Mucosal-associated Invariant T cell activation and accumulation after *in vivo* infection depends on microbial riboflavin synthesis and co-stimulatory signals

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Abstract

Despite recent breakthroughs in identifying MAIT cell antigens, the precise requirements for *in vivo* MAIT cell responses to infection remain unclear. Using MR1 tetramers, the MAIT cell response was investigated in a model of bacterial lung infection employing riboflavin gene-competent and deficient bacteria. MAIT cells were rapidly enriched in the lungs of C57BL/6 mice infected with *Salmonella* Typhimurium, comprising up to 50% of αβT cells after one week. MAIT cell accumulation was MR1-dependent, required antigen derived from the microbial riboflavin synthesis pathway and did not occur in response to synthetic antigen, unless accompanied by a TLR agonist or by co-infection with riboflavin pathway-deficient *S.* Typhimurium. MAIT cell accumulation involved proliferation in the lungs and regional LN, and was associated with their long-term retention in the lungs. Lung MAIT cells from infected mice were mainly $CD4^{-}CD8^{-}$ double negative or $CD8^{+}$, displayed an activated/memory phenotype, and most expressed the transcription factor RORγt. T-bet expression increased following infection. The majority produced IL-17 while smaller subsets produced IFNγ or TNF, detected directly *ex vivo*. Thus, the activation and expansion of MAIT cells coupled with their pro-inflammatory cytokine production, occurred in response to antigens derived from microbial riboflavin synthesis and was augmented by co-stimulatory signals.

INTRODUCTION

Mucosal-Associated Invariant T (MAIT) cells are emerging as an important class of T cell with features of both innate and adaptive immunity. The function of MAIT cells is still being elucidated, but a key role is suggested by their conservation across many mammalian species and their high abundance in human blood and mucosal tissues. MAIT cells recognize conserved microbial metabolites generated during riboflavin synthesis, captured by the monomorphic MHC related protein 1 (MR1)[1-6]. MAIT cells express a conserved αβ T cell antigen receptor (TCR), consisting of a near-invariant TCRα-chain coupled with a restricted set of β-chains that are relatively conserved in

humans and mice[1-6]. In mice, MAIT TCRs use TRAV1-TRAJ33 (Vα19-Jα33) generally assembled with TRBV19 (Vβ6) or TRBV13 (Vβ8)[7-9]. In humans most MAIT cells express TRAV1-2-TRAJ33 (Vα7.2-Jα33), but TRAV1-2-TRAJ12 or TRAV1-2-TRAJ20 are also used[8]. These TCRα-chains predominantly form receptors with TRBV20 (Vβ2) or TRBV6 (Vβ13) β-chains[9], although many other TRBV genes are also used at lower frequency[8].

To date, characterisation of MAIT cells in models of infectious diseases has largely depended on analysis of the DN T cell population and PCR detection of the Vα19Jα33 invariant TCR-α chain as there is no Vα19-specific mAb, or the use of Vα19Jα33 TCR transgenic.Cα$^{-/-}$ mice[5,10-13]. While Vα19 is characteristic of MAIT cells, it is also expressed by non-MAIT T cells. In addition, most conventionally-housed inbred mice, with the exception of the wild-derived inbred CAST/EiJ strain [17], have very low numbers of blood and lymphoid MAIT cells compared with humans[9], and while TCR transgenic models have been used to help circumvent this problem[18,19], the relationship between MAIT cells generated in TCR transgenic versus wild-type mice is unclear.

We recently identified by-products of microbial riboflavin synthesis as a major natural source of antigen (Ag) driving MAIT cell activation *in vitro*[2,4,20]. It is unclear whether this pathway is the exclusive source of antigen *in vivo*, as Vβ chain and complementarity determining region (CDR)3β diversity in MAIT TCRs and differential responses to diverse pathogens might reflect diversity in MAIT antigens[21-23]. Indeed, a number of MAIT cell antigens were identified, including 5-(2-oxoepropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe) and 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH), which vary considerably in potency, as well as non-stimulatory MR1 ligands including 6-formyl-pterin (6-FP), a natural breakdown product of folic acid

[2,4]. 5-OP-RU and 5-OE-RU, the most potent MAIT cell antigens, are created from the riboflavin precursor 5-amino-6-D-ribitylaminouracil (5-A-RU) by non-enzymatic condensation with small ubiquitous metabolites methylglyoxal and glyoxal, respectively[2]. This finding has enabled the production of highly specific MR1-Ag-tetramers that stain all MAIT cells in mice and humans[2]. Here we have examined the behaviour, functionality and phenotype of mouse MAIT cells, detected using MR1-5-OP-RU tetramers, in a bacterial lung infection model in C57BL/6 mice. Our data reveal that MAIT cell activation and accumulation *in vivo* was dependent on the presence of a functional riboflavin synthesis pathway in the microbial pathogen and expression of MR1 in the host mice. The cell-surface phenotype and functional properties of MAIT cells indicate a responsiveness of MAIT cells to infection. These studies have substantially advanced our understanding of the functional capacity of MAIT cells *in vivo*.

