

Research Article

TLR signaling in human antigen-presenting cells regulates MR1-dependent activation of MAIT cells

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Mucosal-associated invariant T (MAIT) cells are an abundant innate-like T lymphocyte population that are enriched in liver and mucosal tissues. They are restricted by MR1, which presents antigens derived from a metabolic precursor of riboflavin synthesis, a pathway present in many microbial species, including commensals. Therefore, MR1-mediated MAIT cell activation must be tightly regulated to prevent inappropriate activation and immunopathology. Using an *in vitro* model of MR1-mediated activation of primary human MAIT cells, we investigated the mechanisms by which it is regulated. Uptake of intact bacteria by antigen presenting cells (APCs) into acidified endolysosomal compartments was required for efficient MR1-mediated MAIT cell activation, while stimulation with soluble ligand was inefficient. Consistent with this, little MR1 was seen at the surface of human monocytic (THP1) and B-cell lines. Activation with a TLR ligand increased the amount of MR1 at the surface of THP1 but not B-cell lines, suggesting differential regulation in different cell types. APC activation and NF- κ B signaling were critical for MR1-mediated MAIT cell activation. In primary cells, however, prolonged TLR signaling led to downregulation of MR1-mediated MAIT cell activation. Overall, MR1-mediated MAIT cell activation is a tightly regulated process, dependent on integration of innate signals by APCs.

Keywords: Activation · Antigen presenting cell · Endotoxin tolerance · MAIT cells · MR1 · NF- κ B



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Introduction

Mucosal-associated invariant T (MAIT) cells are innate-like T lymphocytes that are restricted by the non-classical MHC class

Ib-related protein, MHC-related protein 1 (MR1) [1, 2]. MR1 is nonpolymorphic and highly evolutionarily conserved [3, 4]. MAIT cells predominantly express a canonical semi-invariant T-cell receptor, V α 7.2-J α 33 [1, 5]; in addition minor populations of MR1-restricted MAIT cells with V α 7.2-J α 12 and V α 7.2-J α 20 T-cell receptors have recently been described [6, 7]. MAIT cells are abundant in humans, comprising ~5% of T lymphocytes in

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adult blood, with up to 95% expressing CD8 α [8, 9]. MAIT cells are further enriched in the liver, where they comprise ~18% of all lymphocytes [10], and are also found at mucosal sites [1].

MR1 has recently been shown to present a microbially derived metabolic precursor of riboflavin biosynthesis [11, 12]. MR1 captures and binds unstable ribityllumazine intermediates 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)6-D-ribitylaminouracil (5-OE-RU), which are formed by the condensation of 5-amino-6-D-ribitylaminouracil (5-A-RU), an intermediate in riboflavin biosynthesis, with methylglyoxal or glyoxal respectively [12]. 5-OP-RU was found in the supernatant of a *Salmonella* sp. culture and was able to activate MAIT cells [11, 12]. Consistent with this, MAIT cells are activated by riboflavin-synthesizing microorganisms in an MR1-dependent manner [13]. In addition, MAIT cells can be activated by both riboflavin-synthesizing and nonriboflavin-synthesizing bacterial species, independently of TCR stimulation, by the pro-inflammatory cytokines, interleukin-12 and interleukin-18 [14, 15].

Given the abundance of MAIT cells at mucosal surfaces and in liver [1, 10], the wide range of microorganisms, including commensals, that are able to produce the ligand for MR1 [13], the small molecular size of the ligand [11, 12], which may encourage diffusion, and the rapid response of MAIT cells to MR1-mediated activation [14], we hypothesized that MR1-mediated MAIT cell activation must be tightly regulated to prevent immunopathology while ensuring activation in the setting of infection. To investigate this we used an in vitro model which we have recently described which separates early MR1-mediated MAIT cell activation from later MR1-independent, IL-12 and IL-18-dependent, activation [14]. In this paper we demonstrate that efficient MR1-mediated MAIT cell activation requires uptake of intact bacteria by antigen presenting cells (APCs), as well as activation of the APC via NF- κ B activation or interferon signaling. Furthermore, MR1-mediated MAIT cell activation is negatively regulated in endotoxin tolerance, suggesting tight regulation.

Results

Early activation of MAIT cells is MR1 dependent and occurs independently of IL-12 and IL-18

We have previously shown that there are two mechanisms of primary human MAIT cell activation: MR1-dependent activation (TCR-dependent), which occurs early, and IL-12- and IL-18-mediated activation, which occurs later and is independent of TCR signaling [14]. As THP1 cells were used as the APCs in the previous experiments, we assessed whether these findings were generalizable to other APC types. Primary human monocytes were incubated overnight with fixed *Escherichia coli* [13, 14, 16] and CD8⁺ T cells were either added for the final 5 h of incubation (Fig. 1A–C) or co-incubated overnight (Fig. 1D–F); MAIT cell activation was detected by intracellular cytokine staining for IFN- γ production; the gating strategy is shown in Supporting Information Fig. 1.

While the magnitude of responses was lower with primary human monocytes than with THP1s (Fig. 1B and 1E), the experiment confirmed that early MAIT cell activation (5-h co-incubation) was MR1-dependent (Fig. 1C), while late MAIT cell activation (20-h co-incubation) was dependent upon both MR1 and IL-12 and IL-18 (Fig. 1F). The MR1 dependence of early MAIT cell activation was confirmed using primary B cells (Supporting Information Fig. 2A and B), or an EBV-transformed B-cell line (BCL) (Supporting Information Fig. 2C and D) as APCs. Therefore early (5 h) production of IFN- γ by MAIT cells can be used as a read-out of MR1-mediated MAIT cell activation, irrespective of the APC.

Bacterial uptake into acidified endosomes is required for efficient MR1-driven MAIT cell activation

The MAIT cell-activating MR1 ligand, 5-OP-RU, was recently identified from a *Salmonella enterica* subsp. *enterica* culture supernatant [11, 12]. C1R.hMR1 cells, which express large amounts of MR1 at the cell surface [17], efficiently activated MAIT cells when treated with *Salmonella* sp. culture supernatant or the synthetic ligand, rRL-6-CH₂OH [11]. In contrast, in an earlier study the activation of murine MAIT cells by *E. coli* infected bone marrow-derived dendritic cells was dependent upon phagocytosis and endosomal acidification [13]. Also, surface expression of MR1 in nontransduced cells has been reported to be transient and difficult to detect [18]. Therefore, we hypothesized that nontransduced APCs treated with bacterial culture supernatant would only weakly stimulate MAIT cells.

To test this THP1s were treated with bacterial culture supernatant, cell lysate, or fixed intact bacteria and their ability to stimulate MAIT cells assessed; equivalent proportions of a stationary phase culture were used. Robust MAIT cell activation was only seen with intact bacteria. With both *E. coli* and non-typhoidal *Salmonella*, weak activation was seen with both the supernatant and cell lysate (Fig. 2A). Confirming the superior stimulatory ability of intact bacteria, robust MR1-mediated MAIT cell activation was still seen with lower numbers of intact bacteria per APC (Supporting Information Fig. 3A), while the response to supernatant was rapidly lost (Supporting Information Fig. 3B). Primary human monocyte-derived macrophages (MDMs) (Fig. 2B) and primary human monocytes (Fig. 2C) treated with intact *E. coli* also stimulated a more robust response from MAIT cells than those treated with *E. coli* supernatant. Therefore, while the ligand is present in culture supernatant, more robust MR1-mediated MAIT cell activation is seen with an equivalent amount of intact bacteria.

The robust stimulatory ability of THP1s treated with fixed intact bacteria but not supernatant or bacterial cell lysate, suggested that uptake of intact bacteria into the cell is important for subsequent MAIT cell activation. Consistent with this, treatment of THP1s with cytochalasin D, an inhibitor of actin polymerization, partially inhibited the ability of THP1s treated with intact *E. coli* to stimulate MAIT cells (Fig. 2D). Furthermore, inhibition of endolysosomal acidification with bafilomycin A1, an inhibitor of the H⁺ ATPase pump, partially inhibited the ability of THP1s to

