Atypical TRAV1-2\textsuperscript{−} T cell receptor recognition of the antigen-presenting molecule MR1

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MR1 presents vitamin B\textsubscript{12}–related metabolites to mucosal associated invariant T (MAIT) cells, which are characterized, in part, by the TRAV1-2\textsuperscript{−} αβ T cell receptor (TCR). In addition, a more diverse TRAV1-2\textsuperscript{−} MR1-restricted T cell repertoire exists that can possess altered specificity for MR1 antigens. However, the molecular basis of how such TRAV1-2\textsuperscript{−} TCRs interact with MR1–antigen complexes remains unclear. Here, we describe how a TRAV12\textsuperscript{−} TCR (termed D462-E4) recognizes an MR1–antigen complex. We report the crystal structures of the unliganded D462-E4 TCR and its complex with MR1 presenting the riboflavin-based antigen 5-OP-RU. Here, the TRBV29-1 b-chain of the D462-E4 TCR binds over the F′-pocket of MR1, whereby the complementarity-determining region (CDR) 3\β loop surrounded and projected into the F′-pocket. Nevertheless, the CDR3\β loop anchored proximal to the MR1 A′-pocket and mediated direct contact with the 5-OP-RU antigen. The D462-E4 TCR footprint on MR1 contrasted that of the TRAV1-2\textsuperscript{+} and TRAV36\textsuperscript{+} TCRs’ docking topologies on MR1. Accordingly, diverse MR1-restricted T cell repertoire reveals differential docking modalities on MR1, thus providing greater scope for differing antigen specificities.

MR1 is a monomorphic antigen (Ag)–presenting molecule that captures and presents a broad range of small organic compounds. Several MR1 antigens have been described, including folate (vitamin B\textsubscript{9})–derived antigens, such as 6-formylpterin (6-FP) and its synthetic derivative acetyl-6-FP (Ac-6-FP) (1–3). Likewise, MR1 presents microbial metabolites of riboflavin precursors, which are derived from a wide range of riboflavin-producing microbes (1, 2, 4–6). These riboflavin derivatives include the ribityl-lumazines 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH) and 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe), and some extremely potent uracil-based antigens such as 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU). Furthermore, MR1 was recently found to display a diverse panel of small chemical scaffolds such as drugs, druglike, and drug metabolites, including analogues of the anti-inflammatory drugs aspirin and diclofenac (7). Interestingly, a recent report suggested that MR1 presents nonmicrobial tumor cell–derived metabolites, although the ligand identity is yet to be identified (8). Accordingly, MR1 exhibits sufficient plasticity to present a chemically diverse range of small hydrophobic organic molecules. These ligands are sequestered within the aromatic cradle residues of the MR1 A′-pocket, whereas no physiological ligands have been described to bind within the neighboring F′-pocket of MR1. Notably, many MR1 antigens form a Schiff base covalent bond with Lys-43 within the A′-pocket of MR1, an interaction that plays a prominent role in stabilizing the ligands inside the pocket and allowing MR1 to egress to the cell surface (1, 2, 6, 9, 10).

MR1 presents such antigens to defined populations of αβ and γδ T cells, with the majority of MR1-restricted T cells being mucosal associated invariant T (MAIT) cells (11–17). MAIT cells are an abundant innate-like T cell population in mammals and are mainly localized at the mucosa, liver, peripheral blood, and lung (12, 18–25). Human MAIT cells express a semi-invariant T cell receptor (TCR), comprised of an α-chain of an invariant TRAV1-2 gene frequently recombined with TRAJ33, TRAJ12, or TRAJ20 segments, paired with a biased set of TRBV genes including TRBV6 and TRBV20 (12). Much of the diversity in the classical MAIT TRAV1-2\textsuperscript{+} TCR repertoire is found within the complementarity determining region (CDR) 3\β loop, which can fine-tune MR1–antigen recognition (3, 14, 26, 27). Riboflavin-related antigens can activate MAIT cells with varying degrees of potency, with 5-OP-RU representing the most potent MAIT agonist described to date. In contrast, folate-based ligands do not activate MAIT cells in general (1, 2). Structural investigations show that these TRAV1-2\textsuperscript{+} MAIT TCRs dock similarly atop the A′-pocket of MR1, where the ribityl 2′-OH group of the stimulating ligands forms an

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“interaction triad” with the MR1–Tyr-152 and the evolutionary conserved Tyr-95α from the CDR3α loop. Disruption of this interaction triad has been linked to a loss of potency for known MAIT cell antigens (2, 3, 6, 28, 29).

