Promiscuous recognition of MR1 drives self-reactive mucosal-associated invariant T cell responses

Chancellor, Andrew; Alan Simmons, Robert; Khanolkar, Rahul C; Nosi, Vladimir; Beshirova, Aisha; Berloffa, Giuliano; Colombo, Rodrigo; Karuppiah, Vijaykumar; Pentier, Johanne M; Tubb, Vanessa; Ghadbane, Hemza; Suckling, Richard J; Page, Keith; Crean, Rory M; Vacchini, Alessandro; De Gregorio, Corinne; Schaefer, Verena; Constantin, Daniel; Gligoris, Thomas; Lloyd, Angharad

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2 **self-reactive Mucosal-Associated Invariant T cell responses**

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4 Andrew Chancellor[1]†‡*, Robert Simmons[2]†, Rahul C. Khanolkar[2], Vladimir Nosi[1,3], Aisha

5 Beshirova[1], Giuliano Berloffa[1], Rodrigo Colombo[1], Vijaykumar Karuppiah[2], Johanne M.

6 Pentier[2], Vanessa Tubb[2], Hemza Ghadbane[2], Richard J. Suckling[2], Keith Page[2], Rory M.

7 Crean[4,5], Alessandro Vacchini[1], Corinne De Gregorio[1], Verena Schaefer[1], Daniel Constantin[1],

8 Thomas Gligoris[2], Angharad Lloyd[2], Miriam Hock[2], Velupillai Srikannathasan[2], Ross A.

9 Robinson[2], Gurdyal S. Besra[7], Marc W. van der Kamp[6], Lucia Mori[1], Raffaele Calogero[3],

10 David K. Cole[2#], Gennaro De Libero[1*#], Marco Lepore[2*#]

11

12 [1]Experimental Immunology, Department of Biomedicine, University Hospital Basel,

13 University of Basel, 4031 Basel, Switzerland.

14 [2]Immunocore Ltd., Milton Park, Abingdon, UK.

15 [3]Department of Molecular Biotechnology and Health Sciences, University of Torino, 10126

16 Torino, Italy.

17 [4]Department of Biology and Biochemistry, University of Bath, Bath, UK.

18 [5]Doctoral Training Centre in Sustainable Chemical Technologies, University of Bath, Bath,

19 UK.

20 [6]School of Biochemistry, University of Bristol, Biomedical Sciences Building, University

21 Walk, Bristol, UK.

22 [7]School of Biosciences, Institute of Microbiology and Infection, University of Birmingham,

23 Edgbaston, B15 2TT Birmingham, United Kingdom.

24 †These authors contributed equally.

25 [#] These authors contributed equally.

26   ‡Primary corresponding author

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

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

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

30

31   **Non-standard abbreviations:** 5-OP-RU, 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil;

32   MAIT cell, mucosal associated invariant T cell; MR1, MHC-class-I-related protein 1; moDC,

33   monocyte-derived dendritic cell; RU, response unit; SPR, surface plasmon resonance.

34

35   **Summary**

36   Promiscuous recognition of MR1 by canonical MAIT TCRs endowed with dual reactivity to

37   both microbial and self-antigens enables MAIT cell responses in the absence of microbial

38   infection.

39

40

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.

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).

89   Several MAIT TCR-MR1 crystal structures demonstrated a highly conserved binding mode

90   that is similar to the conformation adopted by canonical HLA class I- and HLA class II-

91   restricted TCRs (Awad et al., 2020; Corbett et al., 2014; Eckle et al., 2014; Patel et al., 2013).

92   This binding mode positions the invariant MAIT TCRα orthogonally above the Ag,

93   establishing a conserved and essential hydrogen bond with the ribityl moiety of the riboflavin-

94   related agonists via the residue Y95α (encoded by the TRAJ gene segments) (Corbett et al.,

95   2014). Despite the conserved mode of MR1 binding of the invariant TCR Vα, the TCRβ chain

96   has an important role, in influencing Ag recognition and magnitude of the T cell activation

97   (Eckle et al., 2014; Gherardin et al., 2016)

98

99   Although MR1 is ubiquitously expressed, the majority of MR1 molecules remain unfolded

100   within the endoplasmic reticulum (ER) until an abundance of Ags is available for loading

101   (McWilliam et al., 2016). MR1 stabilization is achieved through neutralization of K43 within

102   the Ag binding pocket, allowing egress to the surface. However, small quantities of MR1 are

103   commonly expressed on the cell surface, consistent with the recognition of different cell types

104   such as dendritic cells, epithelial cells and cancer cells by MR1T cell clones that do not react

105   to microbial Ags (Lepore et al., 2017). This implies a wider role for MR1-restricted T cells in

106   immunity (Crowther et al., 2020; Lepore et al., 2017). Recently, additional MAIT cell agonists

107   beyond 5-OP-RU have been identified (Harriff et al., 2018; Keller et al., 2017) and MAIT cells

108   can distinguish between different Ags through their TCR Vβ-chain, indicating the MAIT TCR

109   repertoire may be shaped by available Ags (Gherardin et al., 2016; Gold et al., 2014; Howson

110   et al., 2018; Lopez-Sagaseta et al., 2013).

111

112   The reactivity of MAIT cells toward self has not been systematically investigated since initial

113   experiments, in which mouse MAIT cells were stimulated by uninfected fibroblasts and B cells

114 (Huang et al., 2008). Recent studies extended these observations to humans using MR1

115 tetramers and structural analyses, and suggested that MAIT TCRs can be cross-reactive toward

116 other MR1 ligands, including some common drugs and potential self-Ags (Gherardin et al.,

117 2016; Huang et al., 2008; Keller et al., 2017; Young et al., 2013). Further reports on MAIT

118 cells have indicated that while bacterial colonization of the gut is required for their thymic

119 selection and peripheral expansion, MAIT cells are still present in the periphery of germ-free

120 mice, albeit at a much lower frequency (Koay et al., 2019; Legoux et al., 2019), implying

121 microbial-independent thymic selection. Consistently, MAIT cell alterations in inflammatory

122 diseases with no direct microbial aetiology, such as diabetes, multiple sclerosis and obesity,

123 have been reported (Croxford et al., 2006; Miyazaki et al., 2011 34; Rouxel et al., 2018; Toubal

124 et al., 2020). Furthermore, activation of MAIT cells upon challenge with SARS-CoV-2 infected

125 macrophages has been observed (Flament et al., 2021). Overall, these data suggest that some

126 MAIT cells could become activated in a microbial-independent manner.

127

128 Here, we set out to define the extent and mechanisms of MR1-mediated MAIT cell self-

129 reactivity. We demonstrate that microbial-independent MR1 reactivity of a canonical MAIT

130 cell subset is commonly observed in healthy individuals. Furthermore, we describe a novel

131 mode of promiscuous MR1 recognition by a MAIT TCR, associated with a CDR3β-motif

132 enriched in circulating self-reactive MAIT cells.