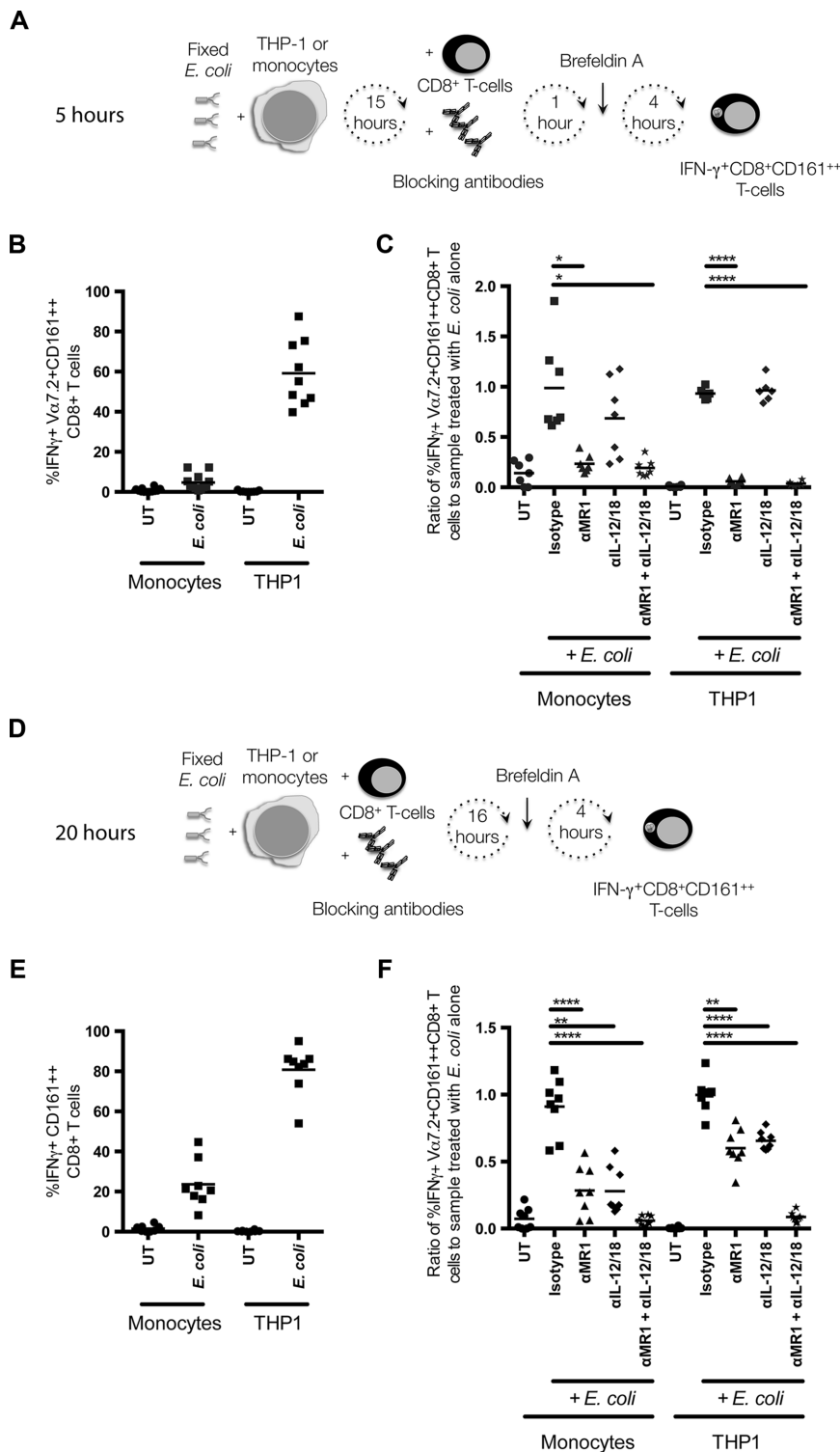


Figure 1. Early MAIT cell activation is MR1-dependent and IL-12 plus IL-18-independent. Primary human monocytes or THP1 cells were incubated for 20 h with fixed *E. coli*. CD8⁺ T cells (autologous to the monocytes) were either (A–C) added for the final 5 h of culture, or (D–F) co-cultured for the entire 20 h. (A, D) Blocking antibodies were added as indicated. (B, E) Brefeldin A was added for the final 4 h of culture. Cells were stained with fluorochrome-labeled antibodies (anti-CD3-PECy7, anti-CD8-eFluor450, anti-CD161-APC, anti-V α 7.2-PE) and Live/Dead Fixable Near IR dye, and the percentage of MAIT cells making IFN- γ determined by intracellular cytokine staining (anti-IFN- γ -FITC). (C, F) The effect of the blocking antibodies on production of IFN- γ by MAIT cells, normalized to the production of IFN- γ in the absence of blocking antibodies, was assessed. Data shown are pooled from 3 (C, E, F) or 4 (B) independent experiments. Means and each data point, representing different donors, are shown ((B) primary human monocytes, $n = 12$, THP1 cells, $n = 9$; (C) primary human monocytes, $n = 7$, THP1 cells, $n = 6$; (E, F) both cell types, $n = 8$). Comparisons were made with a repeated measures one-way ANOVA with Dunnett's multiple comparison test, comparing all conditions to isotype. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. See Supporting Information Fig. 1 for the gating strategy.

activate MAIT cells (Fig. 2E). Neither inhibitor adversely affected THP1 viability compared with DMSO (Supporting Information Fig. 4A and 4C), although the relative proportion of nonadherent cells recovered was reduced with cytochalasin D (Supporting Information Fig. 4B) but not bafilomycin A (Supporting Information Fig. 4D). Furthermore, neither inhibitor effected the stimulation of MAIT cells by THP1 cells treated with *E. coli* supernatant

(Fig. 2F). While uptake of riboflavin-synthesizing bacteria led to robust MAIT cell activation (Fig. 2A), induction of phagocytosis with *Enterococcus faecalis*, which does not synthesize riboflavin, did not enhance the MAIT cell stimulatory capacity of *E. coli* culture supernatant (Fig. 2G). Therefore, uptake of intact ligand-producing bacteria into an acidic compartment is required for efficient MR1-mediated antigen presentation.

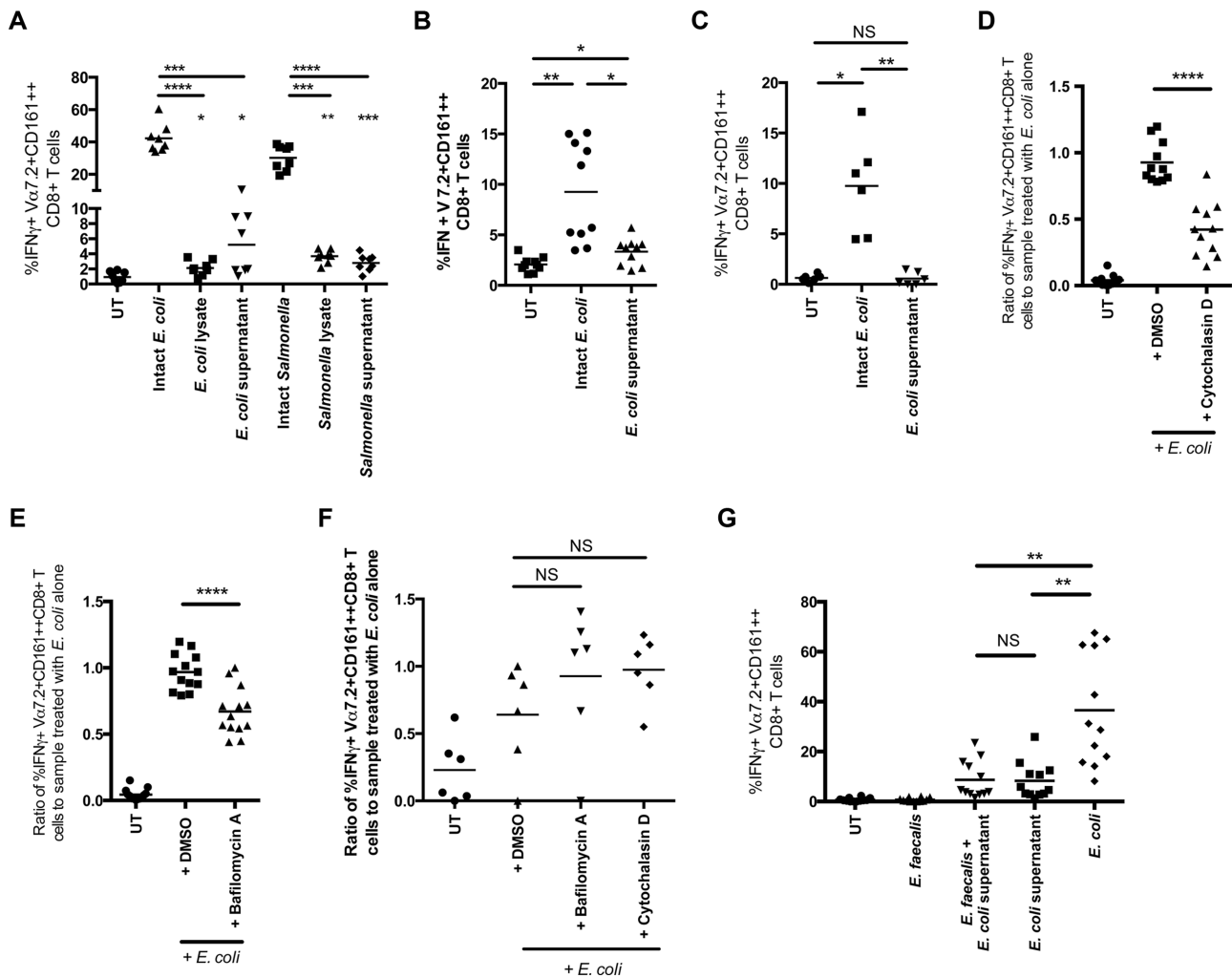


Figure 2. Uptake of intact bacteria into an acidified endosomal compartment is required for efficient MR1-mediated MAIT cell activation. (A) THP1 cells were incubated for 20 h with equivalent amounts of intact *E. coli*, *E. coli* cell lysate, *E. coli* culture supernatant, intact *Salmonella*, *Salmonella* cell lysate, or *Salmonella* culture supernatant, then CD8⁺ T cells added for the final 5 h of culture and the production of IFN- γ by MAIT cells determined; as THP1 cells were washed prior to the addition of CD8⁺ T-cells, supernatant and lysate were replaced. Data shown are pooled from two independent experiments with eight different donors. (B) Primary human MDMs or (C) primary human monocytes (isolated by fluorescence activated cell sorting) were incubated for 20 h with equivalent amounts of intact *E. coli* or *E. coli* supernatant, then CD8⁺ T cells added for the final 5 h of culture and the production of IFN- γ by MAIT cells determined. (B, C) Pooled data from three independent experiments with 10 (B) or 6 (C) different donors is presented. (D) The effect of cytochalasin D and (E) bafilomycin A1, added at the start of culture, on the ability of *E. coli*-treated APCs to subsequently stimulate IFN- γ production by MAIT cells was assessed. Six (D) and 7 (E) independent experiments were performed with 11 (D) and 13 (E) different donors and pooled data are presented. (F) THP1 cells were incubated overnight with *E. coli* supernatant, *Enterococcus faecalis*, or both, and IFN- γ production by MAIT cells at 5 h was assessed. Three independent experiments were performed with 12 different donors and pooled data are presented. In all experiments, THP1s/MDMs/monocytes were washed three times prior to the addition of CD8⁺ T cells (isolated with CD8 microbeads (A, B, D–F) or CD8-PE and anti-PE microbeads (C)) for the final 5 h of culture; brefeldin A was added for the final 4 h. Cells were stained with fluorochrome-labeled antibodies ((A, B, D–F): anti-CD3-PECy7, anti-CD8-eFluor450, anti-CD161-APC, anti-V α 7.2-PE; (C): anti-CD3-PECy7, anti-CD8-PE, anti-CD161-APC, anti-V α 7.2-FITC) and Live/Dead Fixable Near IR dye, and the percentage of MAIT cells making IFN- γ determined by intracellular cytokine staining ((A, B, D–F): anti-IFN- γ -FITC; (C): anti-IFN- γ -PerCPy5.5). Means and each data point, representing different donors, are shown. Comparisons were made with repeated measures one-way ANOVA with Tukey's multiple comparison test when all conditions were compared with one another (A) or with Sidak's multiple comparison test, when preselected pairs of columns were compared (B–D). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Surface expression of MR1 is differentially regulated in different cell lines

The previous report of robust activation of MAIT cells by *Salmonella* culture supernatant used C1R cells transduced with MR1 (C1R.hMR1) as the APC [11]. C1R.hMR1 cells express high

levels of MR1 at the cell surface [17]. Therefore, we hypothesized that the level of surface MR1 expression is critical for the ability of an APC to present soluble ligand.

