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The balance of interleukin-12 and interleukin-23 determines the bias of MAIT1 *versus* MAIT17 responses during bacterial infection

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Abstract

Mucosal-associated invariant T (MAIT) cells are a major subset of innate-like T cells mediating protection against bacterial infection through recognition of microbial metabolites derived from riboflavin biosynthesis. Mouse MAIT cells egress from the thymus as two main subpopulations with distinct functions, namely, T-bet-expressing MAIT1 and RORγt-expressing MAIT17 cells. Previously, we reported that inducible T-cell costimulator and interleukin (IL)-23 provide essential signals for optimal MHC-related protein 1 (MR1)-dependent activation and expansion of MAIT17 cells *in vivo*. Here, in a model of tularemia, in which MAIT1 responses predominate, we demonstrate that IL-12 and IL-23 promote MAIT1 cell expansion during acute infection and that IL-12 is indispensable for MAIT1 phenotype and function. Furthermore, we showed that the bias toward MAIT1 or MAIT17 responses we observed during different bacterial infections was determined and modulated by the balance between IL-12 and IL-23 and that these responses could be recapitulated by cytokine coadministration with antigen. Our results indicate a potential for tailored immunotherapeutic interventions via MAIT cell manipulation.

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INTRODUCTION

T cells provide immunity to a broad range of pathogens through recognition of a diverse range of ligands by conventional T helper (Th) cells, cytotoxic T lymphocytes, natural killer T cells, mucosal-associated invariant T (MAIT) cells and γδ T cells, as well as by differentiation into subsets with diverse functional responses such as secretion of different combinations of cytokines. Th subsets provide tailored responses to different pathogens, such as viruses, bacteria, fungi and parasites. When the quality of T-cell immunity is unsuited to protecting the host,

immunopathology can ensue.[1,2] The differentiation of T cells into Th effector cell subsets is tightly regulated by lineage-specific transcription factors, for example, T-bet for Th1, GATA3 for Th2 and RORγt for Th17 cells. In addition, the polarizing cytokines interleukin (IL)-12 and interferon-gamma (IFNγ) promote Th1 cell generation, while IL-1β, IL-6, IL-23 and transforming growth factor beta are required for differentiation and maintenance of Th17 cells.[3]

MAIT cells are a subset of highly conserved innate-like T cells. They display a restricted repertoire of T-cell receptors (TCRs), consisting of a relatively invariant TCRα chain, TRAV1-2-TRAJ33 (Vα7.2-Jα33) in humans and homologous TRAV1-TRAJ33 (Vα19-Jα33) in mice, paired with one of a limited number of TCRβ chains.[4-6] MAIT cells recognize derivatives of a riboflavin precursor presented by the monomorphic MHC-related protein 1 (MR1), and respond to a wide range of bacteria and fungi capable of riboflavin biosynthesis.[7-10] The antimicrobial function of MAIT cells has been demonstrated using MAIT cell-deficient mice where bacterial infections, including with the pathogens *Francisella tularensis*, *Escherichia coli* and *Legionella longbeachae*, are less efficiently controlled.[11-13] MAIT cells can also be activated *in vitro* by cytokines in an MR1-independent manner, and are implicated in immunity to viral infections, autoimmune diseases and cancer in the absence of known microbial antigens.[14-17]

MAIT cells in mice are mostly $CD4^{-}CD8^{-}$, with smaller proportions of cells expressing CD8 or CD4 coreceptors.[6] They exist as T-bet^{+} MAIT1 and RORγt^{+} MAIT17 subsets, analogous to the Th1 and Th17 subsets of conventional T cells, respectively.[6,18] In contrast to conventional $CD4^{+}$ T cells, which branch into different subsets upon antigen priming in secondary lymphoid tissues, MAIT cells emerge from the thymus with an "effector memory" phenotype ($CD44^{hi}CD62L^{low}$).[19] They encounter their cognate antigen in the thymus, and the differentiation of these two MAIT cell subsets also occurs in the thymus during late stages of development.[19-21] Once mature, MAIT cell subsets egress from the thymus and populate peripheral tissues with distinct tropism.[18] MAIT1 or MAIT17 cells are preferentially activated by distinct pathogens and accumulate at sites of infection where they contribute to host defense. For instance, pulmonary infection with *Salmonella* Typhimurium or *L. longbeachae* triggers an expansion of mostly MAIT17 cells in the lungs, whereas during systemic *F. tularensis* infection, MAIT1 responses dominate and can mediate effective antibacterial protection.[13,22,23] Given MAIT cells are innate-like cells, which differ from conventional T cells in their thymic development, functional subsets, phenotype, tissue location and rapid response to infection, it is important to understand the factors driving the response of each subset. We previously showed that IL-23 and inducible T-cell costimulator (ICOS) costimulate MAIT17 cell responses during bacterial infection.[24] In another study, application of commensal bacteria to the skin induced homeostatic expansion and functional consolidation of MAIT17 cells, which was dependent on IL-18 and IL-1 signaling, respectively.[25] By contrast, the mechanisms, including costimulatory factors, involved in MAIT1 cell activation *in vivo*, have not yet been fully defined, despite the importance of these cells in protective immunity.

In this study, we sought to define the costimulatory requirements that drive the MAIT1 cell response following systemic infection with *F. tularensis*, in a mouse model of tularemia. We found that, as with MAIT17 cells, expansion of MAIT1 cells was not dependent on costimulatory interactions through the CD80/86 pathway. However, unlike MAIT17 cells, expansion of MAIT1 cells was also independent of the ICOS costimulatory pathway. Our results demonstrated that both IL-12 and IL-23 promoted MAIT1 expansion during acute bacterial infection, but IL-12 and not IL-23 was indispensable for maintaining the MAIT1 phenotype and corresponding cytokine production. Accordingly, MAIT1 cells could be systemically expanded in normal mice through antigen challenge with synthetic 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU)[8,26] in combination with IL-12. Moreover, we demonstrated that the bias toward MAIT1 or MAIT17 responses, as observed during different bacterial infections, could be predictably reproduced by varying the balance of IL-12 and IL-23 during antigen exposure. These data suggest that the balance of IL-12 and IL-23 defines the type of MAIT cell responses following pathogen challenge.

RESULTS

MAIT1 cell responses during systemic infection with *F. tularensis* are independent of ICOS and CD80/86

MAIT1 (defined as T-$bet^{+}ROR\gamma t^{-}$) and MAIT17 (RORγt^{+}) cells preferentially expand in the lungs and liver in response to infection with different bacterial pathogens.[13,22,23] Here, we directly compared the MAIT cell response in C57BL/6 mice infected with either *S.* Typhimurium, *L. longbeachae* or *F. tularensis* live vaccine stain (LVS). Consistent with previous studies, *S.* Typhimurium or *L. longbeachae* induced a mostly MAIT17 response, which was evident in the lungs, whereas *F. tularensis* induced a predominantly MAIT1 response in the lungs and liver (Figure 1a and Supplementary figure 1a, b). Considering that distinct patterns of costimulatory molecules and cytokines might be elicited by these pathogens, we hypothesized that these

MAIT1- or MAIT17-skewed responses may reflect the different costimulatory requirements of MAIT1 and MAIT17 cells *in vivo*. A recent study showed that MAIT1 cells were distinguished from MAIT17 counterparts by relatively higher expression of CD28 and lower expression of ICOS molecules,[27] indicating that these costimulatory molecules might be selectively involved in the activation of MAIT cell subsets. To test this hypothesis, we utilized an *F. tularensis* LVS infection model in which robust MAIT1 responses dominate.[23] Here, wild-type (C57BL/6), *Cd80/86*$^{-/-}$ (which lack signaling through CD28) or *Icos*$^{-/-}$ mice were intravenously infected with 10^4 colony-forming units (CFU) of *F. tularensis* LVS and MAIT cells were examined in the lungs at day 7 after the infection (corresponding to the peak expansion[23]). We found that the absolute number of resultant lung MAIT cells remained unaffected by the absence of either ICOS or CD28 signaling, whereas, consistent with our previous study,[24] non-MAIT αβ T cells were significantly reduced in *Cd80/86*$^{-/-}$ mice compared with wild-type mice (Figure 1b). To further assess whether ICOS- or CD28-mediated costimulation was required for MAIT1 responses, we analyzed expanded MAIT1 and MAIT17 subsets based on the expression of their corresponding transcription factors, T-bet and RORγt. Both *Cd80/86*$^{-/-}$ and *Icos*$^{-/-}$ mice had comparable numbers of resultant MAIT1 cells with their wild-type counterparts (Figure 1c), despite having a slight decrease in the proportion of MAIT17 cells and an increase in MAIT1 cells (Figure 1d, e). In addition, the MAIT1 cell responses in the liver from the same mice mirrored those results (Supplementary figure 2a–c). This indicates that signaling through neither ICOS nor CD28 is required for the MAIT1 cell response during infection with *F. tularensis*, which differs from the ICOS dependency of MAIT17 cell responses (Supplementary figure 2d).

