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Abstract

Mucosal-associated invariant T (MAIT) cells are an abundant innate-like T cell subset in humans, enriched in mucosal tissues and the liver. MAIT cells express a semi-invariant T cell receptor (TCR) and recognize microbial-derived riboflavin metabolites presented on the MHC Class I-like molecule MR1. In addition to activation via the TCR, MAIT cells can also be activated in response to cytokines such as IL-12 and IL-18, in contrast to conventional T cells. Here we describe TCR-dependent and -independent methods for MAIT cell activation. The TCR-dependent approaches include stimulation with microbead- or plate-bound anti-CD3/anti-CD28 antibodies, and with 5-OP-RU or paraformaldehyde (PFA)-fixed *E. coli* in the presence of antigen-presenting cells (APCs). The latter method includes a combination of TCR- and cytokine-mediated stimulation. The TCR-independent methods include direct stimulation with the recombinant cytokines IL-12 and IL-18, and indirect stimulation with TLR-4/TLR-8 agonists or influenza A virus in the presence of APCs. Finally, we outline a protocol to analyze activated MAIT cells using flow cytometry.

Keywords (separated by '-')

MAIT cells - Cytokines - MR1 - Invariant T cell receptor - Vα7.2 - Innate - Interleukin-12 - Interleukin-18 - *E. coli* - CD3 - CD28 - Beads - Plate-bound - 5-OP-RU - Toll-like receptor

Chapter 7

Human MAIT Cell Activation In Vitro

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Abstract

Mucosal-associated invariant T (MAIT) cells are an abundant innate-like T cell subset in humans, enriched in mucosal tissues and the liver. MAIT cells express a semi-invariant T cell receptor (TCR) and recognize microbial-derived riboflavin metabolites presented on the MHC Class I-like molecule MR1. In addition to activation via the TCR, MAIT cells can also be activated in response to cytokines such as IL-12 and IL-18, in contrast to conventional T cells. Here we describe TCR-dependent and -independent methods for MAIT cell activation. The TCR-dependent approaches include stimulation with microbead- or plate-bound anti-CD3/anti-CD28 antibodies, and with 5-OP-RU or paraformaldehyde (PFA)-fixed *E. coli* in the presence of antigen-presenting cells (APCs). The latter method includes a combination of TCR- and cytokine-mediated stimulation. The TCR-independent methods include direct stimulation with the recombinant cytokines IL-12 and IL-18, and indirect stimulation with TLR-4/TLR-8 agonists or influenza A virus in the presence of APCs. Finally, we outline a protocol to analyze activated MAIT cells using flow cytometry.

Key words MAIT cells, Cytokines, MR1, Invariant T cell receptor, Vα7.2, Innate, Interleukin-12, Interleukin-18, *E. coli*, CD3, CD28, Beads, Plate-bound, 5-OP-RU, Toll-like receptor

1 Introduction

Innate-like subsets of T cells, including Natural Killer T (NKT), Mucosal-Associated Invariant T (MAIT), and γδ-T cells, operate at the interface of the innate and adaptive immune responses. In contrast to T cell receptors (TCRs) of conventional T cells, the TCRs of innate-like T cells show more limited diversity, and function in a similar manner to pattern recognition receptors through recognition of common pathogen-associated molecular motifs [1–3]. A prominent example is the semi-invariant MAIT cell TCR (Vα7.2-Jα33/20/12 TCRα chain paired with a limited Vβ repertoire) that recognizes microbial riboflavin metabolites presented by the MHC class Ib molecule MR1 on antigen-presenting cells

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(APCs) [4–6]. This allows MAIT cells to respond rapidly to public antigens and thus bridge the temporal gap between the responses of innate and adaptive immunity [2]. Compared with the TCR expressed by conventional T cells, the MAIT cell TCR is hyporesponsive. This may be necessary to ensure tolerance to commensal microorganisms, given the promiscuity of the MAIT cell TCR in regard to pathogen recognition [7, 8]. The majority of human MAIT cells (about 80%) are CD8+ and almost all MAIT cells express the marker CD161 [9–12], which is highly expressed by innate-like T cell subsets compared with conventional T cells [3, 12–16]. An important characteristic of MAIT cells is their rapid activation in response to cytokines such as IL-12 and IL-18, which they share with other CD161-expressing T cells [11, 17]. Such cytokines are produced by activated APCs in the context of bacterial or viral infections and induce MAIT cell responses either on their own or in combination with TCR signals [11, 18].

Here we describe several TCR-dependent and -independent experimental approaches for the activation of MAIT cells. Protocols to activate MAIT cells via their TCR include using anti-CD3 and anti-CD28 antibodies coated on microbead particles or immobilized on a plate. Moreover, we describe an activation protocol using the riboflavin intermediate 5-OP-RU that can be bound and presented on MR1 by APCs to induce specific MAIT cell activation. We also outline how MAIT cells can be activated by combined TCR and cytokine stimulation using an in vitro experimental model in which APCs (within Peripheral Blood Mononuclear Cells (PBMCs) or THP-1 cells) are challenged with PFA-fixed *E. coli*. MAIT cells are activated by the riboflavin metabolites presented on MR1, and cytokines produced by activated APCs. Furthermore, we explain how to stimulate MAIT cells in a TCR-independent manner using cytokines such as IL-12 and IL-18, either directly by adding recombinant cytokines or indirectly by stimulating APCs to produce IL-12 and IL-18 with Toll-like receptor (TLR) agonists. Finally, we extend this MR1-independent activation to an in vitro viral infection model in which virus-activated APCs produce cytokines that activate MAIT cells. As one possible way to analyze TCR-dependent or -independent MAIT cell activation, we provide a protocol to assess expression of IFNγ, TNFα, GzmB, and CD69 by flow cytometry.

2 Materials

2.1 TCR-Dependent MAIT Cell Activation

1. R10 medium: RPMI 1640 Medium, 10% heat-inactivated fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin/streptomycin.

2. Phosphate Buffered Saline (PBS), pH 7.4, sterile.
3. MACS Buffer: PBS, pH 7.2, 0.5% BSA (or alternatively 2% FCS), 2 mM EDTA.
4. PBMCs (peripheral blood mononuclear cells).
5. CD8 MicroBeads (Miltenyi Biotec).
6. THP-1 cells (THP-1 ECACC 88081201), European Collection of Authenticated Cell Cultures.
7. T Cell Activation/Expansion Kit, human (contains anti-Biotin MACSiBead Particles and anti-CD3 and anti-CD28 antibodies to generate immobilized anti-CD3 and anti-CD28 on MACSiBead Particles, Miltenyi Biotec).
8. 96-well U-bottom cell culture plates.
9. 96-well flat-bottom Nunc™ MaxiSorp™ ELISA Plate.
10. PMA (Phorbol 12-myristate 13-acetate).
11. Ionomycin 1mM in DMSO.
12. Brefeldin A Solution, 1000×.
13. Anti-human CD3 antibody, clone UCHT1.
14. Anti-human CD28 antibody, clone CD28.2.
15. Isotype control antibodies for anti-human CD3 and CD28.
16. 5-OP-RU (5-(2-oxopropylideneamino)-6-dribitylaminouracil) obtained from Prof. David P. Fairlie, University of Queensland, Australia.
17. DMSO.
18. LB Broth with agar (Lennox) tablet or powder microbial growth medium.
19. LB Broth (Lennox) powder microbial growth medium.
20. Petri dishes (100 × 15 mm).
21. DH5α or TOP10 *E. coli* cells.
22. 0.25 L Erlenmeyer flasks.
23. Rattler Plating Beads, 4.5 mm.
24. Paraformaldehyde solution 2% in PBS.
25. Tube rotator.

2.2 TCR-Independent MAIT Cell Activation

1. R10 medium: RPMI 1640 Medium, 10% heat-inactivated fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin/streptomycin.
2. PBMCs.
3. CD8 MicroBeads (Miltenyi Biotec).
4. THP-1 cells (THP-1 ECACC 88081201), European Collection of Authenticated Cell Cultures.

