Polyclonal MAIT Cells Have Unique Innate Functions in Bacterial Infection

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Abbreviations: MR1, MHC-related protein 1; MAIT cells, mucosal-associated invariant T cells
ABSTRACT

Mucosal associated invariant T (MAIT) cells are a unique population of αβ T cells in mammals that reside preferentially in mucosal tissues and express an invariant Vα paired with limited Vβ TCR chains. Furthermore, MAIT cell development is dependent upon the expression of the evolutionarily conserved MHC class Ib molecule MR1. Using in vitro assays, recent reports have shown that mouse and human MAIT cells are activated by APCs infected with diverse microbes including numerous bacterial strains, and yeast, but not viral pathogens. However, whether MAIT cells play an important, and perhaps unique, role in controlling microbial infection has remained unclear. To probe MAIT cell function, we show here that purified polyclonal MAIT cells potently inhibit intracellular bacterial growth of Mycobacterium bovis BCG in macrophages (M) in co-culture assays; and this inhibitory activity was dependent upon MAIT cell selection by MR1, secretion of IFN-γ, and an innate IL-12 signal from infected M. Surprisingly, however, the cognate recognition of MR1 by MAIT cells on the infected M was found to play only a minor role in MAIT cell effector function. We also report that MAIT cell-deficient mice had higher bacterial loads at early times after infection compared to wild type (WT) mice, demonstrating that MAIT cells play a unique role among innate lymphocytes in protective immunity against bacterial infection.
INTRODUCTION

Innate and adaptive components of the cellular immune system function sequentially to provide protection against microbial infection. Unlike conventional CD4 and CD8 T cells that must undergo clonal expansion in response to infection, NK cells expressing germline-encoded receptors respond rapidly to kill infected cells or release cytokines, and provide the initial defense during infection. To kinetically bridge the innate and adaptive responses, innate-like T cells, such as iNKT cells, and M3-restricted T cells, respond more quickly than conventional T cells to infection. Innate T cells are less dependent upon clonal expansion than conventional CD4/CD8 T cells, because they appear to detect molecular patterns or danger signals rather than specific peptides bound to polymorphic MHC molecules. For example, iNKT cells use an invariant TCRα chain (Vα24-Jα18 in humans, and the homologous Vα14-Jα18 in mice) combined with a limited set of TCRβ chains to detect the non-classical MHC class I (class Ib) molecule CD1d that binds an assortment of self and microbial lipid antigens. However, iNKT cells display little antigen discrimination since the invariant TCRα chain primarily engages the CD1d-lipid complex while the TCRβ chain makes limited contact (6). Additionally, iNKT cell activation can occur by various pathways some of which do not require the iNKT detection of CD1d (8,74). Therefore, with a limited TCR repertoire, iNKT cells can rapidly secrete cytokines in response to a wide range of infections by bacterial, viral, protozoan, or fungal pathogens. The immunomodulatory role of iNKT cells is well established, but the physiological role of iNKT cells in host immunity remains incompletely understood (4,69). Mucosal-associated invariant T (MAIT) cells, a recently identified T cell subset in mammals, share striking similarities and interesting differences with iNKT cells (73). MAIT cells have been implicated recently in
detection of diverse microbial infections (33,50) raising important questions of whether and how they might function in pathogen protection as an addition to the innate T cell population.

Unlike iNKT cells, MAIT cells preferentially reside in mucosal tissues, and their homotypic expansion is dependent upon commensal microbes (72). In addition, MAIT cells are more abundant in humans than mice, whereas iNKT cells are more abundant in mice than humans (73). Similar to iNKT cells, MAIT cells express a semi-invariant TCR that recognizes a novel non-polymorphic class Ib molecule. More specifically, MAIT cells express an invariant TCRα chain (Vα7.2-Jα33 in humans, and the homologous Vα19-Jα33 in mice) that is preferentially associated with select TCRβ chains (Vβ2, Vβ13, or Vβ22 in humans, and Vβ6 or Vβ8 in mice) (45,71). Most mouse MAIT cells are CD4/CD8 double negative (DN), but some are also singly positive for CD8 or CD4 co-receptors (45,55,72). Human MAIT cells have been reported in either the DN or CD8⁺ subsets (33,55,71). The developmental selection and peripheral activation of MAIT cells is restricted by the non-polymorphic class Ib molecule MHC-related protein 1 (MR1) (72). The selection of MAIT cells in the thymus requires the expression of MR1 on hematopoietic cells likely including double positive (DP) thymocytes, macrophages (M) and dendritic cells (DCs) (13,55,72). The Mr1 gene is highly conserved among mammals (64,78,79). The predicted amino acid sequences of mouse and human MR1 are 89%/90% identical in their 𝛼1/𝛼2 domains. In contrast, mouse and human CD1 𝛼1/𝛼2 domains are only 60%/62% identical (38). Interestingly, the sequence analysis of Mr1 gene orthologs has indicated their strong purifying selection during evolution, suggesting their physiological importance (41). In functional support of this conclusion, mouse and human MAIT cells, like iNKT cells, have the ability to be activated in a cross-species manner (41).
The notion that MR1 presents antigen to MAIT cells is supported by extensive mutagenesis studies (39,56) as well as by the fact that all αβ T cells characterized thus far detect MHC or MHC-like molecules bound by antigen. What is known about the MR1 presentation pathway is that MAIT cell development and activation is independent of TAP (40,71). Moreover, the activation of mouse MAIT cell hybridomas by MR1-overexpressing cell lines was found to be proteasome independent and enhanced by endocytic trafficking (40). Thus, MR1 clearly does not use the same antigen presentation pathway as classical MHC class I (class Ia) molecules. More interestingly, unlike class Ia or CD1 molecules that are constitutively expressed on the plasma membrane of antigen presenting cells (APCs), MR1 proteins reside mostly in the endoplasmic reticulum (ER) and only low levels have been detected at the plasma membrane (13,35,40). However, surface expression of mouse MR1 can be stabilized at the cell surface using selective monoclonal antibodies (mAbs) (13), whereas human MR1 was shown to be inducible on a human lung epithelial cell line after in vitro infection with Mycobacterium tuberculosis (33). Collectively, these findings demonstrate that MR1 activation of MAIT cells represents a novel and unique mechanism of αβ T cell activation that has been conserved throughout mammalian evolution suggesting physiologic importance in host immunity.

