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**1 A population of pro-inflammatory TRAV1-2<sup>+</sup> CD8<sup>+</sup> T-cells including oligoconal  
2 expansions of MAIT cells is enriched in the airways in human tuberculosis**

3  
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57

58

59

60 **Abstract.**

61

62 Mucosal-associated invariant T (MAIT) cells typically express a TRAV1-2<sup>+</sup> semi-invariant  
63 TCR $\alpha$  that enables recognition of bacterial, mycobacterial, and fungal riboflavin  
64 metabolites presented by MR1. MAIT cells are associated with immune control of  
65 bacterial and mycobacterial infections in murine models. Here, we report that a  
66 population of pro-inflammatory TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells are present in the airways and  
67 lungs of healthy individuals and are enriched in bronchoalveolar fluid of patients with  
68 active pulmonary tuberculosis (TB). High-throughput T cell receptor analysis reveals  
69 oligoclonal expansions of canonical and donor-unique TRAV1-2<sup>+</sup> MAIT-consistent TCR $\alpha$   
70 sequences within this population. Some of these cells demonstrate MR1-restricted  
71 mycobacterial reactivity and phenotypes suggestive of MAIT cell identity. These findings  
72 demonstrate enrichment of TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells with MAIT or MAIT-like features in  
73 the airways during active TB and suggest a role for these cells in the human pulmonary  
74 immune response to *Mycobacterium tuberculosis*.

75

76 **Introduction**

77

78 Mucosal-associated invariant T (MAIT) cells are unconventional lymphocytes that use  
79 semi-invariant T cell receptor-alpha (TCR $\alpha$ ) chains to recognize non-peptide small  
80 molecule ligands presented by the HLA-Ib molecule MR1<sup>1-6</sup>. In mice, MAIT cells have  
81 been shown to play a protective role in models of respiratory infection<sup>7-10</sup>. In humans,  
82 MAIT cells are abundant in the peripheral blood of healthy individuals, where they  
83 produce cytolytic enzymes and pro-inflammatory cytokines and typically express a  
84 TRAV1-2<sup>+</sup> TCR $\alpha$  chain and the CD8 coreceptor<sup>1-4,11-13</sup>. MAIT cells are depleted in the  
85 blood of humans with TB<sup>4,5,14</sup>. However, little is known about the function and phenotype  
86 of MAIT cells in the human lung, especially in the setting of pulmonary tuberculosis (TB).  
87 We postulated that MAIT cells are recruited to and/or expand at sites where  
88 *Mycobacterium tuberculosis* (Mtb) antigens are present, potentially acting as sentinels of  
89 infection in the respiratory mucosa.

90

91 Here we report that a population of pro-inflammatory TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells are present  
92 in the airways and lungs of healthy individuals and are enriched in bronchoalveolar fluid  
93 of patients with active pulmonary TB. Some of these cells demonstrate MR1-restricted  
94 mycobacterial reactivity, phenotypic features and/or TCR $\alpha$  chain usage suggestive of  
95 MAIT cell identity. We conclude that TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells with MAIT or MAIT-like  
96 features are oligoclonally expanded in the airways during active TB suggesting that they  
97 play a role in the human pulmonary immune response to *Mycobacterium tuberculosis*.

98

## 99 **Results**

### 100 **TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in human lung and intestine tissues**

101 To explore the role of MAIT cells in healthy mucosal tissues, we first determined the  
102 frequency of TRAV1-2<sup>+</sup> CD8<sup>+</sup> cells in the respiratory tract of an individual organ donor  
103 (Fig. 1a). Dramatic enrichment was observed in the trachea, where nearly half of all  
104 CD8<sup>+</sup> T cells expressed TRAV1-2 (Fig. 1a). TRAV1-2<sup>+</sup> cells were also enriched in the  
105 proximal and distal bronchi (35% and 22% of CD8<sup>+</sup> T cells respectively) and in the lung  
106 parenchyma (17% of CD8<sup>+</sup> T cells), relative to the draining mediastinal lymph node  
107 where the frequency (6% of CD8<sup>+</sup> T cells) approximated levels typically found in  
108 peripheral blood<sup>11,15</sup>. To determine the anatomical localization of TRAV1-2<sup>+</sup> CD8<sup>+</sup> cells in  
109 the airway, we used immunohistochemistry to quantify CD8<sup>+</sup> and TRAV1-2<sup>+</sup> cells in 1<sup>st</sup>  
110 and 2<sup>nd</sup> order bronchial sections from three additional organ donors (Fig. 1b, left).  
111 Although the number of CD8<sup>+</sup> cells was similar in tissue sections from the proximal and  
112 distal airways, TRAV1-2<sup>+</sup> cells were more frequent in the proximal compared to distal  
113 airway (Fig. 1b, right). As expression of TRAV1-2<sup>+</sup> TCRs is insufficient to define MAIT  
114 cells, we also performed *ex vivo* functional assays in which cytokine-production by  
115 TRAV1-2<sup>+</sup> CD8<sup>+</sup> cells upon exposure to HLA mis-matched *M. smegmatis*-infected  
116 antigen-presenting cells is used to define mycobacterial-reactive MAIT cells<sup>4,12,16,17</sup>. In a  
117 single donor for whom paired tissues were available, we evaluated lymphocytes from  
118 lung parenchyma, the small intestinal lamina propria (LP), and the small intestinal  
119 intraepithelial lymphocytes (IEL) for *M. smegmatis*-dependent release of the pro-  
120 inflammatory cytokine TNF. Interestingly, TNF-producing TRAV1-2<sup>+</sup> cells were found  
121 only in the lung (Fig.1c, left). It is also notable that CD161, a C-type lectin highly  
122 expressed on peripheral MAIT cells<sup>11,13,17</sup>, was not detected on TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells  
123 from the lung but was found in abundance on small intestinal TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells  
124 (Fig.1c, right). We next compared the frequencies of TRAV1-2<sup>+</sup> and TNF-producing cells

125 in the lung (n=9) and intestinal mucosa (n=8, unmatched samples) where MAIT cells  
126 were initially found to be enriched<sup>3</sup>. The frequencies of TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells were  
127 similar across mucosal sites, associated lymphoid tissues and unmatched peripheral  
128 blood samples (n=6) (Fig. 1d). In contrast, significantly higher frequencies of TRAV1-2<sup>+</sup>  
129 cells from the lung produced TNF in response to *M. smegmatis*-infected cells compared  
130 with TRAV1-2<sup>+</sup> cells from lymphoid tissues, small intestine, or peripheral blood (P =  
131 0.035, 0.0025, 0.0023 and 0.0005 (Mann Whitney U-test), Fig. 1e). Cell yields from  
132 these tissues were insufficient to establish functional dependence on MR1 as has been  
133 shown previously with this assay<sup>18</sup>. Nonetheless, these data demonstrate that  
134 mycobacterial stimulation results in TNF production by donor-unrestricted, lung resident  
135 TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells.

136

### 137 **TRAV1-2<sup>+</sup> CDR3 $\alpha$ usage in Mtb-infected lung tissue**

138 On the basis of these results, we hypothesized that pulmonary infection with Mtb leads  
139 to the migration to and/or expansion of TRAV1-2<sup>+</sup> CD8<sup>+</sup> cells in the lung, potentially  
140 driven by Mtb-derived MR1 ligands. A hallmark of the human immune response to Mtb is  
141 the formation of lung granulomas. We therefore sought to determine the relevance of  
142 TRAV1-2<sup>+</sup> T cell receptor (TCR) usage in lung granulomas from patients with TB. Single  
143 cell suspensions were prepared from diseased lung parenchyma from individuals (n = 5)  
144 undergoing clinically indicated surgical resection for complications of TB<sup>19</sup>. The most  
145 highly diseased lung granuloma (LG) tissues were designated "A" and the least  
146 diseased tissues designated "C." CD4<sup>+</sup> T cells from these samples were sorted by flow  
147 cytometry and subjected to high-throughput repertoire analysis using the bias-controlled  
148 immunoSEQ TCR sequencing platform<sup>20</sup>. In the 12 samples that yielded the minimal  
149 necessary sequencing data for analysis (>10<sup>4</sup> productive reads, yielding a median of  
150 3,919 unique productive TCR $\alpha$  reads (range 397-28,792) and a median of 167 TRAV1-  
151 2-utilizing unique productive TCR $\alpha$  reads (range 19-1,081), the overall frequency of  
152 TRAV1-2<sup>+</sup> TCR sequences in granulomas ranged from 3.1 to 5.9% across all donors  
153 and tissue samples (Fig. 2a and Supplementary Table 1). These frequencies are similar  
154 to those observed in peripheral blood and lymph nodes. We then developed an  
155 algorithm based on published MAIT CDR3 $\alpha$  amino acid (aa) sequences<sup>16,21</sup> to determine  
156 which of these TRAV1-2<sup>+</sup> CDR3 $\alpha$  sequences represented MAIT cell-consistent TCR $\alpha$ s.  
157 A CDR3 $\alpha$  sequence similarity analysis was performed using "MAIT Match"