5. 96-well U-bottom cell culture plates.
6. Recombinant human IL-12.
7. Recombinant human IL-18.
8. PMA (Phorbol 12-myristate 13-acetate).
9. Ionomycin, 1 mM in DMSO.
10. Brefeldin A Solution, 1000×.
11. TLR8 agonist ssRNA40/Lyovec.
12. TLR4 agonist *E. coli* K12 LPS.
13. Influenza virus strain A/WSN/1933(H1N1).

2.3 Analysis of MAIT Cell Activation Via Flow Cytometry

1. 96-well U-bottom or 96-well V-bottom cell culture plates.
2. PBS, pH 7.4.
3. PBS with 5% FCS.
4. LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (Invitrogen).
5. Antibodies: refer to Tables 1, 2, and 3
6. Formaldehyde solution 2% in PBS.
7. Permeabilization Buffer 10× (Thermo Fisher Scientific).

3 Methods

3.1 TCR-Dependent MAIT Cell Activation

3.1.1 MAIT Cell Activation Using Anti-CD3/Anti-CD28: MACSiBead Particles

Here we describe how to activate MAIT cells in a non-specific way by exposing MAIT cells within PBMCs or isolated CD8+ T cells to anti-CD3 and anti-CD28 antibodies immobilized on MACSiBead Particles. The anti-CD3 and anti-CD28 antibodies bind CD3 and CD28, respectively, on T cells including MAIT cells, inducing CD3 and CD28 clustering and T cell activation [19–22] (Figs. 1a and 2).

1. Anti-CD3/anti-CD28—MACSiBead Particles (T Cell Activation/Expansion Kit, human) are prepared according to manufacturer's instructions with a few alterations.
   (a) Add 100 µL anti-CD3-Biotin, 100 µL anti-CD28-Biotin, and 100 µL MACS Buffer to a sterile and sealable 2 mL tube under aseptic conditions (*see* **Note 1**).
   (b) Vortex anti-Biotin MACSiBead Particles thoroughly for 30 s as they have a tendency to sediment quickly.
   (c) Add 500 µL anti-Biotin MACSiBead Particles to the antibody mix.
   (d) Add 200 µL MACS Buffer to obtain a final volume of 1 mL.
   (e) To allow the beads to be loaded with the antibodies, incubate at 2–8 °C for at least 2 h under constant, gentle rotation using a tube rotator.

Table 1
Example antibody panel 1 for analysis with MACSQuant Analyzer 10, Miltenyi Biotec

Antibody	Clone	Color	Manufacturer	Dilution
Antibodies for surface antigens				
CD3	OKT3	eFluor 450	Invitrogen	1:100
CD8	REA734	VioGreen	Miltenyi Biotec	1:25
Vα7.2 TCR	3C10	FITC	BioLegend	1:50
CD161	191B8	PE	Miltenyi Biotec	1:50
Antibodies for intracellular antigens				
TNFα	MAb11	PerCP-Cy5.5	BioLegend	1:25
IFNγ	4S.B3	PE-Cy7	BioLegend	1:100
Granzyme B	GB12	APC	Invitrogen	1:100

Table 2
Example antibody panel 2 for analysis with MACSQuant Analyzer 10, Miltenyi Biotec

Antibody	Clone	Color	Manufacturer	Dilution
Antibodies for surface antigens				
CD3	OKT3	eFluor 450	Invitrogen	1:100
CD8	REA734	VioGreen	Miltenyi Biotec	1:25
CD69	FN50	FITC	eBioscience	1:50
CD161	191B8	PE	Miltenyi Biotec	1:50
Vα7.2 TCR	3C10	PerCP-Cy5.5	BioLegend	1:50
Antibodies for intracellular antigens				
IFNγ	4S.B3	PE-Cy7	BioLegend	1:100
Granzyme B	GB12	APC	Invitrogen	1:100

(f) Do not remove the antibody mix from the loaded beads. Loaded beads can be stored for up to 4 months at 2–8 °C.

2. Count PBMCs or isolated CD8+ T cells (For CD8+ T cell isolation we recommend using CD8 MicroBeads and following manufacturer's instructions).
3. Resuspend cells in R10 medium at a concentration of 10 million PBMCs or 2 million CD8+ T cells per 1 mL.
4. Seed 100 µL of cells per well into a 96-well U-bottom cell culture plate. This results in a final cell density of 1 million PBMCs or 0.2 million CD8+ T cells per well. Cell numbers can be adapted to your needs (*see* **Note 2**).

t.1 **Table 3**
Example antibody panel 3 for analysis with BD LSR II, BD Biosciences

t.2 Antibody	Clone	Color	Manufacturer	Dilution
t.3 *Antibodies for surface antigens*				
t.4 CD69	FN50	eFluor 450	eBioscience	1:50
t.5 CD8	REA734	VioGreen	Miltenyi Biotec	1:25
t.6 CD3	UCHT1	BV605	BioLegend	1:100
t.7 CD161	191B8	PE	Miltenyi Biotec	1:50
t.8 Vα7.2 TCR	3C10	PE-Cy7	BioLegend	1:50
t.9 CD4	SK3	APC	BioLegend	1:100
t.10 *Antibodies for intracellular antigens*				
t.11 IFNγ	45-15	FITC	Miltenyi Biotec	1:50
t.12 Granzyme B	GB11	AF700	BioLegend	1:100
t.13 TNFα	MAb11	PerCP-Cy5.5	BioLegend	1:50

t.14 The panel was optimized using a BD LSR II setup including violet (405 nm), blue (488 nm), and red (633 nm) lasers with the following filter configuration: Violet array filters: 450/50, 525/50, 560/20, 585/15, 605/12, 655/8. Blue array filters: 530/30, 575/26, 610/20, 670/14, 695/40, 780/60. Red array filters: 670/14, 730/45, 780/60

5. To prepare the loaded MACSiBead particles, resuspend or vortex beads thoroughly, as beads tend to sediment quickly to the bottom of the container.
6. Take an aliquot of loaded MACSiBead particles (50 μL per 1 million PBMCs or isolated CD8+ T cells, which equals 0.5 million beads; bead-to-cell ratio 1:2 for PBMCs and isolated CD8+ T cells) and transfer into a new sterile Eppendorf tube.
7. Add at least 100–200 μL R10 medium and centrifuge at 500 × *g* for 5 min to separate the beads from any unbound antibody.
8. Aspirate the supernatant completely and resuspend in 100 μL R10 medium per 0.5 million beads.
9. Add 100 μL of the bead mix to the wells containing the cells and mix well. This results in a total culture medium volume of 200 μL (*see* **Note 3**).
10. As a negative control, add 100 μL R10 medium instead of beads. Further controls can include conditions with unloaded or isotype-loaded MACSiBead particles.
11. As a positive control, add 100 μL 2× PMA + Ionomycin mix, diluted in R10 medium, to the cells and mix well (final concentration: PMA = 5–50 ng/mL, we recommend 10 ng/mL; Ionomycin: 0.66–1 μM, we recommend 0.66 μM) (*see* **Note 4**).

