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IL-23 costimulates antigen-specific MAIT cell activation and enables vaccination against bacterial infection

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Title: IL-23 co-stimulates antigen-specific MAIT cell activation and enables vaccination against bacterial infection[1]

Short title: Signals driving Ag-specific MAIT cell activation

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One Sentence Summary: ICOS and IL-23 co-stimulation are both important for driving MR1-antigen-specific MAIT cell activation and that combined vaccination with IL-23 plus 5-OP-RU enhances protection against bacterial infection

ABSTRACT

MAIT cells are activated in a TCR-dependent manner by antigens derived from the riboflavin synthesis pathway, including 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil, bound to MHC-related protein-1 (MR1). However, MAIT cell activation *in vivo* has not been studied in detail. Here, we have discovered and characterised additional molecular signals required for optimal activation and expansion of MAIT cells following pulmonary *Legionella* or *Salmonella* infection in mice. We show that either bone marrow-derived APCs or non-bone marrow-derived cells can activate MAIT cells *in vivo,* depending upon the pathogen. Optimal MAIT cell activation *in vivo* requires signalling through the inducible co-stimulator of T cells (ICOS), which is highly expressed on MAIT cells. Subsequent expansion and maintenance of MAIT-17/1-type responses are dependent on IL-23. Vaccination with IL-23 plus 5-OP-RU augments MAIT cell-mediated control of *Legionella* infection. These findings reveal cellular and molecular targets for manipulating MAIT cell function under physiological conditions.

INTRODUCTION

Mucosal-associated Invariant T (MAIT) cells respond to a range of bacteria and fungi by recognizing compounds derived from riboflavin biosynthesis, the most potent of which is 5-(2-

oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), presented by MHC-related protein-1 (MR1) (*1, 2*). MAIT cells express a conserved αβ-TCR, consisting of a relatively invariant TCRα-chain coupled with a broad repertoire of β-chains, but with preferential Vβ-usage (*3-7*). In mice, MAIT TCRs use TRAV1-TRAJ33 (Vα19-Jα33) generally assembled with TRBV19 (Vβ6) or TRBV13 (Vβ8), and homologous TCRs exist in humans. MAIT cells have been shown to respond to a wide range of bacteria and fungi, including both pathogenic and commensal species, correlating with the presence of genes involved in riboflavin biosynthesis in these organisms, and thus with their capacity for Ag production (*2, 8-10*), and multiple studies suggest that MAIT cells have evolved for host defence against microbial pathogens. However, MR1 is ubiquitously expressed at low levels (*11-13*), and the cells responsible for presenting Ag *via* MR1 to MAIT cells during *bona fide* infection have not been described. Moreover, the requirements for activation of MAIT cells and their role in protecting against pathogens are still being unravelled.

MAIT cells that egress from the thymus display memory/effector cell properties (*14-17*), making them distinct from naïve conventional T cells. However, following TCR-dependent stimulation, MAIT cells acquire an activated status, where they expand and rapidly produce cytokines, including IL-17, IFNγ, TNF and GM-CSF in an "innate-like" manner in response to bacteria (*15, 18*). Recently, we demonstrated that MAIT cells differ from conventional circulating cells as they appear to be activated to proliferate at the site of infection, contributing substantially to the accumulation of MAIT cells at these sites (*19, 20*). Like conventional T cells, MAIT cells also require other co-stimulatory signals in addition to TCR recognition of synthetic 5-OP-RU antigen (Ag), for their activation (*19, 21*). These additional signals can be provided by TLR-agonists or bacterial products (*19*), but the nature of such obligatory signals for MAIT cell priming *in vivo* are not well understood. Certain cytokines are known to be important in TCR-dependent activation of MAIT cells (*22-25*), with IL-12 and IL-18 in particular having been reported to drive MAIT cell activation independently of MR1-TCR ligation in *in vitro* assays (*8, 26*).

Activation of MAIT cells through prior vaccination with 5-OP-RU in the presence of TLR agonists, or adoptive transfer of activated MAIT cells, each confer improved protection against bacterial infection (*20*). To understand the signals required to stimulate MAIT cells for optimal function *in vivo,* we embarked on a study of their activation requirements in mouse models of infection with two pathogens, *Legionella longbeachae* and *Salmonella enterica* var Typhimurium (*S.* Typhimurium). Here we use BM chimeras, in which either BM-derived (WT→*Mr1*$^{-/-}$) or non-BM-derived (*Mr1*$^{-/-}$→WT) cells, or both (WT→WT) express MR1 and hence could potentially present Ag through MR1, to show that either bone marrow-derived APCs or non-bone marrow-derived cells can activate MAIT cells *in vivo* depending upon the pathogen. ICOS is highly expressed on MAIT cells and is required for optimal MAIT cell activation *in vivo.* We demonstrate that subsequent expansion and maintenance of MAIT-17/1-type responses depend on IL-23, and that combined vaccination of mice with IL-23 and 5-OP-RU augments MAIT cell-mediated control of *Legionella* infection.

RESULTS

Differential requirement for bone-marrow (BM) derived and non-BM derived cells to activate MAIT cells *via* MR1, depending on the biology of the bacterial infection.

The low level, ubiquitous expression of MR1 (*11-13*) has made it difficult to identify the cells that drive MAIT cell responses *in vivo*. Studies reporting MAIT cell activation *in vitro* have used both BM-derived and non-BM-derived cells or cell lines (*23, 27-29*) suggesting that either cell type is capable of presenting Ag on MR1 for MAIT cell activation. To assess the ability of different MR1-expressing cells that are BM-derived or not to activate and drive proliferation of MAIT cells *in vivo* in an MR1-dependent manner, we employed two models of intranasal infection of C57BL/6 mice with *S.* Typhimurium BRD509 or *L. longbeachae* NSW150, where we have previously reported activation and expansion of MAIT cells (*19, 20*). We first confirmed that MAIT cells accumulated in the infected site (lungs) during pulmonary infection. Seven days after intranasal infection with *S.*

Typhimurium BRD509 or *L. longbeachae,* MAIT cells represented on average 46% and 24%, respectively, of TCRβ$^+$ lymphocytes in the lungs as detected with MR1-5-OP-RU tetramers **(Fig. 1a**, gating strategy shown in **Supplementary Fig. 1a)**. Although the MAIT cells observed in infected lungs could derive from contaminating MAIT cells from the circulation (*30*), this is not a plausible explanation considering the low numbers of MAIT cells in the circulation of mice (<0.1% of T cells) (*19, 31*) and the documented capacity for MAIT cell proliferation in the lungs following infection (*20*). Hence, we consider that circulating MAIT cells may make only a minor contribution to the expansion of MAIT cells. To determine the source of APC required to present bacterial Ag *via* MR1 to MAIT cells in these models, we generated three sets of bone marrow (BM) chimeric mice: 1) WT C57BL/6 (CD45.1) BM transplanted into recipient MR1-deficient (CD45.2) mice or 2) WT C57BL/6 (CD45.1) BM transplanted into recipient WT congenic (CD45.2) mice or 3) MR1-deficient BM (*Mr1*$^{-/-}$, CD45.2) transplanted into recipient WT C57BL/6 mice (CD45.1). These BM transfers resulted in chimeric mice that could present Ag to MAIT cells only *via* BM-derived cells (WT→*Mr1*$^{-/-}$), via both BM-derived and non-BM-derived cells (WT→WT) **(Fig. 1b)** or only via non-BM-derived cells (*Mr1*$^{-/-}$→WT) **(Fig 1f)**. Congenic markers (CD45.1 or CD45.2) were used to track the donor- and recipient-derived cells in these mice. We validated reconstitution of cells in the lungs of chimeric mice 8 weeks later, where >99% of CD3$^-$TCRβ$^-$CD45$^+$ cells were derived from donor BM **(Supplementary Fig. 1b)**.

BM chimeric mice were then infected intranasally with either *S.* Typhimurium or *L. longbeachae*, and MAIT cells from the lungs examined on day 7 post infection (p.i). Following *L. longbeachae* infection, MAIT cells expanded similarly in WT→*Mr1*$^{-/-}$ mice and WT→WT controls (% of αβ-T cells or absolute number of MAIT cells) **(Fig. 1c, d),** suggesting that MR1 expression on BM-derived APCs is sufficient for activation of MAIT cells in this infection model. However, in mice infected with *S.* Typhimurium BRD509, there were significantly fewer MAIT cells in the lungs of WT→*Mr1*$^{-/-}$ chimeric mice on day 7 p.i. compared with WT→WT controls **(Fig. 1c, d)**, indicating that expression of MR1 on BM-derived cells is insufficient for complete MAIT cell activation in response

to *S.* Typhimurium pulmonary infection. There were no significant differences in the non-MAIT T cell response to either bacterial infection in WT→*Mr1*$^{-/-}$ *vs* WT→WT BM chimeras **(Supplementary Fig. 1c)**.

To determine whether non-BM-derived cells could provide APC function for MAIT cell activation, we created (*Mr1*$^{-/-}$→WT) BM chimeric mice. However, no donor MAIT cells were detected in the lungs of these mice **(Fig. 1e)**, consistent with the requirement for MR1^{+} DP thymocytes in MAIT cell development (*32*). Hence, to examine MAIT cells in this setting, where only non-BM-derived cells expressed MR1, we adoptively transferred MAIT cells sorted from the lungs of *S.* Typhimurium BRD509-infected WT (CD45.2) mice **(Fig. 1f)**. This transfer reconstituted MAIT cells to a similar frequency as compared to naïve C57BL/6 mice two weeks post transfer **(Fig. 1e)**. We then infected these mice intranasally with *S.* Typhimurium BRD509 or *L. longbeachae*. In *S.* Typhimurium-infected mice, we found a selective enrichment of transferred MAIT cells in the lungs: proportionally expanding from ~1% to ~30% of all αβ-T cells (**Fig. 1g)** with an ~80-fold increase in the absolute number of MAIT cells (**Fig. 1h)**. In contrast, following *L. longbeachae* infection, no significant increase in the proportion of MAIT cells was observed (**Fig. 1g)**, and the expansion of absolute MAIT cell numbers was limited (~6-fold) **(Fig. 1h)**. To further validate the use of transferred MAIT cells in this model, we transferred MAIT cells (bearing distinct allotypes of CD45 from endogenous MAIT cells) into WT→*Mr1*$^{-/-}$ chimeras. These mice were then infected with either *S.* Typhimurium or *L. longbeachae* two weeks post transfer, and examined for the accumulation of transferred and endogenous MAIT cells using CD45 congenic markers at 7 days p.i. The transferred MAIT cells expanded with a similar pattern to endogenous MAIT cells (**Fig. 1d** *vs* **Supplementary Fig. 1d**) following infection. Together these data suggest that MR1 expressing non-BM-derived cells could activate MAIT cells in response to *S.* Typhimurium BRD509, but not *L. longbeachae* infection, which required MR1 expression on BM-derived cells for MAIT cell expansion.

