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# 1 **A three-stage intrathymic development pathway for the mucosal-associated** 2 **invariant T cell lineage**

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44

45 Running title: MAIT cell development.

46 **Mucosal-associated invariant T (MAIT) cells detect microbial vitamin B2 derivatives presented**  
47 **by the antigen-presenting molecule, MR1. Here, we define three developmental stages and**  
48 **checkpoints for the MAIT cell lineage in humans and mice. Stage 1 and 2 MAIT cells predominate**  
49 **in thymus, while stage 3 cells progressively increase extrathymically. Transition through each**  
50 **checkpoint is regulated by MR1, whereas the final checkpoint that generates mature functional**  
51 **MAIT cells is controlled by multiple factors, including the transcription factor PLZF and**  
52 **microbial colonisation. Furthermore, stage 3 MAIT cells are expanded in CD1d-deficient mice,**  
53 **suggesting a niche shared between MAIT cells and NKT cells. Accordingly, this study maps the**  
54 **developmental pathway and checkpoints that control the generation of functional MAIT cells.**

55  
56 MAIT cells are a specialized T cell lineage that detects vitamin-B derivatives such as 5-(2-  
57 oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), presented by the major histocompatibility  
58 complex (MHC) class-I-related protein, MR1<sup>1,2,3,4,5</sup>. Both the MAIT T cell receptor (TCR) and MR1 are  
59 evolutionarily conserved suggesting strong selective pressure to maintain MAIT cell-mediated  
60 immunity<sup>6,7</sup>. These cells express a semi-invariant TCR comprising an invariant TRAV1-2 TRAJ33  
61 (TCR alpha variable 1-2<sup>+</sup> TCR alpha joining 33) chain in humans (TRAV1<sup>+</sup>TRAJ33<sup>+</sup> in mice) and a  
62 constrained range of TCR $\beta$  chains, with a bias toward TCR beta variable (TRBV)6 and TRBV20 in  
63 humans and TRBV19 and TRBV13 in mice. MAIT cells are abundant, representing ~5-50% of T cells  
64 in different human tissues, although their numbers vary widely between individuals<sup>8,9,10,11</sup>. Moreover,  
65 they play important roles in the immunity to infection with a broad range of pathogens<sup>9,12,13</sup>, and are also  
66 involved in autoimmunity and other inflammatory responses<sup>24</sup>. Given the functional significance of  
67 MAIT cells, and their wide variability, it is important to understand the factors that control their  
68 development and maturation.

69  
70 MAIT cells develop in the thymus, where they undergo positive selection and lineage commitment upon  
71 interaction with MR1-expressing CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) thymocytes<sup>1,4,14</sup>. While  
72 phenotypically distinct subsets of MAIT cells have been defined, including CD4<sup>+</sup>, CD4<sup>-</sup>CD8<sup>-</sup> and CD4<sup>-</sup>  
73 CD8<sup>+</sup> populations<sup>11,15</sup>, the developmental pathway that underpins the production and maturation of  
74 MAIT cell remains unclear. In humans, blood MAIT cells are often defined by expression of TRAV1-2  
75 in conjunction with CD161 and CD218 (IL-18R $\alpha$ )<sup>8,16,17,18</sup>. However, when these markers are acquired  
76 during the development of MAIT cells is unknown, so it is unclear if these can be used to study MAIT  
77 cell development in the thymus. With the recent development of MR1 tetramers it is now possible to  
78 specifically detect and isolate MAIT cells in mice and humans<sup>2,15,19,20</sup>. Here, we identify previously  
79 unknown populations of MAIT cells in mouse and human thymus and delineate a three-stage  
80 developmental pathway in both species, defining key control points and factors that regulate the

81 generation of these cells. Thus, we have mapped the development of MAIT cells in mice and humans  
82 and demonstrated the potential for this model to understand how this process is regulated.

83

## 84 **RESULTS**

### 85 **Identification of mouse thymic MAIT cell progenitors**

86 To understand MAIT cell development, MR1-5-OP-RU tetramers were used to characterize MAIT cells  
87 in the thymus and peripheral lymphoid tissues of mice. This identified a novel population of  
88 CD24<sup>+</sup>CD44<sup>-</sup> MAIT cells that were exclusively present in thymus, whereas in peripheral organs  
89 essentially all MAIT cells were CD24<sup>-</sup>CD44<sup>+</sup> (**Fig. 1a**). Control MR1 tetramers loaded with the non-  
90 agonist folate-derivative acetyl-6-formylpterin Ac-6-FP<sup>21</sup> failed to stain MAIT cells from these organs  
91 (**Fig. 1a**), confirming specific staining. Single cell TCR sequence analysis of both CD24<sup>+</sup>CD44<sup>-</sup> and  
92 CD24<sup>-</sup>CD44<sup>+</sup> populations indicated that both populations utilized an invariant TRAV1-TRAJ33 TCR  
93  $\alpha$ -chain, paired with a limited range of TCR  $\beta$ -chains (TRBV13 and TRBV19), characteristic of the  
94 MAIT cell lineage (**Supplementary Table 1**)<sup>4</sup>. Comparison of CD24<sup>+</sup>CD44<sup>-</sup> and CD24<sup>-</sup>CD44<sup>+</sup> MAIT  
95 cells within the thymus indicated that CD24<sup>+</sup>CD44<sup>-</sup> MAIT cells had low expression of CD103, CD122  
96 (IL-2R $\beta$ ), CD127 (IL-7R $\alpha$ ), CD218 (IL-18R), CD278 (ICOS) and NK1.1 (CD161), but higher  
97 expression of CD62L and CD69 compared to CD24<sup>-</sup>CD44<sup>+</sup> MAIT cells (**Fig. 1b**). Staining of thymus  
98 MAIT cells enriched by MR1-5-OP-RU tetramer and magnetic beads indicated an additional  
99 subpopulation of CD24<sup>-</sup>CD44<sup>-</sup> MAIT cells (**Fig. 1c**). Thus, based on their cell-surface phenotype, we  
100 tentatively defined these populations as stage 1 (CD24<sup>+</sup>CD44<sup>-</sup>), stage 2 (CD24<sup>-</sup>CD44<sup>-</sup>) and stage 3  
101 (CD24<sup>-</sup>CD44<sup>+</sup>) MAIT cells (**Fig. 1c**). The vast majority of stage 1 thymic MAIT cells were small (FSC  
102 (forward scatter)<sup>10</sup>) CD4<sup>+</sup> or CD4<sup>+</sup>CD8<sup>+</sup> DPs, a phenotype that is typically associated with immature T  
103 cells in the thymus, and which also defines the earliest stage in the development of CD1d-restricted  
104 natural killer T (NKT) cells<sup>22</sup>. Stage 2 cells were larger, mostly CD4<sup>+</sup>CD8<sup>-</sup>, whereas stage 3 MAIT cells  
105 were the largest and resembled mature MAIT cells in peripheral organs, being mostly CD4<sup>-</sup>CD8<sup>-</sup> or  
106 CD8<sup>+</sup> (**Fig. 1a,c**). Most CD8<sup>+</sup> MAIT cells in thymus expressed CD8 $\alpha\beta$  heterodimers, while those in the  
107 periphery express CD8 $\alpha\alpha$  homodimers or CD8 $\alpha\beta$  heterodimers (**Fig. 1a**), suggesting further extrathymic  
108 maturation of MAIT cells. Collectively, these data support the presence of three distinct stages of MAIT  
109 cell development within mouse thymus.