133

134

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.

160   S1D) and upregulation of additional T cell activation markers in response to both 5-OP-RU-

161   pulsed THP-1 and A375b-wtMR1 cells (Fig. 1C). Notably, both stimuli also induced an MR1-

162   dependent decrease of TCR surface levels, as detected with anti-Vα7.2 mAb staining,

163   suggestive of activation-induced TCR downregulation (Fig. 1D).

164

165   We next asked whether self-reactive MAIT cells could also be identified *ex vivo* following

166   short-term stimulation. A small but detectable fraction of freshly purified MAIT cells

167   upregulated CD137 and CD69 after overnight culture with A375b-MR1 cells (mean 1.1%, Fig.

168   1E) in the absence of microbial Ags. As expected, a large majority of MAIT cells responded

169   instead to 5-OP-RU (Fig. 1E). Anti-MR1 blocking mAb prevented MAIT cell activation in the

170   control culture (mean 0.1%). Remarkably, *ex vivo* self-reactive MR1-restricted responses were

171   consistently detected in circulating MAIT cells from all the seven donors investigated.

172

173   Finally, we compared the functional response of *in vitro* expanded self-reactive MAIT cells to

174   either 5-OP-RU or A375b-wtMR1 cells and did not observe major qualitative differences

175   between the two stimuli (Fig. 1F, G and Fig. S1E).

176

177   Taken together, these data indicated that MAIT cells endowed with dual reactivity to microbial

178   Ags and cell-endogenous MR1-Ag complexes are present in circulating T cells of healthy

179   donors, as they expand *in vitro* and are detectable *ex vivo* following co-culture with MR1-

180   overexpressing tumor cells. Furthermore, microbial and cell-endogenous stimulation elicited a

181   comparable effector program within *in vitro*-expanded self-reactive MAIT cells.

182

183

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 Vα7.2$^+$ cells and subsequent expansion with PHA stimulation were challenged with 4 sets

234   of MR1 tetramers loaded with either 5-OP-RU, 6-FP, 5-F-SA, and 3-F-SA (Fig S3D-G). MAIT

235   cells were identified as CD161$^+$ CD3$^+$ MR1-5-OP-RU tetramer$^+$ (Fig. S3E, F and G) whereas

236   CD161$^-$ CD3$^+$ MR1-5-OP-RU tetramer$^-$ cells represented control non-MAIT cells (Fig. S3E, F

237   and G). Within the MAIT group, 23.9% (donor 3) and 11.4% (donor 4) of cells bound to at

238   least one of the other 3 tetramers bearing different ligands (Fig. 3E and S3F, G). Distinct

239   patterns of tetramer cross-reactivity were observed, with some cells binding to all 4 tetramer

240   sets (Fig. 3E and S3F, G). In contrast, non-MAIT cells infrequently reacted to any of the

241   tetramers (3.4%, donor 3, and 3.1%, donor 4; (Fig. 3E and S3F, G).

242

243   Finally, we estimated the proportion of cross-reactive MAIT cells able to bind MR1-tetramers

244   loaded with non-microbial Ags directly *ex vivo* (Fig. 3F). As previous studies have reported

245   that CD8 has an important impact on MR1-dependent MAIT cell reactivity to microbial

246   metabolites due to its ability to bind to MR1 (Souter et al., 2022), we used the same mutated

247   MR1 tetramers devoid of the CD8 binding capacity (CD8-null). With these reagents, we could

248   strictly assess TCR-dependent binding and rule out any potential contribution of CD8.

249   Significantly more MAIT cells bound to at least one of the three CD8-null tetramers loaded

250   with 6-FP, 5-F-SA, or 3-F-SA ligands (mean 2.7% of MAIT cells) as compared to the control

251   non-MAIT cell group (0.4% of non-MAIT) (Fig. 3F).

252

253   Taken together, these data sets indicated that a proportion of MAIT cells feature different

254   degrees of promiscuous TCR interaction with MR1 complexes presenting non-microbial

255   ligands, thus suggesting that TCR cross-reactivity is not uncommon within MAIT cells.

256

257

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

283   nanomolar to low micromolar affinities (K_D range = 0.002 – 0.6 μM) with MR1-5-OP-RU

284   being the strongest affinity ligand (Fig. 4D). In contrast, the AF-7 TCR bound to MR1-5-OP-

285   RU with K_D = 1.1 μM, but not to other immobilized MR1-ligand complexes (Fig. 4E). Thus,

286   the E8 TCR is an extremely promiscuous classical MAIT TCR that displays self-reactivity

287   towards healthy and tumor cells and binding to a range of soluble MR1 ligand complexes.

288

289   **Structural basis of promiscuous MR1 recognition by the E8 TCR**

290   To understand the molecular basis of the broad E8 TCR reactivity, we solved the crystal

291   structures of the E8 TCR in complex with MR1-5-OP-RU, -6-FP, -3-F-SA, -5-F-SA, -3-F-BA,

292   -4-F-BA and empty MR1-K43A between 1.84 Å and 2.4 Å resolution (Fig. 5A, Fig. S4A and

293   Table S1). The structures of the E8 TCR were aligned to the structure of the classical MAIT

294   TCR AF-7 (PDB: 6PUC) bound to MR1-5-OP-RU (Awad et al., 2020), (Fig. 5A). The E8 and

295   AF-7 TCRs bound to MR1 in a very similar mode (Fig. 5, A, B and C). They possess the same

296   chain pairing and virtually identical amino acid sequences in their complementarity

297   determining region (CDR) loops (Table 1) that adopted identical positions and made a very

298   similar network of contacts with both the MR1 surface and 5-OP-RU (Fig. 5, A, B, C and D

299   and Table S2). Like the AF-7 TCR, the E8 TCR contacted the ribityl chain of 5-OP-RU with

300   the Y95α residue in the CDR3α loop (Fig. 5D and table S2). The only difference in contacts

301   with MR1-5-OP-RU between the E8 TCR and the AF-7 MAIT TCR was mediated by the E8

302   TCR residue R96β in the CDR3β loop, which made additional salt bridges with MR1 residues

303   E76 and E149 (Fig. 5E and Table S2). The structures of the E8 TCR in complex with MR1-5-

304   OP-RU, -K43A, -6-FP, -3-F-SA, -5-F-SA, -3-F-BA and -4-F-BA revealed an almost identical

305   network of contacts (Table S3). No ligands except 5-OP-RU contributed to TCR binding,

306   providing a possible explanation of the stronger affinity and greater potency of this TCR for

307   MR1-5-OP-RU complexes (Table S3). Together these results suggest that the TCR residue

308   R96β drives broad recognition of MR1-ligand complexes by forming salt bridges to the MR1

309   heavy chain.