Active invasion by *S.* Typhimurium is essential for its capacity to activate MAIT cells *in vivo*

Although priming and activation of conventional T cells by epithelial cells presenting peptide Ag has been demonstrated by others (*33, 34*), it was surprising that non-BM-derived cells appeared to be dominant in driving MAIT responses to *S.* Typhimurium infection. To understand why non-BM-derived cells are required for the accumulation of MAIT cells during *S.* Typhimurium pulmonary infection, we used the *S.* Typhimurium SL1344Δ*invA* mutant, which lacks the ability to actively invade epithelial cells (*35*). Presentation of Ag in this infection is largely restricted to professional APCs that are capable of phagocytosing the bacteria (*36*). First, we tested the SL1344Δ*invA* mutant in an MR1-dependent *in vitro* activation assay (*37*) to confirm its ability to produce MAIT-stimulatory Ag. Bacterial culture supernatants from *S.* Typhimurium SL1344Δ*invA* and SL1344 were equally capable of activating a reporter cell line expressing a MAIT TCR; Jurkat.MAIT (*2, 37*), as detected by up-regulation of CD69 **(Fig. 2a)**, suggesting no defect in MAIT cell Ag production by the SL1344Δ*invA* mutant. The defect in invasion of the SL1344Δ*invA* mutant (*35*) was confirmed in both human and murine macrophage versus epithelial cell lines. Thus, after 1 h co-culture with bacteria, we found that both human and murine epithelial cells contained significantly fewer SL1344Δ*invA* mutant colony forming units (CFU) compared to the SL1344 control, whereas no difference in bacterial load per cell was observed in macrophage cell lines, which actively phagocytose bacteria **(Fig. 2b)**.

To test the effect of this invasion defect on the accumulation of MAIT cells *in vivo*, we intranasally infected C57BL/6 mice with either SL1344 or SL1344Δ*invA* bacteria and examined MAIT cells from the lungs at day 6 p.i. Additionally, we determined the bacterial load in these mice by CFU assay from homogenized lungs. In contrast to SL1344, the SL1344Δ*invA* mutant strain failed to evoke significant MAIT cell accumulation in the lungs of these infected mice **(Fig. 2c)**, despite having equivalent bacterial loads **(Fig. 2d)**. Together, these data demonstrate that active bacterial invasion is essential to *S.* Typhimurium stimulation of an optimal MAIT cell response *in vivo* consistent with our finding that non-BM-derived cells are important APCs presenting Ag to MAIT cells in infection by

this facultative intracellular pathogen. Thus, taken together, these data demonstrate that, depending on the pathogen biology, either BM-derived or non-BM-derived cells are capable of acting as APCs and activating MAIT cells in an MR1-dependent manner.

ICOS is highly expressed by MAIT cells and critical for optimal MAIT cell expansion and RORγt expression

Previously, we showed that synthetic 5-OP-RU antigen was insufficient for MAIT cell activation *in vivo* and that other required stimulatory signals could be provided through co-inoculation of mice with TLR agonists CpG, poly I:C or Pam2Cys (*19*). Here, we investigated the direct requirements for co-stimulatory molecules (signal 2) for MAIT cell activation, hypothesising that TLR agonists may not directly activate MAIT cells. Rather they might act indirectly through APCs, either by enhancing MR1 presentation (*38*) or by stimulating cytokine production or upregulation of co-stimulatory ligands. First, we examined the expression of a range of co-stimulatory molecules on MAIT and non-MAIT TCRβ$^+$ cells from the lungs of naïve C57BL/6 wild-type mice. Interestingly, ICOS was highly expressed by MAIT cells from naïve mice, compared with non-MAIT T cells **(Fig. 3a)**. In addition, MAIT cells expressed intermediate levels of CD154 (CD40L) and CD27 and similar levels of CD28 to non-MAIT T cells, whereas CD137 (4-1BB) was not detected on lung MAIT cells. Thus, we hypothesised that ICOS could play a significant role in the co-stimulation of MAIT cells.

To further investigate whether these co-stimulatory molecules were required for optimal accumulation and activation of MAIT cells during bacterial infection, we studied gene KO mice (*Icos*$^{-/-}$ mice, and *Cd80*$^{-/-}$/*Cd86*$^{-/-}$ double KO mice; the latter lack signalling through CD28). WT (C57BL/6), *Icos*$^{-/-}$, and *Cd80*$^{-/-}$/*Cd86*$^{-/-}$ double KO mice were intranasally infected with *S.* Typhimurium BRD509 or *L. longbeachae* NSW150 and then examined for MAIT cell and CD4$^+$ T cell accumulation in the lungs after 7 days **(Fig. 3b, Supplementary Fig 2a, b)**. Compared with WT mice, we observed a significant reduction in the proportion and absolute number of MAIT cells responding to infection in *Icos*$^{-/-}$ mice for both *S.* Typhimurium and *L. longbeachae* infections. There was no significant

difference in MAIT cell numbers detected between WT mice and *Cd80*$^{-/-}$/*Cd86*$^{-/-}$ mice **(Fig. 3b, Supplementary 2a, b)** nor in the number of CD4^{+} T cells in WT versus *Icos*$^{-/-}$ mice following pulmonary infection with *S.* Typhimurium BRD509 or *L. longbeachae* NSW150 **(Supplementary Fig. 2a, b)**.

We next examined the expression of key transcription factors, T-bet and RORγt, which drive Th-1 and Th-17 type responses, respectively (*39, 40*), by the responding MAIT cells, to determine whether different co-stimulatory signals may drive phenotypic differences in MAIT cells. We have previously observed an increase in T-bet expression on lung MAIT cells following infection with *S.* Typhimurium or *L. longbeachae* (*19, 20*). Consistent with our previous data, in WT C57BL/6 mice there was an increase in the proportion of both T-bet^{+}RORγt^{+} and T-bet^{+}RORγt^{-} MAIT cells following infection **(Fig. 3c, d)**. A similar effect was observed in *Cd80*$^{-/-}$*Cd86*$^{-/-}$ mice. In contrast, in the absence of ICOS, there was a reduction in the numbers of RORγt^{+}T-bet^{-} MAIT cells following *S.* Typhimurium or *L. longbeachae* infection, and proportionally T-bet^{+}RORγt^{-} MAIT cells were increased **(Fig. 3d, Supplementary Fig. 2c, d).** These data suggest that ICOS is required for MAIT cell maintenance of RORγt expression and, in the absence of ICOS, more MAIT cells were skewed towards a Th-1 like phenotype (MAIT-1) with high expression of T-bet, presumably reflecting other signals in play.

IL-23 is required for optimal MAIT cell accumulation and activation during pulmonary bacterial infection

Optimal MAIT cell responses require additional signals concurrent with TCR-Ag recognition (*19*). However, while previous studies have shown that cytokines, including IL-12 and IL-18, can act in a TCR-independent manner to stimulate MAIT cells (*8*), the requirement and type of cytokines that enhance MR1-dependent MAIT cell activation (*22-25, 41*), particularly *in vivo*, is less well understood. To probe the cytokine requirements for MAIT cell activation *in vivo,* mice genetically deficient in one or more individual cytokines were infected intranasally with *S.* Typhimurium

BRD509, and MAIT cell accumulation in the lungs was analysed at day 7 p.i. Mice deficient in IL-18, IL-6 or IFNγ, or *Il-12p35*$^{-/-}$ mice, which lack IL-12 and IL-35 (*42*), were all capable of mounting a robust MAIT cell response similar to WT mice **(Fig. 4a, b)**. In contrast, both *Il-12p40*$^{-/-}$ mice, which lack both IL-12 and IL-23, and *Il-23p19*$^{-/-}$ mice, which lack IL-23 (*42-45*), had a marked reduction in their MAIT cell accumulation (percentage of T cells and absolute number) in the lungs compared to WT mice **(Fig. 4a, b)**, indicating that the lack of IL-23 significantly compromises the expansion of MAIT cells in the lungs in response to *S.* Typhimurium infection. A reduction in MAIT cell accumulation in *Il-23p19*$^{-/-}$ mice was also observed when mice were infected with *L. longbeachae* **(Supplementary Fig. 3a, b)**. In contrast, there was no significant difference in non-MAIT αβ-T cell numbers in WT vs *Il-23p19*$^{-/-}$ mice (**Supplementary Fig. 3c**). Additionally, we found no significant effect of either ICOS or IL-23 deficiency on numbers of NKT cells, γδ-T cells or ILCs in the lungs of *Legionella*-infected mice (**Supplementary Fig. 3c**). There was no significant difference on bacterial load (CFU) at day 7 in the lungs of *Icos*$^{-/-}$ or *Il-23p19*$^{-/-}$ mice compared to WT mice, although *Il-23p19*$^{-/-}$ mice had slightly higher load by 10 days post infection **(Supplementary Fig. 3d)**.

The expression of RORγt and T-bet appear to be mutually exclusive in naïve MAIT cells (*17, 31*), but intranasal infection with *S.* Typhimurium induced expression of T-bet, resulting in a large proportion of MAIT cells expressing both transcription factors (*19*). Given the requirement for IL-23 in the expansion of MAIT cells **(Fig. 4a, b)**, and its known role in driving differentiation of naïve conventional T cells to Th17 subsets (*46-54*), we determined how IL-23 was involved in altering transcription factor expression in MAIT cells following infection. Upon infection with *S.* Typhimurium BRD509, the proportion of MAIT cells expressing T-bet was lower in *Il-12p35*$^{-/-}$ and *Il-12p40*$^{-/-}$ mice compared with WT mice **(Fig. 4c)**. In *Il-23p19*$^{-/-}$ mice the proportion of T-bet^{+}RORγt^{-} MAIT cells was significantly increased during bacterial infection whereas in WT mice most MAIT cells co-expressed T-bet and RORγt **(Fig. 4c)**. Additionally, we observed similar changes in the transcriptional factor expression by the MAIT cell population in *Il-23p19*$^{-/-}$ mice infected with *L. longbeachae* NSW150 **(Supplementary Fig. 3e)**.

In *S.* Typhimurium-infected WT mice, ~50% of MAIT cells, and 12% of non-MAIT αβ-T cells isolated from the lungs produced IL-17A, as detected by intracellular cytokine staining 7 days post infection **(Fig. 4d, e)**. *Il-6*$^{-/-}$ mice showed a significant decrease in non-MAIT T cells, but not MAIT cells, producing IL-17A **(Fig. 4d, e)**. In contrast, *Il-23p19*$^{-/-}$ mice had lower numbers of MAIT cells **(Fig. 4b)**, and only 5-15% of these MAIT cells produced IL-17A, consistent with a lower expression of RORγt **(Fig. 4c, e)**. Non-MAIT T cells required both IL-6 and IL-23 for optimal IL-17 production consistent with previous reports (*55*) **(Fig. 4d, e)**. Overall, these data suggest that IL-23, but not IL-6 or other cytokines tested, is essential for MAIT cell accumulation in the lungs and for maintaining a MAIT-17 profile following bacterial lung infection.

