Chancellor, A., Simmons, R. A., Crean, R. M., Van der Kamp, M. W., De Libero , G., & Lepore, M. (2023). Promiscuous recognition of MR1 drives self-reactive Mucosal-Associated Invariant T cell responses. *Journal of Experimental Medicine*, *220*(9), [e20221939]. https://doi.org/10.1084/jem.20221939

Promiscuous recognition of MR1 drives self-reactive Mucosal-Associated Invariant T cell responses

Andrew Chancellor[1]†‡*, Robert Simmons[2]†, Rahul C. Khanolkar[2], Vladimir Nosi[1,3], Aisha Beshirova[1], Giuliano Berloffa[1], Rodrigo Colombo[1], Vijaykumar Karuppiah[2], Johanne M. Pentier[2], Vanessa Tubb[2], Hemza Ghadbane[2], Richard J. Suckling[2], Keith Page[2], Rory M. Crean[4,5], Alessandro Vacchini[1], Corinne De Gregorio[1], Verena Schaefer[1], Daniel Constantin[1], Thomas Gligoris[2], Angharad Lloyd[2], Miriam Hock[2], Velupillai Srikannathasan[2], Ross A. Robinson[2], Gurdyal S. Besra[7], Marc W. van der Kamp[6], Lucia Mori[1], Raffaele Calogero[3], David K. Cole[2#], Gennaro De Libero[1*#], Marco Lepore[2*#]

[1]Experimental Immunology, Department of Biomedicine, University Hospital Basel, University of Basel, 4031 Basel, Switzerland.

[2]Immunocore Ltd., Milton Park, Abingdon, UK.

[3]Department of Molecular Biotechnology and Health Sciences, University of Torino, 10126 Torino, Italy.

[4]Department of Biology and Biochemistry, University of Bath, Bath, UK.

[5]Doctoral Training Centre in Sustainable Chemical Technologies, University of Bath, Bath, UK.

[6]School of Biochemistry, University of Bristol, Biomedical Sciences Building, University Walk, Bristol, UK.

[7]School of Biosciences, Institute of Microbiology and Infection, University of Birmingham, Edgbaston, B15 2TT Birmingham, United Kingdom.

†These authors contributed equally.

[#] These authors contributed equally.

‡Primary corresponding author

*Correspondence: andrew.chancellor@unibas.ch (A.C. ORCID 0000-0001-9658-4124),

gennaro.delibero@unibas.ch (G.D.L., ORCID 0000-0003-0853-7868),

lepore.mmarco@gmail.com (M.L., ORCID 0000-0003-1353-8224).

Non-standard abbreviations: 5-OP-RU, 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil; MAIT cell, mucosal associated invariant T cell; MR1, MHC-class-I-related protein 1; moDC, monocyte-derived dendritic cell; RU, response unit; SPR, surface plasmon resonance.

Summary

Promiscuous recognition of MR1 by canonical MAIT TCRs endowed with dual reactivity to both microbial and self-antigens enables MAIT cell responses in the absence of microbial infection.

Abstract

Mucosal-associated-invariant-T (MAIT) cells use canonical semi-invariant T cell receptors (TCR) to recognize microbial riboflavin precursors displayed by the antigen-presenting molecule MR1. The extent of MAIT TCR cross-reactivity toward physiological, microbially-unrelated antigens remains underexplored. We describe MAIT TCRs endowed with MR1-dependent reactivity to tumor and healthy cells in the absence of microbial metabolites. MAIT cells bearing TCRs cross-reactive toward self are rare but commonly found within healthy donors and display T-helper-like functions *in vitro*. Experiments with MR1-tetramers loaded with distinct ligands revealed significant cross-reactivity among MAIT TCRs both *ex vivo* and upon *in vitro* expansion. A canonical MAIT TCR was selected on the basis of extremely promiscuous MR1 recognition. Structural and molecular dynamic analyses associated promiscuity to unique TCRβ-chain features, that were enriched within self-reactive MAIT cells of healthy individuals. Thus, self-reactive recognition of MR1 represents a functionally relevant indication of MAIT TCR cross-reactivity suggesting a potentially broader role of MAIT cells in immune homeostasis and diseases, beyond microbial immunosurveillance.

Keywords: MAIT cells, MR1, T cell cross-reactivity, T cell receptor (TCR), auto-reactivity, autoimmunity.

Running title: Self-reactive MAIT cells

Introduction

MR1 is a non-polymorphic MHC class I-like molecule that presents small metabolites to T cells. In humans, the MR1-restricted T cell pool consists of two populations, mucosal-associated invariant T (MAIT) cells (Porcelli et al., 1993; Treiner et al., 2003) and MR1T cells (Harriff et al., 2018; Lepore et al., 2017), which significantly differ in antigen (Ag) specificity and TCR repertoire. While MR1T cells react to not yet identified self and potential tumor-associated Ags (Harriff et al., 2018; Lepore et al., 2017), and display polyclonal TCRs, MAIT cells recognize microbial metabolites and are uniquely defined by an almost invariant TCRα chain paired with a restricted TCRβ chain repertoire (Lepore et al., 2014; Porcelli et al., 1993; Tilloy et al., 1999). The canonical MAIT TCRα is made by the *TRAV1-2* variable gene rearranged with either the *TRAJ33*, *TRAJ12* or *TRAJ20* gene segments and with remarkably limited junctional variability (Lepore et al., 2014; Porcelli et al., 1993; Tilloy et al., 1999). Other MAIT cell defining features include high expression of CD161, CD26 (Sharma et al., 2015) and IL-18Rα, expression of the transcription factor PLZF and reactivity to microbial Ags (Le Bourhis et al., 2010) although very rare $CD161^-$ MAIT cells have also been described (Koay et al., 2019). Microbial Ags include the potent pyrimidine agonists 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU), intermediates of riboflavin biosynthesis that convert to 6,7-Dimethyl-8-ribityllumazine (RL-6,7-diMe) and 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH), which also stimulate MAIT cells, although with lower potency. 5-OE-RU and 5-OP-RU are captured by MR1 *via* a Schiff base with MR1 residue K43, whereas the RL-6,7-diMe and RL-6-Me-7-OH metabolites do not form a Schiff base (Corbett et al., 2014; Kjer-Nielsen et al., 2012). Thus, MR1 binds distinct microbial Ags that show different stimulatory capacity (Schmaler et al., 2018).

Several MAIT TCR-MR1 crystal structures demonstrated a highly conserved binding mode that is similar to the conformation adopted by canonical HLA class I- and HLA class II-restricted TCRs (Awad et al., 2020; Corbett et al., 2014; Eckle et al., 2014; Patel et al., 2013). This binding mode positions the invariant MAIT TCRα orthogonally above the Ag, establishing a conserved and essential hydrogen bond with the ribityl moiety of the riboflavin-related agonists via the residue Y95α (encoded by the TRAJ gene segments) (Corbett et al., 2014). Despite the conserved mode of MR1 binding of the invariant TCR Vα, the TCRβ chain has an important role, in influencing Ag recognition and magnitude of the T cell activation (Eckle et al., 2014; Gherardin et al., 2016)

Although MR1 is ubiquitously expressed, the majority of MR1 molecules remain unfolded within the endoplasmic reticulum (ER) until an abundance of Ags is available for loading (McWilliam et al., 2016). MR1 stabilization is achieved through neutralization of K43 within the Ag binding pocket, allowing egress to the surface. However, small quantities of MR1 are commonly expressed on the cell surface, consistent with the recognition of different cell types such as dendritic cells, epithelial cells and cancer cells by MR1T cell clones that do not react to microbial Ags (Lepore et al., 2017). This implies a wider role for MR1-restricted T cells in immunity (Crowther et al., 2020; Lepore et al., 2017). Recently, additional MAIT cell agonists beyond 5-OP-RU have been identified (Harriff et al., 2018; Keller et al., 2017) and MAIT cells can distinguish between different Ags through their TCR Vβ-chain, indicating the MAIT TCR repertoire may be shaped by available Ags (Gherardin et al., 2016; Gold et al., 2014; Howson et al., 2018; Lopez-Sagaseta et al., 2013).

The reactivity of MAIT cells toward self has not been systematically investigated since initial experiments, in which mouse MAIT cells were stimulated by uninfected fibroblasts and B cells

(Huang et al., 2008). Recent studies extended these observations to humans using MR1 tetramers and structural analyses, and suggested that MAIT TCRs can be cross-reactive toward other MR1 ligands, including some common drugs and potential self-Ags (Gherardin et al., 2016; Huang et al., 2008; Keller et al., 2017; Young et al., 2013). Further reports on MAIT cells have indicated that while bacterial colonization of the gut is required for their thymic selection and peripheral expansion, MAIT cells are still present in the periphery of germ-free mice, albeit at a much lower frequency (Koay et al., 2019; Legoux et al., 2019), implying microbial-independent thymic selection. Consistently, MAIT cell alterations in inflammatory diseases with no direct microbial aetiology, such as diabetes, multiple sclerosis and obesity, have been reported (Croxford et al., 2006; Miyazaki et al., 2011 34; Rouxel et al., 2018; Toubal et al., 2020). Furthermore, activation of MAIT cells upon challenge with SARS-CoV-2 infected macrophages has been observed (Flament et al., 2021). Overall, these data suggest that some MAIT cells could become activated in a microbial-independent manner.

Here, we set out to define the extent and mechanisms of MR1-mediated MAIT cell self-reactivity. We demonstrate that microbial-independent MR1 reactivity of a canonical MAIT cell subset is commonly observed in healthy individuals. Furthermore, we describe a novel mode of promiscuous MR1 recognition by a MAIT TCR, associated with a CDR3β-motif enriched in circulating self-reactive MAIT cells.

Results

A subset of MAIT cell responds *in vitro* and *ex vivo* to stimulation by MR1-overexpressing tumor cells

We asked whether canonical MAIT cells that recognize microbial metabolites could also respond to MR1-mediated stimulation in the absence of microbial ligands. Purified, circulating Vα7.2$^+$ T cells from healthy donors were cultured with β2-microglobulin (B2M)-deficient A375 melanoma cells (A375b) engineered to display high levels of surface wild-type MR1 (A375b-wtMR1; Fig S1A). We selected MR1-overexpressing A375 cells as APCs because of their capacity to broadly stimulate MAIT and MR1T cells through an efficient presentation of both microbial and self-Ags, respectively (Lepore et al., 2017). Freshly isolated T cells were stimulated with A375b-wtMR1 cells and proliferative responses as well as MR1-dependent activation upon rechallenge, were analyzed. Most of the proliferating Vα7.2$^+$/CD161$^+$ MAIT cells upregulated the activation marker CD137 following rechallenge with A375b-wtMR1 cells, contrary to non-proliferating cells that remained unresponsive (Fig. 1A, B and S1B). Activation was prevented by anti-MR1 mAb and did not occur when the cultures were rechallenged with A375b cells that lack wtMR1 on their surface (Fig. 1A, B and S1B). Both proliferating and non-proliferating MAIT cells recognized 5-OP-RU-pulsed THP-1 cells in an MR1-dependent fashion (Fig. 1A and S1B) and bound 5-OP-RU-loaded MR1 tetramers (Fig. S1C). Of note, a proportion of proliferating non-MAIT cells (defined as Vα7.2$^+$ and CD161$^-$) exhibited an MR1-dependent response to A375b-wtMR1 cells (Fig. 1A), representing MR1T cells that do not recognize microbial Ags and express distinct TCRs as previously reported (Lepore et al., 2017).

The dual reactivity of proliferating Vα7.2$^+$/CD161$^+$ MAIT cells in the presence and absence of microbial-Ags was further confirmed by the detection of MR1-dependent IFN-γ release (Fig.

S1D) and upregulation of additional T cell activation markers in response to both 5-OP-RU-pulsed THP-1 and A375b-wtMR1 cells (Fig. 1C). Notably, both stimuli also induced an MR1-dependent decrease of TCR surface levels, as detected with anti-Vα7.2 mAb staining, suggestive of activation-induced TCR downregulation (Fig. 1D).

We next asked whether self-reactive MAIT cells could also be identified *ex vivo* following short-term stimulation. A small but detectable fraction of freshly purified MAIT cells upregulated CD137 and CD69 after overnight culture with A375b-MR1 cells (mean 1.1%, Fig. 1E) in the absence of microbial Ags. As expected, a large majority of MAIT cells responded instead to 5-OP-RU (Fig. 1E). Anti-MR1 blocking mAb prevented MAIT cell activation in the control culture (mean 0.1%). Remarkably, *ex vivo* self-reactive MR1-restricted responses were consistently detected in circulating MAIT cells from all the seven donors investigated.

Finally, we compared the functional response of *in vitro* expanded self-reactive MAIT cells to either 5-OP-RU or A375b-wtMR1 cells and did not observe major qualitative differences between the two stimuli (Fig. 1F, G and Fig. S1E).

Taken together, these data indicated that MAIT cells endowed with dual reactivity to microbial Ags and cell-endogenous MR1-Ag complexes are present in circulating T cells of healthy donors, as they expand *in vitro* and are detectable *ex vivo* following co-culture with MR1-overexpressing tumor cells. Furthermore, microbial and cell-endogenous stimulation elicited a comparable effector program within *in vitro*-expanded self-reactive MAIT cells.