In support of their physiologic importance, two recent studies showed that human and mouse MAIT cells can detect microbial infection in an MR1-restricted manner (33,50). In a human study, Gold et al. cloned M. tuberculosis-reactive CD8+ T cells from infected and uninfected individuals, and found in both populations that a high frequency of the M. tuberculosis-reactive T cells are human MR1-restricted MAIT cells (33). Furthermore, these M. tuberculosis-reactive human MAIT cells are also activated by APCs infected with other bacterial strains including Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, but not...
Listeria monocytogenes. A concomitant study by Le Bourhis et al. showed that both mouse and human MAIT cells display activation markers after in vitro co-culture with APCs infected with various strains of bacteria and yeast, but not viral pathogens (50). This highly cross-reactive recognition of microbial-infected APCs by MAIT cells suggests their activation may be more similar to pattern recognition than ligand discrimination. However, the up-regulation of activation markers on MAIT cells was found not to be dependent upon known innate receptors or adaptors including Toll-like receptors (TLRs), Nod-like receptors, MyD88, and TRIF (50). By contrast, innate receptor pathways have been shown to be important in iNKT cell activation both in vivo and in vitro (8,9,15,59,74,75). Regarding the clinical relevance of MAIT cells, the two studies mentioned above reported that patients with ongoing M. tuberculosis infection have fewer MAIT cells in the blood and higher levels in the lung compared with healthy individuals (33,50). This observation suggested that MAIT cells could migrate from the periphery into infected tissues to provide protection during infection.

To probe the role of MAIT cells in infection in vivo, anti-bacterial immune responses have been compared in MR1-deficient (MR1-/-) and wild type (WT) mice; however, findings using this approach thus far have been ambiguous, complicating mechanistic interpretations. For example, Le Bourhis et al. reported that there was no significant difference in bacterial burden between MR1-/- and WT mice infected with M. abscessus (50). This finding was discordant with their observation that M. abscessus activated MAIT cells in vitro. In a subsequent study, Georgel et al. reported that MR1 was not required for bacterial clearance after infection with E. coli, Shigella dysenteriae or Yersinia enterocolitica, but MAIT cells were required for clearance of Klebsiella pneumonia (32). It is unclear why in vitro activation experiments showed that mouse MAIT cells respond to APCs infected with diverse microbes, yet most in vivo
experiments showed that MR1−/− mice which lack MAIT cells are not impaired in their ability to control infection with most bacterial strains tested. A possible explanation of this conundrum is that WT mice lack a sufficient number of MAIT cells to show an anti-bacterial phenotype. Consistent with this explanation, Vα19-Jα33 TCR transgenic mice that overexpress MAIT cells had lower bacterial loads than control mice after infection with *E. coli* or *M. abscessus* (50). However, because the TCR transgenic mice used in these comparisons have few, if any, conventional CD4+ and CD8+ T cells, this model system lacks physiologic context or the ability to address the question of whether MAIT cells have a non-redundant role in controlling bacterial infection (12). Another possible explanation to the inconsistency between *in vivo* and *in vitro* studies is that perhaps an infection model in mucosal tissues, where MAIT cells mainly reside, needs to be employed. To our knowledge, *in vivo* infections that have been reported so far to compare MR1−/− and WT mice are conducted through intraperitoneal injection (32,50). The *in vivo* role of MAIT cells may be overlooked in the systemic challenge, and therefore did not provide agreeable results with the *in vitro* reactivity to diverse microbes.

To determine whether MAIT cells function as innate T cells and what unique role they might play in bacterial infection, we employed *in vitro* and *in vivo* assays and identified a robust phenotype. Using the *M. bovis* Bacillus Calmette-Guerin (BCG) infection *in vitro*, we showed that MAIT cells secrete IFN-γ that enhances the anti-bacterial activity of infected M in a manner dependent upon IL-12/23p40. Unexpectedly, the anti-bacterial activity in these *in vitro* assays was found to be largely independent of MR1 expression by infected M, even though MR1 expression is required during ontogeny for MAIT cells to acquire their innate function. Furthermore, our *in vivo* studies using low-dose aerosol challenge showed that MR1−/− mice had significantly higher bacterial loads in the lung after mycobacterial infection than WT mice.
These findings demonstrate that the antibacterial activity of MAIT cells is unique and not redundant with other innate T cell populations in the immune system.
MATERIALS AND METHODS

Mice

C57BL/6 and IL-12/23p40−/− mice were purchased from The Jackson Laboratory. MR1−/−, Vα19iTgMR1+/+, and Vα19iTgMR1−/− mice (all C57BL/6 background) were gifts from S. Gilfillan (Washington University). The Vα19i transgene in these mice is the canonical TCRVα of MAIT cells share by >90% of mouse MAIT cells; and MAIT cells isolated from the transgenic mice used in this study have limited endogenous TCRVβ usage and surface marker characteristics of polyclonal MAIT cells (45). Mice were bred and maintained under specific pathogen-free conditions and were used by protocols in the studies approved by the Animal Studies Committee of Washington University.

Antibodies and cytokine

Anti-MR1 mAbs 26.5 and 8F2.F9 were reported previously (13,39) and used as a mixture in the in vitro cultures. Anti-IFN-γ (H22) was a gift from R. Schreiber (Washington University). Anti-IL-12/23p40 mAb (C17.8) was purchased from the R&D Systems (Minneapolis, MN). Anti-IL17A (18H10) was purchased from Mabtech (Mariemont, OH). All antibodies were used at the concentration of 10 µg/mL in the in vitro cultures. Recombinant mouse IL-12 was purchased from PeproTech (Rocky Hill, NJ) and reconstituted according to the instruction.

T cell isolation

Spleen cells from naïve mice were aseptically removed and used as the source for T cell isolation. A single-cell suspension was prepared, and red blood cells were lysed with ammonium chloride. Cells were washed, counted, and subjected to magnetic labeling and separation.
according to the instructions of the Pan T cell Isolation Kit II (#130-095-130, Miltenyi Biotec, Auburn, CA). The enriched T cells were used in the \textit{in vitro} cultures.

\textbf{In vitro analysis of the inhibition of intracellular \textit{M. bovis} BCG growth in M by T cells}

The procedures including the \textit{in vitro} culture of bone marrow-derived M, the infection of mycobacteria, the co-culture of M and T cells, and the measurement of intracellular bacterial growth were adopted and modified from published reports and protocols (18,26,61,77). In brief, bone marrow was flushed from femurs of mice with D10 medium which is DMEM (Gibco, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine (Mediatech, Manassas, VA), 10 mM HEPES buffer (Mediatech, Manassas, VA), 1X nonessential amino acids (Mediatech, Manassas, VA), 1 mM sodium pyruvate (Mediatech, Manassas, VA), 100 U/mL penicillin, and 100 µg/mL streptomycin. Red blood cells were lysed, and the remaining bone marrow cells were washed and plated at $2 \times 10^4$ viable cells per well in 96-well plates (flat bottom) in D10 medium supplemented with 20 ng/ml recombinant murine macrophage colony-stimulating factor (rmM-CSF, PeproTech 315-02, Rocky Hill, NJ). Cells were incubated at 37°C in 5% CO$_2$. After 3 days, the attached cells were gently washed with warm antibiotic-free D10$^-$ medium (D10 medium without penicillin and streptomycin). The attached cells were cultured in D10$^-$ medium containing fresh rmM-CSF (20 ng/mL) for an additional 4 days at 37°C in 5% CO$_2$. After the 7-d culture period, bone marrow-derived M formed a confluent monolayer, and the density of M was estimated to be $2 \times 10^5$ cells per well in 96-well plates. M were infected with \textit{M. bovis} BCG (Danish strain) at a multiplicity of infection (MOI) of 3:1 (bacteria:M) in D10$^-$ medium at 37°C in 5% CO$_2$. After overnight culture, infected M were washed with D10$^-$ medium to remove the extracellular \textit{M}. 


