

# Identification and phenotype of MAIT cells in cattle and their response to bacterial infections

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29

30 **Abstract**

31 Mucosal-associated invariant T (MAIT) cells are a population of innate-like T cells that utilise a semi-  
32 invariant T cell receptor (TCR)  $\alpha$  chain and are restricted by the highly conserved antigen presenting  
33 molecule MR1. MR1 presents microbial riboflavin biosynthesis derived metabolites produced by  
34 bacteria and fungi. Consistent with their ability to sense ligands derived from bacterial sources, MAIT  
35 cells have been associated with the immune response to a variety of bacterial infections, such as  
36 *Mycobacterium spp.*, *Salmonella spp.* and *Escherichia coli*. To date, MAIT cells have been studied in

humans, non-human primates and mice. However, they have only been putatively identified in cattle by PCR based methods; no phenotypic or functional analyses have been performed. Here, we identified a MAIT cell population in cattle utilising MR1 tetramers and high-throughput TCR sequencing. Phenotypic analysis of cattle MAIT cells revealed features highly analogous to those of MAIT cells in humans and mice, including expression of an orthologous TRAV1-TRAJ33 TCR  $\alpha$  chain, an effector memory phenotype irrespective of tissue localisation, and expression of the transcription factors PLZF and EOMES. We determined the frequency of MAIT cells in peripheral blood and multiple tissues, finding that cattle MAIT cells are enriched in mucosal tissues as well as in the mesenteric lymph node. Cattle MAIT cells were responsive to stimulation by 5-OP-RU and riboflavin biosynthesis competent bacteria *in vitro*. Furthermore, MAIT cells in milk increased in frequency in cows with mastitis. Following challenge with virulent *Mycobacterium bovis*, a causative agent of bovine tuberculosis and a zoonosis, peripheral blood MAIT cells expressed higher levels of perforin. Thus MAIT cells are implicated in the immune response to two major bacterial infections in cattle. These data suggest that MAIT cells are functionally highly conserved and that cattle are an excellent large animal model to study the role of MAIT cells in important zoonotic infections.

52

## 53 1 Introduction

54 Mucosal-associated invariant T (MAIT) cells represent the largest antigen specific  $\alpha/\beta$  T cell  
55 population in humans, comprising up to 10% of all T cells in the periphery and 50% in the liver (1-3).  
56 Unlike conventional  $\alpha/\beta$  T cells that recognize peptides presented by MHC class I (MHC-I) or MHC  
57 class II (MHC-II) molecules, MAIT cells recognise microbial riboflavin biosynthesis derived  
58 metabolites presented by a monomorphic MHC-I like molecule, MHC related protein 1 (MR1) (4-6).  
59 To date the most potent ligand presented by MR1 is 5-(2-oxopropylideneamino)-6-D-  
60 ribitylaminouracil (5-OP-RU), which is a derivative of a key riboflavin biosynthetic intermediate (7,  
61 8). MAIT cells are unconventional T cells that emerge from the thymus in a “pre-primed” state and  
62 express an effector memory phenotype (in humans CD45RA $^-$ CD45RO $^+$ CD95 $^{hi}$ CD62L $^{lo}$ ) (9). This  
63 facilitates rapid innate-like responses to bacterial infections, such as *Francisella tularensis* (10),  
64 *Legionella longbeachae* (11) and *Mycobacterium tuberculosis* (TB) (12, 13).

65 MR1 is estimated to have appeared around 170 Mya ago in a common ancestor of marsupial  
66 and placental mammals (14, 15) and is the most highly conserved MHC molecule in its ligand-binding  
67  $\alpha 1/\alpha 2$ -domains (14, 16-18). Despite this, MR1 has been lost in a select number of species, including  
68 carnivores and lagomorphs (15). MAIT cells express an essentially invariant T cell receptor (TCR)  $\alpha$

69 chain (TRA), composed in humans of the variable gene segment 1-2 (*TRA1-2*) (also known as V $\alpha$ 7.2  
70 in Arden nomenclature) rearranged with the TRA joining gene segment 33 (*TRAJ33*) (also known as  
71 J $\alpha$ 33), or less commonly *TRAJ12* (J $\alpha$ 12) and *TRAJ20* (J $\alpha$ 20), paired with TCR  $\beta$  chains (TRB) featuring  
72 dominant usage of specific TRBV gene segments (12, 19-22). Species that possess the MR1 gene also  
73 express a homologue of the canonical MAIT TCR *TRA1-2* gene segment (15) and the entire canonical  
74 MAIT TCR  $\alpha$  chain is conserved across mammalian species, with homologous TCR  $\alpha$  chains detected  
75 in mouse (20), cattle (20, 23), sheep (23), pigs (24) and macaques (25). MAIT cells follow similar  
76 developmental pathways in humans and mice (26, 27) however, mice have much smaller MAIT cell  
77 populations than humans with differences in phenotype and tissue distribution (26). Thus, whilst mice  
78 have provided valuable information on MAIT cells in protection against infections (1, 11, 28, 29),  
79 pathology (30) and tissue repair (31), this might not always be fully representative of MAIT cell  
80 function in humans.

81 Beyond primates and mice, information on MAIT cells is limited. Cattle are an economically important  
82 livestock species and are also a relevant large animals model for human infections, including  
83 tuberculosis (32, 33) and respiratory syncytial virus (RSV) (34). Cattle express the MR1 gene and the  
84 canonical MAIT cell TRA (17, 20, 23, 35). However, MAIT cells have not been characterised directly  
85 and there is no knowledge of the phenotype and function of MAIT cells in cattle. In humans and mice,  
86 as well as more recently in macaques, fluorescently labelled MR1 tetramers loaded with the MAIT cell  
87 activating ligand 5-OP-RU have become the gold standard to identify MAIT cells (7, 19, 36-40).  
88 Tetramers loaded with the MR1 ligand 6-formylpterin (6-FP) (6, 41) or its acetylated analogue, acetyl-  
89 6-FP (Ac-6-FP) (41), typically do not bind to MAIT cells and are often used as negative controls for  
90 MR1-5-OP-RU tetramer staining in humans (42). Here we used human MR1 tetramers and synthetic  
91 5-OP-RU antigen to identify and characterise MAIT cells in cattle. Further, we show that cattle MAIT  
92 cells can be activated by bacteria *in vitro* and that MAIT cells respond in the context of mastitis and  
93 *Mycobacterium bovis* (*M. bovis*) infection in cattle, suggestive of a role for MAIT cells in these  
94 diseases, caused by riboflavin biosynthesis competent pathogens.

95

## 96 2 Material and Methods

### 97 2.1 Animals

98 All animal experiments were conducted within the limits of a United Kingdom Home office license  
99 under the Animal (Scientific Procedures Act 1986) (ASPA) and were reviewed and approved by the  
100 Animal Welfare and Ethical Review Bodies of the institutes where the experiments were performed

101 (the Roslin Institute and the Animal and Plant Health Agency). Sampling milk from cattle is below the  
102 threshold of pain, suffering, distress or lasting harm that requires A(SP)A licensing and the procedure  
103 was reviewed by the Veterinary Ethical Review Committee of the Royal Dick School of Veterinary  
104 Studies (RDSVS), Edinburgh University.

105 Healthy, Holstein-Frisian cattle aged between three to fifty-six months were housed at the Edinburgh  
106 University farms or at the Animal Plant and Health Agency (APHA) facilities at Weybridge. Blood  
107 was sampled from the jugular vein and peripheral blood mononuclear cells (PBMC) were isolated from  
108 blood by density gradient centrifugation and cryopreserved. To harvest tissues, seven male cows (aged  
109 10, 10, 10, 10, 20, 55 and 56 months) were culled by schedule 1 methods under the ASPA followed by  
110 auscultation of the heart to confirm cessation of the circulation.

111 The BCG-vaccination and *M. bovis*-challenge study was described previously (43). In short, six bovine  
112 tuberculosis-free 6-months old male Holstein-Friesian (cross) calves were vaccinated subcutaneously  
113 with  $4.6 \times 10^6$  CFU *M. bovis* BCG Danish SSI 1331 (Statens serum Institute) and two calves served as  
114 controls. Nine weeks later all calves were infected with  $10^4$  CFU virulent *M. bovis* AF2122/97 via the  
115 endobronchial route. Twenty weeks post BCG vaccination all animals were euthanised and post-  
116 mortem examination was performed as described by Vordermeier et al. (44). Gross visible lesions of  
117 lungs and the lymph-nodes of the head and pulmonary regions were scored semi-quantitatively  
118 resulting in a total gross pathology score. Blood was sampled regularly and PBMC were isolated and  
119 cryopreserved.

120 Milk was sampled from Holstein-Friesian cattle housed at Langhill Dairy Farm, the teaching farm of  
121 the RDSVS, Edinburgh University.

## 122 **2.2 Tissue sampling and processing.**

123 Single cell suspensions were obtained from peripheral blood, prescapular lymph node (Ln), mesenteric  
124 Ln, lung, bronchial alveolar lavage (BAL), ilium, spleen, liver, and milk. Peripheral blood was diluted  
125 1:1 in PBS and layered over Histopaque-1077 (Sigma-Aldrich) before centrifugation at 1200 g for 20  
126 minutes. Cells were washed and resuspended in RPMI supplemented with 10% FCS and 1% penicillin  
127 streptomycin (Sigma Aldrich) (complete media) or in PBS buffer supplemented with 2% FCS and  
128 0.01% Azide. Prescapular Ln and mesenteric Ln were suspended in complete media before being  
129 manually disrupted and passed through a 100  $\mu$ M cell strainer. For lung and BAL, a lung lobe was  
130 removed and the main bronchus washed with 750 ml of PBS. Lungs were massaged for 30 seconds  
131 before BAL fluid was collected. The BAL fluid was transferred into 50 ml Falcon tubes and centrifuged  
132 at 400 g for 10 minutes and resuspended in complete media. A piece of lung was dissected in  $\sim$ 0.5 cm

133 cubes and resuspended in 7 ml serum free RPMI containing 30 µg/ml DNase and 700 µg/ml  
134 collagenase (Sigma Aldrich) and in C Tubes (Miltenyi Biotec) disassociated using the gentleMACS  
135 Octo Dissociator (Miltenyi Biotec). The C tubes were then incubated for 60 minutes at 37 °C, before  
136 being dissociated a second time, cells re-suspended in complete media and passed through a 100 µM  
137 cell strainer. Ileum, spleen and liver were also dissected and, samples were suspended in C Tubes in  
138 complete media and disassociated using the gentleMACS Octo Dissociator. Following disassociation,  
139 the resulting ileum and spleen cell suspension was passed through a 100 µM cell strainer. For liver, the  
140 cell suspension was resuspended in a 50 ml Falcon tube in 20 ml 35% PERCOL which had previously  
141 been made isotonic with 10x PBS and diluted with complete media. The 35% PERCOL was underlaid  
142 with 10 ml 70% PERCOL and centrifuged at 1200 g for 20 minutes. Cells were collected at the interface  
143 and resuspended. For all tissues other than liver and peripheral blood, the obtained cell suspensions  
144 were layered onto Histopaque-1077 (Sigma-Aldrich) before centrifugation at 1200 g for 20 minutes  
145 and the lymphocytes collected at the interphase. All cells were finally filtered through a 100 µM cell  
146 strainer, red blood cells lysed with an Ammonium Chloride Lysis buffer if required, washed and if not  
147 immediately used for assays, cryopreserved in FCS containing 10% DMSO. To isolate cells from milk,  
148 milk was centrifuged (400 g, 4 °C, 15 minutes). The resulting fay layer was removed with a pipette tip  
149 and the supernatant discarded. The pellet was resuspended in PBS and moved to a clean tube and the  
150 procedure was repeated. For the second PBS wash, the cell suspension was filtered with a 70 µM  
151 strainer and after centrifugation the cell pellet was resuspended in PBS + 2% FCS for downstream  
152 procedures.

### 153 2.3 ELISPOT

154 Frequencies of IFN-γ secreting cells were determined by ELISPOT IFN-γ assay. MultiScreen-HA  
155 ELISPOT plates (Merck Millipore) were coated with primary anti-IFN-γ clone CC330 (Serotec, 2  
156 µg/ml) and incubated at 4°C overnight. Plates were washed and blocked with complete media for two  
157 hours. Plates were seeded with  $2.5 \times 10^5$  PBMC and stimulated with either 1 µM 5-OP-RU (produced  
158 in house as previously described (8)), 4 µg/ml ConA (Sigma-Aldrich) or medium control. Plates were  
159 incubated overnight at 37 °C before washing with PBS containing 0.05% Tween 20 and addition of  
160 secondary biotinylated IFN-γ detection Ab (clone CC302 (Serotec, 2 µg/ml)). Plates were incubated  
161 for 2 hours at room temperature, washed a further five times, and streptavidin–alkaline phosphatase  
162 (Invitrogen) was added for 1 hour. Spots were visualised using alkaline phosphatase substrate kit (Bio-  
163 Rad) and the reaction stopped using water. Immunospots were enumerated using the AID ELISPOT  
164 reader (AID Autoimmun Diagnostika). Results are expressed as the total number of IFN-γ producing

165 cells per  $10^6$  input PBMC following subtraction of the average number of IFN- $\gamma$  positive cells in  
166 medium control wells.

167 **2.4 MR1 tetramers**

168 The MR1 tetramer technology was developed jointly by Dr. James McCluskey, Dr. Jamie Rossjohn,  
169 and Dr. David Fairlie (7) and the human MR1 tetramers (human MR1-5-OP-RU and human MR1-6-  
170 FP) were obtained from the NIH Tetramer Core Facility as permitted to be distributed by the University  
171 of Melbourne.

172 **2.5 Flow cytometry**

173 For phenotyping, *ex vivo* isolated or thawed cryopreserved cells were seeded into a 96 well plate at 1-  
174  $3 \times 10^6$  cells / well. Cells were stained with pre-diluted tetramer in PBS + 2% FCS for 40 minutes at  
175 room temperature. Following tetramer staining, primary antibodies (Table 1) were added in PBS buffer  
176 supplemented with 2% FCS and 0.01% Azide, and Near-Infrared or Yellow Fixable LIVE/DEAD stain  
177 (Invitrogen or Molecular probes) for 15-30 minutes at 4 °C. If required, cells were washed, and  
178 secondary antibodies added for 15-30 minutes at 4 °C. Cells were resuspended in buffer supplemented  
179 with 2% FCS and 0.01% sodium Azide and either immediately analysed or fixed in 4%  
180 paraformaldehyde and resuspended in PBS prior to analysis.