Self-reactive MAIT cells can recognize physiological levels of MR1 in healthy cells and display T-helper-like functions *in vitro*

To validate the reactivity and further investigate the function of self-reactive MAIT cells, we interrogated T cell clones. Each clone displayed phenotypic and functional features of canonical MAIT cells, including expression of the classical invariant TCRα chain (Table 1), dose dependent IFN-γ release by 5-OP-RU stimulation (Fig. 2A) and binding to 5-OP-RU-loaded MR1 tetramers (Fig. S2A). When challenged with A375b-wtMR1 cells in the absence of foreign Ags, the clones BC75B31 and BC75B38 released IFN-γ in an MR1-dependent manner (Fig. 2B). TCR transfer experiments confirmed self-reactive recognition of A375b-wtMR1 (Fig. 2C). These results indicated that MAIT clones BC75B31 and BC75B38 possess TCRs endowed with dual, MR1-dependent reactivity to microbial and cell-endogenous stimulation. We, therefore, selected these two clones as models to investigate whether MAIT cell self-reactivity also occurs toward healthy cells, expressing physiological MR1 levels on the cell surface. We focused on monocyte-derived dendritic cells (moDCs) and asked whether the low constitutive levels of MR1 they express (Lepore et al., 2017) could be sufficient to drive immunologically relevant recognition by self-reactive MAIT cells. Co-culture experiments revealed that moDCs were able to support MR1-dependent IFN-γ release by the BC75B31 and BC75B38 self-reactive MAIT clones, whereas they failed to stimulate a control non-self-reactive MAIT clone (MRC25) (Fig. 2D). A third clone (SMC3) weakly recognized moDCs (Fig. S2B). Notably, MR1-dependent interaction between the MAIT clones and moDCs resulted in the upregulation of markers associated to DC maturation and licensing, including CD83, CD86 and CD40 (Fig. 2E). These *in vitro* results suggested a potential role of self-reactive MAIT cells in modulating DC function.

Canonical MAIT TCRs display various degrees of cross-reactive MR1 recognition

Combined microbial and self-reactivity of canonical MAIT cells is an indication of TCR cross-reactivity toward different MR1-presented ligands. To further investigate the extent of MAIT TCR cross-reactivity**,** four different approaches were used. In the first, bulk MAIT cell lines were generated from two donors after stimulation with the microbial agonist 5-OP-RU. A fraction of 5-OP-RU-expanded MAIT cells displayed self-reactivity upon challenge with A375b-MR1 cells, as detected by CD137 upregulation (Fig. 3A). This activation was prevented by anti-MR1 blocking mAb (Fig. 3A), thus further indicating that this group of MAIT cells were able to recognise both endogenous MR1-ligand complexes displayed by A375b-wtMR1 cells as well as exogenous 5-OP-RU presented by MR1. These experiments also suggested that self-reactive MAIT cells may accumulate *in vivo* following any microbial infection that drives significant MAIT cell expansion.

The second assay consisted of staining the same MAIT cell lines with MR1-tetramers loaded with either 5-OP-RU, 3-formylsalycilic acid (3-F-SA) or 6-formylpterin (6-FP). All MAIT cells ($CD3^{+}CD161^{high}$) bound to 5-OP-RU tetramers (Fig. S3A and C), as expected. A very small fraction of non-classical MAIT cells ($CD3^{+}CD161^{-}$) also reacted to 5-OP-RU tetramers (Fig. S3A and B), representing 5-OP-RU-reactive MR1-restricted T cells lacking the canonical MAIT TCR, as previously reported (Gherardin et al., 2018; Gold et al., 2010). Surprisingly, up to 47.1% of MAIT cells were stained by 3-F-SA tetramers (Fig. 3B), and up to 29.6% by 6-FP tetramers (Fig. 3C). In addition, a significant number of MAIT cells (~15% and ~19% in two donors, respectively) even bound to all three tetramers (Fig. 3D).

In the third experiment, MAIT cell lines generated from additional 2 donors by enrichment of TCR $V\alpha7.2^{+}$ cells and subsequent expansion with PHA stimulation were challenged with 4 sets

of MR1 tetramers loaded with either 5-OP-RU, 6-FP, 5-F-SA, and 3-F-SA (Fig S3D-G). MAIT cells were identified as $CD161^{+}$ $CD3^{+}$ MR1-5-OP-RU $tetramer^{+}$ (Fig. S3E, F and G) whereas $CD161^{-}$ $CD3^{+}$ MR1-5-OP-RU $tetramer^{-}$ cells represented control non-MAIT cells (Fig. S3E, F and G). Within the MAIT group, 23.9% (donor 3) and 11.4% (donor 4) of cells bound to at least one of the other 3 tetramers bearing different ligands (Fig. 3E and S3F, G). Distinct patterns of tetramer cross-reactivity were observed, with some cells binding to all 4 tetramer sets (Fig. 3E and S3F, G). In contrast, non-MAIT cells infrequently reacted to any of the tetramers (3.4%, donor 3, and 3.1%, donor 4; (Fig. 3E and S3F, G).

Finally, we estimated the proportion of cross-reactive MAIT cells able to bind MR1-tetramers loaded with non-microbial Ags directly *ex vivo* (Fig. 3F). As previous studies have reported that CD8 has an important impact on MR1-dependent MAIT cell reactivity to microbial metabolites due to its ability to bind to MR1 (Souter et al., 2022), we used the same mutated MR1 tetramers devoid of the CD8 binding capacity (CD8-null). With these reagents, we could strictly assess TCR-dependent binding and rule out any potential contribution of CD8. Significantly more MAIT cells bound to at least one of the three CD8-null tetramers loaded with 6-FP, 5-F-SA, or 3-F-SA ligands (mean 2.7% of MAIT cells) as compared to the control non-MAIT cell group (0.4% of non-MAIT) (Fig. 3F).

Taken together, these data sets indicated that a proportion of MAIT cells feature different degrees of promiscuous TCR interaction with MR1 complexes presenting non-microbial ligands, thus suggesting that TCR cross-reactivity is not uncommon within MAIT cells.

Extreme promiscuity in MR1 recognition by a canonical MAIT TCR

The significant extent of promiscuity in MR1 recognition we observed among MAIT cells prompted us to investigate to what extent unique TCRβ chains contribute to MR1 cross-reactivity. For this purpose, a MAIT TCR phage library was generated using a canonical MAIT TCRα chain complemented with random TCRβ chains. When phages were screened for binding to MR1-K43A monomers, a canonical MAIT TCR, called E8 was isolated, expressing the *TRAV1-2-TRAJ33* and a chimera of *TRBV6-1/ TRBV6-5* gene pairs (Table 1). The E8 TCR displayed MR1-dependent self-reactivity toward healthy moDCs, monocytes and B cells, but not T cells when transduced into NFAT-Luciferase TCR-null B2M knock-out Jurkat cells (Jurkat-E8) (Fig. 4A). The microbial Ag 5-OP-RU increased their reactivity to monocytes and B cells but did not enhance the already strong Jurkat-E8 cell response to moDCs (Fig. 4A). A control, non-self-reactive MAIT TCR VT001 (*TRAV1-2-TRAJ33*/*TRBV6-2* gene pairs) also expressed in NFAT-Luciferase TCR-null B2M knock out Jurkat cells (Jurkat-VT001) did not respond to these APCs, unless they were pulsed with 5-OP-RU (Fig. 4B). In addition, Jurkat-E8, but not Jurkat-VT001 cells reacted to multiple tumor cell lines of different tissue origin in MR1-dependent-manner and in the absence of any microbial Ag (Fig. 4C). Taken together, these data indicated a dual reactivity of the E8 TCR, exemplified by recognition of microbial metabolites and self-reactivity to multiple MR1-expressing cell types.

We next expressed the E8 TCR in a soluble format and further assessed the extent of cross-reactivity by surface plasmon resonance using MR1 monomers loaded with 5-OP-RU or a range of non-microbial ligands, including 6-FP, 3-F-SA, 5-F-SA, 3-formylbenzoic acid (3-F-BA), 4-formylbenzoic acid (4-F-BA). We also included the empty MR1-K43A mutant of MR1. A previously characterized and non-cross-reactive MAIT TCR AF-7 (Eckle et al., 2014) was used as a reference (Table 1). The E8 TCR bound to all MR1-ligand complexes with low

nanomolar to low micromolar affinities (K_D range = 0.002 – 0.6 μM) with MR1-5-OP-RU being the strongest affinity ligand (Fig. 4D). In contrast, the AF-7 TCR bound to MR1-5-OP-RU with K_D = 1.1 μM, but not to other immobilized MR1-ligand complexes (Fig. 4E). Thus, the E8 TCR is an extremely promiscuous classical MAIT TCR that displays self-reactivity towards healthy and tumor cells and binding to a range of soluble MR1 ligand complexes.

Structural basis of promiscuous MR1 recognition by the E8 TCR

To understand the molecular basis of the broad E8 TCR reactivity, we solved the crystal structures of the E8 TCR in complex with MR1-5-OP-RU, -6-FP, -3-F-SA, -5-F-SA, -3-F-BA, -4-F-BA and empty MR1-K43A between 1.84 Å and 2.4 Å resolution (Fig. 5A, Fig. S4A and Table S1). The structures of the E8 TCR were aligned to the structure of the classical MAIT TCR AF-7 (PDB: 6PUC) bound to MR1-5-OP-RU (Awad et al., 2020), (Fig. 5A). The E8 and AF-7 TCRs bound to MR1 in a very similar mode (Fig. 5, A, B and C). They possess the same chain pairing and virtually identical amino acid sequences in their complementarity determining region (CDR) loops (Table 1) that adopted identical positions and made a very similar network of contacts with both the MR1 surface and 5-OP-RU (Fig. 5, A, B, C and D and Table S2). Like the AF-7 TCR, the E8 TCR contacted the ribityl chain of 5-OP-RU with the Y95α residue in the CDR3α loop (Fig. 5D and table S2). The only difference in contacts with MR1-5-OP-RU between the E8 TCR and the AF-7 MAIT TCR was mediated by the E8 TCR residue R96β in the CDR3β loop, which made additional salt bridges with MR1 residues E76 and E149 (Fig. 5E and Table S2). The structures of the E8 TCR in complex with MR1-5-OP-RU, -K43A, -6-FP, -3-F-SA, -5-F-SA, -3-F-BA and -4-F-BA revealed an almost identical network of contacts (Table S3). No ligands except 5-OP-RU contributed to TCR binding, providing a possible explanation of the stronger affinity and greater potency of this TCR for MR1-5-OP-RU complexes (Table S3). Together these results suggest that the TCR residue

R96β drives broad recognition of MR1-ligand complexes by forming salt bridges to the MR1 heavy chain.

To further explore the hypothesis that the salt bridge interaction network mediated by E8 TCR residue R96β was important for the broad recognition of multiple MR1 ligands, MR1 residues E76 and E149 were mutated to Q76 and Q149. E (Glu) and Q (Gln) have very similar physiochemical properties, but Q lacks the anionic carboxylate group that is required to form a salt bridge. Thus, we reasoned that these mutations would abrogate the salt bridge interaction whilst maintaining the overall binding mode of the E8 TCR, and likely still enable some interaction with MR1 residues 76 and 149. The mutated form of MR1 (MR1 E76Q E149Q) was refolded in complex with 5-OP-RU, 6-FP, and 5-FSA, and the soluble protein was used to investigate the binding affinity of the E8 TCR. In addition, we measured the binding affinities to a version of the E8 TCR engrafted with TRBV6-1 (to control for the chimeric output of the phage display) and the canonical AF7 TCR (Table 2). In line with the structural analysis, the binding affinity of the AF7 TCR was not substantially affected by the MR1 mutations: we observed a K_D = 0.6 μM for MR1 E76Q E149Q in complex with 5-OP-RU compared to a K_D = 1 μM for MR1-5-OP-RU. In contrast, the binding affinity of the E8 TCR to MR1 E76Q E149Q was substantially reduced compared to its binding affinity to MR1 wildtype bound to all ligands tested (8 to 10-fold reduction in binding affinity) (Table 2 and source data file). Similar observations were made with the E8 TRBV6-1 TCR, with an 8-fold reduction in binding affinity for MR1 E76Q E149Q. Previous studies have shown that affinity reductions within this range can abrogate MAIT cell recognition of MR1 ligands (Patel et al., 2013), indicating that the salt bridge interactions mediated by R96β are likely to be central to biologically relevant recognition of MR1 by the E8 TCR. Altogether, these data support the

structural analysis and demonstrate that the salt bridge interaction between E8 TCR residue R96β and MR1 residues E76 and E149 plays a central role in cross-ligand recognition.

Energetic basis of promiscuous MR1 recognition by the E8 TCR

To further explore the basis for the promiscuous behavior of the E8 TCR, we performed molecular dynamics (MD) simulations and binding free energy calculations. These were made on both the AF-7 and E8 TCR bound to wtMR1 loaded with 5-OP-RU and to empty MR1-K43A. The MD simulations allowed us to sample the conformational space available to each complex and snapshots from these simulations were used to calculate the binding free energy using the molecular mechanics Poisson-Boltzmann surface area (MMPBSA) approach (Genheden and Ryde, 2015; Miller et al., 2012). We utilized two different MMPBSA protocols to predict $\Delta\Delta G$ (Table 3 and Table S4). For the AF-7 TCR-MR1 complex with and without 5-OP-RU bound we obtained $\Delta\Delta G$ values of -19.6 and -8.7 kcal mol^{-1} respectively, with the 5-OP-RU bound complex consistently predicted to have a substantially higher affinity (Table 2). In contrast, using the same two protocols for the E8 TCR, we obtained $\Delta\Delta G$ values of +2.8 and -0.9 kcal mol^{-1} (Table 3), demonstrating our calculations can reproduce the ligand dependent behaviour of the AF-7 TCR and promiscuous behaviour of E8 TCR.

The MMPBSA approach also allows the decomposition of calculated binding free energy differences into per-residue contributions, which we and others have successfully utilized to identify the key residues and interactions across the binding interface that drive affinity (Crean et al., 2020; Holland et al., 2020; Xiao et al., 2019; Zoete et al., 2010). Thus, we used the MMPBSA approach to understand why the AF-7 and the E8 TCRs have differing ligand dependencies. To this end, the per-residue binding contributions of each TCR to MR1 loaded with and without 5-OP-RU were investigated (Fig. 5F and Fig. S4B). For the AF-7 TCR-MR1

357 complex, several MR1 residues (Y62, Q64 and Q71) and TCR residues on the CDR3α loop
358 (S93α, N94α, Y95α), on the CDR2β loop (Y48β and D56β), and on the CDR3β loop (T97β
359 and E99β) showed a substantial loss in binding energy in the absence of 5-OP-RU **(**Fig. 5F,
360 left panels). This was consistent with previous literature that identified the Y95α residue as a
361 key driver of the AF-7 TCRs MR1 5-OP-RU specificity (Eckle et al., 2014). In contrast, for
362 the E8 TCR-MR1 complex, only two residues (Y62 on MR1 and D56β on the CDR2β loop)
363 across the entire binding interface showed large differences with and without 5-OP-RU bound
364 **(**Fig. 5F right panels**).** Irrespective of 5-OP-RU, the preservation of the binding energy
365 "footprint", is consistent with our structural and SPR data on the E8 TCR. Further analysis of
366 the most favourable contributions of the E8 TCR revealed they were within the CDR3β loop
367 (particularly residues R96β and Y98β) (Fig. 5G and Fig. S4B). For both complexes, MR1
368 residues Q64, Y152, and N155 provided particularly strong interactions (Fig. 5G and Fig.
369 S4B). However, the E8 TCR-MR1 complex was more focused towards MR1 residues located
370 on the α1-helix (R61, W69 and E76) (Fig. 5G and Fig. S4B).