310

311   To further explore the hypothesis that the salt bridge interaction network mediated by E8 TCR

312   residue R96β was important for the broad recognition of multiple MR1 ligands, MR1 residues

313   E76 and E149 were mutated to Q76 and Q149. E (Glu) and Q (Gln) have very similar

314   physiochemical properties, but Q lacks the anionic carboxylate group that is required to form

315   a salt bridge. Thus, we reasoned that these mutations would abrogate the salt bridge interaction

316   whilst maintaining the overall binding mode of the E8 TCR, and likely still enable some

317   interaction with MR1 residues 76 and 149. The mutated form of MR1 (MR1 E76Q E149Q)

318   was refolded in complex with 5-OP-RU, 6-FP, and 5-FSA, and the soluble protein was used to

319   investigate the binding affinity of the E8 TCR. In addition, we measured the binding affinities

320   to a version of the E8 TCR engrafted with TRBV6-1 (to control for the chimeric output of the

321   phage display) and the canonical AF7 TCR (Table 2). In line with the structural analysis, the

322   binding affinity of the AF7 TCR was not substantially affected by the MR1 mutations: we

323   observed a K_D = 0.6 µM for MR1 E76Q E149Q in complex with 5-OP-RU compared to a K_D

324   = 1 µM for MR1-5-OP-RU. In contrast, the binding affinity of the E8 TCR to MR1 E76Q

325   E149Q was substantially reduced compared to its binding affinity to MR1 wildtype bound to

326   all ligands tested (8 to 10-fold reduction in binding affinity) (Table 2 and source data file).

327   Similar observations were made with the E8 TRBV6-1 TCR, with an 8-fold reduction in

328   binding affinity for MR1 E76Q E149Q. Previous studies have shown that affinity reductions

329   within this range can abrogate MAIT cell recognition of MR1 ligands (Patel et al., 2013),

330   indicating that the salt bridge interactions mediated by R96β are likely to be central to

331   biologically relevant recognition of MR1 by the E8 TCR. Altogether, these data support the

332   structural analysis and demonstrate that the salt bridge interaction between E8 TCR residue

333   R96β and MR1 residues E76 and E149 plays a central role in cross-ligand recognition.

334

335   **Energetic basis of promiscuous MR1 recognition by the E8 TCR**

336   To further explore the basis for the promiscuous behavior of the E8 TCR, we performed

337   molecular dynamics (MD) simulations and binding free energy calculations. These were made

338   on both the AF-7 and E8 TCR bound to wtMR1 loaded with 5-OP-RU and to empty MR1-

339   K43A. The MD simulations allowed us to sample the conformational space available to each

340   complex and snapshots from these simulations were used to calculate the binding free energy

341   using the molecular mechanics Poisson-Boltzmann surface area (MMPBSA) approach

342   (Genheden and Ryde, 2015; Miller et al., 2012). We utilized two different MMPBSA protocols

343   to predict $\Delta\Delta G$ (Table 3 and Table S4). For the AF-7 TCR-MR1 complex with and without 5-

344   OP-RU bound we obtained $\Delta\Delta G$ values of -19.6 and -8.7 kcal mol^{-1} respectively, with the 5-

345   OP-RU bound complex consistently predicted to have a substantially higher affinity (Table 2).

346   In contrast, using the same two protocols for the E8 TCR, we obtained $\Delta\Delta G$ values of +2.8 and

347   -0.9 kcal mol^{-1} (Table 3), demonstrating our calculations can reproduce the ligand dependent

348   behaviour of the AF-7 TCR and promiscuous behaviour of E8 TCR.

349

350   The MMPBSA approach also allows the decomposition of calculated binding free energy

351   differences into per-residue contributions, which we and others have successfully utilized to

352   identify the key residues and interactions across the binding interface that drive affinity (Crean

353   et al., 2020; Holland et al., 2020; Xiao et al., 2019; Zoete et al., 2010). Thus, we used the

354   MMPBSA approach to understand why the AF-7 and the E8 TCRs have differing ligand

355   dependencies. To this end, the per-residue binding contributions of each TCR to MR1 loaded

356   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.

407   S5C). No significant difference among proliferating and non-proliferating cells were found in

408   the usages of *TRBJ* (Fig. S5D), *TRBD* (Fig. S5E) genes, nor in the lengths of CDR3β (Fig.

409   S5F). In addition, no significant differences were seen when combinations of genes were

410   analyzed Fig. S5G). Thus, the motif outlined by the E8 TCR and represented by the

411   combination of *TRBV6*, CDR3L13 with R96 might be used to identify and track a population

412   of *bona fide* self-reactive MAIT cells.

413

414   To validate the presence of MAIT cells displaying this motif, we searched within previously

415   acquired TCRβ-chain datasets from healthy donor's circulating TRAV1-2$^+$/TRBV6$^+$/CD161$^+$

416   (MAIT cells) and TRAV1-2$^+$/TRBV6$^+$/CD161$^-$ (non-MAIT) (Lepore et al., 2014). In 7

417   different donors, the motif was observed in 1.73-2.4% of MAIT TCR transcripts (median

418   1.99%) (Fig. 6C). In contrast, the same motif was detected in 1.28-1.88% (median 1.52%)of

419   TCR transcripts from T cells expressing Vα7.2$^+$ and lacking CD161 (Fig. 6C). Taken together,

420   these findings suggested that in healthy individuals a small fraction of MAIT cells express the

421   E8-like *TRBV6*, CDR3L13-R96β motif, which we found associated with self-reactive

422   recognition of MR1.

423

424   **Discussion**

425   Here we describe a population of cross-reactive human MAIT cells that are activated upon

426   recognition of MR1 in the absence of microbial Ags. The functional responses we investigated

427   did not qualitatively diverge from those induced by the microbial Ag 5-OP-RU and non-

428   transformed target cells expressing low, physiological levels of MR1 were sufficient for

429   productive stimulation of tested clones. Our data revealed that self-reactive recognition of MR1

430   is a feature of some MAIT cell TCRs, enabling recognition of both tumor and healthy cells.

431   We estimated that self-reactive MAIT cells are rare within circulating T cells of healthy

432   individuals, and whether their frequency is increased and/or their function altered in patients

433   with autoimmunity, inflammatory diseases, and cancer deserves appropriate clinical

434   investigation.