181 For activation experiments, cryopreserved PBMC were thawed and stimulated with titrating amounts  
182 of 5-OP-RU, fixed *E.coli* or *S. aureus* at 30 bacteria per cell (BpC) for 7 hours. Stimulation with human  
183 IL-1212 (Miltenyi) and IL-18 (Biolegend), either alone (50 ng/ml) or in combination (50 ng/ml), and  
184 *M. bovis* strain BCG (3BpC) were performed over 18 hours. For mitogen stimulation, PMA and  
185 Ionomycin (eBioscience cell stimulation cocktail) were added to cells for 6 hours. Ac-6-FP (Schircks  
186 Laboratories) was used as a blocking reagent for some 5-OP-RU stimulations and was added 30  
187 minutes prior to 5-OP-RU. For all stimulation conditions, Golgi plug (BD Biosciences) was added for  
188 the final 6 hours of stimulation. Cells were stained with human MR1-5-OP-RU tetramer for 40 minutes  
189 at RT in the dark prior to the addition of anti-bovine CD8-biotin (Clone ILA51) and Near-Infrared  
190 Fixable LIVE/DEAD stain (Invitrogen or Molecular Probes) for 20 minutes at 4 °C. BV421 conjugated  
191 streptavidin was added for 20 minutes at 4 °C prior to fixation and permeabilisation (BD Fix perm kit).  
192 Anti IFN- $\gamma$ -FITC (clone CC302) and TNF-APC (clone CC327) were added for 20 minutes at 4 °C.  
193 Cells were washed and re-suspended in PBS prior to analysis.

194 For transcription factor stains, thawed PBMC were stained with tetramer, live/dead stain and primary  
195 and secondary antibodies as described. Cells were fixed in Fix perm buffer (eBioscience) for one hour

196 followed by 1 hour in perm/wash buffer (BD). Conjugated antibodies specific for transcription factors  
197 PLZF (clone R17-809), EOMES (clone WD1928), ROR- $\gamma$ T (clone Q21-559) and T-bet (clone 4B10)  
198 diluted in perm/wash buffer were added for 1 hour at 4 °C. Cells were resuspended in PBS prior to  
199 analysis. Positive staining for each marker was determined by appropriate isotype controls and non-  
200 MAIT cell control populations.

201 All cells were acquired on a MACSquant analyser 10 (Miltenyi) or LSRFortessa (BD Biosciences).

## 202 2.6 Bovine T cell receptor $\alpha$ and $\beta$ chain sequencing

203 To obtain CD8 $^+$ MR1-5-OP-RU tetramer $^-$  (non-MAIT) and CD8 $^+$ MR1-5-OP-RU tetramer $^+$  (MAIT) cell  
204 populations for TCR sequencing, sequential MACS isolation and cell sorting were performed. Freshly  
205 isolated PBMC from four 13 months old heifer calves were stained with biotinylated mAb ILA51 (anti-  
206 CD8, produced in-house) in PBS supplemented with 0.5% FCS and 2 mM EDTA for 15 minutes on  
207 ice with resuspending every 5 minutes. Following washing, cells were stained with magnetic anti-  
208 biotin beads (Miltenyi Biotec) and labelled cells were isolated using MS columns (Miltenyi Biotec)  
209 according to the manufacturer's instruction. Isolated cells were stained with MR1-5-OP-RU tetramer,  
210 followed by staining with mAbs MM1a (anti-CD3) and ILA105 (anti-CD8) and secondary antibodies  
211 and Fixable Yellow Dead Cell Stain (Thermofisher Scientific). These cells were sorted using a  
212 FACSAria III (BD), gating on FSC and SSC, singlets and live cells, respectively. Within these gates  
213 CD8 $^+$ MR1-5-OP-RU $^-$  and CD8 $^+$ MR1-5-OP-RU $^+$  T cell populations were sorted directly into lysis  
214 buffer for RNA isolation (RNeasy Plus Micro kit, Qiagen). Purity of ungated sorted CD8 $^+$ humMR1-5-  
215 OP-RU $^+$  cells was between 91-99% (Fig. S1). RNA was isolated from 20,000 cells using the RNeasy  
216 Plus Micro kit according to manufacturer's instruction, with addition of 4 ng/ $\mu$ l carrier RNA. cDNA  
217 was generated using the SuperScript IV kit (ThermoFisher Scientific) with a SMART oligo containing  
218 unique molecular identifiers (AAG CAG UGG TAU CAA CGC AGA  
219 GTUNNNNUNNNNUUNNNNUCTTgggg (where N represents a mixture of A, T, G and C and lower  
220 case 'g' represents RNA bases) and the uracil-containing primers subsequently removed by treatment  
221 with UDG (NEB, Hitchin, UK). TRA and TRB sequences were amplified using a pair of 5' 'step-out'  
222 primers specific for the SMART oligo (long 5' primer - CTA ATA CGA CTC ACT ATA GGG CAA  
223 GCA GTG GTA TCA ACG CAG AGT, and short 5' primer - CTA ATA CGA CTC ACT ATA GGG  
224 CAA GCA G) in combination with *TRAC*- (TGG GGT TGG GGT CCT TGA CTT) and *TRBC*- (GAC  
225 SYG GCT CAG ATC ATC ) specific 3'primers. PCR amplification was conducted using Phusion HF  
226 reagents (NEB) and employed the following cycling conditions: manual hot start, 30 seconds at 98 °C,  
227 35 cycles of (98 °C for 10s, 65 °C for 30s, 72 °C for 30s) and a final extension period of 5 minutes at

228 72 °C. PCR products of the anticipated size were excised following agarose gel electrophoresis,  
229 purified using AMPure beads (Beckman Coulter, Indiana, US) and sent to Edinburgh Genomics for  
230 sequencing on the Illumina MiSeq v3. Platform. Analysis of the TCR repertoire was conducted with  
231 the MiXCR package (45) using an external database of bovine TRAV, TRBV, TRAJ and TRBJ genes  
232 derived from a combination of published (46, 47) and unpublished data.

233 **2.7 Bacteria for MAIT cell stimulation**

234 Tryptic soy broth (TSB) was inoculated with *Escherichia coli* strain DH5 $\alpha$  and *Staphylococcus aureus*  
235 strain RF122 from glycerol stocks and cultured overnight at 37 °C and 200 RPM. The next morning a  
236 1:100 dilution in TSB was cultured for two hours and OD at 600 nm measured to estimate CFU/ml  
237 (OD 600 nm of 1.0 = 8 x 10<sup>8</sup> CFU/ml). Bacteria were washed with PBS, fixed in 1% formaldehyde for  
238 5 minutes at RT, washed thrice with PBS, resuspended in CM and stored at -20 °C.

239 *M. bovis* strain BCG Danish SSI 1331 (Statens Serum Institute) was cultured in glass vials in  
240 Middlebrook 7H9 broth supplemented with Tween 80, Amphotericin B (all Sigma-Aldrich), and BD  
241 Difco™ BBL™ Middlebrook ADC Enrichment and incubated at 37 °C with agitation with a magnetic  
242 stir bar. After 25 days the bacterial culture was vortexed vigorously and after 1 minutes the  
243 'supernatant' was harvested and passed several times through a 21g needle to obtain a single bacteria  
244 suspension. Bacteria were pelleted and resuspended in 7H9 broth supplemented with 30% glycerol,  
245 aliquoted and stored at -80 °C. Thawed aliquots were serially diluted 1:10 in 7H9 broth and 100  $\mu$ l of  
246 these suspensions were cultured on Middlebrook 7H11 agar, supplemented with OADC (both BD), to  
247 determine CFU/ml.

248 **2.8 Data analyses and statistics**

249 Flow cytometry data were analysed using FlowJo v10. Descriptive and statistical analyses were  
250 performed using Prism software version 8 (GraphPad). Data presented in the text and figures are means  
251 with standard error of the mean (SEM). P-values corrected for multiple comparisons  $\leq 0.05$  were  
252 considered significant. \*  $p \leq 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ .

253

254 **3 Results**

255 **3.1 Identification of MAIT cells in cattle.**

256 Due to the high level of MR1 (14, 15, 17, 35) and MAIT TCR  $\alpha$  chain (14, 15, 20, 23, 24, 40)  
257 conservation between species, we hypothesised that human MR1 tetramers would likely cross react

258 with cattle MAIT cells. We isolated PBMC from a cohort of cattle (n = 17) of varying age (3 to 56  
259 months) and indeed staining with MR1-5-OP-RU tetramers identified a clear population of CD3<sup>+</sup>  
260 tetramer<sup>+</sup> ‘putative’ MAIT cells (**Fig. 1A-B**) with a mean frequency of 0.6% amongst CD3<sup>+</sup> cells,  
261 which was comparable to previous qPCR estimates of MAIT cell frequency in cattle (~0.2% of  
262 transcribed TRA) (23). A much lower frequency was identified by the control MR1-6-FP tetramer (**Fig.**  
263 **1A-B**). The frequency of MAIT cells varied greatly between individuals with a range of 0.18-1.72%  
264 and an interquartile range (IQR) of 0.33-0.66% of total T cells. Within this age cohort (3-56 months),  
265 there was no correlation between age and MAIT cell frequency. Whilst in humans MAIT cells make  
266 up a higher proportion of T cells (mean 3.1%), frequencies of MAIT cells in humans also vary widely  
267 between individuals with an IQR of 1.3-4.5% (38). The most potent MAIT cell ligand identified to  
268 date is 5-OP-RU (7, 8), which specifically induces cytokine secretion, including IFN- $\gamma$ , in MAIT cells  
269 but not in other T cells (7, 41). To corroborate the identification of MAIT cells using tetramers, we  
270 next determined whether a 5-OP-RU reactive population was present in cattle PBMC by IFN- $\gamma$  ELISpot  
271 following stimulation with synthetic 5-OP-RU (8) (**Fig. 1C**). Following stimulation with 5-OP-RU a  
272 mean of 125 IFN- $\gamma$  secreting cells /10<sup>6</sup> PBMC were detected, demonstrating that there was a 5-OP-RU  
273 reactive T cell population in cattle. In summary, we identified a population of MR1-5-OP-RU tetramer<sup>+</sup>  
274 T cells and 5-OP-RU reactive cells in cattle peripheral blood, strongly suggesting that we identified a  
275 MAIT cell population in cattle.

### 276 **3.2 Phenotype of MAIT cells in bovine peripheral blood.**

277 MAIT cells are unconventional T lymphocytes with functional and phenotypic features that  
278 distinguishes them from conventional T lymphocytes, including an effector memory phenotype prior  
279 to antigen exposure (48), enrichment in mucosa (2) and expression of specific transcription factors  
280 such as PLZF (49). According to co-receptor expression (**Fig. 2A**), cattle peripheral blood MAIT cells  
281 were predominantly CD8<sup>+</sup> (mean 73.9%, IQR 64-87%) or double negative (mean 19.7%, IQR 12.7-  
282 27.5%) with a low frequency of CD4<sup>+</sup> (2.7% IQR 0.9-3.4%) MAIT cells identified. This disagrees with  
283 the earliest report of MAIT cells in cattle suggesting that cattle MAIT cells were not CD8 positive (20).  
284 Interestingly, some of the tetramer positive cells were TCR $\delta$ <sup>+</sup> (mean 8.33% IQR 2.3-13.3% of total  
285 MR1-5-OP-RU tetramer<sup>+</sup> population in PBMC) (**Fig. 2A**), equating to a mean frequency of 0.08% of  
286 total  $\gamma\delta$ <sup>+</sup> T cells in cattle. Cattle are a  $\gamma\delta$  T cell high species and in adult cattle ~10-20% of circulating  
287 lymphocytes are TCR $\gamma\delta$  positive (50). This observation mirrors a recent report of human MR1 reactive  
288  $\gamma\delta$  T cells (51).

289 In contrast to non-MAIT T cells, peripheral blood MAIT cells featured almost exclusively an effector  
290 memory phenotype (CD45RO<sup>+</sup> CCR7<sup>-</sup>) (**Fig. 2B**), as in humans (48). We also compared transcription  
291 factor expression in CD8<sup>+</sup> MAIT cells to CD8<sup>+</sup> non-MAIT cells. Whilst cattle specific antibodies  
292 against the transcription factors PLZF, EOMES, ROR $\gamma$ T and T-bet are not available, transcription  
293 factors are highly conserved between species and monoclonal antibodies have previously been shown  
294 to cross react between species. ROR $\gamma$ T and T-Bet showed little expression above isotype control likely  
295 due to insufficient cross reactivity. However, PLZF and EOMES-expression was significantly higher  
296 in bovine CD8<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup> MAIT cells compared to CD8<sup>+</sup> non-MAIT T cells (**Fig. 2C**).  
297 Similarly, antibodies specific for human TRAV1-2 (clone 3C10) and CD161 (clone 191B8) did not  
298 appear to cross react (data not shown). Together these data showed that MAIT cells in cattle almost  
299 exclusively had an effector memory phenotype and were predominantly CD8, PLZF and EOMES  
300 positive.

301 **3.3 MAIT cells in cattle can be activated by 5-OP-RU and by cytokines.**

302 As the majority of MAIT cells were CD8<sup>+</sup> (**Fig. 2A**), activation experiments focussed on comparing  
303 human MR1-5-OP-RU tetramer positive and negative CD8<sup>+</sup> populations (MAIT and non-MAIT CD8  
304 T cells, respectively). CD8<sup>+</sup> MAIT cells were specifically stimulated to express IFN- $\gamma$  (mean 32%  
305 IFN- $\gamma$ <sup>+</sup>) and TNF (mean 29% TNF<sup>+</sup>) by the canonical MAIT cell ligand 5-OP-RU (**Fig. 3A**) at  
306 concentrations as low as 50 pM (**Fig. S2A-B**). Increased concentrations of 5-OP-RU (**Fig. S2C**) and  
307 prolonged incubation time with 5-OP-RU (**Fig S2D**) correlated with declining fractions of MR1-5-OP-  
308 RU tetramer<sup>+</sup> cells (**Fig. S2C**), suggesting that the TCRs of bovine MAIT cells are downregulated upon  
309 binding to cognate ligand, as described previously (52). The residual fraction of cytokine positive CD8<sup>+</sup>  
310 tetramer negative cells following 5-OP-RU stimulation (**Fig 3A, S2**) are therefore most likely activated  
311 MAIT cells with downregulated TCRs. The 5-OP-RU-mediated activation of cattle MAIT cells was  
312 competitively inhibited by the inhibitory MR1 ligand Ac-6-FP (**Fig. 3B**), as is the case with human  
313 (41) and mouse MAIT cells (53), and strongly suggests that activation is mediated through MR1-TCR  
314 interactions.