371

372 In summary, our MD analysis revealed that the CDR3β loop of the E8 TCR acts as an anchor,
373 helping to lock in place the remainder of the binding interface independent of the ligand loaded.
374 This is in agreement with structural data that showed the R96β in the E8 CDR3β loop likely
375 forms a salt bridge with the MR1 E76 and E149 residues and is the key driver of promiscuous
376 MR1 binding.

377

378 **Self-reactive MAIT TCRs can use E8-like CDR3βs**

379 Next, we asked whether a portion of self-reactive MAIT cell TCRβ chains contain E8-like
380 features that could account for the self-recognition of MR1. According to the structural
381 analysis, we reasoned the position of R96β within the CDR3β loop structure would be most

382 influenced by TRBV usage (*TRBV6* gene), and CDR3β length of 13 amino acids (CDR3L13).
383 These three features were the basis to search for TCRs with similar motifs.
384
385 We initially addressed whether this motif was increased in *in vitro* expanded autoreactive
386 MAIT cells. Proliferating and non-proliferating MAIT cells stimulated with A375b-wtMR1
387 cells (from four donors), were sorted into two pools, and their TCRβ-chains sequenced (Fig.
388 S5A). A significant increase in the number of TCRβ sequences concomitantly expressing the
389 *TRBV6* gene, CDR3L13 and R96β was observed within self-reactive proliferating MAIT cells
390 as compared to non-self-reactive non-proliferating MAIT cells (p=0.04; Fig. 6A). Importantly,
391 the frequency of TCRs also co-expressing *TRBV6* and CDR3L13 but displaying the Arg in a
392 distinct position nearby the CDR3β residue 96 (R95β, R97β or R98β) was not significantly
393 different between the two MAIT cell groups (Fig. 6A), indicating that the E8-like motif is
394 enriched within the TCRβ repertoire of healthy donors' circulating self-reactive MAIT cells.
395
396 To functionally validate these findings, a TCRβ chain bearing this motif was co-expressed with
397 a canonical MAIT TCRα chain (clone SMC3) in J.RT3-T3.5 cells (TCR 393). The expression
398 of this hybrid MAIT TCR pair enabled MR1-dependent self-reactivity toward A375b-wtMR1
399 cells, in addition to conferring the expected canonical reactivity to 5-OP-RU (Fig. 6B).
400
401 We then performed additional analysis of both proliferating and non-proliferating cells, to
402 investigate other unique sequences enriched in self-reactive MAIT cells. TRBV gene usage
403 was biased towards *TRBV6*, *TRBV20* and *TRBV4* gene families, which are also the most
404 frequently used in the classical MAIT TCRs (Treiner et al., 2003). In proliferating cells, *TRBV6*
405 was significantly over-represented compared to non-proliferating cells (p = 0.03) (Fig. S5B)
406 with a significantly increased use of *TRBV6-3* (p=0.013) and *TRBV6-6* (p=0.013) genes (Fig.

S5C). No significant difference among proliferating and non-proliferating cells were found in the usages of *TRBJ* (Fig. S5D), *TRBD* (Fig. S5E) genes, nor in the lengths of CDR3β (Fig. S5F). In addition, no significant differences were seen when combinations of genes were analyzed Fig. S5G). Thus, the motif outlined by the E8 TCR and represented by the combination of *TRBV6*, CDR3L13 with R96 might be used to identify and track a population of *bona fide* self-reactive MAIT cells.

To validate the presence of MAIT cells displaying this motif, we searched within previously acquired TCRβ-chain datasets from healthy donor's circulating TRAV1-2^{+}/TRBV6^{+}/CD161^{+} (MAIT cells) and TRAV1-2^{+}/TRBV6^{+}/CD161^{-} (non-MAIT) (Lepore et al., 2014). In 7 different donors, the motif was observed in 1.73-2.4% of MAIT TCR transcripts (median 1.99%) (Fig. 6C). In contrast, the same motif was detected in 1.28-1.88% (median 1.52%)of TCR transcripts from T cells expressing Vα7.2^{+} and lacking CD161 (Fig. 6C). Taken together, these findings suggested that in healthy individuals a small fraction of MAIT cells express the E8-like *TRBV6*, CDR3L13-R96β motif, which we found associated with self-reactive recognition of MR1.

Discussion

Here we describe a population of cross-reactive human MAIT cells that are activated upon recognition of MR1 in the absence of microbial Ags. The functional responses we investigated did not qualitatively diverge from those induced by the microbial Ag 5-OP-RU and non-transformed target cells expressing low, physiological levels of MR1 were sufficient for productive stimulation of tested clones. Our data revealed that self-reactive recognition of MR1 is a feature of some MAIT cell TCRs, enabling recognition of both tumor and healthy cells. We estimated that self-reactive MAIT cells are rare within circulating T cells of healthy

individuals, and whether their frequency is increased and/or their function altered in patients with autoimmunity, inflammatory diseases, and cancer deserves appropriate clinical investigation.

As self-reactive MAIT cells are present in healthy individuals, an important question arises regarding their regulation *in vivo*. MAIT cells exhibit an effector memory phenotype seemingly due to the abundance of microbial Ags that leads to a continual stimulation (Legoux et al., 2019; Seach et al., 2013). Such frequent stimulation promotes the expression of several regulatory molecules, including NK inhibitory receptors and immune checkpoint controls (McMahon and Raulet, 2001), that may allow self-reactivity of certain MAIT cells only in circumstances when they are not engaged. The regulation of MR1 levels on APCs could also play a role, only enabling MAIT cell stimulation when enough stimulatory self-Ags are available for loading, or when the 'correct' Ags for self-reactive MAIT cells are present. Another possibility supported by our *in vitro* data, suggests that self-reactive MAIT cells can expand during microbial infections. Thus, after peripheral expansion by abundant microbial Ags, rare high-affinity self-reactive MAIT TCRs might increase in frequency and subsequently respond to MR1-mediated stimulation in non-infectious settings. Finally, MAIT cell self-reactivity could also be promoted by high SYK Tyrosine kinase expression, which facilitates productive TCR signalling as shown with recognition of CD1d-self lipids by self-reactive human iNKT cells (Perroteau et al., 2020).

Self-reactive MR1 recognition is a clear indication of MAIT TCR cross-reactivity, which we found to be represented at different degrees within MAIT cells, thus confirming and extending previous results (Gherardin et al., 2016; Keller et al., 2017). A significant fraction of both *in vitro* expanded and *ex-vivo* analyzed 5-OP-RU-reactive MAIT TCRs appear to be promiscuous, and able to bind MR1 tetramers loaded with distinct non-microbial ligands. Of

note, a gradient of promiscuity is observed, where some MAIT TCRs can bind either one, two, or three different MR1 tetramers. An elegant study recently reported a key role of CD8 in enhancing MAIT cell response to 5-OP-RU via lateral binding to MR1 (Souter et al., 2022). The same study also indicated the CD8-MR1 interaction as crucial for the recognition of the weak folate Ags (*e.g.,* 6-FP) by MR1-restricted T cells. Thus, both MR1- and conventional MHC-I-restricted T cells use the CD8 co-receptor to amplify TCR-depended responses, with the greatest impact in the case of low-affinity interactions with Ags (Laugel et al., 2011). Our experiments with both CD8-enabled and CD8-disabled MR1 tetramers, whilst supporting these findings, also highlight the presence of cross-reactive MAIT TCRs that are less influenced by CD8 for the binding of MR1-ligand complexes. Accordingly, CD8-negative MAIT cells are commonly found in both humans and mice, albeit at much lower frequencies (Gherardin et al., 2018; Martin et al., 2009).

An important consideration relates to the non-microbial Ags we and others used to load MR1-tetramers. To our knowledge, these are all physiologically uncommon small molecules that are buried within the MR1 pocket, and therefore not easily accessible for direct contact with TCRs. Thus, a hypothesis that deserves further investigation is whether this type of cross-reactive recognition relies on particular ligand-depended conformations of MR1 sensed by specific TCRs rather than direct TCR-ligand interactions, as already observed within the CD1 system (Cotton et al., 2018). In addition, unique TCR features could also enable broad productive interactions with distinct MR1-ligand complexes as exemplified by the extremely promiscuous E8 TCR mode of MR1 recognition. Here, classical MAIT TCR primary interactions with both 5-OP-RU and MR1 led to robust cell activation, as previously described (Corbett et al., 2014). In addition, key interactions between the CDR3β loop and the MR1 α-helices enabled recognition of a variety of MR1-Ag complexes and contributed to the high affinity for 5-OP-

RU. The E8 TCR residue R96β forms a double salt bridge with MR1 residues E76 and E149, allowing productive recognition of MR1 without direct ligand interaction or further stabilizing the complex in the presence of 5-OP-RU. Thus, the two MR1 residues behave as a tweezer, anchoring the TCR residue R96β. MD simulations supported the importance of R96β-mediated interaction in enabling promiscuous and 5-OP-RU-enhanced MR1 recognition compared to the canonical MAIT TCR, AF-7 (Eckle et al., 2014). In addition, biophysical analysis, using MR1 with conservative mutations at residues E76 and E149 to disrupt the salt bridge, confirmed the central role of R96β in driving biologically relevant Ag cross-recognition, and the MD simulations recapitulated the binding affinities for the TCRs to different tested ligands and demonstrated a major energetic role mediated by the E8 TCR CDR3β residue R96.

Our findings fit with the current view of MAIT TCR-MR1-Ag interaction. An elegant study using a series of Ag analogs revealed the importance of a network of polar interactions between TCR, MR1 and Ag, called the interaction triad, that is critical for MAIT cell activation (Awad et al., 2020). Major contributions of different residues within the CDR3β loop were revealed, in some instances directly interacting with the MR1-bound Ag. Another study assigned a degree of autoreactivity to the M33.64 MAIT TCR in which two residues in the CDR3β loop (Thr100β and Asn99β) pinched residue Glu149 of MR1, forming a stable bond (Gherardin et al., 2016). Instead, in the case of the E8 TCR described here, two MR1 residues (E76 and E149) clamp the R96β within the CDR3β. These different modes of binding underline a variety of mechanisms enabling CDR3β-mediated MAIT cell autoreactivity, further describing the unusual plasticity of this interaction. The role of the TCRβ chain in modulating and fine tuning the response of invariant TCRs to Ag in the context of non-polymormic molecules has been previously highlighted within the CD1d/semi-invariant iNKT TCR system (Mallevaey et al., 2011). In that study, a hydrophobic motif promoting association with CD1d was found in the

CDR3β loops of TCR from self-reactive iNKT cells. This motif facilitated the iNKT response to a broad range of CD1d-restricted self-Ags. It was also described that by transferring this CDR3 β loops into the Vβ6 chain, the resulting TCR started to interact with self-CD1d tetramers. Analogous findings related to the contribution of TCRβ in fine-tuning responses were also subsequently reported for MAIT TCR recognition of the 5-OP-RU Ag (Eckle et al., 2014; Narayanan et al., 2020).

Thus, the immune system deploys distinct mechanisms to extend its breadth of Ag recognition in the case of non-polymorphic Ag-presenting molecules and maximize its ability to interact with these targets.

Last, but not least, the potential Ags involved in the self-reactivity of some MAIT TCRs to both tumor and healthy cells deserve further consideration. Whilst we cannot exclude that some Ag recognition is shared between these cell types, we anticipate that promiscuous recognition of different Ags plays a large role. Some outstanding questions remain, including the identity of recognized Ags, their abundance, expression in healthy *vs*. tumor cells, and their impact on the immune function of MAIT cells.

In conclusion, our data indicate that a discrete population of MAIT cells is endowed with MR1-dependent self-reactivity toward tumor and healthy cells. In addition, our work provides compelling evidence that TCR cross-reactivity is not infrequent within MAIT cells and is characterized by degrees of promiscuity toward distinct MR1-Ag complexes. The self-reactive recognition of MR1 by MAIT cells may have important physiological and immunological implications. Within T cells restricted to non-polymorphic Ag-presenting molecules self- and tumor-reactivity is also observed towards CD1a, CD1b, CD1c, and CD1d molecules (Bagchi et al., 2017; Bendelac et al., 1995; de Jong et al., 2010; Lepore et al., 2014; Porcelli et al.,

1989). On one hand, these autoreactive T cells might participate in inflammatory and autoimmune diseases, sustained by unbalanced immune homeostasis and/or play a role in cancer surveillance/progression. On the other hand, the consistent presence of these self-reactive cells in healthy individuals and their T-helper-like properties *in vitro* suggest they may also have regulatory and/or homeostatic functions, as previously proposed for other non-MHC restricted T cell populations such as iNKT cells, canonical non-self-reactive MAIT cells and MR1T cells (Cerundolo et al., 2009; Lepore et al., 2017; Salio et al., 2017). Future studies in different cohorts of patients will address the immunological roles of promiscuous Ag recognition by MAIT TCRs.

Materials and methods

Study design

The objectives of this study were to identify whether MAIT cells possess reactivity beyond that toward microbial Ags. To enable these goals, we designed and performed experiments in cellular immunology, protein biochemistry, and crystallography. The number of independent experiments is outlined in the figure legends, where applicable.

Study approval

All human blood samples collected at Immunocore were processed in accordance with the guidelines of Immunocore's Human Tissue Act compliance team, to conform to the United Kingdom Human Tissue Act 2004, (under ethical approval license IMCres02). Blood specimens obtained from the University Hospital Basel were approved by the local ethical review board (EKNZ, Ethics Committee North-West & Central Switzerland, EKNZ 2017-01888).