Exogenously provided IL-23 restores MAIT cell capacity to proliferate in *Il-23p19*$^{-/-}$ mice

To confirm the requirement for IL-23 in driving the accumulation of MAIT cells in the lungs following infection, we investigated whether restoring IL-23 would rescue MAIT cell accumulation following infection in *Il-23p19*$^{-/-}$ mice. To do this, we administered a plasmid construct coding for the p19 and p40 subunits of IL-23 fused to an immunoglobulin Fc region, (IL-23-Ig (*56*)) to *Il-23p19*$^{-/-}$ mice *via* hydrodynamic injection one day prior to intranasal infection with *S.* Typhimurium BRD509 and then assessed MAIT cell accumulation in the lungs at day 7 p.i. Notably, IL-23 was readily detectable in bronchoalveolar lavage fluid (BALF) of mice receiving IL-23-Ig plasmid by 8h, and at 24 h, post hydrodynamic injection **(Supplementary Fig. 4a)**, consistent with the expected kinetics of protein expression by this method (*57*). In *Il-23p19*$^{-/-}$ mice administered with IL-23-Ig, but not a control plasmid, accumulation of MAIT cells comparable to that found in WT mice at day 7 p.i. was observed **(Fig. 5a, b)**. The transcription factor profile of these accumulating cells shifted towards co-expression of RORγt and T-bet (MAIT17/MAIT-1 phenotype) and resembled that observed in WT mice **(Fig. 5c)**. These data demonstrate that MAIT cells in *Il-23p19*$^{-/-}$ mice are capable of proliferating and maintaining expression of RORγt and this depends on the presence of

IL-23. Interestingly, the administration of IL-23-Ig plasmid DNA, but not control plasmid, also increased the expression of ICOS on MAIT cells relative to untreated control mice **(Fig. 5d)**.

MAIT cells express high levels of IL-23 receptor and respond to IL-23 in a direct manner

To determine whether IL-23 also affected MAIT cells directly during bacterial infection, we examined the expression of IL-23 receptor (IL-23R) by utilizing the *Il-23r*$^{gfp/+}$ heterozygous mice (reporter mice in which GFP expression is linked to IL-23 expression) (*58*). Flow cytometric detection of GFP in lung αβ-T cells showed that >85% of MAIT cells expressed IL-23R in naïve reporter mice **(Fig. 6a)**. During acute bacterial infection (day 7), MAIT cells that accumulated in the lungs down-regulated expression of IL-23R, with expression restored to ~74% in mice long-term post infection **(Fig. 6a)**. Next, we adoptively transferred WT MAIT cells (sourced from the lungs of long-term *S.* Typhimurium-infected mice) into either *Il-23r*$^{-/-}$ mice, as a model in which only the transferred MAIT cells could respond to IL-23, or into *Il-23p19*$^{-/-}$ mice, and infected these mice with *S.* Typhimurium **(Fig. 6b)**. After infection, adoptively transferred WT MAIT cells accumulated to a much greater extent (~143 fold) in the lungs of *Il-23r*$^{-/-}$ mice compared with those in *Il-23p19*$^{-/-}$ mice (~7 fold) and compared to the expansion of endogenous MAIT cells present in *Il-23r*$^{-/-}$ mice (~15 fold) **(Fig. 6b, c, Supplementary Fig. 5)**. Endogenous MAIT cells in either *Il-23p19*$^{-/-}$ or *Il-23r*$^{-/-}$ mice expanded similarly (23-fold and 15-fold, respectively). This finding suggests that both the availability of IL-23 and the expression of IL-23R on MAIT cells are essential for optimal MAIT cell expansion during bacterial infection, although other cytokines may drive a milder response. Together, our data demonstrate that MAIT cells express high levels of IL-23R and respond to IL-23 directly, resulting in their expansion at the site of infection.

IL-23 drives MAIT cell expansion in an MR1-Ag-dependent manner

We next tested whether IL-23 would be a sufficient co-stimulatory signal for MAIT cell accumulation and activation *in vivo* in response to synthetic Ag. To address this question, we infused IL-23-Ig

plasmid DNA hydrodynamically into WT mice 1 day before intranasal administration of synthetic 5-OP-RU. We detected a significant expansion of MAIT cells in the lungs of mice treated with IL-23-Ig, but not with a control plasmid, 7 days post initial 5-OP-RU inoculation **(Fig. 7a, Supplementary Fig. 4b)**. This enrichment of MAIT cells occurred in a dose- (of 5-OP-RU) dependent manner **(Fig. 7a, Supplementary 4b**), although there was a small, but significant effect on the absolute number of MAIT cells with IL-23-Ig alone **(Fig 7a)**. None of the inoculations had any obvious effect on the absolute number of non-MAIT T cells in the lungs **(Fig. 7b)**. In addition, either IL-23-Ig or 5-OP-RU alone induced some up-regulation of T-bet in MAIT cells, but this effect was enhanced when IL-23-Ig was combined with 5-OP-RU **(Fig. 7c)** such that the profile of transcription factor expression, an indicator of cytokine production, resembled that in mice infected with *S.* Typhimurium **(Fig 3c)**. Thus, IL-23 provides a sufficient co-stimulatory signal to trigger MAIT cell expansion and activation *in vivo*, and skews MAIT cells towards a MAIT-17/1 phenotype.

We next sought to determine whether these findings could be recapitulated with human MAIT cells. To this end, we stimulated healthy donor PBMCs *in vitro* with 5-OP-RU in the presence of IL-2 with and without IL-23, for 13 days to allow time for the cells to proliferate to high enough numbers and consolidate their cytokine profiles for analysis. Cell were then stained intracellularly to detect production of IL-17 and IFNγ. In the absence IL-23, 5-OP-RU induced a strong IFNγ response, whereas when 5-OP-RU was combined with IL-23, MAIT cells increased IL-17 production and the percentage of IFNγ-producing MAIT cells was reduced **(Supplementary Fig. 6a, b)**. IL-23 alone induced minimal cytokine production. Thus, similar to mouse MAIT cells the presence of IL-23 during Ag-stimulation drives human MAIT cells towards a MAIT-17-type response.

IL-23 plus 5-OP-RU-primed MAIT cells can enhance control of *Legionella longbeachae*

To determine whether MAIT cells primed with 5-OP-RU in combination with IL-23 could augment protective immunity to bacterial infection, we first vaccinated mice with IL-23-Ig *via* hydrodynamic injection (once, day 0) *via* tail vein combined with intranasal administration of synthetic 5-OP-RU (4

times, day 1, 2, 3, 4) (MAIT cell vaccination). After 4-5 weeks these mice, and control mice receiving IL-23-Ig alone or control-Ig DNA together with 5-OP-RU, were challenged intranasally with 10^4 CFU *L. longbeachae*, and the bacterial load in the lungs was measured by CFU assay at days 5 and 7 post infection **(Fig. 7d).** In control mice, the bacterial load decreased significantly between days 5-7 post infection, indicating bacterial clearance, consistent with our previous data in WT C57BL/6 mice (*20*). Importantly, bacterial loads were significantly reduced in mice that had received Ag-specific MAIT cell vaccination with 5-OP-RU in combination with IL-23-Ig, compared to control mice (untreated, treated with IL-23-Ig alone, or treated with control-Ig plus 5-OP-RU) **(Fig. 7e)**. The difference was most evident 5 days post infection, where a 50-fold reduction in bacterial load was observed in vaccinated (IL-23-Ig+5-OP-RU) mice relative to controls.

DISCUSSION

Our findings demonstrate that MAIT cell activation *in vivo* is not just dependent on MR1 and antigen, but also requires costimulatory signals and that ultimate expansion of MAIT cells is augmented by the inflammatory cytokine IL-23. Although MR1 is ubiquitously expressed at low levels (*11-13*) and both haematopoietic and epithelial cell lines can activate MAIT cells *in vitro* (*29, 59*), the source of cells responsible for presenting Ag to MAIT cells *in vivo* have not been described. Using various combinations of WT↔*Mr1*$^{-/-}$ BM chimeras, we show here that both BM-derived and non-BM-derived are capable of driving MAIT cell activation, but the predominant APC type is determined by the nature of the bacterial infection. *L. longbeachae* does not actively invade cells, but replicates in alveolar macrophages following phagocytosis (*60*). Consequently, *L. longbeachae* is confined to BM-derived lung macrophages which provide the necessary signals for MAIT cell activation. In contrast, for *S.* Typhimurium, which actively infects many cell types, including epithelial cells (*61*), non-BM-derived cells were more influential than BM-derived APC for the activation of MAIT cells. The low capacity of BM-derived APC to drive activation of MAIT cells in this context may be due to inhibitory mechanisms evolved by *S.* Typhimurium (*62-64*).

In addition to Ag, T cell activation requires a secondary "co-stimulatory" signal and we found that ICOS, but not CD80 or CD86, exerted a significant impact on MAIT cell activation *in vivo.* This finding is consistent with the high constitutive level of surface expression of ICOS on MAIT cells compared to non-MAIT T cells in naïve mice. ICOS co-stimulation is involved in a number of other processes during adaptive immune responses, including the formation of T-follicular helper cells and enhancing or dampening Th1 and Th2 inflammatory responses, depending on the pathogen (*65, 66*), as well as co-stimulation of cytokine production by memory $CD4^+$ T cells (*67*). The significantly lower accumulation of MAIT cells in *Icos*$^{-/-}$ mice following either *Salmonella* or *Legionella* infection suggests that ICOS signalling promotes MAIT cell proliferation. This finding contrasted with non-MAIT T cell and ILC numbers that were unaffected by ICOS deficiency. ICOS-L can be expressed on both BM-derived APCs and non-BM-derived cells (*67-71*). The greater dependence of MAIT cell expansion in *Icos*$^{-/-}$ mice during infection with *Salmonella* compared with *Legionella* may reflect the greater importance of ICOS as a co-stimulatory signal on epithelial versus BM-derived cells.

In contrast to conventional naïve T cells (*72*) and CD1d-restricted invariant NKT cells (*73*) where CD28-CD80/CD86 interactions are critical to activation, CD80/86-CD28 signalling did not appear to be important for MAIT cell activation, perhaps consistent with the description of MAIT cells as "effector-memory" cells (*14-17*). Other co-stimulatory molecules such as CD137 and CD154, are expressed at low levels on naïve MAIT cells though a proportion of human MAIT cells up-regulated CD137 (4-1BB) when cultured with *Mycobacterium tuberculosis* lysate (*74*). Thus, our study does not rule out a role for CD137 during infection.

The majority of MAIT cells in naïve mice express RORγt, and the expression of RORγt and T-bet can be mutually exclusive (*17*) but following infection the majority express both transcription factors (*19*). Double positive RORγt and T-bet MAIT cells were also observed in *Icos*$^{-/-}$ mice, but with an increase in proportion of MAIT-1 (T-bet$^+$RORγt$^-$, Th1-like) cells, suggesting a role for ICOS in maintaining a MAIT-17 phenotype during infection, as for conventional $CD4^+$ and $CD8^+$ T cells (*66,*

75). Notably too, an ICOS agonist confers more potent T cell function through an increase in inflammatory cytokines and IL-23R expression (*66, 75*).

IL-23 is required for the functional differentiation of IL-17 producing Th17 cells, stabilizing their phenotype through the transcription factor STAT3 pathway (*76, 77*). Our findings indicate that IL-23 is also required for MAIT cell proliferation and differentiation, the addition of exogenous IL-23 fully reconstituting the MAIT cell proliferation and phenotype in IL-23 deficient mice. Of note, STAT3 loss of function mutations result in deficiency of human MAIT cells and other cells (*78*). Although not directly tested here, we speculate that IL-23 also acts through STAT3 in driving the differentiation of MAIT-17 cells.