To address this question we derived two cell lines overexpressing MR1: THP1.hMR1 and BCL.hMR1. Overexpression of MR1 was confirmed by detection of the C-terminal HA tag (Fig. 3A).

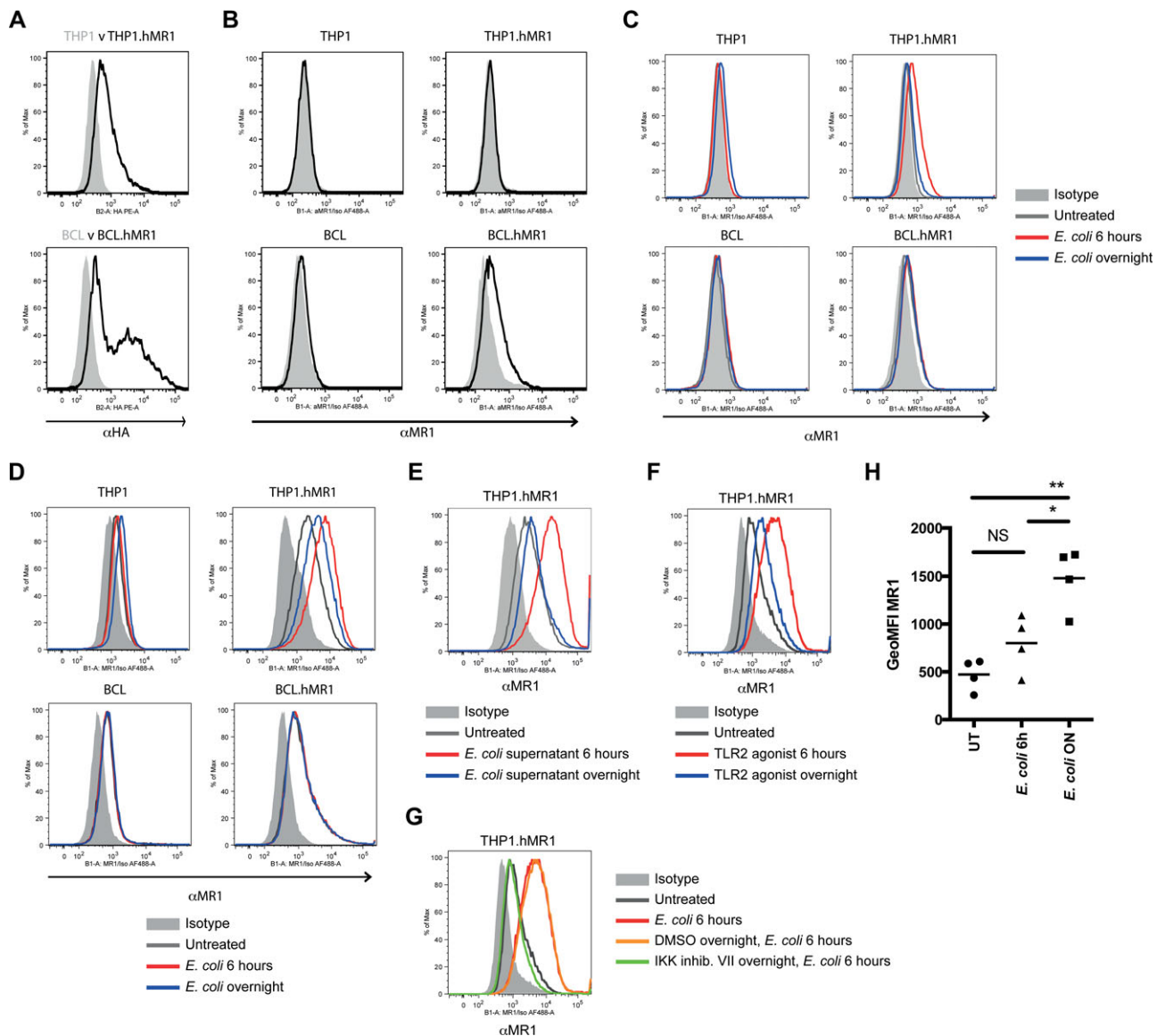


Figure 3. Regulation of MR1 surface expression. (A) Intracellular expression of the HA tag in THP1.hMR1 cells and BCL.hMR1 cells (black lines), with the parental THP1 and BCL cells (gray shaded) shown for comparison. Cells were stained with Live/Dead Fixable Near IR dye, fixed, permeabilized, and stained with anti-HA-PE. (B) Traditional surface stain of MR1 on unstimulated THP1 cells, THP1.hMR1 cells, BCL cells and BCL.hMR1 cells (all black line) compared to isotype (gray shaded). Cells were stained with Live/Dead Fixable Near IR dye and anti-MR1-AlexaFluor488 or an AlexaFluor488-labeled isotype control. (C) Traditional surface stain of MR1 on THP1, THP1.hMR1, BCL, and BCL.hMR1 cells after treatment with *E. coli* for 6 h or 20 h (overnight, ON), or without treatment. The shaded histogram is cells stained with the isotype control. Cells were stained with Live/Dead Fixable Near IR dye and anti-MR1-AlexaFluor488 or an AlexaFluor488-labeled isotype control for 4 h at 37°C on THP1, THP1.hMR1, BCL, and BCL.hMR1 cells after treatment with *E. coli* for 6 h or overnight, or without treatment. (D) MR1 capture assay (incubation with anti-MR1-AlexaFluor488 or AlexaFluor488-labeled isotype control for 4 h at 37°C) on THP1, THP1.hMR1, BCL, and BCL.hMR1 cells after treatment with *E. coli* for 6 h or overnight, or untreated. (E, F) MR1 capture assay on THP1.hMR1 cells after treatment with (E) *E. coli* supernatant or (F) a TLR2 agonist (HKLM) for 6 h or overnight, or untreated. (G) MR1 capture assay on THP1.hMR1 cells pretreated overnight with IKK inhibitor VII or DMSO, or untreated prior to treatment with *E. coli* for 6 h. Cells that were not exposed to *E. coli* are shown. (H) Geometric mean fluorescent intensity (GeomFI) of MR1 (capture assay) on the parental THP1 cell line after treatment with *E. coli* for 6 h or overnight. (A–G) Representative plots are shown; experiments were performed independently at least twice in triplicate. (H) Each data point represents the mean of technical triplicates from one of four independent experiments; the mean of the independent experiments is shown. Comparisons were made with a repeated measures one-way ANOVA with Tukey's multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

In unstimulated cells MR1 could only be detected at the surface of BCL.hMR1 cells; no surface expression could be detected on THP1, THP1.hMR1, or BCL cells (Fig. 3B). This likely reflects the higher expression levels of the MR1.HA transgene in BCL.hMR1 cells (Fig. 3A), probably due to variable transduction and the

nonclonal nature of the cell lines. In addition, the low levels of surface expression in both cell lines could be due to the C-terminal HA tag impeding MR1 trafficking.

To investigate whether treatment with bacteria affected the surface expression of MR1, cells were treated for 6 h or overnight

with fixed *E. coli* and the cell surface stained for MR1. No change in surface expression of MR1 was seen in BCL.hMR1 cells (Fig. 3C). In contrast, a small increase in MR1 surface expression was seen in THP1.hMR1 cells, which was more evident after 6 h treatment with *E. coli* than overnight. No MR1 surface expression was detectable in the parental BCL and THP1 cell lines (Fig. 3C).

We hypothesized that MR1 might not be detectable by regular immunostaining of the cell surface due to trafficking to and from the surface, with little present at the surface at any one time. To detect MR1 that might be trafficking to and from the surface, anti-MR1 antibody was added to the media for the final 4 h of culture (MR1 capture assay). Specific staining with anti-MR1 was seen with all cell lines (Fig. 3D). Furthermore, upon treatment with *E. coli* up-regulation of MR1 surface expression was detected in THP1 and THP1.hMR1 cells but not BCL or BCL.hMR1 cells (Fig. 3D), despite the higher levels of intracellular MR1 in the BCL.hMR1 cells (Fig. 3A). In THP1.hMR1 cells, this increase occurred early (6 h) with decreased amounts of MR1 detected at the cell surface with prolonged (overnight) incubation with *E. coli* (Fig. 3D). Similar changes in MR1 expression were seen with *E. coli* culture supernatant (Fig. 3E), with a TLR2 agonist (heat-killed *Listeria monocytogenes*, which lacks the riboflavin synthetic pathway) (Fig. 3F) and with *E. faecalis* (Supporting Information Fig. 5A), suggesting changes in surface expression were dependent upon cellular activation and did not require the presence of ligand. Using a conventional surface immunostain, no upregulation of surface expression of MR1 was seen when THP1.hMR1 cells were treated with the TLR2 agonist (Supporting Information Fig. 5B). To confirm the specificity of the MR1 capture assay on TLR2-treated THP1.hMR1 cells, the parental un-transduced THP1 cell line was treated with TLR2 agonist overnight and the MR1 capture assay performed: no staining was seen, suggesting antibody 26.5 was indeed binding to MR1 on TLR2-treated THP1.hMR1 cells (Supporting Information Fig. 5C).