bovis BCG before co-culture with purified T cells. Purified T cells were obtained as described above and added to the wells of infected M at the ratio of 1:1 in D10− medium at 37°C in 5% CO2 for 72 h. At the end of co-culture, supernatants were collected for the measurement of the production of cytokines and nitric oxide. Cells were lysed with 0.2% saponin to release intracellular M. bovis BCG. Mycobacteria were radiolabeled with tritiated uridine as previously described (77) to determine the viability of bacteria under different co-culture conditions. Briefly, [5,6-3H]uridine (Perkin Elmer, Waltham, MA) at 1 µCi/mL prepared in Middlebrook 7H9 broth supplemented with 10% ADC enrichment medium (BD BBL™ 211887, Franklin Lakes, NJ) was added to saponin lysates. After incubation at 37°C for 72 h, the tritiated uridine-pulsed mycobacteria were harvested (Tomtec Harvester 96 MACH III M, Hamden, CT) onto glass fiber filters (Perkin Elmer, Waltham, MA) and radioactivity was quantitated by liquid scintillation counting (Wallac MicroBeta TriLux 1450, Perkin Elmer, Waltham, MA). The percentages of M. bovis BCG growth inhibition under different co-culture conditions were calculated by the formula: percent inhibition = 100 − {100 × (disintegrations per minute from T cell-infected M co-cultured wells/ disintegrations per minute from infected M alone wells)}.

Measurement of cytokine and nitric oxide (NO) production

Culture supernatants were collected after 72 hours of co-culture. The concentration of IFN-γ and IL-17A was quantified by standard sandwich ELISAs according to the manufacturer's instruction (R&D Systems, Minneapolis, MN). NO production was measured by the Griess reaction, using commercial Griess reagent (G4410, Sigma-Aldrich, St. Louis, MO). Serially diluted NaNO2 (S-2252, Sigma-Aldrich, St. Louis) (0 to 100 µM) was used to generate a standard curve to quantify the amount of nitrite (NO2−) in supernatants (37,57,62).
**In vivo infection of M. bovis BCG**

WT and MR1<sup>−/−</sup> mice were infected with *M. bovis* BCG (Danish strain) via aerosol or intranasal routes. In the aerosol infection, *M. bovis* BCG was diluted to $10^7$ CFU/mL in PBS with 0.04% Tween 80. A nebulizer (BioAerosol Nebulizing Generator (BANG), CH Technologies, USA) was set to liquid feed at 1 mL/min and air flow at 1L/min to expose mice to *M. bovis* BCG. 20 minutes of exposure to the aerosol resulted in the delivery of 100 to 300 infectious bacilli per mouse lung. In the intranasal infection, WT and MR1<sup>−/−</sup> mice were injected with *M. bovis* BCG via nares ($10^7$ CFU per mouse) under anesthesia laid on their dorsal side on a plastic mouse holder (60). On day 10 and 30 post infection, the lungs were harvested and homogenized. To determine the mycobacterial colonization, the lung homogenate was serially diluted (1:10 and 1:100) and plated on Middlebrook 7H10 agar plates supplemented with 10% OADC enrichment medium (BD BBL<sup>™</sup> 212240, Franklin Lakes, NJ). 2 to 3 weeks later, mycobacteria colony-forming units (CFUs) formed on the agar plates and were counted.

**Ex vivo stimulation of bronchoalveolar lavage (BAL) cells by mycobacterial antigens and IFN-γ ELISPOT analysis**

To determine the IFN-γ response elicited in the lung by *M. bovis* BCG infection, bronchoalveolar lavage (BAL) cells from WT and MR1<sup>−/−</sup> mice were harvested. BAL cells from the same genotype were pooled and used in the ELISPOT assay. The BAL cells were stimulated *ex vivo* with *M. bovis* BCG (MOI = 3) or various antigens derived from *M. tuberculosis* including culture filtrate proteins (CF) (10 µg/mL; Colorado State University, Fort Collins, CO), cell wall (CW) fraction (50 µg/mL; Colorado State University, Fort Collins, CO) and Ag85B
peptide pool (Ag85Bpp) (2 µg/mL; JPT Peptide Technologies GmbH, Berlin, Germany). The frequency of IFN-γ-producing cells in the BAL was measured by ELISPOT analysis. Anti-IFN-γ mAbs, clones AN18 and R4-6A2 (biotinylated), were purchased from eBioscienc (San Diego, CA) and used as the pair to capture the release of IFN-γ of BAL cells. Streptavidin-ALP (Mabtech, Mariemont, OH) and BCIP/NBT substrate (Fisher Scientific, Pittsburgh, PA) were used to visualize the IFN-γ spot-forming units (SFUs). CTL-ImmunoSpot® S6 Micro Analyzer and ImmunoSpot® Professional Software (Shaker Heights, OH) were used to quantitate the SFUs.
RESULTS

MR1-restricted MAIT cells exert innate function and inhibit mycobacterial growth in M

To define the potential role of MAIT cells in controlling bacterial infection, we established an in vitro infection assay whereby purified MAIT cells were co-cultured with M. bovis BCG-infected M. In these co-culture assays, purified transgenic MAIT cells composed of 50% of DN, 25% CD8+ and 25% CD4+ T cells (45, and data not shown) from naïve Vα19iTgMR1+/+ mice were used. As a negative control, purified naïve T cells from Vα19iTgMR1−/− mice were included (45). In these latter mice, T cells that express the Vα19-Jα33 transgene are found in the periphery, but due to the lack of selection by MR1 during development, these T cells do not display MAIT cell characteristics including cytokine secretion, effector/memory phenotype, and bias in the TCRβ usage (45,55). For comparisons, we also included naïve T cells from WT mice that have very low frequency of MAIT cells, and from MR1−/− mice that lack detectable MAIT cells (72). These various populations of naïve T cells were co-cultured with M. bovis BCG-infected M. As shown in Figure 1a and 1b, MAIT cells inhibited the intracellular growth of M. bovis BCG in M by 40-80% (varying in several independent experiments) compared to bacterial growth in M in the absence of MAIT cells. Importantly, control transgenic T cells from Vα19iTgMR1−/− mice showed no inhibition of bacterial growth in M. bovis BCG-infected M (Fig. 1a,b). Thus, the anti-bacterial function of Vα19-Jα33 TCR bearing T cells requires MR1 selection during development. T cells from naïve MR1−/− and WT mice also failed to inhibit the intracellular growth of M. bovis BCG in M suggesting that normal levels of MAIT cells or other innate T cells are not sufficient to control the mycobacterial growth in these co-culture assays (Fig. 1a,b). In addition, MAIT cells, but not control transgenic T cells, enhanced nitric oxide (NO) production by infected M (Fig. 1c) indicating an increase in microbicidal...
activity of M and providing further evidence of the ability of MAIT cells to control bacterial infection.

