Technical Advance: Human MAIT-cell responses to *Escherichia coli*: activation, cytokine production, proliferation, and cytotoxicity

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ABSTRACT

Mucosa-associated invariant T cells are a large and relatively recently described innate-like antimicrobial T-cell subset in humans. These cells recognize riboflavin metabolites from a range of microbes presented by evolutionarily conserved major histocompatibility complex, class I-related molecules. Given the innate-like characteristics of mucosa-associated invariant T cells and the novel type of antigens they recognize, new methodology must be developed and existing methods refined to allow comprehensive studies of their role in human immune defense against microbial infection. In this study, we established protocols to examine a range of mucosa-associated invariant T-cell functions as they respond to antigen produced by *Escherichia coli*. These improved and dose- and time-optimized experimental protocols allow detailed studies of MR1-dependent mucosa-associated invariant T-cell responses to *Escherichia coli* pulsed antigen-presenting cells, as assessed by expression of activation markers and cytokines, by proliferation, and by induction of apoptosis and death in major histocompatibility complex, class I-related-expressing target cells. The novel and optimized protocols establish a framework of methods and open new possibilities to study mucosa-associated invariant T-cell immunobiology, using *Escherichia coli* as a model antigen. Furthermore, we propose that these robust experimental systems can also be adapted to study mucosa-associated invariant T-cell responses to other microbes and types of antigen-presenting cells. *J. Leukoc. Biol.* 100: 233-240; 2016.

Introduction

MAIT cells are a novel and evolutionarily conserved subset of innate-like antimicrobial T cells that are highly abundant in mucosal tissues, the liver, and peripheral blood [1]. In humans, MAIT cells are mostly CD8αα or CD8αβ, they express a semi-invariant TCR with an invariant Vα7.2 segment coupled with restricted Ja and Vβ repertoires and recognize antigens in complex with the MR1 protein [2-6]. MR1 displays an extraordinary level of evolutionary conservation among mammals [2, 3, 7], strongly supporting the notion that MR1 and MAIT cells perform critical functions in the immune system. Human MAIT cells have high expression of CD161, IL-18Rα, and the promyelocytic leukemia zinc finger transcription factor [8, 9]. Recent seminal studies discovered that MAIT cells recognize vitamin B2 (riboflavin) metabolites from a wide range of microbes [10, 11]. Once activated by such antigens, MAIT cells rapidly mediate broad and potent antimicrobial activity [4, 5, 8, 12-17], which most likely contributes to the MAIT cells' potential role in the protection against diverse bacterial infections in animal models and in humans [8, 18-25]. Furthermore, recent studies have shown the involvement of MAIT cells in metabolic disorders, inflammatory diseases, and malignancies, therefore highlighting their potential significance in noninfectious diseases and their role beyond antimicrobial effector T cells [24-29].

The study of MAIT-cell function and their role in human antimicrobial immunity is still in its infancy and there is a great need to study their role in a range of diseases where MAIT-cell deficiency or dysfunction is implicated. The recent definition of the structure of MR1-presented MAIT-cell antigens allows the detailed study of MAIT-cell specificity to such antigens [10, 11, 30, 31]. However, the synthesis of these ligands is technically challenging and is not widely available to most immunologic research laboratories. Another limitation is that it may be hard to directly interpret MAIT-cell antimicrobial activity against a certain microbe when such synthetic ligands are used to assess their effector function. Furthermore, it is possible that there are other MR1-presented

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Abbreviations: Ctrl = control, CTV = cell trace violet, DCM = live/dead cell marker, E:T ratio = effector:target cell ratio, FLICA = fluorescent inhibitor of caspases, Grz = granzyme, MAIT = mucosa-associated invariant T (cell), MR1 = major histocompatibility complex, class I-related

The online version of this paper, found at www.jleukbio.org, includes supplemental information.
antigenic structures that have yet to be identified. In this study, we developed improved methodologies to study MAIT-cell antimicrobial effector functions in detail based on the widely available *Escherichia coli* as a model microbe and natural source of MAIT-cell–activating ligands. These methods allowed us to study MAIT-cell activation, cytokine production, and proliferative responses in the context of defined APCs, as well as killing capacity against bacteria-pulsed target cells. In addition, these adaptable methods also offer the flexibility to assess various aspects of MAIT-cell antimicrobial activity against different microbes and, therefore, to unravel their role in different immunologic contexts.