A diverse population of MR1-restricted αβ T cells that do not utilize the conventional TRAV1-2 chain, called atypical TRAV1-2–MR1-restricted T cells, have been identified through MR1-Ag tetramer staining of human peripheral blood mononuclear cells (14, 16, 30, 31). Moreover, other identified TRAV1-2–MR1-restricted T cells exhibit differing reactivities to folate, riboflavin, MR1 autoreactivity, or reactivities to unknown ligands that appear to be a function of the αβTCR gene usage (5, 15, 16, 19, 30–33). Recent studies identified TRAV36+ MR1–5-OP-RU reactive T cell clones that have been detected in various unrelated human donors, suggesting a public TRAV1-2–MR1-reactive TCR repertoire (16, 30). The crystal structure of one of these TCRs, MAV36 (TRAV36/TRAJ34–TRBV28), complexed with MR1–5-OP-RU was determined and showed a more central docking of MAV36 TCR on the MR1 cleft than for TRAV1-2+ MAIT TCRs (30). Other TRAV1-2+ MR1-restricted T cell clones have been described (31) and one of these clones (D462–E4) carries the TRAV12/2–TRAJ39 paired with TRBV29-1/TRBJ01-05 gene. Interestingly, the D462–E4 T cell clone showed a distinct pattern of ligand and microbial recognition in detecting infection by riboflavin-producing microbes and certain riboflavin-auxotroph Streptococcus pyogenes microbes in a TCR-dependent manner (31). Accordingly, this TCR could recognize specific riboflavin-based, and unidentified non-riboflavin based, ligands (5, 31). How this particular TCR interacted with MR1 remained unknown.

To expand our molecular understanding of how human TRAV1-2–TCRs recognize MR1, we determined the crystal structure of unliganded D462–E4 TCR and its complex with the MR1–5-OP-RU adduct. Our data reveal that the TRAV12–TRBV29-1 TCR adopts a distinctly different molecular footprint atop MR1 compared with TRAV1-2+ MAIT TCR-MR1-Ag and MAV36+ TCR-MR1-Ag ternary complexes. Accordingly, we provide a new understanding of how diverse αβTCR usage manifests in differing MR1 docking geometries.

Results
Characteristics of MR1-restricted TRAV12-2/TRBV29-1 T cell clone

Previously, we identified T cell clones that bound to the MR1–5-OP-RU tetramer, yet expressed atypical TRAV1-2–MR1-restricted TCRs; one of these T cell clones is referred to as D462–E4 (31). Both D462–E4 and MAIT TRAV1-2+ T cell clones expressed CD8α and stained with the MR1–5-OP-RU tetramer to similar levels of intensity, but not to the MR1–6-FP tetramer (Fig. 1a). TCR sequencing of the D462–E4 T cell clone showed that the TCR α-chains and TCR β-chains were composed of TRAV12-2/TRAJ39-01 and the TRBV29-1/TRBJ01-05 gene segments, respectively (Table 1). The D462–E4 TCR does not contain the critical Tyr-95α residue from the CDR3α loop, which interacts with 5-OP-RU in the TRAV1-2+ TCR–MR1–5-OP-RU ternary structures (2).

To investigate the MR1-restricted function and activation potency of the D462–E4 T cell clone, we used a plate-bound tetramer ELISPOT (tetraSPOT) assay to measure the IFN-γ release upon T cell activation. Here, both MR1–5-OP-RU and MR1–6-FP tetramers were immobilized onto the plate and IFN-γ production of both TRAV1-2– (D462–E4) and MAIT TRAV1-2+ (D426–G11) T cell clones was quantified against titrated tetramer concentrations (Fig. 1b). Both TRAV1-2– and TRAV1-2+ T cell clones responded to MR1–5-OP-RU (not to MR1–6-FP) and showed similar maximal efficacy (IFN-γ release) (Fig. 1b). The antigen concentration of half-maximal response (EC_{50}) of MR1–5-OP-RU for the atypical MR1-restricted D462–E4 T cell clone was 0.11 nM, whereas the EC_{50} for the control MAIT cell clone was 0.002 nM. The response to MR1–5-OP-RU by D462–E4 was inhibited by an MR1-blocking antibody, confirming MR1 restricted T cell function (Fig. 1c).

Collectively, the D462–E4 TRAV1-2– TCR displayed similar maximal efficacy, but lower TCR avidity and potency of 5-OP-RU antigen dose, compared with the TRAV1-2+ MAIT TCRs.