MAIT cells are a potent source of innate IFN-γ

IFN-γ is a key cytokine for optimal anti-mycobacterial immunity (16,28,29). In the early stage of infection, innate T cells and NK cells are activated by mycobacteria-infected M to release IFN-γ (27,49,66). This innate source of IFN-γ is indispensable for enhancement in bactericidal activity of M including the induction of inducible nitric oxide synthase (iNOS) (54). It has been shown that human MAIT cells produce IFN-γ in response to mycobacteria-infected APCs (33,50). However, whether or not IFN-γ production is required for MAIT cells to control mycobacterial growth was not previously investigated. To address this question, we first determined whether mouse MAIT cells secrete IFN-γ when co-cultured with M. bovis BCG-infected M. As shown in Figure 2a, MAIT cells responding to infected M had high levels of IFN-γ production. By contrast, the supernatant from the co-cultures of Vα19/TgMR1−/−, WT, or MR1−/− T cells showed low, if any, IFN-γ production (Fig. 2a). To test whether IFN-γ production by MAIT cells was required for the control of mycobacterial growth, anti-IFN-γ blocking antibody was added to the co-culture. Anti-IFN-γ antibody ablated the inhibition of bacterial growth by MAIT cells (Fig. 2b), and also suppressed the enhancement of NO production by MAIT cells (Fig. 2c). These findings suggested that MAIT cells are a potent source of innate IFN-γ which is required for their ability to control mycobacterial growth in M.

Activated MAIT cells produce IL-17A in response to infection
In addition to IFN-γ, MAIT cells have also been reported to produce IL-17 in response to mitogenic or cytokine stimulations (10,25). These findings raise the question of whether MAIT cells secrete IL-17 when encountering bacteria-infected APCs. As shown in Figure 3a, MAIT cells produced high levels of IL-17A when co-cultured with *M. bovis* BCG-infected, but not uninfected, M. To determine whether IL-17A secretion was required for MAIT cell control of the intracellular growth, an anti-IL-17A blocking antibody was added to the co-culture. Interestingly, the blockage of IL-17 in the co-culture did not affect the ability of MAIT cells to control the intracellular growth of *M. bovis* BCG in M (Fig. 3b). The combined results (Fig. 2 and 3) suggest that during infection, MAIT cells produce IFN-γ to provide direct control of bacterial growth in infected APCs, whereas their IL-17 production may help stimulate the adaptive immune response (discussed later).

**Effector function of MAIT cells is dependent on the innate signal IL-12 from infected M**

Next, we sought to dissect the mechanism by which APCs activate MAIT cells during infection. iNKT cells can receive two signals from APCs during infection (8,74). One signal is the cognate interaction between CD1d/lipid complexes on APCs and TCRs on iNKT cells (6), whereas the more dominant signal was recently shown to be IL12 secretion by the microbe-infected APCs (9). Our results have demonstrated that MR1 is required for MAIT cells during development to obtain their effector function (Fig. 1 and 2a, and Fig. S1). To determine if cognate recognition of MR1 on infected M is required for MAIT cell activation, anti-MR1 blocking mAbs (13,33,39,50) or MR1−/− M were used in the co-culture assay. Surprisingly, anti-MR1 mAbs did not block MAIT cell production of IFN-γ and IL-17A (Fig. 4a,c) nor enhancement of NO production (Fig. 4e) when co-cultured with WT M infected with *M. bovis*.
BCG. Furthermore, MR1−/− M infected with *M. bovis* BCG elicited comparable MAIT cell responses as infected WT M (Fig. 4b,d,f). Thus, the cognate recognition of MR1 on infected cells was not required for MAIT cytokine responses to infected APCs.

To determine whether the IL-12 signal from infected APCs was responsible for MAIT cell activation, we took advantage of anti-IL-12/23p40 mAb blocking mAb and M from mice deficient in the IL-12/23p40 gene (p40−/−). The co-culture experiments showed that WT M infected with *M. bovis* BCG failed to activate MAIT cells in the presence of anti-IL-12/23p40 mAb (Fig. 4a,c,e). Furthermore, p40−/− M infected with *M. bovis* BCG did not elicit IFN-γ or IL-17A production (Fig. 4b,d) nor support enhancement of NO production by MAIT cells (Fig. 4f).

We next tested whether MR1-recognition and IL-12 secretion were required for the ability of MAIT cells to control the intracellular bacterial growth in infected M. As shown in Figures 5a and c, anti-IL-12/23p40 mAb blocked the anti-bacterial activity of MAIT cells, but anti-MR1 mAbs did not (Fig. 5a,c). Furthermore, MAIT cells were not able to control bacterial growth in p40−/− M, but were still effective in inhibiting bacterial growth in MR1−/− M (Fig. 5b,d). The MAIT cell dependence on IL-12 was also revealed by the addition of anti-IL-12/23p40 mAb in the co-culture with MR1−/− M infected with *M. bovis* BCG where MAIT cell activation and effector function was blocked (data not shown). Thus, these *in vitro* findings highlight the importance of MAIT cell control of intracellular bacterial growth in M by a mechanism that is IL-12-dependent; and that in the presence of an innate IL-12 signal, the requirement of cognate engagement of MR1 by MAIT cells was not detected.

The above observations indicated that the IL-12 signal rather than MR1 recognition may dominate MAIT cell activation and effector function in bacterial infection. However, it
remained possible that an MR1-restricted interaction between MAIT cells and M may be masked by a strong IL-12 signal. To test this possibility, we added anti-MR1 mAbs in the coculture of MAIT cells and p40\textsuperscript{-/-} M infected with \textit{M. bovis} BCG. As shown in Figures 6a and 6b, anti-MR1 mAbs further decreased the already defective production of IFN-\(\gamma\) and IL-17A by MAIT cells in the absence of IL-12 signal from infected M. However, the decrease in MAIT cell cytokine production by anti-MR1 mAbs was not reflected in the control of \textit{M. bovis} BCG growth in p40\textsuperscript{-/-} M (Fig. 6c). These data suggested that the MR1/TCR mediated signal may be weak and/or transient. IL-12 was shown to amplify weak TCR-mediated activation of iNKT cells (9). The addition of recombinant IL-12 stimulated MAIT cells co-cultured with uninfected WT M to release IFN-\(\gamma\) in a dose-dependent manner (Fig. 7). In comparison, control T cells from V\(\alpha\)19i\textsuperscript{+}\textsuperscript{+}MR1\textsuperscript{-/-} mice did not respond to recombinant IL-12. We concluded from these mechanistic studies that the innate signal IL-12 is indispensable for MAIT cell function of controlling bacterial infection. By contrast, certain effector functions of MAIT cells may be MR1 independent, in spite of the critical role of MR1 expression during development.