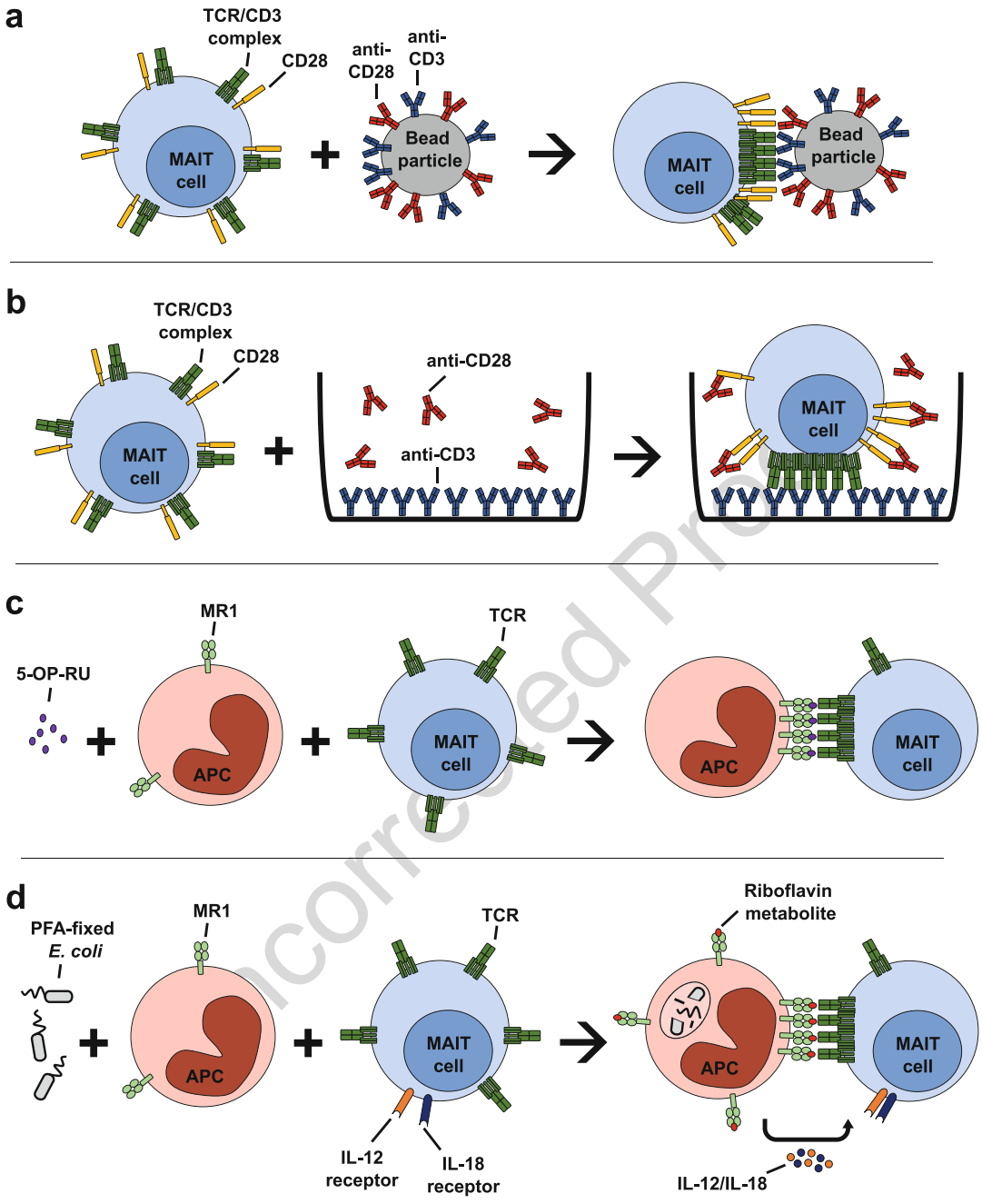

Fig. 1 Methods to activate MAIT cells in a TCR-dependent manner. (**a**) MAIT cells can be activated by anti-CD3 and anti-CD28 antibodies that are coupled to beads. The beads bind CD3 and CD28 on MAIT cells, which results in clustering of those molecules leading to MAIT cell activation. (**b**) MAIT cells can be activated by anti-CD3 antibody that is immobilized on a plate in combination with soluble anti-CD28 antibody. The plate-immobilized anti-CD3 antibodies bind and cluster CD3, and the soluble anti-CD28 antibodies bind and crosslink CD28 on MAIT cells. This results in MAIT cell activation. (**c**) MAIT cells can be activated in the presence of APCs by the administration of the riboflavin intermediate 5-OP-RU. 5-OP-RU can be bound by MR1

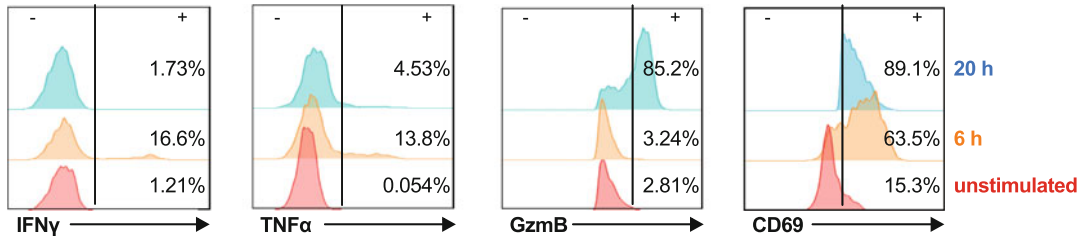

Fig. 2 MAIT cell activation using anti-CD3/anti-CD28 beads. Isolated CD8+ T cells were stimulated using anti-CD3/anti-CD28 beads and subsequently analyzed by flow cytometry. Example histograms show the kinetics of IFNγ, TNFα, GzmB, and CD69 expression by MAIT cells at 6 and 20 h post-stimulation. Half-offset histograms show modal *y*-axis scaling (% max. count per condition)

12. Incubate at 37 °C, 5% CO_2 for 24 h (the incubation time can be varied depending on the experimental question, *see* **Note 5**).
13. If the experiment is to be analyzed by flow cytometry including staining for intracellular cytokines, add Brefeldin A to all conditions 4 h before termination of the assay. Prepare a 1:50 dilution of Brefeldin A 1000× stock solution in R10 medium. Add 10 µL to each well containing 200 µL cultured cell suspension and mix well. The final working concentration of Brefeldin A is 3 µg/mL. Incubate at 37 °C and 5% CO_2 for the remaining 4 h and then continue with Subheading 3.3.

3.1.2 MAIT Cell Activation Using Plate-Bound Anti-CD3/Anti-CD28

This protocol describes how to activate MAIT cells within PBMCs or isolated CD8+ T cells by exposing them to anti-CD3 antibodies, which are immobilized on a plate, and soluble anti-CD28 antibodies. The anti-CD3 antibodies bind CD3 on T cells, resulting in CD3 clustering and the soluble anti-CD28 antibodies crosslink CD28. This results in the activation of T cells, including MAIT cells [20, 23] (Fig. 1b).

1. To coat the plate, prepare a 2.5 µg/mL solution of anti-CD3 antibody or IgG isotype control (negative control) in sterile PBS (*see* **Note 6**).
2. Add 50 µL anti-CD3 or IgG isotype control antibody solution to the wells of a 96-well flat-bottom Nunc™ MaxiSorp™ ELISA Plate. Use aseptic techniques when handling the plate and cover the plate with the lid of a sterile standard 96-well cell culture plate.

Fig. 1 (continued) on the APCs and presented to MAIT cells. The MAIT cell TCR recognizes and binds 5-OP-RU in the context of MR1, which leads to MAIT cell activation. (**d**) MAIT cells can be activated in the presence of APCs by administration of PFA-fixed *E. coli*. The bacteria activate the APCs, which take up and process the *E. coli*. Bacterial-derived riboflavin metabolites are then presented on MR1 to MAIT cells. Additionally, the activated APCs express cytokines including IL-12 and IL-18, which can be recognized by the MAIT cells via IL-12 and IL-18 receptors. The combination of TCR and cytokine signal leads to MAIT cell activation

3. Seal the plate tightly with Parafilm to avoid evaporation and incubate at 4 °C overnight or at 37 °C for 2 h.
4. To wash the wells, add 150 µL sterile PBS or R10 medium under aseptic conditions, then aspirate and discard all the liquid.
5. Wash two more times with 200 µL sterile PBS or R10 medium.
6. Add 200 µL R10 medium containing 10% FCS and incubate for at least 2 h at 37 °C to block the plate.
7. In the meantime, prepare and count PBMCs or isolated CD8+ T cells (For CD8+ T cell isolation we recommend using CD8 MicroBeads and following manufacturer's instructions).
8. Resuspend PBMCs or CD8+ T cells in R10 medium at a concentration of 2 million cells per 1 mL.
9. Seed 100 µL cells per well into a 96-well U-bottom cell culture plate, resulting in a cell density of 0.2 million cells per well (*see* **Note 7**).
10. Prepare a 2× anti-CD28 antibody or IgG isotype antibody mix (2 µg/mL) diluted in R10.
11. Add 100 µL antibody mix to 100 µL cell suspension and mix well, resulting in a final anti-CD28 antibody concentration of 1 µg/mL (*see* **Note 6**) and a total culture medium volume of 200 µL (*see* **Note 3**).
12. Incubate at 37 °C, 5% CO_2 for 24 h (the incubation time can be varied depending on the experimental question, *see* **Note 5**).
13. If the experiment is to be analyzed by flow cytometry including staining for intracellular cytokines, add Brefeldin A to all conditions 4 h before termination of the assay. Prepare a 1:50 dilution of Brefeldin A 1000× stock solution in R10 medium. Add 10 µL to each well containing 200 µL cultured cell suspension and mix well. The final working concentration of Brefeldin A is 3 µg/mL. Incubate at 37 °C and 5% CO_2 for the remaining 4 h and then continue with Subheading 3.3.