158 ([http://www.cbs.dtu.dk/services/MAIT\\_Match](http://www.cbs.dtu.dk/services/MAIT_Match)), a tool based on the method described by  
159 Shen et al,<sup>22</sup> where a score of 1 reflects a perfect match and a score of 0 a perfect  
160 mismatch with published MAIT cell CDR3 $\alpha$  sequences. To determine the validity of this  
161 tool, we compared the proportion of TRAV1-2<sup>+</sup> sequences with the proportion of  
162 TRAV12-2<sup>+</sup> sequences (an unrelated control) for TCRs with scores ranging from 0.85 to  
163 1. MAIT Match scores of 0.95 to 1 were significantly increased among the in TRAV1-2<sup>+</sup>  
164 but not TRAV12-2<sup>+</sup> TCR sequences ( $P = 0.0035$ ,  $P = 0.00046$ , t-test; Fig. 2b). We  
165 therefore chose a MAIT Match score of 0.95 as a conservative threshold to define MAIT  
166 cell-consistent TCRs (Fig. 2b). In one individual with paired samples from the lung and  
167 mediastinal lymph node (LN), TRAV1-2 usage was comparable at both sites, but  
168 similarity analysis revealed MAIT cell-consistent TCR enrichment in the lung ( $P <$   
169  $0.0001$ ; 2-way ANOVA; Fig. 2c).

170

171 To address the possibility that Mtb drives the recruitment and/or expansion of TRAV1-2<sup>+</sup>  
172 T cells with MAIT-consistent CDR3 $\alpha$ 's in granulomatous tissue, we analyzed the MAIT  
173 cell-consistent CDR3 $\alpha$  sequences (MAIT Match score 0.95-1) found in diseased lung  
174 parenchyma ( $n = 5$  individuals, 11 samples). It is established that certain MAIT cell  
175 TCR $\alpha$  chains can be shared among individuals (public sequences)<sup>23</sup>, while donor-unique  
176 (private) CDR3 $\alpha$  sequences can be selected in response to distinct microbes<sup>16</sup>. As  
177 shown in Figure 2d, both private and public CDR3 $\alpha$  sequences were detected among  
178 the MAIT cell-consistent CDR3 $\alpha$  sequences present in granulomatous lung tissue  
179 isolated from patients with TB. Notably, public MAIT cell-consistent CDR3 $\alpha$  were  
180 frequently encoded by multiple synonymous nucleotide sequences within individuals  
181 suggesting the expansion of multiple clones with the same CDR3 $\alpha$  amino acid  
182 sequences (Fig. 2e, right). In contrast, private MAIT cell-consistent CDR3 $\alpha$  sequences  
183 were encoded by individual nucleotide sequences suggesting that these were the result  
184 of expansions of a single MAIT cell clone in each donor (Fig. 2e, left). Private CDR3 $\alpha$   
185 sequences were not restricted to infrequent clonotypes and in some tissue samples  
186 occurred as the dominant MAIT cell-consistent TCR.

187

### 188 **Bronchoalveolar TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in active pulmonary TB**

189 Diminished frequencies of circulating MAIT cells have consistently been observed in  
190 people with TB<sup>4,5</sup>. This apparent peripheral depletion may occur as a consequence of

191 selective MAIT cell migration to the lung or may reflect increased host vulnerability to  
192 infection with Mtb. Having found that TRAV1-2<sup>+</sup> CD8<sup>+</sup> cells are enriched in healthy  
193 airways and respond to mycobacteria (Fig. 1e)<sup>4</sup>, we hypothesized that pulmonary  
194 infection with Mtb drives the accumulation and expansion of TRAV1-2<sup>+</sup> CD8<sup>+</sup> cells in the  
195 lung in response to Mtb-derived MR1 ligands. To address this possibility, we measured  
196 the frequency of TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in bronchoalveolar (BAL) fluid samples obtained  
197 from individuals with untreated, active pulmonary TB and controls with no evidence of  
198 infectious or inflammatory pulmonary disease (Supplementary Table 2). In BAL fluid,  
199 TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells were significantly enriched in patients with TB at frequencies  
200 approximately 3-fold higher than controls (P = 0.0022, Mann-Whitney U test, Fig 3a).  
201 Conversely, in matched peripheral blood samples, TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells were  
202 significantly diminished in patients with TB at frequencies approximately 2-fold lower  
203 compared to healthy controls (P = 0.0028, Mann-Whitney U test, Fig 3a). To assess the  
204 functional capacity of TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in the BAL fluid and matched peripheral  
205 blood samples, we utilized  $\alpha$ -CD2/CD3/CD28 beads as a stimulant to trigger responses  
206 via the TCR. Cell yields were insufficient to explore ligand-specific activation, which may  
207 also be subject to bias arising from compartment-specific differences in MR1-expression  
208 by antigen-presenting cells<sup>24</sup>. MAIT cells have been reported to produce IFN- $\gamma$ , TNF,  
209 granzymes, granulysin, IL-17 and IL-22.<sup>25-27</sup> Among these, we chose to measure TNF, a  
210 representative Th1 effector cytokine essential for immune control of Mtb<sup>28</sup> and IL-17, an  
211 immunomodulatory cytokine reportedly produced in a TCR-independent manner by  
212 MAIT cells<sup>29</sup>. A significantly greater proportion of TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in BAL fluid  
213 produced TNF (median 40%, range 36-91%) compared with TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in  
214 matched peripheral blood samples (median 15%, range 4.7-27%) (P = 0.004, Mann-  
215 Whitney U test, Fig. 3b, 3c and Supplementary Figure 1). In contrast fewer than 1% of  
216 TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in the BAL fluid and only 2% in matched peripheral blood  
217 samples produced IL-17 (Supplementary Figure 2). We therefore concluded that TCR  
218 triggering of these BAL-resident TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells does not evoke IL-17  
219 production, though other mitogenic or cytokine-associated stimulations may do so. Next,  
220 we characterized the phenotype of BAL resident TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells. MAIT cells can  
221 be defined in peripheral blood by TRAV1-2 usage in conjunction with high-level  
222 expression of the c-type lectin CD161, and the di-peptidase CD26.<sup>13,27</sup> In BAL fluid  
223 obtained from patients with TB, TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells expressed low levels of CD161  
224 compared with peripheral blood TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells (Fig. 3d), consistent with the



225 data from healthy lung tissue (Fig. 1c) and the prior demonstration that CD161 can be  
226 down-regulated as a result of MAIT cell activation<sup>17,29,30</sup>. In contrast, TRAV1-2<sup>+</sup> CD8<sup>+</sup> T  
227 cells in the BAL fluid more consistently expressed CD26, which is abundantly present on  
228 all functional MR1-restricted MAIT cells in peripheral blood<sup>17,25</sup>. CD103, the  $\alpha$ E integrin  
229 associated with tissue-resident memory T cells<sup>31</sup> was expressed variably but exclusively  
230 on BAL TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells.