MAIT cell transfer experiments also demonstrated that IL-23 acts directly on MAIT cells consistent with the high expression level of IL-23R on MAIT cells (*14, 79*) and with previous studies, where the cytokine appeared critical for prolonged survival of Th17 cells, consolidated the Th1 phenotype and conferred full Th17 pathogenic function potential (*39, 80, 81*). Consistent with findings in other T cell subsets (*82*), IL-23, from plasmid DNA, enhanced ICOS expression on MAIT cells, suggesting an indirect effect, via augmented ICOS expression, on MAIT cell activation. In contrast, unlike $CD4^+$ Th17 cells (*39, 83*) cells, MAIT cell development was not dependent on IL-6, demonstrating a key difference in the regulation of MAIT cell activation compared to conventional Th17 cells. MAIT cell expansion appeared to be independent of bacterial antigen load since CFU counts were similar at the peak of infection in WT, *Icos*$^{-/-}$ and *Il-23*$^{-/-}$ mice. Moreover, neither IL-12 nor IL-18 were required for MAIT cell expansion after infection, consistent with the fact that *in vitro* observations do not always reflect *in vivo* responses (*84*). However, MAIT cells expanded, albeit at a much lower level, in IL-23-deficient mice suggesting there may be other activation pathways for MAIT cells and that IL-23 may play a synergistic role in the maximal activation of MAIT cells. In addition to their role in clearing bacterial infection, demonstrated by our previous studies (*20*), and by others for different bacteria (*85-87*), it has also been proposed that MAIT cells act to protect barrier integrity (*88*). and have a tissue repair phenotype (Hinks *et al.* bioRivx preprint

doi: https://doi.org/10.1101/490649). At this stage the mechanisms linking MAIT cell expansion and pathogen clearance are unclear.

Vaccination of mice with 5-OP-RU in combination with IL-23 augmented bacterial clearance at early time points (most evident at day 5 post infection), consistent with the MAIT cell vaccination effect shown in our previous study where we primed MAIT cells with 5-OP-RU and CpG (*20*). In contrast, the effect of MAIT cell deficiency on bacterial loads was most evident at day 10 in a primary *L. longbeachae* infection (*20*). Thus, priming of MAIT cells is consistent with the concept that MAIT cells can form a population of cells with memory-like recall properties. Understanding the signals that drive MAIT cells during infection is fundamental to strategies that might enhance protection against subsequent pulmonary infections. Accordingly, our findings not only verify the role of MAIT cells in protective immunity but also suggest that this might be boosted for specific benefit under some circumstances. Since MAIT cells can produce both IFNγ and IL-17, both of which have been shown to be important for clearance of *Legionella* infections (*89-91*), it will be important to determine the mechanisms by which MAIT cells contribute to bacterial clearance in this setting.

Our data demonstrate that MAIT cell activation requirements are broadly similar but distinct from those of other T cells. Ag presented by MR1 constitutes an indispensable signal 1 and both BM-derived and non-BM-derived cells could act as APCs, depending on the pathogen. Among co-stimulating receptors (signal 2), ICOS appears critical for optimal MAIT cell activation. IL-23 acts directly on MAIT cells and may also act indirectly through upregulating ICOS expression, promoting cell proliferation and skewing MAIT cells to a MAIT-17/1 profile. Combined vaccination with IL-23-Ig and 5-OP-RU was able to activate MAIT cells and confer enhanced protection against bacterial infection, demonstrating the therapeutic potential of modulating MAIT cells by harnessing the molecular signals that drive this innate-like T cell subset.

MATERIALS AND METHODS

Mice and infections. Mice were bred and housed in the Biological Research Facility of the Peter Doherty Institute for Infection and Immunity (Melbourne, Victoria, Australia). *Mr1*$^{-/-}$ mice were generated by breeding Vα19*i*C$\alpha^{-/-}$*Mr1*$^{-/-}$ mice (*92*) (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 as previously described (*19*). IL-23RGFP reporter mice (*Il-23r*$^{+/GFP}$) were F1 mice from breeding of C57BL/6 and homozygous *Il-23r*$^{GFP/GFP}$ mice. To generate BM chimeras, recipient mice were lethally irradiated (2 x 550 rad, 3 h apart) to deplete BM-derived cells. Donor BM cells (5 x 10^7) were injected to the recipient mice *via* the tail vein. The mice were then rested for 8 weeks before infection, to allow full reconstitution of their BM-derived cells. Mice aged 6–12 weeks (except BM chimeras) were used in experiments, after approval by the University of Melbourne Animal Ethics Committee.

Compounds, immunogens and tetramers. 5-OP-RU was prepared as described previously (*93*) and diluted to the desired concentration in PBS just prior to use. Murine and human MR1 and β2-microglobulin genes were expressed in *Escherichia coli* inclusion bodies, refolded, and purified as described previously (*2, 94*). MR1-5-OP-RU and MR1-6-FP tetramers were generated as described previously (*1*). CD1d-αGalCer tetramer-PE was kindly provided by Prof. Dale Godfrey (University of Melbourne).

Bacterial strains. Cultures of *Legionella longbeachae* NSW150 were grown at 37°C in buffered yeast extract (BYE) broth supplemented with 30-50 μg/ml streptomycin for ~16 h with shaking at 180 rpm to reach log-phase (OD_{600nm} 0.2-0.6). For the infecting inoculum, bacteria were re-inoculated in pre-warmed medium for a further 2–4 h culture (OD_{600} 0.2–0.6), and with the estimation that 1 OD_{600}=5x10^8/ml, sufficient bacteria were washed and diluted in phosphate buffered saline (PBS) with 2% BYE for i.n. delivery to mice. A sample of inoculum was plated onto buffered charcoal yeast

extract (BCYE) agar plates with streptomycin for verification of bacterial inoculum dose by counting colony-forming units (CFU).

For infection of adoptive transfer donor-mice with *Salmonella* Typhimurium BRD509 cultures were prepared as previously described (*19*). SL1344 and SL1344Δ*invA* (χ4370; originally provided by Prof. R. Curtiss III, Department of Biology, Washington University, St. Louis, MO) were grown in Luria broth (LB) or on Luria agar plates containing appropriate antibiotics. Inoculum preparation and intranasal infection of mice were performed as previously described (*19*).

For infection of cultured cells with *S.* Typhimurium or mutants, human and murine cells (10^6/well) of either epithelial (h: BSB-II; m: LET1 (*95*)) or macrophage (h: THP-1; m: BMDM) origin were cultured in 24-well plates in triplicates, washed once with DMEM without antibiotics, resuspended in 10% FCS DMEM medium and co-cultured with bacteria prepared at log-phase at a defined multiplicity of infection (MOI) for 1 h. The cells were then washed three times with DMEM medium containing 10% FCS and antibiotics, resuspended in 0.5 ml of 10% FCS DMEM containing 100 μg/ml gentamicin and cultured for 1.5 h at 37 ºC to kill extracellular bacteria. Cells were then harvested, washed twice with PBS, lysed with 0.2 ml of 0.1% digitonin, spread on Luria agar plates containing appropriate antibiotics and incubated at 37 ºC overnight for bacterial counts.

Intranasal infection. Intranasal (i.n.) inoculation with *S.* Typhimurium, *L. longbeachae* or antigens (76 pmol 5-OP-RU) in 50 μl per nares was performed on isofluorane-anesthetized mice. Mice were killed by CO_2 asphyxia at different time points post-infection and the lungs perfused with 10 ml cold RPMI via heart injection before being taken for further processing.

To prepare single-cell suspensions, lungs were finely chopped with a scalpel blade and digested with 3 mg/ml collagenase III (Worthington, Lakewood, NJ), 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. Red blood cells were lysed with hypotonic buffer TAC (Tris-based amino chloride) for 5 min

at 37°C. Approximately 1.5x10^6 cells were stained with MR1-tetramers and antibodies, washed and filtered (40 μm) before flow cytometric analysis.

Determination of bacterial counts in infected lungs. Bacterial load in the lungs was determined by counting CFU obtained from plating homogenized lungs in duplicate from infected mice (x5 per group) on buffered charcoal yeast extract (BCYE) agar containing 30 μg/ml streptomycin and colonies counted after 4 days at 37°C under aerobic conditions.

Adoptive transfer. As MAIT cell frequencies are low in naïve C57BL/6 mice, prior to adoptive transfer experiments MAIT cell populations were expanded by intranasal infection with 10^6 CFU *S.* Typhimurium BRD509 in 50 μl PBS for 7 days as previously described (*19*). After 7 days, mice were sacrificed, single cell suspensions prepared and live TCRβ$^+$CD45$^+$MR1-5-OP-RU tetramer$^+$ cells sorted using a BD FACS Aria III. 10^5 MAIT cells were injected into recipient mice through the lateral tail vein. Mice were rested for 2 weeks post transfer to allow the MAIT cell population to settle to homeostasis prior to subsequent infectious challenge.

Jurkat.MAIT reporter cell activation assays. MAIT cell reporter activation assays were performed essentially as previously reported (*2, 37*). Jurkat cells overexpressing the MAIT TCR clone AF-7 (Jurkat.MAIT), were tested for activation by co-incubation with compounds and C1R cells over-expressing MR1 (CIR.MR1) for 16 h. Cells were subsequently stained with PE-Cy7-conjugated anti-CD3 (UCHT1, eBioscience, 1:300), and APC-conjugated anti-CD69 (BD, 1:25) before analysis by on a FACS CantoII (BD) flow cytometer. Activation of Jurkat.MAIT was measured by an increase in surface CD69 expression.

***In vitro* human PBMC assays.** In four independent experiments PBMCs from healthy donors were stimulated with 0.1 μM 5-OP-RU in the absence or presence of 20 ng/ml recombinant human IL-23

for 10-12 days (10 u/ml IL-2 was added at Day 5) prior to re-stimulation with C1R-MR1 APCs in the presence or absence of 5-OP-RU for 5 h. Brefeldin A (BD Biosciences) was added during the last 4 h. Viable cells (defined using Aqua Live/Dead Dye, ThermoFisher) were then stained with anti-CD3 (PE-CF594), anti-CD8α (APC), anti-CD4 (APC-Cy7), anti-Vα7.2 (BV785) mAbs and MR1-5-OP-RU-Streptavidin PE tetramer; after which cells were permeabilised and fixed (eBioscience) prior to intracellular staining with anti-IL-17A (PE-Cy7) and anti-IFNγ (AF700) mAbs. Stained cells were analysed on an LSR Fortessa flow cytometer (BD Biosciences).