D462–E4 TCR exhibits moderate affinity for MR1–5-OP-RU

To establish how the D462–E4 TRAV1-2– TCR binds to the MR1 molecule, we expressed the extracellular domains of the D462–E4 TCR as inclusion bodies in Escherichia coli, and the D462–E4 TCR was refolded into its native conformation and purified as summarized under “Experimental procedures.” Next, we conducted surface plasmon resonance (SPR) experiments to compare the specificities and binding affinities of both of A-F7 (TRAV1-2–TRBV6-1) and D462–E4 (TRAV12–TRBV29-1) TCRs against MR1 presenting the 5-OP-RU, 6-FP, or Ac-6-FP ligands (Fig. 2). As previously reported, A-F7 TCR recognized MR1–5-OP-RU with high affinity (K_D = 2.2 ± 0.4 μM) and showed weak binding to MR1–Ac-6-FP and MR1–6-FP proteins (K_D = 88.2 ± 20 and 101.2 ± 30 μM, respectively).

Consistent with the activation data (Fig. 1), whereas the D462–E4 and A-F7 TCRs exhibited similar affinity against MR1–Ac-6-FP and MR1–6-FP (∼80–100 μM), the affinity of the D462–E4 TCR for MR1–5-OP-RU was a 7-fold lower affinity than that of the A-F7 TCR (K_D = 16.3 ± 1.1 μM). Collectively, both A-F7 and D462–E4 TCRs were riboflavin-based ligand reactive TCRs, yet they exhibited differing affinities toward MR1–5-OP-RU.

Overview of the D462–E4 TCR–MR1–5-OP-RU ternary complex

Next, we determined the crystal structure of the D462–E4 TCR–MR1–5-OP-RU ternary complex to 2.9 Å resolution (Table 2 and Figs. 3 and 4). The D462–E4 TCR docked orthogonally atop the MR1 antigen binding cleft (Fig. 3, d and e). The buried surface area (BSA) at the interface between D462–E4 TCR and MR1 was ∼1070 Å², a value that falls within the range of BSA values of TRAV1-2+ MAIT TCR-MR1-Ag complexes (1050–1200 Å²) (6, 29). Although the α- and β-chains of MR1-restricted TCRs contributed almost equally to the BSA of the interfaces of most TCR-MR1-Ag complexes, the α- and β-chains of the D462–E4 TCR contributed to ∼39 and 61%, respectively, with this difference being attributed to a differing MR1 docking modality of the D462–E4 TCR (Fig. 3).
The electron density of 5-OP-RU and residues at the D462-E4 TCR/MR1 molecular interface was unambiguous. Here, the 5-OP-RU ligand in the D462-E4 TCR-MR1–5-OP-RU complex was typically sequestered within the MR1 A9-pocket and formed a Schiff base covalent bond with MR1–Lys-43 (Fig. 4c). The interactions made with the ligand uracil ring, as well as the 29- and 39-OH groups of the ribityl moiety of 5-OP-RU were conserved compared with the TRAV1-2 TCR-MR1–5-OP-RU complexes. Nevertheless, the 49-OH and 59-OH groups of the ribityl chain in the D462-E4 TCR-MR1–5-OP-RU structure exhibited two alternate conformations within the pocket, each having 50% occupancy (Fig. 4c, d, and e). This resulted in greater surface exposure and accessibility of the ribityl moiety, where one conformation was oriented toward the F-pocket of MR1 and contacted the CDR3b loop. Further, structural modifications within the MR1 antigen binding cleft were observed upon D462-E4 TCR binding compared with the TRAV1-2+ TCR-MR1 complexes (Fig. 4). Here, part of the MR1 a2-helix (Trp-143 to Asn-155) was slightly displaced (root mean square deviation (rmsd), 0.53 Å) (Fig. 4b), as a result of its interactions with the CDR3b loop compared with the TRAV1-2+ TCR-MR1 complexes (described below). Collectively, this reflects the adaptability of the MR1 Ag-binding cleft, and its sequestered ligand, upon ligation with the MR1-reactive TCRs.