**MATERIALS AND METHODS**

**Peripheral blood**

Peripheral blood was obtained from healthy individuals recruited at the Blood Transfusion Clinic (Karolinska University Hospital, Huddinge, Sweden). Written informed consent was obtained from all individuals, in accordance with study protocols conforming to the provisions of the Declaration of Helsinki and approved by the Regional Ethics Review Board in Stockholm.

**Cell isolation procedures and bacterial culture**

PBMCs were isolated from peripheral blood by Ficol-Hypaque density gradient centrifugation (Lymphoprep;Axis-Shield, Oslo, Norway) and rested overnight in RPMI-1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine (all from Thermo Fisher Scientific, Waltham, MA, USA), 10% FBS (Sigma-Aldrich, St. Louis, MO, USA), 50 µg/ml gentamicin (Thermo Fisher Scientific), and 100 µg/ml Normocin (InvivoGen, San Diego, CA, USA) (complete medium). Va7.2+ cells were isolated from PBMCs with anti-Va7.2 PE- or APC-conjugated mAb (BioLegend, San Diego, CA, USA), followed by positive selection with MACS anti-PE or anti-APC microbeads, respectively (Miltenyi Biotec, San Diego, CA, USA), according to manufacturer’s instructions. Monocytes were obtained from peripheral blood by negative selection with the RosetteSep human monocyte enrichment cocktail (StemCell Technologies), according to the manufacturer’s instructions. The Va7.2+ strain D21 was cultured overnight to late stationary phase at 37°C in Luria-Bertani broth. Live bacteria were counted by the standard plate-counting method, and counts were adjusted, taking into account the proportion of MAIT cells within the Vα7.2+ T cells. Supplemental Fig. 2 depicts the gating strategy to identify MAIT cells and activation status in a multicolor, dual-laser flow cytometer. Supplemental Fig. 3A shows representative flow cytometry data with MAIT cell killing of 293T-hMR1 cells. To determine the proportion of *E. coli* internalization by target cells, we labeled *E. coli* with 5 µM pHrodo Red dye (Thermo Fisher Scientific Life Sciences), according to the manufacturer’s instructions. Briefly, *E. coli* was washed once in 100 mM NaHCO₃ buffer and then incubated with the dye for 45 min at room temperature. The bacteria were washed twice in 100% methanol to remove excess dye, followed by 2 washes in PBS. Human 293T cells stably transduced with human MR1 (293T-hMR1 cells; a kind gift from Dr. Ted Hansen, Washington University, St. Louis, MO, USA) [3] were maintained in complete medium. For the bacteria internalization experiments, 293T-hMR1 cells were incubated for 3 h with pHrodo Red-labeled or nonlabeled 5 min-fixed *E. coli* at the indicated dose. In the pHrodo Red-labeled *E. coli* experiments as indicated, 293T-hMR1 cells were incubated in complete medium alone or supplemented with 25 ng/ml recombinant human IL-7 (R&D Systems, Minneapolis, MN, USA) for 72 h and then added to the 293T-hMR1 cells at the indicated E:T (MAIT:293T-hMR1) ratio and for the indicated length of time. The MAIT:293T-hMR1 ratio was adjusted, taking into account the proportion of MAIT cells within the Vα7.2+ cell population. Apoptosis of 293T-hMR1 cells was determined by adding the FLICA reagent (Vybrant FAM Poly Caspases Assay Kit; Thermo Fisher Scientific) at a final concentration of 0.2% (v/v) to the MAIT-293T-hMR1 cell culture at the beginning of the assay. Anti-CD107a PECy7 at a final concentration of 0.4 µg/ml was added to detect MAIT-cell degranulation in the MR1-blocking experiments, 20 µg/ml anti-MR1 mAb or IgG2a isotype ctrl were added for 60 min before the addition of the Vα7.2+ cells. The 293T-hMR1 cells were cultured for 3 d in the presence of 0.2% (v/v) anti-MR1 mAb or IgG2a isotype ctrl were added for 60 min before the addition of the Vα7.2+ cells. The 293T-hMR1 cells were detached from the flat-bottom 96-well plates (Falcon; Corning Inc., Corning, NY, USA) by gentle pipetting at the end of the experiment.

**Flow cytometry**

The antibodies used are listed in Supplemental Table 1. Cell surface and intracellular staining was performed as has been described previously [4, 15, 16]. Samples were acquired on an LSRLFortessa flow cytometer (BD Biosciences) equipped with 405, 488, 561, and 639 nm lasers. Single-stained polystyrene beads (BD Biosciences) were used for compensation, which was performed using the compensation platform in FlowJo software v. 9.8 (Tree Star).

