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A three-stage intrathymic development pathway for the mucosal-associated invariant T cell lineage

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43
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-ribitylamino-uracil (5-OP-RU), presented by the major histocompatibility
58 complex (MHC) class-I-related protein, MR1^{1,2,3,4,5}. Both the MAIT T cell receptor (TCR) and MR1 are
59 evolutionarily conserved suggesting strong selective pressure to maintain MAIT cell-mediated
60 immunity^{6,7}. These cells express a semi-invariant TCR comprising an invariant TRAV1-2 TRAJ33
61 (TCR alpha variable 1-2⁺ TCR alpha joining 33) chain in humans (TRAV1⁺TRAJ33⁺ in mice) and a
62 constrained range of TCR β 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^{8,9,10,11}. Moreover,
65 they play important roles in the immunity to infection with a broad range of pathogens^{9,12,13}, and are also
66 involved in autoimmunity and other inflammatory responses²⁴. 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⁺CD8⁺ double positive (DP) thymocytes^{1,4,14}. While
72 phenotypically distinct subsets of MAIT cells have been defined, including CD4⁺, CD4⁻CD8⁻ and CD4⁻
73 CD8⁺ populations^{11,15}, 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 α)^{8,16,17,18}. 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^{2,15,19,20}. 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

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

RESULTS

Identification of mouse thymic MAIT cell progenitors

To understand MAIT cell development, MR1-5-OP-RU tetramers were used to characterize MAIT cells in the thymus and peripheral lymphoid tissues of mice. This identified a novel population of CD24⁺CD44⁻ MAIT cells that were exclusively present in thymus, whereas in peripheral organs essentially all MAIT cells were CD24⁻CD44⁺ (**Fig. 1a**). Control MR1 tetramers loaded with the non-agonist folate-derivative acetyl-6-formylpterin Ac-6-FP²¹ failed to stain MAIT cells from these organs (**Fig. 1a**), confirming specific staining. Single cell TCR sequence analysis of both CD24⁺CD44⁻ and CD24⁻CD44⁺ populations indicated that both populations utilized an invariant TRAV1-TRAJ33 TCR α -chain, paired with a limited range of TCR β -chains (TRBV13 and TRBV19), characteristic of the MAIT cell lineage (**Supplementary Table 1**)⁴. Comparison of CD24⁺CD44⁻ and CD24⁻CD44⁺ MAIT cells within the thymus indicated that CD24⁺CD44⁻ MAIT cells had low expression of CD103, CD122 (IL-2R β), CD127 (IL-7R α), CD218 (IL-18R), CD278 (ICOS) and NK1.1 (CD161), but higher expression of CD62L and CD69 compared to CD24⁻CD44⁺ MAIT cells (**Fig. 1b**). Staining of thymus MAIT cells enriched by MR1-5-OP-RU tetramer and magnetic beads indicated an additional subpopulation of CD24⁻CD44⁻ MAIT cells (**Fig. 1c**). Thus, based on their cell-surface phenotype, we tentatively defined these populations as stage 1 (CD24⁺CD44⁻), stage 2 (CD24⁻CD44⁻) and stage 3 (CD24⁻CD44⁺) MAIT cells (**Fig. 1c**). The vast majority of stage 1 thymic MAIT cells were small (FSC (forward scatter)^{lo}) CD4⁺ or CD4⁺CD8⁺ DPs, a phenotype that is typically associated with immature T cells in the thymus, and which also defines the earliest stage in the development of CD1d-restricted natural killer T (NKT) cells²². Stage 2 cells were larger, mostly CD4⁺CD8⁻, whereas stage 3 MAIT cells were the largest and resembled mature MAIT cells in peripheral organs, being mostly CD4⁻CD8⁻ or CD8⁺ (**Fig. 1a,c**). Most CD8⁺ MAIT cells in thymus expressed CD8 $\alpha\beta$ heterodimers, while those in the periphery express CD8 $\alpha\alpha$ homodimers or CD8 $\alpha\beta$ heterodimers (**Fig. 1a**), suggesting further extrathymic maturation of MAIT cells. Collectively, these data support the presence of three distinct stages of MAIT cell development within mouse thymus.