315 Viruses can stimulate MAIT cells in a TCR independent manner via cytokine stimulation (54).  
316 Following stimulation with IL-18, bovine MAIT cells produced IFN- $\gamma$  (mean 31 % IFN- $\gamma$ <sup>+</sup>) and low  
317 frequencies of TNF producing cells (mean 2 % TNF<sup>+</sup>) were observed (**Fig. 3C**). There was no  
318 significant response to stimulation with IL-12 only by MAIT or non-MAIT T cells, but in conjunction  
319 with IL-18, IL-12 did significantly increase the frequency of MAIT cells producing IFN- $\gamma$  (mean 56%  
320 IFN- $\gamma$ <sup>+</sup>) compared to IL-18 stimulation alone. Unlike 5-OP-RU stimulation, cytokine stimulation did

321 not appear to affect MR1-5-OP-RU tetramer binding, suggesting that it did not induce TCR  
322 downregulation (data not shown).

323 **3.4 Cattle MAIT cells express a conserved T cell receptor alpha chain and show low beta chain**  
324 **diversity.**

325 The canonical MAIT TCR $\alpha$  chain has previously been identified in cattle by sequencing of a limited  
326 number of unsorted T cells with no pre-identification of MAIT cells (20, 23). Here we performed deep  
327 TCR profiling of sorted bovine CD8 $^{+}$  MR1-5-OP-RU tetramer $^{+}$  MAIT cells in comparison to MR1-5-  
328 OP-RU tetramer $^{-}$  CD8 $^{+}$  T cells. The sorted MAIT cell populations were highly enriched for the  
329 canonical *TRAV1* (73.3%) and *TRAJ33* (72%) gene segments which were not enriched in the CD8 $^{+}$   
330 non-MAIT cell population (**Figs. 4A-B, S3 and S4**). The TCR $\beta$  chain usage of bovine MAIT cells was  
331 more diverse than the TCR $\alpha$  chain, though an enrichment of *TRBV4*, *TRBV7* and *TRBV20* was seen  
332 in the MAIT cell population compared to the non-MAIT CD8 $^{+}$  population (**Figs. 4A-B, S3 and S4**),  
333 accounting for 35 %, 13 % and 23 % of total TRBV sequences in MAIT cells respectively. This mirrors  
334 findings in humans where the critical residues for MR1 recognition are found in the TCR $\alpha$  chain (5,  
335 55) and the TCR $\beta$  chains are more variable, though specific TRBV, particularly *TRBV6* and *TRBV20*,  
336 dominate (19, 21, 38, 41, 56, 57). The nomenclature of cattle TCR gene segments is based on human  
337 orthologues and *TRBV20* is, therefore, enriched in both human and cattle MAIT cells. The CDR3 $\alpha$   
338 loops of CD8 $^{+}$  MAIT TCRs were primarily 12aa long, similar to the canonical human MAIT cell TCR  
339 (**Fig. 4C**), accounting for 76 % of all MAIT cells, whereas CDR3 $\alpha$  loops of non-MAIT TCRs varied  
340 much more in length and most (73 %) were longer. The CDR3 $\beta$  loops of CD8 $^{+}$  MAIT TCRs were more  
341 varied in length than the CDR3 $\alpha$  loops and displayed a similar length distribution as compared to those  
342 of non-MAIT CD8 $^{+}$  TCRs. In humans the MAIT TCR $\alpha$  rearrangements *TRAV1-2-TRAJ33/20/12*  
343 account for the majority (~95%) of MAIT TCR clonotypes in blood (19, 21, 38, 56, 58).

344 When analyzing the TCR sequences at the clonal level, 62-76% of CD8 $^{+}$  MAIT cells expressed the  
345 canonical *TRAV1-TRAJ33* TCR rearrangement (**Fig. 3B, S3 and S4**), similar to what Greene et al. (25)  
346 previously described in macaques where 70% of TCRs of MR1 tetramer positive peripheral blood T  
347 cells were *TRAV1-2* $^{+}$ . The non-canonical TCR rearrangements were highly diverse, with no CDR3  
348 sequences shared between all four donors (**Supplementary Data 1**). The non-canonical TCR may be  
349 the result of nonspecific binding of tetramer to non-MAIT cells, sorting impurities or non-MAIT cell  
350 MR1 reactive T cells, which are rare populations identified in mice and humans (59). *TRAV1-TRAJ33* $^{+}$   
351 cattle MAIT TCRs featured two similar CDR3 sequences, the predominant sequence of which was  
352 CVVMDGNYQWIW with a secondary sequence observed in all animals with a single aa substitution

353 of methionine to isoleucine at position 91 (**Fig. 3D, Supplementary Data 1**). The same position in  
354 addition to the neighbouring residue also varies in human *TRAJ1-2-TRAJ33<sup>+</sup>* MAIT cell TCRs  
355 (CAXXDSNYQLIW )(20, 38, 57, 58). Both of the cattle CDR3 $\alpha$  sequences are conserved in Tyr95,  
356 which is critical for MR1 and antigen binding in humans (55). While cattle have orthologues for the  
357 human *TRAJ12* and *TRAJ20* gene segments, including conservation of a tyrosine at the same position,  
358 these *TRAJ* segments were not enriched in cattle MAIT cells. Altogether, the identified TCR CDR3 $\alpha$   
359 sequences are in agreement with previously predicted putative cattle MAIT TCR CDR3 $\alpha$  sequences  
360 (20, 23). The deep TCR profiling, together with the functional and phenotypical analyses, confirmed  
361 that the MR1-5-OP-RU tetramer positive cells in cattle are bona fide MAIT cells.

362 **3.5 Distribution and phenotypic comparison of MAIT cells in tissues.**

363 In cattle PBMC, approximately 0.6% of CD3 $^{+}$  lymphocytes (**Fig. 1B**) or 4% of CD8 $^{+}$  lymphocytes  
364 were MAIT cells (**Fig. 5A-B**). MAIT cells in humans are highly enriched in mucosal tissues and liver  
365 (2). This was also true in cattle (**Fig. 5A-B**), with greater frequencies of MR1-5-OP-RU tetramer  
366 positive cells detected in lung, spleen, liver and BAL when compared to PBMC (mean 1.74%, 1.02%,  
367 2.71% and 1.84% of CD3 $^{+}$  lymphocytes respectively). MAIT cell frequencies in the ileum were  
368 comparable to those in PBMC (mean 0.6%). Of note, an enrichment of MAIT cells was observed in  
369 the mesenteric Ln (mean 3.5%), but not in the pre-scapular Ln (mean 0.6%) (**Fig. 5A-B**). The high  
370 frequency of MAIT cells in the mesenteric Ln was not seen in a recent study of the pigtail macaque  
371 (39) and may be specific to cattle. One could speculate that this difference is due to the large microbial  
372 populations in the rumen and large intestines of cattle, which are drained by the mesenteric lymph  
373 node. Although in all tissues a low fraction of T cells bound MR1-6-FP tetramer compared to MR1-5-  
374 OP-RU tetramer, relatively more MR1-6-FP tetramer positive T cells were identified in spleen and  
375 liver (**Fig. 5A-B**). The percentage of effector memory non-MAIT T cells varied greatly across tissues,  
376 whereas MAIT cells predominantly had an effector memory phenotype irrespective of origin (**Fig. 5C**).  
377 Non-MAIT T cells in lymph nodes were predominantly CCR7 high. In contrast, MAIT cells in  
378 prescapular and mesenteric lymph nodes had an effector memory phenotype with low expression of  
379 CCR7, as was reported for human MAIT cells in thoracic duct lymph (60). Migration of  $\gamma\delta$  T cells  
380 from tissue to lymph nodes was shown to be CCR7 independent in cattle (61) and it has been  
381 hypothesized that CCR7 low MAIT cells enter the lymphatics from tissues in a CCR7 independent  
382 manner (60).

383 Differences in MAIT cell co-receptor usage between blood and tissues were observed (**Fig. 5D**).  
384 MAIT cells from peripheral blood had the largest CD8 $^{+}$  population (mean 76% CD8 $^{+}$ ) with no

385 significant difference in the frequency of MAIT cells expressing CD8 in lung, spleen and liver (mean  
386 74%, 68% and 68% CD8<sup>+</sup> respectively). Significantly lower fractions of CD8<sup>+</sup> MAIT cells were  
387 identified in the pre-scapular Ln. (mean 54% CD8<sup>+</sup>) and in ileum, which showed the lowest frequency  
388 of CD8 expression (mean 37% CD8<sup>+</sup>). There was a trend for a lower fraction of CD8<sup>+</sup> MAIT cells in  
389 mesenteric Ln. (mean 52% CD8<sup>+</sup>, p = 0.079) and in BAL (mean 58%, p = 0.054). There was a strong  
390 negative correlation between the fraction of CD8<sup>+</sup> and CD8<sup>-</sup>CD4<sup>-</sup>TCR $\gamma$ <sup>-</sup> MAIT cells ( $R^2 = 0.90$ ) and  
391 the fraction of these triple negative MAIT cells was proportionally increased in tissues with low CD8  
392 expression. Differences in IL-2R $\alpha$  chain (CD25) expression were also noted (Fig. 5D), with a trend for  
393 a greater frequency of CD25 high MAIT cells in BAL (mean 61%, p = 0.066), compared to MAIT  
394 cells in peripheral blood (mean 25%). A significantly higher proportion of CD25 high MAIT cells was  
395 seen in prescapular (mean 69%) and mesenteric (mean 81.2%) lymph nodes, which is more comparable  
396 to other tissues than to blood, potentially due to MAIT cell recirculation between tissues and lymph  
397 nodes (60, 61).

### 398 3.6 Cattle MAIT cells respond to bacterial infections *in vivo* and bacterial stimulation *in vitro*

399 Next, we sought to characterise MAIT cells in cattle directly *ex vivo* during infection as well as in an  
400 immunisation-challenge model. Mastitis is an inflammation of the mammary gland and is most often  
401 due to bacterial infections by riboflavin biosynthesis proficient bacteria, such as *Escherichia coli* (*E.*  
402 *coli*) and *Staphylococcus aureus* (*S. aureus*). It is the most frequent disease in dairy cattle, presents a  
403 major impact on animal welfare, and is associated with economic losses (62). Milk contains many  
404 different blood derived immune cells (63, 64), and MR1-tetramer staining of cells in milk from healthy  
405 cows revealed a distinct MAIT cell population consistently present in bovine milk (mean 0.8% of  
406 CD3<sup>+</sup>, IQR 0.4-1.4%, n = 6, Fig 6A, S5A). The number of cells in milk, also referred to as somatic cell  
407 count (SCC) is used as a biomarker for mastitis, where animals with an elevated SCC (>200,000  
408 cells/ml) are considered to have mastitis (65). Cattle with an elevated SCC had on average a greater  
409 than 5-fold increase in MAIT cells as a percentage of CD3<sup>+</sup> T cells, indicating increased migration of  
410 MAIT cells relative to other T cells from blood to milk during mastitis (Fig. 6A) and suggesting a  
411 possible direct or bystander role of MAIT cells in this inflammatory condition.

412 We next assessed CD8<sup>+</sup> MAIT cell responses to *Mycobacterium bovis* (*M. bovis*) infection  
413 longitudinally in cattle vaccinated with the attenuated *M. bovis* strain Bacillus Calmette–Guérin (BCG)  
414 and following endobronchial challenge with the virulent *M. bovis* strain AF2122/97. Perforin and  
415 granzyme production can be used as activation markers of MAIT cells (2, 66). In cattle, no changes in  
416 the frequency of perforin expressing MAIT cells were found *ex vivo* in PBMC following BCG

417 vaccination (**Fig. 6B**). However, two weeks following endobronchial challenge with *M. bovis* the  
418 fraction of perforin expressing CD8<sup>+</sup> MAIT cells was significantly higher in animals that showed  
419 tuberculosis associated lesions in the lungs and lymph nodes compared to animals without lesions (**Fig.**  
420 **6C**). While perforin expression did not change in tetramer negative (non-MAIT) CD8<sup>+</sup> T cells, there  
421 was a significant, transient, increase in perforin expression amongst CD8<sup>+</sup> MAIT cells in animals with  
422 lesions (**Fig. S5B**). In macaques, activation of MAIT cells was much more pronounced locally at the  
423 site of BCG vaccination (25). Vaccination with the attenuated *M. bovis* BCG strain causes a local  
424 infection and it is therefore not surprising that MAIT cell activation was not detectable in peripheral  
425 blood. We hypothesize that severe infection with virulent *M. bovis* resulting in lesions in multiple  
426 organs leads to more widespread MAIT cell activation that can be detected in peripheral blood. No  
427 changes in CD69 expression, or in CD8<sup>+</sup> MAIT cell frequencies were detected in peripheral blood of  
428 BCG vaccinated or *M. bovis* challenged animals (**Fig. S5C-F**), which is in agreement with findings  
429 after BCG vaccination in humans (67) and *M. tuberculosis* challenge in macaques (25). Overall, these  
430 data demonstrate that *M. bovis* infection in cattle can lead to activation of MAIT cells *in vivo*.

431 Having established that MAIT cells may respond to bacterial infections in cattle *in vivo*, we went on  
432 to validate whether cattle MAIT cells were activated by riboflavin biosynthesis competent bacteria.  
433 PBMC were stimulated with *E. coli*, and the attenuated *M. bovis* strain BCG (**Figs. 6D-E**). Stimulation  
434 with *E. coli* for 7 hours led to robust IFN- $\gamma$  and TNF upregulation by MAIT cells, while tetramer  
435 negative CD8<sup>+</sup> T cells showed limited activation (**Fig. 6D**), which may include activated MAIT cells  
436 that have downregulated their TCRs (**Fig. S2**). When stimulated overnight with BCG, MAIT cells  
437 displayed robust IFN- $\gamma$  production whilst TNF expression was limited (**Fig. 6E**), comparable with the  
438 cytokine profile observed upon IL-12/IL-18-stimulation (**Fig. 3C**). This is in agreement with human  
439 MAIT cell responses to BCG stimulation, which were reported to be mediated primarily by IL-12/IL-  
440 18 rather than TCR-antigen-MR1 stimulation and yielded INF- $\gamma$ , but not TNF production (67). *S.*  
441 *aureus* also stimulated IFN- $\gamma$  and TNF production in bovine MAIT cells (data not shown). Together  
442 these data illustrate that cattle MAIT cells respond to bacterial infections *in vivo* and are activated by  
443 bacteria *in vitro*.