**Online supplemental material**

Supplemental Table 1 lists antibodies used in flow cytometry. Supplemental Fig. 1 depicts the gating strategy to identify MAIT cells and activation status in PBMCs and in MACS-purified Vα7.2+ T cells. Supplemental Fig. 2 depicts CD161 downregulation after MAIT-cell proliferation. Supplemental Fig. 3 depicts cytokolytic protein profile by IL-7-treated MAIT cells and the strategy to measure the MAIT-cell killing of 293T-hMR1 cells.

**RESULTS AND DISCUSSION**

Single variable optimization of the assay for activation of MAIT cells by *E. coli* fed monocytes

In vitro activation of MAIT cells can be assessed by cytokine production after overnight incubation of PBMCs with *E. coli* [4].
However, the composition of PBMCs in type and amount of APCs is variable, and differences in the composition between individuals could in part lead to the interindividual variability in MAIT-cell activation. Therefore, we sought to establish an activation assay where the type and amount of APC and antigen can be controlled, using monocytes as APCs, *E. coli* as the source of antigen, and Vα7.2+ cells positively selected from PBMCs as the source of MAIT cells.

Initial experiments were performed to ascertain that MAIT cells enriched through magnetic bead-based positive selection were not phenotypically altered. The proportions of CD8+ and CD4/8 double-negative MAIT-cell subsets in the Vα7.2+ cell fraction were similar to those among MAIT cells within the paired PBMCs after a 6 h incubation at 37°C/5% CO2 (Supplemental Fig. 1A, B). Purified MAIT cells displayed no signs of activation by the isolation procedure, as evidenced by the lack of change in expression of CD69, CD25, CD38, HLA-DR, and T-cell immunoglobulin and mucin domain-3 (Supplemental Fig. 1C). These data indicate that positive selection of Vα7.2+ cells is a suitable method for enriching MAIT cells while maintaining their phenotype and resting status and is appropriate for detailed studies of MAIT-cell functions.

To establish the activation assay, we performed single variable optimization for each of the variables in the experiment, including the time of *E. coli* fixation, microbial dose (defined as the *E. coli* CFU:monocyte ratio), concentration of anti-CD28, Vα7.2+ cell:monocyte ratio, and duration of the culture. The coexpression of CD69 and IFNγ (CD69/IFNγ) by MAIT cells was used as readout for activation of these cells (Fig. 1A) [4].

To avoid bacterial overgrowth during the 24 h culture with monocytes and Vα7.2+ cells, *E. coli* was fixed in 1% formaldehyde before addition to the monocytes. *E. coli* fixed for 1–10 min supported similar levels of MAIT-cell activation, whereas levels decreased when *E. coli* was fixed longer than 30 min (Fig. 1B). This pattern suggests that the MAIT-cell activating antigens present in *E. coli* are not destroyed by short periods of fixation, and this notion was further confirmed by comparing the activation induced by 3 min-fixed *E. coli* with that induced by live *E. coli*. In our study, MAIT-cell activation and IFNγ production were comparable between the 2 conditions (Fig. 1C). In contrast, activation of MAIT cells by heat-killed *E. coli* was close to zero (Fig. 1B), consistent with the notion that MAIT-cell-activating antigens are heat sensitive. No observable bacterial growth in the culture supernatant was found after 24 h stimulation with heat-killed or formaldehyde-fixed bacterial preparations (data not shown). Using the 3 min-fixed *E. coli* stimulation, we next determined the optimal microbial dose

![Figure 1. Optimization of an *E. coli* mediated MAIT-cell activation assay.](image-url)