110

### 111 **Functionally mature stage 3 MAIT cells arise through ontogeny**

112 To investigate the developmental progression of the three stages of thymic MAIT cells, we performed  
113 an ontogeny study for mouse thymic MAIT cell subsets at 2, 4 and 8 weeks of age. Stage 1 CD24<sup>+</sup>CD44<sup>-</sup>  
114 MAIT cells were the major subset (>50%) at 2 weeks, declining to ~30% at 4 weeks and ~10% of thymic  
115 MAIT cells in the adult 8-week-old mouse thymus (**Fig. 2a,b**). Conversely, the proportion of stage 3  
116 CD24<sup>-</sup>CD44<sup>+</sup> MAIT cells was low (~20%) at 2 weeks and gradually increased with age (~60% at 4

117 weeks and ~80% at 8 weeks) (**Fig 2a,b**), supporting the concept of developmental progression of MAIT  
118 cells from stage 1 to stage 3.

119

120 To determine the stage where MAIT cells gained functional maturity, we examined the expression of  
121 transcription factors known to regulate their cytokine production. Stage 1 and 2 cells had low expression  
122 of ROR $\gamma$ t and T-bet (**Fig. 2c**), suggesting little capacity to secrete IL-17 and IFN- $\gamma$ <sup>15</sup>, while stage 3 MAIT  
123 cells expressed either ROR $\gamma$ t or T-bet (**Fig. 2c**), suggesting functional competence. Analysis of ROR $\gamma$ t-  
124 GFP reporter mice confirmed that only stage 3 MAIT cells expressed GFP (**Supplementary Fig. 1**).  
125 The proportion of stage 3 MAIT cells that expressed ROR $\gamma$ t or T-bet did not significantly change  
126 throughout ontogeny (**data not shown**). Stimulation of MAIT cells with PMA and ionomycin revealed  
127 that stage 3 MAIT cells, but not stage 1 and 2 MAIT cells, were capable of IL-17 and IFN- $\gamma$  production  
128 (**Fig. 2d**). Accordingly, the transition to stage 3 correlated with the acquisition of functional potential of  
129 mouse MAIT cells (**Fig. 2d**), supporting the concept that stage 1 and 2 MAIT cells are immature MAIT  
130 cell precursors.

131

### 132 **Stage 1 and stage 2 MAIT cells develop into stage 3 MAIT cells**

133 To directly investigate the precursor-progeny relationship of the three stages of thymic MAIT cells we  
134 established an *in vitro* MAIT cell development system using an adaptation of the OP9 cell-thymocyte  
135 co-culture system<sup>23</sup>. Because the numbers of thymus MAIT cell subsets in wild-type mice were very  
136 low, we used transgenic mice that express the TRAV1-TRAJ33 TCR $\alpha$  chain on a TCR-constant region-  
137 null background (C $\alpha$ <sup>-/-</sup>), in which thymic MAIT cells are far more abundant (**Supplementary Figure 2**).  
138 After 5 days of co-culture with OP9 cells, FACS-sorted stage 1 MAIT cells had started to differentiate  
139 into stage 2 and stage 3 cells (**Fig. 3a,b**). Similarly, many stage 2 MAIT cells had progressed to stage 3,  
140 whereas stage 3 cells maintained their mature CD24<sup>-</sup>CD44<sup>+</sup> phenotype (**Fig. 3a,b**). Optimal  
141 differentiation from stage 1 and stage 2 MAIT cells into stage 3 MAIT cells required the presence OP9  
142 cells (**Fig. 3a,b**), indicating a key role for stromal cell-derived factors in this process. We also cultured  
143 stage 1, 2 and 3 MAIT cells with OP9 cells engineered to express Notch ligand, Delta-like 1, (OP9-DL1)  
144 and found that signalling via DL1 was not required for maturation of TCR<sup>+</sup> MAIT cell precursors (**data**  
145 **not shown**). Addition of an MR1-blocking antibody completely abrogated the progression of stage 1 to  
146 stage 3 MAIT cells, and partially inhibited the differentiation or survival of stage 2 and stage 3 cells, as  
147 reflected by a sharp reduction in cell numbers by day 5 (**Fig.3a,b**). These data are consistent with the  
148 lack of MAIT cells in MR1-deficient mice<sup>1,11,15</sup>, and indicate that the expression of MR1 is critical for  
149 both the initial development and further differentiation and/or survival of MAIT cells.

150

151 We also isolated MAIT cell subsets from wild-type mice and cultured them with OP9 cells. Because  
152 these cells were less frequent, we were unable to isolate sufficient numbers of the intermediate stage 2

153 cells. Nonetheless, stage 1 cells progressed to stages 2 and 3 after 5 days of co-culture with OP9 cells,  
154 whereas stage 3 cells maintained their mature phenotype (**Fig.3a,b**), indicating a similar developmental  
155 profile and kinetics as the TRAV1-TRAJ33 TCR-transgenic cells. These data indicate that  
156 differentiation of mouse MAIT cells can be defined by a three-stage sequential pathway from  
157 CD24<sup>+</sup>CD44<sup>-</sup> (stage 1), via CD24<sup>-</sup>CD44<sup>-</sup> (stage 2), to CD24<sup>-</sup>CD44<sup>+</sup> (stage 3).

### 158 159 **PLZF controls the maturation and function of MAIT cells**

160 The transcription factor PLZF, known to be important for the development of NKT cells, innate  
161 lymphoid cells (ILC) and some  $\gamma\delta$  T cells<sup>24,25,26,27</sup> is expressed by MAIT cells in humans and mice<sup>11,15</sup>.  
162 The production of normal numbers of MAIT cells depends upon this factor, although notably, a residual  
163 population of MAIT cells remained in mice that were deficient for PLZF (PLZF-null mice)<sup>15</sup>. In wild-  
164 type mice, PLZF was not detected in stage 1, showed heterogeneous expression in stage 2, and was fully  
165 expressed in stage 3 MAIT cells (**Fig. 4a**). In PLZF-null mice stage 1 and stage 2 MAIT thymic cells  
166 were intact, whereas stage 3 MAIT cells were completely absent, in contrast to wild-type mice where  
167 stage 3 MAIT cells were the major population (**Fig. 4b,c**). Residual MAIT cells in the periphery of  
168 PLZF-null mice were predominantly CD24<sup>-</sup>CD44<sup>-</sup> and CD4<sup>+</sup>CD8<sup>-</sup> (**Fig. 4b**), and lacked CD218, CD127  
169 and CD103 expression (**data not shown**), indicating they were blocked at stage 2. Stimulation (PMA  
170 and ionomycin) of the residual MAIT cells isolated from thymus, spleen and lymph nodes of PLZF-null  
171 mice did not induce cytokine secretion, compared to wild-type MAIT cells, which expressed either IL-  
172 17A or IFN- $\gamma$  (**Fig. 4d**). This suggests that progression to stage 2 is PLZF-independent, but maturation  
173 to stage 3 and acquisition of effector function is PLZF-dependent, and can occur after stage 2 cells leave  
174 the thymus, because in the absence of the PLZF-driven maturation, MAIT cells remain immature in the  
175 thymus and peripheral tissues.

### 176 177 **miRNA controls the development of MAIT cells**

178 Because microRNAs (miRNAs) play important roles in regulating gene expression and profoundly  
179 influence development and function of NKT cells<sup>28</sup>, we next examined the role of Drosha, a member of  
180 the ribonuclease-III superfamily that initiates miRNA processing in the nucleus<sup>28,29</sup> in MAIT cell  
181 maturation. We used Drosha-floxed mice crossed with transgenic mice expressing *Cre* under the control  
182 of the CD4 promoter (*Drosha<sup>fl/fl</sup> CD4-Cre*) mice, which have a marked Drosha deficiency from the  
183 CD4<sup>+</sup>CD8<sup>+</sup> DP thymocyte stage<sup>30</sup>. The vast majority of MAIT cells in the thymus of *Drosha<sup>fl/fl</sup> CD4-  
184 Cre* mice were stage 1 cells, whereas stage 2 and stage 3 MAIT cells were both significantly reduced  
185 compared to *Drosha<sup>fl/+</sup> CD4-Cre* heterozygous control mice (**Fig. 5a,b**), suggesting that development  
186 beyond stage 1 is dependent on miRNAs. Despite the developmental impairment in the *Drosha<sup>fl/fl</sup> CD4-  
187 Cre* thymus, stage 3 CD44<sup>+</sup> MAIT cells were still detected in spleen and lymph nodes of these mice,  
188 albeit at diminished numbers compared to heterozygous controls (**Fig. 5a,b**). We also observed a

189 significant reduction in NKT cells within thymus, spleen and lymph nodes of *Drosha*<sup>fl/fl</sup> *CD4-Cre* mice  
190 compared to control mice (**Supplementary Fig. 3a**), which is consistent with observations in Dicer-  
191 deficient mice (another member of the ribonuclease-III superfamily<sup>31</sup>). Accordingly, miRNAs control  
192 MAIT cell development beyond stage 1.

