

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

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

3
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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

41 **Abstract**

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

56

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

59

60 Running title: Self-reactive MAIT cells

61

62

63

64 **Introduction**

65 MR1 is a non-polymorphic MHC class I-like molecule that presents small metabolites to T
66 cells. In humans, the MR1-restricted T cell pool consists of two populations, mucosal-
67 associated invariant T (MAIT) cells (Porcelli et al., 1993; Treiner et al., 2003) and MR1T cells
68 (Harriff et al., 2018; Lepore et al., 2017), which significantly differ in antigen (Ag) specificity
69 and TCR repertoire. While MR1T cells react to not yet identified self and potential tumor-
70 associated Ags (Harriff et al., 2018; Lepore et al., 2017), and display polyclonal TCRs, MAIT
71 cells recognize microbial metabolites and are uniquely defined by an almost invariant TCR α
72 chain paired with a restricted TCR β chain repertoire (Lepore et al., 2014; Porcelli et al., 1993;
73 Tilloy et al., 1999). The canonical MAIT TCR α is made by the *TRAV1-2* variable gene
74 rearranged with either the *TRAJ33*, *TRAJ12* or *TRAJ20* gene segments and with remarkably
75 limited junctional variability (Lepore et al., 2014; Porcelli et al., 1993; Tilloy et al., 1999).
76 Other MAIT cell defining features include high expression of CD161, CD26 (Sharma et al.,
77 2015) and IL-18R α , expression of the transcription factor PLZF and reactivity to microbial
78 Ags (Le Bourhis et al., 2010) although very rare CD161⁻ MAIT cells have also been described
79 (Koay et al., 2019). Microbial Ags include the potent pyrimidine agonists 5-(2-
80 oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU) and 5-(2-oxopropylideneamino)-6-d-
81 ribitylaminouracil (5-OP-RU), intermediates of riboflavin biosynthesis that convert to 6,7-
82 Dimethyl-8-ribityllumazine (RL-6,7-diMe) and 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-
83 6-Me-7-OH), which also stimulate MAIT cells, although with lower potency. 5-OE-RU and 5-
84 OP-RU are captured by MR1 *via* a Schiff base with MR1 residue K43, whereas the RL-6,7-
85 diMe and RL-6-Me-7-OH metabolites do not form a Schiff base (Corbett et al., 2014; Kjer-
86 Nielsen et al., 2012). Thus, MR1 binds distinct microbial Ags that show different stimulatory
87 capacity (Schmalzer et al., 2018).

88

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 TCR α 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 Y95 α (encoded by the TRAJ gene segments) (Corbett et al.,
95 2014). Despite the conserved mode of MR1 binding of the invariant TCR V α , the TCR β 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 V β -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 CDR3 β -motif
132 enriched in circulating self-reactive MAIT cells.

133

134

135 **Results**

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

138 We asked whether canonical MAIT cells that recognize microbial metabolites could also
139 respond to MR1-mediated stimulation in the absence of microbial ligands. Purified, circulating
140 $V\alpha 7.2^+$ T cells from healthy donors were cultured with $\beta 2$ -microglobulin (B2M)-deficient
141 A375 melanoma cells (A375b) engineered to display high levels of surface wild-type MR1
142 (A375b-wtMR1; Fig S1A). We selected MR1-overexpressing A375 cells as APCs because of
143 their capacity to broadly stimulate MAIT and MR1T cells through an efficient presentation of
144 both microbial and self-Ags, respectively (Lepore et al., 2017). Freshly isolated T cells were
145 stimulated with A375b-wtMR1 cells and proliferative responses as well as MR1-dependent
146 activation upon rechallenge, were analyzed. Most of the proliferating $V\alpha 7.2^+/CD161^+$ MAIT
147 cells upregulated the activation marker CD137 following rechallenge with A375b-wtMR1
148 cells, contrary to non-proliferating cells that remained unresponsive (Fig. 1A, B and S1B).
149 Activation was prevented by anti-MR1 mAb and did not occur when the cultures were
150 rechallenged with A375b cells that lack wtMR1 on their surface (Fig. 1A, B and S1B). Both
151 proliferating and non-proliferating MAIT cells recognized 5-OP-RU-pulsed THP-1 cells in an
152 MR1-dependent fashion (Fig. 1A and S1B) and bound 5-OP-RU-loaded MR1 tetramers (Fig.
153 S1C). Of note, a proportion of proliferating non-MAIT cells (defined as $V\alpha 7.2^+$ and $CD161^-$)
154 exhibited an MR1-dependent response to A375b-wtMR1 cells (Fig. 1A), representing MR1T
155 cells that do not recognize microbial Ags and express distinct TCRs as previously reported
156 (Lepore et al., 2017).