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The electron density of 5-OP-RU and residues at the D462-E4 TCR/MR1 molecular interface was unambiguous. Here, the 5-OP-RU ligand in the D462-E4 TCR-MR1–5-OP-RU complex was typically sequestered within the MR1 A’-pocket and formed a Schiff base covalent bond with MR1–Lys-43 (Fig. 4c). The interactions made with the ligand uracil ring, as well as the 2’- and 3’-OH groups of the ribityl moiety of 5-OP-RU were conserved compared with the TRAV1-2+ TCR-MR1–5-OP-RU complexes. Nevertheless, the 4’-OH and 5’-OH groups of the ribityl chain in the D462-E4 TCR-MR1–5-OP-RU structure exhibited two alternate conformations within the pocket, each having 50% occupancy (Fig. 4c, d, and e). This resulted in greater surface exposure and accessibility of the ribityl moiety, where one conformation was oriented toward the F’ pocket of MR1 and contacted the CDR3b loop. Further, structural modifications within the MR1 antigen binding cleft were observed upon D462-E4 TCR binding compared with the TRAV1-2+ TCR-MR1 complexes (Fig. 4). Here, part of the MR1 a2-helix (Trp-143 to Asn-155) was slightly displaced (root mean square deviation (rmsd), 0.53 Å) (Fig. 4b), as a result of its interactions with the CDR3b loop compared with the TRAV1-2+ TCR-MR1 complexes (described below). Collectively, this reflects the adaptability of the MR1 Ag-binding cleft, and its sequestered ligand, upon ligation with the MR1-reactive TCRs.

**Atypical TRAV1-2+ TCR-MR1 and TRAV1-2+ MAIT TCR-MR1 contacts are disparate**

All MR1-restricted αβ TCRs docked atop the MR1 antigen binding cleft whereby the TCRs α- and β-chains resided over the α2- and α1-helix of MR1, respectively (29). The D462-E4 TCR also docked similarly atop the MR1 antigen binding cleft,
but more toward the MR1 F'-pocket (Fig. 3d), whereas the TRAV1-2 MAIT TCRs sat orthogonally to the A'-pocket of MR1 (Fig. 3, a–c) (29). Here, the center of gravity of the D462-E4 TCR β-chain was displaced ~5 Å toward the MR1 F'-pocket compared with the β-chain of TRAV1-2 MAIT TCR-MR1 complexes. Nevertheless, the TRAV12-2 α-chain adopted a similar position to the TRAV1-2 chain of MAIT TCRs (Fig. 4, a and b). Further, the D462-E4 TCR was rotated ~11° compared...
The CDR1α, CDR2α loops and the Vα framework region sat close to the α2-helix and contributed only 6%, 10 and 7% to the complex BSA, respectively. Here, Gln32α of CDR1α formed hydrogen bonds with MR1-Asn155 and Glu160. While Tyr52α from the CDR2α loop extensively interacted with MR1-His148, Tyr152 and Asn155 (Fig. 5c and Table 3). Altogether, the TRAV12-2 TCR α-chain interactions was distinct from that of the TRAV1-2+ TCR footprint on MR1.

Role of TRBV29-1 β-chain and its CDR3β loop in MR1 recognition

The TRBV29-1 β-chain contributed almost two thirds of the interface between D462-E4 TCR and MR1 (61% BSA). The Vβ framework region, CDR1β, and CDR2β extended above the α1-helix of MR1 and contributed 7%, 10 and 7% BSA, respectively, to the binding interface. The Vβ framework and the CDR1β loop bound MR1 mainly by hydrophobic interactions, whereas Asn51β from the CDR2β loop H-bonded to MR1–Gln64 (Table 3 and Fig. 5d).

Surprisingly, the CDR3β loop wedged between the helical jaws of the MR1 cleft, surrounding the F’-pocket, although proximal to the bound antigen in A’-pocket (Fig. 5, e and f). Notably, the CDR3β loop was the principal contributor to the D462-E4 TCR-MR1 interface, providing ~37% of the BSA and thus played a prominent role in the interaction and recognition of D462-E4 TCR by MR1. Moreover, the CDR1β loop played an important role in consolidating the CDR3β loop in a fixed configuration atop the F’-pocket of MR1 by forming H-bonds between Met32β and Thr31β from the CDR1β, and the Gly97β and Asp99β residues from the CDR3β, respectively.