### 193 194 **MAIT cell development is impaired in germ-free mice**

195 MAIT cells were previously reported to be absent in germ-free mice<sup>1</sup>, thus we examined whether a  
196 developmental block occurred at a specific stage, comparing germ-free mice to specific-pathogen-free  
197 (SPF) control mice. The numbers of MAIT cells in the thymus and spleen of germ-free mice were  
198 significantly reduced (**Fig. 5a,b**). Moreover, thymic stage 3 CD44<sup>+</sup> MAIT cells were diminished (**Fig.**  
199 **5a,b**), while stage 1 CD24<sup>+</sup>CD44<sup>-</sup> MAIT cells were increased in frequency, but not number, in germ-  
200 free mice (**Fig.5a and data not shown**). In contrast, there was no difference in the number of NKT cells  
201 in germ-free mice compared to SPF controls (**Supplementary Fig. 3b**).

202  
203 Since IL-18 expression is diminished in germ-free mice<sup>32</sup> and because MAIT cells expressed IL-18R  
204 during their intrathymic maturation (**Fig. 1**), we examined whether the loss of IL-18 would impact on  
205 MAIT cell development. Similar to germ-free mice, IL-18-deficient mice displayed reduced thymic  
206 MAIT cell numbers and a decrease in the frequency of thymic stage 3 CD24<sup>-</sup>CD44<sup>+</sup> MAIT cells, with  
207 an increase in the frequency, but not number, of stage 1 CD24<sup>+</sup>CD44<sup>-</sup> MAIT cells compared to wild-  
208 type mice (**Supplementary Fig. 4a,b**). Furthermore, MAIT cells were significantly reduced in the spleen  
209 and lymph nodes of IL-18-deficient mice compared to wild-type mice (**Supplementary Fig. 4a**).  
210 Paradoxically, IL-18R $\alpha$ -deficient mice showed normal MAIT cell development and maturation within  
211 the thymus, spleen and lymph nodes (**Supplementary Fig. 4c,d**). Comparison of IL-18-deficient and  
212 IL-18R $\alpha$ -deficient mice may be complicated because IL-18 can signal through another receptor, the Na-  
213 Cl co-transporter<sup>33</sup> whereas another cytokine, IL-37, can signal through IL-18R<sup>34</sup>. Thus, MAIT cell  
214 development and maturation is impaired in germ-free mice, suggesting an important role for commensal  
215 bacteria and IL-18 in this process.

### 216 217 **MAIT cells are over-represented in CD1d-deficient mice**

218 Because MAIT cells and NKT cells have some similarities, such as a dependence on PLZF and selection  
219 by DP thymocytes, we examined MAIT cell development in CD1d-deficient mice that lack both CD1d  
220 and CD1d-restricted NKT cells. CD1d-deficient mice showed increased numbers of MAIT cells in  
221 thymus, spleen (**Figure 6a,b and Supplementary Fig. 5**) and liver (**data not shown**) compared to wild-  
222 type controls on both BALB/c and C57BL/6 backgrounds, and this was particularly apparent on the  
223 BALB/c background (**Fig. 6a,b**). As previously reported<sup>15</sup>, a higher percentage of MAIT cells expressed  
224 the co-receptor CD8 in BALB/c compared to C57BL/6 mice, and this was seen in both wild-type and



225 CD1d-deficient mice (**Fig. 6a and Supplementary Fig. 5**). In terms of MAIT cell development, while  
226 numbers of stage 1 and 2 MAIT cells were not altered in CD1d-deficient mice, stage 3 CD24<sup>+</sup>CD44<sup>+</sup>  
227 MAIT cells were far more numerous in thymus and spleen of CD1d-deficient mice compared to wild-  
228 type controls (**Figure 6a,b and Supplementary Fig. 5**). Conversely, NKT cell numbers were similar in  
229 thymus and spleen of MR1-deficient and wild-type mice (**data not shown**). Taken together, these data  
230 suggest that MAIT cells might compete with NKT cells for factors or a shared niche during their  
231 maturation in the thymus.

232

### 233 **Identification of MAIT cell precursors in the human thymus**

234 We next determined whether MAIT cell development in humans follows a similar developmental  
235 pathway as in mice. We analysed MAIT cells in human thymus, umbilical cord blood, young peripheral  
236 blood (matched from thymus donors) and adult peripheral blood, using human MR1-5-OP-RU tetramers  
237 in combination with other markers commonly used to identify MAIT cells, including TRAV1-2, CD161  
238 and CD218<sup>8,16,17</sup>. MAIT cells (defined as TRAV1-2<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup>) were detected in all  
239 human thymus samples, although they were much less frequent (<0.05%) than in adult human blood,  
240 where they typically represent 2-5% of CD3<sup>+</sup> T cells<sup>15</sup>. Because they were so infrequent in human  
241 thymus, TRAV1-2<sup>+</sup> cells were first enriched using magnetic beads, which yielded a clear population of  
242 MAIT cells, ranging from 0.08-0.45% of TRAV1-2<sup>+</sup> thymocytes (**Fig. 7a,b**). The frequency of MAIT  
243 cells as a percentage of TRAV1-2<sup>+</sup> cells increased in cord blood, young blood and adult blood (**Fig.**  
244 **7a,b**), respectively, suggesting that peripheral expansion is responsible for the abundance of MAIT cells  
245 in adult humans.

246

247 The vast majority of MAIT cells from adult blood co-expressed CD218 and CD161 while most (up to  
248 97%) thymic MAIT cells were CD218<sup>-</sup>CD161<sup>-</sup> (**Fig. 7a,b**). The majority of MAIT cells in young blood  
249 and cord blood were CD218<sup>+</sup>CD161<sup>+</sup> (**Fig. 7a**). Thus, CD218<sup>-</sup>CD161<sup>-</sup> MAIT cells were predominant in  
250 human thymus and they gradually diminished in increasingly mature blood samples, suggesting that they  
251 were immature precursors (**Fig. 7a,c**). Furthermore, whereas blood MAIT cells were predominantly  
252 CD4<sup>-</sup>CD8<sup>-</sup> or CD4<sup>-</sup>CD8<sup>+</sup> (**Fig. 7a**), roughly half of thymus MAIT cells were either CD4<sup>+</sup>CD8<sup>+</sup> or  
253 CD4<sup>+</sup>CD8<sup>-</sup> (**Fig. 7a**). Moreover, analysis of paired thymus and blood samples showed that CD4<sup>+</sup> MAIT  
254 cell populations declined sharply between thymus and matched blood (**Fig. 7d**). Accordingly, most  
255 human thymic MAIT cells were markedly distinct from MAIT cells in blood.

256

257 We also performed an ontogeny study of thymus and blood MAIT cells in matched thymus and blood  
258 samples from 18 different donors ranging from 5 days to 14 years of age. The analysis indicated that  
259 MAIT cell frequencies (as a percentage of TRAV1-2<sup>+</sup> cells) remain relatively low and stable in the  
260 thymus (0.1-0.3% of TRAV1-2<sup>+</sup> cells) regardless of age (**Fig. 7e**), while in contrast, MAIT cell

261 frequency gradually increased in peripheral blood with age (5 days to 14 years), from less than 1% to up  
262 to 60% of TRAV1-2<sup>+</sup> cells and from less than 0.02% to up to 3.5% of CD3<sup>+</sup> T cells (**Fig. 7e,f**). Thus,  
263 the characteristically high numbers of MAIT cells in human blood is a result of gradual peripheral  
264 expansion with age.