IL-12 and IL-23 promote MAIT1 cell responses during bacterial infection

Lung and skin MAIT cells in naïve mice express high levels of IL-23 receptor[24,25] and lung MAIT cells respond to infection in an IL-23-dependent manner.[24] However, the majority of responding MAIT cells following pulmonary infection with *Legionella* or *Salmonella* are MAIT17 cells, and thus, the impact of IL-23 on MAIT1 cell responses has not been examined. Therefore, we next assessed whether IL-23 was required for MAIT1 cell responses *in vivo* using infection of mice with *F. tularensis*. In addition, given the critical role of IL-12 in promoting Th1 responses,[28] we reasoned that exposure to IL-12 could enhance MAIT1 cell activation during infection. To test the role of IL-23 and IL-12 in expansion of MAIT1 cells, mice deficient in either IL-23 (*Il-23p19*$^{-/-}$) or IL-12 (*Il-12p35*$^{-/-}$) or both cytokines (*Il-12p40*$^{-/-}$) were intravenously infected with 10^3 CFU of *F. tularensis* LVS and subsequent MAIT cell responses were analyzed in the lungs and livers. Expansion of non-MAIT αβ T cells was significantly impaired in the lungs of IL-12-deficient mice (*Il-12p35*$^{-/-}$ and *Il-12p40*$^{-/-}$) compared with wild-type or *Il-23p19*$^{-/-}$ mice (Figure 2a), consistent with the Th1-dominant phenotype of these cells (Supplementary figure 3a, b). By contrast, total MAIT cell expansion in the lungs appeared unaffected in *Il-12p35*$^{-/-}$ mice but was significantly reduced in both IL-23-deficient mouse strains (*Il-12p40*$^{-/-}$ and *Il-23p19*$^{-/-}$; Figure 2a). Nevertheless, further analysis of each subset revealed that the number of MAIT1 cells accumulating in the lungs was significantly reduced in the absence of either IL-23 or IL-12 (Figure 2b). In *Il-12p35*$^{-/-}$ mice, the reduced expansion of MAIT1 cells and corresponding increased expansion of MAIT17 cells resulted in a similar accumulation of the total number of MAIT cells (Figure 2a, b), with an increase in the proportion of MAIT17 cells (Figure 2c, d). Notably, *Il-12p40*$^{-/-}$ mice displayed a more severe defect in MAIT1 cell accumulation than mice deficient in either cytokine alone (Figure 2b), indicating nonredundant or synergistic roles for these two cytokines in driving MAIT1 cell expansion.

Both MAIT and non-MAIT T-cell expansion was enhanced in the livers of *Il-12p35*$^{-/-}$ and *Il-12p40*$^{-/-}$ mice compared with wild-type mice (Supplementary figure 3d *versus* Figure 2a). As IL-12 is known to be important for controlling *F. tularensis* LVS infection in mouse models,[29,30] this enhancement was likely a result of the increased antigen levels caused by less controlled bacterial growth in the absence of IL-12, as the bacterial load was higher in *Il-12p35*$^{-/-}$ or *Il-12p40*$^{-/-}$ mice than in wild-type or *Il-23p19*$^{-/-}$ mice (Supplementary figure 3c). As a consequence, impaired expansion of MAIT1 cell numbers in livers, unlike in lungs, was only observed in *Il-23p19*$^{-/-}$ mice (Supplementary figure 3d). However, *Il-12p35*$^{-/-}$ mice displayed a similar reduction in the proportion of MAIT1 cells in livers as seen in the lungs (Supplementary figure 3e, f *versus* Figure 2c, d).

In addition to affecting expansion of MAIT1 cells, deficiency in IL-12 resulted in lower levels of expression of intracellular T-bet in MAIT1 cells in both *Il-12p35*$^{-/-}$ and *Il-12p40*$^{-/-}$ mice (Figure 2e and Supplementary figure 3g), demonstrating that IL-12, but not IL-23, was further required for stabilizing the MAIT1 phenotype. As reflected by lower T-bet expression, both lung and liver MAIT1 cells from *Il-12p35*$^{-/-}$ mice had reduced production of IFNγ compared with wild-type controls when directly assessed by intracellular cytokine staining without any restimulation (Figure 2f, g).