231

232 Although TRAV1-2 usage is a defining feature of MAIT cells, the same gene segment  
233 can be expressed by T cells recognizing mycobacterial ligands presented in the context  
234 of HLA-Ia molecules and CD1b<sup>32</sup>. On the basis that TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells display a  
235 surface phenotype suggestive of tissue-resident MAIT cells in BAL fluid isolated from  
236 patients with active TB, we postulated that the corresponding CDR3 $\alpha$  sequences would  
237 provide a molecular signature reflecting MAIT cell enrichment relative to TRAV1-2<sup>+</sup> CD8<sup>+</sup>  
238 T cells in matched peripheral blood samples. To test this hypothesis, we performed  
239 high-throughput TCR repertoire analysis of TRAV1-2<sup>+</sup> CD4<sup>-</sup> T cells sorted by flow  
240 cytometry from cryopreserved BAL fluid and matched peripheral blood specimens  
241 obtained from three donors with active TB (Supplementary Table 3). MAIT cell-  
242 consistent CDR3 $\alpha$  sequences comprised a higher percentage of the TRAV1-2<sup>+</sup>  
243 repertoire in BAL fluid compared with peripheral blood, irrespective of the parameter  
244 used to define MAIT cell-consistent CDR3 $\alpha$  sequences, including assessment of  
245 similarity to published MAIT cell CDR3 $\alpha$  sequences (MAIT Match score = 0.95 or 1) or  
246 according to usage of TRAJ12, TRAJ20 or TRAJ33 (Fig. 3e; P = 0.0036; 2-way  
247 ANOVA). Among the patients with TB, CDR3 $\alpha$  sequences with the highest MAIT Match  
248 scores ( $\geq 0.95$ ) were enriched in BAL fluid, while those with the lowest MAIT Match  
249 scores ( $< 0.85$ ), were more frequent in peripheral blood (Fig. 3f).

250

251 To determine the extent to which individual MAIT cell-consistent CDR3 $\alpha$  sequences  
252 (MAIT Match Score  $\geq 0.95$ ) were shared between these two anatomical compartments,  
253 we created a TCR Enrichment Analysis (TEA) webtool  
254 (<https://github.com/eisascience/Wong-Gold-Lewinsohn>) to enable visualization and  
255 weighted frequency analysis of the most common MAIT cell-consistent CDR3 $\alpha$   
256 sequences in matched samples (Fig. 3g and Supplementary Table 4). In all three  
257 patients, the most frequent MAIT cell-consistent CDR3 $\alpha$  sequences were present in both

258 compartments, with disproportionate expansion in the BAL fluid compared with the  
259 peripheral blood. In contrast, CDR3 $\alpha$  sequences with low MAIT Match scores (<0.85)  
260 were generally expanded only in one anatomical compartment (Supplementary Figure  
261 4). The selective expansion of MAIT cell-consistent CDR3 $\alpha$  sequences in the lung  
262 compartment relative to peripheral blood suggests antigen-driven clonal expansion in  
263 response to pulmonary infection with Mtb.

264

265 To determine if TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells present in BAL fluid contained MAIT cells, we  
266 examined the MR1-restricted function of T cell clones generated from a BAL fluid sample  
267 obtained from a patient with TB. Six of these TRAV1-2<sup>+</sup> clones (D0033-A1, A2, A3, A6,  
268 A8 and A10) expressed MAIT cell-consistent CDR3 $\alpha$  sequences (MAIT Match score =  
269 0.98-1; Table 1). Stimulation of these clones with HLA-mismatched *M. smegmatis*-  
270 infected or Mtb-infected antigen-presenting cells induced robust IFN- $\gamma$ , while two control  
271 clones from the same patient (D0033-D7 and E7) failed to produce IFN $\gamma$  under identical  
272 conditions. In contrast, stimulation of the TRAV1-2<sup>+</sup> clones with HLA-mismatched *M.*  
273 *smegmatis*-infected or Mtb-infected MR1-KO antigen-presenting cells<sup>33</sup> resulted in  
274 negligible IFN- $\gamma$ -production, thereby demonstrating MR1-dependent cytokine  
275 production consistent with MAIT cell function (Fig. 3h).

276

277 MR1 tetramer loaded with 5-OP-RU ligand has been shown to identify functional MAIT  
278 cells in the human peripheral circulation<sup>13</sup>. To evaluate whether TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells  
279 in the BAL of humans with TB could be stained by MR1/5-OP-RU tetramer, as well as  
280 the relationship between MAIT cell-consistent CDR3 $\alpha$  usage and MR1/5-OP-RU  
281 tetramer staining, we took advantage of two donors with TB with available cryopreserved  
282 specimens. We stained cells from paired BAL and peripheral blood samples with  
283 TRAV1-2 antibody, MR1/5-OP-RU tetramer and MR1/6-FP tetramer (negative control).  
284 As shown in Figure 4a, BAL cells from donor 1020 demonstrated MR1/5-OP-RU  
285 tetramer staining of 33.7% of the TRAV1-2<sup>+</sup> cells, supporting the TCR $\alpha$  sequencing  
286 analysis that found that 40.7% of BAL TRAV1-2 CDR3 $\alpha$  sequences were MAIT cell-  
287 consistent (MAIT Match score > .95). In the peripheral blood of this participant, only  
288 3.06% of the TRAV1-2<sup>+</sup> peripheral cells demonstrated MR1/5-OP-RU tetramer staining,  
289 in line with the TCR $\alpha$  sequencing analysis that had found that 5.09% of peripheral  
290 TRAV1-2 CDR3 $\alpha$ 's were MAIT cell-consistent.

291

292 In contrast to MR1/5-OP-RU tetramer staining in the peripheral blood where positive and  
293 negative populations were clearly discernable, the MR1/5-OP-RU tetramer staining of  
294 BAL cells was of heterogeneous intensity and did not allow unambiguous delineation of  
295 MR1/5-OP-RU tetramer negative and positive populations. As a result, we sorted  
296 TRAV1-2<sup>+</sup> cells based on MR1/5-OP-RU tetramer staining, subjected both positive and  
297 negative subsets to TCR sequencing, and analyzed MAIT cell-consistent CDR3 $\alpha$  usage  
298 in each population (Table 2). CDR3 $\alpha$  chain sequencing of MR1/5-OP-RU tetramer  
299 positive cells from BAL and peripheral blood revealed that 93.9% and 89.2% of these  
300 respectively utilized MAIT cell-consistent TCRs. CDR3 $\alpha$  chain sequencing of the MR1/5-  
301 OP-RU tetramer negative TRAV1-2<sup>+</sup> populations demonstrated that a substantial  
302 proportion (13.7%) of the MR1/5-OP-RU tetramer negative cells in the BAL utilized MAIT  
303 cell-consistent CDR3 $\alpha$  chains. In contrast, only 2.7% of MR1/5-OP-RU tetramer  
304 negative cells from the peripheral blood utilized MAIT cell-consistent CDR3 $\alpha$  chains.  
305 These data suggest that MR1/5-OP-RU tetramer may perform less efficiently in BAL fluid  
306 than in peripheral blood. Notably, in the other donor (91), in whom 28.5% of the TRAV1-  
307 2<sup>+</sup> cells had a MAIT cell-consistent CDR3 $\alpha$ , only 5.09% of the TRAV1-2<sup>+</sup> cells from the  
308 BAL stained MR1/5-OP-RU positive. In this donor, 24.7% of the MR1/5-OP-RU tetramer  
309 negative cells had MAIT cell-consistent CDR3 $\alpha$  chains, suggesting that MR1/5-OP-RU  
310 tetramer staining of BAL cells may underestimate the presence of MAIT cells as  
311 determined by CDR3 $\alpha$  usage.

312

313 To better understand the relationship between MR1/5-OP-RU tetramer staining, CDR3 $\alpha$   
314 usage and MR1-dependent T cell activity, we sorted MR1/5-OP-RU positive cells from  
315 the BAL of an available individual with non-TB pneumonia and performed limiting dilution  
316 cloning using anti-CD3 and IL-2 stimulation. Following rapid expansion<sup>34</sup>, each clone  
317 was characterized functionally for MR1-restriction and antigenic specificity. As shown in  
318 Figure 4c, four clones (D1004-B3, E1, E5, and H3) produced IFN- $\gamma$  when stimulated with  
319 *M. smegmatis*-infected antigen presenting cells (A549) and abrogated IFN- $\gamma$  production  
320 when stimulated with identically infected MR1-KO antigen presenting cells.<sup>33</sup> TCR  
321 sequencing demonstrated that each of these clones utilized a MAIT cell-consistent  
322 CDR3 $\alpha$  (Figure 4b). Surprisingly, despite clear evidence of MR-1 restricted function,  
323 usage of MAIT cell-consistent CDR3 $\alpha$ 's and TRAV1-2 staining of similar intensity (Fig.