444

## 445 4 Discussion

446 The canonical MAIT cell TCR  $\alpha$  chain was first identified in cattle alongside humans and mice over  
447 20 years ago (20), but phenotypic and functional MAIT cells have not been described in any livestock  
448 species. The use of human MR1 tetramers that cross react with cattle have allowed us to identify MAIT

449 cells in cattle and characterise their phenotype and function *in vitro* and directly *ex vivo*. While these  
450 data were generated using a xeno-MR1 reagent, the further phenotypic and functional analysis of cattle  
451 MAIT cells was in great agreement with that of other species and thus gives confidence that the human  
452 MR1-5-OP-RU tetramer identifies a MAIT cell population in cattle. Our data demonstrate that cattle  
453 MAIT cells are phenotypically and functionally similar to their human counterparts, including  
454 expression of an orthologous conserved TRAV1-TRAJ33 T cell receptor  $\alpha$  chain by the majority of  
455 MR1 tetramer $^+$  cells, an effector memory phenotype, expression of transcription factors associated with  
456 innate immunity, enrichment in mucosal tissues and activation by synthetic 5-OP-RU, the cytokines  
457 IL-12 and IL18, and riboflavin biosynthesis competent bacteria.

458 Cattle produce around 20 to 60 litres of milk per day and are milked at least twice a day. The  
459 large volumes and continuous production of milk means there is a huge migration of immune cells  
460 from blood to milk, even in a healthy non-infected non-inflamed mammary gland (63, 64). The  
461 increased MAIT cell frequency in milk in cows with mastitis implies increased trafficking of MAIT  
462 cells relative to other T cells to the mammary gland during infection. Mastitis in cattle is predominantly  
463 bacterial in origin and is characterised by a massive migration of neutrophils to the mammary gland  
464 (68). The major mastitis pathogens *E. coli* and *S. aureus* (69) stimulated bovine MAIT cells *in vitro*.  
465 MAIT cells are a key source of pro-inflammatory cytokines (30, 37, 70) and bacteria induced cytokine  
466 responses by MAIT cells in the context of mastitis could be a driving force in the neutrophil influx and  
467 inflammation associated with intramammary bacterial infections. Further studies tracking MAIT cells  
468 longitudinally in milk and tissues during intramammary infections, will shed light on the role of MAIT  
469 cells in mastitis, including the temporal relation to neutrophil influx. Maternal immune cells in milk  
470 play a role in the development of the neonatal immune system (71, 72) and milk derived CD8 $^+$  T cells  
471 preferentially home to the payers patches of the small intestine (73). MAIT cells have also been  
472 identified in human breast milk (74). Given the monomorphic nature of their restriction element MR1,  
473 MAIT cells are donor-unrestricted and can be activated by MR1 expressing cells from any individual  
474 (75). MAIT cells present in milk and possibly also in colostrum may be transferred to the neonate  
475 where they could play a role in passive immunity in the upper and lower intestinal tract (72).

476 MAIT cells have been shown to be activated by *Mycobacterium tuberculosis* in humans (13,  
477 67), non-human primates (40) and mice (76). Furthermore, MAIT cells are the predominant IFN- $\gamma$   
478 producing T cell population in TB exposed individuals upon restimulation with BCG (67). The  
479 increased proportion of perforin $^+$  MAIT cells in cows with TB lesions combined with the activation of  
480 MAIT cells by BCG *in vitro* indicates that MAIT cells may play a role in bovine TB. Intravenous, but  
481 not intradermal, administration of BCG was shown to transiently (up to 8 weeks) increase MAIT cell

482 frequencies in the BAL of non-human primates, while there was no effect on MAIT cells in peripheral  
483 blood (77). The same study also showed limited responses by MAIT cells in the periphery to  
484 subcutaneous BCG vaccination and demonstrates that route of vaccine administration and tissue  
485 localisation are important factors to consider when studying MAIT cell responses to vaccination.  
486 Recently, 5-OP-RU vaccination of mice was not shown to be protective of TB infection and contributed  
487 to a delayed CD4 response to the infection. However, treatment with 5-OP-RU during chronic TB  
488 infection led to an increase in MAIT cell frequencies and a lowering of bacterial burden, which was  
489 dependent on IL-17 expression (78). The emerging picture suggests that MAIT cells are involved in  
490 immunity against TB infection, although whether their role is protective may well depend on a range  
491 of factors, including stage of infection. As a natural host of TB and with the possibility for repeated  
492 (tissue) sampling and cannulation of lymph nodes, cattle are an appropriate large animal model with  
493 unique potential to study MAIT cells longitudinally in tissues *in vivo*.

494 In summary, we have identified a MAIT cell population in cattle with phenotypic and functional  
495 characteristics closely resembling MAIT cells in mice and humans. We have demonstrated that cattle  
496 MAIT cells respond to bacterial infections of economic and zoonotic importance and the data and tools  
497 presented here will facilitate the use of cattle as a relevant large animal model to study MAIT cell  
498 biology during immunisation and infection.

499

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518

519 **7 Author contribution statement**

520 PK, ET, LB, SE, TKC conceived, designed and coordinated the study. ME, LB, TKC, CV, SJ, MV,  
521 EM, TC designed and performed experiments, processed samples and analyzed the data. JM, LL, DF  
522 generated 5-OP-RU. LB, ME, SE, prepared the manuscript and figures. All authors reviewed the  
523 manuscript and approved the submitted version.

524

525 **8 Conflict of interest statement**

526 SE, JM, LL and DF are inventors on patents describing MR1 tetramers and MR1–ligand complexes.  
527 All other authors declare that the research was conducted in the absence of any commercial or  
528 financial relationships that could be construed as a potential conflict of interest.

529

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723 **10 Tables**

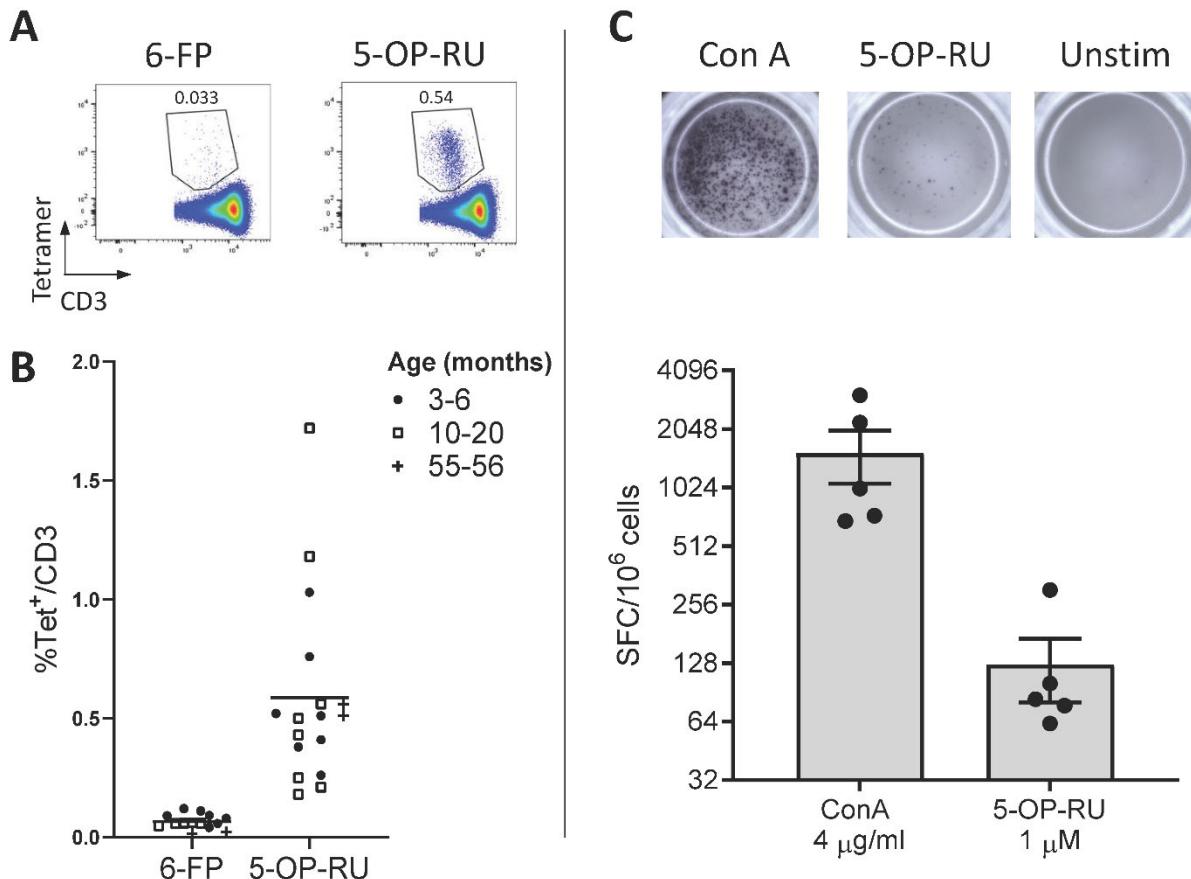
724 **Table 1.** Monoclonal antibodies used in flow cytometry experiments.

Purpose	Antigen	clone	Isotype	Labelling strategy	Fluorochrome	Source primary ab
<b>Identification</b>	/	CD3	MM1A	IgG1	Secondary antibody	AF488
<b>Frequency</b>		MHCII	ILA21	IgG2a	Secondary antibody	AF647
<b>Co receptor phenotyping</b>	CD4	ILA12	IgG2a	Secondary antibody	APC-Cy7	In-house
	TCR $\delta$	GB21a	IgG2b	Secondary antibody	PE-Cy7	In-house
	CD8	ILA51	IgG1	Directly conjugated	AF647	In-house
	CD3	MM1A	IgG1	Directly conjugated	AF488	In-house
<b>Memory / activation status</b>	CD3	MM1A	IgG1	Secondary antibody	AF488	In-house
	CD45RO	ILA116	IgG3	Secondary antibody	PE-Cy7	In-house
	CD25	CACT108a	IgG2a	Secondary antibody	AF647	In-house
	CCR7	3D12	IgG2a (rat)	Directly conjugated	APC-eFlour780	eBioscience
<b>Activation assays</b>	CD8	ILA51	IgG1	biotin-streptavidin	BV-421	In-house
	IFN $\gamma$	CC302	IgG1	Directly conjugated	AF647	BIO-RAD
	TNF	CC327	IgG2b	Directly conjugated	AF488	BIO-RAD
<b>Transcription factor</b>	CD8	ILA51	IgG1	biotin-streptavidin	BV-421	In-house
	T-bet	4B10	IgG1	Directly conjugated	FITC	Biolegend
	EOMES	WD1928	IgG1	Directly conjugated	eFlour661	Invitrogen
	PLZF	R17-809	IgG1	Directly conjugated	AF647	BD
	ROR- $\gamma$ T	Q21-559	IgG2b	Directly conjugated	AF647	BD
<b>CD69</b>	CD8	ILA105	IgG2a	Secondary antibody	AF647	In-house
	CD69	KTSN7A	IgG1	Secondary antibody	AF488	Kingfisher
<b>Perforin</b>	CD8	ILA105	IgG2a	Secondary antibody	APC-Cy7	In-house
	Perforin	dG9	IgG2b	Directly conjugated	Fitc	BD

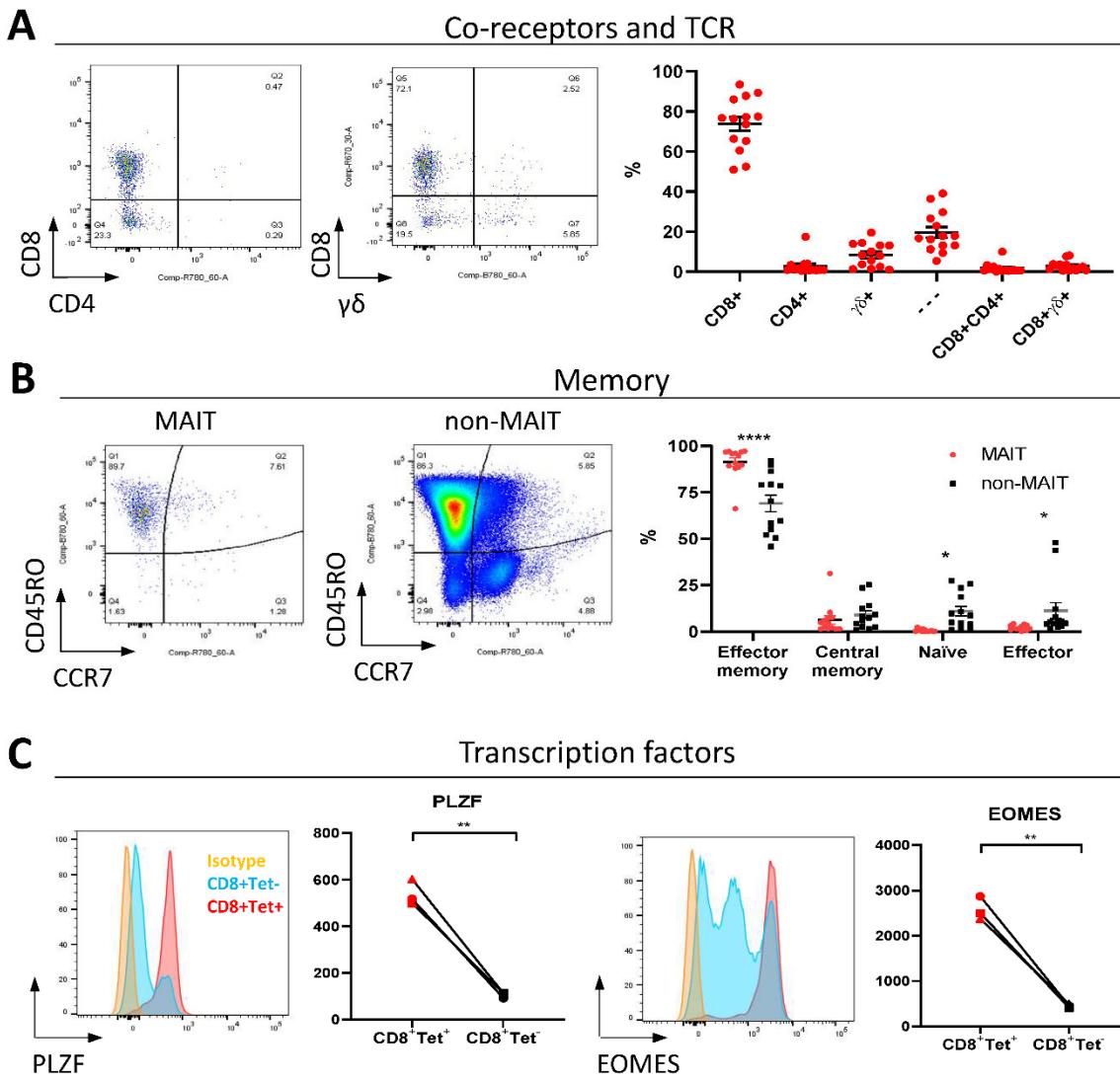
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726 11 Figures