435   As self-reactive MAIT cells are present in healthy individuals, an important question arises

436   regarding their regulation *in vivo*. MAIT cells exhibit an effector memory phenotype seemingly

437   due to the abundance of microbial Ags that leads to a continual stimulation (Legoux et al.,

438   2019; Seach et al., 2013). Such frequent stimulation promotes the expression of several

439   regulatory molecules, including NK inhibitory receptors and immune checkpoint controls

440   (McMahon and Raulet, 2001), that may allow self-reactivity of certain MAIT cells only in

441   circumstances when they are not engaged. The regulation of MR1 levels on APCs could also

442   play a role, only enabling MAIT cell stimulation when enough stimulatory self-Ags are

443   available for loading, or when the 'correct' Ags for self-reactive MAIT cells are present.

444   Another possibility supported by our *in vitro* data, suggests that self-reactive MAIT cells can

445   expand during microbial infections. Thus, after peripheral expansion by abundant microbial

446   Ags, rare high-affinity self-reactive MAIT TCRs might increase in frequency and subsequently

447   respond to MR1-mediated stimulation in non-infectious settings. Finally, MAIT cell self-

448   reactivity could also be promoted by high SYK Tyrosine kinase expression, which facilitates

449   productive TCR signalling as shown with recognition of CD1d-self lipids by self-reactive

450   human iNKT cells (Perroteau et al., 2020).

451

452   Self-reactive MR1 recognition is a clear indication of MAIT TCR cross-reactivity, which we

453   found to be represented at different degrees within MAIT cells, thus confirming and extending

454   previous results (Gherardin et al., 2016; Keller et al., 2017). A significant fraction of both *in*

455   *vitro* expanded and *ex-vivo* analyzed 5-OP-RU-reactive MAIT TCRs appear to be

456   promiscuous, and able to bind MR1 tetramers loaded with distinct non-microbial ligands. Of

457   note, a gradient of promiscuity is observed, where some MAIT TCRs can bind either one, two,

458   or three different MR1 tetramers. An elegant study recently reported a key role of CD8 in

459   enhancing MAIT cell response to 5-OP-RU via lateral binding to MR1 (Souter et al., 2022).

460   The same study also indicated the CD8-MR1 interaction as crucial for the recognition of the

461   weak folate Ags (*e.g.,* 6-FP) by MR1-restricted T cells. Thus, both MR1- and conventional

462   MHC-I-restricted T cells use the CD8 co-receptor to amplify TCR-depended responses, with

463   the greatest impact in the case of low-affinity interactions with Ags (Laugel et al., 2011). Our

464   experiments with both CD8-enabled and CD8-disabled MR1 tetramers, whilst supporting these

465   findings, also highlight the presence of cross-reactive MAIT TCRs that are less influenced by

466   CD8 for the binding of MR1-ligand complexes. Accordingly, CD8-negative MAIT cells are

467   commonly found in both humans and mice, albeit at much lower frequencies (Gherardin et al.,

468   2018; Martin et al., 2009).

469

470   An important consideration relates to the non-microbial Ags we and others used to load MR1-

471   tetramers. To our knowledge, these are all physiologically uncommon small molecules that are

472   buried within the MR1 pocket, and therefore not easily accessible for direct contact with TCRs.

473   Thus, a hypothesis that deserves further investigation is whether this type of cross-reactive

474   recognition relies on particular ligand-depended conformations of MR1 sensed by specific

475   TCRs rather than direct TCR-ligand interactions, as already observed within the CD1 system

476   (Cotton et al., 2018). In addition, unique TCR features could also enable broad productive

477   interactions with distinct MR1-ligand complexes as exemplified by the extremely promiscuous

478   E8 TCR mode of MR1 recognition. Here, classical MAIT TCR primary interactions with both

479   5-OP-RU and MR1 led to robust cell activation, as previously described (Corbett et al., 2014).

480   In addition, key interactions between the CDR3β loop and the MR1 α-helices enabled

481   recognition of a variety of MR1-Ag complexes and contributed to the high affinity for 5-OP-

482   RU. The E8 TCR residue R96β forms a double salt bridge with MR1 residues E76 and E149,

483   allowing productive recognition of MR1 without direct ligand interaction or further stabilizing

484   the complex in the presence of 5-OP-RU. Thus, the two MR1 residues behave as a tweezer,

485   anchoring the TCR residue R96β. MD simulations supported the importance of R96β-mediated

486   interaction in enabling promiscuous and 5-OP-RU-enhanced MR1 recognition compared to the

487   canonical MAIT TCR, AF-7 (Eckle et al., 2014). In addition, biophysical analysis, using MR1

488   with conservative mutations at residues E76 and E149 to disrupt the salt bridge, confirmed the

489   central role of R96β in driving biologically relevant Ag cross-recognition, and the MD

490   simulations recapitulated the binding affinities for the TCRs to different tested ligands and

491   demonstrated a major energetic role mediated by the E8 TCR CDR3β residue R96.

492

493   Our findings fit with the current view of MAIT TCR-MR1-Ag interaction. An elegant study

494   using a series of Ag analogs revealed the importance of a network of polar interactions between

495   TCR, MR1 and Ag, called the interaction triad, that is critical for MAIT cell activation (Awad

496   et al., 2020). Major contributions of different residues within the CDR3β loop were revealed,

497   in some instances directly interacting with the MR1-bound Ag. Another study assigned a

498   degree of autoreactivity to the M33.64 MAIT TCR in which two residues in the CDR3β loop

499   (Thr100β and Asn99β) pinched residue Glu149 of MR1, forming a stable bond (Gherardin et

500   al., 2016). Instead, in the case of the E8 TCR described here, two MR1 residues (E76 and E149)

501   clamp the R96β within the CDR3β. These different modes of binding underline a variety of

502   mechanisms enabling CDR3β-mediated MAIT cell autoreactivity, further describing the

503   unusual plasticity of this interaction. The role of the TCRβ chain in modulating and fine tuning

504   the response of invariant TCRs to Ag in the context of non-polymormic molecules has been

505   previously highlighted within the CD1d/semi-invariant iNKT TCR system (Mallevaey et al.,

506   2011). In that study, a hydrophobic motif promoting association with CD1d was found in the

507   CDR3β loops of TCR from self-reactive iNKT cells. This motif facilitated the iNKT response

508   to a broad range of CD1d-restricted self-Ags. It was also described that by transferring this

509   CDR3 β loops into the Vβ6 chain, the resulting TCR started to interact with self-CD1d

510   tetramers. Analogous findings related to the contribution of TCRβ in fine-tuning responses

511   were also subsequently reported for MAIT TCR recognition of the 5-OP-RU Ag (Eckle et al.,

512   2014; Narayanan et al., 2020).

513   Thus, the immune system deploys distinct mechanisms to extend its breadth of Ag recognition

514   in the case of non-polymorphic Ag-presenting molecules and maximize its ability to interact

515   with these targets.