157

158 The dual reactivity of proliferating $V\alpha 7.2^+/CD161^+$ MAIT cells in the presence and absence of
159 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-V α 7.2 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

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

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

207

208

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

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

221
222 The second assay consisted of staining the same MAIT cell lines with MR1-tetramers loaded
223 with either 5-OP-RU, 3-formylsalicylic acid (3-F-SA) or 6-formylpterin (6-FP). All MAIT
224 cells (CD3⁺CD161^{high}) bound to 5-OP-RU tetramers (Fig. S3A and C), as expected. A very
225 small fraction of non-classical MAIT cells (CD3⁺CD161⁻) also reacted to 5-OP-RU tetramers
226 (Fig. S3A and B), representing 5-OP-RU-reactive MR1-restricted T cells lacking the canonical
227 MAIT TCR, as previously reported (Gherardin et al., 2018; Gold et al., 2010). Surprisingly, up
228 to 47.1% of MAIT cells were stained by 3-F-SA tetramers (Fig. 3B), and up to 29.6% by 6-FP
229 tetramers (Fig. 3C). In addition, a significant number of MAIT cells (~15% and ~19% in two
230 donors, respectively) even bound to all three tetramers (Fig. 3D).

231
232 In the third experiment, MAIT cell lines generated from additional 2 donors by enrichment of
233 TCR Va7.2⁺ 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

258 **Extreme promiscuity in MR1 recognition by a canonical MAIT TCR**

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

276

277 We next expressed the E8 TCR in a soluble format and further assessed the extent of cross-
278 reactivity by surface plasmon resonance using MR1 monomers loaded with 5-OP-RU or a
279 range of non-microbial ligands, including 6-FP, 3-F-SA, 5-F-SA, 3-formylbenzoic acid (3-F-
280 BA), 4-formylbenzoic acid (4-F-BA). We also included the empty MR1-K43A mutant of MR1.
281 A previously characterized and non-cross-reactive MAIT TCR AF-7 (Eckle et al., 2014) was
282 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 μ M) 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 μ M, 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 Y95 α residue in the CDR3 α 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 R96 β in the CDR3 β 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 R96 β 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 R96 β 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 $K_D = 0.6 \mu\text{M}$ for MR1 E76Q E149Q in complex with 5-OP-RU compared to a K_D
324 $= 1 \mu\text{M}$ 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 R96 β 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 R96 β 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 CDR3 α loop
358 (S93 α , N94 α , Y95 α), on the CDR2 β loop (Y48 β and D56 β), and on the CDR3 β loop (T97 β
359 and E99 β) 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 Y95 α 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 D56 β on the CDR2 β 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 CDR3 β loop
367 (particularly residues R96 β and Y98 β) (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 α 1-helix (R61, W69 and E76) (Fig. 5G and Fig. S4B).

371

372 In summary, our MD analysis revealed that the CDR3 β 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 R96 β in the E8 CDR3 β 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 CDR3 β s**

379 Next, we asked whether a portion of self-reactive MAIT cell TCR β 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 R96 β within the CDR3 β loop structure would be most

382 influenced by TRBV usage (*TRBV6* gene), and CDR3 β 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 TCR β -chains sequenced (Fig.
388 S5A). A significant increase in the number of TCR β sequences concomitantly expressing the
389 *TRBV6* gene, CDR3L13 and R96 β 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 CDR3 β residue 96 (R95 β , R97 β or R98 β) was not significantly
393 different between the two MAIT cell groups (Fig. 6A), indicating that the E8-like motif is
394 enriched within the TCR β repertoire of healthy donors' circulating self-reactive MAIT cells.