Functionally mature stage 3 MAIT cells arise through ontogeny

To investigate the developmental progression of the three stages of thymic MAIT cells, we performed an ontogeny study for mouse thymic MAIT cell subsets at 2, 4 and 8 weeks of age. Stage 1 CD24⁺CD44⁻ MAIT cells were the major subset (>50%) at 2 weeks, declining to ~30% at 4 weeks and ~10% of thymic MAIT cells in the adult 8-week-old mouse thymus (**Fig. 2a,b**). Conversely, the proportion of stage 3 CD24⁻CD44⁺ 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 γ t and T-bet (**Fig. 2c**), suggesting little capacity to secrete IL-17 and IFN- γ ¹⁵, while stage 3 MAIT
123 cells expressed either ROR γ t or T-bet (**Fig. 2c**), suggesting functional competence. Analysis of ROR γ 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 γ 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- γ 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 system²³. 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 α chain on a TCR-constant region-
137 null background (C α ^{-/-}), 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⁻CD44⁺ 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⁺ 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^{1,11,15}, 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

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

PLZF controls the maturation and function of MAIT cells

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

miRNA controls the development of MAIT cells

Because microRNAs (miRNAs) play important roles in regulating gene expression and profoundly influence development and function of NKT cells²⁸, we next examined the role of Drosha, a member of the ribonuclease-III superfamily that initiates miRNA processing in the nucleus^{28,29} in MAIT cell maturation. We used Drosha-floxed mice crossed with transgenic mice expressing *Cre* under the control of the CD4 promoter (*Drosha*^{fl/fl} *CD4-Cre*) mice, which have a marked Drosha deficiency from the CD4⁺CD8⁺ DP thymocyte stage³⁰. The vast majority of MAIT cells in the thymus of *Drosha*^{fl/fl} *CD4-Cre* mice were stage 1 cells, whereas stage 2 and stage 3 MAIT cells were both significantly reduced compared to *Drosha*^{fl/+} *CD4-Cre* heterozygous control mice (**Fig. 5a,b**), suggesting that development beyond stage 1 is dependent on miRNAs. Despite the developmental impairment in the *Drosha*^{fl/fl} *CD4-Cre* thymus, stage 3 CD44⁺ MAIT cells were still detected in spleen and lymph nodes of these mice, albeit at diminished numbers compared to heterozygous controls (**Fig. 5a,b**). We also observed a

significant reduction in NKT cells within thymus, spleen and lymph nodes of *Drosha*^{fl/fl} *CD4-Cre* mice compared to control mice (**Supplementary Fig. 3a**), which is consistent with observations in Dicer-deficient mice (another member of the ribonuclease-III superfamily³¹). Accordingly, miRNAs control MAIT cell development beyond stage 1.

MAIT cell development is impaired in germ-free mice

MAIT cells were previously reported to be absent in germ-free mice¹, thus we examined whether a developmental block occurred at a specific stage, comparing germ-free mice to specific-pathogen-free (SPF) control mice. The numbers of MAIT cells in the thymus and spleen of germ-free mice were significantly reduced (**Fig. 5a,b**). Moreover, thymic stage 3 CD44⁺ MAIT cells were diminished (**Fig. 5a,b**), while stage 1 CD24⁺CD44⁻ MAIT cells were increased in frequency, but not number, in germ-free mice (**Fig. 5a and data not shown**). In contrast, there was no difference in the number of NKT cells in germ-free mice compared to SPF controls (**Supplementary Fig. 3b**).