324 4c), these clones demonstrated considerable heterogeneity in MR1/5-OP-RU tetramer  
325 staining, with two of the four clones staining weakly (Figure 4d).

326

### 327 **Discussion**

328 Collectively, our data indicate that donor-unrestricted mycobacterial-reactive TRAV1-2<sup>+</sup>  
329 CD8<sup>+</sup> T cells are present in the human respiratory mucosa and that pulmonary infection  
330 with Mtb leads to an enrichment of airway resident, pro-inflammatory TRAV1-2<sup>+</sup> CD8<sup>+</sup>  
331 cells including oligoclonal expansions of MAIT cells.

332

333 In lung tissue explanted from healthy organ donors, we find that TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells  
334 localize to the respiratory tract mucosal surface. In contrast to their counterparts in the  
335 gut mucosa, TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells from the respiratory mucosa produce TNF in  
336 response to mycobacterial stimulation by donor-unrestricted antigen-presenting cells.  
337 This suggests that TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in the airway mucosa may play a role in anti-  
338 Mtb immunity by initiating a local pro-inflammatory response upon exposure to  
339 aerosolized Mtb.

340

341 In the setting of active pulmonary tuberculosis, we observed striking expansions of  
342 TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in the bronchoalveolar compartment. Compared to paired  
343 peripheral blood TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells, the bronchoalveolar TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells  
344 produced significantly more TNF. Some, but not all, of these expanded bronchoalveolar  
345 TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells could be identified as MAIT cells based on their utilization of  
346 MAIT cell-consistent CDR3 $\alpha$  chains, demonstration of MR1-restricted function or  
347 selective binding of the MR1/5-OP-RU tetramer. It should be noted that among the  
348 TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells that could not be unequivocally confirmed as MAIT cells we  
349 identified subpopulations that displayed certain "MAIT-like" features, such as high-level  
350 expression of CD26 or oligoclonal expansions of TRAV1-2<sup>+</sup> TCR $\alpha$  chains with features  
351 similar to MAIT cell CDR3 $\alpha$  sequences (incorporation of the TRAJ12, TRAJ20 or  
352 TRAJ33 segments, or the presence of the Tyr95 which is known to be critical for MAIT  
353 cell TCR binding to MR1-restricted ligands<sup>35</sup>). Our attempts to clone these populations  
354 have been unsuccessful to date, such that further work will be required to determine if  
355 these TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells with "MAIT-like" features are restricted by MR1. It is also  
356 notable that MR1/5-OP-RU tetramers identified only a subset of TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells  
357 with MAIT cell-consistent CDR3 $\alpha$ 's.

358

359 We postulate that variable MR1-tetramer staining observed on bronchoalveolar TRAV1-  
360 2<sup>+</sup> CD8<sup>+</sup> cells could reflect a state of activation among tissue resident cells. Supporting  
361 this, we note that differential tetramer staining can be observed following expansion of  
362 MAIT cell clones with activating cytokines (data not shown). Alternatively, we postulate  
363 that TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells with MAIT cell-consistent CD3 $\alpha$ 's may have altered  
364 tetramer-binding avidity as a result of differential affinity of their TCRs for MR1-ligands.  
365 This possibility is suggested by the variable magnitude of response to *M. smegmatis* in  
366 the functional assay, and has recently been demonstrated for the photolumazine I  
367 ligand<sup>36</sup>. Further work will be required to better understand the relationship between  
368 TCR-dependent MR1-dependent activation, MR1/5-OP-RU tetramer staining, and ligand  
369 selectivity among bronchoalveolar TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells. At this point we conclude  
370 that MR1/5-OP-RU tetramer staining of bronchoalveolar MAIT cells is weaker and more  
371 variable than MR1/5-OP-RU tetramer staining of peripheral blood MAIT cells and hence  
372 may underestimate MAIT cell prevalence in the BAL.

373

374 In contrast to the bronchoalveolar fluid of active TB patients, analysis of TCR $\alpha$  chain  
375 usage in granulomas of patients undergoing lung-resection for clinically complicated  
376 tuberculosis did not demonstrate dramatic expansions of TRAV1-2<sup>+</sup> TCR $\alpha$ 's. The  
377 contrast between the enrichment of TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells observed in bronchoalveolar  
378 lavage fluid and the relatively low frequencies of TRAV1-2<sup>+</sup> TCR $\alpha$ 's found in the lung  
379 granuloma tissue may be due to differences between cells present in the airway mucosal  
380 environment and in lung parenchymal tissue. It is also possible that the kinetics of  
381 expansion of TRAV1-2<sup>+</sup> CD8<sup>+</sup> cells with MAIT cell-consistent CDR3 $\alpha$ 's varied during the  
382 long course of TB disease and anti-tuberculosis therapy that preceded surgical  
383 treatment in these medically-complex lung-resection patients.

384

385 It is therefore notable that even in the resected granuloma tissue, the subset of TCR $\alpha$ 's  
386 with MAIT cell-consistent sequences was enriched among the TRAV1-2<sup>+</sup> CDR3 $\alpha$ 's in  
387 lung granuloma tissue compared to paired mediastinal lymph node tissue. We postulate  
388 that this relative enrichment of MAIT cell-consistent TCRs among TRAV1-2<sup>+</sup> sequences  
389 from the lung was driven by local antigen exposure, while acknowledging that tissue-  
390 specific non-antigen stimuli could also lead to the independent expansion of clones in  
391 the lung compartment. Further understanding of this will require additional organ-

392 specific datasets to allow comparison of diseased and reference TCR repertoires. We  
393 found both public and private MAIT cell-consistent CDR3 $\alpha$  chains in the TB-infected  
394 human lung tissues we analyzed. Interestingly, public MAIT cell-consistent CDR3 $\alpha$   
395 chains were frequently encoded by multiple synonymous nucleotide sequences within an  
396 individual sample. This finding is consistent with a previous report implicating convergent  
397 recombination as a determinative process in the generation of public MAIT cell CDR3 $\alpha$   
398 sequences<sup>23</sup>, and suggests that tissue-resident public MAIT cell-consistent CDR3 $\alpha$   
399 expansions are the result of multiple individual MAIT cells clonally expanding in infected  
400 tissues. The significance of cells with private MAIT cell-consistent CDR3 $\alpha$  chains in the  
401 context of Mtb-infected tissue remains uncertain. One possibility is that public and  
402 private MAIT cell-consistent CDR3 $\alpha$  chains have similar ligand-binding properties, such  
403 that utilization and expansion of specific clonotypes in individual hosts is the result of  
404 differences in the naive TCR repertoire and is not driven by specific microbial exposures.  
405 Alternatively, the observed clonal expansion of private MAIT cell-consistent clonotypes  
406 within Mtb infected tissue may reflect selective expansions in response to the local  
407 presence of microbe-derived ligands presented by MR1<sup>36</sup>. A third possibility is that  
408 because our sample set is small, sequences that appear to be private in this analysis  
409 could in fact turn out to be public when larger numbers of individual donors are sampled.  
410 In order to determine the significance of private and public MAIT cell-consistent TCRs  
411 in the context of mycobacterial infection, further study of selective ligand specificity in  
412 larger numbers of donors is needed. Nonetheless, the convergence of multiple  
413 nucleotide rearrangements on expanded public MAIT cell-consistent TCR $\alpha$  chains  
414 suggests that in some instances, multiple MAIT cells with genetically unique but  
415 functionally similar TCR $\alpha$  chains clonally expand in the TB-infected lung, potentially in  
416 response to microbe-derived antigenic-stimulation.