RESULTS

MAIT cells accumulate in the lungs of mice following infection with *Salmonella* Typhimurium

Using *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), we previously showed that the ability to stimulate MAIT cells *in vitro* depends on the presence of an intact microbial riboflavin pathway, specifically involving genes necessary for the production of riboflavin precursor compounds[4] including 5-A-RU[2]. We showed how 5-A-RU recombines with small metabolites to form MAIT-activating ligands, including the most potent identified to date, 5-OP-RU[2]. Tetramers of human or mouse MR1 loaded with 5-OP-RU specifically detect MAIT cells in the respective species[2,8,24,25]. Mouse MR1-5-OP-RU tetramers were used here to identify MAIT cells (defined as TCRβ^+MR1-5-OP-RU tetramer$^+$ cells) in single cell suspensions derived from the lungs, reproductive tract, liver, spleen, thymus, blood and mesenteric lymph nodes of uninfected C57BL/6 mice. MAIT cells represented ~2% of αβ-T cells in the reproductive tract, and about 0.5-2% of αβ-T cells in lung, with lower proportions in blood and other organs **(Fig. 1a)**, consistent with recent findings[24]. In

contrast, we could not detect MAIT cells from C57BL/6.MR1$^{-/-}$ mice **(Fig. 1a),** confirming earlier findings that MR1 is vital for MAIT cell development[6,19].

We next examined the impact of lung infection on MAIT cells in a model involving inoculation of mice with live-attenuated vaccine strain *S.* Typhimurium BRD509[26,27]. While *S.* Typhimurium is not a natural pathogen in the lung, it is known to activate MAIT cells *in vitro* and genetic manipulation of this bacterium now allows us to probe the Ag requirements for MAIT cell activation *in vivo*. Following intranasal infection with *S.* Typhimurium BRD509, there was a substantial enrichment of MAIT cells in the lungs of C57BL/6 mice such that they comprised 25-50% of all infiltrating lung αβ-T cells after one week **(Fig. 1b,c, Supplementary Fig. 1)**. MAIT cell enrichment was absent in C57BL/6.MR1$^{-/-}$ mice **(Fig. 1b,c)** and was blocked by treatment with MR1-specific mAbs, 26.5 or 8F2.F9[28,29] **(Fig. 1c, Supplementary Fig. 1).** Thus the MAIT cell accumulation and enrichment in this model was MR1-dependent. The accumulation of lung MAIT cells was also dependent on the size of the bacterial inoculum **(Fig. 1d)** and peaked at day 7 post infection (d7 p.i.) **(Fig. 1e)**.

In vivo enrichment of MAIT cells post-infection requires antigen derived from a bacterial riboflavin pathway

To confirm the requirement for microbial riboflavin metabolites in driving the *in vivo* MAIT cell response, we constructed a mutant strain of BRD509 in which *ribD* and *ribH* genes, which are co-located in the *Salmonella* genome, were deleted; BRD509Δ*ribDH*. While deficiency of the *ribH* gene does not impact on bacterial activation of MAIT cells[2] the *ribD* gene is essential for production of the 5-A-RU precursor to MAIT cell antigens 5-OP-RU and 5-OE-RU[2]. The mutant BRD509Δ*ribDH* bacteria were first tested *in vitro* and bacterial supernatants were found to be deficient in their ability to stimulate reporter cells expressing a MAIT TCR (Jurkat.MAIT)[4] in an MR1-dependent activation assay **(Fig. 2a)**. By comparison, BRD509 supernatant (wt) caused up-regulation of CD69 on

Jurkat.MAIT reporter cells **(Fig. 2a)**. Supernatant from the BRD509Δ*ribDH* bacteria reconstituted with *ribD* and *ribH* genes (BRD509Δ*ribDH*+*ribDH)* activated Jurkat.MAIT cells to wild-type levels **(Fig. 2a)**, confirming previous *in vitro* results obtained with mutants of another *Salmonella* strain[2].

In contrast to *S.* Typhimurium BRD509, infection with BRD509Δ*ribDH* bacteria did not evoke MAIT cell accumulation beyond the resident MAIT cell numbers in uninfected lungs **(Fig. 2b,c)**. This lack of MAIT cell accumulation was unlikely the result of the reduced growth rate of BRD509Δ*ribDH* since a 10-fold higher dose of BRD509Δ*ribDH* bacteria was used, resulting in equivalent bacterial counts at day 7 **(Supplementary Fig. 2)**, and non-MAIT αβ-T cells still accumulated **(Fig. 2b)**. Moreover, the non-responsiveness of MAIT cells to infection with BRD509Δ*ribDH Salmonella* could be rescued if the bacteria were co-administered intranasally with synthetic MAIT cell antigen 5-OP-RU delivered i.n. **(Fig. 2b,c)** or i.v. **(Supplementary Fig. 3)**. In contrast, co-administration of the BRD509 strain and the MR1-ligand 6-FP[4], which does not activate MAIT cells, did not cause MAIT cell accumulation in the lungs **(Fig. 2c, Supplementary Fig. 3)**, confirming the specificity of the response. Of note, 5-OP-RU alone caused no MAIT cell accumulation at any dose or time point tested, when delivered either intranasally or intravenously, suggesting that synthetic Ag alone is insufficient to induce the MAIT cell accumulation **(Fig. 2f, Supplementary Fig. 3)**. This could reflect a requirement for co-stimulation provided by the BRD509Δ*ribDH* bacteria or the unstable nature of free 5-OP-RU failing to produce sustained MAIT cell stimulation. To examine whether a co-stimulus could be furnished through TLRs, we inoculated mice with 5-OP-RU and TLR agonists S-[2,3-bis(palmitoyloxy)propyl] cysteine (Pam2Cys), CpG oligonucleotides or polyinosinic:polycytidylic acid (poly I:C). Pam2Cys is a synthetic mimic of the lipid component of TLR2 and TLR6 ligand macrophage activating lipopeptide-2 (MALP-2) and has been shown to be an agonist for TLR2[30,31]. Poly I:C is a TLR3 agonist similar to double stranded