3.1.3 MAIT Cell Activation by 5-OP-RU

Here we outline a protocol to activate MAIT cells using 5-OP-RU, a riboflavin intermediate. This metabolite can be bound by MR1 on APCs and presented to MAIT cells. Recognition of 5-OP-RU in the context of MR1 by the MAIT cell TCR induces MAIT cell activation (Figs. 1c and 3). This experiment can be performed using MAIT cells and APCs within PBMCs or using MAIT cells within isolated CD8+ T cells in combination with THP-1 cells as APCs [24–26].

1. Prepare and count PBMCs or THP-1 cells and isolated CD8+ T cells (For CD8+ T cell isolation we recommend using CD8 MicroBeads and following manufacturer's instructions).

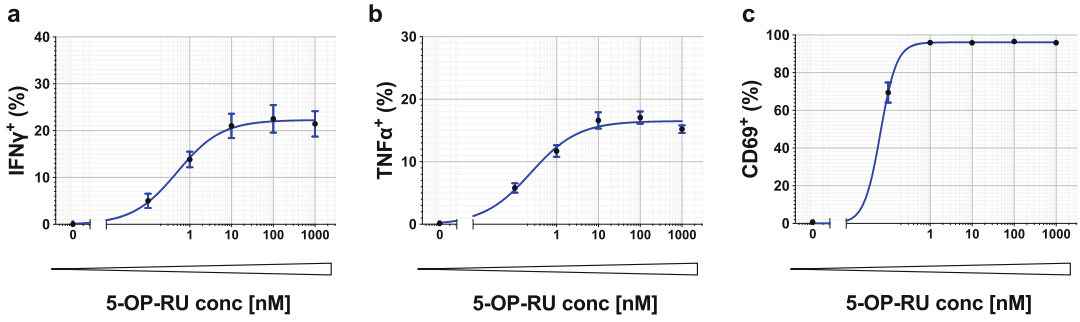

Fig. 3 Stimulation of MAIT cells with 5-OP-RU in the presence of APCs. Increasing concentrations of 5-OP-RU were added to isolated CD8+ T cells and THP-1 cells as indicated. Cells were incubated for 24 h and subsequently analyzed by flow cytometry. (**a**) Frequency of IFNγ+ MAIT cells. (**b**) Frequency of TNFα+ MAIT cells. (**c**) Frequency of CD69+ MAIT cells. Data points represent mean ± SEM of five donors

2. Resuspend cells in R10 medium at a concentration of 10 million PBMCs per 1 mL or 4 million CD8+ T cells per 1 mL and 2 million THP-1 cells per 1 mL.
3. Seed 100 µL of PBMCs or alternatively 50 µL of CD8+ T cells per well into a 96-well U-bottom cell culture plate. When using isolated CD8+ T cells, add 50 µL THP-1 cells into each well. This results in a final cell density of 1 million PBMCs or 0.2 million CD8+ T cells and 0.1 million THP-1 cells per well. Cell numbers can be adapted to your needs (*see* **Notes 2** and **8**).
4. Dilute 5-OP-RU in R10 medium to obtain a 2× mix (20 nM).
5. Add 100 µL of the 2× 5-OP-RU mix to the wells containing 100 µL cell suspension and mix well. This results in a final 5-OP-RU concentration of 10 nM. The concentration of 5-OP-RU can be adapted according to your experimental needs (*see* **Note 9**).
6. As a negative control, prepare a 2× mix of DMSO diluted in R10 medium (because the 5-OP-RU stock is diluted in DMSO), and add 100 µL to the cells instead of 5-OP-RU.
7. Incubate at 37 °C, 5% CO_2 for 24 h (the incubation time can be varied depending on the experimental question, *see* **Note 5**).
8. If the experiment is to be analyzed by flow cytometry including staining for intracellular cytokines, add Brefeldin A to all conditions 4 h before termination of the assay. Prepare a 1:50 dilution of Brefeldin A 1000× stock solution in R10 medium. Add 10 µL to each well containing 200 µL cultured cell suspension and mix well. The final working concentration of Brefeldin A is 3 µg/mL. Incubate at 37 °C and 5% CO_2 for the remaining 4 h and then continue with Subheading 3.3.

3.1.4 MAIT Cell Activation by PFA-Fixed E. coli

In this experimental model, MAIT cells are activated within PBMCs (model 1) or within CD8+ T cells in the presence of THP-1 cells (model 2). APCs within PBMCs or THP-1 cells are activated by PFA-fixed *E. coli*. This results in the processing of the bacteria within the APCs, upregulation of MR1 expression, presentation of bacterial riboflavin metabolites on MR1, and expression of IL-12 and IL-18 by the APCs. MAIT cells are specifically activated by recognizing the riboflavin metabolites in the context of MR1 in combination with binding IL-12 and IL-18 to respective receptors on their surface [5, 6, 11, 27, 28] (Figs. 1d and 4). TCR-mediated MAIT cell activation dominates at early time points (6 h), while both TCR and cytokine signals contribute to later MAIT cell activation (24 h) [11].

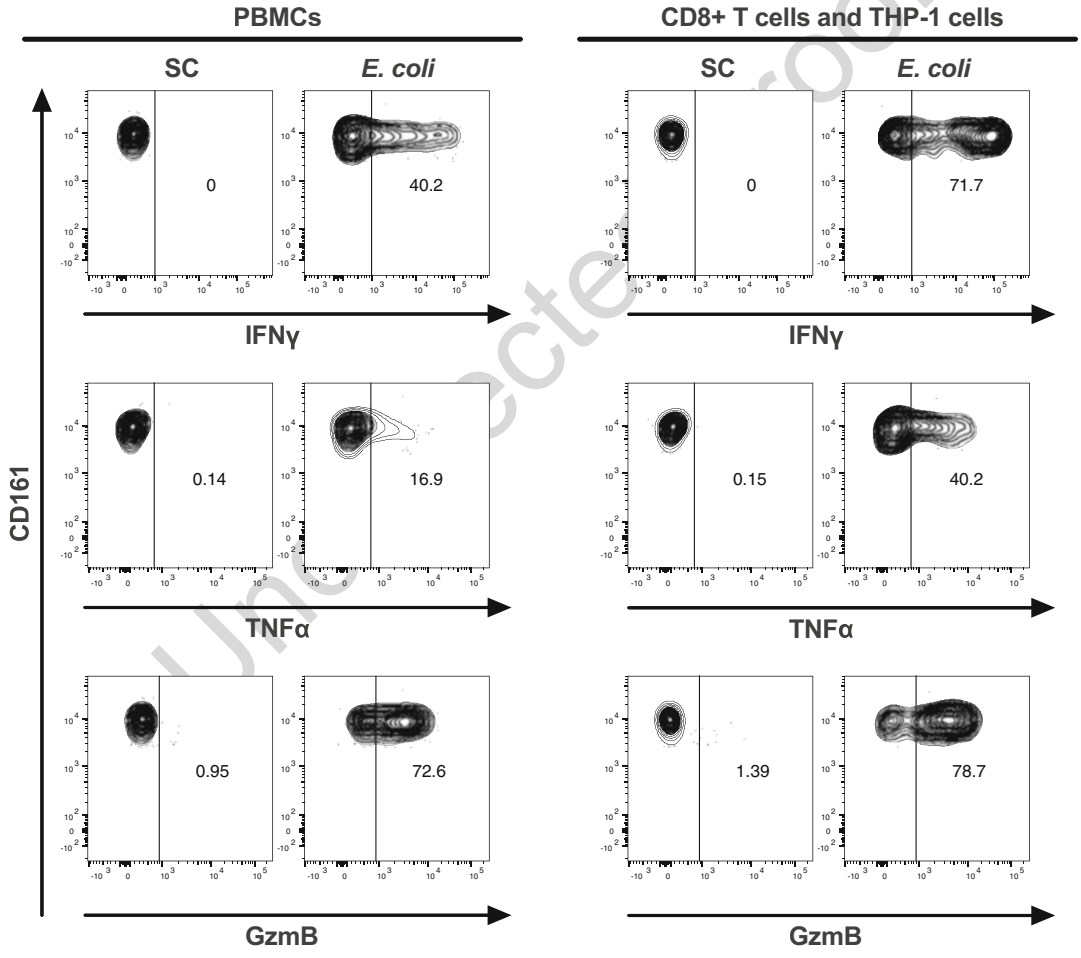

Fig. 4 Activation of MAIT cells with *E. coli*. PFA-fixed *E. coli* or sterility control (SC) was added to PBMCs or CD8 + T cells and THP-1 cells. Cells were incubated for 24 h and subsequently analyzed by flow cytometry. Graph shows an example of flow cytometry plots of IFNγ, TNFα, and GzmB production by CD8+Va7.2+CD161++ MAIT cells

Preparation of PFA-Fixed *E. coli* [27–30]

1. Preparation of LB agar plates

   (a) Add LB Broth with agar powder or tablets to deionized water following manufacturer's instructions and make up the quantity required for the desired number of plates (approximately 10 mL LB agar per Petri dish) within a glass bottle.