Antibodies and flow cytometry. Antibodies against murine CD3 (145-2C11, Pecy7), CD4 (GK1.5, APC-Cy7), CD19 (1D3, PerCP-Cy 5.5), CD137 (1AH2, FITC), CD45.2 (104, FITC), IFNγ (XMG1.2, PE-Cy7), TCRβ (H57-597, APC or PE) and IL-17A (TC11-18H10, PE) were purchased from BD (Franklin Lakes, NJ). Antibodies against CD8a (53-6.7, PE), CD45.1 (A20, PE), CD27 (LG.7F9, APC), RORγt (B2D, APC) and T-bet (4B10, PE-Cy7) were purchased from eBioscience (San Diego, CA). Abs against CD45.1 (A20, FITC), CD28 (E18, FITC), CD154 (MR1, PE), ICOS (7E.17G9, PE), CD86 (PO3, PE), γδTCR (GL3, APC), CD127 (A7R34, APC), CD3 (17A2, PerCPCy5.5) and Lineage cocktail (FITC) were purchased from Biolegend (San Diego, CA). To block non-specific staining, cells were incubated with MR1-6FP tetramer and anti-Fc receptor (2.4G2) for 15 min at room temperature and then incubated at room temperature with Ab/tetramer cocktails in PBS/2% FCS. 7-aminoactinomycin D (Sigma) was added during antibody staining.

Antibodies against human CD3 (UCHT1, PE-AlexaFluor594), TCR-Vα7.2 (3C10, APC), CD161 (HP-3G10, PE-Cy7), TNF (Mab11, Pacific Blue), and viability dye (Zombie Yellow) were purchased from Biolegend. Antibodies against IFNγ (25725.11, FITC) and CD69 (FN50, PE) were purchased from BD, and anti-CD3 (UCHT1, APC) from eBioscience.

Cells were fixed with 1% paraformaldehyde prior to analysis on LSRII, LSR Fortessa or Canto II (BD Biosciences) flow cytometers. For intracellular cytokine staining Golgi plug (BD Biosciences) was used during all processing steps. Cells stimulated with PMA (phorbol 12-myristate 13-

acetate;)/ionomycin (20 ng/ml, 1μg/ml, respectively) for 3 h at 37°C were included as positive controls. FVD (eBioscience) was added for 30 min at 4°C before surface staining. Surface staining was performed at room temperature, and cells were stained for intracellular cytokines using the BD Fixation/Permeabilization Kit (BD, Franklin Lakes, NJ) or transcription factors using the transcription buffer staining set (eBioscience) according to the manufacturers' instructions. Flow cytometric data analysis was performed with FlowJo10 software (Ashland, OR). Gating for MAIT cells was performed as previously described (*20*).

Constructs, hydrodynamic injection and MAIT antigen delivery. IL-23-Ig plasmid (pEF-BOS-IL-23-IgG3) and control-Ig (pEF-BOS-IgG3) constructs were graciously provided by Burkhard Becher, Switzerland. IL-23 expression from the plasmid has been previous characterised (*56, 96*). Hydrodynamic injection was performed as described elsewhere (*97*), by placing prewarmed mice in a prewarmed conical restraining device. 10 μg of a plasmid vector encoding IL-23-Ig or control vector were injected in 1.6–1.8 ml TransIT-EE Hydrodynamic Delivery Solution (MIR 5340, Mirus Bio LLC) over a period of 10 seconds (*98*). MAIT antigen (5-OP-RU, 50 μl, 1 μM) was delivered intranasally 4 times (on day 0, 1, 2 and 4), twice (day 0 and 1) or once (day 0) post hydrodynamic injection. Mice were then killed on day 7 for examination of MAIT cell number and function or left for a month for vaccinated MAIT cells to form memory before challenge with *L. longbeachae as* described previously (*20*).

Detection of IL-23 in bronchoalveolar lavage fluid (BALF) by enzyme-linked immunosorbent assay (ELISA). BAL was performed using an 18 G needle and syringe loaded with 0.6 ml cold PBS. The needle was inserted into the trachea and PBS injected and aspirated 3 times, slowly. About 0.4 ml BALF was recovered from each mouse and kept on ice. Supernatant was collected after centrifugation and aliquots were stored under -80°C for further experiments. The concentration of IL-

23 in BALF was measured using mouse IL-23 Quantikine Elisa Kit (R&D Systems) according to the manufacturers' instructions.

Statistical analysis. Statistical tests were performed using the Prism GraphPad software (version 7.0 La Jolla, CA). Comparisons between groups were performed using Student's t-tests between two groups and one-way ANOVA tests for multiple groups as appropriate. All experiments were performed at least twice independently with similar results.

LIST OF SUPPLEMENTARY MATERIALS

Supplementary Figure 1. Validation of BM chimeras for assessment of MR1-dependent MAIT cell activation. Relates to Figure 1.

Supplementary Figure 2. MAIT cell expansion following bacterial infection is impaired in *Icos*$^{-/-}$ mice. Relates to Figure 3.

Supplementary Figure 3. IL-23 is required for optimal MAIT cell accumulation and activation during pulmonary infection with *L. longbeachae.* Relates to Figure 4.

Supplementary Figure 4. IL-23 is expressed in the lungs from hydrodynamically delivered plasmid DNA and expands MAIT cells in the lungs in combination with 5-OP-RU. Relates to Figures 5 and 7.

Supplementary Figure 5. MAIT cells respond directly to IL-23. Relates to Figure 6.

Supplementary Figure 6. IL-23 drives IL-17 production by human MAIT cells in response to 5-OP-RU. Relates to Figure 7.

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FIGURE LEGENDS

Figure. 1. Both BM-derived and non-BM-derived APCs can drive MAIT cell accumulation in the lungs in response to bacterial infection. **A.** MAIT cell frequency in the lungs of C57BL/6 mice uninfected or at day 7 after intranasal infection with 10^6 *S.* Typhimurium BRD509 (*Salm.*) or 10^4 *L. longbeachae (Leg.)*. Data show individual mice and mean ± SEM. (**, p <0.01; ****, p <0.0001; One-way ANOVA with Dunnett's multiple comparisons). **B.** Schematic of BM chimeras for (C, D). **C.** Frequency, and **D.** absolute number of MAIT cells of WT→WT or WT→ *Mr1*$^{-/-}$ chimeric mice uninfected or on day 7 p.i. intranasally infected with 10^6 *S.* Typhimurium BRD509 (*Salm.*) or 10^4 *L. longbeachae* (*Leg.*). (*, p <0.05; ***, p<0.001; ****; p <0.0001; multiple unpaired t-test). **E.** Lung CD45.2$^+$TCRβ$^+$ cells from *Mr1*$^{-/-}$→WT chimeric mice with or without 10^5 adoptively transferred C57BL/6 MAIT cells 2 weeks after transfer showing reconstitution of MAIT cells in the lungs. **F.** Schematic of protocol and BM chimeras for (G, H). **G.** Frequency, and **H.** absolute number of transferred MAIT cells on day 7 p.i. in *Mr1*$^{-/-}$→ WT chimeric mice, adoptively transferred with 10^5 WT MAIT cells and then intranasally infected with 10^6 *S.* Typhimurium BRD509 (*Salm.*) or 10^4 *L. longbeachae (Leg.)*. (ns, non-significant; ****, p <0.0001; One-way ANOVA with Dunnett's multiple comparisons). Also see Supplementary Figure 1.

Figure 2. Active invasion by *S.* Typhimurium is vital for MAIT cell stimulation *in vivo*. **A.** CD69 expression on Jurkat.MAIT cells, following co-culture (for 16 h) with C1R.MR1 cells and 5-OP-RU or filtered culture supernatant from *S.* Typhimurium SL1344, SL1344Δ*invA* or media control (LB). Data show mean fluorescent intensity (MFI) of gated Jurkat.MAIT cells, with SEM of triplicate samples as error bars. ***, p<0.001; ****, p<0.0001; One-way ANOVA with Tukey's multiple comparisons. The experiment was performed twice with similar results. **B.** Bacterial counts from human and murine cells (10^6/well) of either epithelial (human: BSB-II; mouse: Lung epithelial type 1, LET1 (*95*)) or macrophage (human: THP-1; mouse: Bone-marrow derived macrophages, BMDM) origin after 1 h infection with SL1344 (open circle) or SL1344Δ*invA* (closed circle) *Salmonella* (multiplicity of infection (MOI): 10). **C-D.** MAIT cells as a percentage of αβ-T cells **(C)** and bacterial

load (CFU) **(D)** from lungs on day 6 p.i. of C57BL/6 mice intranasally-infected with 10^5 *S.* Typhimurium SL1344 or SL1344Δ*invA*. Pooled data show mean ± SEM of 7-9 mice per group. (***, p<0.001; ****, p <0.0001; Unpaired t-test). The experiments were carried out independently two times with similar results.

Figure 3. ICOS is highly expressed on MAIT cells and is important for driving their response to infection. **A.** Representative FACS histograms showing expression of CD154, CD28, CD137, CD27 and ICOS on MAIT cells, non-MAIT T cells and B cells from the lungs of uninfected C57BL/6 WT mice. **B.** MAIT cell frequency in the lungs at day 7 p.i. of *Cd80*$^{-/-}$*Cd86*$^{-/-}$, *Icos*$^{-/-}$ and C57BL/6 WT mice, intranasally infected with 10^6 *S.* Typhimurium BRD509 (*Salm.*) or 10^4 *L. longbeachae* (*Leg.*). Data show individual mice and mean ± SEM. (*, p <0.05; ****, p <0.0001; One-way ANOVA with Dunnett's multiple comparisons). **C.** Representative FACS plots and **D.** Stacked plots showing proportion of MAIT cells expressing T-bet and RORγt from naïve, *S.* Typhimurium BRD509- or *L. longbeachae*-infected *Cd80*$^{-/-}$*Cd86*$^{-/-}$, *Icos*$^{-/-}$ and C57BL/6 WT mice (7 days p.i.), mean ± SEM of 5 (naïve) or 8-9 (infected) mice, pooled from 2 experiments. See also Supplementary Figure 2.

Figure 4. IL-23 is required for MAIT cell accumulation and activation during pulmonary infection with *S.* Typhimurium. **A.** Percentage, and **B.** absolute numbers, of MAIT cells isolated from the lungs of gene knockout mice lacking indicated cytokines, following intranasal infection with 10^6 *S.* Typhimurium BRD509 (day 7 p.i.). Data show individual mice and mean ± SEM of 3-15 mice per group. One-way ANOVA with Dunnett's multiple comparisons for infected mice; ****, p <0.0001 (all uninfected mice non-significant). **C.** Representative flow cytometry plots, and stacked plots showing intracellular staining of T-bet and RORγt in MAIT cells from *S.* Typhimurium-infected *Il-12p35*$^{-/-}$, *Il-12p40*$^{-/-}$, *Il-23p19*$^{-/-}$ and WT (C57BL/6) mice (day 7 p.i.). **D.** Flow cytometry plots and **E.** Percentages of pulmonary TCRβ$^+$ lymphocytes (non-MAIT T cells and MAIT cells) producing IL-17A by intracellular staining, directly *ex-vivo* from the lungs of WT (C57BL/6), *Il-23p19*$^{-/-}$ and *Il-6*$^{-}$

$^{-/-}$ mice infected with 10^6 *S.* Typhimurium BRD509 (day 7 p.i.). Data show mean ± SEM as well as individual mice for 8-9 mice per group. (**, p <0.01; ***, p <0.001; ****, p <0.0001; One-way ANOVA with Dunnett's multiple comparisons). See also Supplementary Figure 3.