395

396 To functionally validate these findings, a TCR β chain bearing this motif was co-expressed with
397 a canonical MAIT TCR α 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 CDR3 β (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 TCR β -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 V α 7.2⁺ 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-R96 β 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-dependent 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-dependent 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 CDR3 β 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 R96 β 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 R96 β . MD simulations supported the importance of R96 β -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 R96 β 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 CDR3 β 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 CDR3 β 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 CDR3 β loop
499 (Thr100 β and Asn99 β) 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 R96 β within the CDR3 β . These different modes of binding underline a variety of
502 mechanisms enabling CDR3 β -mediated MAIT cell autoreactivity, further describing the
503 unusual plasticity of this interaction. The role of the TCR β chain in modulating and fine tuning
504 the response of invariant TCRs to Ag in the context of non-polymorphic 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 CDR3 β 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 V β 6 chain, the resulting TCR started to interact with self-CD1d
510 tetramers. Analogous findings related to the contribution of TCR β 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

542 **Materials and methods**

543

544 **Study design**

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

549

550 **Study approval**

551 All human blood samples collected at Immunocore were processed in accordance with the
552 guidelines of Immunocore's Human Tissue Act compliance team, to conform to the United
553 Kingdom Human Tissue Act 2004, (under ethical approval license IMCres02). Blood
554 specimens obtained from the University Hospital Basel were approved by the local ethical
555 review board (EKNZ, Ethics Committee North-West & Central Switzerland, EKNZ 2017-
556 01888).

557

558 **Cell lines and primary T cells**

559 The following cell lines were obtained from American Type Culture Collection: A375 (human
560 melanoma), THP-1 (myelomonocytic leukemia), J.RT3-T3.5 (TCR β -deficient T cell
561 leukemia). The HEK 293 cell line was obtained from the Leibniz-Institute DSMZ-German
562 Collection of Microorganisms and Cell Cultures. J.RT3-T3.5 were engineered to lack
563 endogenous TCR α protein and express an NFAT-driven luciferase reporter. NFAT-Luciferase
564 TCR-null B2M knock-out Jurkat cells were developed from Parental NFAT-Luciferase Jurkat
565 cells from Promega (Part no: J133A). All cells were routinely tested for the absence of
566 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-V α 7.2 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 (20 μ g/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 μ g/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 μ l total volume in triplicates for 18 h. In some
588 experiments, anti-MR1 mAb (20 μ g/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 TCR α
598 and β chains were identified by sequencing and analysis using the ImMunoGeneTics
599 information system (<http://www.imgt.org>). The TCR α 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 TCR α 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 μ g/ml) or anti-human V α 7.2 (2.5 μ g/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 (Cytex) 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™ 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™
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 N₂. 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 N ϵ 2 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, $10 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ 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 $5 \text{ kcal mol}^{-1} \text{ \AA}^{-1}$ positional restraints on only
817 the $C\alpha$ 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 C α 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

827 **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 V β ,
836 J β , CDR3 β lengths as well as the analysis of the sequences carrying Arginine in positions 5, 6,
837 7 or 8 of the CDR3 β 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

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879

880 **Author contributions**

881 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.,
882 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
883 experiments, analyzed data, and provided critical inputs to the manuscript. R.Ca. and L.M.
884 discussed data and revised the manuscript. A.C., D.K.C., G.D.L., and M.L. conceived and
885 directed the project. A.C., D.K.C., G.D.L and M.L. wrote the manuscript. All authors
886 reviewed and commented upon the manuscript.

887

888 **Declaration of interests**

889 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.
890 and M.L. are or have been employees of Immunocore LTD. The authors have no additional
891 financial interests.

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1177 **Figure Legends**

1178

1179 **Figure 1. Self-reactivity and polyfunctionality of circulating MAIT cells from healthy**
1180 **donors.**