   (b) Close the bottle loosely with the cap and label it with autoclave tape.

   (c) Autoclave the LB agar medium at 121 °C to sterilize.

   (d) When the LB agar medium has cooled enough to handle safely, but before it begins to solidify, pour it into sterile Petri dishes under aseptic conditions to form a layer of approximately 5 mm. Avoid bubbles.

   (e) In order to prevent condensation within the plates, leave them to solidify and dry at room temperature. When the agar plates are solidified and free of moisture, store them upside down at 2–8 °C.

2. Obtaining *E. coli* colonies on LB agar plates

   (a) Warm LB agar plates to 37 °C.

   (b) From now on, work near a Bunsen burner flame or within a laminar flow cabinet.

   (c) Take the *E. coli* stock vial (strain DH5α or TOP10) from the −80 °C freezer and transfer it onto ice. Scrape the surface of the frozen bacterial stock with an inoculation loop.

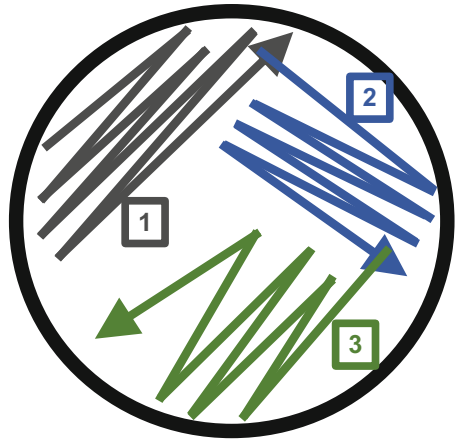

Fig. 5 Streaking technique to obtain single *E. coli* colonies on LB agar plates. Spread bacteria with an inoculation loop on the LB agar plate to obtain streak 1. To further separate the bacteria, use a fresh sterile inoculation loop and streak through streak 1 to create streak 2. Repeat using a fresh sterile inoculation loop, crossing through streak 2 to create streak 3

(d) Streak the bacteria onto a plate with an inoculation loop as shown in Fig. 5. This procedure is necessary to obtain isolated colonies.

(e) Incubate plates in a microbiological incubator overnight (approx.16 h) at 37 °C.

(f) Remove the plates containing the *E. coli* cultures. Single colonies should be visible. Store the plates at 2–8 °C for up to 2 months.

3. Growing up *E. coli* cultures

(a) To prepare LB Broth medium, add LB Broth powder to deionized water within a glass bottle following manufacturer's instructions. Swirl to mix. Powder will not completely dissolve. Loosen the bottle cap for sterilization. Autoclave at 121 °C to sterilize. Allow medium to cool before use.

(b) Add 50 mL of LB Broth medium into a 0.25 L Erlenmeyer flask for the bacterial culture (BC). Prepare a second flask for the sterility control (SC).

(c) Use a pipette with a sterile 200 µL tip and pick five bacterial colonies from the agar plate and eject the tip into the BC flask (*see* **Note 10**).

(d) Take a fresh tip and eject it into the SC flask. This will be the no bacteria control.

(e) Cover the flasks with aluminum foil. NB: cover should not be too tight, as this will prevent gas exchange.

(f) Incubate the flasks in a shaking incubator (225 rpm) at 37 °C for 16 h.

4. Quantification of bacteria: Determination of CFU [31]

(a) Prepare a 1/10 serial dilution from BC and SC ranging from 1/10 to $1/10^{10}$. For the initial 1/10 dilution, take 50 µL of BC or SC and add it to 450 µL sterile PBS. Work under aseptic conditions.

(b) To plate each of the dilutions, add 50 µL to the center of an agar plate.

(c) Pour 4–5 Rattler Plating spherical glass beads into the plate and close the plate lid.

(d) Shake the plate back and forth and from side to side to spread the bacteria over the plate. Ensure that the beads cover the entire plate so that there is an even distribution of bacteria.

(e) To remove the beads, invert the plate. Use fresh beads for each dilution and plate.

(f) Incubate plates in a microbiological incubator overnight (approx. 16 h) at 37 °C.

(g) Remove the plates and count bacterial colonies. The plates containing the dilutions of the SC should not contain any colonies.

(h) For calculation of CFU, use the plate containing from 30 to 300 colonies.

$$\frac{\text{CFU}}{\text{mL}} = \frac{\text{number of colonies} \times \text{dilution factor}}{\text{volume of bacterial culture plated}}$$

$$= \frac{\text{number of colonies} \times \text{dilution factor}}{0.05\ \text{mL}}$$

$$\text{total CFU} = \frac{\text{CFU}}{\text{mL}} \times \text{total volume of bacterial culture}$$

$$= \frac{\text{CFU}}{\text{mL}} \times 50\ \text{mL}$$

5. PFA fixation of *E. coli*

(a) Transfer the BC and SC into 50 mL Falcon tubes. Treat the SC in exactly the same way you treat the BC throughout the whole protocol.

(b) Pellet bacteria through centrifugation (10 min at 4000–6000 × *g*). Discard supernatant.

(c) Resuspend bacteria in 50 mL sterile PBS. Make sure the bacterial pellet is thoroughly resuspended by pipetting up and down (this is necessary for all the washing steps and for the fixation).

(d) Centrifuge (10 min at 4000–6000 × *g*) and discard supernatant.

(e) Resuspend the bacterial pellet in 25 mL 2% PFA, diluted in PBS, and fix at room temperature for 20 min.

(f) Add 25 mL sterile and filtered (0.22 µm) PBS and centrifuge for 10 min at 4000–6000 × *g*. Discard supernatant.

(g) Wash twice with 50 mL sterile filtered PBS (10 min at 4000–6000 × *g*).

(h) Resuspend bacteria in sterile filtered PBS at a concentration of 1 million CFU per 1 µL. Add an equal volume of sterile filtered PBS to the SC tube.

(i) Aliquots should be prepared and stored at 2–8 °C for up to 2 months.

Experimental Setup for MAIT Cell Activation by PFA-Fixed *E. coli*

1. Prepare and count PBMCs or THP-1 cells and isolated CD8+ T cells (For CD8+ T cell isolation we recommend using CD8 MicroBeads and following manufacturer's instructions) (*see* **Note 11**).
2. Resuspend cells in R10 medium at a concentration of 10 million PBMCs per 1 mL or 4 million CD8+ T cells per 1 mL and 2 million THP-1 cells per 1 mL.
3. Seed 100 µL of PBMCs or alternatively 50 µL of CD8+ T cells per well into a 96-well U-bottom cell culture plate. When using isolated CD8+ T cells, add 50 µL THP-1 cells into each well. This results in a final cell density of 1 million PBMCs or 0.2 million CD8+ T cells and 0.1 million THP-1 cells per well. Cell numbers can be adapted to your needs (*see* **Notes 2** and **8**).
4. Dilute PFA-fixed *E. coli* in R10 medium to obtain a 2× mix (we recommend 20 million CFU per 100 µL for the PBMC assay or 6 million CFU per 100 µL for the THP-1 assay) (*see* **Note 12**).
5. Add 100 µL of the 2× *E. coli* mix to the wells containing 100 µL cell suspension and mix well. This results in a final concentration of 10 CFU per PBMC or 30 CFU per THP-1 cell.
6. As a negative control, prepare a 2× mix of the sterility control (SC). Add 100 µL of the 2× SC mix to the control wells containing 100 µL cell suspension and mix well.
7. Incubate at 37 °C, 5% CO_2 for 6 or 24 h (the incubation time can be varied depending on the experimental question, *see* **Note 5**).
8. If the experiment is to be analyzed by flow cytometry including staining for intracellular cytokines, add Brefeldin A to all conditions 4 h before termination of the assay. Prepare a 1:50 dilution of Brefeldin A 1000× stock solution in R10 medium. Add 10 µL to each well containing 200 µL cultured cell suspension and mix well. The final working concentration of Brefeldin A is 3 µg/mL. Incubate at 37 °C and 5% CO_2 for the remaining 4 h and then continue with Subheading 3.3.