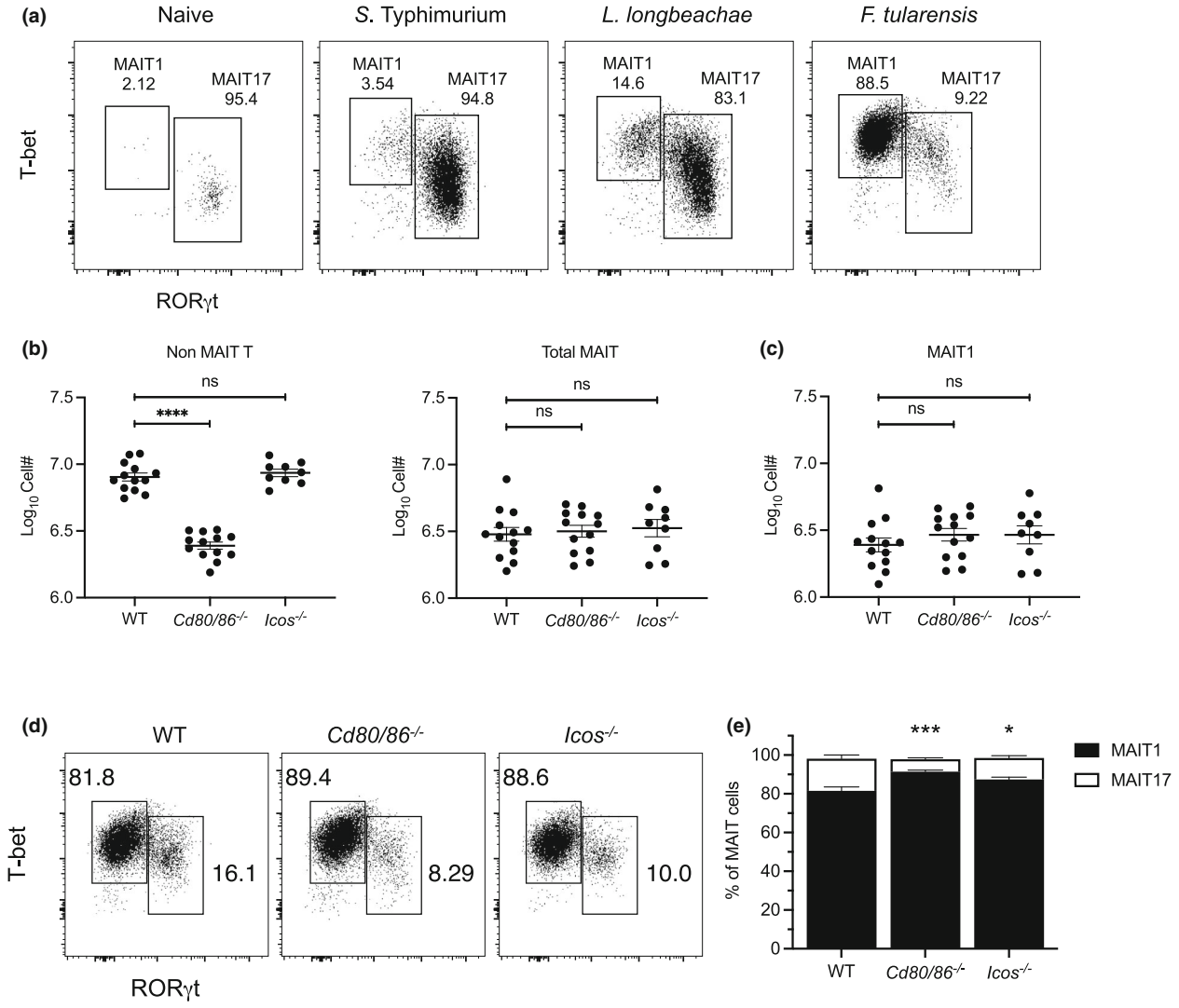

Figure 1. Optimal lung MAIT1 subset response during systemic *Francisella tularensis* infection is independent of inducible T-cell costimulator (ICOS) and CD28. **(a)** Representative flow cytometric plots showing transcription factor profiles of MAIT cells isolated from lungs of C57BL/6 [wild-type (WT)] mice uninfected, or at day 7 after intranasal infection with 10^6 *Salmonella* Typhimurium or 10^4 *Legionella longbeachae*, or at day 7 after intravenous infection with 10^4 CFU *F. tularensis*. Frequency of MAIT1 (T-bet$^+$ RORγt$^-$) and MAIT17 (RORγt$^+$) subsets is indicated. **(b–e)** WT, *Cd80/86*$^{-/-}$ and *Icos*$^{-/-}$ mice were infected with 10^4 *F. tularensis* via the intravenous route. Lungs and livers were harvested for analysis at day 7 after infection. **(b)** Absolute numbers of non-MAIT αβ T cells, MAIT cells and **(c)** MAIT1 cells in the lungs. Data show means ± s.e.m. and individual mice (n = 9 or 13) from three independent experiments. ****$P < 0.0001$, one-way ANOVA with Dunnett's multiple comparisons. **(d)** Representative plots and **(e)** stacked plots showing the frequency of MAIT1 and MAIT17 subsets in the lungs. Data show means ± s.e.m. (n = 9) from two independent experiments. *$P < 0.05$, ***$P < 0.001$, one-way ANOVA with Dunnett's multiple comparisons performed on MAIT1 cell% between WT and each genetic knockout strain. See also Supplementary figures 1 and 2. MAIT, mucosal-associated invariant T; ns, not significant.

Collectively, our results demonstrate that IL-12 and, to a lesser extent, IL-23 are both required for maximum expansion of a MAIT1 population *in vivo*, and that IL-12 additionally contributes to sustaining T-bet expression and Th1 cytokine (IFNγ) production by these cells. This validates our hypothesis that MAIT1 cells have activation requirements that are distinct from those of their MAIT17 siblings.

Exogenous IL-12 and IL-23 restored MAIT1 cell expansion in IL-12- and IL-23-deficient mice during acute *F. tularensis* LVS infection, respectively

Alterations in MAIT cell numbers in naive *Il-23p19*$^{-/-}$, *Il-12p40*$^{-/-}$ and *Il-12p35*$^{-/-}$ mice, compared with wild-type controls (Figure 3a and Supplementary figure 4a, b), suggested that IL-12 and IL-23 may be required for MAIT

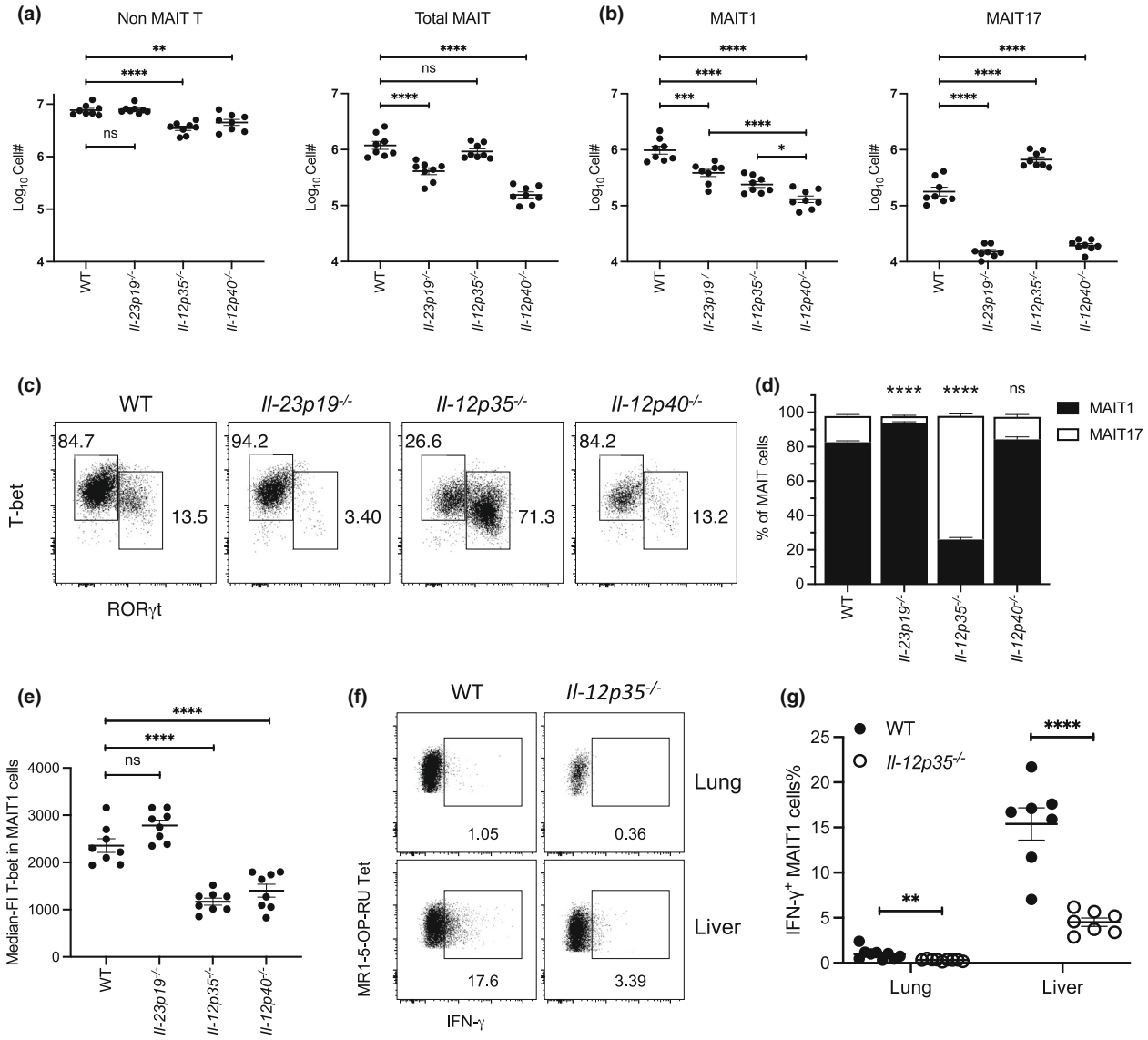

Figure 2. IL-12 and IL-23 promote MAIT1 cell responses during *Francisella tularensis* infection. Wild-type (WT), *Il-23p19*$^{-/-}$, *Il-12p35*$^{-/-}$ and *Il-12p40*$^{-/-}$ mice were infected with 10^3 colony-forming units of *F. tularensis* via the intravenous route. Lungs and livers were harvested for analysis at day 7 after infection. **(a)** Absolute numbers of non-MAIT αβ T cells and MAIT cells in lungs. Data show means ± s.e.m. and individual mice (n = 8) from two independent experiments. **P < 0.01, ****P < 0.0001, one-way ANOVA with Dunnett's multiple comparisons. **(b)** Absolute numbers of MAIT1 and MAIT17 cells in lungs. Data show means ± s.e.m. and individual mice (n = 8). *P < 0.05, ***P < 0.001, ****P < 0.0001, one-way ANOVA with Tukey's multiple comparisons performed between all groups. **(c)** Representative flow cytometry plots and **(d)** stacked plots showing frequency of MAIT1 and MAIT17 subsets in lungs. Data show means ± s.e.m. (n = 8). ****P < 0.0001, one-way ANOVA with Dunnett's multiple comparisons performed on MAIT1 cell percentage between WT and each genetic knockout strain. **(e)** Expression of T-bet in lung MAIT1 cells. Data show means ± s.e.m. and individual mice (n = 8). ****P < 0.0001, one-way ANOVA with Dunnett's multiple comparisons. **(f)** Representative flow cytometric plots and **(g)** scatter plots showing IFNγ production in MAIT1 cells isolated from lungs and livers of WT and *Il-12p35*$^{-/-}$ mice. Cells were cultured with Golgi plug for 4 h without any further stimulation. Data show means ± s.e.m. and individual mice (n = 7) from two independent experiments. **P < 0.01, ****P < 0.0001, unpaired t-test. See also Supplementary figure 3. 5-OP-RU, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil; IL, interleukin; MAIT, mucosal-associated invariant T; MR1, MHC-related protein 1; ns, not significant.