1181 **(A)** CD137 expression by autoreactive MAIT cells expanded for 10 days. Proliferating and not
1182 proliferating MAIT (top row) or non-MAIT (bottom row) cells following stimulation with the
1183 indicated APCs \pm anti-MR1 mAb (aMR1). MAIT cells ($V\alpha 7.2^+/CD161^+$) proliferative status
1184 is revealed by Cell Trace Violet (CTV) emission. Plots are representative of results obtained
1185 with 6 donors. **(B)** Summary of MAIT cell CD137 expression on proliferating cells (CTV dull)
1186 after rechallenge with the indicated condition (numbers as in panel A). Data obtained from 6
1187 donors. Statistical significance was determined using a one-way ANOVA with Friedman test,
1188 * $p \leq 0.05$. **(C)** Effect of aMR1 mAb on surface expression of the indicated activation markers
1189 on CTV-dull MAIT cells stimulated with 5-OP-RU-pulsed THP-1 cells (top row) or with
1190 A375b-wtMR1 cells without exogenous antigens (bottom row). Median fluoresce intensity
1191 (MFI) is indicated \pm aMR1 mAb. Data obtained from 5 donors. Statistical significance was
1192 determined using Student's t-test, * $p \leq 0.05$, ** $p \leq 0.01$. **(D)** $V\alpha 7.2$ surface expression on MAIT
1193 cells stimulated with 5-OP-RU-pulsed THP-1 cells (top row) or with A375b-wtMR1 cells
1194 without exogenous antigens (bottom row). Median fluoresce intensity (MFI) is indicated \pm
1195 aMR1 mAb. Data obtained from 5 donors. Statistical significance was determined using
1196 Student's t-test * $p \leq 0.05$. **(E)** Percentage of *ex vivo* MAIT cells from healthy donors double
1197 positive for CD137 and CD69 after overnight co-culture with A375b-MR1 cells \pm aMR1 mAb.
1198 Stimulation with 5-OP-RU was used as positive control with scale on the right-hand y-axis
1199 (green). Cells were pre-gated as $CD3^+/CD26^+V\alpha 7.2^+/CD161^+$. Data are a summary of all 5
1200 donors tested. Statistical significance was determined using Student's t-test * $p \leq 0.05$. **(F)**
1201 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 \pm 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 \pm aMR1 mAb. 5-OP-RU
1212 pulsed THP-1 cells were used as a positive control. IFN- γ release is shown as mean \pm SD of
1213 triplicate cultures. The data is representative of a 3 independent experiments, *** $p \leq 0.001$.
1214 **(C)** Percentage of J.RT3-T3.5 cells expressing surface CD69 after incubation with A375b-
1215 wtMR1 cells \pm 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, *** $p \leq 0.001$. **(D)** Release of IFN- γ by three
1218 MAIT cell clones stimulated with monocyte-derived DCs (moDCs) \pm 5-OP-RU \pm aMR1 mAb
1219 or \pm Ac-6-FP. moDCs pulsed with 5-OP-RU were used as a positive control. IFN- γ release is
1220 mean \pm SD of triplicate cultures. The data is representative of 3 independent experiments, ***
1221 $p \leq 0.001$. **(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

1227 **Figure 3. Cross-reactivity of circulating MAIT cells from healthy donors.**
1228 **(A)** Percentage of CD137⁺ MAIT cells following activation by A375b-MR1 cells ± aMR1
1229 mAb. MAIT cell lines were previously generated from two donors by *in vitro* expansion with
1230 5-OP-RU. **(B)** Plots of MAIT cell lines stained with MR1-3-F-SA tetramer vs. CTV. Cells were
1231 pre-gated on CD161⁺ cells. Data was obtained from a total of 2 donors. **(C)** Representative
1232 plots of MAIT cell lines stained with MR1-6-FP tetramer vs. CTV. Cells were pre-gated on
1233 CD161⁺ cells. Data was obtained from a total of 2 donors. **(D)** Populations of MAIT cells that
1234 are double positive for MR1-3-F-SA and MR1-6-FP tetramers in the same two donors (B, C
1235 and D). **(E)** Frequency of MR1-5-OP-RU, -6-FP, -3-F-SA or -5-F-SA MR1 tetramer⁺ cells
1236 from two additional MAIT cell lines derived from the peripheral blood of donors 3 and 4. Pie
1237 segments indicate cells positive for any combination of the 4 tetramer sets. Pie arcs indicate
1238 the tetramer positivity of each segment. Percentages indicate the total number of cells positive
1239 for at least one tetramer. **(F)** Percentage of *ex vivo* MAIT cells from healthy donors stained
1240 with at least one of three tetramers: CD8-null MR1-3-F-SA, -5-F-SA or -6-FP. MAIT cells
1241 were pre-gated on live CD3⁺/Vα7.2⁺/CD161⁺/CD26⁺ cells. Non-MAIT cells were pre-gated
1242 on live CD3⁺/Vα7.2⁻ cells. Statistical significance was determined using Student's t-test * p≤
1243 0.05.