Figure 5. Infused IL-23-Ig plasmid restores MAIT cell accumulation in *Il-23p19*$^{-/-}$ mice following infection. **A.** Absolute numbers, and **B.** MAIT cell percentage of T cells isolated from the lungs of WT and *Il-23p19*$^{-/-}$ mice intranasally infected with 10^6 *S.* Typhimurium BRD509 (day 7 p.i.). *Il-23p19*$^{-/-}$ mice were treated with 10 μg of plasmid encoding recombinant IL-23-Ig or control Ig by hydrodynamic injection 1 day before infection. Data show mean ± SEM as well as individual mice. (***, p < 0.001; Unpaired two-tailed Student's t-test). **C.** Expression of T-bet and RORγt in MAIT cells from lungs of mice described above. Plots show gated MAIT cells from one representative mouse per group. **D.** Relative ICOS expression (compared to WT C57BL/6 mice) on pulmonary MAIT cells from untreated (nil) WT, *Il-23p19*$^{-/-}$ or *Icos*$^{-/-}$ mice, or WT mice treated with 10 μg IL-23-Ig or control-Ig plasmid for 24 h. Data show mean ± SEM as well as individual mice (***, p<0.001; ****, p<0.0001; One-way ANOVA with Dunnett's multiple comparisons test). See also, Supplementary Figure 4.

Figure 6. MAIT cells express IL-23R and respond directly to IL-23. **A.** GFP expression (indicating IL-23R expression) in TCRβ$^+$ cells isolated from the lungs of IL-23R$^{GFP/+}$ uninfected mice or following intranasal infection with 10^6 *S.* Typhimurium BRD509 (day 7 or day 100 p.i.). **B.** Schematic of MAIT cell transfer and tracking using CD45 congenic markers. Plots show MAIT cells (CD45$^+$TCRβ$^+$MR1-5-OP-RU-Tetramer$^+$) isolated from the lungs of recipient mice colour-coded to match the donor/recipient cells. Fold increase of MAIT cell number after infection is shown. **C.** Total MAIT cells (CD45.2$^+$ endogenous and CD45.1$^+$ adoptively transferred) isolated from the lungs of *Il-23p19*$^{-/-}$ or *Il-23r*$^{-/-}$ mice that received 10^5 MAIT cells (sorted from CD45.1 congenically-labelled mice primed for 7 days with *S.* Typhimurium BRD509), and 2 weeks later were intranasally infected

with 10^6 *S.* Typhimurium BRD509. Data show mean ± SEM as well as individual mice fold change of normalised data at day 7 post infection (**, p <0.01; ***, p <0.001; ****, p <0.0001; ns, p>0.05; One-way ANOVA with Tukey's multiple comparisons test). See also, Supplementary Figure 5.

Figure 7. IL-23 plus synthetic 5-OP-RU antigen is sufficient to induce MAIT cell activation and accumulation *in vivo* and increase protection against infectious challenge. **A.** Absolute numbers of MAIT cells, and **B.** Absolute number of non-MAIT TCRβ$^+$ cells isolated from lungs on day 8 of naïve WT (C57BL/6) mice, or WT mice treated with 10 μg of plasmid encoding recombinant IL-23-Ig or control plasmid by hydrodynamic injection (day 0) and inoculated intranasally with 1-4 doses (as indicated) of 5-OP-RU on day 0, 1, 2, and 4. Data show mean ± SEM as well as individual mice. **C.** Expression of T-bet and RORγt in MAIT cells from lungs of mice described in (A) Plots show data from one representative mouse from each group. **D.** Experimental scheme for vaccination experiments shown in (E). **E.** Bacterial CFU counts of *Legionella* from the lungs at indicated time points after intranasal infection with 10^4 CFU of mice previously untreated (open diamonds) or primed with IL-23-Ig (hydrodynamic injection) alone (open squares), or IL-23-Ig or control-Ig with 5-OP-RU (4 doses intranasally) (closed and open circles, respectively). Data show mean ± SEM as well as individual mice. (**, p<0.01; ***, p<0.001; ****, p<0.0001; One-way ANOVA with Dunnett's multiple comparisons test). See also Supplementary Figure 4.

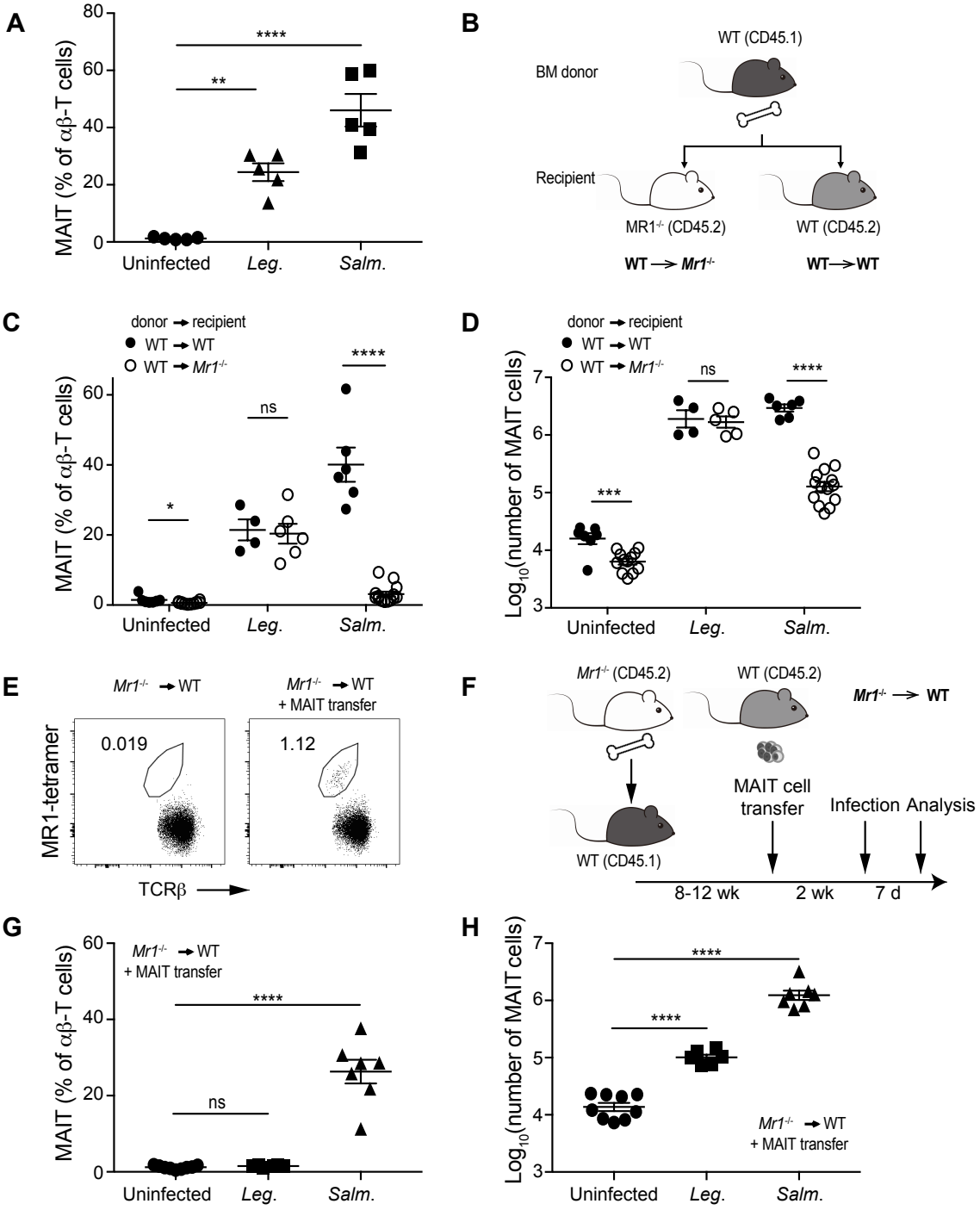
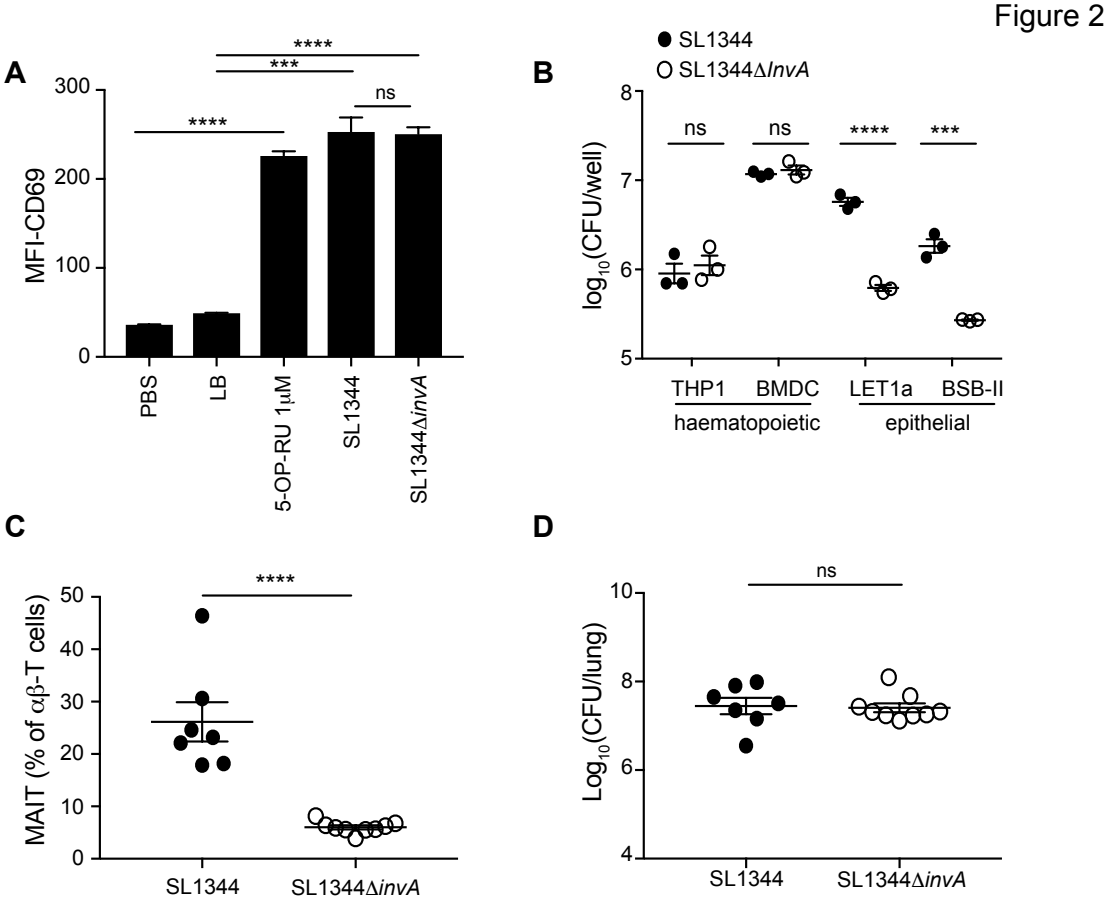