RNA[32]. CpG oligonucleotides stimulate through TLR9[33]. Challenge with each TLR agonist by itself, or with the non-activating MR1 ligand 6-FP, led to a lesser (2-3-fold) enrichment for MAIT cells in the lungs suggesting co-stimuli alone can exert some influence on MAIT cells **(Fig. 2d)**. In addition, although intranasal challenge with antigen alone did not induce accumulation of lung MAIT cells, it did induce rapid up-regulation of CD69 on MAIT cells *in vivo* as early as 2h following inoculation **(Fig. 2e).** However, the intranasal co-administration of synthetic antigen 5-OP-RU and Pam2Cys, poly I:C or CpG led to a 15-25 fold enrichment of MAIT cells in the lung in the absence of any bacterial challenge **(Fig. 2d,f)**. At least for Pam2Cys plus 5-OP-RU, this response was sustained over time (not shown) and was largely blocked by administration of anti-MR1 mAb **(Supplementary Fig. 1b).** Thus, the failure of intranasal 5-OP-RU alone to drive MAIT cell enrichment was not necessarily the result of the lability of the antigen, suggesting that MAIT cell accumulation and enrichment requires stronger signals than those needed for activation. Indeed, repeated (x6) intranasal administration of 5-OP-RU alone over a seven-day period was still an insufficient stimulus to induce MAIT cell accumulation, despite sustained Ag exposure **(Fig. 2f).** Taken together, these findings demonstrate that *Rib*-defective (and thus MAIT cell antigen deficient) bacteria and TLR agonists are independently capable of providing a co-stimulus that helps drive MAIT cell accumulation and enrichment in response to 5-OP-RU antigen *in vivo* **(Fig. 2c,d)**.

Infection induces IL-17 production, an increase in T-bet expression and the long-lived retention of phenotypically effector/memory MAIT cells *in situ*

Upon activation *in vitro*, MAIT cells produce IFNγ, TNF and IL-17[4,8,13,34]. This pattern of cytokine expression was mirrored following intranasal inoculation with *S.* Typhimurium **(Fig. 3, Supplementary Fig. 5, 6)**. Most striking was the production of IL-17, which was detected in approximately 50% of lung MAIT cells directly *ex vivo* at d7 without further stimulation **(Fig. 3)**. Interestingly, MAIT cells still appeared 'primed' to rapidly secrete IL-17 long-term p.i. **(Fig. 3)**,

suggesting that MAIT cells acquire rapid responsiveness as they differentiate into their memory phase. In contrast, a higher proportion of non-MAIT αβ-T cells than MAIT cells produced IFNγ and TNF 7 days p.i. **(Fig. 3, Supplementary Fig. 4).**

The transcriptional factor promyelocytic leukemia zinc finger (PLZF) is a master regulator of innate-like T cell development, including Natural Killer T cells and some γδT cells[35]. Both human and mouse MAIT cells express PLZF[19,24]. Notably, we found that PLZF was expressed by nearly all lung MAIT cells both before and after infection, along with the orphan nuclear receptor, RORγt, which plays a key role in differentiation of Th17 cells[36] and is expressed by human and mouse MAIT cells[24,34] **(Fig. 4a, Supplementary Fig. 5)**. In addition, we examined the expression of the transcription factor T-bet, a key regulator of Th1 type immunity[37]. T-bet is reported to suppress IL-17 expression and promote Th1 development, whereas loss of T-bet expression can induce commitment to Th2 and loss of IFNγ expression[38-40]. The expression of RORγt declined slightly in MAIT cells as T-bet expression levels increased from d7-d142 p.i. Interestingly, the majority of cells at d142 co-expressed the two transcription factors **(Fig. 4b, Supplementary Fig. 5)**. The production of IL-17 by MAIT cells d7 p.i. *ex vivo* or following PMA stimulation, was observed in the presence of either T-bet or RORγt (or both) **(Fig. 4c, Supplementary Fig. 6)**. In contrast, at the same time point, a much smaller proportion of MAIT cells produced IFNγ after PMA stimulation and most of these expressed T-bet. This pattern was also true for IFNγ production by non-MAIT cells **(Fig. 4c)**. IL-17 and IFNγ appeared to be independently expressed by MAIT cells, as shown by co-staining **(Supplementary Fig. 7).**

Even before infection, CD44, a cell adhesion molecule highly expressed on memory T cells, was expressed by most MAIT cells. In contrast, a smaller proportion of non-MAIT αβ-T cells expressed CD44 pre- and post infection **(Fig. 5).** CD62L (L-selectin) is highly expressed on naïve T cells, and

is used to further distinguish central memory ($CD62L^+$) from effector memory ($CD62L^-$). The regulation of CD62L plays a pivotal role in controlling the traffic of conventional T lymphocytes through peripheral lymph nodes, with CD62L shed from the cell membrane following activation[41]. Not surprisingly CD62L remained low on lung MAIT cells at all three phases: homeostatic MAIT cells in the absence of infection, activated MAIT cells (during infection) and antigen experienced MAIT cells (>d30 p.i.) (**Fig. 5**). These observations are consistent with the effector/memory phenotype previously described for human MAIT cells[34.] However, the proportion of MAIT cells expressing the activation marker CD69 increased from ~20% pre-infection to 90% post-infection (**Fig. 5b**) consistent with our other results showing that further activation of MAIT cells occurred following infection. MAIT cells from the lungs of uninfected mice also showed CD25 expression (not shown).