3.2 TCR-Independent MAIT Cell Activation

3.2.1 MAIT Cell Activation by Cytokines IL-12 + IL-18

Here we describe how to activate MAIT cells within PBMCs or isolated CD8+ T cells by recombinant IL-12 and IL-18 [11] (Figs. 6a and 7).

1. Prepare and count PBMCs or isolated CD8+ T cells (For CD8+ T cell isolation we recommend using CD8 MicroBeads and following manufacturer's instructions).
2. Resuspend cells in R10 medium at a concentration of 10 million PBMCs or 2 million CD8+ T cells per 1 mL

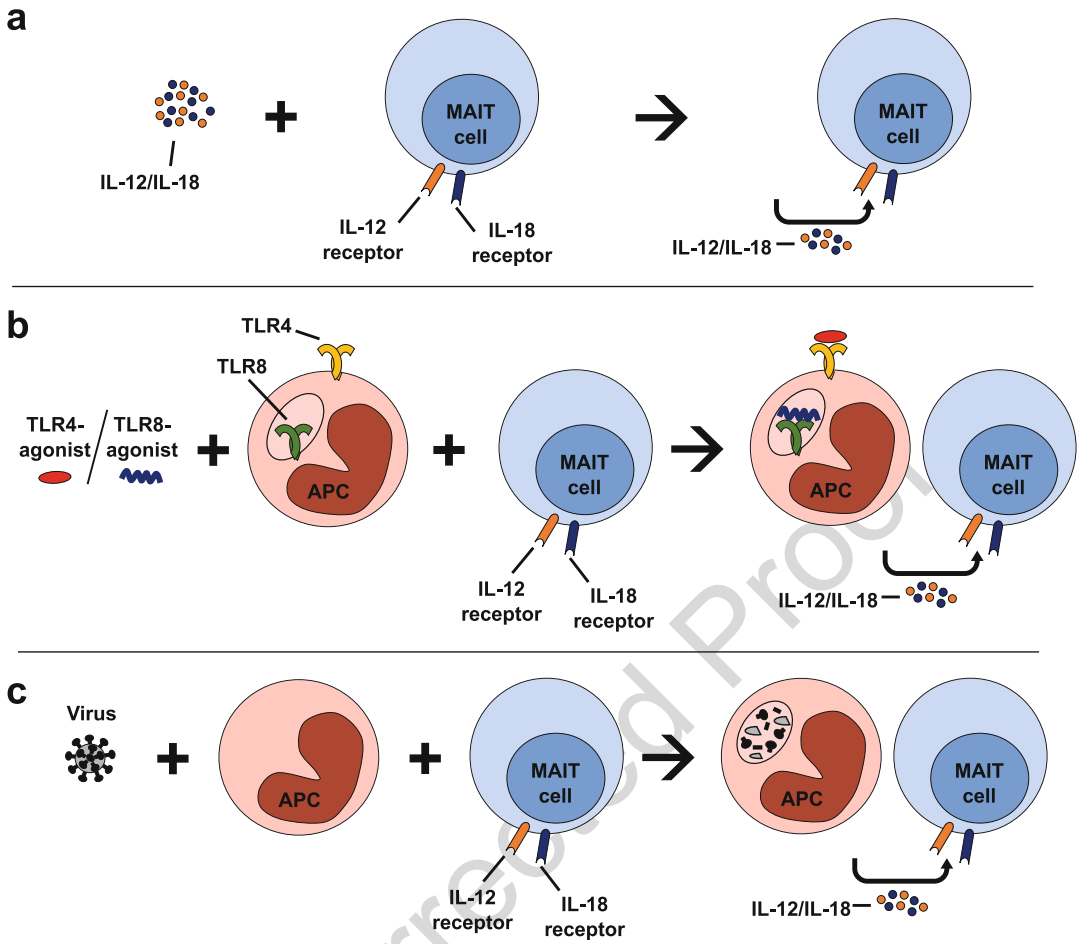

Fig. 6 Methods to activate MAIT cells in a TCR-independent manner. (**a**) MAIT cells can be activated by cytokines such as the combination of IL-12 and IL-18. The cytokines signal through IL-12 and IL-18 receptors on MAIT cells, inducing activation. (**b**) MAIT cells can be indirectly activated by TLR agonists in the presence of APCs. The administration of TLR8-agonist to TLR8-expressing APCs within PBMCs or TLR4-agonist to TLR4-expressing THP-1 cells induces APC activation. Activated APCs produce cytokines including IL-12 and IL-18 that can activate MAIT cells. (**c**) MAIT cells can be activated indirectly by the administration of certain viruses in the presence of APCs. The APCs become activated by sensing pathogen-associated molecular patterns of the viruses resulting in the expression of cytokines such as IL-12 and IL-18 and hence MAIT cell activation

3. Seed 100 µL cells per well into a 96-well U-bottom cell culture plate. This results in a final cell density of 1 million PBMCs or 0.2 million CD8+ T cells per well. Cell numbers can be adapted to your needs (*see* **Note 2**).
4. To generate a 2× IL-12 + IL-18 mix, add 0.5 µL of each cytokine (stock concentration: 100 µg/mL) per 1 mL R10 medium.
5. Add 100 µL of the 2× IL-12 + IL-18 mix to 100 µL cell suspension. This results in a total culture medium volume of

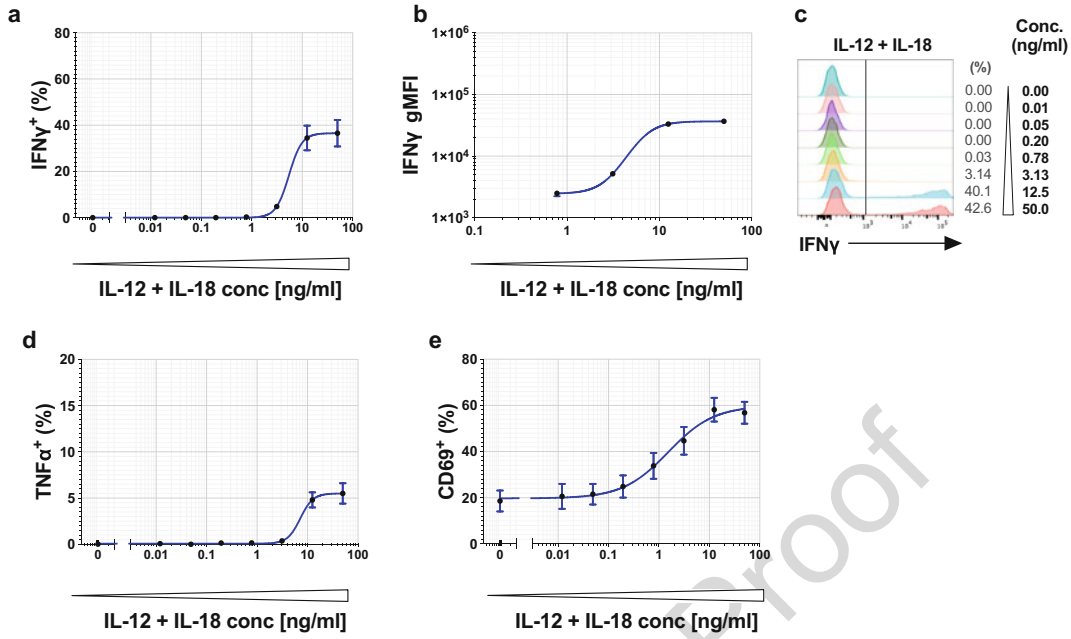

Fig. 7 MAIT cell stimulation with IL-12 + IL-18. CD8+ T cells were stimulated with increasing concentrations of IL-12 + IL-18 as indicated. Cells were incubated for 24 h and subsequently analyzed by flow cytometry. Responses of CD161++ T cells, including CD161++Va7.2+ MAIT cells and CD161++Va7.2- T cells, are shown. (**a**) Frequency of IFNγ+ cells. (**b**) IFNγ gMFI of IFNγ+ cells. (**c**) Representative flow cytometry plot of IFNγ expression by CD161++ T cells. Half-offset histogram shows modal *y*-axis scaling (% max. count per condition). (**d**) Frequency of TNFα+ cells. (**e**) Frequency of CD69+ cells. Graphs show mean ± SEM of four donors

200 µL (*see* **Note 3**) and a final cytokine concentration of 50 ng/mL each. The cytokine concentration can be adapted to your experimental needs (*see* **Note 13**).