417

418 In line with recent studies that have found MAIT cell expansions in the lungs of mice  
419 experimentally infected with Mtb<sup>37</sup> and *S. enterica* serovar Typhimurium (*S.*  
420 Typhimurium)<sup>38</sup>, we found expansions of TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells with MAIT or MAIT-like  
421 features in the BAL and lung parenchyma in patients with TB. Supported by the findings  
422 Chen et al who found that accumulation of MAIT cells in the lungs of mice following  
423 challenge with *S. Typhimurium* is dependent on antigen derived from the microbial  
424 riboflavin synthesis pathway<sup>38</sup>, we postulate that these TRAV1-2<sup>+</sup> CD8<sup>+</sup> cell enrichments

425 contain MAIT cells and are driven by Mtb-derived small molecular ligands. Howson et al  
426 recently reported that MAIT cell clones with more avid ligand binding expand during *S.*  
427 *enterica* serovar Paratyphi A infection and that these clones remain expanded after  
428 treatment of the infection<sup>39</sup>. This finding supports the idea that exposure to microbe-  
429 derived MR1 ligands alters the human MAIT cell TCR repertoire and suggests a role for  
430 MR1-ligand vaccine strategies. Overall our findings suggest a previously unrecognized  
431 and potentially important role for TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells with MAIT or MAIT-like features  
432 in the immune response to aerosolized Mtb infection, and would support exploration of  
433 these cells as targets of either vaccination or immunotherapeutic strategy.

434

## 435 **Methods**

436

### 437 **Human subjects**

438 *Samples from Portland, Oregon, USA.* Airway, lung, small intestine and associated  
439 lymph node tissues ineligible for transplantation were obtained from the Pacific  
440 Northwest Transplant Bank under a protocol approved by the Institutional Review Board  
441 at Oregon Health & Science University. Limited clinical information was available for  
442 these individuals, who were generally considered healthy prior to demise. For  
443 comparison with the organ samples, PBMCs were obtained by apheresis from healthy  
444 adult donors providing informed consent.

445 *Samples from Durban, South Africa.* Explanted granulomatous lung tissue and  
446 associated lymph nodes were obtained under a protocol approved by the University of  
447 KwaZulu Natal Human Biomedical Research Ethics Committee (UKZN BREC) allowing  
448 adults undergoing clinically indicated lung resection for complicated tuberculosis at  
449 Inkosi Albert Luthuli Central Hospital (IALCH) to donate excess tissue for scientific  
450 research.<sup>19</sup> Tissue was isolated from different areas of resected lungs based on the  
451 experience of the operating surgeon and the preoperative radiological data. Clinical  
452 characteristics of the individuals and samples have been described<sup>19</sup>. All donors  
453 provided written informed consent prior to surgery. BAL fluid and paired peripheral  
454 blood samples were obtained under a protocol approved by the UKZN BREC and  
455 Partners Institutional Review Board allowing collection of excess fluid from adult patients  
456 undergoing clinically indicated diagnostic bronchoscopies at IALCH. Active tuberculosis  
457 was defined microbiologically (positive BAL Mtb culture or BAL Mtb PCR by GeneXpert)  
458 and/or histologically (Ziehl-Neelsen positive necrotizing granulomas on transbronchial

459 biopsy obtained at the time of BAL). Uninfected controls were defined as individuals with  
460 no evidence of either infectious or inflammatory lung disease, as determined by a  
461 committee of study physicians on the basis of clinical history, chest x-rays, computerized  
462 tomography scans, and negative BAL microbiology (mycobacterial, bacterial and fungal  
463 cultures, and Mtb PCR). Most controls underwent bronchoscopy for suspected lung  
464 cancer, and a non-cancerous segment was lavaged in these cases. All donors provided  
465 written informed consent prior to bronchoscopy. Cryopreserved peripheral blood  
466 mononuclear cells (PBMCs) from healthy donors (defined as asymptomatic and HIV-  
467 negative with no evidence of Mtb by ELISPOT) were available from the iThimba Cohort  
468 which was approved by the UKZN BREC and Partners Institutional Review Board<sup>40</sup>. All  
469 participants provided written informed consent.

470

#### 471 **Isolation and stimulation of lung and gut T cells**

472 Lymphocytes were isolated from fresh lung tissue as described previously<sup>4</sup>. A two-step  
473 process was used to extract cells from the small intestine. For collection of lymphocytes  
474 from the intraepithelial (IEL) layer, the tissue was washed in HBSS, stripped of muscle,  
475 and incubated with agitation for 30 minutes in 0.15% dithiothreitol (Sigma-Aldrich). IEL  
476 lymphocytes were then harvested, and the remaining tissue was incubated for 30  
477 minutes in PBS. Lamina propria (LP) lymphocytes were released by digestion with 0.1%  
478 collagenase (CLS-3, Worthington) and 0.3% DNase (Roche) for 30 minutes at 37° C.  
479 IEL and LP preparations were further enriched over a discontinuous Percoll gradient.  
480 Lymphocyte stimulations were performed as described previously<sup>4,12,16</sup>. Briefly,  
481 lymphocytes were incubated for 16 hours with uninfected (control) or *M. smegmatis*  
482 strain mc<sup>2</sup>122-infected (multiplicity of infection = 3) A549 cells (ATCC CCL-185) at a  
483 ratio of 3:1 in the presence of  $\alpha$ -CD28 and  $\alpha$ -CD49d (Biolegend), together with an  $\alpha$ -  
484 TNF mAb (Beckman Coulter) and the TNF-Processing Inhibitor 0 (TAPI-0, 10  $\mu$ M)  
485 (Calbiochem). Cells were then stained as described above for surface expression of  
486 CD45, CD3, CD8, TRAV1-2, and CD161<sup>4</sup>. Dead cells were excluded using Aqua  
487 LIVE/DEAD (Invitrogen). Stained samples were acquired on a Fortessa flow cytometer  
488 (BD Biosciences) and data were analyzed with FlowJo software version 10.6 (Tree Star).

489

#### 490 **Immunohistochemistry of airway tissues**

491 Cryosections (10  $\mu$ M) of frozen airway tissues were treated with acetone and air-dried  
492 prior to incubation with  $\alpha$ -TRAV1-2 antibody (clone-3C10; Biolegend) followed by goat  $\alpha$ -



493 mouse IgG1-Alexa Fluor 488 (1:1000), and then  $\alpha$ -CD8 antibody (1:50; LSBio) followed  
494 by goat  $\alpha$ -mouse IgG1-Alexa Fluor 568 (1:1000). Sections were washed and stained  
495 with DAPI. Images were acquired using an Olympus FluoView FV1000 laser scanning  
496 confocal microscope system with a 40 x 1.3 Oil Plan Fluorite objective. Confocal images  
497 were analyzed using Imaris Analysis Software.

498

### 499 **Isolation and TCR sequencing of T cells from lung granulomas**

500 Diseased lung tissue (approximately 3 cm<sup>3</sup>) was isolated from surgically resected  
501 explants. Each sample was washed in multiple changes of Hank's Balanced Salt  
502 Solution (HBSS), diced into smaller pieces (approximately 1 mm<sup>3</sup>), strained,  
503 resuspended in pre-warmed R10 supplemented with 0.5 mg/ml collagenase D (Roche)  
504 and 40 U/ml DNaseI (Roche), and transferred to GentleMACS C-tubes (Miltenyi Biotec)  
505 for mechanical digestion per the manufacturer's instructions. The resulting suspension  
506 was incubated for 60 minutes at 37°C, subjected to an additional mechanical digestion  
507 step, strained through a 70  $\mu$ m filter, washed twice in HBSS, and stained prior to sorting  
508 CD4<sup>-</sup> T-cells using a FACSARIA flow cytometer (BD Biosciences). Cells were gated as  
509 live (nearIR<sup>-</sup>, Invitrogen), single lymphocytes (determined on the basis of light scatter),  
510 then sorted as CD45<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>-</sup> events directly into RLT buffer. Genomic DNA was  
511 extracted using a DNeasy Minikit (Qiagen) and high-throughput TCR $\alpha$  sequencing was  
512 performed using the ImmunoSEQ assay (Adaptive Biotechnologies Corp.)<sup>41</sup>. Data were  
513 analyzed using the ImmunoSEQ Analyser.