Cell lines and primary T cells

The following cell lines were obtained from American Type Culture Collection: A375 (human melanoma), THP-1 (myelomonocytic leukemia), J.RT3-T3.5 (TCRβ-deficient T cell leukemia). The HEK 293 cell line was obtained from the Leibniz-Institute DSMZ-German Collection of Microorganisms and Cell Cultures. J.RT3-T3.5 were engineered to lack endogenous TCRα protein and express an NFAT-driven luciferase reporter. NFAT-Luciferase TCR-null B2M knock-out Jurkat cells were developed from Parental NFAT-Luciferase Jurkat cells from Promega (Part no: J133A). All cells were routinely tested for the absence of mycoplasma contamination. None of the cell lines used in this study are present in the database

of commonly misidentified cell lines. Cells lines were not authenticated. All primary cell lines and clones used in this study were isolated from PBMCs obtained using Lymphoprep (Stemcell Technologies) from blood of blood bank donors and maintained in culture as previously described (Lepore et al., 2014). Briefly, MAIT cell lines were generated by magnetic bead enrichment using biotinylated anti-Vα7.2 mAb (Clone 3C10, Biolegend) or specific expansion using 5-OP-RU. Enriched MAIT cells were prelabelled with Cell Trace Violet according to manufacturer instructions and then cultured with irradiated A375b-wtMR1 cells for the indicated number of days in a 1:1 ratio. Human rIL-2 (5 IU/ml, Peprotech) was added at day 5 and thereafter every two days. Cells were washed and rechallenged as indicated (ratio 2:1) in the presence or absence of purified anti-MR1 mAb (20µg/ml, Ultra-LEAF™ Purified Clone 26.5, Biolegend). From these lines, self-reactive MAIT clones were derived by limiting dilution in the presence of PHA (1 µg/ml), human rIL-2 (100 U/ml) and irradiated PBMC (5×10^5 cells/ml), and screened for reactivity toward indicated cell. $CD14^+$ monocytes were isolated from PBMCs by positive selection using magnetic beads (Stemcell Technologies) and cultured in the presence of 25 ng/ml GM-CSF and 20 ng/ml IL-4 (both from Peprotech). Activation of monocytes was achieved by treating them with 50 ng/ml LPS (from *Escherichia coli* O111:B4, Sigma) overnight.

T cell activation

T cell clones (5×10^4 cells/well unless otherwise indicated) were co-cultured with indicated target cells (5×10^4 cells /well) in 130 µl total volume in triplicates for 18 h. In some experiments, anti-MR1 mAb (20 µg/ml, Ultra-LEAF™ Purified Clone 26.5, Biolegend) were added and incubated for 30 min prior to the addition of T cells. In other experiments, APCs were pulsed for 2 h at 37°C with indicated concentrations of Ags or freshly-prepared 5-OP-RU as described in (Schmaler et al., 2018). J.RT3-T3.5 activation assays were performed in a 1:1

ratio with the indicated APC for 18 h. Cells were then either harvested and stained for surface CD69 upregulation or luciferase was measured using Bio-Glo (Promega).

TCR gene transfer

Total RNA was extracted from snap-frozen cell pellets from each clone. SMARTer RACE 5'/3' kit (Takara) was used for cDNA synthesis and generation of TCR transcripts. Functional TCRα and β chains were identified by sequencing and analysis using the ImMunoGeneTics information system (http://www.imgt.org). The TCRα and β sequences were either synthesized at Integrated DNA Technologies (TCR 393) or amplified from cDNA with gene specific primers (TCRs BC75B31, BC75B38, MRC25) containing cloning adaptors. In both cases the insert was cloned by In-Fusion HD (Takara) to a lentiviral vector for co-transfection of HEK 293 T LX cells. The endotoxin-free vectors were co-transfected together with the lentivirus packaging plasmids pMD2.G, pMDLg/pRRE and pRSV-REV (all from Addgene) to HEK 293 T LX cells with Metafectene PRO reagent from Biontex. Lentiviral supernatants of the corresponding TCRα and β sequences were combined and used to transduce J.RT3-T3.5 cells overnight. TCR-expressing J.RT3-T3.5 cells were sorted for CD3 expression before functional analysis. For the experiments described in Fig. 4, TCR genes were transduced in NFAT-Luciferase TCR-null B2MKO Jurkat cells developed from the parental NFAT-Luciferase Jurkat line (Promega, part n. J133A).

Flow cytometry

When staining with MR1 tetramers (20 μg/ml) or anti-human Vα7.2 (2.5μg/ml Clone 3C10, Biolegend), the cells were pre-treated for 30 min at 37°C with 50 nM dasatinib (Sigma) in PBS. All mAb for staining were titrated on appropriate cells before use. Tetramers were added first for 20 min at RT and anti-human mAb were added for a further 20 min in PBS with dasatinib:

mAb specific for CD3 (Clone UCHT1), CD4 (Clone OKT4), CD8 (Clone RPA-T8), CD161 (Clone HP-3G19) and for activation markers CD137 (Clone 4B4-1), CD69 (Clone FN50), CD25 (Clone BC96), ICOS (Clone DX29), all from Biolegend. DAPI was used to exclude dead cells. Doublets were excluded by FSC-A, FSC-W, SSC-A and SSC-H.

Intracellular cytokine staining was performed by the addition of Brefeldin A (5 µg/ml, Biolegend), monensin (2 µM, Biolegend) and 4 µg/ml anti-human CD107a mAb (Clone H43A, Biolegend) 1 h after co-culture with indicated APCs. Cells were then harvested and treated with fixable LIVE/DEAD BLUE stain (ThermoFisher) for 20 min followed by anti-CD137, CD161 and CD3 mAb before fixation and permeabilization (Buffers from Biolegend). After permeabilization the following mAb specific for intracellular cytokines were added for 40 min on ice: IFN-γ (Clone 4S-B3), TNF-α (Clone MAb.11), GM-CSF (Clone BVD2-21C11), IL-17a (Clone BL168), IL-13 (Clone JES10-5A2), Granzyme B (Clone QA16A02) all from Biolegend. All cells were acquired on a Fortessa (BD) or Aurora spectral analyzer (Cytek) and analyzed using FlowJo v10 software (LLC).

Cytokine analysis

The following human cytokines were assessed by ELISA as previously described (Lepore et al., 2017): human IFN-γ (capture MD-1 mAb; revealing biotinylated 4S.B3 mAb, Biolegend), human IL-13 (capture clone JES10-5A2; revealing biotinylated clone SB126d 1090, SouthernBiotech).

Identification of VT001 MAIT TCR

TCR was identified from a MAIT T cell clone generated from normal human PBMC as previously described (Lepore et al., 2017; Lepore et al., 2014). Briefly, MAIT T cells ($CD3^+$ $CD161^+$ $TRAV1\text{-}2^+$ $V\delta2^-$) were sorted by flow cytometry (BD FACS Aria) and expanded on

PHA, IL-2 and irradiated allogenic PBMCs to establish a T cell line from which clones were subsequently generated by limiting dilution. Individual clones were assessed for CD161, TRAV1-2 and CD137 expression by flow cytometry following overnight co-culture with 5-OP-RU loaded THP-1 cells. Positive clones were selected for TCR genes sequencing. Briefly, this involves first-strand cDNA generation and universal amplification using SmartSeq2 chemistry (Picelli et al., 2014), followed by targeted amplification of TCR chains and MiSeq Next Generation Sequencing. Sequencing data were analyzed using a bespoke bioinformatics pipeline (unpublished).

Identification of E8 TCR

TCRs were isolated from phage libraries based on healthy donor MAIT cell TCR repertoire**.** TCR isolation to produce E8 TCR has been described previously (Li et al., 2005; Liddy et al., 2012). In brief, phage display panning was performed using the MR1-K43A to select for an MR1 specific TCR.

Construct design, protein expression and purification

The sequences coding for the TCR chains were cloned into the pGMT7 vector. TCR constructs were designed to include the variable and constant domains of both α and β chains with an engineered inter-chain disulphide bond as previously described (Boulter et al., 2003). The sequences coding for wtMR1, MR1-K43A, MR1-E76Q-E149Q and B2M and were cloned into the pET23d vector. CD8-null MR1 coding sequence was generated by introducing the mutations Q223A and E224K as previously described (Souter et al., 2022). The proteins were expressed in the BL21 (DE3) Rosetta pLysS strain (Novagen), refolded from inclusion bodies and purified as previously described (Boulter et al., 2003; Reantragoon et al., 2013; (Garboczi et al., 1992). For SPR measurements a C-terminal AVI-tag was added to the wtMR1, MR1-K43A and MR1-E76Q-E149Q constructs and biotinylated after purification using the Avidity

Bir A Biotinylation kit, then purified again using a size exclusion column to remove the biotin and Bir A.

SPR single cycle kinetic analysis

Purified TCRs, MR1-K43A, MR1-E76Q-E149Q and wtMR1 loaded with 6-FP (Schircks Laboratories), 3-F-BA (Fluorochem), 4-F-BA (Sigma Aldrich), 3-F-SA (Thermo Scientific Acros Organics) and 5-F-SA (Fluorochem) were subjected to SPR analysis using a BIAcoreT200™ using steady state affinity analysis. The curves were fitted, and calculations were completed in GraphPad Prism v9 software. Purified E8 TCR and MR1 (WT and E76Q-E149Q mutant) loaded with 5-OP-RU were subjected to SPR analysis using a BIAcore8K™ using single cycle kinetic analysis, which was completed with Biacore Insight Evaluation software.

Crystallization and protein structure determination

E8 TCR and MR1 molecules in 10 mM Tris pH 8.0, 100 mM NaCl were mixed in equimolar ratio and concentrated to 8-10 mg/ml. Sitting drops were set up containing 150 nl of protein solution and 150 nl of reservoir solution in MRC crystallization plates using the Gryphon robot (ART Robbins) and incubated at 20°C. For every E8 TCR-MR1 sample, crystals appeared in many different crystallization conditions. Crystals were cryoprotected using reservoir solution containing 30% ethylene glycol and flash cooled in liquid N_2. Diffraction data were collected at beamlines I03 and I04 at the Diamond Light Source, UK. Datasets used for structure solutions were collected from crystals grown in the following crystallization conditions:

E8-MR1-5-OP-RU: 0.2 M Potassium thiocyanate, 0.1 M Bis-Tris propane pH 8.5 and 20 % w/v PEG 3350;

E8-MR1-K43A: 0.2 M Magnesium chloride hexahydrate, 0.1 M Tris pH 8.5 and 15 % w/v PEG 4000;

E8-MR1-6-FP: 0.2 M Sodium iodide, 0.1 M Bis-Tris propane pH 8.5 and 20 % w/v PEG 3350;
E8-MR1-3-F-SA: 0.2 M Sodium malonate dibasic monohydrate, 0.1 M Bis-Tris propane pH 8.5 and 20 % w/v PEG 3350;
E8-MR1-5-F-SA: 0.2 M Sodium fluoride, 0.1 M Bis-Tris propane pH 8.5 and 20 % w/v PEG 3350;
E8-MR1-3-F-BA: 0.1 M Magnesium acetate tetrahydrate, 0.1 M MOPS pH 7.5 and 12 % w/v PEG 8000;
E8-MR1-4-F-BA: 0.1 M HEPES pH 7.5, 20 % w/v PEG 4000 and 15 % Glycerol.

The diffraction data were integrated and scaled using the xia2 (Winter et al., 2013) automated processing pipeline using XDS (Kabsch, 2010) and XSCALE. The E8 TCR-MR1-5-OP-RU complex structure was solved by molecular replacement using MR1 and TCR coordinates from PDB 4PJA as the search models in Phaser (McCoy et al., 2007) within the CCP4 suite (Winn et al., 2011). The model was built using iterative cycles of manual model building in COOT (Emsley et al., 2010) and refinement using Refmac (Murshudov et al., 2011). The ligand restraints for refinement were generated using AceDRG (Long et al., 2017). All other E8-MR1 complex structures were solved using the E8 TCR-MR1-5-OP-RU structure (with the ligand removed) as the search model for molecular replacement in Phaser. Model building and refinement processes for these complexes were carried out as explained for the E8 TCR-MR1-5-OP-RU complex structure. The stereochemical properties and validation of the models were assessed using PDB-REDO (Joosten et al., 2012) and MolProbity (Williams et al., 2018). Buried surface area and TCR docking geometry statistics based on those described previously (Rudolph et al., 2006) were generated using Molecular Operating Environment (Chemical Computing Group) (Molecular Operating Environment (MOE)) The structural Figures were generated using Pymol (Schrödinger).The diffraction data were integrated and scaled using the

719 xia2 (Winter et al., 2013) automated processing pipeline using XDS (Kabsch, 2010) and
720 XSCALE. The E8 TCR-MR1-5-OPRU complex structure was solved by molecular
721 replacement using MR1 and TCR coordinates from PDB 4PJA as the search models in Phaser
722 (McCoy et al., 2007) within the CCP4 suite (Winn et al., 2011). The model was built using
723 iterative cycles of manual model building in COOT (Emsley et al., 2010) and refinement using
724 Refmac (Murshudov et al., 2011). The ligand restraints for refinement were generated using
725 AceDRG (Long et al., 2017). All other E8-MR1 complex structures were solved using the E8
726 TCR-MR1-5-OP-RU structure (with the ligand removed) as the search model for molecular
727 replacement in Phaser. Model building and refinement processes for these complexes were
728 carried out as explained for the E8 TCR-MR1-5-OP-RU complex structure. The stereochemical
729 properties and validation of the models were assessed using PDB-REDO (Joosten et al., 2012)
730 and MolProbity (Williams et al., 2018). Buried surface area and TCR docking geometry
731 statistics based on those described previously (Rudolph et al., 2006) were generated using
732 Molecular Operating Environment (Chemical Computing Group) (Molecular Operating
733 Environment (MOE)). The structural Figures were generated using Pymol (Schrödinger).
734
735 **Molecular dynamics simulations and MMPBSA calculations**
736 Molecular dynamics (MD) simulations were prepared and performed using the Amber18
737 software package (Song et al., 2019). In all cases, X-ray crystal structures were used as the
738 starting point for simulations and those used are listed in the Supplementary Methods.
739 Following structure preparation (His tautomerization states assignments, Asn/Gln flips,
740 protonation states set for an effective pH of 7), each structure was solvated in octahedral water
741 box with all crystallographic water molecules retained. His tautomerization states assignments
742 are provided in (Table S4**)**. The Amber ff14SB (Maier et al., 2015) force field and TIP3P water
743 model were used to describe protein and water molecules, respectively. For simulations with