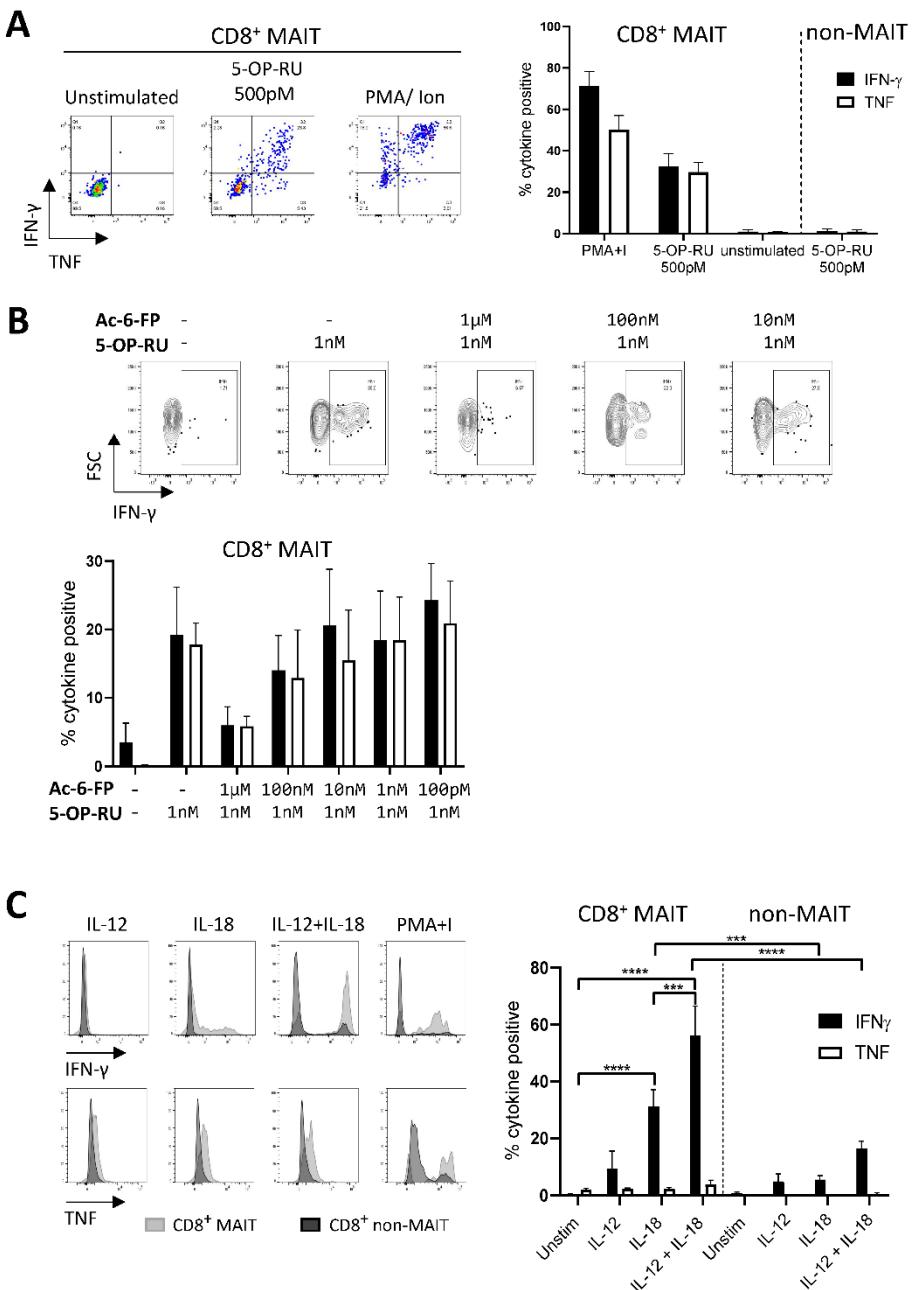
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728 **Figure 1: Identification of MAIT cells in cattle. (A)** Representative flow cytometry plots depicting  
729 human MR1-6-FP and MR1-5-OP-RU tetramer staining of bovine PBMC, gated on CD3<sup>+</sup> cells. **(B)**  
730 Fraction of human MR1-5-OP-RU (mean 0.59, range 0.18-1.72, n = 17) and MR1-6-FP (mean 0.06,  
731 range 0.01-0.12, n = 14) tetramer<sup>+</sup> cells within MHC-II<sup>+</sup> CD3<sup>+</sup> PBMCs from cows of various ages. **(C)**  
732 Representative images and summarized data (mean  $\pm$  SEM, n = 5) of IFN- $\gamma$  secreting cells following  
733 stimulation of PBMC with 5-OP-RU and Concanavalin A (ConA) as a control. Responses are medium  
734 control subtracted.

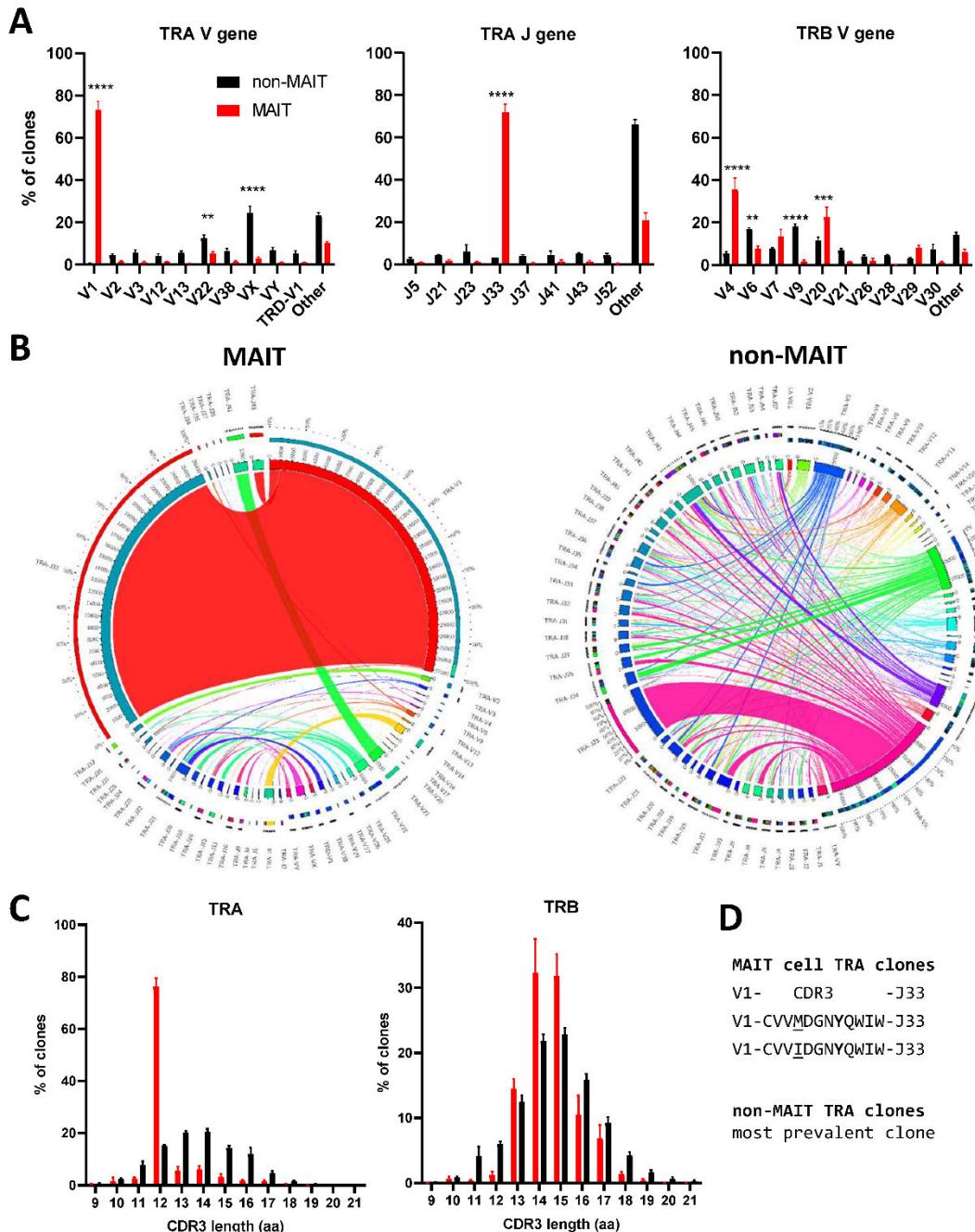


735 **Figure 2. Phenotypic characterisation of bovine MAIT cells in peripheral blood. (A)** Frequencies  
736 of co-receptor and  $\delta$ -TCR expression of  $CD3^+$ MAIT cells ( $CD3^+ MR1-5-OP-RU$  tetramer $^+$ ) in PBMC.  
737 Representative flow cytometry dot plots and summarized data (mean  $\pm$  SEM, n = 14).  $CD4^-CD8^- \gamma \delta^- = -$   
738 ---. **(B)** Memory phenotyping of peripheral blood MAIT and non-MAIT ( $CD3^+ MR1-5-OP-RU$   
739 tetramer $^+$ ) T cells based on  $CD45RO$  and  $CCR7$  expression. Effector memory ( $CD45RO^+ CCR7^-$ ),  
740 central memory ( $CD45RO^+ CCR7^+$ ), naïve ( $CD45RO^- CCR7^+$ ), effector ( $CD45RO^- CCR7^-$ ) phenotype.  
741 Data was analyzed using two-way ANOVA with T cell population as repeated measures, followed by  
742 Sidak's multiple comparisons post-hoc test comparing memory phenotype between populations.  
743 Representative flow cytometry plots and summarized data (mean  $\pm$  SEM, n = 13) are shown. **(C)**  
744 Expression of the transcription factors PLZF and EOMES in  $CD8^+$   $MR1-5-OP-RU$  tetramer $^+$  MAIT  
745 cells ( $CD8^+ Tet^+$ ) and non-MAIT T cells ( $CD8^+ Tet^-$ ) from PBMC; representative flow cytometry  
746 histograms and summarized data of mean fluorescence intensities (n = 3) are displayed. Paired T test  
747 within animal.  
748



749 **Figure 3. Antigen and cytokine stimulation of cattle MAIT cells.** (A) Representative flow cytometry plots  
750 and summarized data of IFN- $\gamma$  and TNF expression (mean  $\pm$  SEM, n = 3) of live CD8<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup>  
751 (MAIT) and CD8<sup>+</sup> tetramer<sup>-</sup> (non-MAIT) cells incubated with 5-OP-RU for 7 h at a concentration of 500 pM.  
752 Unstimulated cells and phorbol myristate acetate & ionomycin (PMA+I) stimulated controls are also shown. (B)  
753 Representative flow cytometry plots and summarized data of cattle PBMC incubated with Ac-6-FP prior to the  
754 addition of 1 nM 5-OP-RU (mean  $\pm$  SEM, n = 3). Lymphocytes were gated for FSC&SSC, singlets, live and  
755 CD8 expression followed by tetramer<sup>+</sup> / IFN- $\gamma$ <sup>+</sup> gating. The fraction of IFN- $\gamma$  and TNF positive cells within  
756 MAIT cells (CD8<sup>+</sup> tetramer<sup>+</sup> or IFN- $\gamma$ <sup>+</sup>) was determined. (C) PBMC were stimulated for 18 hours with IL-12,  
757 IL-18 or a combination of both (all at 50 ng/ml). Representative flow cytometry histogram plots show IFN- $\gamma$   
758 and TNF expression within CD8<sup>+</sup> MAIT cells overlaid with CD8<sup>+</sup> non-MAIT cells. Summarized data of the  
759 fraction of cytokine positive CD8<sup>+</sup> MAIT and non-MAIT cells are shown (mean  $\pm$  SEM, n = 4). Data were  
760 analyzed using two-way ANOVA with repeated measures within animal, followed by Sidak's multiple  
761 comparisons post-hoc test comparing the mean of each cytokine stimulation within and between CD8<sup>+</sup> MAIT  
762 cells and CD8<sup>+</sup> non-MAIT cells.