514

### 515 **CDR3 $\alpha$ sequence similarity**

516 Similarity between CDR3 $\alpha$  sequences was calculated as described previously<sup>22</sup>. This  
517 method allows similarities to be assigned between sequences of different length in an  
518 alignment-free manner. An implementation of the similarity matching between CDR3  
519 sequences is publicly available at [http://www.cbs.dtu.dk/services/MAIT\\_Match](http://www.cbs.dtu.dk/services/MAIT_Match). The  
520 server takes as input a list of CDR3 $\alpha$  sequences, and returns for each a score based on  
521 the maximal sequence similarity with a reference database of MAIT cell CDR3 $\alpha$   
522 sequences. A perfect match has a similarity score of 1, and a perfect mismatch a  
523 similarity score of 0.

524

### 525 **Collection, staining and stimulation of BAL lymphocytes**

526 Bronchoscopies were performed by pulmonologists at IALCH. Patients received  
527 sedation and bronchodilators according to the local standard of care; Normal saline (200  
528 mL) was lavaged into the lobe with the highest burden of pathology or, in patients with  
529 diffuse disease, the right middle lobe. Available BAL fluid was combined in a 1:1 ratio  
530 with R10 (RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, penicillin  
531 and streptomycin) and stored on ice. All samples were processed within 3 hours of  
532 collection, BAL fluid was filtered through a 40  $\mu$ m strainer (BD Pharmingen) and  
533 centrifuged. Resuspended BAL cells were aliquoted for staining with Aqua LIVE/DEAD  
534 (Invitrogen) and some or all of the following antibodies:  $\alpha$ -CD3-PE-CF594 (BD Horizon,  
535 clone UCHT1),  $\alpha$ -CD8-APC-H7 (BD Pharmingen, clone SK1),  $\alpha$ -CD14-PerCP-Cy5.5  
536 (BioLegend, clone HCD14),  $\alpha$ -CD235a-PerCP-Cy5.5 (BioLegend, clone HIR2),  $\alpha$ -  
537 TRAV1-2-APC (clone OF-5A12<sup>12</sup>),  $\alpha$ -CD161-PE-Cy7 (BioLegend, clone HP-3G10),  $\alpha$ -  
538 CD26-PE (BioLegend, clone BA5b). All stains were performed at 4°C. Cells were then  
539 fixed with 4% paraformaldehyde. Functional studies were performed if sufficient  
540 numbers of BAL lymphocytes were available. After depletion of macrophages via plastic  
541 adherence for 1 hour,  $1 \times 10^6$  lymphocytes were stimulated for 18 hours at 37°C with  $\alpha$ -  
542 CD2/CD3/CD28-loaded Anti-Biotin MACSiBead Particles (Miltenyi Biotec) at a ratio of  
543 2:1 in RPMI 1640 supplemented with 10% fetal bovine serum, L-glutamine, HEPES,  
544 penicillin, and streptomycin. Brefeldin A was added after the first hour to inhibit protein  
545 transport from the endoplasmic reticulum. Stimulated and unstimulated cells were then  
546 stained with Aqua LIVE/DEAD (Invitrogen) and the following antibodies:  $\alpha$ -CD235a-  
547 PerCP-Cy5.5,  $\alpha$ -CD14-PerCP-Cy5.5,  $\alpha$ -CD8-APC-H7,  $\alpha$ -TRAV1-2-APC,  $\alpha$ -CD161-PE-  
548 Cy7. After a wash step, cells were fixed with PERM/FIX Medium A (Invitrogen),  
549 permeabilized with PERM/FIX Medium B (Invitrogen), and stained with the following  
550 antibodies:  $\alpha$ -CD3-PE-CF594,  $\alpha$ -TNF $\alpha$ -PE (Beckman Coulter, clone IPM2), and  $\alpha$ -IL-17-  
551 BV421 (BioLegend, clone BL168). Stained samples were acquired using a Fortessa flow  
552 cytometer (BD Bioscience). Rainbow Fluorescent Particles (BD Bioscience) and  
553 applications settings in FACSDiva7 were used to correct for day-to-day variations in  
554 instrument performance. Cells were gated as live (aqua viability dye negative)  
555 lymphocytes (determined on the basis of light scatter), and CD14<sup>++</sup> cells were excluded  
556 prior to selecting CD3<sup>+</sup> cells for analysis. Paired peripheral blood samples were collected  
557 where possible and freshly isolated PBMC were processed in parallel with matched BAL  
558 cells. Data were analyzed with FlowJo10.6 (Treestar). Background cytokine production

559 was subtracted to calculate percentage of cells producing cytokine in response to  
560 stimulation. When available, paired cryopreserved BAL and PBMC cells were thawed  
561 and stained with some or all of the above antibodies and MR1/5-OP-RU or MR1/6-FP  
562 tetramers (courtesy of the McCluskey Laboratory). Cell suspensions were acquired and  
563 sorted on a FACSARIA flow cytometer (BD Biosciences) into TRIzol (Invitrogen).  
564 Genomic DNA was extracted utilizing the phenol-chloroform method according to  
565 manufacturer protocol, using linear acrylamide (Invitrogen) as a carrier. High-throughput  
566 TCR $\alpha$  sequencing was performed using the ImmunoSEQ assay (Adaptive  
567 Biotechnologies Corp)<sup>41</sup>.

568

### 569 **Visualization of MAIT cell CDR3 $\alpha$ sequences**

570 Data were coded in R using the packages RColorBrewer, Shiny, data.table, ggplot2, and  
571 dplyr. Synonymous nucleotide sequences within a tissue were counted, and the  
572 associated frequencies are summed. These frequencies were visualized using the TCR  
573 Enrichment Analysis (TEA) webtool the code for which is archived at  
574 <https://github.com/eisascience/Wong-Gold-Lewinsohn/tree/v1.0.0>

575

### 576 **Generation and characterization of T cell clones**

577 Cells from BAL samples were stained with Aqua LIVE/DEAD (Invitrogen), MR1/5-OP-RU  
578 tetramer (0.3nM, McCluskey Laboratory),  $\alpha$ -CD4-FITC (clone OKT4; BioLegend), and  $\alpha$ -  
579 CD8-APC-Cy7 (clone SK8; BioLegend). Live tetramer-binding cells were sorted by the  
580 basis of co-receptor expression using an Influx flow cytometer (BD Biosciences), rested  
581 overnight in RPMI 1640 supplemented with 10% heat-inactivated pooled human serum  
582 and 0.5ng/ml rhIL-2, and then distributed in limiting dilution format with irradiated PBMCs  
583 ( $150 \times 10^5$ /well) and irradiated B-lymphoblastoid cells ( $3 \times 10^4$ /well) in a 96-well round  
584 bottom plate. The cultures were stimulated with rhIL-2 (5ng/ml), rhIL-12 (0.5ng/ml), rhIL-  
585 7 (0.5ng/ml), rhIL-15 (0.5ng/ml) and  $\alpha$ -CD3 (0.03  $\mu$ g/ml). Clones were harvested after  
586 incubation for 20 days at 37°C and assessed for clonality by flow cytometry, TCR  
587 sequencing, and MR1-restricted function by ELISPOT.

588

589 Nitrocellulose-backed multiscreen 96-well plates (Millipore) were coated overnight at 4°C  
590 with a 10  $\mu$ g/ml solution of  $\alpha$ -IFN $\gamma$  antibody (clone 1-D1K; Mabtech) in 0.1 M Na<sub>2</sub>CO<sub>3</sub>,  
591 0.1 M NaHCO<sub>3</sub>, pH 9.6. The plate was washed three times with sterile PBS and blocked  
592 for 1 hour at room temperature with RPMI 1640 containing 10% heat-inactivated pooled

593 human serum. Uninfected, *M. smegmatis* mc<sup>2</sup>122-infected (multiplicity of infection = 3),  
594 or *M. tuberculosis* H37Rv-infected (multiplicity of infection = 30) wildtype or MR1-null<sup>33</sup>  
595 A549 cells (1 x 10<sup>4</sup>/well) and clonal T cells (1 x 10<sup>4</sup>/well) were added and incubated  
596 overnight at 37°C. The plates were then washed six times in PBS containing 0.05%  
597 Tween-20, incubated for 2 hours at room temperature with a 1 µg/ml solution of α-IFNγ-  
598 biotin antibody (clone 7-B6-1; Mabtech) in PBS containing 0.5% bovine serum albumin  
599 and 0.05% Tween-20, washed again six times in PBS containing 0.05% Tween-20  
600 followed by PBS alone, and developed using an AEC Vectastain Kit (Vector  
601 Laboratories). Spots were counted using an automated ELISPOT Reader System  
602 (Autoimmun Diagnostika GmbH).