Consistent with a change in MR1 surface expression upon cellular activation, *E. coli*-induced upregulation could be blocked with an inhibitor of the NF- κ B signaling pathway (IKK inhibitor VII, which inhibits the IKK complex) (Fig. 3G). Interestingly, inhibiting NF- κ B signaling also partially inhibited detection of MR1 at the cell surface in BCL.hMR1 cells treated with *E. coli* (Supporting Information Fig. 5D) and in untreated BCL.hMR1 cells (Supporting Information Fig. 5E), and completely inhibited detection in untreated THP1.hMR1 cells (Supporting Information Fig. 5E). These results suggest that NF- κ B signaling is required even for basal surface expression of MR1.

In untransduced THP1 cells, increased trafficking of MR1 to the surface upon treatment with *E. coli* was also evident, with a small increase in surface MR1 6 h after exposure, which further increased with overnight incubation (Fig. 3D and 3H). This contrasted with the kinetics of surface MR1 expression in THP1.hMR1 cells, in which maximal surface expression was seen after 6 h and declined with overnight incubation (Fig. 3D). Inhibition of NF- κ B signaling inhibited detection of MR1 at the cell surface of untransduced THP1 cells treated with *E. coli* (Supporting Information Fig. 5F). Upregulation of MR1 surface expression on MDMs was

also evident after overnight treatment with *E. coli* (Supporting Information Fig. 5G).

Therefore, there is little MR1 at the surface of unstimulated THP1 and BCL cells, even when overexpressed. Surface expression is increased in THP1 cells, but not BCL cells, following exposure to pathogen associated molecular patterns, and signaling via NF- κ B is required. Of note, treatment of THP1.hMR1 cells with cytochalasin D had no effect on *E. coli*-induced upregulation of MR1 surface expression (Supporting Information Fig. 5H).

MR1 expression level limits presentation of ligand from supernatant but not from intact bacteria

To assess whether differences in MR1 surface expression affected the ability of APCs to activate MAIT cells, MR1-overexpressing cell lines (THP1.hMR1 and BCL.hMR1) or parental cell lines (THP1 and BCL) were treated for 7 h or overnight with either *E. coli* culture supernatant or intact fixed *E. coli*. Purified CD8⁺ T cells were co-incubated for the final 5 h of culture and IFN- γ production assessed by intracellular cytokine staining (Fig. 4A). With bacterial culture supernatant, MR1 overexpression increased the ability of APCs to stimulate MAIT cells irrespective of the cell line (Fig. 4B,C). The duration of incubation with supernatant (7 h versus overnight) did not significantly affect the ability of THP1, THP1.hMR1, or BCL.hMR1 cell lines to activate MAIT cells (Fig. 4B,C); with the parental BCL cell line, greater MAIT cell activation was seen with shorter incubation, however the effect was modest (Fig. 4C).

With intact *E. coli* there was no difference in the ability of THP1.hMR1 and parental THP1 cell lines to stimulate MAIT cells (Fig. 4D). BCL.hMR1 cells stimulated more MAIT cells than the parental BCL cells at 7 h but not overnight (Fig. 4E). Strikingly, with prolonged incubation with intact bacteria the ability of all APCs to stimulate MAIT cells increased significantly (Fig. 4D and 4E).

Therefore, the level of MR1 surface expression is limiting for the presentation of ligand from supernatant but not from intact bacteria. With intact bacteria, prolonged incubation markedly increases the ability of APCs to stimulate MAIT cells.

APC activation in vitro increases MR1-mediated MAIT cell activation

Given this delay for maximal MAIT cell stimulatory ability, we hypothesized that APC activation is important for MAIT cell stimulation. Therefore we investigated whether preactivation of THP1s with TLR agonists could enhance their ability to stimulate MAIT cells early after exposure to bacteria (7 h); THP1s have been reported to express TLR1-10 [19]. THP1s were incubated overnight with or without TLR agonists and the following morning intact *E. coli* was added. Two hours later the cells were extensively washed and CD8⁺ T cells were added (Fig. 5A). Preincubation with agonists to TLR1, TLR2, or TLR6 enhanced the early MAIT cell

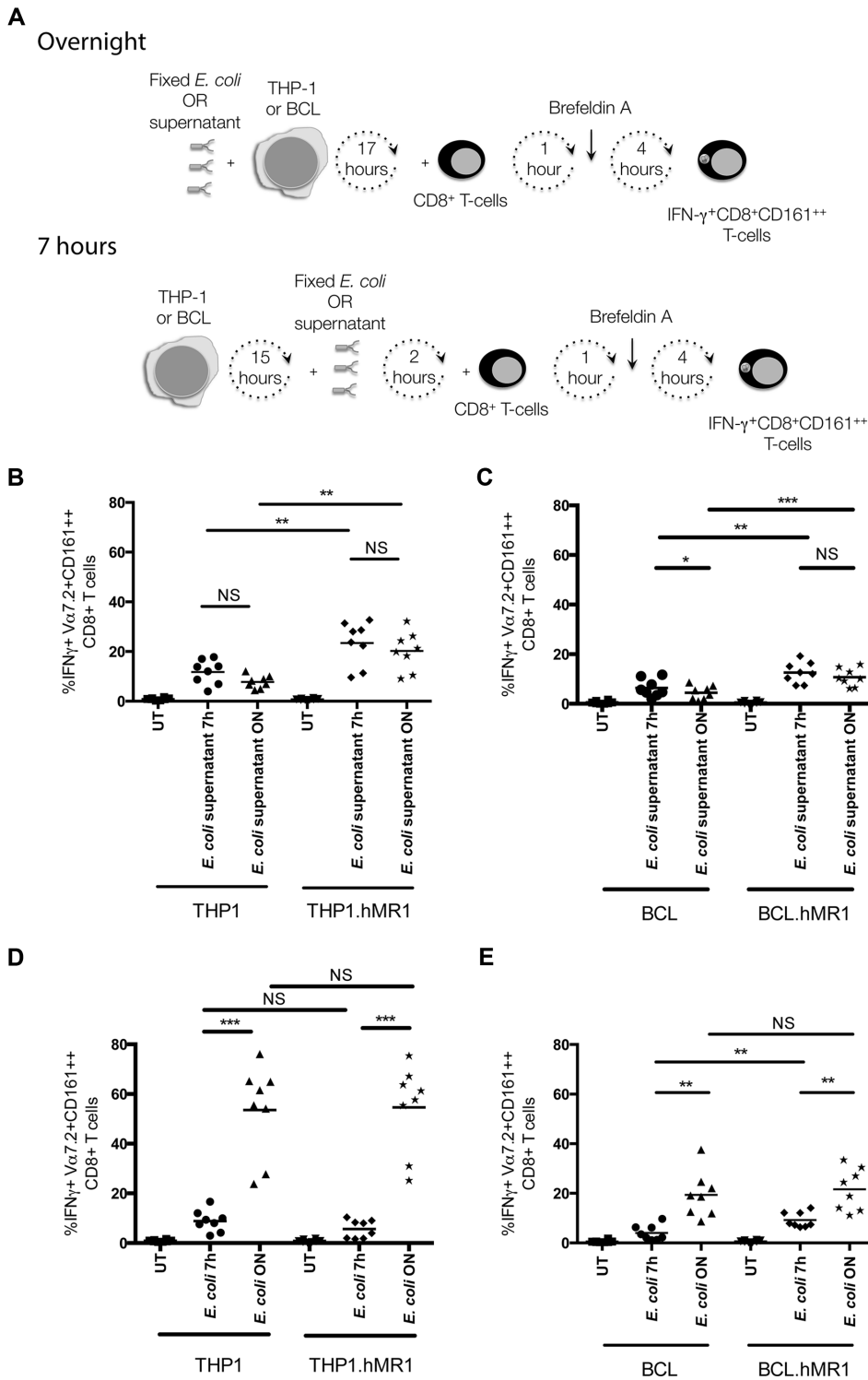


Figure 4. The effect of MR1 expression and time on the MAIT cell stimulatory capacity of APCs treated with supernatant or intact bacteria. (A) APCs were incubated for 7 h or for 20 h (overnight (ON)) with fixed intact *E. coli* or *E. coli* supernatant, and CD8⁺ T cells added for the final 5 h of incubation. Fresh *E. coli* supernatant was added to the appropriate wells along with the CD8⁺ T cells. Cells were stained with fluorochrome-labeled antibodies (anti-CD3-PECy7, anti-CD8-eFluor450, anti-CD161-APC, anti-Vα7.2-PE) and Live/Dead Fixable Near IR dye, and the percentage of MAIT cells making IFN-γ determined by intracellular cytokine staining (anti-IFN-γ-FITC). (B) MAIT cell activation by THP1 or THP.hMR1 cells treated with *E. coli* supernatant. (C) MAIT cell activation by BCL or BCL.hMR1 cells treated with *E. coli* supernatant. (D) MAIT cell activation by THP1 or THP.hMR1 cells treated with intact *E. coli*. (E) MAIT cell activation by BCL or BCL.hMR1 cells treated with intact *E. coli*. All experiments were performed independently twice with eight different donors and pooled data are presented. Means and each data point, representing different donors, are shown. Comparisons were made by repeated measures one-way ANOVA with Sidak's multiple comparison test. **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001, *****p* ≤ 0.0001.

stimulatory capacity of THP1s (Fig. 5B). Similarly preincubation of THP1s with either type I or type II interferons enhanced their early MAIT cell stimulatory capacity (Figs. 5C and 5D). Consistent with our previous findings [14] and with Fig. 1, overnight incubation of THP1 cells with TLR2, TLR4, or IFN-γ alone did not result in early (5 h) MAIT cell activation (Supporting Information Fig. 6A and B), which is MR1-dependent. Therefore preactivation

of THP1s enhances their ability to activate MAIT cells early after exposure to bacteria.