Here, the 99 Asp-Ser-Leu-Ile-Gly-Asn104 segment had a dominant role in mediating CDR3β contacts with MR1 (Table 3 and Fig. 5f). The backbone of this peptide folded upon itself to produce a structural “hairpin turn” motif by forming three intramolecular H-bonds (Fig. 5f). This hairpin turn of CDR3β capped the F’-pocket and extensively interacted with various residues of MR1 α1- and α2-helices. Interestingly, the CDR3β Asp99β and Asn104β residues occupied the space between the A’- and F’-pockets, and their side chains were oriented toward the bound antigen in the A’-pocket. Asp99β and Ser100β formed various hydrophobic contacts with the Trp69, Met72, Val75 and Glu76 of MR1 α1-helix. In addition, the backbone carbonyl of Leu101β formed H-bonds with the side chains of MR1–Arg79 and –Trp143 of α1- and α2-helices, respectively. On the other side of the hairpin turn, Ile102β, Gly103β, and Asn104β residues extensively interacted with MR1–Ala142, –Trp143, –Asn146, and –Glu149 residues from the MR1 α2-helix (Table 3 and Fig. 5f). Accordingly, the CDR3β loop play a prominent role in the interaction and recognition of the D462-E4 TCR by the MR1 molecule.

Divergent recognition of riboflavin derivatives by MR1-restricted TCRs

The D462-E4 TRAV1-2− TCR presented Asn97α in an equivalent position to the Tyr95α of TRAV1-2+ TCRs. However, the entire CDR3α loop of D462-E4 TCR and its Asn97α were positioned >6 Å from 5-OP-RU, with no direct or water-
Figure 3. Structural comparison of ternary complexes of TRAV1-2* and TRAV1-2* TCRs with MR1-5-OP-RU. Crystal structures of ternary complexes. a–c, A-F7 (TRAV1-2/TRBV6-1) TCR-MR1-5-OP-RU (PDB ID: 4NQC). d–f, D426-E4 (TRAV12-2/TRBV29-1) TCR-MR1-5-OP-RU. g–i, MAV36 (TRAV36/TRBV28) TCR-MR1-5-OP-RU (PDB ID: 5D7L). a, d, and g, top panels, depict ribbon diagrams of the ternary complexes and pie charts representing the contribution of each TCR segment toward the MR1-5-OP-RU complex. The MR1 and β2-microglobulin molecules are colored white and pale cyan, respectively, and 5-OP-RU is presented as green sticks. A-F7 TCRα, olive; A-F7 TCRβ, orange; D426-E4 TCRα, blue; D426-E4 TCRβ, light-pink; MAV36 TCRα, violet-purple; MAV36 TCRβ, light brown. b, e, and h, middle panels, show the TCRs and their CDR loops docking into MR1. The center of mass of the respective TRAV and TRBV variable domains are shown as a sphere colored consistent with chain colors in the upper panels. The CDR loops are colored as follows: CDR1α, teal; CDR2α, sky-blue; CDR3α, light-blue; frameworks of α-chain, dark-green; CDR1β, maroon; CDR2β, violet; CDR3β, yellow-orange; frameworks of β-chain, dark-gray. c, f, and i, lower panels, illustrate the TCR footprints on the molecular surface of MR1-5-OP-RU. The atomic footprint is colored according to the TCR segment making contact.
based contacts with ligand observed. Nevertheless, the side chain of MR1–Tyr-152 was H-bonded to the Asn-97α, as well as to the 5′-OH of both 5-OP-RU conformations (Fig. 6a). Asp-99β from the CDR3β loop oriented toward the A′-pocket of MR1 antigen binding cleft, forming a H-bond (2.9 Å) with the 5′-OH of one ribityl conformation (Fig. 6a).

The usage of the TRAV1-2 gene in MR1-restricted TCRs facilitates a consistent docking mode atop the MR1. This docking mode maintains the evolutionary conserved Tyr-95α from the CDR3α loop in a conserved location, so that it protrudes deeply into the A′-pocket of the MR1-Ag-binding cleft. This enabled H-bonding between Tyr-95αOH and the 2′-OH of the ribityl moiety of the antigen, as well as MR1–Tyr-152 (Fig. 6b) (2, 3, 28). Indeed, Tyr-95α, Tyr-152 and 5-OP-RU formed an interaction triad that has been shown to play a prominent role in TRAV1-2+ TCR recognition of MR1-presenting riboflavin derivatives (6). The structure of the MAV36 TCR-MR1–5-OP-RU complex showed that Asn-29α from the CDR1α loop was vital for recognition of 5-OP-RU, by forming a direct H-bond with the 2′-OH of the ribityl moiety (Fig. 6c) (30). Notably, the CDR3α Tyr-95α of TRAV1-2+ TCRs and the CDR1α Asn-29α of MAV36 TCR were closely aligned in a position that enabled their side chains to interact with the 2′-OH of 5-OP-RU in a convergent recognition mechanism of the ligand. In contrast, Asp-99β from CDR3β loop of D426-E4 TCR interacted with the terminal 5′-OH group of 5-OP-RU. This reveals differential recognition of riboflavin-based metabolites by diverse MR1-restricted TCRs.