To measure the contribution of recruitment from blood or other sites, we examined MAIT cell enrichment in lung and draining LN versus possible loss in other organs for 7 weeks p.i. with BRD509 **(Fig. 6a,b)**. The enrichment of MAIT cells as a proportion of all αβ-T cells was most evident in the lungs of infected mice (d7, 35-50% of αβ-T cells), but was only evident in the draining LN and spleen at wk7 **(Fig. 6a,b),** suggesting redistribution to these sites during the resolution phase of the infection (**Fig. 6c)**. No MAIT cell loss was observed in the spleen or draining LN, where the percentage of MAIT cells remained stable throughout the infection course and increased slightly during the late course **(Fig. 6a,b).**

Together, our data show a rapid MAIT cell response to bacterial lung infection, which was dependent on MR1 presentation of riboflavin biosynthesis-derived ligands. MAIT cell accumulation and proliferation, but not CD69 up-regulation, was dependent on second signals, which could be

furnished through TLR ligation or by bacteria. Cell surface markers and cytokine production were consistent with an effector/memory phenotype.

DISCUSSION

We have developed a model bacterial infection in mice to evaluate the importance of the riboflavin synthesis pathway in driving MAIT cell responses *in vivo*, and to define the phenotypic and functional characteristics of the responding MAIT cells. The generation of highly specific MR1-antigen tetramers[2,8] has allowed the precise Ag-dependent definition of MAIT cells, which are much less abundant in wild-type mice than humans[9], overcoming potential confusion engendered by using surrogate markers of MAIT cells or transgenic mice. Using MR1-Ag-tetramers, we show that MAIT cells are readily detectable, albeit in low numbers, in wild-type C57BL/6 mice, being most abundant in the reproductive tract, lung and liver. We exploited the capacity of *S.* Typhimurium, which we previously showed to potently activate MAIT cells *in vitro*[2,4] as a model to analyse MAIT cell responses and activation requirements *in vivo*, with the respiratory tract providing an accessible organ to assess MAIT activation. *S.* Typhimurium is a gastrointestinal pathogen of humans and a systemic pathogen of mice. Clearance of *S.* Typhimurium in mice is achieved through a number of redundant cellular mechanisms, including $CD4^+$, and to a lesser extent $CD8^+$, T cells[26]. In our respiratory model, lung clearance was independent of the presence of MAIT cells, suggesting their redundancy as protective T cells in this model too. It would be interesting to establish whether the type or kinetics of the non-MAIT immune response differed in the absence of MAIT cells. Nevertheless, intranasal infection of mice led to a substantial increase in MAIT cells in the lung to up to 50% of all pulmonary αβ-T cells (a ~200-fold increase in absolute numbers versus ~5 fold for non-MAIT T cells). This enrichment peaked at d7 and was MR1-dependent, as shown genetically (in $MR1^{-/-}$ mice) and by anti-MR1 mAb blocking, consistent with previous *in vitro* data[1-6]. The accumulation of MAIT cells was directly related to the size of the bacterial inoculum. Interestingly, expanded MAIT cells

were long-lived in the lung and other tissues, persisting in higher numbers than found in uninfected mice long after *S.* Typhimurium was cleared to undetectable levels (~10 weeks). It is interesting to speculate that a history of infection in humans could explain the higher proportion of MAIT cells compared to SPF-housed mice, and it will be exciting to discover the role of MAIT cells in immune protection against a range of microbial respiratory pathogens.

Genetic manipulation of *S.* Typhimurium allowed us to demonstrate that responsiveness of MAIT cells *in vivo* required an intact riboflavin pathway, and hence was abolished upon disruption of the key *Rib*D gene responsible for generating the precursor 5-A-RU, which generates the dominant MAIT cell ligand, 5-OP-RU. Notably, the BRD509Δ*ribDH* mutant could still induce weak, transient up-regulation of MAIT cell CD69 *in vitro*, but did not induce accumulation or enrichment of MAIT cells *in vivo*. It remains unclear whether there are natural MAIT cell antigens derived from pathways other than riboflavin synthesis. The variability of MAIT TCRs from the semi-invariant MAIT TCR combinations[7,8,22] suggests that these receptors may be selected by alternate ligands and MAIT cells have an apparent capacity o discriminate between pathogen-derived ligands in a clonotype-dependent manner[21]. On the other hand, the semi-invariant Vα and restricted Vβ chain usage, the importance of the conserved Tyr 95α residue across the TRAJ33, TRAJ20 and TRAJ12 gene segments tested to date and the stereotypic mode of binding of the MAIT TCR to all known ligands and analogues studied[1,2,4,20,22], suggest a limited ligand family is recognized by MAIT cells. Accordingly, the abrogation of MAIT cell accumulation and activation following mutagenesis of the riboflavin synthesis pathway implies that this pathway furnishes the dominant, and likely exclusive, family of natural antigens recognized by MAIT cells at least for *S.* Typhimurium.