cell development. Therefore, to exclude the possibility that defective MAIT1 responses were inherently established during development in cytokine-deficient mice, we examined the effect of reconstituting cytokine deficiency during acute infection. To this end, a plasmid encoding an IL-12-Ig fusion protein was delivered into *Il-12p35*$^{-/-}$

mice via hydrodynamic injection on day 1 after intravenous. infection with 10^3 CFU *F. tularensis* LVS (Figure 3b). Using this delivery system, a single dose of plasmid injection has been shown to result in transient and systemic expression of plasmid-encoded protein.[24,31] Provision of exogenous IL-12-Ig plasmid to *Il-12p35*$^{-/-}$ mice rescued the impaired expansion of MAIT1 cells in the lungs and further enhanced MAIT1 expansion in the livers (Figure 3c and Supplementary figure 4c), rendering MAIT1 cells dominant among MAIT cell responses, as observed in wild-type mice (Figure 3d and Supplementary figure 4d). In addition, expression of T-bet in MAIT1 cells significantly increased in the presence of IL-12-Ig, although this did not reach equivalent levels to those in wild-type mice (Figure 3e and Supplementary figure 4e). A similar effect of IL-23-Ig on MAIT1 responses was observed in *Il-23p19*$^{-/-}$ mice, whereby delivery of IL-23-Ig-encoding plasmid could rescue the MAIT1 expansion in these mice (Figure 3f and g and supplementary figure 4f, g). The mechanism for this is unclear because, although not possible to assess the expression of IL-23 receptor on MAIT subsets during infection with current tools, in naïve mice, IL-23 receptor expression was almost exclusively observed on ICOS$^+$ MAIT17 cells (Supplementary figure 5). These data make it unlikely that defective MAIT1 responses to infection in cytokine-deficient mice were the result of the developmental impact of the deleted cytokines, and validate the role of IL-12 and IL-23 in the process of peripheral MAIT1 cell activation and expansion during *F. tularensis* LVS infection.

Administration of IL-12-Ig and synthetic MAIT antigen drives systemic MAIT1 cell expansion *in vivo*

We have previously demonstrated that a combination of synthetic antigen 5-OP-RU and cytokine IL-23 was sufficient to trigger expansion of pulmonary MAIT cells and provide protection against *Legionella* infection in mice.[24] These expanded MAIT cells were mostly of the RORγt$^+$ MAIT17 subset.[24] To determine whether MAIT1 cells could be similarly enriched *in vivo*, we infused mice with IL-12-Ig-expressing plasmid, followed by multiple doses of 5-OP-RU via intravenous injection during the first 4 days (Figure 4a). In the absence of 5-OP-RU, provision of IL-12-Ig or IL-23-Ig alone induced only minimal MAIT cell expansion—less than fourfold in lungs and sixfold in livers (Supplementary figure 6a, b). With two injections of 5-OP-RU, a systemic expansion of MAIT cells, up to 55-fold, was readily detectable in the lungs and livers of mice receiving 0.1 or 1 μg IL-12-Ig plasmid at day 7 (Figure 4b, c). No MAIT cell enrichment was observed in mice given the lower dose of 0.01 μg plasmid IL-12-Ig, indicating a minimal threshold for costimulatory signals. Two extra injections of 5-OP-RU further promoted MAIT cell expansion (Figure 4b, c). It is worth noting that mice treated with 1 μg IL-12-Ig plasmid exhibited less MAIT cell expansion than the group dosed with 0.1 μg of antigen (Figure 4b, c), likely because of high levels of expression of the proinflammatory cytokine. Indeed, provision of high doses of IL-12-Ig-expressing plasmid (≥1 μg) led to severe pathology, with mice developing splenomegaly, weight loss and in some cases mortality within 7 days (Supplementary figure 6c, d).

As expected, whereas IL-23-Ig + 5-OP-RU promoted mainly RORγt$^+$ MAIT17 cell expansion, nearly all of the responding MAIT cells in both lungs and livers induced by IL-12-Ig + 5-OP-RU were T-bet$^+$ RORγt$^-$ MAIT1 cells (Figure 4d, e), demonstrating that the combination of IL-12 and synthetic MAIT antigen selectively drives MAIT1 cell responses *in vivo*.

The balance of IL-12 and IL-23 dictates the MAIT1-to-MAIT17 ratio of responding cells

We next assessed MAIT cell responses when mice were infused with titrating amounts of plasmids encoding IL-12-Ig or IL-23-Ig, together with 5-OP-RU delivered intravenously on days 0 and 2 (Figure 4a). To vary the IL-12-to-IL-23 ratio, a fixed dose of IL-23-Ig plasmid was mixed with three graded doses of IL-12-Ig plasmid. At day 7, mice from all groups exhibited a comparable level of MAIT cell expansion in their lungs (Supplementary figure 7a), but a reduction of MAIT cell expansion was observed in the livers with the highest dose of 1 μg IL-12-Ig plasmid (Supplementary figure 7b), consistent with the deleterious effects of excess IL-12 (Supplementary figure 6c, d). Strikingly, we found that the proportion of MAIT1 cells among responding MAIT cells directly correlated with the IL-12-to-IL-23 ratio, as it progressively increased from about 1% in the IL-23-Ig-only-treated group to more than 70% in mice receiving the highest amount of IL-12-Ig plasmid (Figure 5a, b). A full spectrum of MAIT cell responses was obtained with different ratios of MAIT1 and MAIT17 cells (Figures 4e and 5b), creating MAIT phenotypes mimicking those observed during infection with *Salmonella, Legionella* and *Francisella* (Figure 1a). These findings demonstrate that the balance between IL-12 and IL-23 can determine the type of MAIT cell response *in vivo*. In addition, consistent with our previous study,[23] we found that the skewing effect on MAIT cells of these treatments, while not permanent, persisted for at least 3 months, gradually returning toward homeostatic phenotypes. Frequencies of each MAIT subset were recalibrated based on their