To confirm the importance of APC activation for MAIT cell stimulation, we assessed the effect of blocking NF-κB signaling. THP1 cells were incubated overnight with intact *E. coli* in the presence of inhibitors of the NF-κB pathway (IKK inhibitor VII or (5Z)-7-oxozeanol, which inhibits transforming growth factor-β

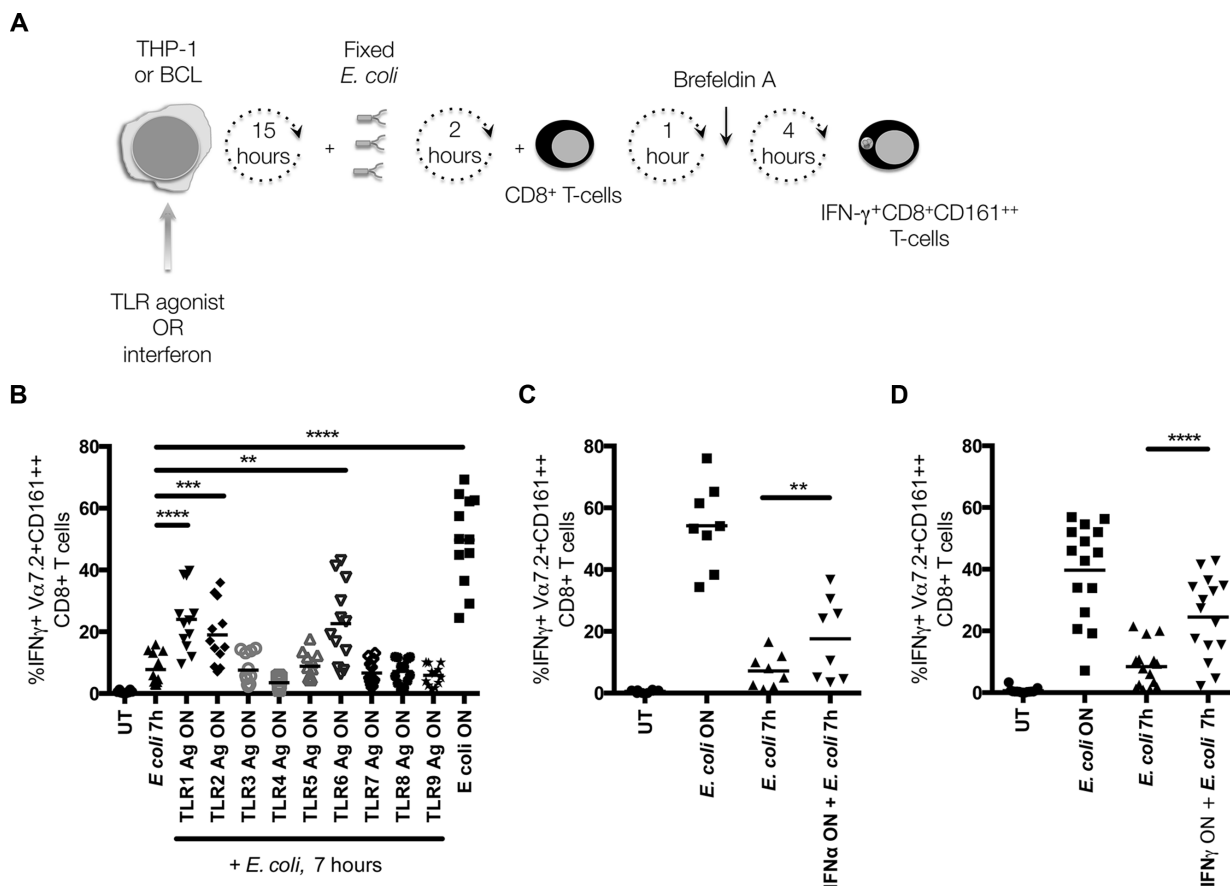


Figure 5. Preactivation of THP1 cells enhances their early stimulatory ability. (A) THP1 cells were preactivated overnight with one of (B) a panel of TLR agonists, (C) interferon α , or (D) interferon γ . Fixed intact *E. coli* was added the following morning and 2 h later the THP1s were washed three times and CD8⁺ T cells added for the final 5 h of incubation; brefeldin A was added for the final 4 h. Cells were stained with fluorochrome-labeled antibodies (anti-CD3-PECy7, anti-CD8-eFluor450, anti-CD161-APC, anti-V α 7.2-PE) and Live/Dead Fixable Near IR dye, and the percentage of MAIT cells making IFN- γ determined by intracellular cytokine staining (anti-IFN- γ -FITC). Two (C), 3 (B), or 4 (D) independent experiments were performed and pooled data are presented. Means and each data point, representing different donors, are shown ((B) $n = 12$; (C) $n = 8$; (D) $n = 15$). Comparisons were made with repeated measures one-way ANOVA with Sidak's multiple comparison test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

activated kinase-1 (TAK1)) or DMSO. Cells were then thoroughly washed and incubated with purified CD8⁺ T cells for 5 h. Inhibition of the NF- κ B pathway potentially inhibited the ability of THP1 cells to activate MAIT cells (Figs. 6A, and 6B). Both inhibitors had a significant but minor ($\leq 5\%$) negative effect on THP1 cell viability; (5Z)-7-oxozeanolol, but not IKK inhibitor VII, had a significant but minor (5.5%) negative impact on THP1 frequency (Supporting Information Fig. 7A–D). Furthermore, treatment with IKK inhibitor VII did not inhibit the ability of THP1 cells to phagocytose *E. coli* (Supporting Information Fig. 7E). Inhibition of THP1 activation was confirmed by immunophenotyping, with inhibition of *E. coli*-induced upregulation of the activation markers CD54, CD80, CD86, HLA2, and HLADR (Supporting Information Fig. 8). Similar results were obtained with BCL.hMR1 cells treated with intact *E. coli* (Fig. 6C) and BCL.hMR1 cells treated with *E. coli* culture supernatant (Fig. 6D). This suggests that not only is NF- κ B signaling important for surface expression of MR1 (Fig. 3G, Supporting Information Fig. 5), it is also essential for the ability of the APC to stimulate MAIT cells.

MAIT cell stimulation by MDMs and monocytes depends on NF- κ B signaling but is inhibited by LPS

Finally, the requirement for APC activation and the kinetics of MR1-mediated MAIT cell activation were assessed using primary human MDMs and monocytes. Consistent with the findings with THP1s, the MAIT cell stimulatory ability of MDMs and monocytes was dependent upon NF- κ B signaling (Fig. 7A, Supporting Information Fig. 9A); inhibition of NF- κ B signaling did not affect the ability of MDMs to phagocytose bacteria (Supporting Information Fig. 10A). In contrast to THP1s, robust MAIT cell activation was seen early after exposure of MDMs to *E. coli* (7 h) and significantly decreased with prolonged exposure (20 h) (Fig. 7B), despite detectable surface expression of MR1 by MDMs after 20 h (Supporting Information Fig. 10B). With monocytes there was a small but significant increase in MAIT cell activation when monocytes were exposed to *E. coli* for 20 h (Supporting Information Fig. 9B); surface expression of MR1 was not detectable in monocytes, even after treatment with *E. coli* (data not shown).