265  
266 Further analysis of human CD161<sup>-</sup> MAIT cells based on CD27 expression, indicated that CD161<sup>-</sup>CD27<sup>-</sup>  
267 subset was exclusively found in thymus, whereas CD161<sup>-</sup>CD27<sup>+</sup> MAIT cells were detected at low  
268 frequency in cord blood (~22%) and young blood (~13%) and were essentially absent from adult blood  
269 (~1%). Conversely, CD161<sup>+</sup>CD27<sup>+/-lo</sup> MAIT cells were rare in thymus (~10%) and abundant in blood  
270 (**Fig. 7a,g**). Thus, we tentatively defined three distinct stages of MAIT cell development in human  
271 thymus: stage 1 (CD161<sup>-</sup>CD27<sup>-</sup>); stage 2 (CD161<sup>-</sup>CD27<sup>+</sup>) and stage 3 (CD161<sup>+</sup>CD27<sup>+/-lo</sup>). Further  
272 analysis of these stages demonstrated the decline in stage 1 and 2 and the rise in stage 3 with increasingly  
273 mature human tissues (**Fig. 7a,g**). In line with this maturation pathway, and similar to that in mice,  
274 human stage 1 MAIT cells were predominantly CD4<sup>+</sup>CD8<sup>+</sup>, stage 2 MAIT cells were CD4<sup>+</sup>, CD4<sup>+</sup>CD8<sup>+</sup>  
275 cells, and CD4<sup>-</sup>CD8<sup>+</sup> cells whereas stage 3 MAIT cells were mostly CD4<sup>-</sup>CD8<sup>-</sup> or CD8<sup>+</sup> (**Fig. 8a**), the  
276 latter more closely resembling MAIT cells found in human blood (**Fig. 8a**). Taken together, the 3-stage  
277 MAIT cell development pathway we have identified in human thymus roughly parallels the pathway we  
278 have mapped for mouse thymus (**Supplementary Fig. 6**).

279  
280 Many peripheral CD8<sup>+</sup> MAIT cells express CD8αα homodimers rather than CD8αβ heterodimers (**Fig.**  
281 **8a**)<sup>17</sup>. Given that most human thymocytes express CD8αβ heterodimers, we determined where in the  
282 three-stage development pathway human MAIT cells began to express CD8αα homodimers. Similar to  
283 MAIT cells in mouse thymus, essentially all CD8<sup>+</sup> MAIT cells in the human thymus expressed the  
284 CD8αβ heterodimer, regardless of whether they were at stage 1, 2 or 3 of development (**Fig. 8a**).  
285 However in thymus-matched blood samples, a high proportion of blood MAIT cells were CD8αα<sup>+</sup> (**Fig.**  
286 **8a**). This distinct CD8 profile of thymic MAIT cells is consistent with earlier reports suggesting that  
287 CD8αα MAIT cells develop from a pool of CD161<sup>+</sup>CD8αβ T cells<sup>17,18</sup>. Furthermore, this also suggests  
288 that stage 3 MAIT cells in human thymus were not recirculating cells from the periphery, but rather a  
289 recently developed population arising from stage 2 CD161<sup>-</sup>CD27<sup>+</sup> MAIT cells.

### 290 291 **Functional maturation begins at stage 3 in MAIT cell development**

292 We next examined if the 3 stages of human thymic MAIT cells displayed a similar pattern of  
293 transcription factors compared to the same stages in mice. Akin to mouse thymic MAIT cells, the  
294 expression of PLZF, T-bet and RORγt increased between stage 2 and 3 (**Fig. 8b**) although RORγt was  
295 not highly expressed in mature MAIT cells (**Fig. 8b**). In contrast to mouse stage 3 MAIT cells where T-  
296 bet and RORγt were mutually exclusive, these factors were co-expressed in human stage 3 MAIT cells

297 (Fig. 8b). A small subset of stage 1 MAIT cells expressed ROR $\gamma$ t, which likely reflected their recent  
298 transition from CD4<sup>+</sup>CD8<sup>+</sup> DP thymic precursors that are ROR $\gamma$ t<sup>+</sup> (Fig. 8b).

299

300 To examine their functional competence, human thymic and matched blood MAIT cells were tested for  
301 IFN- $\gamma$ , TNF and IL-17A following PMA and ionomycin stimulation (Fig. 8c and data not shown).

302 While a subset (<25%) of stage 3 thymic MAIT cells produced IFN- $\gamma$  and TNF, the matched blood stage  
303 3 cells were far superior in their cytokine producing potential, with the majority (>80%) producing both

304 IFN- $\gamma$  and TNF (Fig. 8c,d). We were unable to detect IL-17A production by any of these MAIT cell  
305 subsets (data not shown), which probably reflects the relatively low expression of ROR $\gamma$ t by these cells.

306 Thus, human MAIT cells are functionally competent at stage 3, but continue their functional maturation  
307 extrathymically.

308

## 309 DISCUSSION

310 Here we have identified precursor populations for the MAIT cell lineage in the thymus and mapped a  
311 three-stage pathway in both mice and humans. In both species, only stage 3 cells produced cytokines

312 and these resembled MAIT cells in peripheral organs, although further extrathymic maturation occurred  
313 for human stage 3 cells. Based on the presence of some stage 2 cells in human blood, especially in cord

314 blood, and in PLZF-null mice, we suggest that MAIT cells can first emigrate from the thymus at stage  
315 2 and continue their maturation and expansion extrathymically.

316

317 Our data provide a more complete view of MAIT cell development compared to previous studies that  
318 relied on surrogate phenotypic markers such as TRAV1-2<sup>+</sup>CD161<sup>+</sup> and to identify MAIT cells in

319 humans<sup>18</sup> or the use of transgenic TCR systems or ROR $\gamma$ t to detect MAIT cells in mice<sup>11,35,36</sup>. The  
320 surrogate markers for human MAIT cells exclude CD161<sup>-</sup> stage 1 and stage 2 MAIT cells, limiting

321 earlier studies to the minor subset of mature stage 3 cells in human thymus<sup>18</sup>. Furthermore, we found  
322 that ~50% of TRAV1-2<sup>+</sup>CD161<sup>+</sup> cells from human thymus failed to bind MR1-5-OP-RU tetramer (data

323 not shown) suggesting many of these are not MAIT cells. Similarly, while the use of ROR $\gamma$ t-GFP  
324 combined with CD44 should identify many of the mature stage 3 MAIT cells in mice<sup>36</sup>, this strategy will

325 miss immature stage 1 and stage 2 CD44<sup>-</sup> MAIT cells and mature stage 3 MAIT cells that lack ROR $\gamma$ t.  
326

327 There are some similarities between the development of MAIT cells and CD1d-restricted NKT cells.  
328 Both lineages depend on PLZF expression for normal maturation, and both are dependent on miRNAs

329 regulated by Drosha. However, there are some important differences in the development of these  
330 lineages. Firstly, PLZF is highly expressed very early in NKT cell development and is downregulated

331 in most mature NKT cells<sup>25,27</sup>, with the exception of IL-4-producing NKT2 cells<sup>37,38</sup>. In contrast, PLZF  
332 is expressed late in MAIT cell development, governing their final maturation step to generate functional

333 stage 3 MAIT cells, and is maintained at high levels by these cells. Upon maturation, most NKT cells  
334 upregulate NK1.1 and CD69 in mice and they are either CD4<sup>+</sup> or CD4<sup>-</sup>CD8<sup>-</sup>. Conversely, most mature  
335 mouse MAIT cells lacked NK1.1 and downregulated CD69 upon maturation, and many expressed CD8.  
336 MAIT cell maturation was impaired in germ-free mice, whereas NKT cell maturation was intact, and,  
337 as previously shown, the SLAM adaptor protein (SAP) is critical for NKT cell development<sup>39</sup>, whereas  
338 MAIT cells appear to be SAP-independent<sup>11</sup>. Furthermore, our observation that MAIT cells are over-  
339 represented in CD1d (NKT cell)-deficient mice suggests that these cells may compete for an  
340 environmental niche. This may partly explain why CAST/EiJ mice, which have less NKT cells<sup>40</sup>, have  
341 higher numbers of MAIT cells compared to C57BL/6 controls<sup>36</sup>. It follows that careful consideration is  
342 required when studying NKT cell deficient mice as these will differ from wild-type control mice not  
343 only because they lack NKT cells, but also because they have increased MAIT cells.