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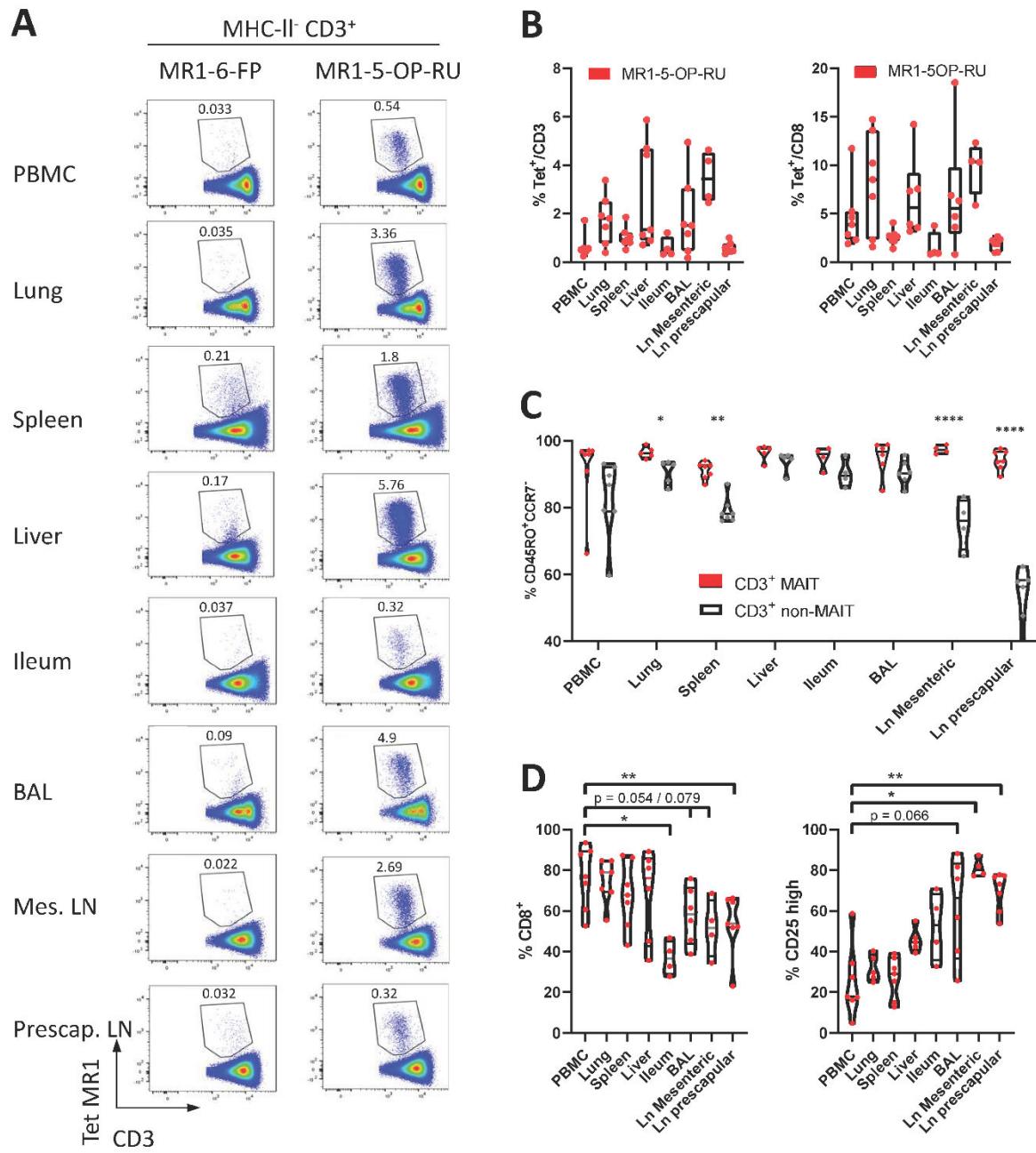
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**Figure 4. Conserved TCR alpha chain and limited beta chain diversity in bovine MAIT cells.** (A) Bar graphs showing TCR alpha (TRA) V and J and TCR beta (TRB) V gene usage in MAIT ( $CD8^+ MR1-5-OP-RU$  tetramer $^+$ ) and non-MAIT ( $CD8^+ MR1-5-OP-RU$  tetramer $^-$ ) T cells. Bars represent the percentage of total TRA or TRB sequences obtained from high throughput TCR sequencing of populations sorted from PBMC (mean  $\pm$  SEM,  $n = 4$ , number of sequences in Supplementary table 1). Data were analyzed using two-way ANOVA with repeated measures within animal, followed by Sidak's multiple comparisons post-hoc test comparing gene usage between MAIT and non-MAIT T cells. (B) Circos plots showing TRAV-J combinatorial diversity within MAIT and non-MAIT T cells. The inner circle shows the number of sequence reads per V and J gene and TRAV/J combinations are indicated by proportional bands linking the genes using the colour of the V gene. The outer ring shows the frequency of pairing for each TCR gene segment to the reciprocal gene segment coloured according to the paired gene. Representative plots from a single individual. (C) Distribution of TRA and TRB CDR3 amino acid (aa) length. (D) Alignment of the TCR alpha CDR3 region of the dominant MAIT cell clones.

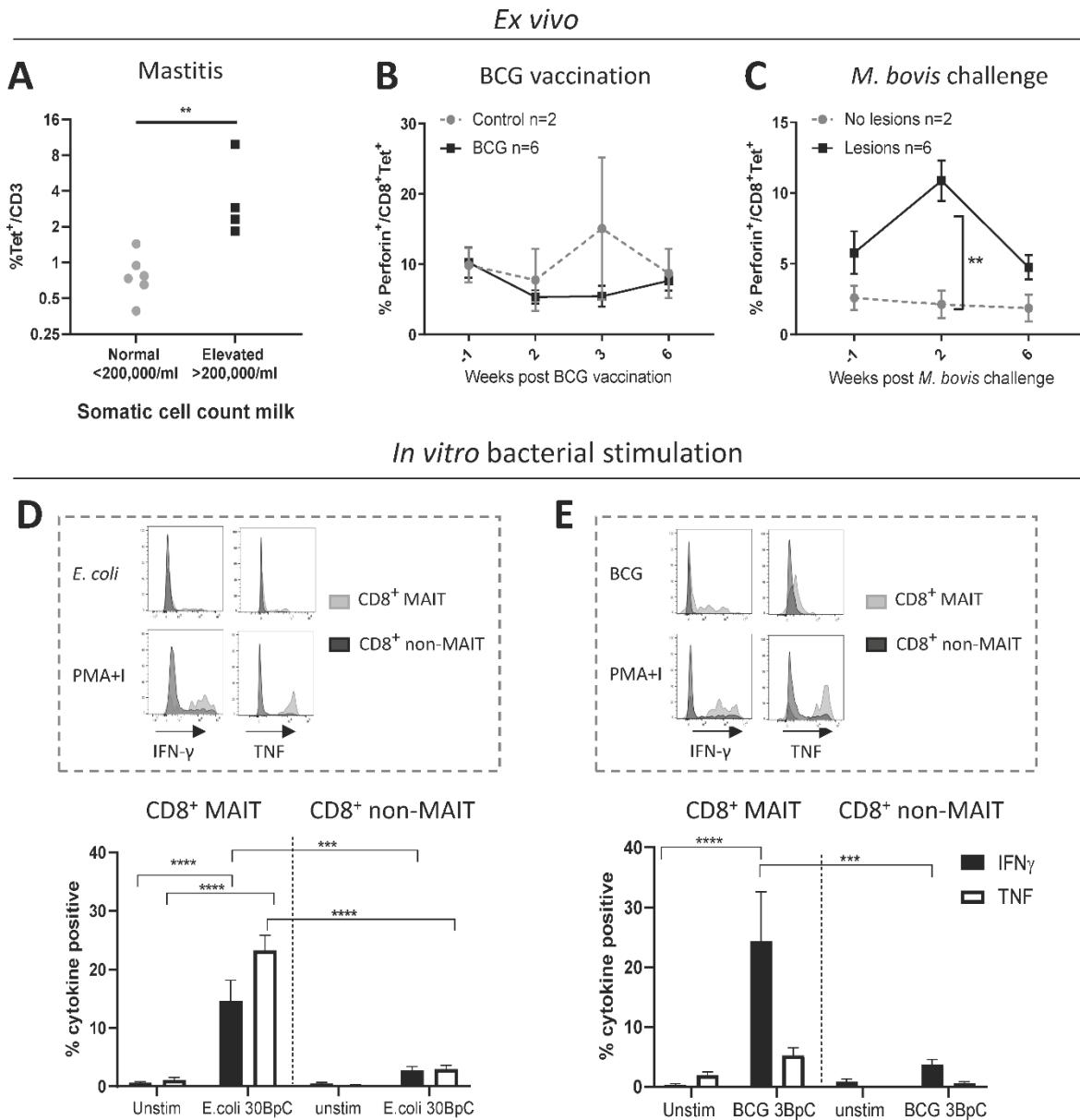


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**Figure 5. Comparisons of the frequencies and phenotype of cattle MAIT cells in different tissues.**

779 (A) Representative flow cytometry plots of MR1-5-OP-RU tetramer gating within MHC-II<sup>+</sup> CD3<sup>+</sup> cells  
780 in different tissues. (B) Frequencies of CD3<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup> MAIT cells and CD8<sup>+</sup> MR1-  
781 5-OP-RU tetramer<sup>+</sup> MAIT cells in different tissues from cattle. Number of animals per tissue are given  
782 in supplementary table 2. (C) Frequency of effector memory (CD45RO<sup>+</sup>CCR7<sup>+</sup>) MAIT (CD3<sup>+</sup>  
783 tetramer<sup>+</sup>) and non-MAIT T cells (CD3<sup>+</sup> tetramer<sup>-</sup>). Mixed effects model with matching across tissue  
784 and cell population, followed by Sidak's multiple comparisons post-hoc test comparing differences  
785 between MAIT and non-MAIT cells within tissues. (D) Frequency of CD8<sup>+</sup> or CD25 high MAIT cells  
786 (CD3<sup>+</sup> MR1-5-OP-RU<sup>+</sup>). Mixed effects model with matching across tissue, followed by Dunnett's  
787 multiple comparisons post-hoc test comparing differences between PBMC and other tissues.  
788



789 **Figure 6. Cattle MAIT cells respond to bacterial infections *in vivo* and *in vitro*.** (A) The percentage of MAIT  
790 cells (MR1-5-OP-RU tetramer<sup>+</sup>) within the total T cell population (CD3<sup>+</sup>) in milk, grouped according to normal  
791 (<200,000 cells/ml) versus elevated milk somatic cell count (SCC) (>200,000 cells/ml, indicating a mastitis).  
792 Groups were compared using a Mann–Whitney U test. (B) Six calves were vaccinated with  $4.6 \times 10^6$  CFU BCG  
793 Danish SSI 1331 subcutaneously and activation of MAIT cells was measured longitudinally as the percentage  
794 of perforin<sup>+</sup> CD8<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup> MAIT cells in PBMC. (C) MAIT cell activation was measured  
795 longitudinally, as in B, following *Mycobacterium bovis* challenge. Calves (n = 8) were challenged  
796 endobronchially with  $10^4$  CFU *M. bovis* AF2122/97. Animals were grouped based on the presence of  
797 tuberculosis associated lesions in lungs and lymph nodes at post-mortem examination 11 weeks post-challenge.  
798 (B,C) Data were analyzed using two-way ANOVA with time as repeated measures, followed by Sidak's multiple  
799 comparisons post-hoc test comparing differences between groups at each time point. Mean  $\pm$  SEM is indicated.  
800 (D and E) Cattle PBMC were stimulated for 7 hours with 30 *E. coli* BpC (D) or for 18 hours  
801 with 3 *M. bovis* BpC (E). Representative flow cytometry histograms and summarized data of IFN- $\gamma$  and  
802 TNF expression in live CD8<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup> MAIT and CD8<sup>+</sup> MR1-5-OP-RU tetramer<sup>-</sup> non-MAIT  
803 cells are shown (mean  $\pm$  SEM, n = 4). Data were analysed using two-way ANOVA with repeated measures  
804 within animal, followed by Sidak's multiple comparisons post-hoc test comparing IFN $\gamma$  and TNF expression of  
805 stimulated CD8<sup>+</sup> MAIT cells to unstimulated MAIT cells and to non-MAIT cells.

## Supplementary Material

### 806 12 Supplementary tables

807 **Supplementary table 1.** Number of paired sequence reads after quality control and TCR database  
808 alignment for TCR alpha (TRA) and beta chain (TRB) sequencing of MAIT (CD8<sup>+</sup> MR1-5-OP-RU  
809 tetramer<sup>+</sup>) and non-MAIT (CD8<sup>+</sup> MR1-5-OP-RU tetramer<sup>-</sup>) T cells sorted from PBMC (n = 4).

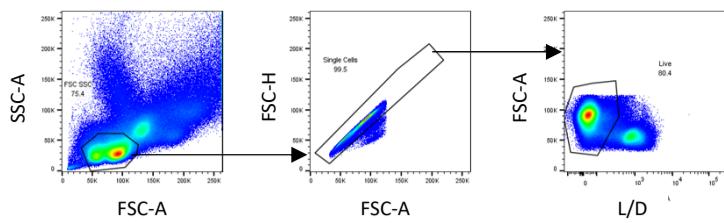
	TRA	TRB	
	non-MAIT	MAIT	non-MAIT
	MAIT		MAIT
<b>Cow 1</b>	97999	68947	80861
<b>Cow 2</b>	95250	34336	33350
<b>Cow 3</b>	46090	31828	48989
<b>Cow 4</b>	68335	46797	126600
			89044

810  
811 **Supplementary table 2.** Number of animals per tissue and staining panel for phenotypic  
812 characterisation of MAIT cells (CD8<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup>) in tissues. Ln Mes. – Mesenteric  
813 lymph node. Ln Prescap. – Prescapular lymph node.

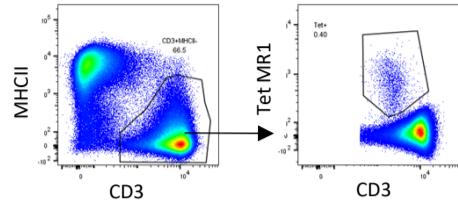
	PBMC	Lung	Spleen	Liver	Ileum	BAL	Ln Mes.	Ln Prescap.
CD3 <sup>+</sup> Tet <sup>+</sup>	7	7	7	7	4	7	4	7
CD8 <sup>+</sup> Tet <sup>+</sup>	7	7	7	6	4	6	4	7
% CD45RO <sup>+</sup> CCR7 <sup>-</sup>	7	5	7	5	4	6	4	7
% CD25 high	7	5	7	5	4	6	4	7
TCR & Co-receptor	7	7	7	6	4	6	4	7

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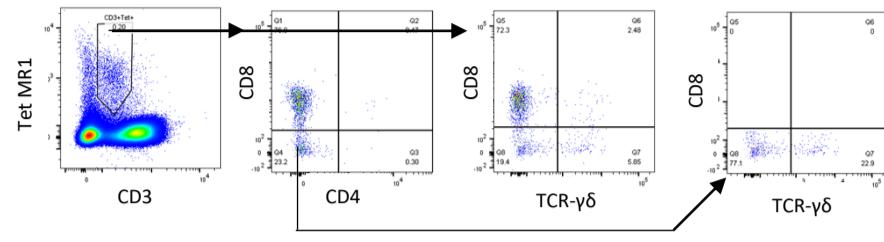
# General