Since IL-18 expression is diminished in germ-free mice³² and because MAIT cells expressed IL-18R during their intrathymic maturation (**Fig. 1**), we examined whether the loss of IL-18 would impact on MAIT cell development. Similar to germ-free mice, IL-18-deficient mice displayed reduced thymic MAIT cell numbers and a decrease in the frequency of thymic stage 3 CD24⁻CD44⁺ MAIT cells, with an increase in the frequency, but not number, of stage 1 CD24⁺CD44⁻ MAIT cells compared to wild-type mice (**Supplementary Fig. 4a,b**). Furthermore, MAIT cells were significantly reduced in the spleen and lymph nodes of IL-18-deficient mice compared to wild-type mice (**Supplementary Fig. 4a**). Paradoxically, IL-18R α -deficient mice showed normal MAIT cell development and maturation within the thymus, spleen and lymph nodes (**Supplementary Fig. 4c,d**). Comparison of IL-18-deficient and IL-18R α -deficient mice may be complicated because IL-18 can signal through another receptor, the Na-Cl co-transporter³³ whereas another cytokine, IL-37, can signal through IL-18R³⁴. Thus, MAIT cell development and maturation is impaired in germ-free mice, suggesting an important role for commensal bacteria and IL-18 in this process.

MAIT cells are over-represented in CD1d-deficient mice

Because MAIT cells and NKT cells have some similarities, such as a dependence on PLZF and selection by DP thymocytes, we examined MAIT cell development in CD1d-deficient mice that lack both CD1d and CD1d-restricted NKT cells. CD1d-deficient mice showed increased numbers of MAIT cells in thymus, spleen (**Figure 6a,b and Supplementary Fig. 5**) and liver (**data not shown**) compared to wild-type controls on both BALB/c and C57BL/6 backgrounds, and this was particularly apparent on the BALB/c background (**Fig. 6a,b**). As previously reported¹⁵, a higher percentage of MAIT cells expressed the co-receptor CD8 in BALB/c compared to C57BL/6 mice, and this was seen in both wild-type and

CD1d-deficient mice (**Fig. 6a and Supplementary Fig. 5**). In terms of MAIT cell development, while numbers of stage 1 and 2 MAIT cells were not altered in CD1d-deficient mice, stage 3 CD24⁺CD44⁺ MAIT cells were far more numerous in thymus and spleen of CD1d-deficient mice compared to wild-type controls (**Figure 6a,b and Supplementary Fig. 5**). Conversely, NKT cell numbers were similar in thymus and spleen of MR1-deficient and wild-type mice (**data not shown**). Taken together, these data suggest that MAIT cells might compete with NKT cells for factors or a shared niche during their 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^{8,16,17}. MAIT cells (defined as TRAV1-2⁺ MR1-5-OP-RU tetramer⁺) 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⁺ T cells¹⁵. Because they were so infrequent in human
241 thymus, TRAV1-2⁺ 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⁺ thymocytes (**Fig. 7a,b**). The frequency of MAIT
243 cells as a percentage of TRAV1-2⁺ 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⁺CD161⁺ (**Fig. 7a,b**). The majority of MAIT cells in young blood
249 and cord blood were CD218⁺CD161⁺ (**Fig. 7a**). Thus, CD218⁺CD161⁺ 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⁺CD8⁺ or CD4⁺CD8⁺ (**Fig. 7a**), roughly half of thymus MAIT cells were either CD4⁺CD8⁺ or
253 CD4⁺CD8⁺ (**Fig. 7a**). Moreover, analysis of paired thymus and blood samples showed that CD4⁺ 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⁺ cells) remain relatively low and stable in the
260 thymus (0.1-0.3% of TRAV1-2⁺ cells) regardless of age (**Fig. 7e**), while in contrast, MAIT cell

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

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

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

Functional maturation begins at stage 3 in MAIT cell development

We next examined if the 3 stages of human thymic MAIT cells displayed a similar pattern of transcription factors compared to the same stages in mice. Akin to mouse thymic MAIT cells, the expression of PLZF, T-bet and RORγt increased between stage 2 and 3 (**Fig. 8b**) although RORγt was not highly expressed in mature MAIT cells (**Fig. 8b**). In contrast to mouse stage 3 MAIT cells where T-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 γ t, which likely reflected their recent
298 transition from CD4⁺CD8⁺ DP thymic precursors that are ROR γ t⁺ (Fig. 8b).