603

#### 604 **TCR sequence analysis of CD8+ T cell clones isolated from BAL fluid**

605 For some clones, total RNA was extracted using an RNeasy Mini Kit (Qiagen).  
606 Unbiased amplification of all expressed *TRA* and *TRB* gene products was then  
607 conducted using a template-switch anchored RT-PCR with chain-specific constant  
608 region primers<sup>42</sup>. Amplicons were sub-cloned, sampled, sequenced and analyzed as  
609 described previously<sup>43</sup>. Gene usage was assigned according to the IMGT nomenclature.  
610 For other clones, genomic DNA was extracted using a DNeasy Mini Kit (Qiagen) and  
611 high-throughput TCRα and TCRβ sequencing was performed using the ImmunoSEQ  
612 assay (Adaptive Biotechnologies Corp)<sup>41</sup>. Data were analyzed using the ImmunoSEQ  
613 Analyser.

614

#### 615 **Statistics and Reproducibility**

616 Statistical analyses were performed using Prism 6 (GraphPad Software Inc). The non-  
617 parametric Mann-Whitney U test was used to assess differences between groups unless  
618 indicated otherwise. All statistical tests were two-sided unless indicated otherwise. P  
619 values < 0.05 were considered significant for direct comparisons. In cases of multiple  
620 comparisons the Bonferonni correction was applied. All antibodies were obtained  
621 commercially and not validated independently. Cell lines tested negative for  
622 mycoplasma contamination.

623

#### 624 **Code Availability**

625 Custom code for the MAIT Match tool is available at  
626 [http://www.cbs.dtu.dk/services/MAIT\\_Match/](http://www.cbs.dtu.dk/services/MAIT_Match/). Custom code for the visualization of

627 frequencies of specific TCR $\alpha$ 's in the TB granuloma tissues and the TCR Enrichment  
628 Analysis (TEA) webtool are archived at [https://github.com/eisascience/Wong-Gold-](https://github.com/eisascience/Wong-Gold-Lewinsohn)  
629 Lewinsohn

630

### 631 **Data Availability Statement**

632 The datasets generated during and/or analyzed during the current study are archived at  
633 <https://github.com/eisascience/Wong-Gold-Lewinsohn> or available from the  
634 corresponding author on reasonable request.

635

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670

671 **Author contributions:**

672

673 EBW, MCG, SMB, AL, TN and DML designed the experiments; EBW, MCG, BZX, EWM,  
674 SK, ZAS, HK, PKS, AHW, JEM, KL, KLM and MN performed experiments; EBW, MCG,  
675 EWM, SK, JEM, DAP, SMB, AL, TN, EM and DML analyzed results. UL, ZR, PB, AA,  
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677 MCG, DAP, EM, TN and DML wrote the manuscript. All co-authors provided comments  
678 and approved the content.

679

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681

682 **FIGURE LEGENDS**

683

684 **Figure 1. TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells from the lung but not the intestine of healthy**  
685 **organ donors respond to mycobacterial infection by producing TNF.**

686 **a.** Dot plots showing the frequency of TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells among live CD3<sup>+</sup> cells in  
687 the indicated tissue samples from one donor.

688 **b.** Tissue sections from the 1<sup>st</sup> and 2<sup>nd</sup> order bronchi were obtained from healthy  
689 individuals (n = 3 biologically independent samples). Immunohistochemistry was  
690 performed to quantify CD8<sup>+</sup> (median 1.6 x 10<sup>4</sup> vs. 2x10<sup>4</sup> cells /mm<sup>3</sup>) and TRAV1-2<sup>+</sup> cells  
691 (7,000 vs. 4,000 cells/mm<sup>3</sup>). Representative sections from 1<sup>st</sup> and 2<sup>nd</sup> order bronchi are  
692 depicted (left), showing CD8<sup>+</sup> cells (red), TRAV1-2<sup>+</sup> cells (green) and cell nuclei (DAPI;  
693 blue).

694 **c.** Histograms depicting TNF production (left) and CD161 expression (right) by TRAV1-  
695 2<sup>+</sup> CD8<sup>+</sup> T cells from matched lung parenchyma (green), small intestine lamina propria  
696 (LP; blue) and the small intestinal intraepithelial layer (IEL; violet) after overnight  
697 stimulation with *M. smegmatis*-infected antigen-presenting cells (dotted black line  
698 indicates the unstimulated control).

699 **d.** Frequency of TRAV1-2<sup>+</sup> cells among CD8<sup>+</sup> T cells from lung (n=9 biologically  
700 independent samples), mediastinal lymph node (Med LN; n=11 biologically independent  
701 samples), IEL (n=7 biologically independent samples), LP (n=8 biologically independent  
702 samples), mesenteric lymph node (Mes LN, n=5 biologically independent samples), and  
703 peripheral blood (PBMC; n=6 biologically independent samples). Medians and  
704 interquartile ranges are displayed.

705 **e.** Frequency of TNF-producing TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells after exposure to *M. smegmatis*-  
706 infected antigen-presenting cells: lung (n=7 biologically independent samples), Med LN  
707 (n=6 biologically independent samples), IEL (n=5 biologically independent samples), LP  
708 (n=6 biologically independent samples), Mes LN (n=2 biologically independent samples),  
709 PBMC (n=12 biologically independent samples). From top to bottom, P = 0.035, 0.0025,  
710 0.0023 and 0.0005 (Mann Whitney U-test). Medians and interquartile ranges are  
711 displayed.

712

713 **Figure 2. Expansions of MAIT cell-consistent CDR3 $\alpha$ 's are present in tuberculous**  
714 **lung granulomas.**

715 **a.** Frequency of TRAV1-2<sup>+</sup> sequences as a percentage of all productive TCR $\alpha$   
716 sequences. LG, lung granuloma; LN, lymph node. In some cases, multiple areas of  
717 tissue were sampled, ranging from closest (A) to furthest (C) from the site of disease.

718 **b.** MAIT cell TCR $\alpha$  sequences are consistent with similarity scores of 0.95 and 1. Each  
719 symbol represents the frequency of TRAV1-2<sup>+</sup> or TRAV12-2<sup>+</sup> sequences within each  
720 similarity score for each donor sample (n= 12 biologically independent samples).

721 **c.** Frequency of total TRAV1-2<sup>+</sup> sequences or those with similarity scores of 0.95 and 1  
722 in the lung and mediastinal lymph node (LN) from donor 23.

723 **d.** Frequencies among TRAV1-2<sup>+</sup> sequences of the top 10 public and private MAIT cell  
724 CDR3 $\alpha$  sequences (MAIT Match score  $\geq$  0.95) across individual donors and lung  
725 samples.

726 **e.** Variation in the number of synonymous nucleotide sequences encoding the five most  
727 frequent private (left) and public (right) MAIT cell CDR3 $\alpha$  amino acid (aa) sequences

728 from all samples displayed in Fig. 3d. For each aa sequence, each colored bar  
729 represents a different nucleotide sequence. The 13 different nucleotide sequences used  
730 to generate the shared MAIT cell CDR3 $\alpha$  aa sequence CAVLDSNYQLIW are displayed.  
731 Text color represents nucleotide origin: purple (TRAV), black (TRAD or n insertion), red  
732 (TRAJ).

733

734 **Figure 3. TNF-producing TRAV1-2<sup>+</sup> CD8<sup>+</sup> cells including oligoclonally expanded**  
735 **MAIT cells are enriched in BAL fluid from patients with TB.**

736 **a.** Frequency of TRAV1-2<sup>+</sup> cells among CD8<sup>+</sup> T cells from the BAL fluid from patients  
737 with TB (n=6 biologically independent samples) and cancer controls (n=6 biologically  
738 independent samples), and among CD8<sup>+</sup> T cells in matched peripheral blood samples  
739 (PBMC) from patients with TB (n=5 biologically independent samples) and unmatched  
740 peripheral blood samples from healthy controls (n=13 biologically independent samples).  
741 Medians and interquartile ranges are displayed. \*\*P < 0.01; Mann-Whitney U-test.