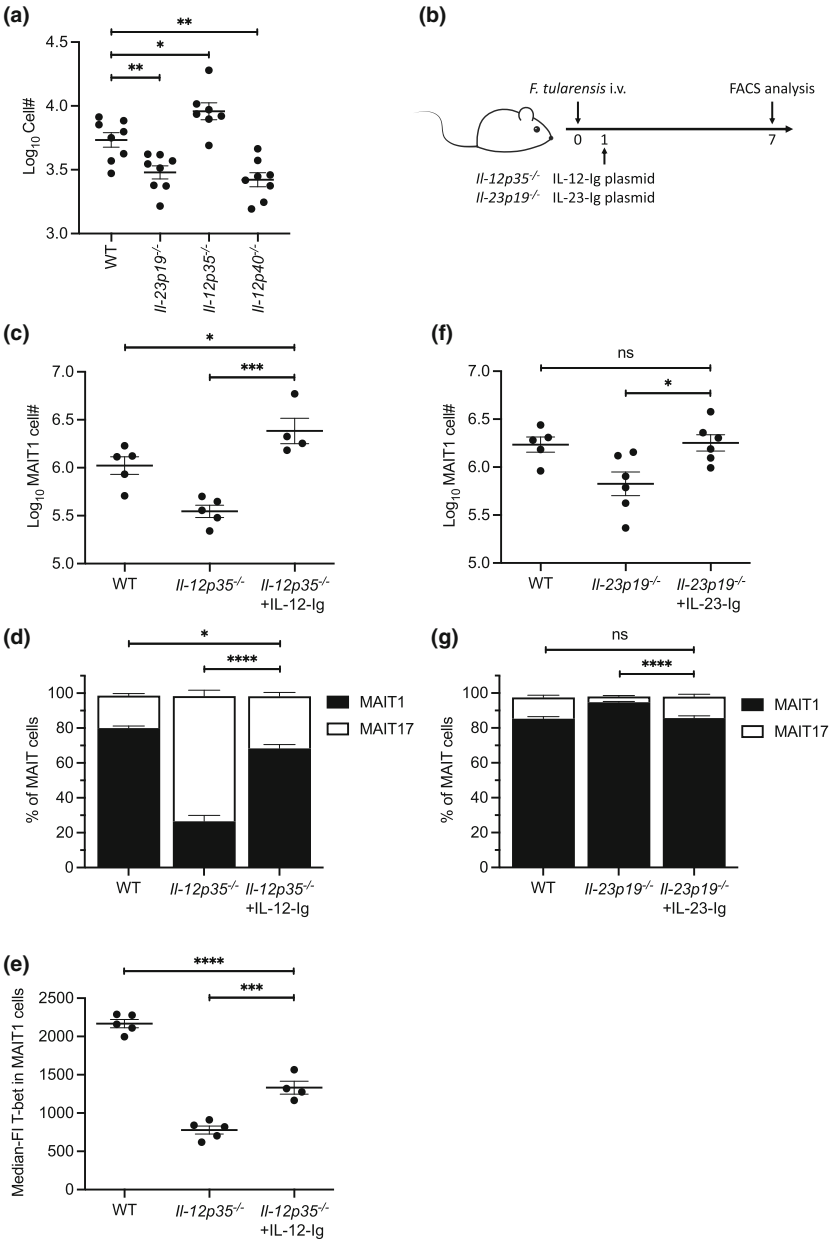

Figure 3. Exogenous IL-12 and IL-23 restores lung mucosal-associated invariant T (MAIT) cell expansion in IL-12- and IL-23- deficient mice respectively. **(a)** Absolute numbers of MAIT cells in the lungs of naïve wild-type (WT), *Il-23p19*$^{-/-}$, *Il-12p35*$^{-/-}$ and *Il-12p40*$^{-/-}$ mice. Data show means ± s.e.m. and individual mice (n = 7 or 8) from two independent experiments. *P < 0.05, **P < 0.01, one-way ANOVA with Dunnett's multiple comparisons. **(b)** Experimental scheme for infection and cytokine reconstitution. **(c–e)** *Il-12p35*$^{-/-}$ and (F-G) *Il-23p19*$^{-/-}$ mice were intravenously infected with 10^3 *Francisella tularensis* and then received 0.02 μg IL-12-Ig- and 2 μg IL-23-Ig-encoding plasmid, respectively, via hydrodynamic injection on day 1 after infection. Lungs and livers were harvested for analysis at day 7 after infection. **(c)** Absolute numbers of MAIT cells in lungs of mice from indicated groups. Data show means ± s.e.m. and individual mice (n = 4 or 5) from two independent experiments. *P < 0.05, ***P < 0.001, one-way ANOVA with Dunnett's multiple comparisons. **(d)** Stacked plots showing frequency of MAIT1 and MAIT17 subsets in lungs. Data show means ± s.e.m. *P < 0.05, ****P < 0.0001, one-way ANOVA with Dunnett's multiple comparisons was performed on MAIT1 cell%. **(e)** Expression of T-bet protein in lung MAIT1 subsets. Data show means ± s.e.m. and individual mice. ***P < 0.001, ****P < 0.0001, one-way ANOVA with Dunnett's multiple comparisons. **(f)** Absolute numbers of MAIT cells in lungs of mice from indicated groups. Data show means ± s.e.m. and individual mice (n = 5 or 6) from two independent experiments. *P < 0.05, one-way ANOVA with Dunnett's multiple comparisons. **(g)** Stacked plots showing frequency of MAIT1 and MAIT17 subsets in lungs. Data show means ± s.e.m. ****P < 0.0001, one-way ANOVA with Dunnett's multiple comparisons was performed on MAIT1 cell%. See also Supplementary figure 4. IL, interleukin; ns, not significant.

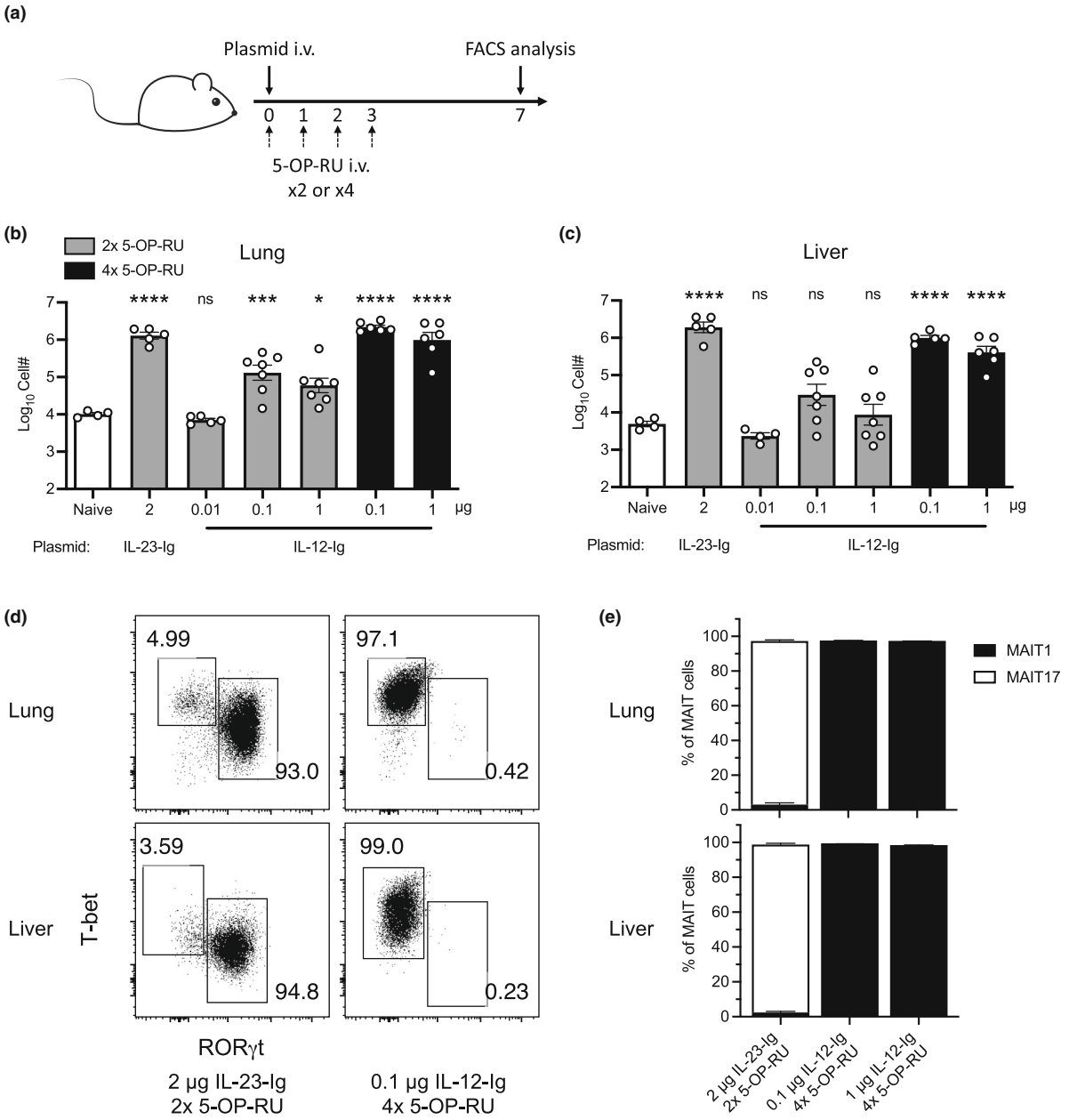

Figure 4. Administration of interleukin-12 (IL-12) and synthetic mucosal-associated invariant T (MAIT) antigen drives systemic MAIT1 cell expansion. **(a)** Experimental scheme for MAIT cell expansion. Wild-type mice were infused with plasmids coding IL-12-Ig or IL-23-Ig via hydrodynamic injection on day 0, followed by two or four doses of synthetic antigen 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) injection (Two doses: days 0 and 2; four doses: days 0, 1, 2 and 3). Lungs and livers were collected and analyzed on day 7. **(b, c)** Absolute numbers of MAIT cells in **(b)** lungs and **(c)** livers of mice untreated or administered with indicated doses of cytokine-coding plasmids and synthetic antigen. Data show means ± s.e.m. and individual mice (n = 4, 5, 6 or 7) from three independent experiments. Data show means ± s.e.m. and individual mice. *P < 0.05, ***P < 0.001, ****P < 0.0001, one-way ANOVA with Dunnett's multiple comparisons was performed between naïve and each treated group. **(d)** Representative flow cytometric plots and **(e)** stacked plots showing frequency of MAIT1 and MAIT17 subsets in lungs and livers of mice with indicated treatments. Data show means ± s.e.m. (n = 3 or 6) from two independent experiments. See also Supplementary figure 5. IL, interleukin; i.v., intravenous; ns, not significant.

locations (Supplementary figure 8), suggesting that some tissue-specific signals foster different polarization of MAIT cell populations.