299

300 To examine their functional competence, human thymic and matched blood MAIT cells were tested for
301 IFN- γ , 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- γ 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- γ 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 γ 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⁺CD161⁺ and to identify MAIT cells in
319 humans¹⁸ or the use of transgenic TCR systems or ROR γ t to detect MAIT cells in mice^{11,35,36}. The
320 surrogate markers for human MAIT cells exclude CD161⁻ stage 1 and stage 2 MAIT cells, limiting
321 earlier studies to the minor subset of mature stage 3 cells in human thymus¹⁸. Furthermore, we found
322 that ~50% of TRAV1-2⁺CD161⁺ 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 γ t-GFP
324 combined with CD44 should identify many of the mature stage 3 MAIT cells in mice³⁶, this strategy will
325 miss immature stage 1 and stage 2 CD44⁻ MAIT cells and mature stage 3 MAIT cells that lack ROR γ 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^{25,27}, with the exception of IL-4-producing NKT2 cells^{37,38}. In contrast, PLZF
332 is expressed late in MAIT cell development, governing their final maturation step to generate functional

stage 3 MAIT cells, and is maintained at high levels by these cells. Upon maturation, most NKT cells upregulate NK1.1 and CD69 in mice and they are either CD4⁺ or CD4⁺CD8⁻. Conversely, most mature mouse MAIT cells lacked NK1.1 and downregulated CD69 upon maturation, and many expressed CD8. MAIT cell maturation was impaired in germ-free mice, whereas NKT cell maturation was intact, and, as previously shown, the SLAM adaptor protein (SAP) is critical for NKT cell development³⁹, whereas MAIT cells appear to be SAP-independent¹¹. Furthermore, our observation that MAIT cells are over-represented in CD1d (NKT cell)-deficient mice suggests that these cells may compete for an environmental niche. This may partly explain why CAST/EiJ mice, which have less NKT cells⁴⁰, have higher numbers of MAIT cells compared to C57BL/6 controls³⁶. It follows that careful consideration is required when studying NKT cell deficient mice as these will differ from wild-type control mice not only because they lack NKT cells, but also because they have increased MAIT cells.

While the developmental sequence for MAIT cells in mice and humans has many parallels, there were also some distinctions. Although we detected stage 3 MAIT cells in human thymus, these were in the minority and were only partially functional compared to stage 3 MAIT cells in mouse thymus. Human peripheral blood MAIT cells accumulate with age, and also acquire greater cytokine producing capacity and many switch from CD8αβ to CD8αα expressing cells. In further contrast to mouse MAIT cells, neither thymus nor blood MAIT cells from humans produced IL-17. Given that human MAIT cells isolated from liver and female genital tract can produce IL-17^{10,41}, this suggests that further environment-dependent extrathymic maturation influences human MAIT cell function. We hypothesize that this peripheral expansion and maturation is shaped by exposure to microbial antigens over time, which is also supported by our studies with germ-free mice where stage 3 MAIT cells were significantly reduced in thymus and periphery and also by a recent paper that demonstrated microbial mediated expansion of peripheral MAIT cells in a mouse model of Salmonella infection⁴².