344

345 While the developmental sequence for MAIT cells in mice and humans has many parallels, there were  
346 also some distinctions. Although we detected stage 3 MAIT cells in human thymus, these were in the  
347 minority and were only partially functional compared to stage 3 MAIT cells in mouse thymus. Human  
348 peripheral blood MAIT cells accumulate with age, and also acquire greater cytokine producing capacity  
349 and many switch from CD8 $\alpha\beta$  to CD8 $\alpha\alpha$  expressing cells. In further contrast to mouse MAIT cells,  
350 neither thymus nor blood MAIT cells from humans produced IL-17. Given that human MAIT cells  
351 isolated from liver and female genital tract can produce IL-17<sup>10,41</sup>, this suggests that further environment-  
352 dependent extrathymic maturation influences human MAIT cell function. We hypothesize that this  
353 peripheral expansion and maturation is shaped by exposure to microbial antigens over time, which is  
354 also supported by our studies with germ-free mice where stage 3 MAIT cells were significantly reduced  
355 in thymus and periphery and also by a recent paper that demonstrated microbial mediated expansion of  
356 peripheral MAIT cells in a mouse model of Salmonella infection<sup>42</sup>.

357

358 In summary, we have defined thymic precursors for the MAIT cell lineage and mapped a three-stage  
359 pathway for MAIT cell maturation in mice and humans that is controlled by developmental checkpoints.  
360 We have identified key factors that control this process and most importantly, this study provides a  
361 foundation for future studies to better understand the factors that regulate the highly abundant, yet highly  
362 variable, MAIT cell lineage.

363 **Figure Legends**

364

365 **Figure 1. Identification of distinct MAIT cell subsets in mouse thymus**

366 (a) Flow cytometric analysis of TCR $\beta$ <sup>+</sup>MR1-5-OP-RU<sup>+</sup> MAIT cells in mouse thymus, spleen, lung and  
367 lymph nodes for expression of CD24, CD44, CD4, CD8 $\alpha$  and CD8 $\beta$ . MR1-Ac-6-FP tetramer was used  
368 as a negative control. (b) Phenotypic analysis of CD24<sup>+</sup>CD44<sup>-</sup> and CD24<sup>-</sup>CD44<sup>+</sup> thymic MAIT cells for  
369 ICOS, NK1.1 (CD161), CD62L, CD69, CD103, CD122 (IL-2R), CD127 (IL-7R) and CD218 (IL-18R),  
370 CD24<sup>+</sup>CD44<sup>-</sup> MAIT cells in blue, CD24<sup>-</sup>CD44<sup>+</sup> MAIT cells in red, CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP)  
371 thymocytes in black. (c) Identification of three populations of thymic MAIT cells following magnetic  
372 bead enrichment. Flow cytometric analysis of 3 stages of MAIT cells defined using CD24 and CD44.  
373 Stage 1 (S1, CD24<sup>+</sup>CD44<sup>-</sup>) in blue, stage 2 (S2, CD24<sup>-</sup>CD44<sup>-</sup>) in green and stage 3 (S3, CD24<sup>-</sup>CD44<sup>+</sup>)  
374 in red. Flow cytometric analysis of stage 1, stage 2 and stage 3 MAIT cells for forward scatter (FSC)  
375 and CD4/CD8 co-receptor expression. Data are representative from a total of 6 mice from 3 independent  
376 experiments (a), or from 2 independent experiments from 5-pooled thymi (b) or from at least 10  
377 independent experiments (c).

378

379 **Figure 2. Ontogeny, transcriptional and functional potential of mouse MAIT cells**

380 (a) Flow cytometric analysis of MR1-5-OP-RU tetramer enriched pooled thymi from 2, 4 or 8-week-old  
381 C57BL/6 mice for CD24 and CD44 expression. (b) Percentages of stage 1, stage 2 and stage 3 MAIT  
382 cells in 2, 4 and 8-week-old mice thymi. (c) ROR $\gamma$ t and T-bet expression on stage 1, stage 2 and stage 3  
383 MAIT cells from enriched C57BL/6 wild-type (WT) mouse thymi. (d) Flow cytometric analysis of stage  
384 1, stage 2, and stage 3 thymic MAIT cells stimulated with PMA/ionomycin, and examined for IFN- $\gamma$   
385 and IL-17A expression. Data are representative of 2 independent experiments with a total of 4 separate  
386 samples (pools of 5 thymi) per age group (a, b; mean  $\pm$  SEM), or of 3 independent experiments from 4-  
387 5 week old mice (c, d).

388

389 **Figure 3. Precursor product relationship of mouse MAIT cells**

390 (a) Flow cytometric analysis of stage 1, stage 2, and stage 3 TRAV1-TRAJ33 TCR transgenic C $\alpha$ <sup>-/-</sup>  
391 thymic MAIT cells; and stage 1 and stage 3 WT thymic MAIT cells purified by flow cytometric sorting  
392 (day 0) and at the end of culture (day 5) in the presence or absence of OP9 cells, with or without anti-  
393 MR1 antibody ( $\alpha$ -MR1), and analyzed for the expression of CD24 and CD44. (b) Percentages and  
394 numbers of stage 3 MAIT cells at the end of culture. Data are representative of 3 independent  
395 experiments (a, b; mean  $\pm$  SEM).

396

397 **Figure 4. PLZF controls development of MAIT cells**

398 (a) Flow cytometric analysis of stage 1, stage 2 and stage 3 cells thymic MAIT cells from WT mice for  
399 PLZF expression. (b) Flow cytometric analysis of MAIT cells from MR1-5-OP-RU enriched thymus,  
400 spleen, and lymph nodes from WT and PLZF-null mice for CD24, CD44, and CD4/CD8 co-receptor  
401 expression. (c) Numbers of MAIT cells in thymus, spleen and lymph nodes of WT and PLZF-null mice,  
402 and percentages of stage 1, stage 2 and stage 3 MAIT cells in enriched thymus, spleen and lymph nodes  
403 of WT and PLZF-null mice. (d) Flow cytometric analysis of MAIT cells in unstimulated (Unstim) and  
404 PMA/ionomycin stimulated (Stim) enriched thymus, spleen and lymph nodes from WT and PLZF-null  
405 mice, examined for IFN- $\gamma$  and IL-17A expression. \*P<0.1 \*\*P<0.01 \*\*\*P<0.001 (Mann-Whitney rank  
406 sum U test (c)). ND = not detectable. Data are representative of 3 independent experiments with a total  
407 of 9 mice per group (b, c; mean  $\pm$  SEM) or of 2 independent experiments with a total of 6 mice per group  
408 (d).