Figure 4. MR1 conformational changes upon interactions with D426-E4 TCR. a, comparison of TRAV1-2+ D426-E4 ternary complex relative to the TRAV1-2+ TCR docking positions. Arrows illustrate TCR rotation around the center of mass of the MR1, as well as displacement of β-chain along the MR1 binding cleft. The colors of the TCR chains are consistent with Fig. 3a, b, superposition of the CDR loops of A-F7 (yellow) and D426-E4 (green) TCRs sitting atop MR1. c, working (2Fo − Fc) map of 5-OP-RU inside MR1 pocket, showing two alternate conformations of the antigen. d, comparison of the MR1 antigen binding pocket and the position of Tyr-152 in both of A-F7 (yellow) and D426-E4 (green) ternary structures. e, interactions of 5-OP-RU and the residues of MR1 A′ portal in the D426-E4 TCR-MR1-5-OP-RU structure.
To investigate the TCR conformational changes upon D462-E4 TCR recognition of MR1–5-OP-RU, we determined the crystal structure of the D462-E4 TCR in its unliganded state (Table 2 and Fig. 7a) and compared it to the structure of the D462-E4 TCR-MR1–5-OP-RU ternary complex. There was little displacement (rmsd, 0.94 Å) of the variable domain of the α-chain (Vα) and all CDRα loops upon binding to MR1 molecule, but with minimal changes to their side chain locations (Fig. 7b). Interestingly, with the exception for CDR3β, no appreciable movement or changes within the CDRβ loops were observed after MR1 engagement, suggesting that little conformational adjustments of the CDRβ loops were required for recognition of MR1 (Fig. 7, c and d). Indeed, the tip of the CDR3β loop slightly moved (rmsd 0.3 Å) to make favorable contacts with the F’-pocket of MR1. Collectively, the relatively rigid D462-E4 TCR recognized MR1 molecule by a lock and key mechanism.

Discussion

MR1 molecules present metabolite-related antigens to diverse populations of T cells, which are biased toward the usage of the TRAV1-2 gene (TRAV1-2⁺) frequently paired with TRBV6 and 20. We and others have previously described diverse populations of TRAV1-2⁺ MR1-restricted T cells in humans that use a broad range of TRAV, TRAJ, TRBV, and TRBJ genes including TRAV19⁺, TRAV36⁺, and TRAV12-2⁺ subsets among others (8, 16, 26, 30, 31). These TRAV1-2⁺ TCRs can exhibit altered specificity toward microbial and/or
nonmicrobial ligands associated with MR1 (5, 15, 32). It is now established that the evolutionary conserved Tyr-95\alpha from the CDR3\alpha loop of TRAV1-2\alpha TCRs forms an interaction triad with antigen 5-OP-RU and MR1 Tyr-152, and that this plays a prominent role in TRAV1-2\alpha TCR recognition of the riboflavin-related antigens (2, 3, 6). For the CDR1\alpha loop of the MAV36 TCR (TRAV36/TRAJ34), Asn-29\alpha directly interacts with 5-OP-RU and MR1 Tyr-152 in a manner analogous to Tyr-95\alpha (30). As such, the TRAV1-2\alpha and MAV36\alpha TCRs showed convergent mechanisms for recognition of riboflavin-related metabolites.

To examine how other TCRs might bind to MR1, we investigated here the recognition of a TRAV1-2\alpha TCR (TRAV12-2\alpha–TRBV29-1). This D462-E4 TCR was able to recognize riboflavin-related as well as non-riboflavin-related MR1-bound ligands produced by S. pyogenes microbes (31), which suggested novel D462-E4 TCR docking and recognition strategies for metabolite antigens. Here, we found that this relatively rigid D462-E4 TCR docks centrally onto the MR1 antigen binding cleft but tilts toward and fully caps the F’ portal. Minor changes occurred for the TCR upon complexation with MR1. However, adaptable conformational changes within both the MR1-binding pocket and the 5-OP-RU ligand were observed upon TCR engagement.