516

517   Last, but not least, the potential Ags involved in the self-reactivity of some MAIT TCRs to

518   both tumor and healthy cells deserve further consideration. Whilst we cannot exclude that some

519   Ag recognition is shared between these cell types, we anticipate that promiscuous recognition

520   of different Ags plays a large role. Some outstanding questions remain, including the identity

521   of recognized Ags, their abundance, expression in healthy *vs*. tumor cells, and their impact on

522   the immune function of MAIT cells.

523

524   In conclusion, our data indicate that a discrete population of MAIT cells is endowed with MR1-

525   dependent self-reactivity toward tumor and healthy cells. In addition, our work provides

526   compelling evidence that TCR cross-reactivity is not infrequent within MAIT cells and is

527   characterized by degrees of promiscuity toward distinct MR1-Ag complexes. The self-reactive

528   recognition of MR1 by MAIT cells may have important physiological and immunological

529   implications. Within T cells restricted to non-polymorphic Ag-presenting molecules self- and

530   tumor-reactivity is also observed towards CD1a, CD1b, CD1c, and CD1d molecules (Bagchi

531   et al., 2017; Bendelac et al., 1995; de Jong et al., 2010; Lepore et al., 2014; Porcelli et al.,

532   1989). On one hand, these autoreactive T cells might participate in inflammatory and

533   autoimmune diseases, sustained by unbalanced immune homeostasis and/or play a role in

534   cancer surveillance/progression. On the other hand, the consistent presence of these self-

535   reactive cells in healthy individuals and their T-helper-like properties *in vitro* suggest they may

536   also have regulatory and/or homeostatic functions, as previously proposed for other non-MHC

537   restricted T cell populations such as iNKT cells, canonical non-self-reactive MAIT cells and

538   MR1T cells (Cerundolo et al., 2009; Lepore et al., 2017; Salio et al., 2017). Future studies in

539   different cohorts of patients will address the immunological roles of promiscuous Ag

540   recognition by MAIT TCRs.

541

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

567 of commonly misidentified cell lines. Cells lines were not authenticated. All primary cell lines

568 and clones used in this study were isolated from PBMCs obtained using Lymphoprep (Stemcell

569 Technologies) from blood of blood bank donors and maintained in culture as previously

570 described (Lepore et al., 2014). Briefly, MAIT cell lines were generated by magnetic bead

571 enrichment using biotinylated anti-Vα7.2 mAb (Clone 3C10, Biolegend) or specific expansion

572 using 5-OP-RU. Enriched MAIT cells were prelabelled with Cell Trace Violet according to

573 manufacturer instructions and then cultured with irradiated A375b-wtMR1 cells for the

574 indicated number of days in a 1:1 ratio. Human rIL-2 (5 IU/ml, Peprotech) was added at day 5

575 and thereafter every two days. Cells were washed and rechallenged as indicated (ratio 2:1) in

576 the presence or absence of purified anti-MR1 mAb (20µg/ml, Ultra-LEAF™ Purified Clone

577 26.5, Biolegend). From these lines, self-reactive MAIT clones were derived by limiting dilution

578 in the presence of PHA (1 µg/ml), human rIL-2 (100 U/ml) and irradiated PBMC

579 (5×10^5 cells/ml), and screened for reactivity toward indicated cell. CD14$^+$ monocytes were

580 isolated from PBMCs by positive selection using magnetic beads (Stemcell Technologies) and

581 cultured in the presence of 25 ng/ml GM-CSF and 20 ng/ml IL-4 (both from Peprotech).

582 Activation of monocytes was achieved by treating them with 50 ng/ml LPS (from *Escherichia*

583 *coli* O111:B4, Sigma) overnight.

584

585 **T cell activation**

586 T cell clones (5×10^4 cells/well unless otherwise indicated) were co-cultured with indicated

587 target cells (5×10^4 cells /well) in 130 µl total volume in triplicates for 18 h. In some

588 experiments, anti-MR1 mAb (20 µg/ml, Ultra-LEAF™ Purified Clone 26.5, Biolegend) were

589 added and incubated for 30 min prior to the addition of T cells. In other experiments, APCs

590 were pulsed for 2 h at 37°C with indicated concentrations of Ags or freshly-prepared 5-OP-RU

591 as described in (Schmaler et al., 2018). J.RT3-T3.5 activation assays were performed in a 1:1

592 ratio with the indicated APC for 18 h. Cells were then either harvested and stained for surface

593 CD69 upregulation or luciferase was measured using Bio-Glo (Promega).

594

595 **TCR gene transfer**

596 Total RNA was extracted from snap-frozen cell pellets from each clone. SMARTer RACE 5'/3'

597 kit (Takara) was used for cDNA synthesis and generation of TCR transcripts. Functional TCRα

598 and β chains were identified by sequencing and analysis using the ImMunoGeneTics

599 information system (http://www.imgt.org). The TCRα and β sequences were either synthesized

600 at Integrated DNA Technologies (TCR 393) or amplified from cDNA with gene specific

601 primers (TCRs BC75B31, BC75B38, MRC25) containing cloning adaptors. In both cases the

602 insert was cloned by In-Fusion HD (Takara) to a lentiviral vector for co-transfection of HEK

603 293 T LX cells. The endotoxin-free vectors were co-transfected together with the lentivirus

604 packaging plasmids pMD2.G, pMDLg/pRRE and pRSV-REV (all from Addgene) to HEK 293

605 T LX cells with Metafectene PRO reagent from Biontex. Lentiviral supernatants of the

606 corresponding TCRα and β sequences were combined and used to transduce J.RT3-T3.5 cells

607 overnight. TCR-expressing J.RT3-T3.5 cells were sorted for CD3 expression before functional

608 analysis. For the experiments described in Fig. 4, TCR genes were transduced in NFAT-

609 Luciferase TCR-null B2MKO Jurkat cells developed from the parental NFAT-Luciferase

610 Jurkat line (Promega, part n. J133A).

611

612 **Flow cytometry**

613 When staining with MR1 tetramers (20 µg/ml) or anti-human Vα7.2 (2.5µg/ml Clone 3C10,

614 Biolegend), the cells were pre-treated for 30 min at 37°C with 50 nM dasatinib (Sigma) in PBS.

615 All mAb for staining were titrated on appropriate cells before use. Tetramers were added first

616 for 20 min at RT and anti-human mAb were added for a further 20 min in PBS with dasatinib:

617   mAb specific for CD3 (Clone UCHT1), CD4 (Clone OKT4), CD8 (Clone RPA-T8), CD161

618   (Clone HP-3G19) and for activation markers CD137 (Clone 4B4-1), CD69 (Clone FN50),

619   CD25 (Clone BC96), ICOS (Clone DX29), all from Biolegend. DAPI was used to exclude dead

620   cells. Doublets were excluded by FSC-A, FSC-W, SSC-A and SSC-H.