409

410 **Figure 5. MAIT cell development is impaired in Droscha KO mice and germ-free mice.**

411 (a) Flow cytometric analysis of MAIT cells from MR1-5-OP-RU tetramer enriched thymus, spleen,  
412 lymph nodes from *Droscha*<sup>fl/+</sup> *CD4-Cre* heterozygous control mice and *Droscha*<sup>fl/fl</sup> *CD4-Cre* mice for  
413 CD24, CD44 and CD4/CD8 co-receptor expression. (b) Percentages of stage 1, stage 2 and stage 3  
414 MAIT cells in enriched thymus of *Droscha*<sup>fl/+</sup> *CD4-Cre* and *Droscha*<sup>fl/fl</sup> *CD4-Cre* mice. Absolute numbers  
415 and percentage of stage 3 MAIT cells from thymus of *Droscha*<sup>fl/+</sup> *CD4-Cre* and *Droscha*<sup>fl/fl</sup> *CD4-Cre* mice.  
416 Absolute numbers and percentage of MAIT cells of TCR $\beta$ <sup>+</sup> cells in spleen and lymph nodes of *Droscha*<sup>fl/+</sup>  
417 *CD4-Cre* and *Droscha*<sup>fl/fl</sup> *CD4-Cre* mice. (c) Flow cytometric analysis of MAIT cells from MR1-5-OP-  
418 RU tetramer enriched thymus and spleen from control specific-pathogen-free (SPF) and germ-free (GF)  
419 mice for CD24, CD44, and CD4/CD8 co-receptor expression. (d) Absolute numbers and percentage of  
420 stage 3 MAIT cells of TCR $\beta$ <sup>+</sup> in thymus of SPF and GF mice. Absolute numbers and percentage of  
421 MAIT cells of TCR $\beta$ <sup>+</sup> cells in spleens of SPF and GF mice. \*P<0.1 \*\*P<0.01 \*\*\*P<0.001 (Mann-  
422 Whitney rank sum U test (b, d)). Data are representative of 3 independent experiments with a total of 8  
423 mice per group (a, b; mean  $\pm$  SEM) or of 2 independent experiments with a combined total of 11-15  
424 mice per group (c, d; mean  $\pm$  SEM).

425

426 **Figure 6. MAIT cell numbers are increased in CD1d-deficient mice.**

427 (a) Flow cytometric analysis of MAIT cells from thymus and spleen from BALB/c WT and BALB/c  
428 CD1d-deficient mice for CD24, CD44 and CD4/CD8 co-receptor expression. (b) Percentages of CD44<sup>+</sup>  
429 MAIT cells in the thymus of BALB/c WT, BALB/c CD1d-deficient, C57BL/6 WT and C57BL/6 CD1d-  
430 deficient mice. Absolute numbers and percentages of MAIT cells of TCR $\beta$ <sup>+</sup> cells in thymus and spleen  
431 from BALB/c WT, BALB/c CD1d-deficient, C57BL/6 WT and C57BL/6 CD1d-deficient mice. \*P<0.1  
432 \*\*P<0.01 \*\*\*P<0.001 (Mann-Whitney rank sum U test (b)). Data are representative of 3 independent  
433 experiments with a total of 6 mice per group (a, b; mean  $\pm$  SEM).

434

435 **Figure 7. Identification of distinct MAIT cell subsets in humans**

436 (a) First two panels: Flow cytometric analysis of CD3<sup>+</sup> cells from adult blood, young blood from thymus  
437 donors, cord blood and human thymus samples enriched for TRAV1-2<sup>+</sup> cells for MR1-Ac-6FP<sup>+</sup> or MR1-  
438 5-OP-RU<sup>+</sup> cells. CD3<sup>+</sup> TRAV1-2<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup> MAIT cells were analyzed for CD4/CD8,  
439 CD161/CD218 and CD161/CD27 expression. (b) Percentage MR1-5-OP-RU tetramer<sup>+</sup> MAIT cells of  
440 total TRAV1-2<sup>+</sup> population in thymus, cord blood, young and adult blood. (c) Stage 1 (CD161<sup>-</sup>CD218<sup>-</sup>)  
441 MAIT cells expressed as a percentage of total TRAV1-2<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup> MAIT cells in  
442 thymus, cord blood, young and adult blood. (d) Percentages of CD4<sup>+</sup>CD8<sup>+</sup> (DP), CD4<sup>+</sup>CD8<sup>-</sup>, and CD4<sup>-</sup>  
443 CD8<sup>+</sup> TRAV1-2<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup> MAIT cells in human thymus and young blood. Donor  
444 matched samples are indicated with a line. (e) Percentage MR1-5-OP-RU tetramer<sup>+</sup> MAIT cells of total  
445 TRAV1-2<sup>+</sup> population in donor matched thymus and young blood samples. (f) Percentage MR1-5-OP-  
446 RU tetramer<sup>+</sup> MAIT cells of total CD3<sup>+</sup> population in young blood samples. (g) Percentages of stage 1,  
447 stage 2, and stage 3 MR1-5-OP-RU tetramer<sup>+</sup> MAIT cells in thymus, cord blood, young blood and adult  
448 blood. \*P<0.1 \*\*P<0.01 \*\*\*P<0.001 (Mann-Whitney rank sum U test (d)). Data show 9 samples for  
449 cord, young and adult blood and 12 thymus samples (a, b, c) or 5 thymus, 4 cord blood, 4 young blood  
450 and 5 adult blood (g). Stage 1 (S1, CD161<sup>-</sup>CD27<sup>-</sup>), stage 2 (S2, CD161<sup>-</sup>CD27<sup>+</sup>), and stage 3 (S3  
451 CD161<sup>+</sup>CD27<sup>+/lo</sup>) (a, g).

452

453 **Figure 8. Comparison of human MAIT cells from thymus and blood**

454 (a) Flow cytometric analysis of thymic stage 1 (S1, CD161<sup>-</sup>CD27<sup>-</sup>, blue), stage 2 (S2, CD161<sup>-</sup>CD27<sup>+</sup>,  
455 green) and stage 3 cells (S3, CD161<sup>+</sup>CD27<sup>+/lo</sup>, red) TRAV1-2<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup> MAIT cells,  
456 young blood TRAV1-2<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup> MAIT cells, and adult blood TRAV1-2<sup>+</sup> MR1-5-OP-  
457 RU tetramer<sup>+</sup> MAIT cells for CD4/CD8, and CD8α/CD8β expression. (b) Flow cytometric analysis of  
458 double positive (DP) thymocytes, stage 1, stage 2, stage 3 thymic MAIT cells from thymus, and MAIT  
459 cells from matched donor young blood for PLZF, RORγt and T-bet expression. (c) Flow cytometric  
460 analysis of stage 1, stage 2, and stage 3 MAIT cells following PMA/ionomycin stimulation (Stim) from  
461 enriched thymus and young blood, analyzed for IFN-γ and TNF. (d) IFN-γ and TNF producing stage 1,  
462 stage 2, and stage 3 MAIT cells expressed as a percentage of cytokine producing MAIT cells. ND = not  
463 detectable. Data are representative of 6 thymus samples and 6 young blood samples, and 5 adult blood  
464 samples (a), or of 5 thymus and 5 young blood samples (b), or of 4 thymus and 4 young blood donor  
465 samples (c, d; mean ± SEM).

466

467

468

469

470 **Online Methods**

471 **Mice**

472 C57BL/6 (B6) mice, MR1-deficient mice, TRAV1-TRAJ33 TCR transgenic  $C\alpha^{-/-}$  mice, IL-18-deficient  
473 mice were all male, on a C57BL/6 background. Male CD1d-deficient mice on C57BL/6 and BALB/c  
474 backgrounds were bred in house at the Department of Microbiology and Immunology Animal House,  
475 University of Melbourne. PLZF-null male and female mice were generated and bred in house at the John  
476 Curtin School of Medical Research as previously described<sup>15</sup>. Germ-free male C57BL/6 and control SPF  
477 male C57BL/6 mice were generated at the Walter and Eliza Hall Institute Animal Facility. All  
478 procedures using mice were approved by the University of Melbourne Animal Ethics Committees, the  
479 Australian National University Animal Experimentation Ethics Committee, or by the Walter and Eliza  
480 Hall Institute Animal Ethics Committee. *Drosha*<sup>fl/+</sup> *CD4-Cre* heterozygous control mice and  
481 *Drosha*<sup>fl/fl</sup> *CD4-Cre* mice, male and female, were generated and bred as previously described in<sup>30</sup>. Male  
482 IL-18R $\alpha$ -deficient mice were generated and bred as previously described<sup>34</sup>. Single cell suspensions from  
483 mouse thymus, spleen, lung and inguinal lymph nodes were prepared as previously described<sup>15</sup>.