(A) A representative example from 11 donors of the MAIT-cell population and CD69 and IFNγ expression by resting and activated MAIT cells. (B-G) Unless otherwise indicated, MAIT cells were stimulated with 3 min-fixed *E. coli* (100 CFU/monocyte) for 24 h, in the presence of 1.25 μg/ml anti-CD28 and at a Vα7.2+ cell:monocyte ratio of 2:1. (B, D–G) Frequency of CD69/IFNγ+ MAIT cells. Lines represent individual donors. (B) *E. coli* was fixed for 1–60 min or heat killed at 95°C for 10 min. Data were normalized to 1 min-fixed *E. coli*. (C) Monocytes were fed live or 3 min-fixed *E. coli*. FACS plots are representative of 7 donors. *E. coli* CFU:monocyte ratio (D) and anti-CD28 concentration (E) ranged from 0.01 to 300 and 0 to 5 μg/ml, respectively. (F) The Vα7.2+ cell:monocyte ratio ranged from 0.25:1 to 64:1. (G) MAIT cells were harvested every 2 h between 6 and 24 h of activation. (H) Timeline of the MAIT-cell activation assay.
for MAIT-cell activation. We found the frequency of CD69+IFNγ+ MAIT cells increased up to a dose of 100 E. coli CFU per monocyte, whereas it consistently decreased at higher doses in all 3 donors (Fig. 1D). At this optimal microbial dose, addition of anti-CD28 increased the MAIT-cell response to E. coli stimulation, whereas anti-CD28 alone did not activate MAIT cells (Fig. 1E). Using 1.25 μg/ml anti-CD28, we found that MAIT-cell activation peaked at Vo7.2’ cell:monocyte ratios of 1:1 and 2:1 (Fig. 1F), whereas cultures with Vo7.2’ cell:monocyte ratios <1 and ≥4 gave rise to lower MAIT-cell responses (Fig. 1F). Finally, we determined the kinetics of MAIT-cell activation after E. coli stimulation. The frequency of CD69+IFNγ+ MAIT cells in 2 of 3 donors examined increased with time up to 16 h of coincubation, after which it reached a plateau (Fig. 1G). However, in 1 donor, the MAIT-cell response continued to increase until 24 h (Fig. 1G). Therefore, the 24 h culture condition was selected for further experiments. Taken together, these experiments allowed us to establish an optimized MAIT-cell activation assay, the timeline of which is depicted in Fig. 1H.

**Activation of MAIT cells by E. coli can be assessed by induction of CD25 expression**

We next sought to identify measures of activation other than the aforementioned CD69 expression and IFNγ production through which MAIT-cell responses may be assessed. To that end, MAIT cells stimulated with E. coli fed monocytes were surface stained for activation markers, and their expression was determined by flow cytometry. Similar to CD69, CD25 was highly upregulated by MAIT cells after 24 h stimulation with fixed E. coli (Fig. 2A). However, CD38, T-cell immunoglobulin and mucin domain-3, and HLA-DR were only modestly upregulated at this time point (Fig. 2A). Thus, upregulation of CD25 expression can be used to track MAIT-cell activation in response to E. coli. CD25 expression, alone or along with CD69, appeared to be more MR1-dependent after MAIT-cell activation with E. coli than did CD69 expression alone, in that MR1 blocking potently inhibited both CD25 and CD25-CD69 coexpression (Fig. 2A–C). Similar results were observed for the production of IFNγ (Fig. 2D, E), indicating that IFNγ production by MAIT cells after E. coli stimulation was likewise predominantly MR1 dependent. These results indicate that CD25, particularly in combination with CD69, may be suitable as an alternative marker for assessment of MR1-dependent MAIT-cell activation.

**Assay for MR1-dependent MAIT-cell proliferation in response to E. coli**

We next wanted to optimize an assay for in vitro proliferation of MAIT cells. Vo7.2’ cells were labeled with the proliferation-tracing dye CTV before 3, 5, or 7 d culture with monocytes and different doses of E. coli (Fig. 3A). MAIT cells proliferated in response to E. coli in a time- and microbial dose-dependent manner, as assessed by CTV dilution (Fig. 3A) and by the index of expansion, which determines the fold expansion, of the overall MAIT-cell population (Fig. 3B). At day 3, MAIT cells displayed only limited proliferation at any E. coli dose, whereas after 7 d in culture, a substantial proportion of MAIT cells had proliferated to an extent that CTV dilution peaks were difficult to discern (Fig. 3A, B). The 5 d E. coli stimulation yielded...
clear CTV dilution peaks and was thus the most suitable time point to study MAIT-cell proliferation in response to *E. coli*. For all the incubation periods, the CTV dilution and expansion index peaked at the highest microbial dose (Fig. 3A, B). There was slight downregulation of CD161 by MAIT cells concomitant with proliferation. However, such downregulation was mitigated by increasing times in culture (Supplemental Fig. 2A, B). Notably, we observed stronger downregulation of CD161 by proliferating MAIT cells within bulk PBMC cultures (Supplemental Fig. 2A). Such strong downregulation is in agreement with results of another study [13] and may potentially be induced by other APCs present in the bulk PBMCs. Proliferation of MAIT cells in the Va7.2−monocyte coculture system led to an enrichment of this cell population across microbial doses and across incubation times over the Va7.2+CD1612 cells, which did not proliferate in response to *E. coli* (Supplemental Fig. 2A). CTV dilution and the expansion index of the MAIT-cell population were strongly inhibited by MR1 blocking (Fig. 3A, B), indicating that MAIT-cell proliferation in response to *E. coli* was predominantly MR1 dependent. The timeline of the optimized proliferation assay layout is depicted in Fig. 3C.