621   Intracellular cytokine staining was performed by the addition of Brefeldin A (5 µg/ml,

622   Biolegend), monensin (2 µM, Biolegend) and 4 µg/ml anti-human CD107a mAb (Clone H43A,

623   Biolegend) 1 h after co-culture with indicated APCs. Cells were then harvested and treated

624   with fixable LIVE/DEAD BLUE stain (ThermoFisher) for 20 min followed by anti-CD137,

625   CD161 and CD3 mAb before fixation and permeabilization (Buffers from Biolegend). After

626   permeabilization the following mAb specific for intracellular cytokines were added for 40 min

627   on ice: IFN-γ (Clone 4S-B3), TNF-α (Clone MAb.11), GM-CSF (Clone BVD2-21C11), IL-

628   17a (Clone BL168), IL-13 (Clone JES10-5A2), Granzyme B (Clone QA16A02) all from

629   Biolegend. All cells were acquired on a Fortessa (BD) or Aurora spectral analyzer (Cytek) and

630   analyzed using FlowJo v10 software (LLC).

631

632   **Cytokine analysis**

633   The following human cytokines were assessed by ELISA as previously described (Lepore et

634   al., 2017): human IFN-γ (capture MD-1 mAb; revealing biotinylated 4S.B3 mAb, Biolegend),

635   human IL-13 (capture clone JES10-5A2; revealing biotinylated clone SB126d 1090,

636   SouthernBiotech).

637

638   **Identification of VT001 MAIT TCR**

639   TCR was identified from a MAIT T cell clone generated from normal human PBMC as

640   previously described (Lepore et al., 2017; Lepore et al., 2014). Briefly, MAIT T cells (CD3^{+}

641   CD161^{+} TRAV1-2^{+} Vδ2^{-}) were sorted by flow cytometry (BD FACS Aria) and expanded on

642   PHA, IL-2 and irradiated allogenic PBMCs to establish a T cell line from which clones were

643   subsequently generated by limiting dilution. Individual clones were assessed for CD161,

644   TRAV1-2 and CD137 expression by flow cytometry following overnight co-culture with 5-

645   OP-RU loaded THP-1 cells. Positive clones were selected for TCR genes sequencing. Briefly,

646   this involves first-strand cDNA generation and universal amplification using SmartSeq2

647   chemistry (Picelli et al., 2014), followed by targeted amplification of TCR chains and MiSeq

648   Next Generation Sequencing. Sequencing data were analyzed using a bespoke bioinformatics

649   pipeline (unpublished).

650

651   **Identification of E8 TCR**

652   TCRs were isolated from phage libraries based on healthy donor MAIT cell TCR repertoire.

653   TCR isolation to produce E8 TCR has been described previously (Li et al., 2005; Liddy et al.,

654   2012). In brief, phage display panning was performed using the MR1-K43A to select for an

655   MR1 specific TCR.

656

657   **Construct design, protein expression and purification**

658   The sequences coding for the TCR chains were cloned into the pGMT7 vector. TCR constructs

659   were designed to include the variable and constant domains of both α and β chains with an

660   engineered inter-chain disulphide bond as previously described (Boulter et al., 2003). The

661   sequences coding for wtMR1, MR1-K43A, MR1-E76Q-E149Q and B2M and were cloned into

662   the pET23d vector. CD8-null MR1 coding sequence was generated by introducing the

663   mutations Q223A and E224K as previously described (Souter et al., 2022). The proteins were

664   expressed in the BL21 (DE3) Rosetta pLysS strain (Novagen), refolded from inclusion bodies

665   and purified as previously described (Boulter et al., 2003; Reantragoon et al., 2013; (Garboczi

666   et al., 1992). For SPR measurements a C-terminal AVI-tag was added to the wtMR1, MR1-

667   K43A and MR1-E76Q-E149Q constructs and biotinylated after purification using the Avidity

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

669   and Bir A.

670

671   **SPR single cycle kinetic analysis**

672   Purified TCRs, MR1-K43A, MR1-E76Q-E149Q and wtMR1 loaded with 6-FP (Schircks

673   Laboratories), 3-F-BA (Fluorochem), 4-F-BA (Sigma Aldrich), 3-F-SA (Thermo Scientific

674   Acros Organics) and 5-F-SA (Fluorochem) were subjected to SPR analysis using a

675   BIAcoreT200^{TM} using steady state affinity analysis. The curves were fitted, and calculations

676   were completed in GraphPad Prism v9 software. Purified E8 TCR and MR1 (WT and E76Q-

677   E149Q mutant) loaded with 5-OP-RU were subjected to SPR analysis using a BIAcore8K^{TM}

678   using single cycle kinetic analysis, which was completed with Biacore Insight Evaluation

679   software.

680

681   **Crystallization and protein structure determination**

682   E8 TCR and MR1 molecules in 10 mM Tris pH 8.0, 100 mM NaCl were mixed in equimolar

683   ratio and concentrated to 8-10 mg/ml. Sitting drops were set up containing 150 nl of protein

684   solution and 150 nl of reservoir solution in MRC crystallization plates using the Gryphon robot

685   (ART Robbins) and incubated at 20°C. For every E8 TCR-MR1 sample, crystals appeared in

686   many different crystallization conditions. Crystals were cryoprotected using reservoir solution

687   containing 30% ethylene glycol and flash cooled in liquid N_2. Diffraction data were collected

688   at beamlines I03 and I04 at the Diamond Light Source, UK. Datasets used for structure

689   solutions were collected from crystals grown in the following crystallization conditions:

690   E8-MR1-5-OP-RU: 0.2 M Potassium thiocyanate, 0.1 M Bis-Tris propane pH 8.5 and 20 %

691   w/v PEG 3350;

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

693   PEG 4000;

694   E8-MR1-6-FP: 0.2 M Sodium iodide, 0.1 M Bis-Tris propane pH 8.5 and 20 % w/v PEG 3350;

695   E8-MR1-3-F-SA: 0.2 M Sodium malonate dibasic monohydrate, 0.1 M Bis-Tris propane pH

696   8.5 and 20 % w/v PEG 3350;

697   E8-MR1-5-F-SA: 0.2 M Sodium fluoride, 0.1 M Bis-Tris propane pH 8.5 and 20 % w/v PEG

698   3350;

699   E8-MR1-3-F-BA: 0.1 M Magnesium acetate tetrahydrate, 0.1 M MOPS pH 7.5 and 12 % w/v

700   PEG 8000;

701   E8-MR1-4-F-BA: 0.1 M HEPES pH 7.5, 20 % w/v PEG 4000 and 15 % Glycerol.

702

703   The diffraction data were integrated and scaled using the xia2 (Winter et al., 2013) automated

704   processing pipeline using XDS (Kabsch, 2010) and XSCALE. The E8 TCR-MR1-5-OP-RU

705   complex structure was solved by molecular replacement using MR1 and TCR coordinates from

706   PDB 4PJA as the search models in Phaser (McCoy et al., 2007) within the CCP4 suite (Winn

707   et al., 2011). The model was built using iterative cycles of manual model building in COOT

708   (Emsley et al., 2010) and refinement using Refmac (Murshudov et al., 2011). The ligand

709   restraints for refinement were generated using AceDRG (Long et al., 2017). All other E8-MR1

710   complex structures were solved using the E8 TCR-MR1-5-OP-RU structure (with the ligand

711   removed) as the search model for molecular replacement in Phaser. Model building and

712   refinement processes for these complexes were carried out as explained for the E8 TCR-MR1-

713   5-OP-RU complex structure. The stereochemical properties and validation of the models were

714   assessed using PDB-REDO (Joosten et al., 2012) and MolProbity (Williams et al., 2018).