484  
485 **Human blood and tissue**

486 Adult peripheral human blood samples were obtained from the Australian Red Cross Blood Service  
487 under agreement number 13-04VIC-07. Young human peripheral blood samples and matching thymus  
488 (donors ranged from 5 days to 14 years of age) were obtained from the Royal Children's Hospital (RCH),  
489 Victoria, Australia. Umbilical cord blood samples were obtained from the Mercy Hospital for Women,  
490 Victoria, Australia. Experiments were conducted in accordance with University of Melbourne Human  
491 Research and Ethics committee guidelines (reference numbers 1035100 and 1443540), Mercy Health  
492 Human Research Ethics Committee Approval (reference number R14/25) and RCH Human Research  
493 Ethics Committee Approval (reference number 24131 G). Blood mononuclear cells were isolated by  
494 Ficoll-Paque Plus<sup>TM</sup> density gradient centrifugation (GE Healthcare). Donor thymi were cut into small  
495 pieces and passed through a 70 micron cell strainer into ice-cold RPMI-1640 medium containing 2mM  
496 EDTA before being washed into PBS + 2% Fetal Calf Serum (FACS buffer).

497  
498 **Magnetic bead enrichment of thymic MAIT cells.**

499 Mouse and human MR1 tetramers were generated and biotinylated as previously described<sup>2,19</sup>.  
500 Biotinylated MR1-5-OP-RU or control or Ac-6-FP monomers were tetramerized with streptavidin  
501 conjugated to either PE (SA-PE) (BD Pharmingen) or Brilliant Violet 421 (SA-BV) (Biolegend). Single  
502 cell suspensions of mouse thymus were prepared and stained with PE-mouse MR1-5-OP-RU tetramers  
503 prior to magnetic bead enrichment using anti-PE microbeads as per manufacturer's instructions  
504 (Miltenyi Biotec). One independent enriched sample represents 3 pooled thymi unless otherwise  
505 specified. Single cell suspensions of human thymus were enriched for TRAV1.2<sup>+</sup> cells by staining for



506 TRAV1.2-PE antibody, followed by magnetic bead enrichment using anti-PE microbeads (Miltenyi  
507 Biotec).

508

### 509 **Single cell TCR sequencing.**

510 MR1-5-OP-RU tetramer<sup>+</sup> cells were single cell sorted based on CD24 and CD44 expression and cDNA  
511 prepared using SuperScript VILO (Invitrogen) as per manufacturer's instructions. Transcripts encoding  
512 different V $\alpha$  and V $\beta$  genes were amplified using multiplex nested PCR as previously described<sup>43</sup>. PCR  
513 products were separated using a 1.5% agarose gel and sequenced by The Molecular Diagnostics Unit,  
514 University of Melbourne.

515

### 516 **Flow Cytometry.**

517 Mouse and human cells were stained with viability dye 7-aminoactinomycin D (7-AAD; Sigma) and the  
518 cell surface antibodies as listed in Supplementary Table 2. Cells were analyzed using a BD LSR Fortessa  
519 equipped with a 561nm yellow-green laser and data processed using FlowJo software (Treestar). Mouse  
520 cells are gated on B220<sup>-</sup> lymphocytes and human cells on CD14<sup>-</sup>CD19<sup>-</sup> lymphocytes after dead cell and  
521 doublet exclusion. Mouse MAIT cells were sorted using a BD FACSAriaIII cell sorter.

522

### 523 **Intracellular cytokine and intracellular transcription factor staining.**

524 Briefly, magnetic bead enriched MR1-5-OP-RU tetramer<sup>+</sup> cells from mouse thymus and enriched  
525 TRAV1.2<sup>+</sup> cells from human thymus were stimulated for 4h with PMA (10 ng/ml) and ionomycin (1  
526  $\mu$ g/ml) in the presence of GolgiStop (BD Biosciences). Surface staining of the cells was then performed,  
527 before the cells were fixed and permeabilized using BD Cytotfix/Cytoperm kit (BD Biosciences) as per  
528 manufacturer's instructions. Cells were then stained for intracellular cytokines as listed in  
529 Supplementary Table 3. Transcription factors were assessed by staining with antibodies as listed in  
530 Supplementary Table 3 after the cells were surface-stained and permeabilized with the eBioscience  
531 Foxp3 Fixation/Permeabilization kit, according to the manufacturer's instructions.

532

### 533 **OP9 co-culture differentiation assay.**

534 To verify the precursor-product relationship of mouse MAIT cells, an adaptation of the OP9 co-culture  
535 protocol similar to that previously described was used<sup>1</sup>. Briefly, OP9 cells were plated in flat 96-well  
536 plates at sub-confluency in complete tissue culture media (DMEM media supplemented with 10% (v/v)  
537 Fetal Calf Serum (FCS), 1x GlutaMAX<sup>TM</sup> (2mM L-Glutamine, Gibco) 15mM HEPES (Gibco), 0.1mM  
538 NEAA (non-essential amino acids, Invitrogen), 100U/ml penicillin (sodium salt, Gibco), 1mM sodium  
539 pyruvate (Invitrogen), 100 $\mu$ g/ml streptomycin sulfate (Gibco) and 50 $\mu$ M 2-mercaptoethanol (Sigma).  
540 1x10<sup>3</sup> sort purified mouse thymus MAIT cells from stage 1 (CD24<sup>+</sup>CD44<sup>-</sup>), stage 2 (CD24<sup>-</sup>CD44<sup>-</sup>) or  
541 stage 3 (CD24<sup>-</sup>CD44<sup>+</sup>) were cultured in the presence or absence of OP9 stromal cells with media

542 supplemented with mouse IL-2 (50ng/ml, Peprotech). 10µg/ml of α-MR1 blocking antibody (clone  
543 8F2.F9)<sup>2</sup> was added in blocking experiments. Co-cultures were kept at 37°C at 5% CO<sub>2</sub> for 5 days. MAIT  
544 cells were harvested, stained with antibodies and analyzed via flow cytometry.

545

#### 546 **Methods Only References**

547

548 1. Dash, P. *et al.* Paired analysis of TCRalpha and TCRbeta chains at the single-cell level in mice.  
549 *J Clin Invest* **121**, 288-295 (2011).

550

551 2. Chua, W.J. *et al.* Endogenous MHC-related protein 1 is transiently expressed on the plasma  
552 membrane in a conformation that activates mucosal-associated invariant T cells. *Journal of*  
553 *immunology* **186**, 4744-4750 (2011).

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557

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575

#### 576 **Author contributions**

577 HK, DGP and NAG performed experiments and HK prepared figures. AE, LLoH, LKM, BER, CAN-P,  
578 MFN, SB, ZC, AJC, SBGE, BM, YdU, IK, ML, LLiu, CCG, DPF, JR, MCC, SJT, KK, SPB, GTB and  
579 JM facilitated experiments and/or provided key reagents and tissue samples. HK, APU, DIG and DGP  
580 planned experiments, interpreted data and prepared the manuscript. DIG and DGP led the investigation.