744 5-OP-RU covalently bound to K43 a custom residue was built using a combination of ff14SB
745 (Maier et al., 2015) for the lysine unit and GAFF2 (Wang et al., 2004) for the connection and
746 5-OP-RU unit (with HF/6-31G(d) RESP fitted charges obtained using the R.E.D. server
747 (Vanquelef et al., 2011). To prepare each system for production MD simulations, a standard
748 procedure of energy minimization, heating, and equilibration was performed. Production MD
749 simulations were performed in the NPT ensemble at 300 K, and 1 atm using a 2 fs time step
750 (with the SHAKE algorithm applied). Production MD simulations were 100 ns long and for
751 each system 5 replicas were performed, with subsequent simulation analysis performed using
752 CPPTRAJ (Roe and Cheatham, 2013). Molecular mechanics Poisson-Boltzmann surface area
753 (MMPBSA) calculations were performed using MMPBSA.py.MPI (Miller et al., 2012) using
754 snapshots from the previously described MD simulations as input (snapshots were taken every
755 50 ps from 5 X 100 ns runs, so 10,000 snapshots per complex in total). The implicit salt
756 concentration was set to 150 mM. Calculated binding free energies were decomposed to their
757 per residue contributions using an internal dielectric constant of 1 (*i.e.* Protocol 1 in Table S4).
758
759 *System preparation.* All simulations were performed starting from crystal structures. For
760 simulations of AF7 MR1 with 5-OP-RU bound we used PDB 6PUC. For simulations of AF7
761 K43A without 5-OP-RU we manually removed the 5-OP-RU unit and mutated K43 to alanine
762 (as no structure exists of this mutant). For simulations of E8 MR1 with 5-OP-RU bound we
763 used PDB 7ZT2 (generated in this study). For simulations of E8 K43A without 5-OP-RU we
764 used PDB 7ZT3 (generated in this study). For the structure of AF7 MR1 with 5-OP-RU (PDB
765 6PUC), we performed simulations using chains A, B, G and H. Missing residues in chain A
766 190-195 were added using chain C of 6PUC (Awad et al., 2020) as the template. For the
767 structure of E8 MR1 K43A (produced in this study), chain A has missing residues 189-196.
768 We used the structure of E8 MR1 with 5-OP-RU (also produced in this study) as the template

for these residues. Optimal histidine tautomerisation states (see Table S4) and asparagine and glutamine side chain orientations were determined using MolProbity (Williams et al., 2018) and all residues except for His90 on MR1 were simulated in their standard protonation states based on pK_a calculations performed using PROPKA 3.1 (for a target pH of 7). His90 on MR1 was simulated as positively charged based on the PROPKA prediction and manual inspection. Further, the tautomerisation state of His58 on MR1 was manually assigned to be singly protonated on its Nε2 atom, with this atom coordinating the Schiff base nitrogen on K43-5-OP-RU, which acts as a hydrogen bond acceptor. Then structures were solvated in an octahedral water box, (with all crystallographic water molecules kept). The system box size was set such that at no solute atom was within 10 Å of the box boundary. To ensure the total system charge was 0, sodium or chloride ions were added as necessary.

Parametrisation of 5-OP-RU. The K43 residue on MR1 which is covalently bound (through a Schiff base) to 5-OP-RU was parameterized for MD simulations as a single (non-standard) amino acid. Here, we followed the recommended procedure as detailed in full here: https://upjv.q4md-forcefieldtools.org/Tutorial/Tutorial-4.php#16. First, we extracted the structure of K43-5-OP-RU from PDB:6PUC (Awad et al., 2020) alongside residues Q42 and E44 which were manually chemically modified in order to acetylate and amidate the backbone of K43. The structure was then optimized with QM (B3LYP/6-31G(d,p), tight convergence criteria) using Gaussian16 (Frisch et al., 2016). The resulting structure was then submitted to the R.E.D. Server (Vanquelef et al., 2011) for the partial charge calculation (with HF/6-31G(d) RESP fitted charges obtained). For these partial charge calculations, we charge-constrained the acetate and amidate groups to each have a total charge of 0. Atom type definitions for all atoms from the Lysine unit with the exception of the side chain nitrogen were described using the Amber ff14SB (Maier et al., 2015) force field, whilst atom types for the Lysine side chain

794 nitrogen and the 5OP-RU unit were described with GAFF2 (Wang et al., 2004) atom types. For
795 bond, angles, dihedrals and improper torsion terms that contained a mixture of ff14SB and
796 GAFF2 atom types, terms were taken from the GAFF2 library by analogy. Complete
797 parameters alongside an exemplar tleap script to use said parameters are deposited on zenodo
798 (https://zenodo.org/record/6651550).
799
800 *MD simulations.* The structure equilibration procedure was used to prepare all systems
801 simulated for production MD simulations in the NPT ensemble at 300 K and 1 atm. All
802 dynamics steps applied the SHAKE algorithm to constrain all bonds containing a hydrogen
803 atom. Replicas simulations were initiated from the second heating step of the following
804 protocol (with each replica therefore assigned different random velocity vectors at this stage).
805 Simulations performed in the NVT ensemble used Langevin temperature control (with a
806 collision frequency of 1 ps^{-1}) and used a simulation timestep of 1 fs. Simulations performed in
807 the NPT ensemble used Langevin temperature control (collision frequency of 1 ps^{-1}) and a
808 Berendsen barostat (1 ps pressure relaxation time).
809
810 The equilibration protocol is as follows: First, hydrogens atoms and solvent molecules were
811 energy minimised (using 500 steps of steepest descent followed by 500 steps of conjugate
812 gradient minimisation). To prevent the movement of non-hydrogen and non-solvent atoms
813 during the minimisation, 10 kcal mol^{-1} $Å^{-1}$ positional restraints were used to keep all heavy
814 atoms fixed. Then the solvent was heated rapidly from 50 K to 300 K (NVT ensemble, 1 fs
815 timestep) over the course of 200 ps, with the previously described restraints still maintained.
816 The positional restraints were then replaced with 5 kcal mol^{-1} $Å^{-1}$ positional restraints on only
817 the Cα carbon atoms of each residue and subjected to another round of energy minimisation
818 (500 steps of steepest descent followed by 500 steps of conjugate gradient). Retaining these

positional restraints, the system was heated from 25 K to 300 K over the course of 50 ps (NVT ensemble, 1 fs time step). Simulations were then performed in the NPT ensemble (1 atm, 300 K, 2 fs time step) by first gradually reducing the 5 kcal mol^{-1} $Å^{-1}$ Cα carbon restraints over the course of 50 ps. This was done by reducing the restraint weight by 1 kcal mol^{-1} $Å^{-1}$ every 10 ps. A final 1 ns long MD simulation with no restraints placed on the system was then performed, with the final structure produced after this run, used as the starting point for production MD simulations.

Informatics analysis of TCRs

Raw sequencing data was demultiplexed using Cutadapt v3.5 and the quality of the reads was checked using FastQC v0.11.9. MiXCR v3.0.13 (Bolotin et al., 2015) was used on the demultiplexed data to align the reads and assemble them into clonotypes, groups of reads sharing the same CDR3 sequence. No further trimming was performed as MiXCR by default handles reads containing low quality nucleotides, performs PCR error correction and conserves only clonotypes that would present a productive TCR. The output tables of MiXCR were loaded into R and sequences corroborated by only 1 read were filtered from the dataset unless they were present in both non-proliferating and proliferating subsets. Exploration of the Vβ, Jβ, CDR3β lengths as well as the analysis of the sequences carrying Arginine in positions 5, 6, 7 or 8 of the CDR3β was performed via custom R scripts. Statistical significance was determined using Fisher's exact test and correcting for multiple testing through the Benjamini-Hochberg method for contingency tables or using the Wilcoxon signed-rank test.

Statistical analysis

Cytokine secretion and luciferase assays as well as flow cytometry data were analyzed for normality using Shapiro-Wilk normality test. The appropriate statistical test is indicated in the figure legend and performed using Prism 9, GraphPad software. For SPICE analysis, multiple comparison Student's *t*-test was performed automatically using the freely available software SPICE 6.

Online supplemental material

Fig. S1 shows functional data relating to Figure 1.

Fig. S2 shows additional clone data.

Fig. S3 shows gating strategy.

Fig. S4 shows crystal structure images.

Fig. S5 shows data relating to TCR usage.

Tables S1 to S5 show additional statistics of crystal structures.

Data and materials availability

The crystal structures of E8 TCR and MR1-ligand complexes have been deposited with the PDB under Accessions # 7ZT2-9. T cell clones are available from G.D.L. under material transfer agreement with the University of Basel. New TCR sequences in this manuscript are available from G.D.L. under a material transfer agreement with the University of Basel. All other data are available in the article itself and in its supplementary materials.

Acknowledgements

The authors would like to thank Diamond Light Source for beam time, and the staff for assistance with crystal testing and data collection and Mariolina Salio for critical reading the manuscript. RMC's studentship is funded by the EPSRC. MWK is a BBSRC David Phillips Fellow (BB/M026280/1). This research made use of the Balena High Performance Computing (HPC) Service at the University of Bath. The MR1 tetramer technology was developed jointly by James McCluskey, Jamie Rossjohn and David Fairlie, and 5-OP-RU- and 6-FP-hMR1 tetramers were produced by the NIH Tetramer Core Facility as permitted to be distributed by the University of Melbourne. **Funding:** This work received funding from the Swiss National Foundation 310030-173240 and 310030B-192828, Krebsliga BeiderBasel KLbB-4779-02-2019, Swiss Cancer League KFS-4707-02-2019, D-BSSE ETH Zürich PMB-02-17 (all to GDL), and from the University of Basel 3MM1055 to AC.

Author contributions

A.C., R.S., R.C.K., V.N., A.B., G.B., R.C., V.K., J.M.P., V.T., H.G., R.J.S., K.P., R.M.C., A.V., C.D.G., V.S., D.C., T.G., A.L., M.H., V.Sri., R.A.R., G.S.B. and M.W.K. performed experiments, analyzed data, and provided critical inputs to the manuscript. R.Ca. and L.M. discussed data and revised the manuscript. A.C., D.K.C., G.D.L., and M.L. conceived and directed the project. A.C., D.K.C., G.D.L and M.L. wrote the manuscript. All authors reviewed and commented upon the manuscript.

Declaration of interests

R.S., R.C.K., V.K., J.M.P., V.T., H.G., R.J.S., K.P., T.G., A.L., M.H., V.Sri., R.A.R., D.K.C. and M.L. are or have been employees of Immunocore LTD. The authors have no additional financial interests.

892 **References**

893 Awad, W., G.J.M. Ler, W. Xu, A.N. Keller, J.Y.W. Mak, X.Y. Lim, L. Liu, S.B.G. Eckle, J.
894 Le Nours, J. McCluskey, A.J. Corbett, D.P. Fairlie, and J. Rossjohn. 2020. The
895 molecular basis underpinning the potency and specificity of MAIT cell antigens. *Nat*
896 *Immunol* 21:400-411.

897 Bagchi, S., Y. He, H. Zhang, L. Cao, I. Van Rhijn, D.B. Moody, J.E. Gudjonsson, and C.R.
898 Wang. 2017. CD1b-autoreactive T cells contribute to hyperlipidemia-induced skin
899 inflammation in mice. *J Clin Invest* 127:2339-2352.

900 Bendelac, A., O. Lantz, M.E. Quimby, J.W. Yewdell, J.R. Bennink, and R.R. Brutkiewicz.
901 1995. CD1 recognition by mouse NK1+ T lymphocytes. *Science* 268:863-865.

902 Bolotin, D.A., S. Poslavsky, I. Mitrophanov, M. Shugay, I.Z. Mamedov, E.V. Putintseva, and
903 D.M. Chudakov. 2015. MiXCR: software for comprehensive adaptive immunity
904 profiling. *Nat Methods* 12:380-381.

905 Cerundolo, V., J.D. Silk, S.H. Masri, and M. Salio. 2009. Harnessing invariant NKT cells in
906 vaccination strategies. *Nat Rev Immunol* 9:28-38.

907 Corbett, A.J., S.B. Eckle, R.W. Birkinshaw, L. Liu, O. Patel, J. Mahony, Z. Chen, R.
908 Reantragoon, B. Meehan, H. Cao, N.A. Williamson, R.A. Strugnell, D. Van Sinderen,
909 J.Y. Mak, D.P. Fairlie, L. Kjer-Nielsen, J. Rossjohn, and J. McCluskey. 2014. T-cell
910 activation by transitory neo-antigens derived from distinct microbial pathways.
911 *Nature* 509:361-365.

912 Cotton, R.N., A. Shahine, J. Rossjohn, and D.B. Moody. 2018. Lipids hide or step aside for
913 CD1-autoreactive T cell receptors. *Curr Opin Immunol* 52:93-99.

914 Crean, R.M., B.J. MacLachlan, F. Madura, T. Whalley, P.J. Rizkallah, C.J. Holland, C.
915 McMurran, S. Harper, A. Godkin, A.K. Sewell, C.R. Pudney, M.W. van der Kamp,
916 and D.K. Cole. 2020. Molecular Rules Underpinning Enhanced Affinity Binding of
917 Human T Cell Receptors Engineered for Immunotherapy. *Mol Ther Oncolytics*
918 18:443-456.