6. Prepare a negative control by adding 100 µL R10 medium instead of cytokines to the cells and mix well.
7. Prepare a positive control by adding 100 µL 2× PMA + Ionomycin mix, diluted in R10 medium, to the cells and mix well (final concentration: PMA = 5–50 ng/mL, we recommend 10 ng/mL; Ionomycin: 0.66–1 µM, we recommend 0.66 µM) (*see* **Note 4**).
8. Incubate at 37 °C, 5% CO_2 for 24 h (the incubation time can be varied depending on the experimental question, *see* **Note 5**).
9. If the experiment is to be analyzed by flow cytometry including staining for intracellular cytokines, add Brefeldin A to all conditions 4 h before termination of the assay. Prepare a 1:50 dilution of Brefeldin A 1000× stock solution in R10 medium. Add 10 µL to each well containing 200 µL cultured cell suspension and mix well. The final working concentration of

Brefeldin A is 3 μg/mL. Incubate at 37 °C and 5% CO_2 for the remaining 4 h and then continue with Subheading 3.3.

3.2.2 Indirect MAIT Cell Activation Using TLR4 or TLR8 Agonists

In this experimental model APCs are activated by TLR4 or TLR8 agonists. The TLR agonists bind to the respective TLR on APCs, stimulating the production of relevant cytokines (e.g., IL-12 and IL-18) for MAIT cell activation [11, 32] (Fig. 6b).

Follow protocol A in order to activate MAIT cells indirectly through TLR8 agonists using PBMCs, or protocol B to activate MAIT cells indirectly through TLR4 agonists using CD8+ T cells in combination with THP-1 cells.

Protocol A: Activate MAIT Cells Indirectly Through TLR8 Agonists Within PBMCs

1. Prepare and count PBMCs.
2. Resuspend cells in R10 medium at a concentration of 10 million PBMCs per 1 mL.
3. Seed 100 μL cells per well into a 96-well U-bottom cell culture plate. This results in a final cell density of 1 million PBMCs per well. Cell numbers can be adapted to your needs (*see* **Note 2**).
4. Prepare a 2× mix of TLR8 agonist (ssRNA40/Lyovec) by adding 2 μL TLR8 agonist (stock: 100 μg/mL) per 100 μL R10 medium.
5. Add 100 μL of the 2× TLR8 agonist mix to 100 μL cell suspension. This results in a total culture medium volume of 200 μL (*see* **Note 3**) and a final TLR agonist concentration of 1 μg/mL each. The final TLR agonist concentration can be adapted to your experimental needs (*see* **Note 14**).
6. Prepare a negative control by adding 100 μL R10 medium instead of TLR agonist to the cells and mix well.
7. Prepare a positive control by adding 100 μL 2× PMA + Ionomycin mix, diluted in R10 medium, to the cells and mix well (final concentration: PMA = 5–50 ng/mL, we recommend 10 ng/mL; Ionomycin: 0.66–1 μM, we recommend 0.66 μM) (*see* **Note 4**).
8. Incubate at 37 °C, 5% CO_2 for 24 h (the incubation time can be varied depending on the experimental question, *see* **Note 5**).
9. If the experiment is to be analyzed by flow cytometry including staining for intracellular cytokines, add Brefeldin A to all conditions 4 h before termination of the assay. Prepare a 1:50 dilution of Brefeldin A 1000× stock solution in R10 medium. Add 10 μL to each well containing 200 μL cultured cell suspension and mix well. The final working concentration of Brefeldin A is 3 μg/mL. Incubate at 37 °C and 5% CO_2 for the remaining 4 h and then continue with Subheading 3.3.

Protocol B: Activate MAIT Cells Indirectly Through TLR4 Agonists Using CD8+ T Cells in the Presence of THP-1 Cells

1. Prepare and count THP-1 cells.
2. Resuspend cells in R10 medium at a concentration of 2 million THP-1 cells per 1 mL.
3. Seed 100 µL THP-1 cell suspension per well into a 96-well U-bottom cell culture plate. This results in a cell density of 0.1 million THP-1 cells per well (*see* **Note 8**).
4. Prepare a 2× mix of TLR4 agonist (*E. coli* K12 LPS) by adding 2 µL TLR4 agonist (stock: 100 µg/mL) per 100 µL R10 medium.
5. Add 100 µL of the 2× TLR4 agonist mix to 100 µL cell suspension. This results in a total culture medium volume of 200 µL (*see* **Note 3**) and a final TLR agonist concentration of 1 µg/mL each. The final TLR agonist concentration can be adapted to your experimental needs (*see* **Note 14**).
6. Prepare a negative control by adding 100 µL R10 medium instead of TLR agonist to the THP-1 cells and mix well.
7. Incubate at 37 °C, 5% CO_2 for 24 h.
8. Before continuing with the plate, prepare and count isolated CD8+ T cells. (For CD8+ T cell isolation we recommend using CD8 MicroBeads and following manufacturer's instructions).
9. Resuspend CD8+ T cells in R10 medium at a concentration of 2 million cells per 1 mL.
10. Centrifuge the plate containing THP-1 cells for 5 min at 350 × *g*. Aspirate and discard supernatant.
11. Wash THP-1 cells by resuspending cells in 200 µL fresh medium. Centrifuge again, and aspirate and discard the supernatant.
12. Resuspend THP-1 cells in 100 µL R10 medium.
13. Add 100 µL of CD8+ T cell suspension per well to the stimulated THP-1 cells. This results in a final cell density of 0.2 million CD8+ T cells and 0.2 million THP-1 cells per well. Cell numbers can be adapted to your needs (*see* **Note 8**).
14. Incubate at 37 °C, 5% CO_2 for 24 h (the incubation time can be varied depending on the experimental question, *see* **Note 5**).
15. If the experiment is to be analyzed by flow cytometry including staining for intracellular cytokines, add Brefeldin A to all conditions 4 h before termination of the assay. Prepare a 1:50 dilution of Brefeldin A 1000× stock solution in R10 medium. Add 10 µL to each well containing 200 µL cultured cell suspension and mix well. The final working concentration of Brefeldin A is 3 µg/mL. Incubate at 37 °C and 5% CO_2 for the remaining 4 h and then continue with Subheading 3.3.

3.2.3 MAIT Cell Activation Using Viruses

MAIT cells can be indirectly activated by certain viruses in the presence of APCs. The viruses stimulate cytokine production by the APCs, for example, IL-12, IL-18, and/or IL-15, which in turn can activate MAIT cells [18] (Fig. 6c). Here we describe a protocol for the activation of MAIT cells within PBMCs using influenza strain A/WSN/1933 (H1N1), which activates MAIT cells mainly through the induction of IL-18 secretion by APCs. Please refer to reference [18] for information about using other viruses such as dengue or hepatitis C virus.