Previous studies have shown that MAIT cells can be directly activated via non-TCR signals including IL-12 and IL-18[44,45], and control of *F. tularensis* intracellular growth required the IL-12 40 kDa

subunit, implying a need for additional signals from infected APCs[13]. These observations are consistent with our finding that a co-stimulus was required to induce MAIT cell accumulation in the lungs following intranasal administration of synthetic MAIT cell antigen, 5-OP-RU, even when delivered repeatedly over 7 days, and despite inducing MAIT cell up-regulation of CD69. In contrast, intranasal administration of 5-OP-RU plus the TLR agonists Pam2Cys (TLR2/6), poly I:C (TLR3) or CpG (TR9) induced enrichment of lung MAIT cells to the approximate level achieved by *S.* Typhimurium infection. Importantly, addition of 5-OP-RU during intranasal challenge with bacteria lacking the riboflavin synthesis pathway (BRD509Δ*ribDH* mutant) restored their capacity to induce MAIT cell accumulation in the lungs, indicating that co-stimulatory capacity is inherent in bacterial infection and plays a role in the expansion and responsiveness of MAIT cells.

The phenotype of MAIT cells that expand in response to infection with *S.* Typhimurium is consistent with previous studies of *in vitro* stimulated MAIT cells, in that they express activation markers even before infection and produce pro-inflammatory cytokines IL-17, IFNγ and TNF upon activation by microbes. This is also consistent with a study of pulmonary infection of mice with *Francisella tularensis*[13], although of note, this study defined MAIT cells as DN thus not addressing a potential role for $CD8^+$ or $CD4^+$ MAIT cells in response to infection. The expression of CD69 on 20% of MAIT cells, compared to ~4% of non-MAIT T cells in the steady state is intriguing, and could suggest that there may be stimulation of MAIT cells through the presence of commensal microbes providing antigen at mucosal sites. As well as being described as an early-activation marker, CD69 expression has been previously shown on human tissue resident memory T cells[46] and to play a role in tissue retention of T cells[47]. However, further stimulation of MAIT cells, and CD69 up-regulation, could be induced by infection or provision of antigen plus co-stimulatory signals. The expansion of MAIT cells following infection may be a consequence of the lower number of MAIT cells in mice versus humans. Interestingly, once expanded, the MAIT cells are long lived in primary tissues,

preserving their activation and homing phenotype, maintaining expression of their transcriptional signature PLZF and RORγt but with gradual increase in the level of T-bet, known to coordinate developmental and effector programs some of which are conserved across adaptive and innate T cells.

Our study presents a picture of MAIT cell responsiveness to pathogens that bridges attributes of innate and adaptive immunity, but that is dependent on specific antigen expressed by the infecting pathogen. It will be fascinating to unravel whether these cells have evolved in response to a range of pathogens with shared antigen(s), or whether they have a more focused role, perhaps involving a single pathogen family possibly at a key site.

METHODS

***In vitro* activation assay.** Jurkat cells expressing a MAIT TCR comprising the TRAV1-2-TRAJ33 α-chain, and TRBV6-1 β-chain (Jurkat.MAIT), were co-incubated with filtered bacterial culture supernatant and C1R APCs expressing MR1 (CIR.MR1) for 16 h, and stained with anti-CD3-PE and anti-CD69-APC antibodies before flow cytometric analysis. Activation of Jurkat.MAIT cells was measured by an increase in surface CD69 expression.

Compounds and immunogens. 5-OP-RU was prepared as described previously[2]. 6-FP was purchased from Schircks Laboratories. TLR2/6 agonist Pam2Cys[48] were chemically synthesized and functionally verified. CpG1688 (Sequence: T*C*C*A*T*G*A*C*G*T*T*C*C*T*G*A*T*G*C*T (* phosphorothioate linkage) nonmethylated cytosine-guanosine oligonucleotides was purchased from Geneworks (Australia). Poly (I:C) (HMW VacciGrade) was purchased from InvivoGen (USA).

Bacterial strains and mutants. *S.* Typhimurium BRD509, (previously described[49,50]) harbours deletions in *aroA* and *aroD,* resulting in limitation of replication of bacteria[27,49] and has an intact

riboflavin synthesis pathway. The BRD509Δ*ribDH* mutant, lacking a gene segment containing *ribD* and *ribH*, which encode key enzymes in the riboflavin synthesis pathway, was constructed by lamda red-recombinase mediated allelic replacement followed by transduction using phage P22 as previously described[27]. BRD509Δ*rib*DH cultures were supplemented with 20 µg/ml riboflavin.

BRD509Δ*ribDH* complemented by transformation of *ribDH* genes resulted in strain BRD509Δ*ribDH+ribDH*. Mutation and reconstitution were verified by lack of growth or growth on Luria Agar, and by PCR. Bacteria were cultured at 37 °C statically, in Luria Bertani broth (LB) with appropriate antibiotics for 16-18 h to log-phase (OD_{600} 0.6-0.9). Supernatant was collected by centrifugation, filtered (0.22 µM) and stored at -80ºC until use. For the infecting inoculum, bacteria were re-inoculated in pre-warmed medium for a further 2-4 h static culture (OD_{600} 0.4-0.6). With the estimation that 1 OD_{600}=$5x10^8$/ml, sufficient bacteria were washed and diluted in PBS for intranasal delivery to mice. A sample of inoculum was plated onto Luria Agar with appropriate antibiotics for verification of bacterial concentration by counting CFU.