A
MAIT (% of αβ-T cells)
Uninfected
Leg.
Salm.
B
WT (CD45.1)
BM donor
Recipient
MR1-/- (CD45.2)
WT (CD45.2)
WT → Mr1-/-
WT→WT
C
donor → recipient
WT → WT
WT → Mr1-/-
ns
D
Log10(number of MAIT cells)
E
Mr1-/- → WT
Mr1-/- → WT + MAIT transfer
0.019
1.12
MR1-tetramer
TCRβ
F
Mr1-/- (CD45.2)
WT (CD45.2)
Mr1-/- → WT
MAIT cell transfer
Infection
Analysis
WT (CD45.1)
8-12 wk
2 wk
7 d
G
H

Figure 2


A
MFI-CD69
PBS
LB
5-OP-RU 1µM
SL1344
SL1344ΔinvA
****
***
ns
B
SL1344
SL1344ΔInvA
log10(CFU/well)
THP1
BMDC
LET1a
BSB-II
haematopoietic
epithelial
C
MAIT (% of αβ-T cells)
D
Log10(CFU/lung)

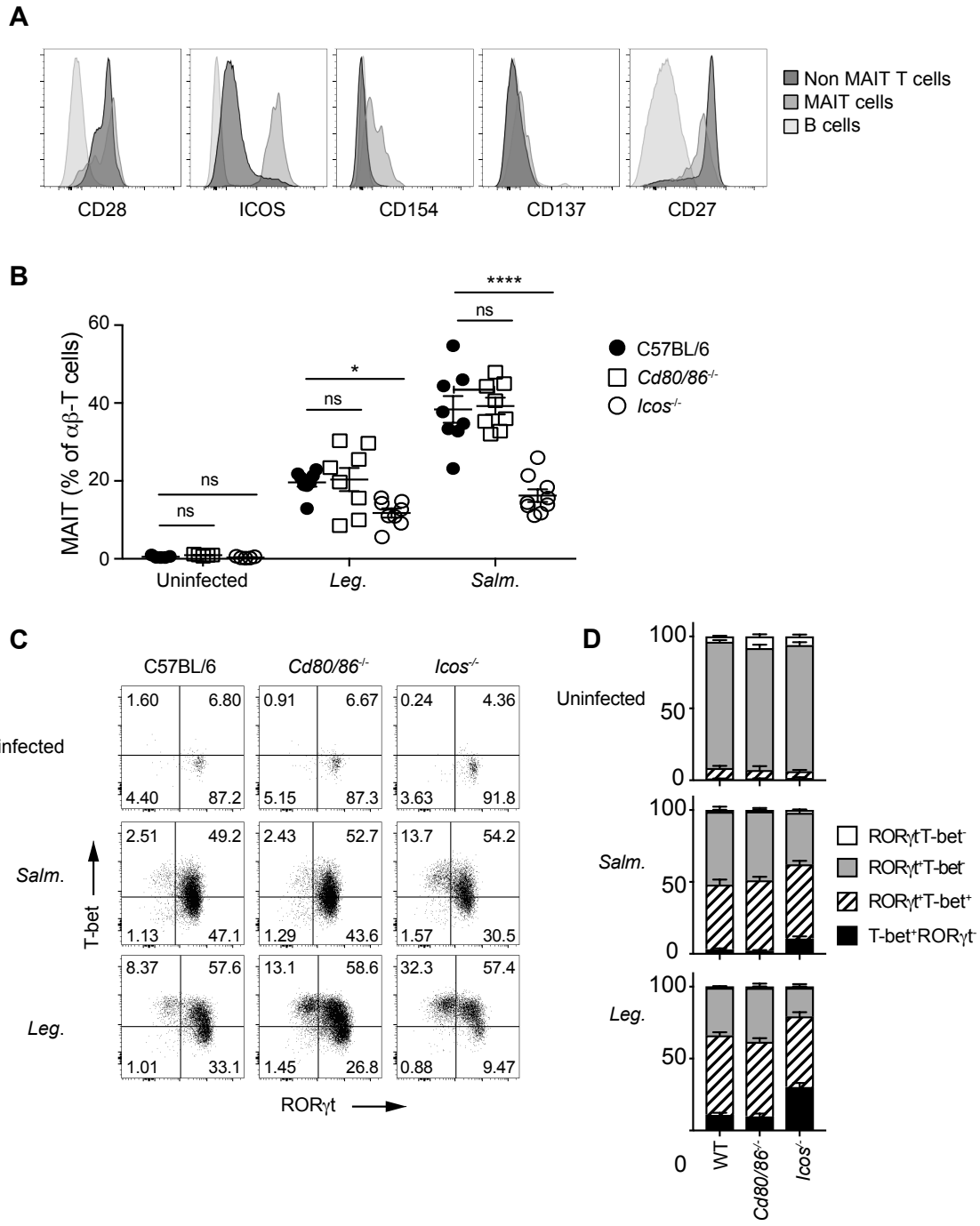

A
Non MAIT T cells
MAIT cells
B cells
CD28
ICOS
CD154
CD137
CD27
B
MAIT (% of αβ-T cells)
60
40
20
0
ns
ns
*
ns
****
C57BL/6
Cd80/86-/-
Icos-/-
Uninfected
Leg.
Salm.
C
C57BL/6
Cd80/86-/-
Icos-/-
Uninfected
Salm.
Leg.
1.60
6.80
4.40
87.2
0.91
6.67
5.15
87.3
0.24
4.36
3.63
91.8
2.51
49.2
1.13
47.1
2.43
52.7
1.29
43.6
13.7
54.2
1.57
30.5
8.37
57.6
1.01
33.1
13.1
58.6
1.45
26.8
32.3
57.4
0.88
9.47
T-bet
RORγt
D
Uninfected
Salm.
Leg.
100
50
0
RORγt+T-bet-
RORγt+T-bet+
T-bet+RORγt-
WT
Cd80/86-/-
Icos-/-

Figure 4

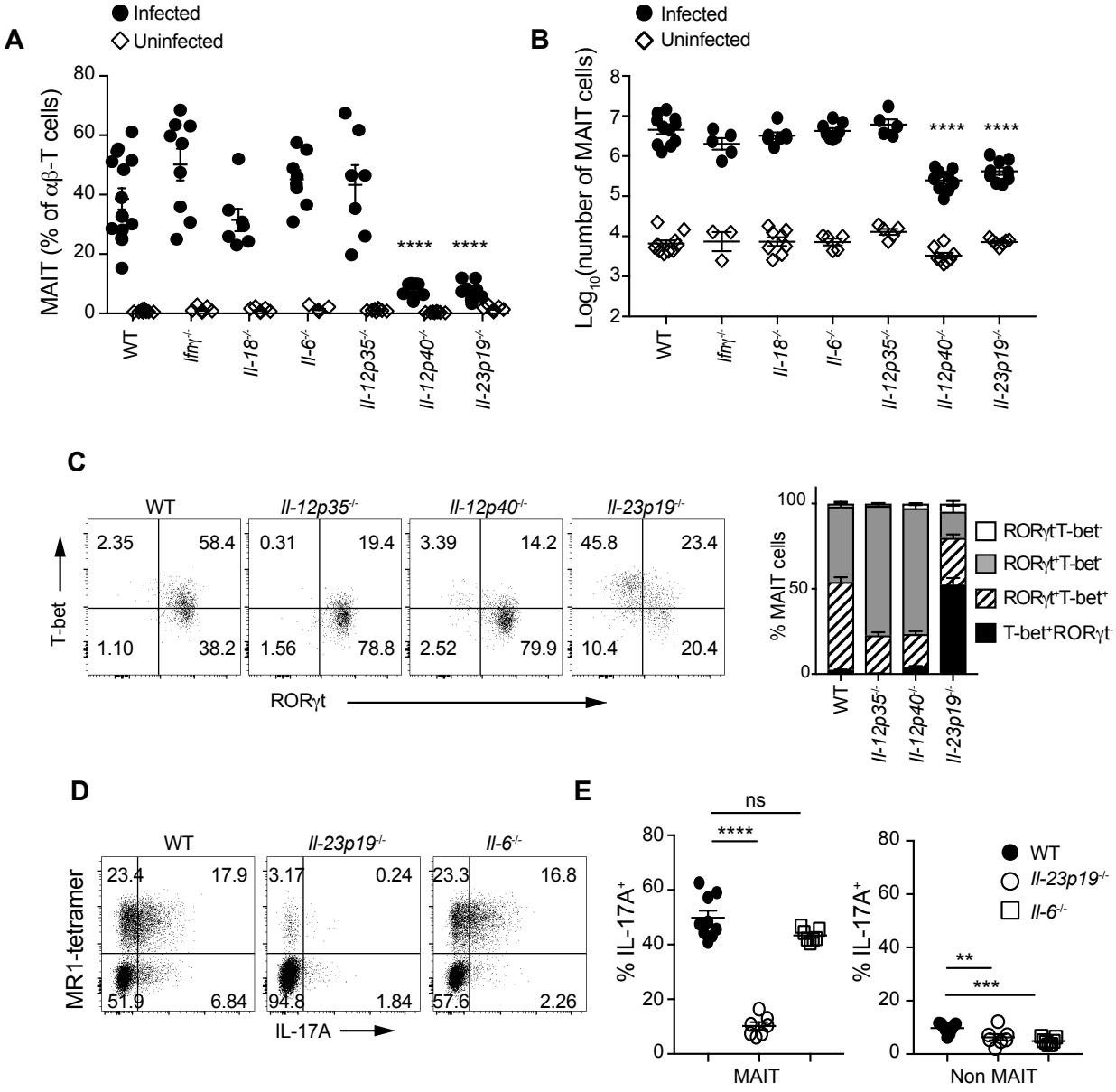
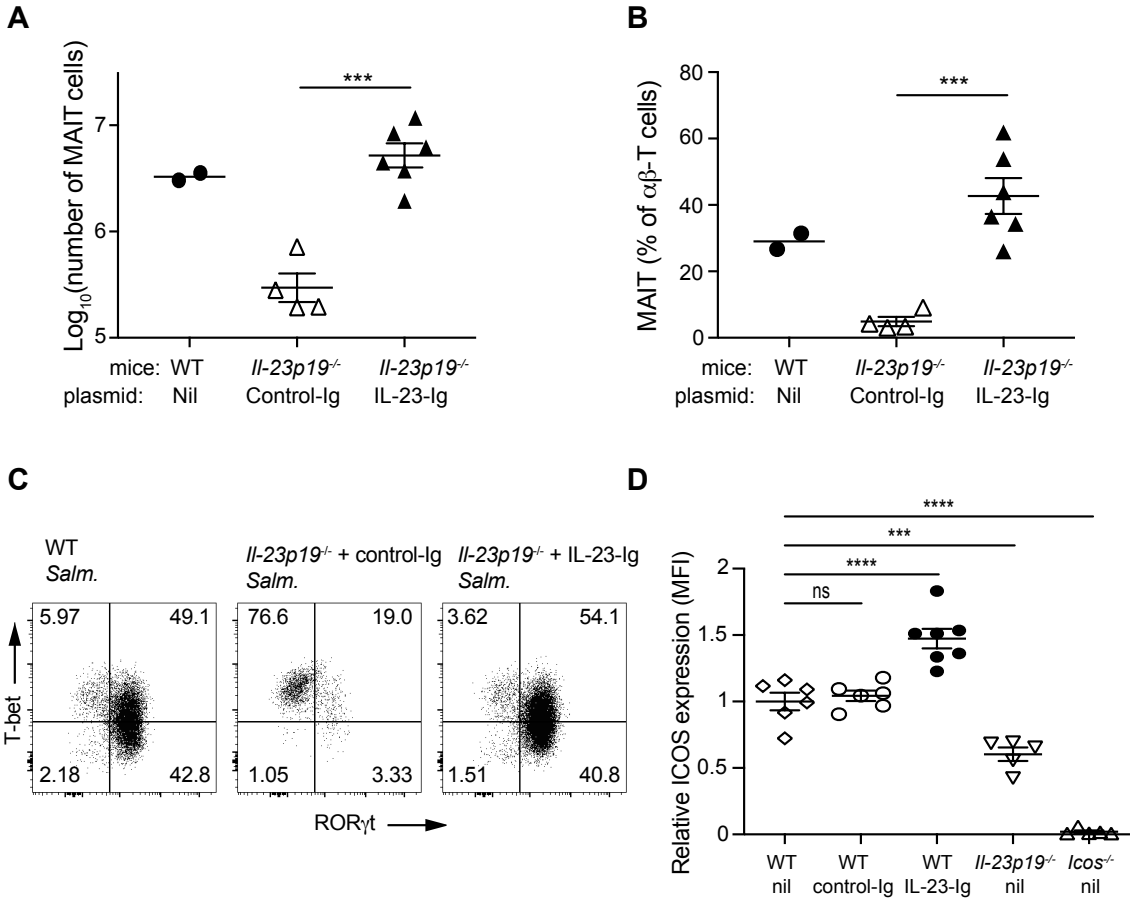