715   Buried surface area and TCR docking geometry statistics based on those described previously

716   (Rudolph et al., 2006) were generated using Molecular Operating Environment (Chemical

717   Computing Group) (Molecular Operating Environment (MOE)) The structural Figures were

718   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

769   for these residues. Optimal histidine tautomerisation states (see Table S4) and asparagine and

770   glutamine side chain orientations were determined using MolProbity (Williams et al., 2018)

771   and all residues except for His90 on MR1 were simulated in their standard protonation states

772   based on pK_a calculations performed using PROPKA 3.1 (for a target pH of 7). His90 on MR1

773   was simulated as positively charged based on the PROPKA prediction and manual inspection.

774   Further, the tautomerisation state of His58 on MR1 was manually assigned to be singly

775   protonated on its Nϵ2 atom, with this atom coordinating the Schiff base nitrogen on K43-5-

776   OP-RU, which acts as a hydrogen bond acceptor. Then structures were solvated in an

777   octahedral water box, (with all crystallographic water molecules kept). The system box size

778   was set such that at no solute atom was within 10 Å of the box boundary. To ensure the total

779   system charge was 0, sodium or chloride ions were added as necessary.

780

781   *Parametrisation of 5-OP-RU.* The K43 residue on MR1 which is covalently bound (through a

782   Schiff base) to 5-OP-RU was parameterized for MD simulations as a single (non-standard)

783   amino acid. Here, we followed the recommended procedure as detailed in full here:

784   https://upjv.q4md-forcefieldtools.org/Tutorial/Tutorial-4.php#16. First, we extracted the

785   structure of K43-5-OP-RU from PDB:6PUC (Awad et al., 2020) alongside residues Q42 and

786   E44 which were manually chemically modified in order to acetylate and amidate the backbone

787   of K43. The structure was then optimized with QM (B3LYP/6-31G(d,p), tight convergence

788   criteria) using Gaussian16 (Frisch et al., 2016). The resulting structure was then submitted to

789   the R.E.D. Server (Vanquelef et al., 2011) for the partial charge calculation (with HF/6-31G(d)

790   RESP fitted charges obtained). For these partial charge calculations, we charge-constrained the

791   acetate and amidate groups to each have a total charge of 0. Atom type definitions for all atoms

792   from the Lysine unit with the exception of the side chain nitrogen were described using the

793   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

819   positional restraints, the system was heated from 25 K to 300 K over the course of 50 ps (NVT

820   ensemble, 1 fs time step). Simulations were then performed in the NPT ensemble (1 atm, 300

821   K, 2 fs time step) by first gradually reducing the 5 kcal mol^{-1} Å^{-1} Cα carbon restraints over the

822   course of 50 ps. This was done by reducing the restraint weight by 1 kcal mol^{-1} Å^{-1} every 10

823   ps. A final 1 ns long MD simulation with no restraints placed on the system was then

824   performed, with the final structure produced after this run, used as the starting point for

825   production MD simulations.

826

Informatics analysis of TCRs

828   Raw sequencing data was demultiplexed using Cutadapt v3.5 and the quality of the reads was

829   checked using FastQC v0.11.9. MiXCR v3.0.13 (Bolotin et al., 2015) was used on the

830   demultiplexed data to align the reads and assemble them into clonotypes, groups of reads

831   sharing the same CDR3 sequence. No further trimming was performed as MiXCR by default

832   handles reads containing low quality nucleotides, performs PCR error correction and conserves

833   only clonotypes that would present a productive TCR. The output tables of MiXCR were

834   loaded into R and sequences corroborated by only 1 read were filtered from the dataset unless

835   they were present in both non-proliferating and proliferating subsets. Exploration of the Vβ,

836   Jβ, CDR3β lengths as well as the analysis of the sequences carrying Arginine in positions 5, 6,

837   7 or 8 of the CDR3β was performed via custom R scripts. Statistical significance was

838   determined using Fisher's exact test and correcting for multiple testing through the Benjamini-

839   Hochberg method for contingency tables or using the Wilcoxon signed-rank test.

840

841

842 **Statistical analysis**

843 Cytokine secretion and luciferase assays as well as flow cytometry data were analyzed for

844 normality using Shapiro-Wilk normality test. The appropriate statistical test is indicated in the

845 figure legend and performed using Prism 9, GraphPad software. For SPICE analysis, multiple

846 comparison Student's *t*-test was performed automatically using the freely available software

847 SPICE 6.

848

849 **Online supplemental material**

850 Fig. S1 shows functional data relating to Figure 1.

851 Fig. S2 shows additional clone data.

852 Fig. S3 shows gating strategy.

853 Fig. S4 shows crystal structure images.

854 Fig. S5 shows data relating to TCR usage.

855 Tables S1 to S5 show additional statistics of crystal structures.

856

857

858 **Data and materials availability**

859 The crystal structures of E8 TCR and MR1-ligand complexes have been deposited with the

860 PDB under Accessions # 7ZT2-9. T cell clones are available from G.D.L. under material

861 transfer agreement with the University of Basel. New TCR sequences in this manuscript are

862 available from G.D.L. under a material transfer agreement with the University of Basel. All

863 other data are available in the article itself and in its supplementary materials.

864

865

866

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.

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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)** $V\alpha7.2$ 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

1202 molecules within self-reactive MAIT cell lines stimulated with A375b-wtMR1 cells or **(G)** 5-

1203 OP-RU-loaded THP-1 cells. Pie segments indicate cells positive for any combination of the

1204 indicated cytokines or activation markers. Pie arcs indicate the cytokine positivity of each

1205 segment. Data is averaged from 5 donors.