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

Figure Legends

364

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

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

378

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

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

388

Figure 3. Precursor product relationship of mouse MAIT cells

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

396

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- γ 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 Drosha 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 *Drosha*^{fl/+} *CD4-Cre* heterozygous control mice and *Drosha*^{fl/fl} *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 *Drosha*^{fl/+} *CD4-Cre* and *Drosha*^{fl/fl} *CD4-Cre* mice. Absolute numbers
 415 and percentage of stage 3 MAIT cells from thymus of *Drosha*^{fl/+} *CD4-Cre* and *Drosha*^{fl/fl} *CD4-Cre* mice.
 416 Absolute numbers and percentage of MAIT cells of TCR β ⁺ cells in spleen and lymph nodes of *Drosha*^{fl/+}
 417 *CD4-Cre* and *Drosha*^{fl/fl} *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 β ⁺ in thymus of SPF and GF mice. Absolute numbers and percentage of
 421 MAIT cells of TCR β ⁺ 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⁺
 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 β ⁺ 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⁺ cells from adult blood, young blood from thymus
 437 donors, cord blood and human thymus samples enriched for TRAV1-2⁺ cells for MR1-Ac-6FP⁺ or MR1-
 438 5-OP-RU⁺ cells. CD3⁺ TRAV1-2⁺ MR1-5-OP-RU tetramer⁺ MAIT cells were analyzed for CD4/CD8,
 439 CD161/CD218 and CD161/CD27 expression. (b) Percentage MR1-5-OP-RU tetramer⁺ MAIT cells of
 440 total TRAV1-2⁺ population in thymus, cord blood, young and adult blood. (c) Stage 1 (CD161⁻CD218⁻)
 441 MAIT cells expressed as a percentage of total TRAV1-2⁺ MR1-5-OP-RU tetramer⁺ MAIT cells in
 442 thymus, cord blood, young and adult blood. (d) Percentages of CD4⁺CD8⁺ (DP), CD4⁺CD8⁻, and CD4⁻
 443 CD8⁺ TRAV1-2⁺ MR1-5-OP-RU tetramer⁺ MAIT cells in human thymus and young blood. Donor
 444 matched samples are indicated with a line. (e) Percentage MR1-5-OP-RU tetramer⁺ MAIT cells of total
 445 TRAV1-2⁺ population in donor matched thymus and young blood samples. (f) Percentage MR1-5-OP-
 446 RU tetramer⁺ MAIT cells of total CD3⁺ population in young blood samples. (g) Percentages of stage 1,
 447 stage 2, and stage 3 MR1-5-OP-RU tetramer⁺ 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⁻CD27⁻), stage 2 (S2, CD161⁻CD27⁺), and stage 3 (S3
 451 CD161⁺CD27^{+/lo}) (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⁻CD27⁻, blue), stage 2 (S2, CD161⁻CD27⁺,
 455 green) and stage 3 cells (S3, CD161⁺CD27^{+/lo}, red) TRAV1-2⁺ MR1-5-OP-RU tetramer⁺ MAIT cells,
 456 young blood TRAV1-2⁺ MR1-5-OP-RU tetramer⁺ MAIT cells, and adult blood TRAV1-2⁺ MR1-5-OP-
 457 RU tetramer⁺ 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).

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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 described¹⁵. 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*^{fl/+} *CD4-Cre* heterozygous control mice and
481 *Drosha*^{fl/jf} *CD4-Cre* mice, male and female, were generated and bred as previously described in³⁰. Male
482 IL-18R α -deficient mice were generated and bred as previously described³⁴. Single cell suspensions from
483 mouse thymus, spleen, lung and inguinal lymph nodes were prepared as previously described¹⁵.

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 PlusTM 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).