Mice and inoculations. Mice were bred and housed in the Biological Research Facility of the Peter Doherty Institute. $MR1^{-/-}$ mice were generated by breeding $V\alpha19i.C\alpha^{-/-}.MR1^{-/-}$ mice[18] (from Susan Gilfillan, Washington University, St. Louis School of Medicine, St. Louis, MO) with C57BL/6 mice, and inter-crossing of F1 mice. The genotype was determined by tail DNA PCR at the MR1 locus and by the presence of intact αβ-T cells in blood using Ab against TCRβ. The absence of MAIT cells was verified by flow cytometry in the retired founder breeder mice. Male mice aged 6-12 weeks were used in experiments, after approval by the University of Melbourne Animal Ethics Committee.

Intranasal inoculation with *S.* Typhimurium (BRD509: 10^6 or BRD509Δ*ribDH:* 10^7 unless otherwise stated) or antigens (76 pmol 5-OP-RU or 6-FP alone, or plus 20 nmol Pam2Cys, 20 μg CpG or 50 μg poly I:C) in 50 μl per nares was performed on anesthetized mice. For blocking experiments, mice were given 250 μg anti-MR1 (26.5 or 8F2.F9)[28,29] or isotype control mAbs in 200 μl PBS, once (i.v or i.p) 1 day prior to inoculation and twice (d1, d4) post inoculation. Mice were killed by administration of CO_2 and organs: lung (following heart perfusion with 10 ml cold RPMI), liver (following portal vein perfusion with 10 ml PBS), mediastinal lymph nodes, blood, spleen, thymus, vagina and uterus were taken.

Preparation of organs and isolation of cells. To prepare single cell suspensions, lungs, uterus and vagina (pooled from 3 mice) were finely chopped with a scalpel blade and treated with 3 mg/ml collagenase III (Worthington), 5 μg/ml DNAse and 2% FCS in RPMI for 90 min at 37 °C with gentle shaking. Cells were then filtered (70 μM) and washed with PBS/2% FCS. Liver lymphocytes were prepared by pushing through metal mesh, followed by Percoll (33.75%) density gradient centrifugation (693 *g*, 12 mins, RT). LNs, spleen and thymus were prepared by pushing the tissues through 70 μM cell strainers. RBCs were lysed from lung, liver and spleen preparations with hypotonic buffer TAC (Tris-based Amino Chloride) for 5 min at 37 °C. Approximately 1.5×10^6 cells were filtered (40 μm) and used for flow cytometric analysis. Blood cells were prepared by centrifugation and serum removal. RBC lysis was by Lysing buffer (BD Biosciences) after flow cytometric staining.

Determination of bacterial counts in infected lungs. Bacterial colonisation was determined by counting CFU obtained from plating homogenized organs from infected mice (5 or more per group) on Luria agar containing appropriate antibiotics and supplements.

Generation of soluble MR1 tetramers. Murine MR1 and β2-Microglobulin genes were expressed in *E. coli* inclusion bodies, refolded and purified as described previously[20]. MR1-5-OP-RU and MR1-6-FP tetramers were generated as described previously[2].

Antibodies and flow cytometry. Ab against CD19 (1D3, PerCP-Cy 5.5), CD3 (UCHT1, PE or 145-2C11, PE-Cy7), CD4 (GK1.5, APC-Cy7), CD45.2 (104, FITC), CD69 (FN50, APC), CD8α (53-6.7, PE), IFNγ (XMG1.2, PE-Cy7), TCRβ (H57-597, APC or FITC), CD44 (IM7, PE), TNF (MP6-XT22, PE) and IL-17A (TC11-18H10, AlexaFluor700) were purchased from BD. Ab against MHC II (M5, AlexaFluor700), CD69 (H1.2F3, PE), CD25 (PC61.5, APC), PLZF (Mags.21F7, PE), RORγt (B2D, APC) and T-bet (4B10, PE-Cy7) were purchased from eBioscience. Ab against CD62L (Mel-14, FITC) was purchased from Biolegend. Blocking Ab (26.5, 8F2.F9) and isotype controls (3E12, 8A5) were prepared in house. To block non-specific staining, cells were incubated with MR1-6FP tetramer and anti-Fc receptor (2.4G2) for 15 min at RT, then incubated at RT with antibody/tetramer cocktails in PBS/2% FCS. 7-AAD (5 µL/sample) was added for the last 10 min. Cells were fixed with 1% PFA prior to analysis on LSRII or LSR Fortessa (BD Bioscience) flow cytometers. Data analysis was performed with FlowJo software. For intracellular cytokine staining (ICS), Golgi plug (BD Biosciences) was used during all processing steps. Cells stimulated with PMA/Ionomycin (20 ng/ml, 1 µg/ml respectively) for 3 h at 37° were included as positive controls. Surface staining was performed at 37°C and cells stained for intracellular cytokines using the BD Fixation/Permeabilisation kit or transcription factors using the transcription buffer staining set (eBioscience) according to the manufacturers' instructions.