Despite the usage of different TRAV genes, both TRAV12-2 of D462-E4 TCR and TRAV1-2 of A-F7 TCRs exhibit similar docking positions atop the A’-pocket of MR1, yet TRAV12-2 contributed much less to the MR1-binding interface (415 Å²) compared with TRAV1-2 (580 Å²). Further, none of the TRAV12-2 loops make contacts with the ligand. Indeed, the \beta-chain of TRBV29-01, in particular the CDR3\beta loop, plays a prominent role in D462-E4 recognition of the MR1-5-OP-RU complex. Here, the CDR3\beta loop extensively interacts with the empty F’-pocket of the MR1 antigen binding cleft. Moreover, Asp-99\beta from the CDR3\beta loop of the D462-E4 TCR interacted with the 5’-OH group of 5-OP-RU. As such our data demonstrate that the diverse MR1-reactive T cell repertoire exhibits varied docking strategies that enable divergent mechanisms to be used to recognize antigens bound to MR1.
**Experimental procedures**

**Cells and flow cytometry**

MR1 restricted T cell clones were expanded using anti-CD3 and IL-2 and maintained as previously described (21, 31). Prior to sequencing TCRs, D462-E4 T cell clone was FACS purified after staining with LIVE/DEAD Fixable Dead Cell Stain Kit (Life Technologies) and an antibody for CD3. TRA and TRB TCR sequencing was performed by immunoSEQ (Adaptive Biotechnologies). T cell clones were stained with MR1 tetramers (NIH Tetramer Core) for 1 h at room temperature. Cells were then washed with PBS 1% FBS buffer and stained with LIVE/DEAD Fixable Dead Cell Stain Kit (Life Technologies) and surface stained with antibodies specific for CD3, CD4, CD8, and TRAV1-2 for 20 min at 4°C. Samples were fixed with 4% paraformaldehyde for 15 min and washed with PBS 1% FBS buffer, and acquisition was performed using a Fortessa flow cytometer with FACSDiva software (BD Biosciences). All flow cytometry data were analyzed using FlowJo software (Treestar) and Prism (GraphPad).

**ELISPOT assays**

For the plate-bound tetramer ELISPOT (tetraSPOT) assay, ELISPOT plates were coated with an anti–IFN-γ antibody, as described previously (Ref. 31). At the time of coating, MR1 tetramers (NIH Tetramer Core) were added to wells at concentrations between 0 and 5 nM per well. After overnight incubation at 4°C, ELISPOT plates were washed three times with sterile PBS and then blocked with RPMI 1640 + 10% human serum for 1 h. MAIT cell clones (2 × 10⁵) were added to wells overnight. IFN-γ ELISPOTs were enumerated following development as described previously (Ref. 31). For the MR1-blocking tetraSPOT assays, MR1 blocking antibody (clone 26.5) or isotype control (mouse IgG2a) was added at 5 mg/ml in additional wells with the MR1-5-OP-RU tetramer.

**Expression and preparation of denatured inclusion bodies of MR1 and TCRs proteins**

Genes encoding soluble human MR1, β2m, TRAV1-2/TRAJ33, TRAV12-2/TRAJ39, TRBV6-1, and TRBV29-1...
chains were expressed for 4 h in BL21 E. coli after induction with 1 mM isopropyl β-D-1-thiogalactopyranoside as described previously (1, 28). E. coli were pelleted and resuspended in a buffer containing 50 mM Tris-HCL, 10 mM DTT, 25% (w/v) sucrose, and 1 mM EDTA, pH 8.0. Then, the inclusion body protein was extracted by lysis of bacteria in a lysis buffer containing 50 mM Tris-HCL, pH 8.0, 10 mM DTT, 1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate, 100 mM NaCl, 5 mM MgCl₂, and 1 mg DNase I, and 2 mg lysozyme per liter of starting culture. Inclusion body protein was homogenized with a polytron homogenizer, then centrifugation and washing inclusion body protein sequentially, with 1) a buffer containing 50 mM Tris, pH 8.0, 1 mM DTT, 0.5% Triton X-100, 100 mM NaCl, and 1 mM EDTA and 2) a buffer containing 50 mM Tris, pH 8.0, 1 mM DTT, and 1 mM EDTA. Inclusion body was next resuspended in a buffer containing 20 mM Tris, pH 8.0, 1 mM DTT, 8 M urea, and 0.5 mM EDTA, and after centrifugation the supernatant containing solubilized, denatured inclusion body protein was collected and stored at −80°C.