\textbf{MR1-restricted MAIT cells play a unique role in protective immunity against mycobacterial infection in vivo}

From our \textit{in vitro} observations, MAIT cells appear to function as innate lymphocytes and are capable of controlling mycobacterial infection. To test the hypothesis that MAIT cells play a non-redundant role in providing immunity against infection, we infected MR1\textsuperscript{-/-} mice with a low-dose of \textit{M. bovis} BCG via the aerosol route. At day 10 post infection, MR1\textsuperscript{-/-} mice showed significantly higher bacterial burdens in the lung compared with WT mice (Fig. 8a). However, MR1\textsuperscript{-/-} and WT mice showed similar bacterial loads in the lung at day 30 post infection (Fig. 8a).
These results suggest that MAIT cells contribute to controlling bacterial growth at the early stage of infection. To detect a greater cytokine response, we increased the infection dose and delivered *M. bovis* BCG via the intranasal route. Consistent with the result in the aerosol challenge, MR1<sup>−/−</sup> mice had higher bacterial burden in the lung than WT mice at day 10 after intranasal infection (data not shown). Furthermore, when stimulated *ex vivo* with *M. bovis* BCG and various antigens derived from *M. tuberculosis*, bronchoalveolar lavage (BAL) cells from infected MR1<sup>−/−</sup> mice showed a lower IFN-γ response than BAL cells from WT mice (Fig. 8b). The antigens tested included culture filtrate proteins (CF) and cell wall (CW) fractions from *M. tuberculosis* H37Rv. Of note, a previous study suggested the CW fraction of *M. tuberculosis* stimulated CD8<sup>+</sup> human MAIT cell clones (33). Thus, the lower IFN-γ response of MR1<sup>−/−</sup> BAL cells after infection may result from the lack of MAIT cells responding to mycobacterial antigens *ex vivo*. However, it is difficult to interpret these findings without definitive evidence that MR1 binds a ligand, and if so, whether it is microbial-derived or an infection-induced self-ligand. Alternatively, BAL cells from infected MR1<sup>−/−</sup> mice also showed a lower IFN-γ response to Ag85Bpp, the peptide pool of known immunodominant epitopes of Ag85B recognized by CD4<sup>+</sup> and CD8<sup>+</sup> T cells (1,44,53) suggesting a poorer adaptive immune response in MR1<sup>−/−</sup> mice during *M. bovis* BCG infection. Because IL-17A is known to be important for driving TH1 immunity in the lung after the infections of intracellular pathogens (36,46,47,51), we speculated that the defective IL-17 response in MR1<sup>−/−</sup> mice due to the lack of MAIT cells results in failure to recruit CD4<sup>+</sup> and CD8<sup>+</sup> T cells to the site of infection. In fact, BAL cells from infected MR1<sup>−/−</sup> mice produced less IL-17A than infected WT mice in response to the CW fraction of *M. tuberculosis* (Fig. 8c). The functional relevance of the published reports that MAIT cells accumulated at the site of infections (33,50) is supported by the evidence reported here that MAIT cells are a
potentially significant source of IFN-γ and IL-17A. In any case, the overall implication of these
findings is that MAIT cells not only play a unique role among innate lymphocytes but may also
predispose protective adaptive immune responses during infection.
DISCUSSION

In this study, we provide *in vitro* and *in vivo* evidence that MAIT cells exert unique innate functions to control mycobacterial growth. Using a co-culture assay of purified MAIT cells and mycobacteria-infected M, MAIT cells were found to curtail growth of intracellular bacterial replication associated with the secretion of IFN-\(\gamma\) and IL-17A, both known mediators of immunity against intracellular and extracellular bacterial infection (17,19,22,42,47,63,80).

Furthermore, the "first-line" production of IFN-\(\gamma\) and IL-17A by innate lymphocytes is known to play an important role in bridging innate and adaptive immunity (3,21,36,46,51,67). Although both IFN-\(\gamma\) and IL-17A of MAIT cells were induced during the co-culture with mycobacteria-infected M, the control of mycobacterial growth was only dependent upon IFN-\(\gamma\). Thus, IL-17A secretion by MAIT cells likely has a different role other than the direct impact on bacterial intracellular growth in the mycobacterial infection model used here.

Importantly, the anti-bacterial activity of MAIT cells in our *in vitro* assays was found to be critically dependent upon their *in vivo* development in MR1 positive mice. Although in WT mice MAIT cell development is MR1-dependent, in transgenic mice expressing the MAIT cell canonical V\(\alpha\)19-J\(\alpha\)33 TCR \(\alpha\) chain, V\(\alpha\)19i T cells can develop in both \(Mrl^{+/+}\) and \(Mrl^{-/-}\) mice. However, only MR1-educated V\(\alpha\)19i T cells have a MR1-dependent bias in TCR\(\beta\) chain usage and exhibit effector/memory and cytokine secretion profiles like non-transgenic MAIT cells (45,55). In complete agreement with this conclusion from previous studies, only V\(\alpha\)19i transgenic T cells developing in the MR1-positive mice were found to control intracellular bacterial growth in our *in vitro* assays. Surprisingly, however, these MAIT cells selected by MR1 *in vivo* were found not to rely on MR1 engagement to impair intracellular bacterial replication *in vitro*. Indeed, this conclusion was supported by both the failure of anti-MR1 mAbs
to block the antibacterial activity of MAIT cells and the observation that MAIT cells controlled bacterial growth in MR1⁻/⁻ MΦ. Thus, the MAIT cell effector function in vitro did not require TCR recognition of MR1 on infected APCs. By contrast, our observations suggest a non-cognate pathway of MAIT cell activation mediated by the innate signal IL-12 during mycobacterial infection. Abolishing the IL-12 signal from infected APCs (with the use of anti-IL-12/23p40 mAb or p40⁻/⁻ MΦ) diminished the effector function of MAIT cells including IFN-γ and IL-17A production as well as the inhibition of mycobacterial growth. Of note, when the IL-12 signal was prevented, anti-MR1 mAbs partially inhibited the anti-bacterial activity of MAIT cells, suggesting an MR1-dependent component may be masked by a strong innate IL-12 signal.