Crowther, M.D., G. Dolton, M. Legut, M.E. Caillaud, A. Lloyd, M. Attaf, S.A.E. Galloway, C. Rius, C.P. Farrell, B. Szomolay, A. Ager, A.L. Parker, A. Fuller, M. Donia, J. McCluskey, J. Rossjohn, I.M. Svane, J.D. Phillips, and A.K. Sewell. 2020. Author Correction: Genome-wide CRISPR-Cas9 screening reveals ubiquitous T cell cancer targeting via the monomorphic MHC class I-related protein MR1. *Nat Immunol*

Croxford, J.L., S. Miyake, Y.Y. Huang, M. Shimamura, and T. Yamamura. 2006. Invariant V(alpha)19i T cells regulate autoimmune inflammation. *Nat Immunol* 7:987-994.

de Jong, A., V. Pena-Cruz, T.Y. Cheng, R.A. Clark, I. Van Rhijn, and D.B. Moody. 2010. CD1a-autoreactive T cells are a normal component of the human alphabeta T cell repertoire. *Nat Immunol* 11:1102-1109.

Eckle, S.B., R.W. Birkinshaw, L. Kostenko, A.J. Corbett, H.E. McWilliam, R. Reantragoon, Z. Chen, N.A. Gherardin, T. Beddoe, L. Liu, O. Patel, B. Meehan, D.P. Fairlie, J.A. Villadangos, D.I. Godfrey, L. Kjer-Nielsen, J. McCluskey, and J. Rossjohn. 2014. A molecular basis underpinning the T cell receptor heterogeneity of mucosal-associated invariant T cells. *J Exp Med* 211:1585-1600.

Emsley, P., B. Lohkamp, W.G. Scott, and K. Cowtan. 2010. Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* 66:486-501.

Flament, H., M. Rouland, L. Beaudoin, A. Toubal, L. Bertrand, S. Lebourgeois, C. Rousseau, P. Soulard, Z. Gouda, L. Cagninacci, A.C. Monteiro, M. Hurtado-Nedelec, S. Luce, K. Bailly, M. Andrieu, B. Saintpierre, F. Letourneur, Y. Jouan, M. Si-Tahar, T. Baranek, C. Paget, C. Boitard, A. Vallet-Pichard, J.F. Gautier, N. Ajzenberg, B. Terrier, F. Pene, J. Ghosn, X. Lescure, Y. Yazdanpanah, B. Visseaux, D. Descamps, J.F. Timsit, R.C. Monteiro, and A. Lehuen. 2021. Outcome of SARS-CoV-2 infection is linked to MAIT cell activation and cytotoxicity. *Nat Immunol* 22:322-335.

Frisch, M.J., G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, G. Scalmani, V. Barone, G.A. Petersson, H. Nakatsuji, X. Li, M. Caricato, A.V. Marenich, J. Bloino, B.G. Janesko, R. Gomperts, B. Mennucci, H.P. Hratchian, J.V. Ortiz, A.F. Izmaylov, J.L. Sonnenberg, Williams, F. Ding, F. Lipparini, F. Egidi, J.

947 Goings, B. Peng, A. Petrone, T. Henderson, D. Ranasinghe, V.G. Zakrzewski, J. Gao,
948 N. Rega, G. Zheng, W. Liang, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J.
949 Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, K.
950 Throssell, J.A. Montgomery Jr., J.E. Peralta, F. Ogliaro, M.J. Bearpark, J.J. Heyd,
951 E.N. Brothers, K.N. Kudin, V.N. Staroverov, T.A. Keith, R. Kobayashi, J. Normand,
952 K. Raghavachari, A.P. Rendell, J.C. Burant, S.S. Iyengar, J. Tomasi, M. Cossi, J.M.
953 Millam, M. Klene, C. Adamo, R. Cammi, J.W. Ochterski, R.L. Martin, K. Morokuma,
954 O. Farkas, J.B. Foresman, and D.J. Fox. 2016. Gaussian 16 Rev. C.01. In
955 Wallingford, CT.

956 Garboczi, D.N., D.T. Hung, and D.C. Wiley. 1992. HLA-A2-peptide complexes: refolding
957 and crystallization of molecules expressed in Escherichia coli and complexed with
958 single antigenic peptides. *Proc Natl Acad Sci U S A* 89:3429-3433.

959 Genheden, S., and U. Ryde. 2015. The MM/PBSA and MM/GBSA methods to estimate
960 ligand-binding affinities. *Expert Opin Drug Discov* 10:449-461.

961 Gherardin, N.A., A.N. Keller, R.E. Woolley, J. Le Nours, D.S. Ritchie, P.J. Neeson, R.W.
962 Birkinshaw, S.B.G. Eckle, J.N. Waddington, L. Liu, D.P. Fairlie, A.P. Uldrich, D.G.
963 Pellicci, J. McCluskey, D.I. Godfrey, and J. Rossjohn. 2016. Diversity of T Cells
964 Restricted by the MHC Class I-Related Molecule MR1 Facilitates Differential
965 Antigen Recognition. *Immunity* 44:32-45.

966 Gherardin, N.A., M.N. Souter, H.F. Koay, K.M. Mangas, T. Seemann, T.P. Stinear, S.B.
967 Eckle, S.P. Berzins, Y. d'Udekem, I.E. Konstantinov, D.P. Fairlie, D.S. Ritchie, P.J.
968 Neeson, D.G. Pellicci, A.P. Uldrich, J. McCluskey, and D.I. Godfrey. 2018. Human
969 blood MAIT cell subsets defined using MR1 tetramers. *Immunol Cell Biol* 96:507-
970 525.

971 Gold, M.C., S. Cerri, S. Smyk-Pearson, M.E. Cansler, T.M. Vogt, J. Delepine, E. Winata,
972 G.M. Swarbrick, W.J. Chua, Y.Y. Yu, O. Lantz, M.S. Cook, M.D. Null, D.B. Jacoby,
973 M.J. Harriff, D.A. Lewinsohn, T.H. Hansen, and D.M. Lewinsohn. 2010. Human
974 mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol*
975 8:e1000407.

976 Gold, M.C., J.E. McLaren, J.A. Reistetter, S. Smyk-Pearson, K. Ladell, G.M. Swarbrick,
977 Y.Y. Yu, T.H. Hansen, O. Lund, M. Nielsen, B. Gerritsen, C. Kesmir, J.J. Miles, D.A.
978 Lewinsohn, D.A. Price, and D.M. Lewinsohn. 2014. MR1-restricted MAIT cells
979 display ligand discrimination and pathogen selectivity through distinct T cell receptor
980 usage. *J Exp Med* 211:1601-1610.

981 Harriff, M.J., C. McMurtrey, C.A. Froyd, H. Jin, M. Cansler, M. Null, A. Worley, E.W.
982 Meermeier, G. Swarbrick, A. Nilsen, D.A. Lewinsohn, W. Hildebrand, E.J. Adams,
983 and D.M. Lewinsohn. 2018. MR1 displays the microbial metabolome driving
984 selective MR1-restricted T cell receptor usage. *Sci Immunol* 3:

985 Holland, C.J., R.M. Crean, J.M. Pentier, B. de Wet, A. Lloyd, V. Srikannathasan, N. Lissin,
986 K.A. Lloyd, T.H. Blicher, P.J. Conroy, M. Hock, R.J. Pengelly, T.E. Spinner, B.
987 Cameron, E.A. Potter, A. Jeyanthan, P.E. Molloy, M. Sami, M. Aleksic, N. Liddy,
988 R.A. Robinson, S. Harper, M. Lepore, C.R. Pudney, M.W. van der Kamp, P.J.
989 Rizkallah, B.K. Jakobsen, A. Vuidepot, and D.K. Cole. 2020. Specificity of bispecific
990 T cell receptors and antibodies targeting peptide-HLA. *J Clin Invest* 130:2673-2688.

991 Howson, L.J., G. Napolitani, D. Shepherd, H. Ghadbane, P. Kurupati, L. Preciado-Llanes, M.
992 Rei, H.C. Dobinson, M.M. Gibani, K.W.W. Teng, E.W. Newell, N. Veerapen, G.S.
993 Besra, A.J. Pollard, and V. Cerundolo. 2018. MAIT cell clonal expansion and TCR
994 repertoire shaping in human volunteers challenged with Salmonella Paratyphi A. *Nat*
995 *Commun* 9:253.

996 Huang, S., S. Gilfillan, S. Kim, B. Thompson, X. Wang, A.J. Sant, D.H. Fremont, O. Lantz,
997 and T.H. Hansen. 2008. MR1 uses an endocytic pathway to activate mucosal-
998 associated invariant T cells. *J Exp Med* 205:1201-1211.

999 Joosten, R.P., K. Joosten, G.N. Murshudov, and A. Perrakis. 2012. PDB_REDO: constructive
1000 validation, more than just looking for errors. *Acta Crystallogr D Biol Crystallogr*
1001 68:484-496.

1002 Kabsch, W. 2010. Xds. *Acta Crystallogr D Biol Crystallogr* 66:125-132.

1003 Keller, A.N., S.B. Eckle, W. Xu, L. Liu, V.A. Hughes, J.Y. Mak, B.S. Meehan, T. Pediongco,
1004 R.W. Birkinshaw, Z. Chen, H. Wang, C. D'Souza, L. Kjer-Nielsen, N.A. Gherardin,
1005 D.I. Godfrey, L. Kostenko, A.J. Corbett, A.W. Purcell, D.P. Fairlie, J. McCluskey,
1006 and J. Rossjohn. 2017. Drugs and drug-like molecules can modulate the function of
1007 mucosal-associated invariant T cells. *Nat Immunol* 18:402-411.

1008 Kjer-Nielsen, L., O. Patel, A.J. Corbett, J. Le Nours, B. Meehan, L. Liu, M. Bhati, Z. Chen,
1009 L. Kostenko, R. Reantragoon, N.A. Williamson, A.W. Purcell, N.L. Dudek, M.J.
1010 McConville, R.A. O'Hair, G.N. Khairallah, D.I. Godfrey, D.P. Fairlie, J. Rossjohn,
1011 and J. McCluskey. 2012. MR1 presents microbial vitamin B metabolites to MAIT
1012 cells. *Nature* 491:717-723.

1013 Koay, H.F., N.A. Gherardin, C. Xu, R. Seneviratna, Z. Zhao, Z. Chen, D.P. Fairlie, J.
1014 McCluskey, D.G. Pellicci, A.P. Uldrich, and D.I. Godfrey. 2019. Diverse MR1-
1015 restricted T cells in mice and humans. *Nat Commun* 10:2243.

1016 Laugel, B., D.K. Cole, M. Clement, L. Wooldridge, D.A. Price, and A.K. Sewell. 2011. The
1017 multiple roles of the CD8 coreceptor in T cell biology: opportunities for the selective
1018 modulation of self-reactive cytotoxic T cells. *J Leukoc Biol* 90:1089-1099.

1019 Le Bourhis, L., E. Martin, I. Peguillet, A. Guihot, N. Froux, M. Core, E. Levy, M. Dusseaux,
1020 V. Meyssonnier, V. Premel, C. Ngo, B. Riteau, L. Duban, D. Robert, S. Huang, M.
1021 Rottman, C. Soudais, and O. Lantz. 2010. Antimicrobial activity of mucosal-
1022 associated invariant T cells. *Nat Immunol* 11:701-708.

1023 Legoux, F., D. Bellet, C. Daviaud, Y. El Morr, A. Darbois, K. Niort, E. Procopio, M. Salou,
1024 J. Gilet, B. Ryffel, A. Balvay, A. Foussier, M. Sarkis, A. El Marjou, F. Schmidt, S.
1025 Rabot, and O. Lantz. 2019. Microbial metabolites control the thymic development of
1026 mucosal-associated invariant T cells. *Science* 366:494-499.

1027 Lepore, M., A. Kalinichenko, S. Calogero, P. Kumar, B. Paleja, M. Schmaler, V. Narang, F.
1028 Zolezzi, M. Poidinger, L. Mori, and G. De Libero. 2017. Correction: Functionally
1029 diverse human T cells recognize non-microbial antigens presented by MR1. *Elife* 6:

1030 Lepore, M., A. Kalinichenko, A. Colone, B. Paleja, A. Singhal, A. Tschumi, B. Lee, M.
1031 Poidinger, F. Zolezzi, L. Quagliata, P. Sander, E. Newell, A. Bertoletti, L.
1032 Terracciano, G. De Libero, and L. Mori. 2014. Parallel T-cell cloning and deep
1033 sequencing of human MAIT cells reveal stable oligoclonal TCRbeta repertoire. *Nat*
1034 *Commun* 5:3866.

1035 Li, Y., R. Moysey, P.E. Molloy, A.L. Vuidepot, T. Mahon, E. Baston, S. Dunn, N. Liddy, J.
1036 Jacob, B.K. Jakobsen, and J.M. Boulter. 2005. Directed evolution of human T-cell
1037 receptors with picomolar affinities by phage display. *Nat Biotechnol* 23:349-354.

1038 Liddy, N., G. Bossi, K.J. Adams, A. Lissina, T.M. Mahon, N.J. Hassan, J. Gavarret, F.C.
1039 Bianchi, N.J. Pumphrey, K. Ladell, E. Gostick, A.K. Sewell, N.M. Lissin, N.E.
1040 Harwood, P.E. Molloy, Y. Li, B.J. Cameron, M. Sami, E.E. Baston, P.T. Todorov,
1041 S.J. Paston, R.E. Dennis, J.V. Harper, S.M. Dunn, R. Ashfield, A. Johnson, Y.
1042 McGrath, G. Plesa, C.H. June, M. Kalos, D.A. Price, A. Vuidepot, D.D. Williams,
1043 D.H. Sutton, and B.K. Jakobsen. 2012. Monoclonal TCR-redirected tumor cell killing.
1044 *Nat Med* 18:980-987.

1045 Long, F., R.A. Nicholls, P. Emsley, S. Graaeulis, A. Merkys, A. Vaitkus, and G.N.
1046 Murshudov. 2017. AceDRG: a stereochemical description generator for ligands. *Acta*
1047 *Crystallogr D Struct Biol* 73:112-122.

1048 Lopez-Sagaseta, J., C.L. Dulberger, A. McFedries, M. Cushman, A. Saghatelian, and E.J.
1049 Adams. 2013. MAIT recognition of a stimulatory bacterial antigen bound to MR1. *J*
1050 *Immunol* 191:5268-5277.