**Refolding and purification of MR1 and MR1-restricted TCRs**

A-F7 (TRAV1-2/TRBV6-1) MAIT TCR and human MR1-β2m-6-FP, Ac-6-FP, and 5-OP-RU were refolded in the cold room overnight in the presence of 0.1 M Tris, pH 8.5, 5 M urea, 2 mM EDTA, 0.4 M l-arginine, 0.5 mM oxidized GSH, and 5 mM reduced GSH as described previously (1, 28). Similarly, D462-E4 (TRAV12-2/TRBV29-1) TCR was refolded at 4°C in 1 liter of refold buffer but with three injections of 50 mg of both α TRAV12-2 and β TRBV29-1 chains over 3 days. 5-OP-RU ligand was generated in situ in water from the addition of 5-A-RU and methylglyoxal as previously described (Ref. 2). Next, the refolded MR1-β2m-Ag and TCR proteins were then dialyzed against three changes of buffer containing 10 mM Tris-HCl, pH 8, over 24 h and purified by sequential crude DEAE anion exchange, size exclusion chromatography then HiTrap-Q HP anion exchange chromatography. The purity of the resulting protein was assessed using SDS-PAGE and further quantified by A280 absorbance.

**Surface plasmon resonance measurements and analysis**

All SPR experiments were conducted at 25°C, in duplicate (n = 3), on a BIAcore 3000 instrument using HBS buffer (10 mM HEPES-HCl, pH 7.4, 150 mM NaCl, and 0.005% surfactant P20) as described previously (28). Biotinylated MR1-β2m-Ag was immobilized on SA-Chip (GE Healthcare) with a surface density of ∼2000 response units. Various concentrations (0–150 μM) of two MR1-restricted TCRs, A-F7 (TRAV1-2/TRBV6-1) and D462-E4 (TRAV12-2/TRBV29-1), were injected over the captured MR1-β2m-Ag at 5 μl/min and equilibrium data were collected. The final response was calculated by subtracting the response of the blank flow cell alone from the TCR-MR1-β2m-Ag complex. The SPR sensorgrams, equilibrium curves, and steady state K_D values (μM) were prepared using GraphPad Prism 7.

**Crystallization, structure determination and refinement**

Purified D462-E4 TCR was mixed with MR1-5-OP-RU in a 1:1 molar ratio at a concentration of 8–10 mg/ml and kept on ice for 2 h. Crystals of D462-E4 TCR-MR1-5-OP-RU ternary complex were grown by hanging-drop vapor diffusion method at 20°C, with a reservoir solution containing 18–26% PEG3350, 100 mM Bis-Tris Propane (pH 8.0–8.6) and 200 mM sodium bromide. Similarly, the binary D462-E4 TCR crystals were obtained at concentration of 5 mg/ml with a precipitant consisting of 16–24% PEG3350, 100 mM Bis-Tris Propane (pH 6.0–6.6), and 200 mM sodium fluoride. Both binary and ternary complexes crystals grew within 5–10 days, and then were flash frozen in liquid nitrogen after quick soaking in reservoir solution with 10–12% glycerol for cryo-protection. X-ray diffraction data were collected at 100 K on the Australian Synchrotron at MX2 beamline (34). Diffraction images were processed using XDS (35) and programs from the CCP4 suite (36) and Phenix package (37). The D462-E4 TCR crystal structure was determined by molecular replacement using PHASER program (38) using an A-F7 TCR as search model (PDB ID: 4L4T). Next, we used the solved binary D462-E4 TCR and MR1 coordinates (PDB ID: 4L4T) as search model to solve the ternary structure of D462-E4 TCR-MR1-5-OP-RU complex. Manual model building was conducted using COOT (39), followed by iterative rounds of refinement using Phenix.refine (37). The Grade Webserver and Phenix tools were used to build and to generate ligand restraints. The models were validated using MolProbity (40) and the final refinement statistics are summarized in Table 2. All molecular graphic representations were generated using PyMOL Molecular Graphics System, Version 1.8, (Schrödinger, LLC, New York, NY). The buried surface area is calculated using ArealMol program, the contacts generated by the Contact program, both from the CCP4 suite (36).

**Data availability**

The coordinates of the D462-E4 TCR and D462-E4 TCR-MR1-5-OP-RU crystal structures have been deposited in the Protein Data Bank under accession codes 6XQQ and 6XQP, respectively.

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Atypical TCR recognition of MR1

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Conflict of interest—J. R., J. M., L. L., and D. P. F. are named inventors on patent applications (PCT/AU2013/000742, WO2014005194) (PCT/AU2015/050148, WO2015149130) involving MR1 ligands for MR1-restricted MAIT cells owned by University of Queensland, Monash University, and University of Melbourne.

Abbreviations—The abbreviations used are: MAIT, mucosal associated invariant T; TCR, T cell receptor; CDR, complementarity-determining region; Ag, antigen; SPR, surface plasmon resonance; BSA, buried surface area; rmsd, root mean square deviation.

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