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587 **References**

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1. Treiner, E. *et al.* Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. *Nature* **422**, 164-169 (2003).
2. Corbett, A.J. *et al.* T-cell activation by transitory neo-antigens derived from distinct microbial pathways. *Nature* **509**, 361-365 (2014).
3. Kjer-Nielsen, L. *et al.* MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature* **491**, 717-723 (2012).
4. Tilloy, F. *et al.* An invariant T cell receptor alpha chain defines a novel TAP-independent major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in mammals. *J Exp Med* **189**, 1907-1921 (1999).
5. Gherardin, N.A. *et al.* Diversity of T Cells Restricted by the MHC Class I-Related Molecule MR1 Facilitates Differential Antigen Recognition. *Immunity* **44**, 32-45 (2016).
6. Tsukamoto, K., Deakin, J.E., Graves, J.A. & Hashimoto, K. Exceptionally high conservation of the MHC class I-related gene, MR1, among mammals. *Immunogenetics* **65**, 115-124 (2013).
7. Gold, M.C. & Lewinsohn, D.M. Co-dependents: MR1-restricted MAIT cells and their antimicrobial function. *Nature reviews* **11**, 14-19 (2013).
8. Dusseaux, M. *et al.* Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood* **117**, 1250-1259 (2011).
9. Le Bourhis, L. *et al.* Antimicrobial activity of mucosal-associated invariant T cells. *Nat Immunol* **11**, 701-708 (2010).
10. Tang, X.Z. *et al.* IL-7 Licenses Activation of Human Liver Intrahepatic Mucosal-Associated Invariant T Cells. *J Immunol* **190**, 3142-3152 (2013).
11. Martin, E. *et al.* Stepwise Development of MAIT Cells in Mouse and Human. *PLoS Biol* **7**, e54 (2009).
12. Gold, M.C. *et al.* Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biol* **8**, e1000407 (2010).
13. Meierovics, A., Yankelevich, W.J. & Cowley, S.C. MAIT cells are critical for optimal mucosal immune responses during in vivo pulmonary bacterial infection. *Proc Natl Acad Sci U S A* **110**, E3119-3128 (2013).
14. Seach, N. *et al.* Double-positive thymocytes select mucosal-associated invariant T cells. *J Immunol* **191**, 6002-6009 (2013).
15. Rahimpour, A. *et al.* Identification of phenotypically and functionally heterogeneous mouse mucosal-associated invariant T cells using MR1 tetramers. *J Exp Med* **212**, 1095-1108 (2015).
16. Ussher, J.E. *et al.* CD161(++) CD8(+) T cells, including the MAIT cell subset, are specifically activated by IL-12+IL-18 in a TCR-independent manner. *Eur J Immunol* **44**, 195-203 (2014).

- 639 17. Walker, L.J. *et al.* Human MAIT and CD8 $\alpha$  cells develop from a pool of type-17  
640 precommitted CD8 $^{+}$  T cells. *Blood* **119**, 422-433 (2012).  
641
- 642 18. Leeansyah, E., Loh, L., Nixon, D.F. & Sandberg, J.K. Acquisition of innate-like microbial  
643 reactivity in mucosal tissues during human fetal MAIT-cell development. *Nature*  
644 *communications* **5**, 3143 (2014).  
645
- 646 19. Eckle, S.B. *et al.* A molecular basis underpinning the T cell receptor heterogeneity of mucosal-  
647 associated invariant T cells. *J Exp Med* **211**, 1585-1600 (2014).  
648
- 649 20. Reantragoon, R. *et al.* Antigen-loaded MR1 tetramers define T cell receptor heterogeneity in  
650 mucosal-associated invariant T cells. *J Exp Med* **210**, 2305-2320 (2013).  
651
- 652 21. Eckle, S.B. *et al.* Recognition of Vitamin B Precursors and Byproducts by Mucosal Associated  
653 Invariant T Cells. *J Biol Chem* **290**, 30204-30211 (2015).  
654
- 655 22. Benlagha, K., Wei, D.G., Veiga, J., Teyton, L. & Bendelac, A. Characterization of the early  
656 stages of thymic NKT cell development. *J Exp Med* **202**, 485-492 (2005).  
657
- 658 23. Schmitt, T.M. & Zuniga-Pflucker, J.C. Induction of T cell development from hematopoietic  
659 progenitor cells by delta-like-1 in vitro. *Immunity* **17**, 749-756 (2002).  
660
- 661 24. Constantinides, M.G., McDonald, B.D., Verhoef, P.A. & Bendelac, A. A committed precursor  
662 to innate lymphoid cells. *Nature* **508**, 397-401 (2014).  
663
- 664 25. Kovalovsky, D. *et al.* The BTB-zinc finger transcriptional regulator PLZF controls the  
665 development of invariant natural killer T cell effector functions. *Nat Immunol* **9**, 1055-1064  
666 (2008).  
667
- 668 26. Kreslavsky, T. *et al.* TCR-inducible PLZF transcription factor required for innate phenotype of  
669 a subset of  $\{\gamma\}$   $\{\delta\}$  T cells with restricted TCR diversity. *Proc Natl Acad Sci U S A*  
670 **106**, 12453-12458 (2009).  
671
- 672 27. Savage, A.K. *et al.* The Transcription Factor PLZF Directs the Effector Program of the NKT  
673 Cell Lineage. *Immunity* **29**, 391-403 (2008).  
674
- 675 28. Heno-Mejia, J. *et al.* The microRNA miR-181 is a critical cellular metabolic rheostat essential  
676 for NKT cell ontogenesis and lymphocyte development and homeostasis. *Immunity* **38**, 984-  
677 997 (2013).  
678
- 679 29. Lee, Y. *et al.* The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415-  
680 419 (2003).  
681
- 682 30. Chong, M.M., Rasmussen, J.P., Rudensky, A.Y. & Littman, D.R. The RNaseIII enzyme  
683 Drosha is critical in T cells for preventing lethal inflammatory disease. *J Exp Med* **205**, 2005-  
684 2017 (2008).  
685
- 686 31. Zhou, L. *et al.* Tie2cre-induced inactivation of the miRNA-processing enzyme Dicer disrupts  
687 invariant NKT cell development. *Proc Natl Acad Sci U S A* (2009).  
688
- 689 32. Levy, M. *et al.* Microbiota-Modulated Metabolites Shape the Intestinal Microenvironment by  
690 Regulating NLRP6 Inflammasome Signaling. *Cell* **163**, 1428-1443 (2015).  
691

- 692 33. Wang, J. *et al.* Interleukin 18 function in atherosclerosis is mediated by the interleukin 18  
693 receptor and the Na-Cl co-transporter. *Nat Med* **21**, 820-826 (2015).  
694
- 695 34. Nold-Petry, C.A. *et al.* IL-37 requires the receptors IL-18Ralpha and IL-1R8 (SIGIRR) to carry  
696 out its multifaceted anti-inflammatory program upon innate signal transduction. *Nat Immunol*  
697 **16**, 354-365 (2015).  
698
- 699 35. Kawachi, I., Maldonado, J., Strader, C. & Gilfillan, S. MR1-restricted V alpha 19i mucosal-  
700 associated invariant T cells are innate T cells in the gut lamina propria that provide a rapid and  
701 diverse cytokine response. *J Immunol* **176**, 1618-1627 (2006).  
702
- 703 36. Cui, Y. *et al.* Mucosal-associated invariant T cell-rich congenic mouse strain allows functional  
704 evaluation. *J Clin Invest* **125**, 4171-4185 (2015).  
705
- 706 37. Engel, I. *et al.* Innate-like functions of natural killer T cell subsets result from highly divergent  
707 gene programs. *Nat Immunol* (2016).  
708
- 709 38. Lee, Y.J., Holzapfel, K.L., Zhu, J., Jameson, S.C. & Hogquist, K.A. Steady-state production of  
710 IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT  
711 cells. *Nat Immunol* **14**, 1146-1154 (2013).  
712
- 713 39. Godfrey, D.I., Stankovic, S. & Baxter, A.G. Raising the NKT cell family. *Nat Immunol* **11**,  
714 197-206 (2010).  
715
- 716 40. Borg, Z.D. *et al.* Polymorphisms in the CD1d promoter that regulate CD1d gene expression are  
717 associated with impaired NKT cell development. *J Immunol* **192**, 189-199 (2014).  
718
- 719 41. Gibbs, A. *et al.* MAIT cells reside in the female genital mucosa and are biased towards IL-17  
720 and IL-22 production in response to bacterial stimulation. *Mucosal immunology* (2016).  
721  
722  
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