1051 Maier, J.A., C. Martinez, K. Kasavajhala, L. Wickstrom, K.E. Hauser, and C. Simmerling.
1052 2015. ff14SB: Improving the Accuracy of Protein Side Chain and Backbone
1053 Parameters from ff99SB. *J Chem Theory Comput* 11:3696-3713.

1054 Mallevaey, T., A.J. Clarke, J.P. Scott-Browne, M.H. Young, L.C. Roisman, D.G. Pellicci, O.
1055 Patel, J.P. Vivian, J.L. Matsuda, J. McCluskey, D.I. Godfrey, P. Marrack, J. Rossjohn,
1056 and L. Gapin. 2011. A molecular basis for NKT cell recognition of CD1d-self-
1057 antigen. *Immunity* 34:315-326.

1058 Martin, E., E. Treiner, L. Duban, L. Guerri, H. Laude, C. Toly, V. Premel, A. Devys, I.C.
1059 Moura, F. Tilloy, S. Cherif, G. Vera, S. Latour, C. Soudais, and O. Lantz. 2009.
1060 Stepwise development of MAIT cells in mouse and human. *PLoS Biol* 7:e54.

1061 McCoy, A.J., R.W. Grosse-Kunstleve, P.D. Adams, M.D. Winn, L.C. Storoni, and R.J. Read.
1062 2007. Phaser crystallographic software. *J Appl Crystallogr* 40:658-674.

1063 McMahon, C.W., and D.H. Raulet. 2001. Expression and function of NK cell receptors in
1064 CD8+ T cells. *Curr Opin Immunol* 13:465-470.

1065 McWilliam, H.E., S.B. Eckle, A. Theodossis, L. Liu, Z. Chen, J.M. Wubben, D.P. Fairlie,
1066 R.A. Strugnell, J.D. Mintern, J. McCluskey, J. Rossjohn, and J.A. Villadangos. 2016.
1067 The intracellular pathway for the presentation of vitamin B-related antigens by the
1068 antigen-presenting molecule MR1. *Nat Immunol* 17:531-537.

1069 Miller, B.R., 3rd, T.D. McGee, Jr., J.M. Swails, N. Homeyer, H. Gohlke, and A.E. Roitberg.
1070 2012. MMPBSA.py: An Efficient Program for End-State Free Energy Calculations. *J*
1071 *Chem Theory Comput* 8:3314-3321.

1072 Miyazaki, Y., S. Miyake, A. Chiba, O. Lantz, and T. Yamamura. 2011. Mucosal-associated
1073 invariant T cells regulate Th1 response in multiple sclerosis. *Int Immunol* 23:529-535.

1074 Molecular Operating Environment (MOE), C.C.G.U., 1010 Sherbooke St. West, Suite #910,
1075 Montreal, QC, Canada, H3A 2R7, 2022.

1076 Murshudov, G.N., P. Skubak, A.A. Lebedev, N.S. Pannu, R.A. Steiner, R.A. Nicholls, M.D.
1077 Winn, F. Long, and A.A. Vagin. 2011. REFMAC5 for the refinement of
1078 macromolecular crystal structures. *Acta Crystallogr D Biol Crystallogr* 67:355-367.

1079 Narayanan, G.A., J.E. McLaren, E.W. Meermeier, K. Ladell, G.M. Swarbrick, D.A. Price,
1080 J.G. Tran, A.H. Worley, T. Vogt, E.B. Wong, and D.M. Lewinsohn. 2020. The MAIT
1081 TCRbeta chain contributes to discrimination of microbial ligand. *Immunol Cell Biol*
1082 98:770-781.

1083 Patel, O., L. Kjer-Nielsen, J. Le Nours, S.B. Eckle, R. Birkinshaw, T. Beddoe, A.J. Corbett,
1084 L. Liu, J.J. Miles, B. Meehan, R. Reantragoon, M.L. Sandoval-Romero, L.C. Sullivan,
1085 A.G. Brooks, Z. Chen, D.P. Fairlie, J. McCluskey, and J. Rossjohn. 2013.
1086 Recognition of vitamin B metabolites by mucosal-associated invariant T cells. *Nat*
1087 *Commun* 4:2142.

1088 Perroteau, J., B. Navet, M.C. Devilder, L. Hesnard, E. Scotet, L. Gapin, X. Saulquin, and L.
1089 Gautreau-Rolland. 2020. Contribution of the SYK Tyrosine kinase expression to
1090 human iNKT self-reactivity. *Eur J Immunol* 50:1454-1467.

1091 Picelli, S., O.R. Faridani, A.K. Bjorklund, G. Winberg, S. Sagasser, and R. Sandberg. 2014.
1092 Full-length RNA-seq from single cells using Smart-seq2. *Nat Protoc* 9:171-181.

1093 Porcelli, S., M.B. Brenner, J.L. Greenstein, S.P. Balk, C. Terhorst, and P.A. Bleicher. 1989.
1094 Recognition of cluster of differentiation 1 antigens by human CD4-CD8-cytolytic T
1095 lymphocytes. *Nature* 341:447-450.

1096 Porcelli, S., C.E. Yockey, M.B. Brenner, and S.P. Balk. 1993. Analysis of T cell antigen
1097 receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells
1098 demonstrates preferential use of several V beta genes and an invariant TCR alpha
1099 chain. *J Exp Med* 178:1-16.

1100 Roe, D.R., and T.E. Cheatham, 3rd. 2013. PTRAJ and CPPTRAJ: Software for Processing
1101 and Analysis of Molecular Dynamics Trajectory Data. *J Chem Theory Comput*
1102 9:3084-3095.

1103 Rouxel, O., J. DaSilva, L. Beaudoin, I. Nel, C. Tard, L. Cagninacci, B. Kiaf, M. Oshima, M.
1104 Diedisheim, M. Salou, A. Corbett, J. Rossjohn, J. McCluskey, R. Scharfmann, M.
1105 Battaglia, M. Polak, O. Lantz, J. Beltrand, and A. Lehuen. 2018. Author Correction:
1106 Cytotoxic and regulatory roles of mucosal-associated invariant T cells in type 1
1107 diabetes. *Nat Immunol* 19:1035.

1108 Rudolph, M.G., R.L. Stanfield, and I.A. Wilson. 2006. How TCRs bind MHCs, peptides, and
1109 coreceptors. *Annu Rev Immunol* 24:419-466.

1110 Salio, M., O. Gasser, C. Gonzalez-Lopez, A. Martens, N. Veerapen, U. Gileadi, J.G. Verter,
1111 G. Napolitani, R. Anderson, G. Painter, G.S. Besra, I.F. Hermans, and V. Cerundolo.
1112 2017. Activation of Human Mucosal-Associated Invariant T Cells Induces CD40L-
1113 Dependent Maturation of Monocyte-Derived and Primary Dendritic Cells. *J Immunol*
1114 199:2631-2638.

1115 Schmaler, M., A. Colone, J. Spagnuolo, M. Zimmermann, M. Lepore, A. Kalinichenko, S.
1116 Bhatia, F. Cottier, T. Rutishauser, N. Pavelka, A. Egli, E. Azzali, M. Pieroni, G.
1117 Costantino, P. Hruz, U. Sauer, L. Mori, and G. De Libero. 2018. Modulation of
1118 bacterial metabolism by the microenvironment controls MAIT cell stimulation.
1119 *Mucosal Immunol* 11:1060-1070.

1120 Seach, N., L. Guerri, L. Le Bourhis, Y. Mburu, Y. Cui, S. Bessoles, C. Soudais, and O. Lantz.
1121 2013. Double-positive thymocytes select mucosal-associated invariant T cells. *J*
1122 *Immunol* 191:6002-6009.

1123 Sharma, P.K., E.B. Wong, R.J. Napier, W.R. Bishai, T. Ndung'u, V.O. Kasprowicz, D.A.
1124 Lewinsohn, D.M. Lewinsohn, and M.C. Gold. 2015. High expression of CD26
1125 accurately identifies human bacteria-reactive MR1-restricted MAIT cells.
1126 *Immunology* 145:443-453.

1127 Song, L.F., T.S. Lee, C. Zhu, D.M. York, and K.M. Merz, Jr. 2019. Using AMBER18 for
1128 Relative Free Energy Calculations. *J Chem Inf Model* 59:3128-3135.

1129 Souter, M.N.T., W. Awad, S. Li, T.J. Pediongco, B.S. Meehan, L.J. Meehan, Z. Tian, Z.
1130 Zhao, H. Wang, A. Nelson, J. Le Nours, Y. Khandokar, T. Praveena, J. Wubben, J.
1131 Lin, L.C. Sullivan, G.O. Lovrecz, J.Y.W. Mak, L. Liu, L. Kostenko, K. Kedzierska,
1132 A.J. Corbett, D.P. Fairlie, A.G. Brooks, N.A. Gherardin, A.P. Uldrich, Z. Chen, J.
1133 Rossjohn, D.I. Godfrey, J. McCluskey, D.G. Pellicci, and S.B.G. Eckle. 2022. CD8
1134 coreceptor engagement of MR1 enhances antigen responsiveness by human MAIT
1135 and other MR1-reactive T cells. *J Exp Med* 219:

1136 Tilloy, F., E. Treiner, S.H. Park, C. Garcia, F. Lemonnier, H. de la Salle, A. Bendelac, M.
1137 Bonneville, and O. Lantz. 1999. An invariant T cell receptor alpha chain defines a

novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. *J Exp Med* 189:1907-1921.

Toubal, A., B. Kiaf, L. Beaudoin, L. Cagninacci, M. Rhimi, B. Fruchet, J. da Silva, A.J. Corbett, Y. Simoni, O. Lantz, J. Rossjohn, J. McCluskey, P. Lesnik, E. Maguin, and A. Lehuen. 2020. Mucosal-associated invariant T cells promote inflammation and intestinal dysbiosis leading to metabolic dysfunction during obesity. *Nat Commun* 11:3755.

Treiner, E., L. Duban, S. Bahram, M. Radosavljevic, V. Wanner, F. Tilloy, P. Affaticati, S. Gilfillan, and O. Lantz. 2003. Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* 422:164-169.

Vanquelef, E., S. Simon, G. Marquant, E. Garcia, G. Klimerak, J.C. Delepine, P. Cieplak, and F.Y. Dupradeau. 2011. R.E.D. Server: a web service for deriving RESP and ESP charges and building force field libraries for new molecules and molecular fragments. *Nucleic Acids Res* 39:W511-517.

Wang, J., R.M. Wolf, J.W. Caldwell, P.A. Kollman, and D.A. Case. 2004. Development and testing of a general amber force field. *J Comput Chem* 25:1157-1174.

Williams, C.J., J.J. Headd, N.W. Moriarty, M.G. Prisant, L.L. Videau, L.N. Deis, V. Verma, D.A. Keedy, B.J. Hintze, V.B. Chen, S. Jain, S.M. Lewis, W.B. Arendall, 3rd, J. Snoeyink, P.D. Adams, S.C. Lovell, J.S. Richardson, and D.C. Richardson. 2018. MolProbity: More and better reference data for improved all-atom structure validation. *Protein Sci* 27:293-315.

Winn, M.D., C.C. Ballard, K.D. Cowtan, E.J. Dodson, P. Emsley, P.R. Evans, R.M. Keegan, E.B. Krissinel, A.G. Leslie, A. McCoy, S.J. McNicholas, G.N. Murshudov, N.S. Pannu, E.A. Potterton, H.R. Powell, R.J. Read, A. Vagin, and K.S. Wilson. 2011. Overview of the CCP4 suite and current developments. *Acta Crystallogr D Biol Crystallogr* 67:235-242.

1164 Winter, G., C.M. Lobley, and S.M. Prince. 2013. Decision making in xia2. *Acta Crystallogr*
1165 *D Biol Crystallogr* 69:1260-1273.

1166 Xiao, Z., Y. Cong, K. Huang, S. Zhong, J.Z.H. Zhang, and L. Duan. 2019. Drug-resistance
1167 mechanisms of three mutations in anaplastic lymphoma kinase against two inhibitors
1168 based on MM/PBSA combined with interaction entropy. *Phys Chem Chem Phys*
1169 21:20951-20964.

1170 Young, M.H., L. U'Ren, S. Huang, T. Mallevaey, J. Scott-Browne, F. Crawford, O. Lantz,
1171 T.H. Hansen, J. Kappler, P. Marrack, and L. Gapin. 2013. MAIT cell recognition of
1172 MR1 on bacterially infected and uninfected cells. *PLoS One* 8:e53789.

1173 Zoete, V., M.B. Irving, and O. Michielin. 2010. MM-GBSA binding free energy
1174 decomposition and T cell receptor engineering. *J Mol Recognit* 23:142-152.
1175

1176

Figure Legends

Figure 1. Self-reactivity and polyfunctionality of circulating MAIT cells from healthy donors.

(A) CD137 expression by autoreactive MAIT cells expanded for 10 days. Proliferating and not proliferating MAIT (top row) or non-MAIT (bottom row) cells following stimulation with the indicated APCs ± anti-MR1 mAb (aMR1). MAIT cells ($V\alpha7.2^+/CD161^+$) proliferative status is revealed by Cell Trace Violet (CTV) emission. Plots are representative of results obtained with 6 donors. **(B)** Summary of MAIT cell CD137 expression on proliferating cells (CTV dull) after rechallenge with the indicated condition (numbers as in panel A). Data obtained from 6 donors. Statistical significance was determined using a one-way ANOVA with Friedman test, * $p \leq 0.05$. **(C)** Effect of aMR1 mAb on surface expression of the indicated activation markers on CTV-dull MAIT cells stimulated with 5-OP-RU-pulsed THP-1 cells (top row) or with A375b-wtMR1 cells without exogenous antigens (bottom row). Median fluoresce intensity (MFI) is indicated ± aMR1 mAb. Data obtained from 5 donors. Statistical significance was determined using Student's t-test, * $p \leq 0.05$, ** $p \leq 0.01$. **(D)** Vα7.2 surface expression on MAIT cells stimulated with 5-OP-RU-pulsed THP-1 cells (top row) or with A375b-wtMR1 cells without exogenous antigens (bottom row). Median fluoresce intensity (MFI) is indicated ± aMR1 mAb. Data obtained from 5 donors. Statistical significance was determined using Student's t-test * $p \leq 0.05$. **(E)** Percentage of *ex vivo* MAIT cells from healthy donors double positive for CD137 and CD69 after overnight co-culture with A375b-MR1 cells ± aMR1 mAb. Stimulation with 5-OP-RU was used as positive control with scale on the right-hand y-axis (green). Cells were pre-gated as $CD3^+$/ $CD26^+V\alpha7.2^+/CD161^+$. Data are a summary of all 5 donors tested. Statistical significance was determined using Student's t-test * $p \leq 0.05$. **(F)** Average frequency of cells expressing one or more of the indicated activation-associated

molecules within self-reactive MAIT cell lines stimulated with A375b-wtMR1 cells or **(G)** 5-OP-RU-loaded THP-1 cells. Pie segments indicate cells positive for any combination of the indicated cytokines or activation markers. Pie arcs indicate the cytokine positivity of each segment. Data is averaged from 5 donors.