Interestingly, besides infection, MAIT cells have been reported to play a regulatory role in mouse models of autoimmunity (10,20,58,68). For example, Croxford et al. showed that MAIT cells provide protection in murine autoimmune encephalomyelitis (EAE) via inducing the IL-10 production by B cells (20). However, this protective mechanism was shown to be MR1-independent and partly ICOS-dependent. More recently, Chiba et al. showed that MAIT cells adoptively transferred into MR1⁻/⁻ mice augment collagen antibody-induced arthritis (10). To explain how MAIT cells modulate the immune response in the absence of MR1, their in vitro analyses showed that MAIT cells are stimulated to proliferate by IL-1β and to produce IL-17A by IL-23 in the absence of TCR signals (10). In the present study, MAIT cells were found to release IFN-γ by the stimulation of IL-12 in the absence of infection. In accordance with these examples of cytokine driven activation, human MAIT cells express high levels of IL-12Rβ2 and IL-23R (2,5). These combined findings point toward a model that after selection by MR1 in thymus, MAIT cells are programmed to respond to innate signals, at least some of which can occur without concurrent MR1 recognition. Alternatively, after egress from the thymus, MAIT
cells adapt by quickly responding to infection (34). During infection, MR1 on either APCs or
MAIT cells themselves may be a sufficient signature to allow MAIT cell activation by an innate
cytokine signal.

Despite the above evidence of the non-MR1-dependent functions of MAIT cells, previously
published studies have provided evidence for cognate interaction between MR1 and MAIT cells
using the same anti-MR1 mAbs used in this study (13,33,39-41,50). In earlier mouse studies,
MAIT cell hybridomas were selected based on TCR usage and a small fraction of these MAIT
hybridomas were found to be activated by MR1-overexpressing cell lines in a manner blockable
by mAbs to MR1 or TCR (13,39,56). More recently, mouse MAIT cells from Vα19 TCR
transgenic mice as well as serologically-isolated polyclonal human MAIT cells were found to
express activation markers after culture with bacteria-infected cells in a manner that was also
blockable with anti-MR1 mAb (50). In addition, numerous CD8+ human MAIT cell clones with
activity against cells infected with *M. tuberculosis* and other bacteria were found to be blocked
by anti-MR1 mAb (33). Although the above studies provide compelling evidence for the
occurrence of the MR1-dependent activation of MAIT cells, its overall importance relative to
non-cognate activation is difficult to assess without a better definition of functional heterogeneity
within polyclonal MAIT cells.

As a notable parallel, the studies of iNKT cells provide a precedent for how innate T cells
bearing limited TCR repertoires employ multiple mechanisms to control microbial infections
(6,8,74). More specifically, iNKT cells react to microbial infections through at least three
activation pathways: microbial antigen driven, cytokine and self-antigen driven, and cytokine
driven (8,74). While microbial and self lipid antigens detected by iNKT cells have been
identified (7,8,48,74), recent studies show that the dominant IL-12 signal from APCs during
microbial infection occurs independent of whether or not microbes express CD1d-presented antigens (9,15). The IL-12 signal is thought to be an essential factor that enables γδ NKT cells to respond to a wide variety of microbes with limited TCR repertoires (8,74). In the case of self-ligand recognition, IL-12 can provide co-stimulation to amplify a weak TCR-mediated signal (8,9,74). Moreover, during viral infection, cytokines including IL-12 and IL-18 dominate the γδ NKT cell activation while CD1d presentation is not required (shown by the use of CD1d−/− mice and/or anti-CD1d mAb) (75). The constitutive expression of high levels of IL-12 receptor on γδ NKT cells provides an explanation for the immediate response of γδ NKT cells to cytokine-driven activation (9). Sharing many innate-like features with γδ NKT cells, it is likely that MAIT cells are also equipped for rapid responses using cognate and non-cognate pathways of activation.

By bridging innate and adaptive immune responses, innate-like T cells are critical for providing the first-line protection against infection (65). In this role, different innate lymphocyte populations share redundant functions. For example, NK cells, γδ T cells, and γδ NKT cells contribute to innate IFN-γ and/or IL-17A production, but mice lacking functional NK cells, γδ T cells, or γδ NKT cells are not more susceptible to mycobacterial infection (24,27,31,43,49,52,69). While having normal development of these other innate lymphocytes (72), we show here that MR1−/− mice are defective in the early control of mycobacterial infection. The inability of MR1−/− mice to elicit a protective immune response is likely due to the defective IFN-γ and IL-17A production in the absence of MAIT cells. These findings demonstrate that MAIT cells have a non-redundant role as innate T cells. The infection model of mycobacteria has several advantages to define innate immune responses including a slow doubling time of mycobacteria after lung infection and a slowly developing adaptive immune response (17).
Our *in vivo* findings supporting a role of MAIT cells in mycobacterial infection are congruent with published observations that MAIT cells are activated *in vitro* by mycobacteria-infected cells (33,50). Similar to our findings, Georgel *et al.* showed that MR1$^{-/-}$ mice infected intraperitoneally with *K. pneumonia* failed to control bacterial burden and the serum cytokine analysis of the infected MR1$^{-/-}$ mice showed a decreased pro-inflammatory response compared to WT mice (32). Again, congruent with these findings, *in vitro* studies showed that MAIT cells displayed activation markers after co-culture with cells infected with *K. pneumonia* (50). Curiously, however, while MAIT cells also showed strong *in vitro* reactivity to *E. coli*, another member of the Enterobacteriaceae family (50), MR1$^{-/-}$ mice were not defective in controlling *E. coli* infection via intraperitoneal route (32). Similarly, cells infected with *M. abscessus* activated MAIT cells *in vitro*, but MR1$^{-/-}$ mice were not defective in controlling bacterial growth *in vivo* (50). However, *Mr1$^{+/+}$* mice in which all T cells expressed the transgene of invariant TCR α chain of MAIT cells, had lower bacterial loads after the infection of *E. coli* or *M. abscessus*, than *Mr1$^{-/-}$* mice with T cells expressing the same transgenes (50). This finding suggested that overexpression of MAIT cells may be required to detect their *in vivo* functions. However, our report and the report by Georgel *et al.* (32) would suggest otherwise. Thus, although MAIT cells are activated by diverse microbes *in vitro*, we speculate that their *in vivo* roles are most prominent when infections are introduced into mucosal tissues, when bacterial replication *in vivo* allows a window to monitor innate effector function, and when appropriate innate effector functions are monitored.

Recent findings and speculations of the importance of MAIT cells raise the question of why they were not detected sooner. Indeed, MAIT cells were likely a factor in previously reported findings comparing β2-microglobulin (β2m)-deficient mice and CD1d-, H2-M3-, or MHC class

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Ia-deficient mice (14,23,30,70). Surface expression of MR1 requires β2m assembly (56) and β2m-/- mice have been shown to be more susceptible to the infections of *K. pneumonia* (14) and *M. tuberculosis* (23) than mice lacking Kb- and Db-restricted CD8+ T cells or CD1d-restricted γδNKT cells. MAIT may also have been involved in the previous studies of non-classical T cells (either CD8+ or CD4/CD8 DN population) in the murine models of bacterial infection. CD8+ T cells presumably restricted by an undefined class Ia molecule represented a high percentage of IFN-γ producing cells in the lung of Kb-/-Db-/- mice after aerosol infection with *M. tuberculosis* (76). A comparative study using Kb-/-Db-/- and Kb-/-Db-/-M3-/- showed that non-H2-M3 MHC class Ib restricted CD8+ T cells express activated surface markers, exhibit cytotoxicity, and produce cytokines including IFN-γ and IL-17A in response to *L. monocytogenes* infection (11).

