain in PBMC samples from donors of different ages, as well as healthy spleen tissues of deceased donors (Fig. 5). Collectively, our study suggests that dual TCRα expression is common among MR1 tetramer binding MAIT cells in different human populations, tissue types, and disease states.

Discussion

In this study, we hypothesized that TCRs with decreased affinity for MR1-5-OP-RU would reveal new TCR motifs that may prefer MR1 ligands other than 5-OP-RU or correlate with TB disease. Our hypothesis was motivated by the reported expansion of diverse MAIT cell clonotypes after *Salmonella* challenge of humans who progress to disease (57) and the discovery of new Ag classes derived from the related *Mycobacterium smegmatis* (28). However, our search for new TCR motifs based on differential binding to the 5-OP-RU–loaded MR1 tetramer was confounded by the coexpression of two TCRα-chains in the same T cell. The phenomenon of dual TCRα coexpression has been described previously for MHC-restricted (58, 59) and CD1d-restricted (49) T cell subsets. Unlike the TCRβ locus, the TCRα counterpart is not subject to strict allelic exclusion, so dual TCRα expression is more common (60, 61). TCRα recombination is also known to occur simultaneously on both alleles to maximize productive TCRαβ recombination and diversity in the TCR repertoire (62).

The simplest explanation for the lower MR1 tetramer staining, which is also supported by these reports of dual TCRα-chains in other systems, is that the canonical MAIT TCR binds to MR1, but the competition of the two TCRα-chains to pair with the same pool of available TCRβ-chains reduces the MR1 tetramer binding intensity by reducing functional TCR expression on the cell surface. Hence, the hypothesis that these TCRs displayed preferential affinity

	Donor	Coded Donor name	TRAV	TRAJ	CDR3α	TRAV	TRAJ	CDR3α	TRBV	TRBJ	CDR3β
CHILD	F3045C37	CH3	6	9	CALEHTGGFKTIF	1-2	33	CAVSDSNYQLIW	20-1	2-1	CSARFASDYNEQFF
	F2072	CH6	1-2	33	CLVMDSNYQLIW	16	33	CALVDSNYQLIW	6-4	2-3	CASSPTSGGATDTQYF
TISSUE	SP234	SP2	1-2	33	CAAMDSNYQLIW	16	16	CALTVGDGQKLLF	28	2-1	CASSRSGEYNEQFF
	SP583	SP1	1-2	33	CAVRDSNYQLIW	8-2	11	CVVTLSPGYSTLTF	4-2/4-3	2-5	CASSPKTSGDIETQYF
OLDER ADULTS	DMC12	ED7	1-2	33	CAVRDSNYQLIW	10	3	CVVTLYSSASKIIF	20-1	1-6	CSARVGGPDSSPLHF
	DMC12	ED7	1-2	33	CAVMDSNYQLIW	13-2	8	CAQEGFQKLVF	29-1	2-1	CSVGSPGQGGNEQFF
	DMC15	ED8	1-2	33	CAVRDSNYQLIW	23/DV6	3	CAASRNQIIF	6-4	2-1	CASSDGSGGNEQFF
	DMC28	ED33	1-2	33	CAATDSNYQLIW	13-2	16	CAENSLLPISDGQKLLF	4-2	2-2	CASNQPTSGWTNTGELFF
ADULT	KK5	AD38	1-2	33	CAVMDSNYQLIW	13-2	27	CAENANNTNAGKSTF	20-1	2-1	CSARLRDNEQFF

FIGURE 5. Examples of dual TCRα-expressing MAIT cell clones detected in different sample types. Codes: AD, adult; CH, child; ED, elderly; SP, spleen.

to different MR1 Ags was not supported by the data. Importantly, our data point to a potentially common artifact in interpreting TCRα sequences, particularly from high-dimensional sequencing data (63). Because research focuses on identifying TCR motifs and Ag specificities of non–MHC-restricted DURT cells, including MAIT cells, new TCR motifs require systematic validation for MR1 specificity through TCR transfer, especially in light of the reported low frequency of TRAV1-2^{-} MAIT cells (13, 20, 33, 34).

We detected dual TCRs or lower tetramer staining in multiple donors studied with different methods in two laboratories. These unexpectedly common observations suggest that T cells with invariant TCRα-chains may even have a higher propensity for expression of two TCRα-chains than conventional MHC-restricted T cells. Several known aspects of conserved TCR gene use on MAIT cells are consistent with this hypothesis. First, innate T cells, including MAIT (24, 64), type I NKT cells (65), and germline-encoded mycolyl lipid-reactive T cells (66), express TCRs that mostly consist of genome-encoded segments and few N nucleotides (7, 67). TCRα recombination starts from the proximal Vα and Jα genes and proceeds outwardly toward distal Vα and Jα segments until a productive rearrangement occurs or the cell undergoes apoptosis (2). TRAV1-2 is the second most distal TCR Vα gene, located near the 5′ end of the TRA/D locus. The reliance of many invariant T cells on distal TCRα rearrangements involving TRAV1-2 raises the possibility that their thymic progenitors had extended survival windows during the CD4^{+}CD8^{+} double-positive thymocyte stage (68), when TCRα recombination took place. However, this hypothesis warrants additional studies. Importantly, the study emphasizes that validation of the MR1 reactivities of new TCR motifs identified in MAIT cells should be a standard practice in the field, because these TCRs may be artifacts of the dual expression of TCRα-chains.