MAIT-cell cytotoxicity assay against *E. coli* pulsed target cells

Next, we sought to establish a cytotoxicity assay to study the ability of MAIT cells to kill *E. coli* pulsed target cells. For this purpose, Va7.2+ cells were used as the source of MAIT cells and 293T-hMR1 as target cells. First, the optimal microbial dose necessary to pulse 293T-hMR1 cells was determined. *E. coli* was labeled with the pHrodo Red dye, and the proportion of pHrodo Red+ 293T-hMR1 cells after a 3 h incubation with labeled *E. coli* was assessed by flow cytometry. pHrodo dye increases in fluorescence in an acidic environment, such as within the cellular lysosomal compartments, and can therefore be used to assess *E. coli* internalization by 293T-hMR1 cells. Our data show that the uptake of *E. coli* increased with increasing doses up to 200 CFU per 293T-hMR1 cell (Fig. 4A, B). Cytochalasin D is an actin polymerization inhibitor and was used to treat 293T-hMR1 cells to confirm that the pHrodo Red-positive signal from *E. coli* pulsed 293T-hMR1 cells was derived from internalized *E. coli* (Fig. 4A, B).

IL-7 is able to license activation of human MAIT cells and boost the production of Th1/Th17 cytokines by MAIT cells after CD3/CD28 stimulation [32]. We recently showed that IL-7 also arms resting MAIT cells, converting them into GrzB-expressing cytotoxic effector T cells capable of killing bacteria-pulsed cells and producing high levels of proinflammatory cytokines in an MR1-dependent fashion [16]. We initially confirmed that pretreatment of PBMCs with IL-7 for 72 h induced GrzB expression and upregulated perforin and GrzA levels by MAIT cells, when compared with levels in untreated cells (Supplemental Fig. 3A, B). Because resting MAIT cells lack GrzB expression (Supplemental Fig. 3A, B) [13, 16], purified Va7.2+ cells were pretreated with IL-7 for 72 h to induce MAIT cells to become cytotoxic effector cells before culture with *E. coli* pulsed target cells. Based on the results in Fig. 4A, B, 30 *E. coli* CFU per 293T-hMR1 cell, a bacterial dose leading 60–70% of 293T-hMR1 cells to internalize at least one *E. coli* CFU per cell, was used to determine the number of effector MAIT cells necessary to efficiently kill *E. coli* pulsed target cells. In this MAIT-293T-hMR1 cell coculture system, the target cells were identified based on their large forward and side-scatter profiles and lack of CD3 expression (Supplemental Fig. 3C). Target cell death was defined as cells positive for both polycaspase activities (FLICA+, an indicator of apoptosis) and amine-reactive DCM+ (Fig. 4C).
An E:T ratio of 10 was sufficient to induce optimal killing of the target cells after a 24 h MAIT-293T-hMR1 cell coculture (Fig. 4D). The minor contaminating Va7.2’CD161’ cell population present in the enriched fraction did not express CD107a, GrzB, or perforin (Supplemental Fig. 3D), suggesting that these cells cannot kill E. coli pulsed target cells. Using this optimal E:T ratio, MAIT cells rapidly induced polycaspase activation in bacteria-pulsed target cells, with maximum apoptosis observed within 6 h, whereas total target cell death (FLICA+ DCM+) did not reach its maximum until 24 h of coculture (Fig. 4E, F). The latter appeared to coincide with optimal MAIT-cell degranulation, as assessed by CD107a expression (Fig. 4E, F). MRI blocking markedly inhibited MAIT-cell degranulation, polycaspase activation, and full target cell death within the first 6 h. Continuous cycling of intracellular MRI to and from the cell surface and high surface MRI expression levels in the transduced 293T cell line could explain why MRI blocking was less effective after 6 h of coculture (Supplemental Fig. 3E and [33]). The addition of MRI-blocking antibody at concentrations higher than the working concentration of 20 μg/ml in 24 h cultures was only somewhat more efficient in inhibiting target cell death. Those higher doses did not further inhibit MAIT-cell degranulation, as the maximum inhibitory effect had already occurred at the lowest concentration tested (2.5 μg/ml; Supplemental Fig. 3F). Neither bacteria feeding of the target cells nor coculture with MAIT cells alone was sufficient to induce observable cell death, confirming the killing specificity of IL-7-treated MAIT cells for bacteria-pulsed target cells (Supplemental Fig. 3G). Altogether, these findings define an optimized MAIT-cell cytotoxicity assay against E. coli pulsed 293T-hMRI cells, as depicted in Fig. 4G, that can be adapted to a range of bacteria and target cells.
CONCLUSIONS