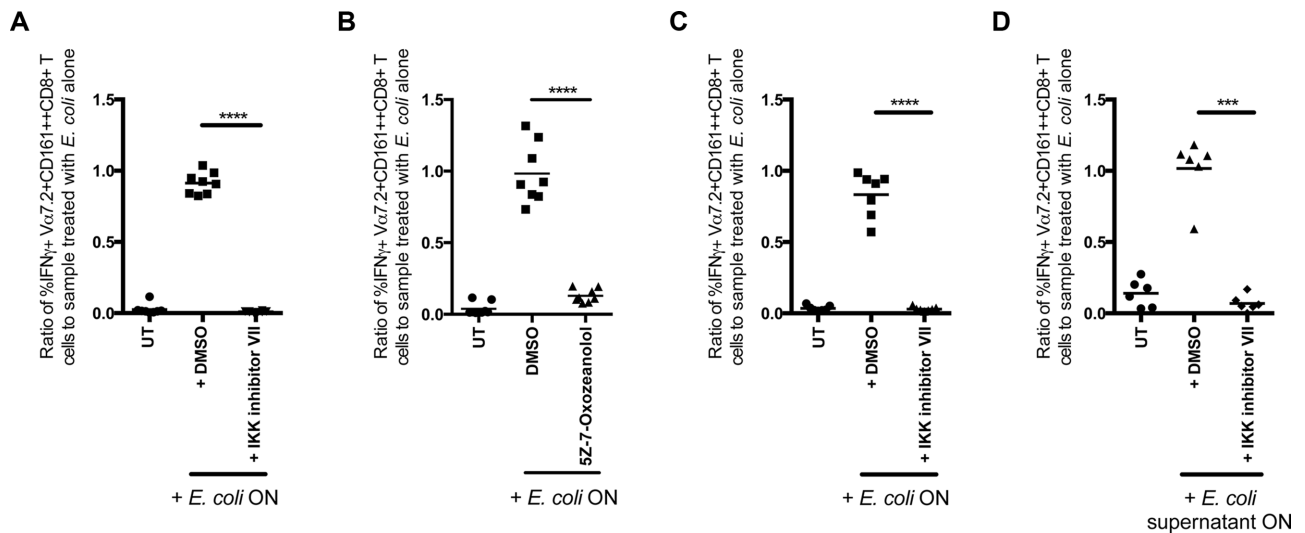


Figure 6. Inhibition of NF- κ B signaling in the APC inhibits MAIT cell activation. (A, B) Inhibition of MAIT cell activation by THP1 cells treated for 20 h (overnight, ON) with intact *E. coli* with or without (A) IKK inhibitor VII or DMSO or (B) (5Z)-7-oxozeanol or DMSO. (C) Inhibition of MAIT cell activation by BCL.hMR1 cells treated overnight with intact *E. coli* with or without IKK inhibitor VII or DMSO. (D) Inhibition of MAIT cell activation by BCL.hMR1 cells treated overnight with *E. coli* supernatant with or without IKK inhibitor VII or DMSO. In all experiments, APCs were washed three times and CD8⁺ T cells added for the final 5 h of culture; brefeldin A was added for the final 4 h. Cells were stained with fluorochrome-labeled antibodies (anti-CD3-PECy7, anti-CD8-eFluor450, anti-CD161-APC, anti-V α 7.2-PE) and Live/Dead Fixable Near IR dye, and the percentage of MAIT cells making IFN- γ determined by intracellular cytokine staining (anti-IFN- γ -FITC). All experiments were performed independently twice and pooled data are presented. Means and each data point, representing different donors, are shown ((A) $n = 8$; (B) $n = 8$; (C) $n = 7$; (D) $n = 6$). Comparisons were made with repeated measures one-way ANOVA with Dunnett's multiple comparison test, with comparison to DMSO. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

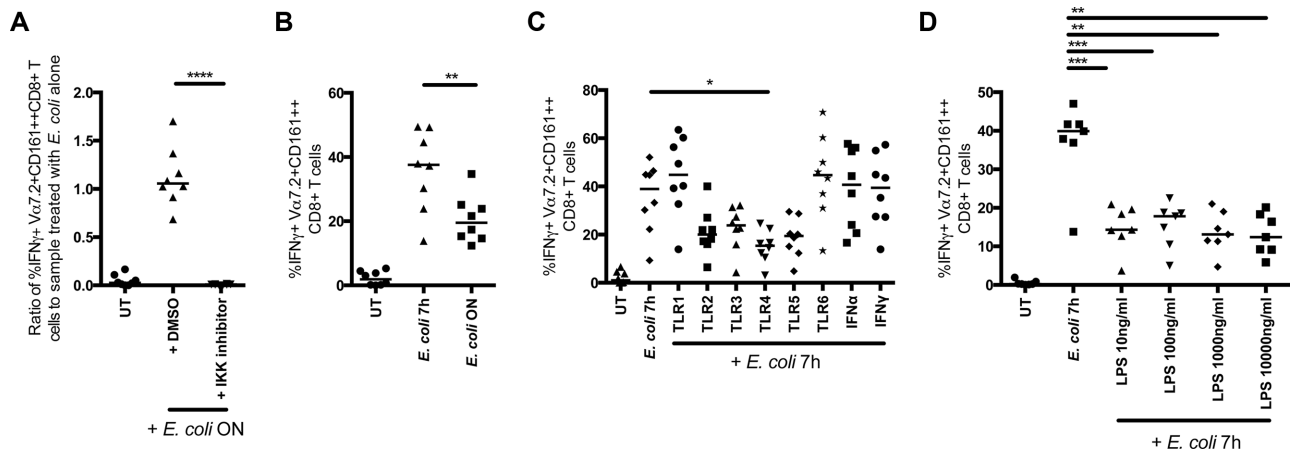


Figure 7. Regulation of MR1-mediated MAIT cell activation by monocyte-derived macrophages. (A) Ability of monocyte-derived macrophages (MDMs) treated for 20 h with intact *E. coli*, with or without DMSO or IKK inhibitor VII, to stimulate IFN- γ production by MAIT cells. (B) Ability of MDMs treated with intact *E. coli* for 7 h or for 20 h to stimulate IFN- γ production by MAIT cells. (C) Ability of MDMs preactivated with TLR agonists or interferons to activate MAIT cells upon subsequent exposure to *E. coli* for 7 h. (D) Ability of MDMs preactivated with various concentrations of LPS to activate MAIT cells upon subsequent exposure to *E. coli* for 7 h. In all experiments MDMs were washed three times prior to the addition of CD8⁺ T cells for the final 5 h of culture; brefeldin A was added for the final 4 h. Cells were stained with fluorochrome-labeled antibodies (anti-CD3-PECy7, anti-CD8-eFluor450, anti-CD161-APC, anti-V α 7.2-PE) and Live/Dead Fixable Near IR dye, and the percentage of MAIT cells making IFN- γ determined by intracellular cytokine staining (anti-IFN- γ -FITC). All experiments were performed independently twice and pooled data are presented. Means and each data point, representing different donors, are shown ((A) $n = 8$; (B) $n = 8$; (C) $n = 8$; (D) $n = 7$). Comparisons were made by repeated measures one-way ANOVA with Dunnett's multiple comparison test (A), Tukey's multiple comparison test (B), or Sidak's multiple comparison test (C, D). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Preactivation of MDMs with TLR agonists or type I or II interferons failed to enhance their ability to stimulate MAIT cells (Fig. 7C); instead, preactivation of MDMs with the TLR4 agonist, LPS, significantly suppressed subsequent MAIT cell activation; there was also a trend to decreased MAIT cell stimulation following preactivation with TLR2, TLR3, or TLR5 agonists (Fig. 7C). Similarly, preactivation of monocytes with LPS reduced their ability to stimulate MAIT cells (Supporting Information Fig. 9C).

Pretreatment of monocytes and macrophages with LPS can inhibit function through endotoxin tolerance [20–22] and activation-induced cell death [23], although M-CSF-derived human macrophages, as used here, are relatively resistant to activation-induced cell death [24]. Endotoxin tolerance is defined as hyporesponsiveness upon rechallenge with LPS. Suppression of MHC class II antigen presentation in endotoxin tolerant monocytes has previously been reported [25]. To address whether MR1-mediated stimulation of MAIT cells was suppressed when MDMs had previously been exposed to LPS, MDMs were pretreated overnight with various concentrations of LPS and their ability to stimulate MAIT cells subsequently assessed. Robust suppression of MAIT cell stimulation was seen with as little as 10 ng/mL of LPS (Fig. 7D); across all concentrations of LPS, a 62% (range 46–78%) reduction in IFN- γ production by MAIT cells was observed. This could be partially explained by activation-induced cell death of MDMs: the viability of MDMs exposed to LPS was reduced by 23% (range 9–33%) (Supporting Information Fig. 10C). Following LPS exposure, MDMs retained their ability to phagocytose bacteria (Supporting Information Fig. 10D), but produced less TNF- α (52% reduction, range 39–62%) upon subsequent exposure to *E. coli* (Supporting Information Fig. 9D), suggesting that the remaining MDMs were endotoxin tolerant. Decreased TNF- α production was also seen following pretreatment with HKLM (a TLR2 agonist) (48% reduction, range 41–60%) but not Pam3CSK4 (a TLR1 agonist) (Supporting Information Fig. 10A), correlating with the ability of MDMs to stimulate MAIT cells (Fig. 7C). When monocytes were pre-exposed to LPS, a 38% reduction (range 19–59%) in IFN- γ production by MAIT cells was observed (Supporting Information Fig. 9C); there was no evidence of activation induced cell death (Supporting Information Fig. 9D,E). Therefore, MR1-mediated MAIT cell activation by primary human MDMs and monocytes is also dependent upon NF- κ B signaling, but is negatively regulated upon prolonged exposure to endotoxin by endotoxin tolerance \pm activation induced cell death.

Discussion

We have shown that MR1-mediated MAIT cell activation is tightly regulated. Using an MR1-dependent model of MAIT cell activation we have found that (i) uptake and processing of intact bacteria by APCs leads to more robust MAIT cell activation than exposure to soluble ligand, (ii) for soluble ligand the amount of MR1 at the cell surface is rate-limiting, (iii) little MR1 is expressed at the cell surface at any one time but that it traffics to and from the cell surface, (iv) the rate at which MR1 accumulates at the cell surface

is increased upon APC activation and that this is dependent upon NF- κ B signaling, (v) MAIT cell stimulatory ability is dependent upon NF- κ B signaling and APC activation, and (vi) in MDMs and monocytes MR1-mediated MAIT cell activation is negatively regulated in endotoxin tolerance. We propose that these mechanisms combine to regulate MR1-mediated MAIT cell activation.

MAIT cells can be activated with or without TCR activation. Consistent with our recent reports [14, 15], we found that early MAIT cell activation by *E. coli*-treated primary human monocytes and B cells was MR1-dependent and IL-12+IL-18-independent, while later activation involved both mechanisms. By restricting observations to the early time-point it allowed us to specifically investigate MR1-mediated antigen presentation. Furthermore, it highlights the importance of considering cytokine-mediated activation when overnight cultures of MAIT cells are performed.

The MR1 ligand was recently identified as unstable ribityllumazine intermediates 5-OP-RU and 5-OE-RU, which are formed by condensation of 5-A-RU with methylglyoxal or glyoxal, respectively [11, 12]. 5-OP-RU was found in the supernatant of a *Salmonella* culture and, when co-incubated with C1R cells transduced with MR1, activated MAIT cells [11, 12]. However, given the number of commensal bacterial strains that can synthesize riboflavin (<http://www.genome.jp/kegg/pathway/map/map00740.html>), the low molecular weight of 5-A-RU and ribityllumazine intermediates, and the potential for these compounds to diffuse away from the site of infection (or even to cross mucosal barriers), we hypothesized that MR1-mediated MAIT cell activation must be tightly regulated to prevent inappropriate activation and subsequent immunopathology. Using non-transduced THP1 cells we found that intact bacteria activate MAIT cells substantially more efficiently than bacterial culture supernatant. While the *E. coli* culture supernatant appears to contain ligand, only weak MR1-mediated activation was seen. This was not due to a difference between bacterial species, as similar results were obtained with non-typhoidal *Salmonella*. Furthermore, it would seem unlikely that there are higher concentrations of the ligand in the bacterium than in the supernatant as little or no stimulation was seen with bacterial cell lysate (although destruction during sonication cannot be excluded). Therefore, loading of MR1 appears to occur most efficiently in an acidified endosomal compartment, and uptake of intact bacteria is the most efficient means of delivering ligand to this compartment. This is consistent with previous studies which have shown MR1 to be in endolysosomes [26] and MR1-mediated MAIT cell activation to be dependent upon endosomal acidification [13].