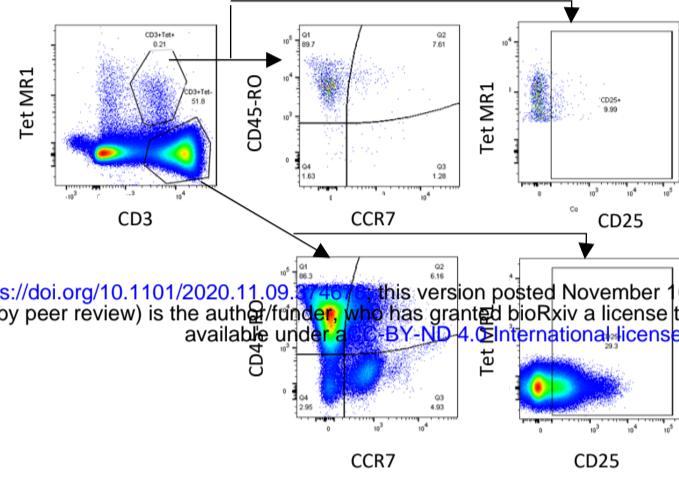
## Enumeration



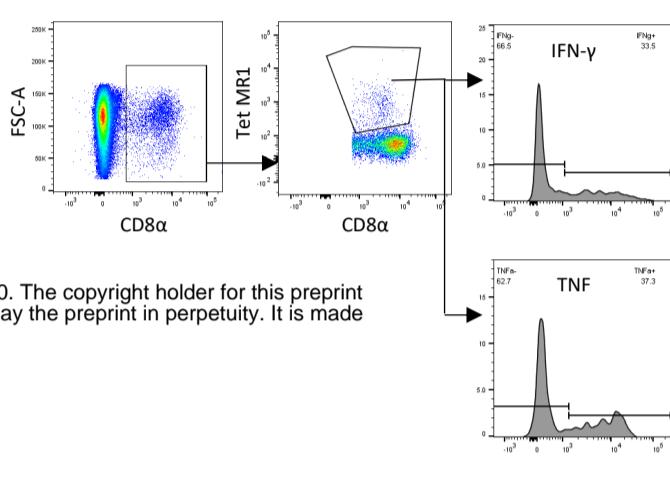
## Phenotype



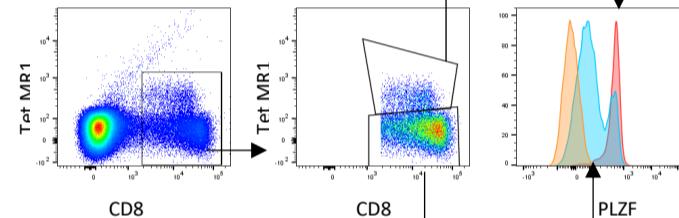
## Memory Panel / IL-2 receptor



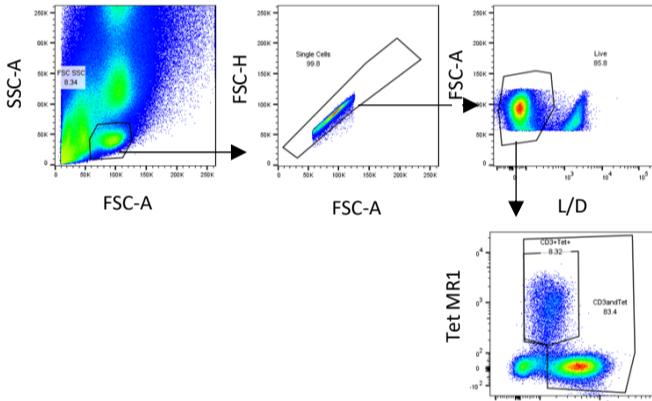
## Activation



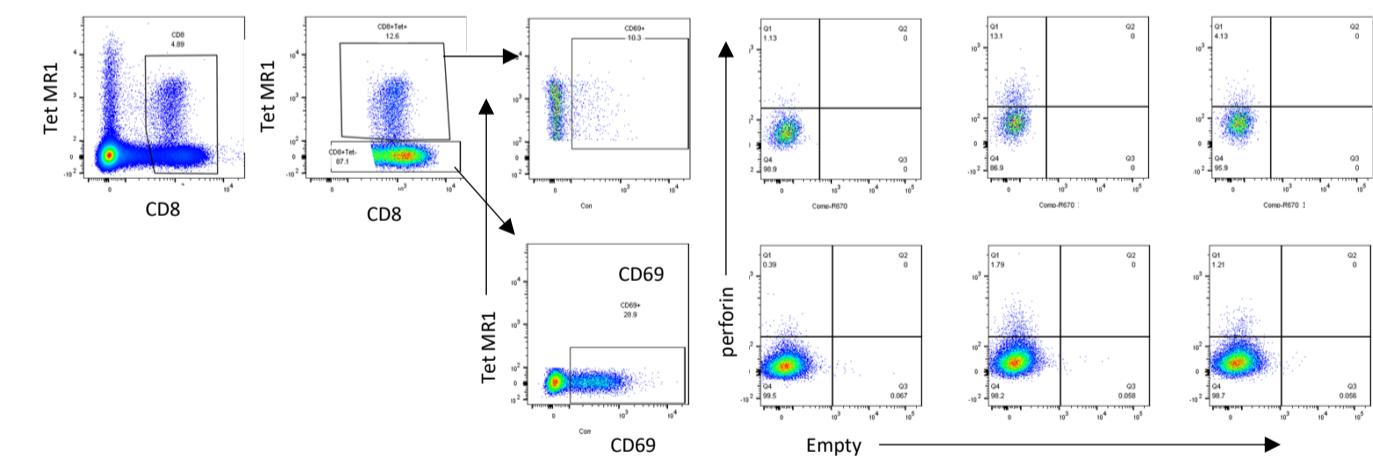
## Transcription factor



## Milk



## CD69 and Perforin



Wk-1

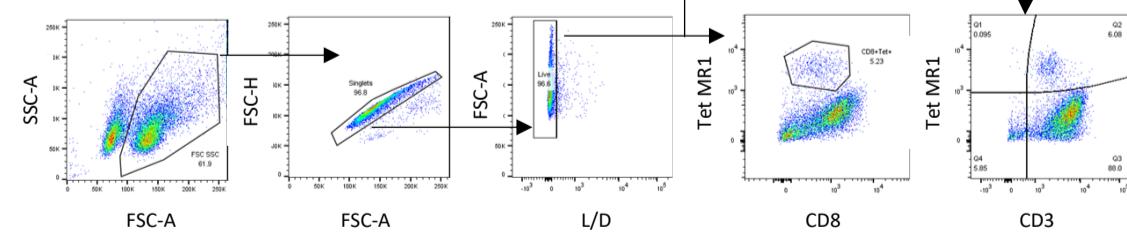
Wk2

Wk6

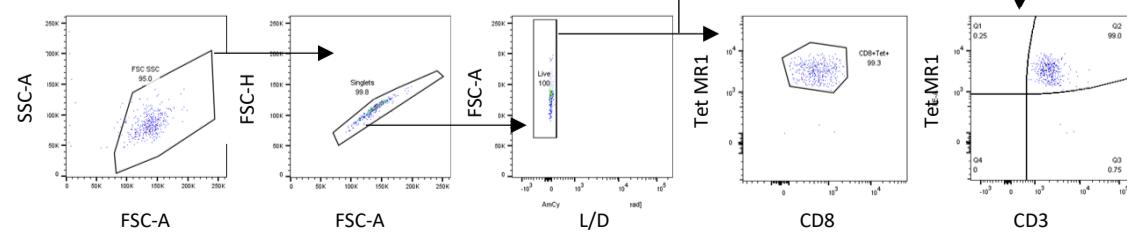
Empty

## Sorting

### Post ILA51 MACS sort

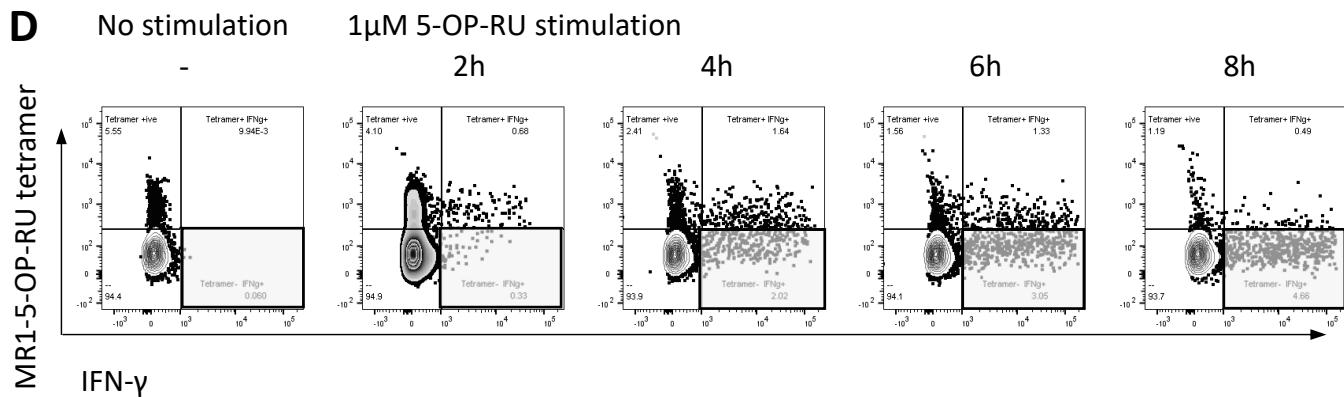
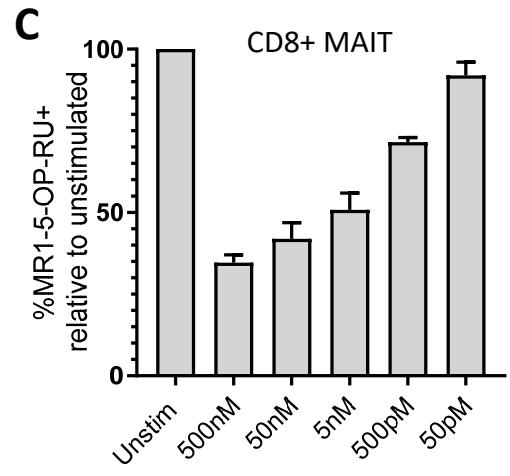
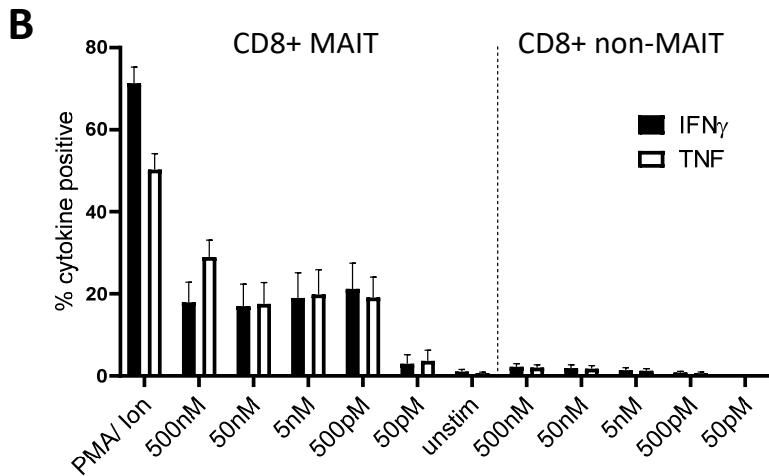
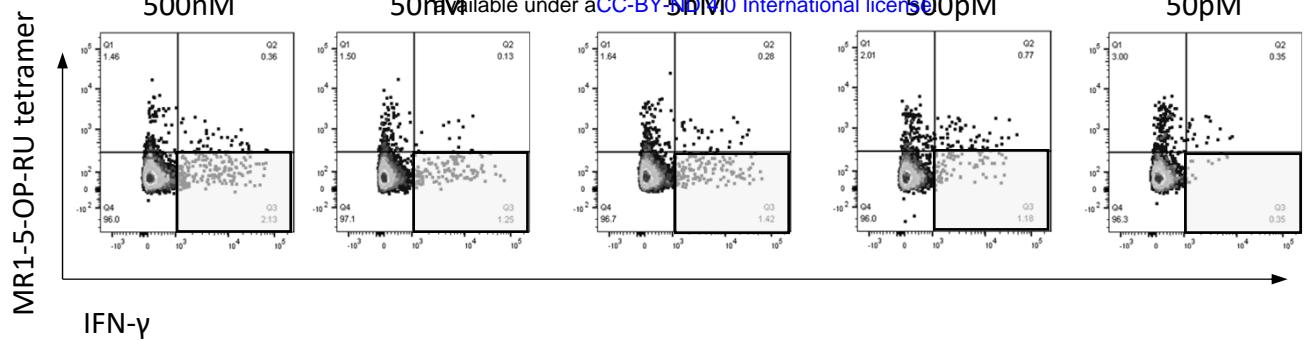


### Post MR1-5-OP-RU tetramer+ ILA105+ flow cytometry sort



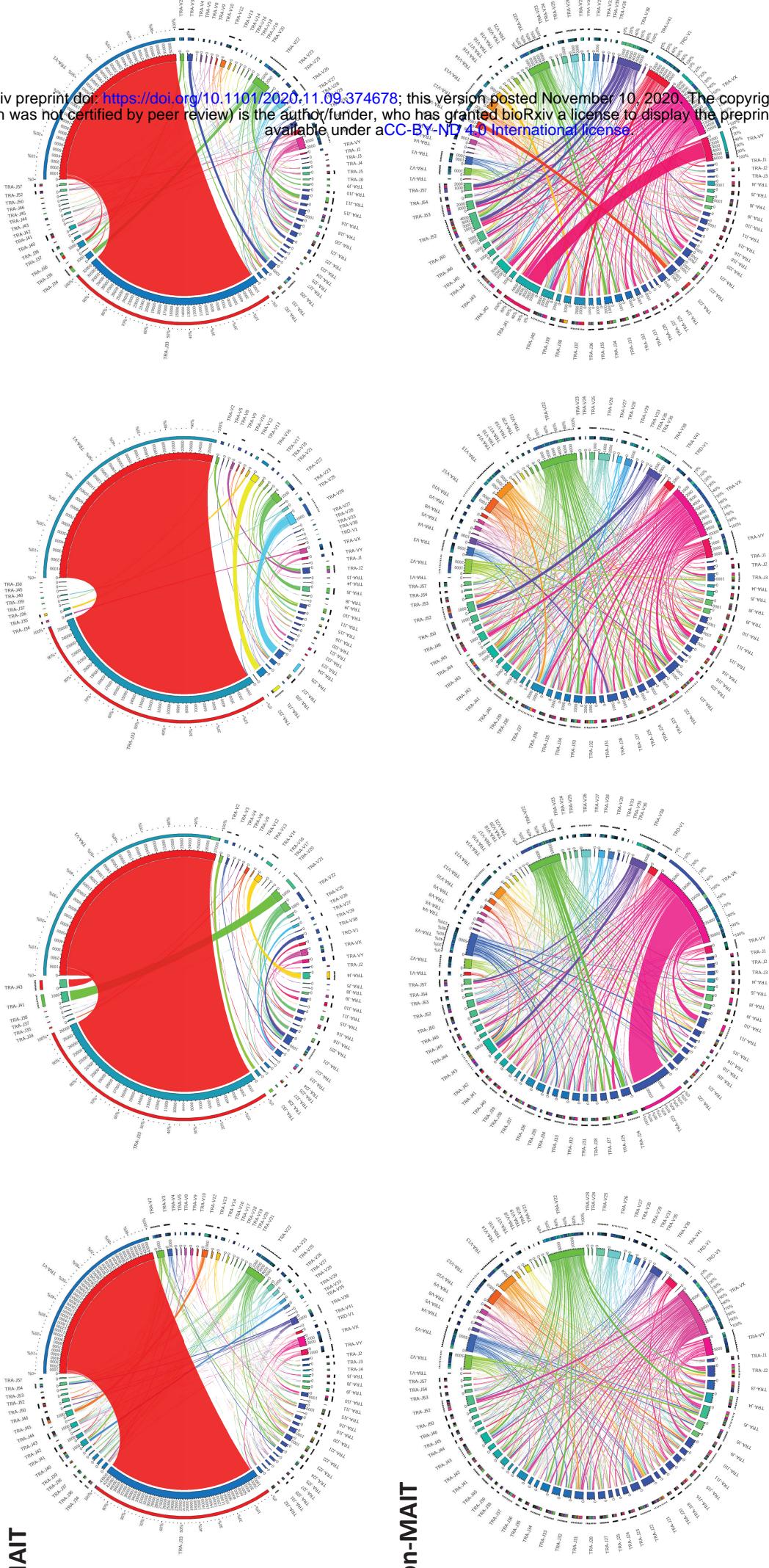
**Supplementary figure 1. Flow cytometry gating strategy.** The general gating strategy was used in all experiments to identify live singlet cells, followed by the specific gating strategies as indicated for each assay / experiment.