1206

1207 **Figure 2. Self-reactivity and T-helper like functions of MAIT cell clones.**

1208 **(A)** Release of IFN-γ by three MAIT cell clones after co-culture with 1×10^5 THP-1 cells and

1209 indicated concentrations of the microbial Ag 5-OP-RU. IFN-γ is reported as mean ± SD of

1210 triplicate cultures. The data is representative of 3 independent experiments. **(B)** Release of IFN-

1211 γ by three MAIT cell clones in A in response to A375b-wtMR1 cells ± aMR1 mAb. 5-OP-RU

1212 pulsed THP-1 cells were used as a positive control. IFN-γ release is shown as mean ± SD of

1213 triplicate cultures. The data is representative of a 3 independent experiments, *** p≤ 0.001.

1214 **(C)** Percentage of J.RT3-T3.5 cells expressing surface CD69 after incubation with A375b-

1215 wtMR1 cells ± aMR1 mAb. A375b-wtMR1 cells pulsed with 5-OP-RU were used as a positive

1216 control. J.RT3-T3.5 cell lines express the TCR of the indicated MAIT cell clones. The data is

1217 representative of 3 independent experiments, *** p≤ 0.001. **(D)** Release of IFN-γ by three

1218 MAIT cell clones stimulated with monocyte-derived DCs (moDCs) ± 5-OP-RU ± aMR1 mAb

1219 or ± Ac-6-FP. moDCs pulsed with 5-OP-RU were used as a positive control. IFN-γ release is

1220 mean ± SD of triplicate cultures. The data is representative of 3 independent experiments, ***

1221 p≤ 0.001. **(E)** Expression levels of the surface maturation markers CD83, CD86 and CD40 on

1222 moDCs after overnight co-culture with the BC75B31 (left panels) and BC75B38 (right panels)

1223 MAIT cell clones. The data is representative of 2 independent experiments. Statistical

1224 significance in all cases was determined using one-way ANOVA with Dunn multiple

1225 comparison test.

1226

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$^+$/ Vα7.2$^+$/CD161$^+$/CD26$^+$ cells. Non-MAIT cells were pre-gated on live CD3$^+$/Vα7.2$^-$ cells. Statistical significance was determined using Student's t-test * p≤ 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

1252   Jurkat cells expressing the E8 TCR (black bars), the VT001 TCR (white bars), or the E8 TCR

1253   in the presence of blocking aMR1 mAb (grey bars). (A, B, C) Luminescence measured

1254   following NFAT-driven luciferase activity is shown as the cumulative relative luminescence

1255   units (RLU) data from 3 experiments with mean ± SD of duplicate cultures. **(D)** Binding

1256   affinities, as measured by surface plasmon resonance, of the E8 TCR interacting with wildtype

1257   MR1 refolded with the indicated range of MR1 ligands, and the empty MR1-K43A mutant.

1258   Dissociation constant values (K_D) are indicated ± standard error. >150μM: the measured K_D of

1259   the TCR MR1 interaction greater than 150μM and therefore is unlikely to elicit a MAIT cell

1260   response. The very high binding affinity of the E8 TCR to MR1 5-OP-RU was measured using

1261   the BIAcore8K^{TM} using single cycle kinetic analysis. The remaining measurements were

1262   performed on a BiacoreT200 and the K_{DS} were calculated using steady state analysis. **(E)**

1263   Binding affinities, as measured by surface plasmon resonance, of the control AF-7 TCR

1264   interacting with wildtype MR1 refolded with the indicated range of MR1 ligands, and MR1-

1265   K43A. Dissociation constant values (K_D) are indicated ± standard error. >150μM: the measured

1266 K_D of the TCR MR1 interaction was greater than 150μM and therefore is unlikely generate a

1267   MAIT cell response.

1268

1269   **Figure 5. Structural and energetic basis of promiscuous recognition of MR1 by the E8**

1270   **TCR.**

1271   **(A)** The structures of the E8 TCR (TRAV in green, TRBV in cyan) bound to MR1 loaded (in

1272   grey) with 5-OP-RU (shown as red sticks) aligned to the AF-7 TCR (TRAV in light blue and

1273   TRBV in dark Blue) bound to MR1 5-OP-RU (PDB 6PUC) (Awad et al., 2020). **(B)** Surface

1274   map of the MR1 binding footprint of the AF-7 TCR (alpha in light blue and beta in dark blue)

1275   as in Ref. (Awad et al., 2020). A vector is drawn connecting the disulphide in the alpha chain

1276   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

1302   THP-1 cells were used as a positive control. Data are representative of 3 individual experiments

1303   each performed in triplicate. Statistical significance was determined using Student's t-test, ***

1304   p≤ 0.001. **(C)** Frequency of TRBV sequences with the E8-like motif within *ex vivo* MAIT cells

1305   (TRAV1-2$^+$/TRBV6$^+$, and CD161$^+$) or non-MAIT cells (TRAV1-2$^+$/TRBV6$^+$, and CD161$^-$)

1306   sorted from the periphery of 7 healthy donors (Lepore et al., 2014).

1307   Statistical significance was determined using Wilcoxon signed-rank test, * p≤0.05.

1308 **Table 1. TCR sequences used in this manuscript**

1309

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

1310

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

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1312 *Paired with TCRα chain from the clone SMC3

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1316 **Table 2. Effects of the MR1 E76Q E149Q salt bridge mutations on the binding affinities**
1317 **of AF-7 TCR, E8 TCR and E8 TRBV6-1 TCR**

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	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

1319

1320 Binding affinities, as measured by surface plasmon resonance, of the AF-7, E8 TCR and E8
1321 TRBV6-1 TCR interacting with wildtype MR1 and MR1 E76Q E149Q refolded with 5-OP-
1322 RU, 6-FP and 5-FSA. >150 μM: the measured K_D of the TCR MR1 interaction was greater
1323 than 150 μM and therefore is unlikely to generate a MAIT cell response. Dissociation constant
1324 values (K_D) are indicated with the standard error in brackets. The binding affinity of the E8
1325 TCR to MR1 5-OP-RU and MR1 E76Q E149Q 5-OP-RU was measured using the
1326 BIAcore8K^{TM} using single cycle kinetic analysis. The remining measurements were completed
1327 on a BiacoreT200^{TM} and the K_DS were calculated using steady state analysis.

1328

1329 **Table 3. Calculated binding free energy differences between the AF7 and E8 TCR**

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TCR	MR1 Complex	MMPBSA Protocol 1		MMPBSA Protocol 2	
		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	

1331

1332 *A negative $\Delta\Delta G$ means the 5-OP-RU bound form is more favourable than the MR1-K43A
1333 form. Errors are presented as the standard deviation from the 5 replicas. MMPBSA Protocols

1334 1 and 2 differ by the choice of internal protein dielectric constant setting. For Protocol 1, the

1335 internal dielectric constant is set to 1, for Protocol 2, the internal dielectric constant is set to 4.