It is unclear why the induction of phagocytosis with intact *Enterococcus faecalis* failed to enhance the MR1-mediated MAIT cell stimulatory ability of *E. coli* supernatant. It may be that insufficient soluble ligand was phagocytosed with the intact bacteria. Alternatively, the formation of ribityllumazine derivatives 5-OP-RU and 5-OE-RU, through the condensation of bacterially derived 5-A-RU with methylglyoxal or glyoxal respectively, may occur more efficiently in the phagolysosome. Indeed, addition of 5-A-RU alone to C1R.MR1 cells was sufficient to increase surface expression of MR1 and to activate Jurkat.MAIT cells [12]. Of note however, the

ribityllumazine derivatives are less stable at <pH6 [12]. Alternatively, the failure to complement *E. coli* supernatant with intact *E. faecalis* could be due to differences in TLR signaling induced by *E. coli* and *E. faecalis*, affecting THP1 phenotype and function, for example the expression of co-stimulatory molecules. TLR signaling differs in response to gram-negative and gram-positive bacteria [27]. While *E. faecalis* stimulates TLR2 [28], it stimulates a less robust pro-inflammatory response by monocyte-derived dendritic cells than *E. coli* [29]. *Enterococcus faecalis* does however stimulate sufficient IL-12 and IL-18 production by THP1 cells to stimulate MAIT cells [14]. Differences in TLR signaling could also contribute to the observed difference between intact *E. coli* and *E. coli* supernatant. *E. coli* supernatant, however, is known to contain multiple TLR agonists, including LPS, the TLR 5 agonist flagellin, and a TLR2 agonist [30–32], and no difference was observed in the upregulation of activation markers (data not shown) or of surface expression of MR1 (Fig. 3D, 3E) between THP1s treated with intact *E. coli* and *E. coli* supernatant.

We hypothesized the discrepancy in the stimulatory ability of bacterial culture supernatant between our study and that of Kjer-Nielsen *et al.* [11] may be due to the levels of MR1 available at the cell surface. Indeed, the C1R.hMR1 cells used by Kjer-Nielsen *et al.* constitutively express large amounts of MR1 at the cell surface [17]. In contrast, untransduced THP1s and BCLs expressed barely detectable levels of MR1 at the surface, even though mRNA could be readily detected in THP1s (data not shown). This is consistent with previous studies that have suggested that MR1 is ubiquitously expressed at the mRNA level [3, 33], but that surface expression is difficult to detect [18]. Even when MR1 was overexpressed in THP1 cells and BCLs, only a small amount of MR1 was seen at the surface at any one time. This may reflect the relatively low levels of intracellular hMR1.HA seen in these nonclonal cell lines. In addition, it is possible that the C-terminal HA tag interferes with MR1 trafficking. The increased staining that was observed when anti-MR1 was added to the culture media for 4 h suggests that there is turnover of MR1 at the surface. Strikingly, this turnover was markedly increased in THP1s, but not in BCLs, upon activation. This increased turnover of MR1 at the cell surface was independent of ligand but dependent upon activation and NF- κ B signaling. Furthermore, it decreased with prolonged activation. Increased transcription of MR1 was seen in THP1 cells upon activation. Therefore, in THP1 cells surface expression of MR1 is increased upon activation. In contrast, in BCL cells, which are similar to C1R cells, increased levels of MR1 were seen at the surface and expression was unaffected by activation. Whether the higher level of MR1 surface expression seen in C1R.MR1 cells compared with BCL.MR1 cells is due to a lack of competition for β 2-microglobulin by MHC class I molecules in C1R cells remains to be determined. In unactivated THP1s and BCLs, inhibition of NF- κ B signaling reduced MR1 trafficking, suggesting basal NF- κ B signaling may indeed be important. Transient trafficking of MR1 to the cell surface of murine double-positive thymocytes, B cells, macrophages, and dendritic cells has previously been reported [18]. Surface expression of MR1 has also previously been seen in the lung epithelial cell line, A549 after infection with

Mycobacterium tuberculosis [34]. Future studies should address whether MR1 trafficking is similarly regulated in epithelial cells and monocytes/macrophages.

NF- κ B-signaling in the APC was required for MR1-mediated MAIT cell activation. While NF- κ B signaling affects MR1 trafficking, NF- κ B-mediated up-regulation of other molecules, such as co-stimulatory molecules, or secretion of cytokines, may also be required for robust MAIT cell-mediated activation. Indeed, Turtle *et al.* [35] found that TCR signaling was detuned in MAIT cells and that co-stimulation or pro-inflammatory cytokines were required for robust MAIT cell responses to TCR stimulation. Consistent with a requirement for APC activation, we found that preactivation of THP1 cells with TLR agonists or interferons enhanced MR1-mediated MAIT cell activation. That type I or II interferons also enhanced MR1-mediated MAIT cell activation, suggests that activation of APCs by pathways other than NF- κ B signaling may also enhance MR1-mediated activation. Activation of MAIT cells by MDMs and primary human monocytes was also dependent upon NF- κ B signaling. However, in contrast to THP1s, MDMs robustly activated MAIT cells early after exposure to bacteria with a later reduction, while in the case of primary human monocytes there was only a small increase in stimulatory ability with prolonged exposure to bacteria. With both MDMs and monocytes preactivation did not enhance MAIT cell activation. This suggests that in contrast to monocytic THP1s, all the proteins required for efficient processing of ligand from intact bacteria, presentation by MR1, and co-stimulation, are already expressed by MDMs and primary human monocytes.

This is consistent with a hypothesis whereby sensing of pathogen-associated molecular patterns by pattern recognition receptors, such as TLRs, is required to license the APC to activate MAIT cells via MR1. While Le Bourhis *et al.* previously found that mouse bone marrow-derived dendritic cells knocked out for MyD88 or TRIF showed only a modest decrease in MAIT cell stimulatory ability [13], cytokine-mediated MAIT cell activation by IL-12 and IL-18 was not excluded in these experiments. In addition, redundancy in pathways for APC activation upon exposure to bacteria may also contribute to these differing results.

MR1-mediated MAIT cell activation by MDMs was also significantly negatively regulated by pretreatment with LPS. Pretreatment with TLR2, TLR3, and TLR5 agonists also appeared to negatively regulate MAIT cell activation, although this did not reach significance. The suppressive effect of TLR4 was partially explained by decreased viability of LPS-treated MDMs, however the significant decrease in TNF- α production upon restimulation and the preservation of phagocytic function suggested that endotoxin tolerance also contributed to the inhibition of MAIT cell activation [36]. Endotoxin tolerance was more evident in primary monocytes, where LPS pretreatment suppressed their ability to subsequently activate MAIT cells, but had no effect on monocyte viability. This combination of activation-induced cell death and endotoxin tolerance may also explain the decreased stimulatory capacity of MDMs with prolonged exposure to bacteria. It is unclear why MDMs were more sensitive to the suppressive effects

of LPS than THP1s. Pretreatment of THP1 cells with LPS did however fail to enhance the early activation of MAIT cells (Fig. 5B), despite the ability of THP1 cells to respond to LPS [14].

Activation-induced cell death and endotoxin tolerance have both been suggested to be negative feedback mechanisms to prevent over-activation in the presence of on-going TLR stimulus [37, 38]. MHC class II surface expression and MHC class II-mediated CD4⁺ T-cell activation have previously been shown to be negatively regulated by endotoxin tolerance [25]. Here we have shown that endotoxin tolerance also negatively regulates both MR1 surface expression and MR1-mediated MAIT cell activation. Given that MR1 presents a microbially derived ligand, downregulation upon prolonged exposure to LPS would limit MAIT cell over-activation in the face of persisting infection.

In conclusion, we have shown that MR1-mediated MAIT cell activation is tightly regulated at several levels. Efficient MR1-mediated MAIT cell activation requires both intact bacteria to access an acidified endolysosomal compartment and activation of the APC through NF- κ B or interferon signaling pathways. Furthermore, MR1-mediated MAIT cell activation is negatively regulated upon prolonged TLR stimulation. We propose that these mechanisms combine to prevent inappropriate or prolonged MR1-mediated activation, which could otherwise cause immunopathology. These findings have implications for the development of MAIT cell based immunotherapies.

Materials and methods

General

These studies were conducted in a laboratory that operates under exploratory research principles and were performed using investigative protocols and assays. The raw data are provided in a supplementary file.