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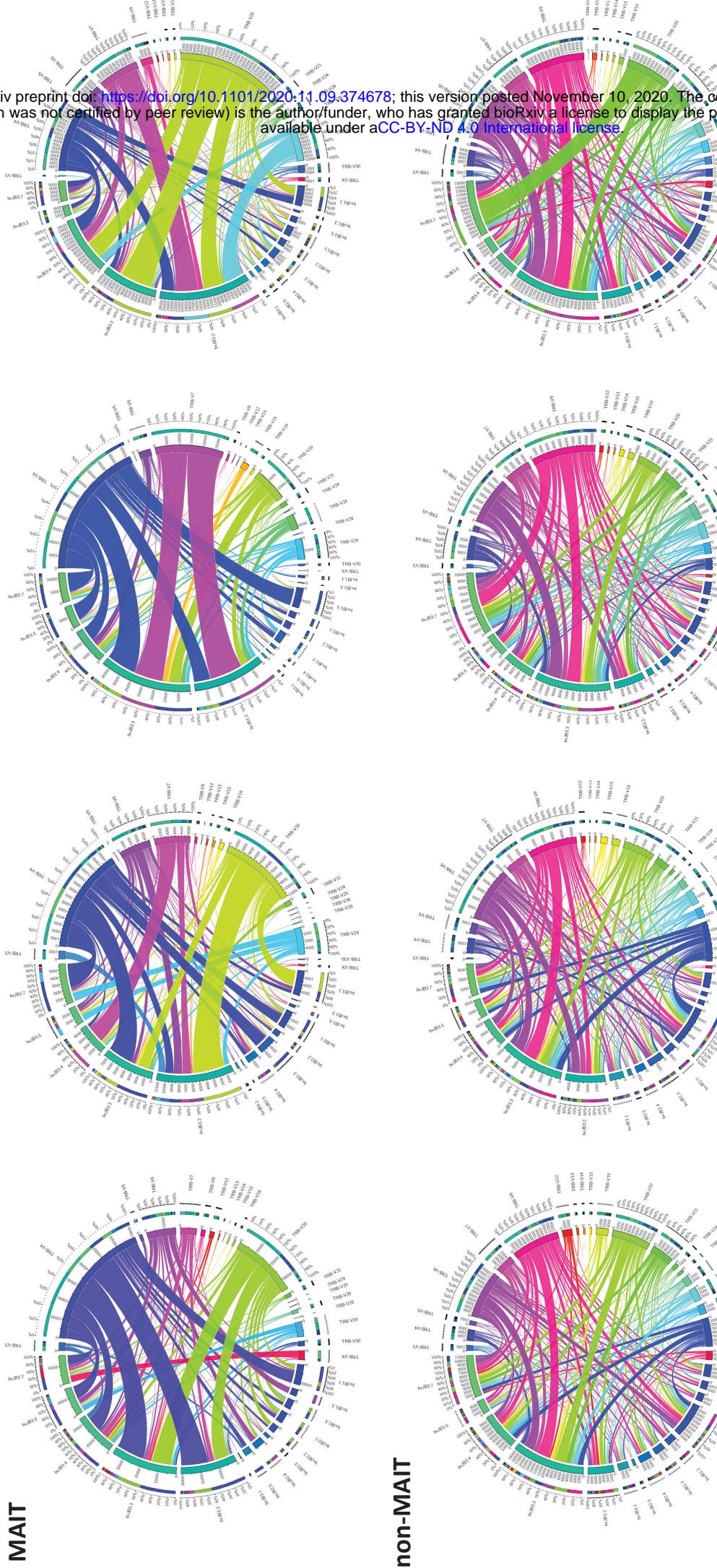


**Supplementary figure 2. TCR downregulation upon activation of MAIT cells.** (A) PBMC were stimulated with various concentrations of 5-OP-RU (50 pM – 500 nM) for 7 hours. Lymphocytes were gated on FSC&SSC, singlets, live and CD8 expression. Flow cytometry plots from a representative animal show MR1-5-OP-RU tetramer vs. IFN- $\gamma$  staining. Grey highlight boxes indicate IFN- $\gamma$  $^+$  T cells which are not detected with MR1-5-OP-RU tetramer. (B) Cattle PBMC were stimulated for 7 hours with 5-OP-RU (50 pM – 500 nM), 1 $\mu$ M Ac-6-FP, medium control or PMA/Ionomycin (PMA/Ion). The fractions of IFN $\gamma$  $^+$  or TNF $^+$  CD8 $^+$ MR1-5-OP-RU tetramer $^+$  MAIT cells and, for comparison, of CD8 $^+$ tetramer $^-$  non-MAIT cells are depicted (data combined from two experiments, mean  $\pm$  SEM, n=6 for each concentration other than 50pM where n = 3). (C) The fraction of MR1-5-OP-RU tetramer $^+$  MAIT cells following stimulation with 5-OP-RU relative to the fraction of MR1-5-OP-RU tetramer $^+$  MAIT cells in unstimulated PBMC from the same animal (mean  $\pm$  SEM, n=6). (D) Bovine PBMC were stimulated with 1  $\mu$ M 5-OP-RU and 50 ng/ml IL-12 and IL-18 over a time course of 2 to 8 hours. Flow cytometry gating as in A. Grey highlight boxes indicate IFN- $\gamma$  $^+$  T cells not detected by MR1-5-OP-RU tetramer.

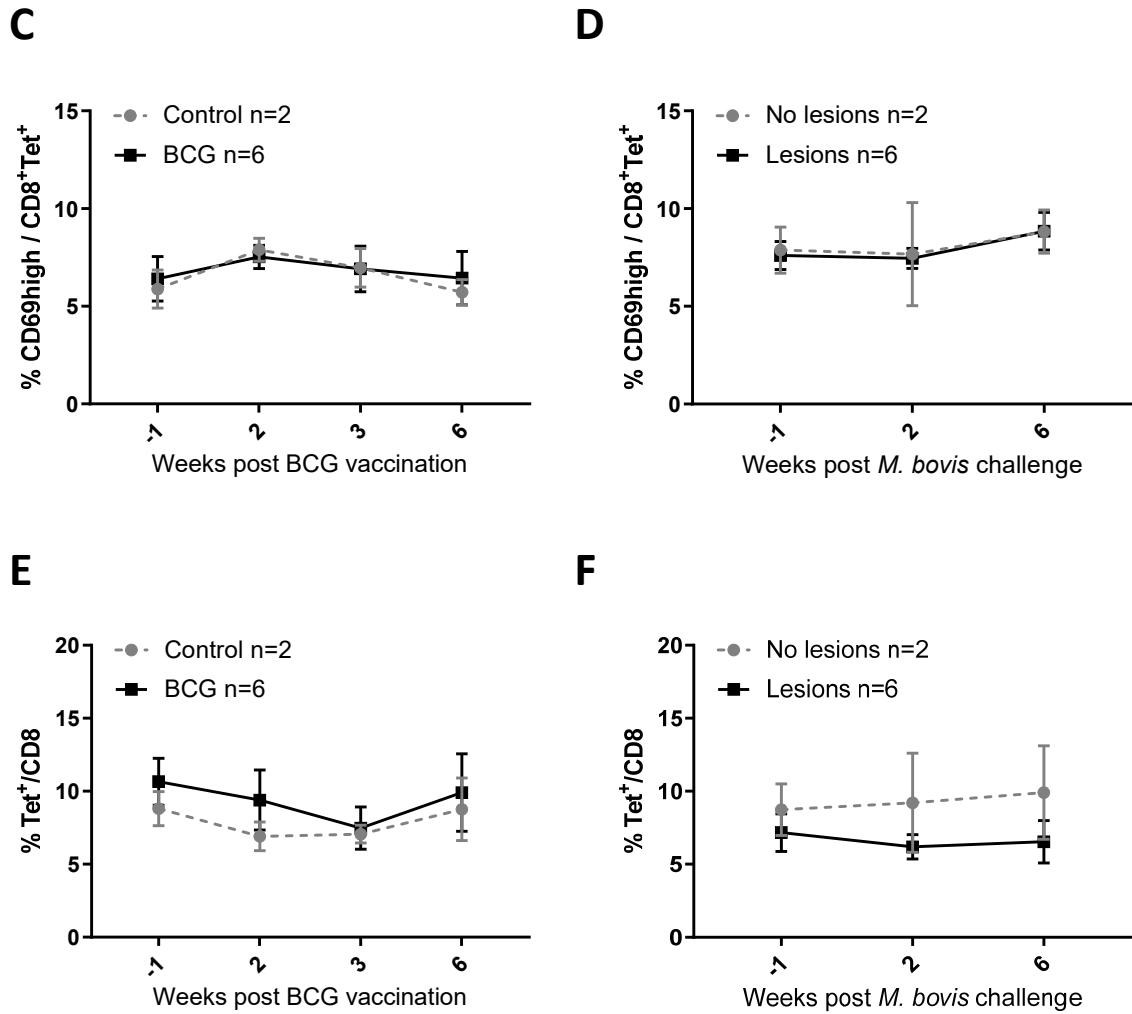
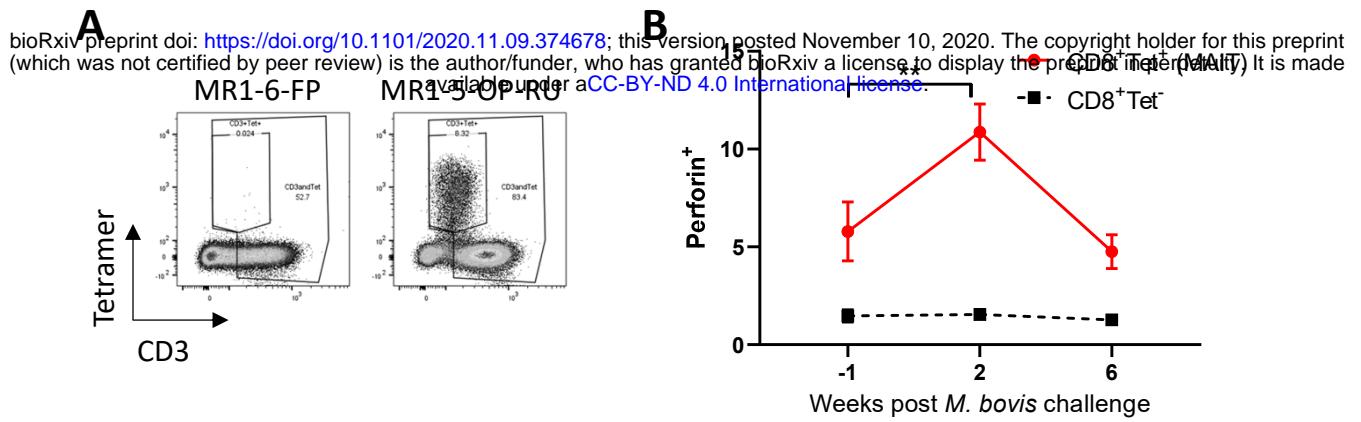
## MAIT



**Supplementary figure 3. Circos plots showing TRAV-TRAJ combinatorial diversity within MAIT (CD8<sup>+</sup> human MR1-5-OP-RU tetramer<sup>+</sup>) and non-MAIT (CD8<sup>+</sup> human MR1-5-OP-RU tetramer<sup>-</sup>) T cells.** The inner circle shows the number of sequence reads per V and J gene. TRAV/J combinations are indicated by proportional bands linking the genes using the colour of the V gene. The outer ring shows the frequency of pairing for each TCR gene segment to the reciprocal gene segment coloured according to the paired gene.



**Supplementary figure 4. Circos plots showing TRBV-TRBJ combinatorial diversity within MAIT (CD8<sup>+</sup> human MR1-5-OP-RU tetramer+) and non-MAIT (CD8<sup>+</sup> human MR1-5-OP-RU tetramer) T cells.** The inner circle shows the number of sequence reads per V and J gene. TRBV/J combinations are indicated by proportional bands linking the genes using the colour of the V gene. The outer ring shows the frequency of pairing for each TCR gene segment coloured according to the paired gene.



**Supplementary figure 5. Bovine MAIT cells respond to infections *in vivo*.** (A) Representative flow cytometry plots of MAIT cell staining in milk. Milk cells were stained with MR1-6-FP or MR1-5-OP-RU tetramer and were gated on FSC&SSC, singlets and live cells. (B-F) Characterization of MAIT cells in cattle PBMC following BCG vaccination (C, E) and *M. bovis* challenge (B, D, F) of calves as in figure 6. (B) Comparison of perforin expression over time by MAIT cells (CD8<sup>+</sup> MR1-5-OP-RU tetramer<sup>+</sup>) and CD8 T cells (CD8<sup>+</sup> MR1-5-OP-RU tetramer<sup>-</sup>) within animals with tuberculosis associated lesions in lungs and lymph nodes at post-mortem examination 11 weeks post *M. bovis* challenge (n = 6). (C, D) Percentage of MAIT cells (CD8<sup>+</sup> tetramer<sup>+</sup>) with high CD69 expression. (E, F) MAIT cell frequencies within CD8 positive cells in peripheral blood over time. Data were analyzed using two-way ANOVA with time as repeated measures, followed by Sidak's multiple comparisons post-hoc test comparing changes in perforin expression over time within MAIT cells and CD8 T cells for B and comparing differences between groups at each time point for C-F. Mean  $\pm$  SEM is indicated in all graphs.