We have developed and optimized a range of methods to study MR1-dependent activation, proliferation, and cytotoxicity of MAIT cells in response to the strongly antigenic microbe \textit{E. coli}. Our activation assay uses a single type of professional APC, the monocyte, to present \textit{E. coli} derived antigens to MAIT cells, added in culture in a MACS-selected Vα7.2+ cell fraction. The expression of CD69 concomitant with the production of IFNγ by MAIT cells (CD69+IFNγ+ MAIT cells) is a good readout for the activation of these cells. However, CD25 coexpression with CD69 may be another viable alternative, good readout for the activation of these cells. However, CD25 coexpression with CD69 may be another viable alternative, because it was also upregulated after 24 h of \textit{E. coli} stimulation. Finally, it should be noted that this assay is not restricted to monocytes and, given the ubiquitous expression of MR1 [34], other types of APCs could be cocultured with MAIT cells. This approach could be used to address the question of whether different APCs differ in their ability to induce activation of MAIT cells, or generate different types of MAIT-cell responses.

The optimized proliferation assay is an effective tool for assessing MAIT-cell proliferation in response to monocytes pulsed with \textit{E. coli}. We used CTV dilution to detect MAIT-cell proliferation by flow cytometry, and software-based analysis allowed us to obtain proliferation-associated parameters, such as the expansion index of the MAIT-cell population. Because multicolor flow cytometry is performed in these assays, the analysis can be further extended to study the characteristics of MAIT cells with different proliferative abilities. We found the 5 d assay to be suitable for quantifying the proliferation of MAIT cells in this system. It is possible that the optimal assay times depend on the type of APC.

In the optimized cytotoxicity assay that we developed, MACS-purified Vα7.2+ cells are the source of MAIT cells and 293T-hMR1 cells are the target cells. The 293T-hMR1 cells were used because they are relatively resistant to bacteria-induced cell death (Supplemental Fig. 3G), and are, therefore, suitable to study the specific killing ability of MAIT cells. In this system, early time points are more appropriate to assess the target cell induction of apoptosis, whereas later time points seem more effective for assessing full cell death and MAIT-cell degranulation. The MR1 blocking experiments also indicate that early time points may be optimal for assessment of the MR1 dependency of MAIT-cell-mediated cytotoxicity against MR1-transduced target cells, although further study is needed for confirmation. In addition, the putative role of MR1-independent MAIT cell killing of bacteria-infected cells can be investigated with our optimized MAIT cell cytotoxicity assay.

The MAIT-cell functions assessed with these assays are probably dependent on a combination of both TCR engagement and the action of APC-derived cytokines directly on MAIT cells [35–37]. To determine the relative contribution of these mechanisms to the overall MAIT-cell response, we used MR1-blocking mAb at specific time points and found activation and proliferation to be mostly, but not completely, MR1 dependent. The MR1 dependency of MAIT-cell responses may vary between different types of stimulatory conditions and the MR1-blocking mAb thus is an effective tool to gauge this factor. However, it should be noted that it is possible that MR1-dependent functions might not be completely blocked at the working concentration of MR1-blocking mAb that we used.

In summary, we have developed and optimized methodologies to study MAIT-cell functional responses to \textit{E. coli} in vitro. These tools can be adapted to microbes other than \textit{E. coli} and to MAIT cells from tissues other than blood. Therefore, the techniques described will facilitate MAIT-cell studies aimed at addressing basic immunobiology questions of this cell population and at unraveling their role in the human immune defense.

AUTHORSHIP

J.D., M.J.S., and E.L. designed the research, performed the experiments, and analyzed the data. J.K.S. and E.L. conceived of and designed the research and supervised the work. J.D, J.K.S., and E. L. wrote the paper.

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DISCLOSURE

The authors declare no conflicts of interests.

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