1244

1245 **Figure 4. Broad self-reactivity and promiscuous recognition of MR1 ligands by the E8**
1246 **TCR.**

1247 **(A)** Recognition of primary immune cells in the absence (black bars) or presence (white bars)
1248 of 5-OP-RU by E8 TCR transduced NFAT-Luciferase TCR-null B2M knock out Jurkat cells.
1249 **(B)** Recognition of primary immune cells in the absence (black bars) or presence (white bars)
1250 of 5-OP-RU by VT001 TCR-transduced NFAT-Luciferase TCR-null B2M knock out Jurkat
1251 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 \pm 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 \pm standard error. $>150\mu\text{M}$: the measured K_D of
1259 the TCR MR1 interaction greater than $150\mu\text{M}$ 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 BIAcore8KTM using single cycle kinetic analysis. The remaining measurements were
1262 performed on a BiacoreT200 and the K_D s 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 \pm standard error. $>150\mu\text{M}$: the measured
1266 K_D of the TCR MR1 interaction was greater than $150\mu\text{M}$ 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

1277 blue sphere). (C) Surface map of the MR1 binding footprint of the E8 TCR (alpha in green and
1278 beta in cyan). A vector is drawn connecting the disulphide in the alpha chain variable domain
1279 (green sphere) to the disulphide in the beta chain variable domain (cyan sphere). (D) The
1280 structures of the AF-7 CDR3 α Y95 residue light (blue sticks) and E8 CDR3 α Y95 residue
1281 (green sticks) showing polar interaction (dotted line) with 5-OP-RU (red sticks) bound to MR1
1282 (grey) (Awad et al., 2020). (E) Superimposed structures of the CDR3 β R96 residue (cyan
1283 sticks) in E8 TCRs that forms salt bridges to the residues E76 and E149 (grey sticks) in MR1
1284 loaded with ligands (5-OP-RU, 6-FP, 3-F-SA, 5-F-SA, 3-F-BA and 4-F-BA). (F) Calculated
1285 per-residue differences (5-OP-RU-wtMR1 minus K43A-MR1) in the binding free energy for
1286 both the AF-7 and E8 TCRs with (5-OP-RU-wtMR1) and without (K43A-MR1) 5-OP-RU
1287 bound to MR1. A blue residue is more favourable in the 5-OP-RU form, whilst a red residue is
1288 more favourable in the MR1-K43A form. Yellow arrows indicate the position of 5-OP-RU.
1289 (G) Calculated per-residue contributions to the binding free energy for the E8 TCR-MR1
1290 complex with 5-OP-RU bound. The MR1 and TCR molecules are shown as surfaces and colour
1291 mapped according to their MMPBSA calculated per residue decomposition energies. Colour
1292 mapping goes from blue (favourable binding) to white (neutral) to red (unfavourable binding)
1293 as indicated by the colour bar.

1294

1295 **Figure 6. Enrichment of R96 in self-reactive MAIT cells.**

1296 (A) Frequencies of TRBV6⁺, CDR3L13⁺ MAIT cells with either R95, R96, R97 or R98 motif
1297 within either self-reactive, proliferated MAIT cells (CTV-) or non-self-reactive, non-
1298 proliferated MAIT cells (CTV+). Statistical significance was determined using Fisher's exact
1299 test, * $p \leq 0.05$. (B) Activation of J.RT3-T3.5 cells transduced with 393 TRBV (a MAIT TRBV
1300 bearing the E8-like motif) or with the control MRC25 TCRBV gene. Percentage of CD69⁺
1301 cells after co-culture with the indicated APC \pm 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 $p \leq 0.001$. (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, * $p \leq 0.05$.

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

1309

TCR α			
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

TCR β				
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	CSAKVTSGQHQTDTQYF
E8	TRBV6-1/5	-	TRBJ1-6	CASSNREYSPLHF
AF-7	TRBV6-1	TRBD2*01	TRBJ2-2	CASSVWTGEGSGELFF
393*	TRBV6-4	-	TRBJ2-3	CASSDREADTQYF

1311

1312 *Paired with TCR α chain from the clone SMC3

1313

1314

1315

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**

1318

TCR	MR1 Wt			MR1 E76Q E149Q		
	5-OP-RU	6-FP	5-FSA	5-OP-RU	6-FP	5-FSA
AF-7	1 ± 0.02 μM	>150 μM	>150 μM	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™ using single cycle kinetic analysis. The remaining measurements were completed
 1327 on a BiacoreT200™ and the K_D s were calculated using steady state analysis.

1328

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

1330

TCR	MR1 Complex	MMPBSA Protocol 1		MMPBSA Protocol 2	
		ΔG (kcal mol ⁻¹)	$\Delta\Delta G_{(5-OP-RU-K43A)}$ (kcal mol ⁻¹)	ΔG (kcal mol ⁻¹)	$\Delta\Delta G_{(5-OP-RU-K43A)}$ (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.