Figure 2. Self-reactivity and T-helper like functions of MAIT cell clones.

(A) Release of IFN-γ by three MAIT cell clones after co-culture with $1x10^5$ THP-1 cells and indicated concentrations of the microbial Ag 5-OP-RU. IFN-γ is reported as mean ± SD of triplicate cultures. The data is representative of 3 independent experiments. **(B)** Release of IFN-γ by three MAIT cell clones in A in response to A375b-wtMR1 cells ± aMR1 mAb. 5-OP-RU pulsed THP-1 cells were used as a positive control. IFN-γ release is shown as mean ± SD of triplicate cultures. The data is representative of a 3 independent experiments, *** $p \leq 0.001$. **(C)** Percentage of J.RT3-T3.5 cells expressing surface CD69 after incubation with A375b-wtMR1 cells ± aMR1 mAb. A375b-wtMR1 cells pulsed with 5-OP-RU were used as a positive control. J.RT3-T3.5 cell lines express the TCR of the indicated MAIT cell clones. The data is representative of 3 independent experiments, *** $p \leq 0.001$. **(D)** Release of IFN-γ by three MAIT cell clones stimulated with monocyte-derived DCs (moDCs) ± 5-OP-RU ± aMR1 mAb or ± Ac-6-FP. moDCs pulsed with 5-OP-RU were used as a positive control. IFN-γ release is mean ± SD of triplicate cultures. The data is representative of 3 independent experiments, *** $p \leq 0.001$. **(E)** Expression levels of the surface maturation markers CD83, CD86 and CD40 on moDCs after overnight co-culture with the BC75B31 (left panels) and BC75B38 (right panels) MAIT cell clones. The data is representative of 2 independent experiments. Statistical significance in all cases was determined using one-way ANOVA with Dunn multiple comparison test.

Figure 3. Cross-reactivity of circulating MAIT cells from healthy donors.

(A) Percentage of $CD137^+$ MAIT cells following activation by A375b-MR1 cells ± aMR1 mAb. MAIT cell lines were previously generated from two donors by *in vitro* expansion with 5-OP-RU. **(B)** Plots of MAIT cell lines stained with MR1-3-F-SA tetramer vs. CTV. Cells were pre-gated on $CD161^+$ cells. Data was obtained from a total of 2 donors. **(C)** Representative plots of MAIT cell lines stained with MR1-6-FP tetramer vs. CTV. Cells were pre-gated on $CD161^+$ cells. Data was obtained from a total of 2 donors. **(D)** Populations of MAIT cells that are double positive for MR1-3-F-SA and MR1-6-FP tetramers in the same two donors (B, C and D). **(E)** Frequency of MR1-5-OP-RU, -6-FP, -3-F-SA or -5-F-SA MR1 $tetramer^+$ cells from two additional MAIT cell lines derived from the peripheral blood of donors 3 and 4. Pie segments indicate cells positive for any combination of the 4 tetramer sets. Pie arcs indicate the tetramer positivity of each segment. Percentages indicate the total number of cells positive for at least one tetramer. (F) Percentage of *ex vivo* MAIT cells from healthy donors stained with at least one of three tetramers: CD8-null MR1-3-F-SA, -5-F-SA or -6-FP. MAIT cells were pre-gated on live $CD3^+$/ $V\alpha7.2^+/CD161^+/CD26^+$ cells. Non-MAIT cells were pre-gated on live $CD3^+/V\alpha7.2^-$ cells. Statistical significance was determined using Student's t-test * $p \leq 0.05$.

Figure 4. Broad self-reactivity and promiscuous recognition of MR1 ligands by the E8 TCR.

(A) Recognition of primary immune cells in the absence (black bars) or presence (white bars) of 5-OP-RU by E8 TCR transduced NFAT-Luciferase TCR-null B2M knock out Jurkat cells. **(B)** Recognition of primary immune cells in the absence (black bars) or presence (white bars) of 5-OP-RU by VT001 TCR-transduced NFAT-Luciferase TCR-null B2M knock out Jurkat cells. **(C)** Recognition of lymphoma cell lines by NFAT-Luciferase TCR-null B2M knock out

Jurkat cells expressing the E8 TCR (black bars), the VT001 TCR (white bars), or the E8 TCR in the presence of blocking aMR1 mAb (grey bars). (A, B, C) Luminescence measured following NFAT-driven luciferase activity is shown as the cumulative relative luminescence units (RLU) data from 3 experiments with mean ± SD of duplicate cultures. **(D)** Binding affinities, as measured by surface plasmon resonance, of the E8 TCR interacting with wildtype MR1 refolded with the indicated range of MR1 ligands, and the empty MR1-K43A mutant. Dissociation constant values (K_D) are indicated ± standard error. >150μM: the measured K_D of the TCR MR1 interaction greater than 150μM and therefore is unlikely to elicit a MAIT cell response. The very high binding affinity of the E8 TCR to MR1 5-OP-RU was measured using the BIAcore8K^{TM} using single cycle kinetic analysis. The remaining measurements were performed on a BiacoreT200 and the K_Ds were calculated using steady state analysis. **(E)** Binding affinities, as measured by surface plasmon resonance, of the control AF-7 TCR interacting with wildtype MR1 refolded with the indicated range of MR1 ligands, and MR1-K43A. Dissociation constant values (K_D) are indicated ± standard error. >150μM: the measured K_D of the TCR MR1 interaction was greater than 150μM and therefore is unlikely generate a MAIT cell response.

Figure 5. Structural and energetic basis of promiscuous recognition of MR1 by the E8 TCR.

(A) The structures of the E8 TCR (TRAV in green, TRBV in cyan) bound to MR1 loaded (in grey) with 5-OP-RU (shown as red sticks) aligned to the AF-7 TCR (TRAV in light blue and TRBV in dark Blue) bound to MR1 5-OP-RU (PDB 6PUC) (Awad et al., 2020). **(B)** Surface map of the MR1 binding footprint of the AF-7 TCR (alpha in light blue and beta in dark blue) as in Ref. (Awad et al., 2020). A vector is drawn connecting the disulphide in the alpha chain variable domain (light blue sphere) to the disulphide in the beta chain variable domain (dark

blue sphere). **(C)** Surface map of the MR1 binding footprint of the E8 TCR (alpha in green and beta in cyan). A vector is drawn connecting the disulphide in the alpha chain variable domain (green sphere) to the disulphide in the beta chain variable domain (cyan sphere). **(D)** The structures of the AF-7 CDR3α Y95 residue light (blue sticks) and E8 CDR3α Y95 residue (green sticks) showing polar interaction (dotted line) with 5-OP-RU (red sticks) bound to MR1 (grey) (Awad et al., 2020). (E) Superimposed structures of the CDR3β R96 residue (cyan sticks) in E8 TCRs that forms salt bridges to the residues E76 and E149 (grey sticks) in MR1 loaded with ligands (5-OP-RU, 6-FP, 3-F-SA, 5-F-SA, 3-F-BA and 4-F-BA). **(F)** Calculated per-residue differences (5-OP-RU-wtMR1 minus K43A-MR1) in the binding free energy for both the AF-7 and E8 TCRs with (5-OP-RU-wtMR1) and without (K43A-MR1) 5-OP-RU bound to MR1. A blue residue is more favourable in the 5-OP-RU form, whilst a red residue is more favourable in the MR1-K43A form. Yellow arrows indicate the position of 5-OP-RU. **(G)** Calculated per-residue contributions to the binding free energy for the E8 TCR-MR1 complex with 5-OP-RU bound. The MR1 and TCR molecules are shown as surfaces and colour mapped according to their MMPBSA calculated per residue decomposition energies. Colour mapping goes from blue (favourable binding) to white (neutral) to red (unfavourable binding) as indicated by the colour bar.

Figure 6. Enrichment of R96 in self-reactive MAIT cells.

(A) Frequencies of TRBV6^{+}, CDR3L13^{+} MAIT cells with either R95, R96, R97 or R98 motif within either self-reactive, proliferated MAIT cells (CTV-) or non-self-reactive, non-proliferated MAIT cells (CTV+). Statistical significance was determined using Fisher's exact test, * $p \leq 0.05$. **(B)** Activation of J.RT3-T3.5 cells transduced with 393 TRBV (a MAIT TRBV bearing the E8-like motif) or with the control MRC25 TCRBV gene. Percentage of CD69^{+} cells after co-culture with the indicated APC ± anti-MR1 mAb are illustrated. 5-OP-RU pulsed

THP-1 cells were used as a positive control. Data are representative of 3 individual experiments each performed in triplicate. Statistical significance was determined using Student's t-test, *** $p \leq 0.001$. **(C)** Frequency of TRBV sequences with the E8-like motif within *ex vivo* MAIT cells (TRAV1-2^{+}/TRBV6^{+}, and CD161^{+}) or non-MAIT cells (TRAV1-2^{+}/TRBV6^{+}, and CD161^{-}) sorted from the periphery of 7 healthy donors (Lepore et al., 2014). Statistical significance was determined using Wilcoxon signed-rank test, * $p \leq 0.05$.

Table 1. TCR sequences used in this manuscript

TCRα			
Clone Name	TRAV	TRAJ	CDR3
MRC25	TRAV1-2	TRAJ33	CAVVDSNYQLI
BC75B31	TRAV1-2	TRAJ33	CAVMDSNYQLI
BC75B38	TRAV1-2	TRAJ33	CAVMDSNYQLI
SMC3	TRAV1-2	TRAJ33	CASMDSNYQLI
E8	TRAV1-2	TRAJ33	CAVKDSNYQLIW
AF-7	TRAV1-2	TRAJ33	CAFLDSNYQLIW

TCRβ				
Clone Name	TRBV	TRBD	TRBJ	CDR3
MRC25	TRBV6-1	TRBD2*01	TRBJ2-7	CASRLMSGSSYEQYF
BC75B31	TRBV4-2	TRBD1*01	TRBJ2-1	CASSHGSTGAYNEQFF
BC75B38	TRBV4-3	TRBD2*02	TRBJ2-1	CASSQDPSGSYNEQFF
SMC3	TRBV20-1	TRBD2*01	TRBJ2-3	CSAKVTSGQHQGTTDTQYF
E8	TRBV6-1/5	-	TRBJ1-6	CASSNREYSPLHF
AF-7	TRBV6-1	TRBD2*01	TRBJ2-2	CASSVWTGEGSGELFF
393*	TRBV6-4	-	TRBJ2-3	CASSDREADTQYF

*Paired with TCRα chain from the clone SMC3

Table 2. Effects of the MR1 E76Q E149Q salt bridge mutations on the binding affinities of AF-7 TCR, E8 TCR and E8 TRBV6-1 TCR

	MR1 Wt			MR1 E76Q E149Q		
TCR	5-OP-RU	6-FP	5-FSA	5-OP-RU	6-FP	5-FSA
AF-7	1 ± 0.02 μM	>150 μM	>150μM	0.6 ± 0.03 μM	>150 μM	>150 μM
E8	1.3 ± 0.1 nM	0.6 ± 0.07 μM	0.2 ± 0.03 μM	11.2 ± 1.5 nM	3.5 ± 0.2 μM	1.8 ± 0.2 μM
E8 TRBV6-1	80.3 ± 8.3 nM	10.8 ± 0.7 μM	6.9 ± 0.9 μM	0.6 ± 0.05 μM	42.1 ± 2.6 μM	32.1 ± 3.7 μM

Binding affinities, as measured by surface plasmon resonance, of the AF-7, E8 TCR and E8 TRBV6-1 TCR interacting with wildtype MR1 and MR1 E76Q E149Q refolded with 5-OP-RU, 6-FP and 5-FSA. >150 μM: the measured K_D of the TCR MR1 interaction was greater than 150 μM and therefore is unlikely to generate a MAIT cell response. Dissociation constant values (K_D) are indicated with the standard error in brackets. The binding affinity of the E8 TCR to MR1 5-OP-RU and MR1 E76Q E149Q 5-OP-RU was measured using the BIAcore8K™ using single cycle kinetic analysis. The remining measurements were completed on a BiacoreT200™ and the K_Ds were calculated using steady state analysis.

Table 3. Calculated binding free energy differences between the AF7 and E8 TCR

		MMPBSA Protocol 1		MMPBSA Protocol 2	
TCR	MR1 Complex	ΔG (kcal mol^{-1})	$\Delta\Delta G_{(5\text{-OP-RU-K43A})}$ (kcal mol^{-1})	ΔG (kcal mol^{-1})	$\Delta\Delta G_{(5\text{-OP-RU-K43A})}$ (kcal mol^{-1})
AF-7	wt-5-OP-RU	-8.6 ± 3.2	*-19.6	-28.9 ± 1.1	*-8.7
	Empty (K43A)	11.1 ± 5.3		-20.2 ± 3.3	
E8	wt-5-OP-RU	-0.8 ± 4.1	2.8	-24 ± 1.6	*-0.9
	Empty (K43A)	-3.6 ± 3.8		-23.1 ± 1.9	

*A negative ΔΔG means the 5-OP-RU bound form is more favourable than the MR1-K43A form. Errors are presented as the standard deviation from the 5 replicas. MMPBSA Protocols

1 and 2 differ by the choice of internal protein dielectric constant setting. For Protocol 1, the internal dielectric constant is set to 1, for Protocol 2, the internal dielectric constant is set to 4.