To test whether MAIT cells were functional when primed *in vivo* using 5-OP-RU in combination with cytokines, we examined cytokine production by MAIT

subsets from the liver, lungs, blood and inguinal lymph nodes using direct *ex vivo* intracellular cytokine staining. IL-17A was exclusively produced by enriched MAIT17 cells (defined by RORγt expression), whereas IFNγ production was mainly found in MAIT1 cells (Figure 5c, d). Despite the expression of T-bet upon activation, only a small fraction (~5%) of MAIT17 (RORγt$^+$) cells produced IFNγ in livers and bloods without further stimulation (Figure 5c, d).

We noticed different co-receptor usage between MAIT1 and MAIT17 cells in MAIT cell-primed mice (Supplementary figure 7c). Although both subsets were mostly CD4$^-$CD8$^-$, the proportion of this population was relatively higher in MAIT17 cells (84% in lungs and 71% in livers) than in MAIT1 cells (60% in both organs). Around 13% of MAIT1 cells in lungs and 21% in livers were found to express CD4, whereas less than 5% of MAIT17 cells were CD4$^+$ (Supplementary figure 7c). In addition, similar patterns of coreceptor expression were observed in MAIT1 and MAIT17 cells from naïve mice (Supplementary figure 7d).

Lastly, we tested whether responses of MAIT cells during *bona fide* infection could be modulated by exogenous IL-12 or IL-23. Mice were intranasally infected with *L. longbeachae*, which drives a MAIT17-skewed response (Figure 1a), then infused with IL-12-Ig plasmid 2 days later. At day 7 after the infection (the peak of MAIT cell expansion[13]), the proportion of MAIT1 cells significantly increased from 19% to 32% and 56% in mice administered 0.1 and 1 μg IL-12-Ig, respectively (Figure 5e, f), indicating that MAIT cell responses during bacterial infection can be further modulated by additional IL-12. Similarly, mice were intravenously infected with the MAIT1-inducing pathogen *F. tularensis*, then infused with IL-23-Ig plasmid in an attempt to elevate the proportion of MAIT17 cells. However, in the face of an extremely polarizing infection with *F. tularensis*, 10 μg IL-23-Ig plasmid failed to shift the subset distribution of MAIT cells in either the lungs or the liver (Supplementary figure 7e, f).

DISCUSSION

While MAIT cells were once regarded as a homogenous polyfunctional population when their cytokine responses were assessed in infection models,[11,13,32] the distinct functions of two MAIT subsets, MAIT1 and MAIT17, have been described for thymic MAIT cells from naïve mice.[6] Our findings reveal that both antigenic stimulation and costimulation with cytokines are required for MAIT1 and MAIT17 cell responses *in vivo*. Although it has been shown that MAIT cells can be activated *in vitro* through cytokines in the absence of TCR stimulation, here IL-12 or IL-23 alone failed to induce robust MAIT cell expansion *in vivo* in the absence of administered synthetic antigen 5-OP-RU. This is consistent with previous studies, where disruptions to microbial riboflavin synthesis or to MR1 antigen presentation nearly abolished MAIT cell expansion in response to bacterial infection or skin commensal association.[22,25] Moreover, antigen (5-OP-RU) alone[22] or with low amounts of IL-12 failed to drive MAIT cell enrichment in lungs and liver, highlighting the necessity for sufficient costimulatory signals. This result is contrary to a report in which topical application of 5-OP-RU was sufficient to induce local expansion of murine cutaneous MAIT cells.[25] Although costimulatory requirements have not been directly assessed for MAIT cells in the skin, the high level of microbial colonization[33] may provide the necessary costimuli at this site. Like conventional T cells, a dual requirement for antigen and cytokines for MAIT cell activation may safeguard against unnecessary MAIT cell responses and may be particularly important, as microbiota-derived antigen is able to pass through intact skin and intestines and circulate systemically.[21] Indeed, a recent multi-omics study revealed that maximal activation of human MAIT cells is only achieved using a combination of cytokines and a TCR stimulus,[34] confirming dual requirements for activating MAIT cells.

IL-12 is a well-defined polarizing cytokine associated with type 1 immune responses, characterized by enhanced IFNγ production and induction of T-bet expression in naïve T cells.[28,35-37] Consistent with this role, we observed impaired expansion and IFNγ production of MAIT1 cells in IL-12-deficient mice, revealing MAIT1 cells as another important target for IL-12. While T-bet expression and concomitant MAIT1 cell commitment occur in the thymus,[27] IL-12 is also required by responding MAIT1 cells for sustained T-bet expression during infection. Consistent with our results, enriched expression of *Il-12rb2* messenger RNA has been detected in murine MAIT1 cells from different organs,[18] suggesting direct IL-12 signaling in MAIT1 cells. Indeed, phosphorylation of the signal transducer and activator of transcription 4 (STAT4), which mediates signal transduction from the IL-12 receptor,[38] has been shown in human MAIT cells in response to IL-12 stimulation.[39] Therefore, we speculate that the IL-12–signal transducer and activator of transcription 4 (STAT4) signaling pathway is also involved in IL-12-induced murine MAIT1 cell responses.

IL-23 is known to facilitate generation of Th17 cells and stabilize IL-17 production,[40] including for both mouse MAIT cells and human MAIT cells,[24] which coexpress T-bet and RORγt,[41-43] while IL-12 has been shown to induce human MAIT cells to produce IFNγ.[44] Surprisingly, we observed that *Il-23p19*$^{-/-}$ mice displayed