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

499 Mouse and human MR1 tetramers were generated and biotinylated as previously described^{2,19}.
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⁺ 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⁺ 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 α and V β genes were amplified using multiplex nested PCR as previously described⁴³. 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⁺ lymphocytes and human cells on CD14⁺CD19⁺ 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⁺ cells from mouse thymus and enriched
525 TRAV1.2⁺ cells from human thymus were stimulated for 4h with PMA (10 ng/ml) and ionomycin (1
526 μ 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 Cytofix/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 used¹. 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 GlutaMAXTM (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 μ g/ml streptomycin sulfate (Gibco) and 50 μ M 2-mercaptoethanol (Sigma).
540 1x10³ sort purified mouse thymus MAIT cells from stage 1 (CD24⁺CD44⁻), stage 2 (CD24⁻CD44⁻) or
541 stage 3 (CD24⁻CD44⁺) 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)² was added in blocking experiments. Co-cultures were kept at 37°C at 5% CO₂ 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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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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586

587 **References**

- 588 1. Treiner, E. *et al.* Selection of evolutionarily conserved mucosal-associated invariant T cells by
589 MR1. *Nature* **422**, 164-169 (2003).
591
- 592 2. Corbett, A.J. *et al.* T-cell activation by transitory neo-antigens derived from distinct microbial
593 pathways. *Nature* **509**, 361-365 (2014).
594
- 595 3. Kjer-Nielsen, L. *et al.* MR1 presents microbial vitamin B metabolites to MAIT cells. *Nature*
596 **491**, 717-723 (2012).
597
- 598 4. Tilloy, F. *et al.* An invariant T cell receptor alpha chain defines a novel TAP-independent
599 major histocompatibility complex class Ib-restricted alpha/beta T cell subpopulation in
600 mammals. *J Exp Med* **189**, 1907-1921 (1999).
601
- 602 5. Gherardin, N.A. *et al.* Diversity of T Cells Restricted by the MHC Class I-Related Molecule
603 MR1 Facilitates Differential Antigen Recognition. *Immunity* **44**, 32-45 (2016).
604
- 605 6. Tsukamoto, K., Deakin, J.E., Graves, J.A. & Hashimoto, K. Exceptionally high conservation of
606 the MHC class I-related gene, MR1, among mammals. *Immunogenetics* **65**, 115-124 (2013).
607
- 608 7. Gold, M.C. & Lewinsohn, D.M. Co-dependents: MR1-restricted MAIT cells and their
609 antimicrobial function. *Nature reviews* **11**, 14-19 (2013).
610
- 611 8. Dusseaux, M. *et al.* Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161^{hi} IL-
612 17-secreting T cells. *Blood* **117**, 1250-1259 (2011).
613
- 614 9. Le Bourhis, L. *et al.* Antimicrobial activity of mucosal-associated invariant T cells. *Nat*
615 *Immunol* **11**, 701-708 (2010).
616
- 617 10. Tang, X.Z. *et al.* IL-7 Licenses Activation of Human Liver Intrasinusoidal Mucosal-Associated
618 Invariant T Cells. *J Immunol* **190**, 3142-3152 (2013).
619
- 620 11. Martin, E. *et al.* Stepwise Development of MAIT Cells in Mouse and Human. *PLoS Biol* **7**, e54
621 (2009).
622
- 623 12. Gold, M.C. *et al.* Human mucosal associated invariant T cells detect bacterially infected cells.
624 *PLoS Biol* **8**, e1000407 (2010).
625
- 626 13. Meierovics, A., Yankelevich, W.J. & Cowley, S.C. MAIT cells are critical for optimal mucosal
627 immune responses during in vivo pulmonary bacterial infection. *Proc Natl Acad Sci U S A* **110**,
628 E3119-3128 (2013).
629
- 630 14. Seach, N. *et al.* Double-positive thymocytes select mucosal-associated invariant T cells. *J*
631 *Immunol* **191**, 6002-6009 (2013).
632
- 633 15. Rahimpour, A. *et al.* Identification of phenotypically and functionally heterogeneous mouse
634 mucosal-associated invariant T cells using MR1 tetramers. *J Exp Med* **212**, 1095-1108 (2015).
635
- 636 16. Ussher, J.E. *et al.* CD161(++) CD8(+) T cells, including the MAIT cell subset, are specifically
637 activated by IL-12+IL-18 in a TCR-independent manner. *Eur J Immunol* **44**, 195-203 (2014).
638

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

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