Although this response was not blocked by anti-MR1 mAb, based on our findings this could be due to non-MR1-dependent activation of MAIT cells. Moreover, it is possible that the role of the MAIT cells in the CD4/CD8 DN T cell population is overlooked in the studies focusing on CD8+ T cells in Kb-/-Db-/- or Kb-/-Db-/-M3-/- mice (11,76). In fact, a rare population of CD4/CD8+ NK1.1- αβ T cells in WT mice was shown to inhibit the intracellular growth of *M. tuberculosis* and *Francisella tularensis* (LVS) (18,19); and these CD4/CD8 DN T cells secreted high levels of IFN-γ and IL-17A in response to *F. tularensis* LVS infection (19). These parallels between previous studies and recently defined features of MAIT cells support the notion that MAIT cells are key innate effectors against infection by diverse microbes.

To our knowledge, this report is the first to investigate the *in vivo* role of MAIT cells in a mucosal infection model. Evidence is provided that MAIT cells exert an innate effector function and play a non-redundant role in the immune system during bacterial infection. Mechanistically, we show that MAIT cells control bacterial growth and produce pro-inflammatory cytokines.
Further characterization of the mechanisms by which MR1 and MAIT cells are involved in the induction of protective immunity may help in the future design of vaccines against bacterial infection.

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Figure 1. Innate MR1-restricted MAIT cells inhibit mycobacterial growth in M.  

A and B, M. bovis BCG-infected M derived from WT mice were cultured alone or co-cultured with naïve T cells from MR1<sup>-/-</sup>, WT, Vα19iTgMR1<sup>-/-</sup>, or Vα19iTgMR1<sup>+/+</sup> mice for 72 hours. The intracellular growth of mycobacteria in M was quantitated by liquid scintillation counting of tritiated uridine uptake (disintegrations per minute) (A). B, The percentage of mycobacterial growth inhibition was assessed from radioactivity in A to demonstrate the extent of suppression of mycobacterial growth by naïve T cells from different mice. See Material and Methods for formula used in the calculation of the percentage. C, Nitric oxide (NO) production of uninfected M, infected M, or infected M co-cultured with naïve T cells (Vα19iTgMR1<sup>-/-</sup> or Vα19iTgMR1<sup>+/+</sup>). Supernatants were collected after 72 hours of culture. The amount of nitrite (NO<sub>2</sub>⁻) in supernatants was measured. Results are given as the mean ± SEM of the triplicate of different culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post-test comparing every culture condition (***, P ≤ 0.001; ns, not significant). The dotted line in C denotes that the uninfected M was not included in statistical comparison. Data represent the results from one of three independent experiments.

Figure 2. MAIT cells are a potent source of innate IFN-γ enhancing the microbicidal activity of infected M.  

A, IFN-γ production of naïve T cells from MR1<sup>-/-</sup>, WT, Vα19iTgMR1<sup>-/-</sup>, or Vα19iTgMR1<sup>+/+</sup> mice when co-cultured with uninfected or M. bovis BCG-infected M. Supernatants were collected after 72 hours of co-culture. The concentration of IFN-γ in supernatants was measured by ELISA. B, Effect of IFN-γ blockage on the inhibition of mycobacterial growth by Vα19iTgMR1<sup>+/+</sup> T cells. M. bovis-infected M was cultured alone, or
co-cultured with \( \alpha \beta 19 \)iTgMR1\(^{-/+}\) T cells in the absence or presence of an anti-IFN-\( \gamma \) mAb for 72 hours. The intracellular growth of mycobacteria in M was quantitated by liquid scintillation counting of tritiated uridine uptake (disintegrations per minute). 

C, Effect of IFN-\( \gamma \) blockage on the enhancement in NO production of infected M by \( \alpha \beta 19 \)iTgMR1\(^{-/+}\) T cells. Supernatants from the cultures described in B were collected before cell lysis for the radiolabeling of mycobacteria. The amount of NO\(_2^-\) in supernatants was measured. Results are given as the mean \( \pm \) SEM of the triplicate of different culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post-test comparing every culture condition (*, \( P \leq 0.05 \); **, \( P \leq 0.01 \); ****, \( P \leq 0.001 \); ns, not significant). Data represent the results from one of three independent experiments.

Figure 3. Innate MAIT cells produce IL-17A in response to infection. 

A, IL-17A production of \( \alpha \beta 19 \)iTgMR1\(^{-/+}\) T cells when co-cultured with uninfected or M. bovis BCG-infected M. Supernatants were collected after 72 hours of co-culture. The concentration of IL-17A in supernatants was measured by ELISA. 