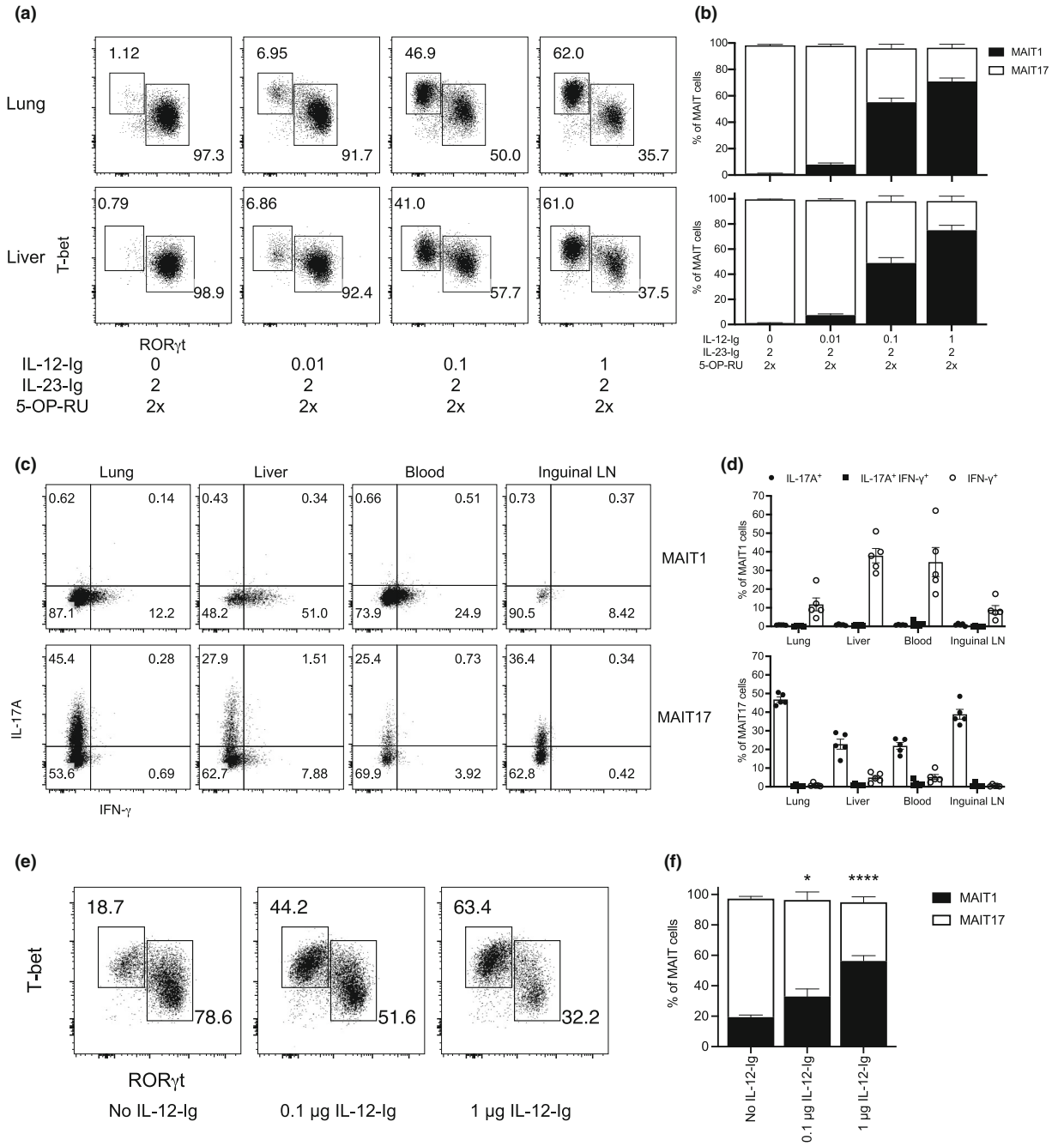

Figure 5. Activation and expansion of mucosal-associated invariant T (MAIT) cell subsets to synthetic antigen or bacterial infection are modulated by the ratio of IL-12 to IL-23. **(a)** Representative flow cytometric plots and **(b)** stacked plots showing frequency of MAIT1 and MAIT17 subsets in lungs and livers at day 7 of wild-type (WT) mice administered with indicated doses of cytokine-coding plasmid and two doses of 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU). Data show means ± s.e.m. and individual mice (n = 7 or 8) from three independent experiments. **(c)** Representative FACS plots and **(d)** scatter plots showing production of IL-17A and interferon-gamma (IFNγ) by MAIT1 and MAIT17 cells, as detected by intracellular cytokine staining, *ex vivo*, from indicated organs of WT mice at day 7 after administration of 0.1 μg IL-12-Ig and 2 μg IL-23-Ig plasmids and two doses of 5-OP-RU. Data show means ± s.e.m. and individual mice (n = 5) from two independent experiments. **(e)** Representative flow cytometric plots and **(f)** stacked plots showing frequency of MAIT1 and MAIT17 subsets in lungs of WT mice at day 7 after intranasal infection with 10^4 *Legionella longbeachae*. Mice were then infused with indicated doses of IL-12-Ig plasmid at day 2 after infection. Data show means ± s.e.m. (n = 6 or 7) from two independent experiments. *P < 0.05, ****P < 0.0001, one-way ANOVA with Dunnett's multiple comparisons performed on MAIT1 cell % compared with the untreated group. See also Supplementary figure 6. IL, interleukin; LN, lymph node.

less MAIT1 cell expansion in lungs and livers during infection and that this defective expansion could be rescued by provision of exogenous IL-23-Ig, suggesting a role for IL-23 in promoting not just MAIT17, but also MAIT1 cell expansion. However, in naïve mice, IL-23 receptor was almost exclusively expressed on MAIT17 cells, consistent with previous RNA-sequencing data showing that murine MAIT1 cells in the liver and spleen express barely detectable *Il-23r* messenger RNA at steady state.[18] A limitation of the current study is that we have not defined the mechanism for costimulatory effects of IL-23 on MAIT1 cells. We speculate that infection may induce expression of the IL-23 receptor on MAIT1 cells upon infection or that IL-23 may act to promote MAIT1 cell expansion in an indirect manner.

In addition to IL-23, other costimulatory effects shared by MAIT1 and MAIT17 cells have been reported. For example, IL-12 not only sustains T-bet expression in MAIT1 cells as described previously, but also triggers a shift in the profile of MAIT17 cells responding to *Legionella* infection from RORγt^{+}T-bet^{-} to RORγt^{+}T-bet^{+}.[24] IL-18 signaling is required for expansion of skin MAIT17 cells during *Staphylococcus epidermidis* association.[25] Although it is dispensable, IL-18 can also augment IFNγ production by MAIT1 cells during *F. tularensis* pulmonary infection.[45] Since there are such similarities in costimulators between MAIT1 and MAIT17 cells, more detailed studies are needed to better understand the downstream signaling pathway involved and genes induced in different MAIT subsets in response to these cytokines. We acknowledge that additional costimulatory signals might exist, as some accumulation of MAIT1 cells was still evident in the IL-12 and IL-23 double-deficient (*Il-12p40*$^{-/-}$) mice.

MAIT cells can express CD4 or CD8 coreceptors, with expression varying between different tissues in mice, although as yet their role in MAIT cell immunity is unknown.[5,6] Here, we show that MAIT1 and MAIT17 cells differ in their coreceptor expression. Interestingly, in human MAIT cells CD4 or CD8 coreceptor expression was found to be associated with different gene expression programs[46] and cytokine production[47] suggesting that, although the proportion of subsets differs between mice and humans,[19] coreceptor expression appears to correlate with functional phenotype in both species. Because the expression of chemokine receptors is driven by the master regulator transcription factors,[48] our results suggest that tissue-specific differences in coreceptor expression may be a consequence of the change in ratio between MAIT1 and MAIT17 cells. Indeed, MAIT17 and double-negative MAIT cells are highly enriched in mucosal and barrier tissues, while MAIT1 cells are mostly found in the liver and spleen where considerable numbers of MAIT cells are either CD8^{+} or CD4^{+}.[6,18,25]

Several groups have now employed similar strategies to boost MAIT cells in mice via synthetic antigen and costimulators (in particular, a range of TLR agonists). Pre-expanded MAIT cells, including with the combination of 5-OP-RU and IL-23 in one study,[24] enhanced the control of infection caused by *L. longbeachae*, *F. tularensis* and *Mycobacterium bovis* BCG, but not by *Mycobacterium tuberculosis*.[13,23,32,49,50] Thus, artificially boosted MAIT cells can effectively contribute to the control of bacterial pathogens. Considering the divergent regulation of cytokine release by TLR signaling,[51] it is reasonable to speculate that different TLR agonists combined with 5-OP-RU induce different MAIT cell subsets, as is observed in response to different bacterial infections. For instance, the TLR9 agonist CpG has been shown to specifically expand MAIT1 cells.[23] Here, we raise a concern that expansion of mismatched MAIT cells may not mediate protective immunity even though they are present in large numbers, or may be harmful. Indeed, MAIT cells have been shown to drive pathology during infection of mice with *Helicobacter pylori*.[52] Our method of boosting MAIT cells with mixed plasmids suggests the possibility of developing customized MAIT cell vaccination strategies to boost protective immunity, while avoiding unwanted pathology.

Collectively, our study demonstrates that MAIT1 and MAIT17 cells have distinct activation requirements. IL-12 and IL-23 are critical for responses of MAIT1 cells and the balance between these two cytokines determines relative MAIT1 and MAIT17 responses generated upon infection or antigen stimulation. Our results highlight the promise of manipulating MAIT cell responses to develop precision immunotherapies.

METHODS

Study design

The aim of the study was to determine the requirements for MAIT1 cell activation *in vivo*, particularly in the context of bacterial infection. Flow cytometry was used to enumerate and characterize MAIT cells, and the bacterial load was measured by CFU counts, from the lungs of wild-type C57BL/6 mice and in different gene-deficient mice. Analyses were conducted after infection with *F. tularensis* LVS, *Legionella* or *Salmonella* and after priming with antigen and cytokines delivered as plasmid DNA. Mouse group sizes were chosen according to the power of the statistical test of each experiment that is depicted in the figures, and the number of independent experiments is listed in the figure captions. Mice were killed at either experimental or humane end points according to institute ethics approvals. The investigators were blinded when performing immunizations and infections but not blinded for analyses. Different groups

of mice were age and sex matched. Both male and female mice were used.

Mice

Mice were bred and housed in the Biological Research Facility of The Peter Doherty Institute for Infection and Immunity (Melbourne, VIC, Australia). IL-23R GFP reporter mice (*Il-23r*$^{+/gfp}$) were F1 mice from breeding of C57BL/6 and homozygous *Il-23r*$^{gfp/gfp}$ mice. Specific pathogen-free, cohoused male or female mice aged 6–12 weeks were used in experiments, after approval by University of Melbourne Animal Ethics Committee (10201).