Statistical analysis. Statistical tests were performed using Prism GraphPad software (version 6). Comparisons between groups were performed using Student's t test, unless otherwise stated.

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DISCLOSURE

ZC, SE, DF, LL, JR, JMc and AC are inventors on patents describing MR1 tetramers. The authors have no other competing financial interests.

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FIGURE LEGENDS

Figure 1. MAIT cells respond to *Salmonella* Typhimurium *in vivo*. **A)** Representative plots and scatter plots showing MAIT cell percentage among TCRβ$^+$ lymphocytes in a range of organs from 5 uninfected C57BL/6 mice. MAIT cells are defined by positive MR1-tetramer and TCRβ staining. Numbers represent MAIT cells as a percentage of TCRβ$^+$ lymphocytes. **B**) Representative plots showing TCRβ$^+$ lymphocytes (with percentage of MAIT cells) harvested from lungs of uninfected C57BL/6, or infected (d7 p.i. with 10^6 *S.* Typhimurium BRD509) C57BL/6 or C57BL/6 MR1$^{-/-}$ mice. **C)** Treatment of mice with anti-MR1 mAb 26.5, but not an isotype control, blocked the accumulation of MAIT cells upon *S.* Typhimurium infection. Three mice per group were injected with 0.25 mg indicated antibodies or no Ab i.p. 1 day prior to infection and three times p.i. (d1, d3 and d5). 10^6 BRD509 were inoculated i.n. at d0. At d7 p.i. mice were killed and lung cells were examined for MAIT cell accumulation. Statistics were performed using Student's t test (**: $p<0.01$, error bars: SEM). Uninfected MR1$^{-/-}$ mice were used as the negative control. The experiment was performed twice with similar results. **D)** Dose (of *S.* Typhimurium BRD509) response of MAIT cells as a percentage of TCRβ$^+$ cells at d7 p.i. Five mice per group were examined (Mean +/- SEM). The experiment was performed three times with similar results. **E)** Absolute numbers of MAIT cells (circle), conventional non-MAIT cells (triangle) and total TCRβ$^+$ cells (square) recovered from lungs were expressed over a time course following intranasal infection with 10^6 *S.* Typhimurium BRD509. Five mice per group were examined (Mean +/- SEM). The experiment was performed twice with similar results.

Figure 2. MAIT cells response depends on both specific riboflavin synthesis-derived antigen and co-stimulation. **A**) *In vitro* activation. Jurkat.MAIT cells were incubated for 6 h with filtered culture supernatant from BRD509 or BRD509Δ*ribDH* mutant (*ΔribDH*) or reconstituted mutant BRD509Δ*ribDH*+*RibDH* S. Typhimurium in the presence of C1R.MR1 cells. Activation was

detected by staining with anti-CD69. Data shows mean MFI of gated Jurkat.MAIT cells with SEM as error bars. The experiment was performed more than three times with similar results. **B)** and **C)** *In vivo* accumulation of MAIT cells by BRD509 and BRD509Δ*ribDH S.* Typhimurium. **B)** Representative plots, and **C)** MAIT cells as a percentage of αβ-T cells, from the lungs of mice immunised with BRD509 (10^6) or BRD509Δ*ribDH* (10^7) *Salmonella* (i.n. once), in combination with 5-OP-RU or 6-FP (50 μl of 1.52 μM i.n.) three times at d1, d3 and d5 p.i. Day 7 p.i. data are shown. Three mice per group were examined (Mean +/- SEM, Student's t test**; $p < 0.05$). The experiment was performed twice with similar results. **D)** MAIT cells as a percentage of αβ-T cells in the lungs of mice immunised i.n. with *S.* Typhimurium BRD509 (10^6), or combinations of Pam2Cys (20 nmol, i.n), CpG (20 μg/mouse, i.n.), or poly I:C (50 μg/mouse, i.n.) and/or 5-OP-RU or 6-FP (50 μl containing 76 pmol μM i.n.). 5-OP-RU or 6-FP was administered twice i.n. 1 and 3 days after Pam2Cys, CpG or poly I:C administration. Day 7 post inoculation data shown for 6 individual mice with mean +/- SEM. The experiment was performed twice with similar results. **E)** Mice were administered i.n with combinations of Pam2Cys (20 nmol/5 μl per mouse) and/or MAIT cell ligands (5-OP-RU, 6-FP, 45 μl of 1.52 μM or 45□μl of PBS, per mouse). Data represents MFI of CD69 expression on MAIT and non-MAIT T cells for individual mice and mean +/- SEM, 2 h post inoculation. **F)** Mice were inoculated i.n. with indicated MR1 ligands and/or co-stimulator as shown in schematic. Percentages of MAIT cells of αβT cells are shown, from 2 mice (control groups): uninfected, Pam2cys (day 0) plus 5-OP-RU/6FP (day 0, 1, 2 and 4); or 3 mice each: 6 times of 5-OP-RU/6FP alone (day 0, 1, 2, 3, 4 and 5).

Figure 3. Cytokine profiling and phenotyping of MAIT cells upon infection with *S.* Typhimurium BRD509. Intracellular cytokine staining of MAIT and non-MAIT αβ-T cells at d7 p.i. detected directly *ex vivo*, or following stimulation with PMA and ionomycin. **A)** representative plots and **B)**

scatter plots showing individual mice and mean +/- SEM from 3 mice (pooled for uninfected). The experiment was performed twice with similar results. MAIT and non-MAIT T cells are defined as live, $CD19^-$, $TCR\beta^+$, Tet^+ and live, $CD19^-$, $TCR\beta^+$, Tet^- cells respectively.