B, Effect of IL-17A blockage on the inhibition of mycobacterial growth by \( \alpha \beta 19 \)iTgMR1\(^{-/+}\) T cells. M. bovis BCG-infected M was cultured alone, or co-cultured with \( \alpha \beta 19 \)iTgMR1\(^{-/+}\) T cells in the absence or presence of an anti-IL-17A mAb for 72 hours. The intracellular growth of mycobacteria in M was quantitated by liquid scintillation counting of tritiated uridine uptake (disintegrations per minute). Results are given as the mean \( \pm \) SEM of the triplicate of different culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post-test comparing every culture (***, \( P \leq 0.001 \); ns, not significant). Data represent the results from one of three independent experiments.
Figure 4. Activation of MAIT cells is dependent on the innate signal IL-12 from infected M. A, C, and E, MAIT cell activation in the presence of anti-MR1 or anti-IL-12/23p40 mAbs. Vα19iTgMR1+/+ T cells were co-cultured with M. bovis BCG-infected M and supernatants were collected after 72 hours of co-culture. The concentration of IFN-γ (A), IL-17A (C), and NO₂⁻ (E) in supernatants were measured. Results are given as the mean ± SEM of the triplicate of different culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post-test comparing every culture. ***, P ≤ 0.001, or, ns, not significant, was assigned when compared to M co-cultured with Vα19iTgMR1+/+ T cells in the absence of blocking mAbs. The dotted line in E denotes that the NO production of M cultured alone was not included in statistical comparison. Data represent the results from one of three independent experiments. B, D, and F, MAIT cell activation when co-cultured with WT, MR1−/−, or p40−/− M infected with M. bovis BCG. Vα19iTgMR1+/+ T cells were co-cultured with WT, MR1−/−, or p40−/− M infected with M. bovis BCG and supernatants were collected after 72 hours of co-culture. The concentration of IFN-γ (B), IL-17A (D), and NO₂⁻ (F) in supernatants were measured. Results are given as the mean ± SEM of the triplicate of different culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post-test comparing every culture. ***, P ≤ 0.001, or, ns, not significant, was assigned when compared to WT M co-cultured with Vα19iTgMR1+/+ T cells. In F, the dotted line denotes that statistical analyses were performed separately for M alone and M co-cultured with Vα19iTgMR1+/+ T cells. There was no statistical difference in NO production among infected M of different genotypes when cultured in the absence of Vα19iTgMR1+/+ T cells. Data represent the results from one of two independent experiments.
Figure 5. Control of mycobacterial growth by MAIT cells depends on IL-12. A and C, The inhibition of mycobacterial growth by MAIT cells in the presence of anti-MR1 or anti-IL-12/23p40 mAbs. *M. bovis*-infected M were cultured alone, or co-cultured with Vα19iTgMR1+/+ T cells in the absence or presence of anti-MR1 mAbs or an anti-IL-12/23p40 mAb for 72 hours. The intracellular growth of mycobacteria in M was quantitated by liquid scintillation counting of tritiated uridine uptake (disintegrations per minute) (A). The percentage of mycobacterial growth inhibition (C) was assessed from radioactivity in A to demonstrate the extent of suppression of mycobacterial growth by Vα19iTgMR1+/+ T cells in the absence or presence of blocking mAbs. See Material and Methods for formula used in the calculation of the percentage. Results are given as the mean ± SEM of the triplicate of different culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post-test comparing every culture. **, P ≤ 0.01, or, ns, not significant, was assigned when compared to M co-cultured with Vα19iTgMR1+/+ T cells in the absence of blocking mAbs. The dotted line in A denotes that M cultured alone was not included in statistical comparison. Data represent the results from one of three independent experiments. B and D, The inhibition of mycobacterial growth in WT, MR1+/−, or p40−/− M by MAIT cells. WT, MR1+/−, or p40−/− M infected with *M. bovis* BCG was cultured alone, or co-cultured with Vα19iTgMR1+/+ T cells for 72 hours. The intracellular growth of mycobacteria in M was quantitated by liquid scintillation counting of tritiated uridine uptake (disintegrations per minute) (B). The percentage of mycobacterial growth inhibition (D) was assessed based on genotype. Results are given as the mean ± SEM of the triplicate of different culture conditions. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison post-test comparing every culture.
***, P ≤ 0.001, or, ns, not significant, was assigned when compared to the co-culture of infected
WT M with Vα19iTgMR1+/+ T cells. In B, the dotted line denotes that statistical analyses were
performed separately for M alone and M co-cultured with Vα19iTgMR1+/+ T cells. There
was no statistical difference in mycobacterial growth among infected M of different genotypes
when cultured in the absence of Vα19iTgMR1+/+ T cells. Data represent the results from one of
two independent experiments.

Figure 6. The role of MR1 in MAIT cell activation. A and B, MAIT cell activation by p40−/−
M in the presence of anti-MR1 mAbs. Vα19iTgMR1+/+ T cells were co-cultured with p40−/−
M infected with M. bovis BCG in the absence or presence of anti-MR1 mAbs. Supernatants
were collected after 24 or 72 hours of co-culture. C, The inhibition of mycobacterial growth in
p40−/− M by Vα19iTgMR1+/+ T cells in the absence or presence of anti-MR1 mAbs. Results are
given as the mean ± SEM of the triplicate of different culture conditions. Statistical analysis was
performed using Student’s t test. Although there was a trend that cytokine secretion of
Vα19iTgMR1+/+ T cells was lower in the presence of MR1 blockage, the difference was not
statistically significant. Data represent the results of two time points from two independent
experiments.

Figure 7. MR1-restricted MAIT cells produce IFN-γ in response to recombinant IL-12 in
the absence of infection. Vα19iTgMR1−/− or Vα19iTgMR1+/+ T cells were incubated with
uninfected WT M in the presence of recombinant IL-12 at various concentrations.
Supernatants were collected after 24 hours of incubation. The concentration of IFN-γ in
supernatants was measured by ELISA. Results are given as the mean ± SEM of the triplicate of
different culture conditions. Statistical analysis was performed using a two-way ANOVA (***, $P \leq 0.001$). Data represent the results from one of two independent experiments.

Figure 8. MR1-restricted MAIT cells play a unique role in providing protective immunity against mycobacterial infection in vivo. A, The bacterial burden in WT and MR1$^{-/-}$ mice in the low-dose aerosol infection of *M. bovis* BCG. WT and MR1$^{-/-}$ mice were infected with *M. bovis* BCG via the aerosol route. Each mouse received 100 to 300 infectious bacilli in lung. 10 and 30 days after infection, lung tissues were harvested to determine the bacterial burden. Results are given as the mean ± SEM of five mice per group. Each dot represents the CFU count of individual mice. The difference in bacterial burden between WT and MR1$^{-/-}$ mice at day 10 post-infection is statistically significant analyzed by Student’s *t* test ($P = 0.0008$). Data represent the results from one of two independent experiments. B, The IFN-γ response of WT and MR1$^{-/-}$ mice in the intranasal infection of *M. bovis* BCG. BAL cells were obtained from WT and MR1$^{-/-}$ mice on day 10 and day 30 after the intranasal infection of *M. bovis* BCG ($10^7$ CFU per mouse). The frequency of IFN-γ-producing cells in the BAL was measured by the ELISPOT assay. BAL cells from the same genotype were pooled and stimulated with *M. bovis* BCG (MOI = 3), and various *M. tuberculosis*-derived antigens: culture filtrate (CF, 10 µg/mL), cell wall (CW, 50 µg/mL), and Ag85b peptide pool (Ag85bpp, 2 µg/mL). The numbers of IFN-γ SFUs per one million BAL cells were assessed. Results are given as the mean ± SEM of the triplicate of different culture conditions. Data represent the results from one of two independent experiments. C, IL-17A production in the supernatant of BAL cells stimulated by the CW fraction of *M. tuberculosis ex vivo*. Results are given as the mean ± SEM of the triplicate. Data represent the results from one of two independent experiments.
Figure 2

Purified T cells:
- MR1<sup>−/−</sup>
- WT
- V<sub>α</sub>19i TgMR1
- V<sub>α</sub>19i TgMR1<sup>+/+</sup>

(a) IFN-γ (pg/mL)

(b) BCG growth (tritiated uridine uptake, DPM)

(c) NO<sub>2</sub> (μM)

Un-infected Mφ | Infected Mφ
Figure 4

(a) IFN-γ (pg/mL)
(b) IFN-γ (pg/mL)
(c) IL-17A (pg/mL)
(d) IL-17A (pg/mL)
(e) NO₂⁻ (µM)
(f) NO₂⁻ (µM)
Figure 6

(a) IFN-γ (pg/mL) levels after 24 h and 72 h.

(b) IL-17A (pg/mL) levels after 24 h and 72 h.

(c) % inhibition of BCG growth.

Legend:
- p40
- p40 + anti-MR1