We restricted the analysis in this study to MR1 tetramer binding MAIT cells, with the aim of identifying unique MAIT TCR motifs, and potentially novel antigenic specificities, as recently described (20, 28, 33–35). To our knowledge, a systematic analysis of the propensities of MHC-restricted T cells and DURTs for expression of dual TCRα-chains has not been formally conducted. Although our analyses were not intended to directly compare the frequency of dual TCRα expression in donor-unrestricted (innate-like) and MHC-restricted T cells, our study calls for caution when identifying new TCR motifs, particularly in DURTs. These DURTs have unique rules for recognition of nonpeptide Ags and Ag-presenting molecules (69), and hence functional validation of new TCR motifs is fundamental to this growing field. Collectively, our findings support that TRAV1-2 is the dominant TCRα gene used for recognition of MR1-5-OP-RU, consistent with the reported low frequency of alternative MAIT TCRα V-genes (13, 20, 33).

Disclosures

L.K.N., A.J.C., J.M., and J.R. are named coinventors on patents describing MR1 tetramers. The MR1 tetramer technology was developed jointly by J.M., J.R., and Prof. David Fairlie, and the material was produced by the National Institutes of Health Tetramer Core Facility as permitted to be distributed by the University of Melbourne. The other authors have no financial conflicts of interest.

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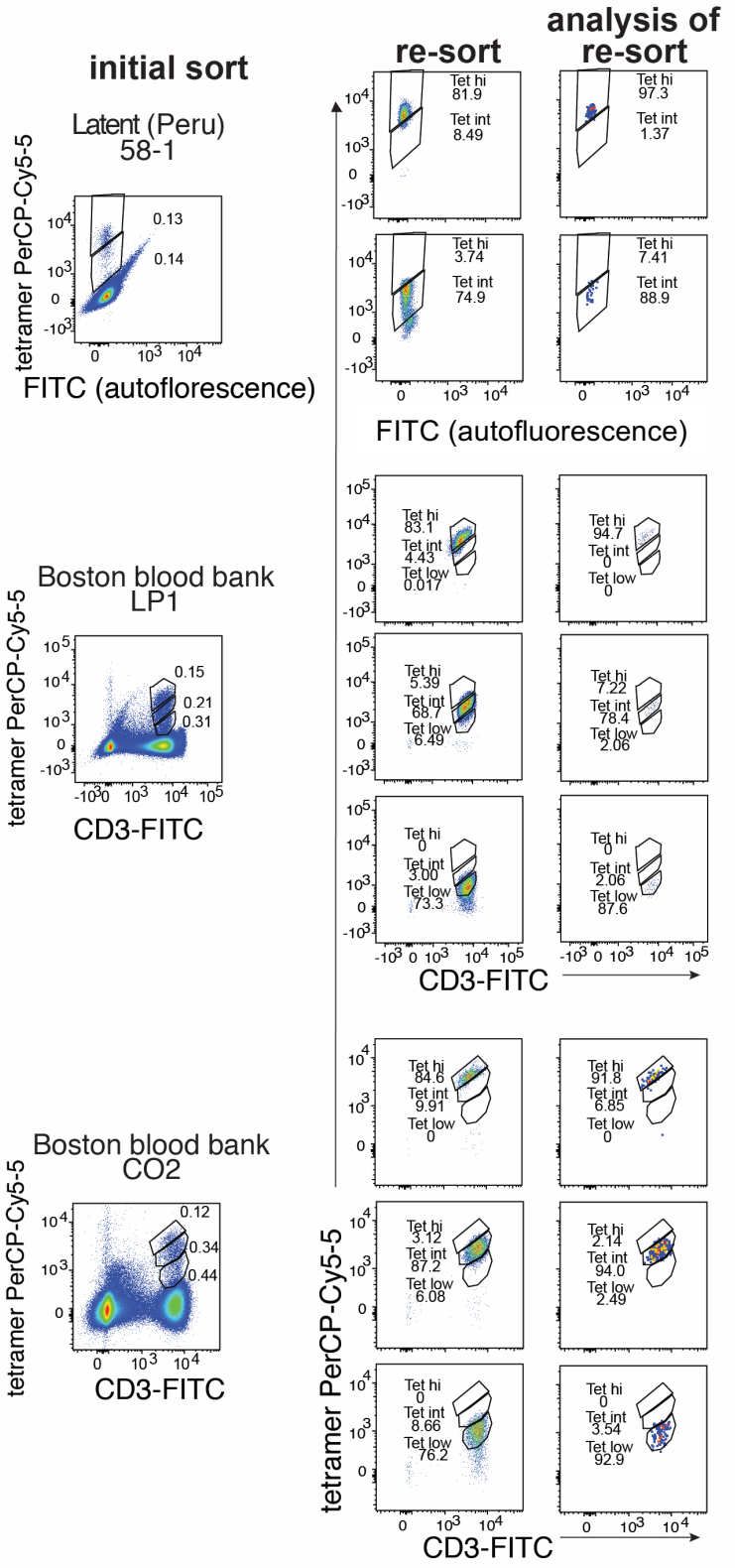

Supplementary Figure 1: Double sorting strategy for bulk sort of MAIT cells.

Left column: flow cytometry gates correspond to initial sort gates set to isolate MAIT cells with different MR1-tetramer staining intensities in samples from latently *Mtb*-infected Peruvian participants (top), or two healthy blood bank donors in Boston (middle and bottom). Gates in the middle column depict repeated sorting of the same population based on gates set in the initial sort. Right column gates correspond to analytical purity check of an aliquot of the double-sorted cells before DNA extraction.