Figure 4. MAIT cell expression of transcription factors PLZF, RORγt and T-bet. **A)** Expression of transcription factors on MAIT cells in uninfected or infected mice at indicated time points. **B)** Co-staining of cells with RORγt and T-bet shown as representative plots and individual mice from one experiment. **C)** Cytokine profile as in Fig. 3, but showing RORγt and T-bet expression. The experiment was performed three times with similar results.

Figure 5. MAIT cells maintain an effector memory phenotype following infection. Analysis of phenotypic and activation marker expression on MAIT cells isolated from the lungs of mice infected i.n. with 10^6 *S.* Typhimurium BRD509 at indicated time points. **A).** Representative plots. **B).** Percentages among MAIT and non-MAIT cells expressing markers for individual mice. The experiment was performed twice with similar results.

Figure 6. MAIT cells proliferate in the lungs and draining LN in response to intranasal infection. **A)** Kinetics of MAIT cell accumulation in lungs, mediastinal draining LN and spleen at indicated times p.i. from C57BL/6 inoculated with BRD509 i.n. The experiment was performed three times with similar results. Data represent mean ± SEM from five mice per group. Data from C57BL/6.$MR1^{-/-}$ mice are not shown. **B)** MAIT cell percentage in various organs 7 weeks after i.n. *S.* Typhimurium BRD509 inoculation. Same data as in A, shown in bar graphs for ease of comparison. **C)** Bacterial load (CFU) recovered from lungs of C57BL/6 or C57BL/6.$MR1^{-/-}$ mice inoculated i.n. with 10^6 *S.* Typhimurium BRD509. Individual mice (5 per group) and mean are shown. The experiment was performed 3 times with similar results.

Supplementary Material

Supplementary Figure 1. Anti-MR1 blocking of MAIT cell accumulation and CD69 upregulation. **A.** Treatment of mice with anti-MR1 mAb 8F2.F9, but not an isotype control, blocked the accumulation of MAIT cells upon *S.* Typhimurium infection. Mice were injected with 0.25 mg indicated antibodies or no Ab i.p. 1 day prior to infection and at d1, d3 and d5 p.i. 10^6 BRD509 were inoculated i.n. at d0. At d7 p.i. mice were killed and lung cells were examined for MAIT cell accumulation. Student's t test (**: $p<0.01$, error bars: SD). The experiment was performed twice with similar results. **B.** Mice were inoculated i.n with combinations of Pam2Cys (Pam) (20 nmol/5 μl per mouse) and/or MAIT cell ligands (5-OP-RU, 6-FP, 45 μl of 1.52 μM or 45 μl of PBS, per mouse). Data represents Mean +/- SEM MFI of CD69 expression on MAIT and non-MAIT T cells 2 h post inoculation. For blocking 0.25 mg anti-MR1 mAb 8F5 was given 1 day prior.

Supplementary Figure 2. Bacterial counts recovered from the lungs following infection. CFU of *S.* Typhimurium recovered from the lungs at indicated time points after infection in C57BL/6 (B6) and $MR1^{-/-}$ mice after infection with rib sufficient (BRD509) and deficient (BRD509ΔribDH) strains. Mean +/- SEM of 5 mice per group. The experiment was performed twice with similar results. For clarity lines are shown for C57BL/6 groups only with markers for both C57BL/6 (open symbols) and $MR1^{-/-}$ mice (closed symbols).

Supplementary Figure 3. *In vivo* accumulation of MAIT cells with ligands delivered i.v. MAIT cells as a percentage of αβ-T cells, from the lungs of mice immunised with BRD509 (10^6) or BRD509Δ*ribDH* (10^7) *Salmonella* (i.n. once), in combination and followed with 5-OP-RU or 6-FP

(76 pmol in 100 μl i.v.) three times at d1, d3 and d5 p.i. Day 7 p.i. data are shown. Two or three mice per group were examined (error bar=SD). The experiment was performed twice with similar results.

Supplementary Figure 4. Cytokine production by non-MAIT T cells upon infection with *S.* Typhimurium BRD509. Percentage of non-MAIT αβ-T cells producing indicated cytokines at d7 p.i. detected by intracellular cytokine staining directly *ex vivo*, or following stimulation with PMA and ionomycin, as in Figure 3. Individual mice with mean +/- SEM are shown.

Supplementary Figure 5. PLZF, RORγt and T-bet expression by MAIT and non-MAIT T cells. Shown is mean fluorescence intensity (MFI) from gated cells from individual mice from the same experiment in Figure 4, direct *ex vivo* samples. Bars = mean +/- SEM.

Supplementary Figure 6. Cytokine production and transcription factor expression by MAIT cells. Shown is percentage of transcription factor positive and negative MAIT cells which produce each cytokine. Individual mice from the same experiment in Figure 4c, direct *ex vivo* samples are shown. Bars = mean +/- SEM.

Supplementary Figure 7. Co-staining of IFNγ and IL-17 from lung MAIT cells following infection. Shown are MAIT cells from one representative mouse per group from the same experiment shown in Figure 4b, direct *ex vivo* and PMA-stimulated samples.