742 **b.** Dot plots showing TNF production by TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in matched BAL and  
743 peripheral blood samples (PBMC) from a patient with TB. Cells were stimulated with  
744  $\alpha$ □□□□□□□□□□□□□□□□beads.

745 **c.** Frequency of TNF or IL-17 production by TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in matched BAL and  
746 peripheral blood samples (PBMC; n=5 biologically independent samples). Medians and  
747 interquartile ranges are displayed. \*\*P < 0.01; Mann-Whitney U-test.

748 **d.** Expression of CD161, CD26 and CD103 on TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells in matched BAL  
749 and peripheral blood samples (PBMC) from patients with TB (n=4). Histograms are  
750 mode-normalized.

751 **e.** Frequency of MAIT cell CDR3 $\alpha$  sequences within TRAV1-2<sup>+</sup> CD4<sup>+</sup> T cells in BAL fluid  
752 and peripheral blood samples (PBMC) from patients with TB (n=3 biologically  
753 independent samples).

754 **f.** Relative frequency of CDR3 $\alpha$  sequences by MAIT Match Score category in BAL fluid  
755 vs. matched peripheral blood (PBMC; n=3 biologically independent samples).

756 **g.** Depiction of the top 10 most frequent MAIT cell CDR3 $\alpha$  sequences (MAIT Match  
757 score  $\geq$  0.95) among TRAV1-2<sup>+</sup> sequences in each compartment. Legend format:  
758 CDR3 $\alpha$  aa (# of synonymous nucleotide sequences in peripheral blood, # of  
759 synonymous nucleotide sequences in BAL fluid).

760 **h.** IFN $\gamma$  spot-forming units (SFU) produced by BAL T cell clones stimulated with *M.*  
761 *smegmatis*-infected or Mtb-infected wildtype (WT) or MR1-KO A549 cells.



762

763 **Figure 4. Heterogeneous MR1/5-OP-RU staining of bronchoalveolar TRAV1-2+**  
764 **CD8<sup>+</sup> T cells with MAIT cell-consistent CDR3 $\alpha$ 's and MR1-restricted function**

765 **a.** Frequency of MR1-tetramer<sup>+</sup> cells (loaded with active (5-OP-RU) and control (6FP)  
766 ligand) in TRAV1-2<sup>+</sup> T cells (gated on live, CD3<sup>+</sup>, CD8<sup>+</sup> lymphocytes) from the BAL fluid  
767 and peripheral blood of a patient with TB. The proportion of cells utilizing MAIT cell-  
768 consistent CDR3 $\alpha$ 's (MAIT Match Score > 95) in MR1/5-OP-RU tetramer positive and  
769 negative populations are shown.

770 **b.** IFN $\gamma$  spot-forming units (SFU) produced by four T cell clones generated from BAL fluid  
771 and stimulated with *M. smegmatis*-infected wildtype (WT) or MR1-KO A549 cells.

772 **c.**  $\alpha$ -TRAV 1-2 staining of four T cell clones generated from BAL fluid demonstrates  
773 consistent staining. Histograms are mode-normalized.

774 **d.** Binding of MR1/5-OP-RU tetramer on the same four T cell clones generated from BAL  
775 fluid demonstrates heterogenous MR1/5-OPRU tetramer staining (left). Binding of  
776 MR1/6-FP (control) and MR1/5-OPRU tetramer is shown for two clones (right).  
777 Histograms are mode-normalized.

778

779 **Supplementary Information:**

780

781 **SUPPLEMENTARY FIGURES**

782 1. Supplementary Figure 1. Representative gating strategy for TRAV1-2<sup>+</sup> CD8<sup>+</sup> T cells  
783 from bronchoalveolar lavage fluid

784 2. Supplementary Figure 2. Extended intracellular cytokine staining

785 3. Supplementary Figure 3. Comparison of CDR3 $\alpha$  sequences with MAIT Match scores  
786 > 0.95 vs. MAIT Match scores < 0.85

787

788 **SUPPLEMENTARY TABLES**

789 1. Supplementary Table 1. Yield of high-throughput repertoire analysis using the  
790 immunoSEQ TCR sequencing platform on lung granuloma samples from humans with  
791 TB infection.

792 2. Supplementary Table 2. Characteristics of tuberculosis patients and BAL controls.

793 3. Supplementary Table 3. Characteristics of tuberculosis patients whose cryopreserved  
794 paired BAL and PBMC samples were sorted to generate the data presented in Figure

795 3e-g.

796 4. Supplementary Table 4. Top 15 MAIT CDR3 $\alpha$  sequences in the bronchoalveolar  
797 lavage of humans with pulmonary tuberculosis.  
798

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945  
946

**Table 1. TCR $\alpha$ / $\beta$  sequences and MAIT Match scores for six MAIT cell clones and two control clones derived from bronchoalveolar fluid from a patient with tuberculosis.**

Clone	TCR $\alpha$ sequencing			MAIT Match score	TCR $\beta$ sequencing		
	TRAV	CDR3 $\alpha$	TRAJ		TRBV	CDR3 $\beta$	TRBJ
<b>MAIT cell clones</b>							
D0033-A1	1-2	CAALDSNYQLIW	33	1.00	4-3	CASSQDMVSITDTQY	2-3
D0033-A2	1-2	CAVTDSNYQLIW	33	1.00	3-1	CASSQAETELNTGELF	2-2
D0033-A3	1-2	CVTMDSNYQLIW	33	0.98	6-1	CASSEAGGGYNEQF	2-1
D0033-A6	1-2	CAVVDSNYQLIW	33	1.00	4-2	CASSHSSGTGGNEQF	2-1
D0033-A8	1-2	CVTMDSNYQLIW	33	0.98	6-1	CASSEAGGGYNEQF	2-1
D0033-A10	1-2	CAVTDSNYQLIW	33	1.00	3-1	CASSGLEVTGELF	2-2
<b>Control T cell clones</b>							
D0033-D7	20	CAARFSDGQKLL	16	0.92	7-9	CASSEGTGVEWDGYT	1-2
D0033-E7	39	CAVPGGGADGLT	45	0.85	2	CASVASGVRDTQY	2-3

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**Table 2. Comparison of MR1/5-OPRU tetramer staining and usage of MAIT cell-consistent CDR3 $\alpha$  sequences within TRAV1-2+ CD8+ T cells in the bronchoalveolar (BAL) and peripheral blood (PBMC) compartments from two patients with active TB.**

Participant ID	Compartment	% MAIT cell-consistent CDR3 $\alpha$	% MR1/5-OPRU tetramer-positive	% MAIT cell-consistent CDR3 $\alpha$ of MR1/5-OPRU tetramer-positive	% MAIT cell-consistent CDR3 $\alpha$ of MR1/5-OPRU tetramer-negative
1020	BAL	40.7	33.7	93.9	13.7
1020	PBMC	5.4	3.1	89.2	2.7
0091	BAL	28.5	5.1	100.0	24.7
0091	PBMC	18.6	6.3	71.4	15.1

950



**Table 3. TCR $\alpha$ / $\beta$  sequences and MAIT Match scores for four T cell clones derived from bronchoalveolar cells.**

Clone	TCR $\alpha$ sequencing			MAIT Match score	TCR $\beta$ sequencing		
	TRAV	CDR3 $\alpha$	TRAJ		TRBV	CDR3 $\beta$	TRBJ
D1004-B3	1-2	CAVTDSNYQLIW	33	1.00	6-5	CASSYEGGGQPQHF	1-5
D1004-E1	1-2	CAALDSNYQLIW	33	1.00	6-4	CASSDGEGQPQHF	1-5
D1004-E5	1-2	CAAMDSNYQLIW	33	1.00	30-1	CAWSHSDRDLNEQYF	2-7
D1004-H3	1-2	CAAMDSNYQLIW	33	1.00	3	CASSQASGGEETQYF	2-5

951