Compounds, immunogens and tetramers

5-OP-RU was prepared as a solution in dimethyl sulfoxide described previously[26,53] and diluted to the desired concentration in phosphate-buffered saline (PBS) immediately before use. 6-FP was purchased from Shircks Laboratories (Bauma, Switzerland). Murine MR1 and β2-microglobulin genes were expressed in *E. coli* inclusion bodies, refolded and purified as described previously.[9] MR1–5-OP-RU and MR1–6-FP tetramers were generated as described previously.[8]

Bacterial strains and inoculation of mice

Cultures of *F. tularensis* LVS were grown in 10 mL of brain heart infusion broth for 16–18 h at 37°C with shaking at 180 rpm. For the infecting inoculum, with the estimation that 1 OD_{600} (optical density at 600 nm) = 2×10^9 per mL, bacteria from overnight cultures [OD_{600}, 0.3–0.5][42,54] were washed and diluted in PBS for instillation to mice. A sample of inoculum was spread onto cystine heart agar plates with 10 μg mL^{-1} ampicillin, 7.5 μg mL^{-1} colistin and 4 μg mL^{-1} trimethoprim for verification of bacterial concentration by counting CFU. To inoculate mice, *F. tularensis* LVS was delivered in 200 μL PBS by intravenous injection via the tail vein. Mice were weighed and assessed for visual signs of clinical disease, including inactivity, ruffled fur, labored breathing and huddling behavior. Animals that had lost ≥ 20% of their original body weight and/or displayed evidence of pneumonia were killed.

Cultures of *L. longbeachae* NSW150 were grown at 37°C overnight in 10 mL buffered yeast extract broth supplemented with streptomycin (30–50 μg mL^{-1}) with shaking at 180 rpm. For the infecting inoculum, bacteria were reinoculated in prewarmed medium and cultured for 2–4 h to reach log phase [OD_{600}, 0.2–0.6] and with the estimation that 1 OD_{600} = 5×10^8 per mL, sufficient bacteria were washed and diluted in PBS containing 2% buffered yeast extract for intranasal delivery to mice. A sample of inoculum was plated onto buffered charcoal yeast extract agar plates with streptomycin for verification of bacterial inoculum dose by counting CFU. To inoculate mice *L. longbeachae* NSW150 was delivered to isoflurane-anesthetized mice by intranasal instillation in 50 μL PBS containing 2% buffered yeast extract. Detailed protocols for inoculum preparation, intravenous injection and intranasal instillation are described elsewhere.[54]

Determination of bacterial counts in infected livers

Bacterial instillation dose or burden in organs was determined by plating homogenized livers from infected mice on agar plates containing appropriate antibiotics (listed above) and counting colonies after 4 days at 37°C under aerobic conditions.

Constructs, hydrodynamic injection and MAIT antigen delivery

IL-23-Ig plasmid (pEF-BOS-IL-23-IgG3) constructs were generously provided by Burkhard Becher, Switzerland. IL-12-Ig plasmid was derived from pEF-BOS-IL-23-IgG3 by replacing the *IL-23p19* gene with the *IL-12p35* gene. IL-23 expression from the plasmid has been previously characterized.[55] Hydrodynamic injection was performed as described elsewhere.[56] Designated amounts of a plasmid vector encoding IL-23-Ig or IL-12-Ig or plasmid mixture were injected in 1.6–1.8 mL of TransIT-EE Hydrodynamic Delivery Solution (MIR 5340, Mirus Bio LLC, Madison, WI, USA) over a period of 10 s. MAIT antigen (5-OP-RU; 200 μL, 1 μM) was delivered intravenously four times (on days 0, 1, 2 and 3) or two times (on days 0 and 2) after hydrodynamic injection. Mice were closely monitored and then killed on day 7 for examination of MAIT cell number and function.

Flow cytometry and antibodies

Detailed protocols for preparation of samples for flow cytometry from various organs and blood were described elsewhere.[54] In brief, for lungs, perfusion through the heart was performed with 10 mL cold PBS and lung single-cell suspensions were prepared by finely chopping the lungs, followed by collagenase (type IV) digestion, pushing through 70-μm cell strainers and red blood cell lysis. Liver single-cell suspensions were prepared by portal vein perfusion with 10 mL cold PBS and pushing the liver tissue through 70-μm cell strainers, followed by gradient (Percoll, 37% *versus* 70%) centrifugation to enrich lymphocytes and red blood cell lysis. Samples for which there was poor perfusion were excluded in an unbiased manner prior to staining. For blood, MAIT cells were analyzed from 200 μL whole blood after red blood cell lysis. For inguinal lymph nodes, single-cell suspensions were prepared by pushing tissue through 70-μm cell strainers.

Prepared samples were kept on ice prior to staining for flow cytometry. To block nonspecific staining, we incubated cells with MR1-6-FP tetramer and anti-Fc receptor (2.4G2) for 15 min at room temperature and then incubated them at room temperature with Ab/tetramer cocktails in PBS/2% FCS for 30 min. 7-Aminoactinomycin D (Sigma-Aldrich, Merck, Darmstadt, Germany) was added during antibody staining to exclude dead cells. Cells were fixed with 1% paraformaldehyde

before analysis on LSRII or LSRFortessa (BD Biosciences, Franklin Lakes, NJ, USA) flow cytometers. For intracellular cytokine staining, Golgi plug (BD Biosciences) was used during all processing steps. Cells were cultured for 3 h at 37°C. Fixable Viability Dye (eBioscience, San Diego, CA, USA) 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 or transcription factors using the transcription buffer staining set (eBioscience) according to the manufacturer's instructions.

Antibodies against murine CD45.2 (clone 104; catalog number 553772, fluorescein isothiocyanate, 1:200), CD19 (clone 1D3; catalog number 551001, PerCPcy5.5, 1:200), TCR (clone H57-597; catalog number 553174, allophycocyanin, 1:200 or catalog number 553172, phycoerythrin, 1:200 or catalog number 612821, BUV737, 1:200), CD44 (clone IM7; catalog number 612799, BUV737, 1:200), CD4 (clone GK1.5, catalog number 552051, APC-Cy7, 1:200 or catalog number 563331, BV786, 1:200), CD8a (clone 53–6.7, catalog number 563786, BUV395, 1:200) and IL-17A (clone TC11-18H10, catalog number 559502, phycoerythrin, 1:200) were purchased from BD Biosciences. Antibodies against murine CD8a (clone 53–6.7, catalog number 12–0081-83, phycoerythrin, 1:1000), RORγt (clone B2D, catalog number 17–6981-82, allophycocyanin, 1:200) and T-bet (clone: 4B10, catalog number 25–5825-82, PE-Cy7, 1:200) were purchased from eBioscience. Antibodies against IFNγ (clone XMG1.2, catalog number 505806, fluorescein isothiocyanate, 1:200), CD45.2 (clone 104, catalog number 109808, phycoerythrin, 1:200) and CD3 (clone 17A2, catalog number 100218, PerCPcy5.5, 1:200) were purchased from BioLegend (San Diego, CA, USA). Blocking Ab (2.4G2, anti-Fc receptor), MR1-6-FP and MR1-5-OP-RU tetramers were prepared in-house.

Statistical analysis

Flow cytometric data analysis was performed with FlowJo10 software (Ashland, OR, USA). Statistical tests were performed using the Prism GraphPad software (version 9.1 La Jolla, CA, USA). Comparisons between groups were performed using Student's *t*-tests or analysis of variance tests as appropriate, unless otherwise stated.

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AUTHOR CONTRIBUTIONS

Huimeng Wang: Conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft; writing – review and editing. **Adam G Nelson:** Investigation. **Bingjie Wang:** Investigation. **Zhe Zhao:** Investigation. **Xin Yi Lim:** Investigation. **Mai Shi:** Investigation. **Lucy Meehan:** Investigation. **Xiaoxiao Jia:** Investigation; methodology. **Katherine Kedzierska:** Methodology; supervision. **Bronwyn S Meehan:** Investigation; project administration. **Sidonia B.G. Eckle:** Resources. **Michael NT Souter:** Resources. **Troi J Pediongo:** Investigation; resources. **Jeffrey Y W Mak:** Resources. **David P. Fairlie:** Resources; supervision, funding acquisition. **James McCluskey:** Funding acquisition; resources; writing – review and editing. **Zhongfang Wang:** Funding acquisition; resources; supervision; writing – review and editing. **Alexandra J Corbett:** Funding acquisition; project administration; resources; supervision; validation; writing – review and editing. **Zhenjun Chen:** Conceptualization; investigation; methodology; project administration; supervision; validation; writing – review and editing.

CONFLICT OF INTEREST

SBGE, JYWM, DPF, JM, AJC and ZC are inventors on patents (WO2014/005194 and WO2015/149130) describing MR1 tetramers and MR1 ligands. The other authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.