A
Infected
Uninfected
MAIT (% of αβ-T cells)
WT
Ifnγ-/-
Il-18-/-
Il-6-/-
Il-12p35-/-
Il-12p40-/-
Il-23p19-/-
****
B
Log10(number of MAIT cells)
C
T-bet
RORγt
2.35
58.4
1.10
38.2
0.31
19.4
1.56
78.8
3.39
14.2
2.52
79.9
45.8
23.4
10.4
20.4
% MAIT cells
RORγt-T-bet-
RORγt+T-bet-
RORγt+T-bet+
T-bet+RORγt-
D
MR1-tetramer
IL-17A
23.4
17.9
51.9
6.84
3.17
0.24
94.8
1.84
23.3
16.8
57.6
2.26
E
ns
% IL-17A+
MAIT
Non MAIT
**
***

Figure 5


A
B
C
D
***
Log10(number of MAIT cells)
MAIT (% of αβ-T cells)
mice: WT Il-23p19-/- Il-23p19-/-
plasmid: Nil Control-Ig IL-23-Ig
WT
Salm.
Il-23p19-/- + control-Ig
Salm.
Il-23p19-/- + IL-23-Ig
Salm.
5.97
49.1
2.18
42.8
76.6
19.0
1.05
3.33
3.62
54.1
1.51
40.8
T-bet
RORγt
****
***
****
ns
Relative ICOS expression (MFI)
WT nil
WT control-Ig
WT IL-23-Ig
Il-23p19-/- nil
Icos-/- nil

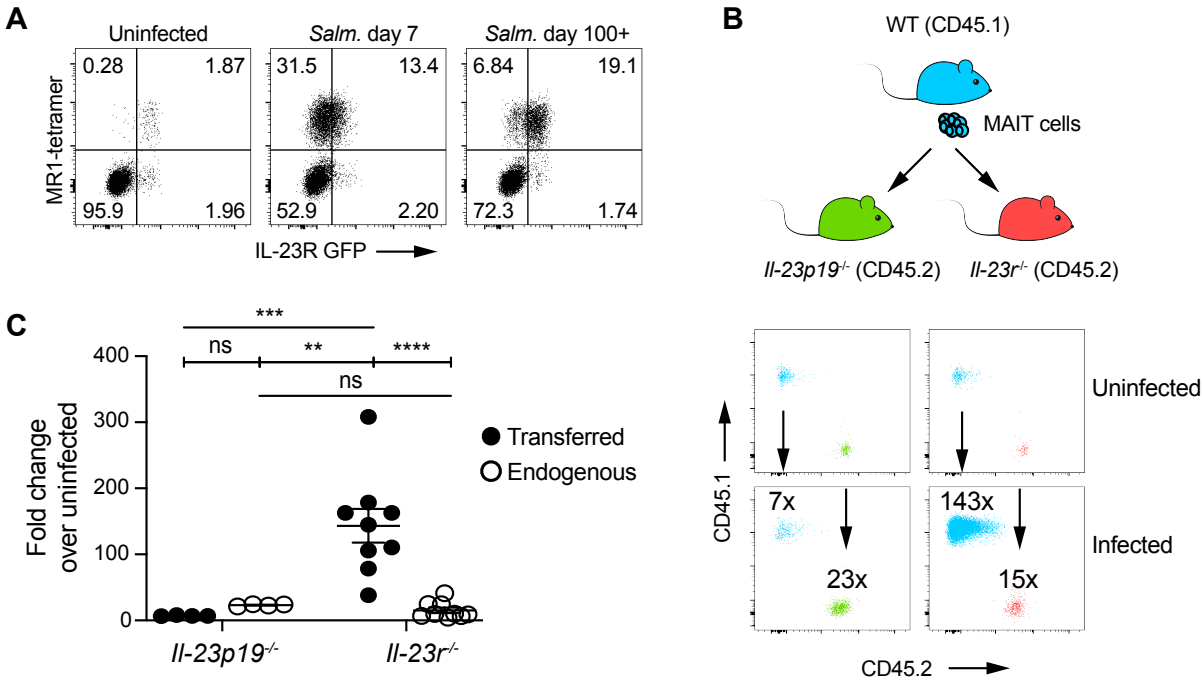
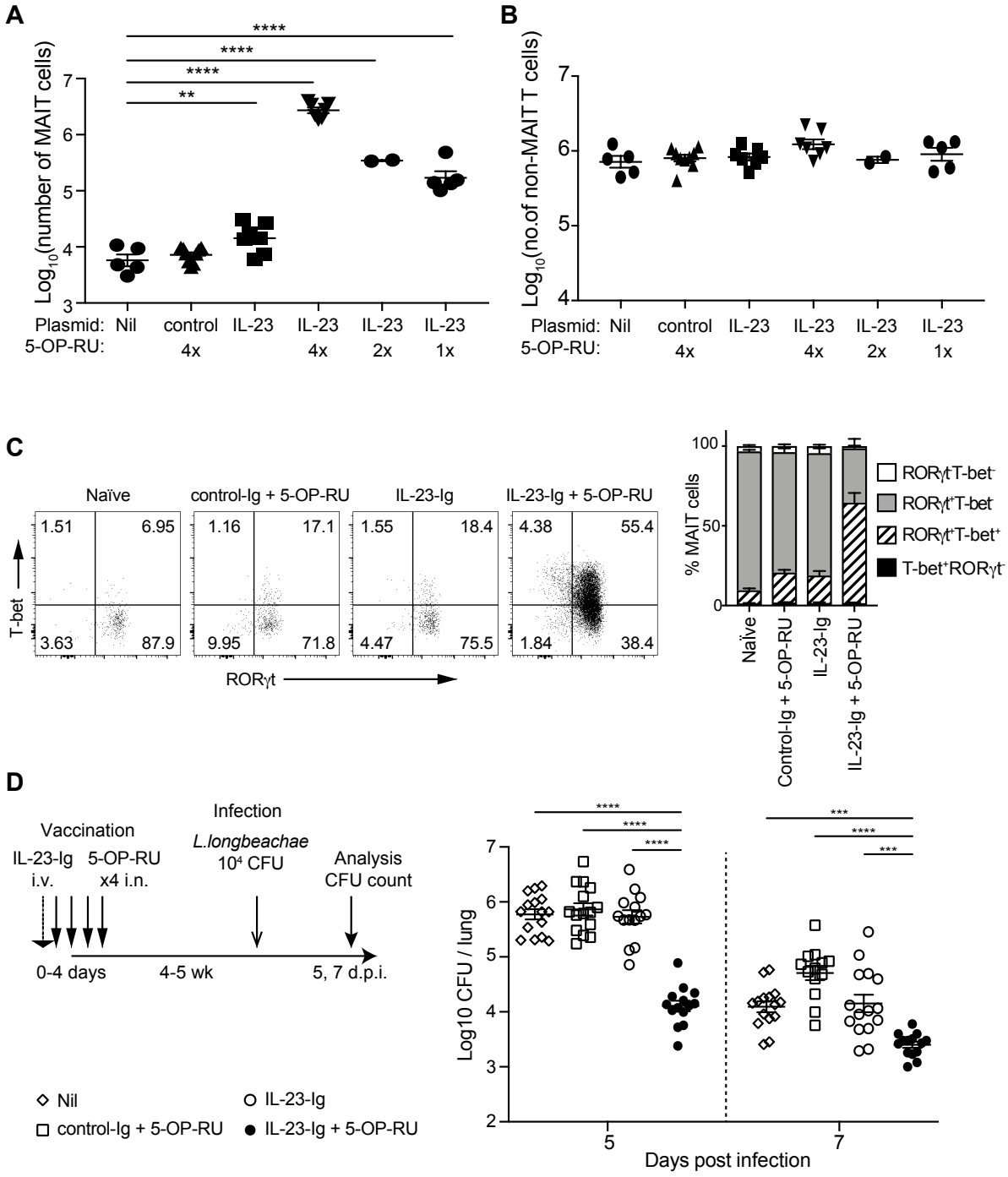

A
Uninfected
Salm. day 7
Salm. day 100+
MR1-tetramer
0.28
1.87
95.9
1.96
31.5
13.4
52.9
2.20
6.84
19.1
72.3
1.74
IL-23R GFP
B
WT (CD45.1)
MAIT cells
Il-23p19-/- (CD45.2)
Il-23r-/- (CD45.2)
CD45.1
CD45.2
Uninfected
Infected
7x
23x
143x
15x
C
Fold change over uninfected
400
300
200
100
0
***
ns
**
****
ns
Transferred
Endogenous
Il-23p19-/-
Il-23r-/-

Figure 7


A
B
C
D
Log10(number of MAIT cells)
Log10(no.of non-MAIT T cells)
Plasmid: Nil control IL-23 IL-23 IL-23 IL-23
5-OP-RU: 4x 4x 2x 1x
Naïve
control-Ig + 5-OP-RU
IL-23-Ig
IL-23-Ig + 5-OP-RU
T-bet
RORγt
% MAIT cells
RORγt-T-bet-
RORγt+T-bet-
RORγt+T-bet+
T-bet+RORγt-
Naïve
Control-Ig + 5-OP-RU
IL-23-Ig
IL-23-Ig + 5-OP-RU
Vaccination
IL-23-Ig i.v.
5-OP-RU x4 i.n.
Infection
L.longbeachae 10⁴ CFU
Analysis CFU count
0-4 days
4-5 wk
5, 7 d.p.i.
Nil
control-Ig + 5-OP-RU
IL-23-Ig
IL-23-Ig + 5-OP-RU
Log10 CFU / lung
Days post infection