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte cones from the NHS Blood Bank or from blood from healthy donors (collected with informed consent with approval from the University of Otago Human Ethics Committee (Health)) using Lymphoprep (Axis-Shield, Dundee, UK). PBMCs were cryopreserved in freezing media (90% fetal calf serum (FCS), 10% DMSO (both Sigma-Aldrich, Dorset, UK)), and stored in liquid nitrogen. Frozen PBMCs were thawed immediately prior to isolation of CD8⁺ T cells, B cells, or monocytes, and were washed in RPMI 1640 media supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin (R10) (all Sigma-Aldrich) with (20 μ g/mL) DNase (Roche, Mannheim, Germany). CD8⁺ T cells were isolated with CD8 microbeads or, where indicated in the figure legend, with anti-PE microbeads (Miltenyi Biotec, Surrey, UK) as per the manufacturer's instructions. Primary human monocytes or B cells were isolated with CD14

or CD19 microbeads respectively (Miltenyi Biotec); in some experiments, where indicated in the figure legend, monocytes were sorted on a FACS Aria (BD Biosciences, San Jose, CA, USA). Monocyte-derived macrophages were derived by culturing primary human monocytes in 50 ng/mL M-CSF (R&D Systems, Abingdon, UK) for 6–7 days. THP1 cells (ECACC, Wiltshire, UK) were cultured in R10 + 0.05 mM 2-mercaptoethanol (Sigma-Aldrich). An EBV-transformed B-cell line (kindly provided by Dr Henrik Kloverpris, University of Oxford, UK [39]) was cultured in R10. The 293Ta lentiviral packaging cell line (GeneCopoeia, Rockville, MD, USA) was maintained in DMEM (Sigma-Aldrich) + 10% FCS + penicillin/streptomycin.

Inhibitors, TLR agonists, cytokines, and functional antibodies

The following inhibitors were used: cytochalasin D (Sigma-Aldrich) 5 μ g/mL; bafilomycin A1 (Sigma-Aldrich) 10 nM; IKK inhibitor VII (Merck-Millipore, Darmstadt, Germany) 5 μ M; (5Z)-7-oxozeaenol (Tocris, Bristol, UK) 0.75 μ M. The effect of inhibitors on THP1 cell viability is shown in Supplementary Fig. 4. The following TLR agonists were used at 1 μ g/mL unless otherwise indicated: Pam3CSK4, HKLM, Poly(I:C), LPS *E. coli* K12, flagellin *Salmonella* Typhimurium, FSL-1, Imiquimod, ssRNA40/Lyovec, and ODN2006 (all InvivoGen, San Diego, CA, USA). The following cytokines were used: M-CSF (R&D Systems) 50 ng/mL; IFN- γ (Miltenyi Biotec) 1000 U/mL; interferon- α 2a (Miltenyi Biotec) 1000 U/mL. The following blocking antibodies were used: anti-MR1 (clone 26.5; TH) 10 μ g/mL; anti-IL-12p40/70 (eBioscience, Hatfield, UK) 5 μ g/mL; anti-IL-18 (MBL International, Woburn, MA, USA) 5 μ g/mL.

Antibodies

The following antibodies were used: V α 7.2-PE, V α 7.2-FITC, CD3-PECy7, CD8-PE, CD14-AF488, CD80-PECy7, CD86-PerCPCy5.5, HLA-A2-PE, HLA-DR-FITC, IFN- γ -PerCPCy5.5 (Biolegend, London, UK), CD161-APC, IFN- γ -FITC, HA-PE (Miltenyi Biotec), CD8-eFluor450, CD54-APC (eBioscience). Samples were stained with Live/Dead Fixable Near IR dye (Invitrogen, Paisley, UK). Anti-MR1 antibody (clone 26.5) and an isotype control (IgG2A, R&D Systems) were labeled with an AlexaFluor488-conjugated anti-mouse IgG Fab fragment (Jackson ImmunoResearch, West Grove, PA, USA), as previously described [40, 41]. The reaction was quenched with normal mouse immunoglobulin (Sigma-Aldrich). In some experiments, where indicated in the figure legend, anti-MR1-PE (clone 26.5) and an IgG2A-PE isotype control were used (both Biolegend).

Bacteria

E. coli (DH5 α), non-typhoidal *Salmonella*, or *Enterococcus faecalis* (clinical isolates obtained from the Microbiology Laboratory, John Radcliffe Hospital, Oxford) were grown overnight in Luria

broth (Sigma-Aldrich). Bacteria were washed twice in phosphate buffered saline (PBS), then fixed for 20 min at room temperature in 2% paraformaldehyde/PBS. After fixation, bacteria were washed two times and resuspended in PBS. Bacterial stocks were quantified by flow cytometry using the MACSQuant. Each batch was standardized to the previous batch. Bacteria were added at 25 bacteria/APC unless otherwise indicated.

Bacterial cell lysates were prepared by sonication (6×30 s pulses at $15 \mu\text{m}$ using an MSE Soniprep 150 (Sanyo Gallenkamp PLC, Leicester, UK)). The resulting lysate was filtered through a $0.2 \mu\text{m}$ filter prior to use. Bacterial supernatant was prepared by centrifugation of an overnight culture, followed by filtration through a $0.2 \mu\text{m}$ filter. Ten microliters of bacterial cell lysate or supernatant were added unless otherwise indicated; this was equivalent to 2×10^6 bacteria (25 bacteria/APC) or the equivalent proportion of the culture supernatant. Bacterial cell lysate and supernatant were freshly prepared for each experiment.

Generation of THP1.hMR1 and BCL.hMR1

The plasmid, pLV120.hMR1-HA, encoding human MR1 with a C-terminal 3x HA tag under the control of a CMV promoter and puromycin resistance, was synthesized by GeneCopoeia (EX-W1295-Lv120). pLV120.hMR1-HA was maintained in GCI-L3 chemically competent cells (GeneCopoeia). The lentiviral vector was produced by co-transfection of pLV120.hMR1-HA and Lenti-Pac HIV mix into 293Ta lentiviral packaging cells with EndoFectin Lenti transfection reagent (all GeneCopoeia). After overnight incubation, media were replaced with fresh DMEM+5% FBS supplemented with 1:500 TitreBoost reagent (GeneCopoeia). Lentiviral-containing supernatant was harvested 2 days post-transfection, centrifuged, and filtered. Lentiviral supernatants were mixed with Lenti-Pac concentration solution (GeneCopoeia) and incubated overnight at 4°C . The solution was centrifuged at $3500 \times g$ for 25 min at 4°C , the supernatant discarded, and the virus pellet resuspended in PBS. Lentiviral vectors were stored at -80°C prior to use.

THP1 and BCL lines overexpressing hMR1.HA were generated by infection with the lentiviral vector. Cells were resuspended in media containing $8 \mu\text{g}/\text{mL}$ polybrene (Sigma-Aldrich). Virus was added and cells centrifuged at 2250 rpm for 30 min. Selection with puromycin-containing media ($4 \mu\text{g}/\text{mL}$) was initiated 24 h post-infection. Transgene expression was determined by flow cytometry for both HA tag and MR1.

MAIT cell activation

APCs were seeded at 8×10^4 /well in U-bottomed (or flat-bottomed for monocyte-derived macrophages) 96-well plates; viable cells were counted using Trypan Blue (Invitrogen) and a Neubauer counting chamber. APCs were incubated overnight for 20 h. Fixed bacteria (25 bacteria per cell unless otherwise stated), bacterial cell lysate, or bacterial culture supernatant (both 10

μL unless otherwise stated) were added at the start of culture or, in some experiments, for the final 7 h of culture. In some experiments pharmacological inhibitors, TLR agonists, interferon- α or IFN- γ , were added at the start of culture as indicated. CD8^+ T cells were added at $1\text{--}2 \times 10^5$ /well either at the start of culture (total 20 h co-culture) or for the final 5 h (total 5 h co-culture); blocking antibodies were added at the same time as the CD8^+ T cells. When CD8^+ T cells were added for the final 5 h of culture, APCs were washed three times in R10 before the addition of the CD8^+ T cells. Brefeldin A ($3 \mu\text{g}/\text{mL}$) was added for the final 4 h of culture. Cells were analyzed for IFN- γ production by intracellular cytokine staining.

Measurement of phagocytosis

Phagocytosis was measured with the pHrodo[®] Red *E. coli* BioParticles[®] Phagocytosis Kit for Flow Cytometry (Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer's protocol. Monocyte-derived macrophages were detached by pipetting following a 30-min incubation on ice in PBS + 5 mM EDTA and 12 mM lidocaine (both Sigma-Aldrich).

Immunostaining

Immunostaining was performed as previously described [16]. Briefly, samples were stained with antibodies against CD3, CD8, CD161, and $\text{V}\alpha 7.2$, and with Live/Dead Fixable Near IR dye for 20 min at 4°C , then washed, then fixed for 20 min in 2% formaldehyde at 4°C , then washed in $1 \times$ Permeabilization Buffer (eBioscience) prior to staining with an antibody against IFN- γ , and restaining CD3, CD8, CD161, and $\text{V}\alpha 7.2$.

To stain for MR1, pre-labeled anti-MR1 (or isotype control) was added at $10 \mu\text{g}/\text{mL}$ for the final 4 h of culture. Cells were then washed, stained with Live/Dead Fixable Near IR dye, and fixed, prior to analysis by flow cytometry.

Flow cytometry

Flow cytometry was performed on a MACSQuant (Miltenyi Biotec) or a FACS Canto (BD Biosciences). Samples were gated on lymphocytes/alive/ $\text{CD3}^+\text{CD8}^+/\text{CD161}^{++}\text{V}\alpha 7.2^+$ cells (Supporting Information Fig. 1). Analysis was performed in FlowJo 9.6 (TreeStar, Ashland, OR, USA). The gate for IFN- γ positivity was defined from the unstimulated sample.

Statistical analysis

Data were analyzed in GraphPad Prism software Version 6.0b (La Jolla, CA, USA). Unless otherwise indicated, means and all data points are shown. Comparisons between groups were made by repeated measures one-way ANOVA followed by Dunnett's,

Tukey's, or Sidak's multiple comparison test, as indicated in the figure legends. Comparisons between two groups were made with a Wilcoxon matched pairs signed rank test. Significance was defined as $p < 0.05$. Where statistical model assumptions were potentially not satisfied, alternative strategies (including transformations and nonparametric analyses, and sensitivity analyses with possible leverage points removed) were investigated to ensure that the reported interpretation of results was robust.

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

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Abbreviations: APC: antigen presenting cell · MAIT cells: mucosal-associated invariant T-cells · MDM: monocyte-derived macrophage

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