1. Prepare and count PBMCs.
2. Resuspend cells in R10 medium at a concentration of 10 million PBMCs per 1 mL.
3. Seed 100 µL cells per well into a 96-well U-bottom cell culture plate. This results in a final cell number of 1 million PBMCs per well. Cell numbers can be adapted to your needs (*see* **Note 2**).
4. Prepare a 2× mix of influenza virus strain A/WSN/1933 (H1N1) in R10 medium (final MOI of PBMC: 0.05–2.5; we recommend a final MOI of 1 for optimal stimulation). Refer to reference [18] for any information about influenza virus preparation.
5. Add 100 µL of the 2× influenza virus mix to 100 µL cell suspension and mix well. This results in a total culture medium volume of 200 µL (*see* **Note 3**).
6. As a negative control, add 100 µL R10 medium instead of influenza virus to the cells and mix well.
7. Prepare a positive control by adding 100 µL 2× PMA + Ionomycin mix, diluted in R10 medium, to the cells and mix well (final concentration: PMA = 5–50 ng/mL, we recommend 10 ng/mL; Ionomycin: 0.66–1 µM, we recommend 0.66 µM) (*see* **Note 4**).
8. Incubate at 37 °C, 5% CO_2 for 24 h (the incubation time can be varied depending on the experimental question, *see* **Note 5**).
9. If the experiment is to be analyzed by flow cytometry including staining for intracellular cytokines, add Brefeldin A to all conditions 4 h before termination of the assay. Prepare a 1:50 dilution of Brefeldin A 1000× stock solution in R10 medium. Add 10 µL to each well containing 200 µL cultured cell suspension and mix well. The final working concentration of Brefeldin A is 3 µg/mL. Incubate at 37 °C and 5% CO_2 for the remaining 4 h and then continue with Subheading 3.3.

3.3 Analysis of MAIT Cell Activation Via Flow Cytometry

3.3.1 Staining for Flow Cytometry

1. Centrifuge the 96-well U-bottom plate containing the cells for 5 min at 350 × *g*. If cells were cultured in a flat-bottom plate, e.g., for a plate-bound anti-CD3/anti-CD28 stimulation assay, transfer cells first to a 96-well U-bottom plate to ensure proper cell pellet formation during centrifugation. For very low cell numbers, V-bottom plates can be used for staining.
2. Discard supernatant.
3. To wash cells, resuspend the pellet in 200 µL PBS and centrifuge (5 min at 350 × *g*). Discard the supernatant.
4. Resuspend cells in 50 µL PBS-diluted viability dye (1:1000 dilution of LIVE/DEAD™ Fixable Near-IR Dead Cell Stain). Incubate for 20–30 min at 2–8 °C.
5. Add 150 µL PBS, centrifuge (5 min at 350 × *g*) and discard the supernatant.
6. To wash cells, resuspend the pellet in 200 µL PBS and centrifuge (5 min at 350 × *g*). Discard the supernatant.
7. Resuspend cells in 50 µL antibody mix containing the antibodies for surface antigens (prepare the antibody mix in advance by diluting antibodies in PBS containing 5% FCS. Tables 1, 2, and 3 lists recommended dilutions of three different panels for use with two different flow cytometers. For optimal performance, we recommend titrating the antibodies before use. This is especially important when using a flow cytometer different from those listed. Refer to **Notes 15** and **16** for further considerations regarding panel design). Incubate for 20–30 min at 2–8 °C.
8. Add 150 µL PBS and centrifuge for 5 min at 350 × *g*. Discard the supernatant.
9. Wash cells in 200 µL PBS, centrifuge (5 min at 350 × *g*) and discard supernatant.
10. Resuspend cells in 100 µL 2% formaldehyde solution (diluted in PBS) and incubate for 10 min at 2–8 °C.
11. Add 100 µL PBS and centrifuge at 500 × *g* for 5 min.
12. Wash cells in 200 µL PBS, centrifuge (500 × *g* for 5 min), and discard supernatant.
13. Resuspend cells in 200 µL 1× Permeabilization Buffer (diluted in distilled water) and incubate for 10 min.
14. Centrifuge (500 × *g* for 5 min) and discard supernatant.
15. Resuspend cells in 50 µL antibody mix containing the antibodies for intracellular antigens (prepare the antibody mix in advance by diluting antibodies in 1× Permeabilization Buffer. Tables 1, 2, and 3 lists recommended dilutions of three different panels for use with two different flow cytometers. For optimal performance, we recommend titrating the antibodies

before use. This is especially important when using a flow cytometer different from those listed. Refer to **Note 16** for further considerations regarding panel design). Incubate for 30 min at 2–8 °C.

16. Add 150 µL 1× Permeabilization Buffer and centrifuge for 5 min at 350 × *g*. Discard the supernatant.
17. Wash cells in 200 µL 1× Permeabilization Buffer, centrifuge (500 × *g* for 5 min), and discard supernatant.
18. Resuspend cells in 100 µL PBS containing 5% FCS.
19. Analyze samples on the flow cytometer.

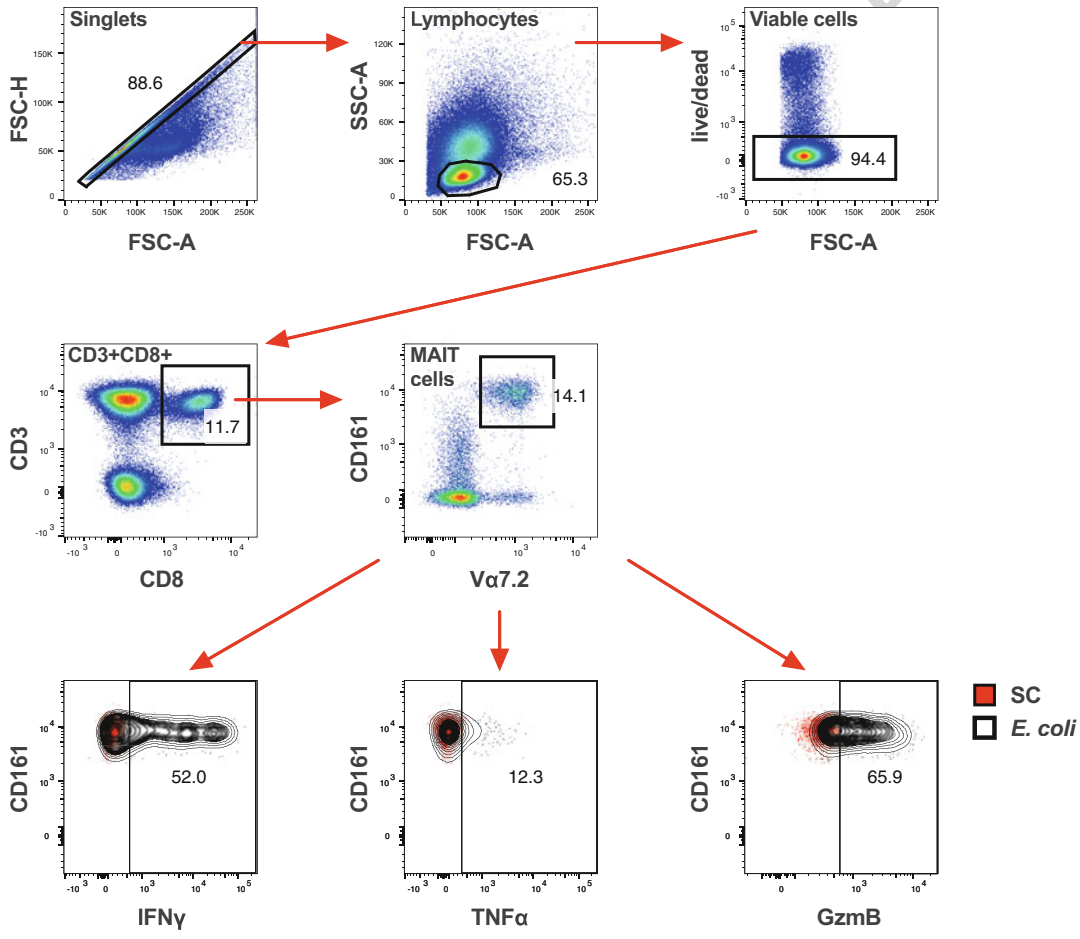

Fig. 8 Gating strategy to identify MAIT cells with their activation profile. The gating strategy is demonstrated on unstimulated (SC, sterility control) and *E. coli*-stimulated PBMCs (24 h). Doublets are first excluded, then lymphocytes are defined by size (FSC-A) and granularity (SSC-A), and viable cells identified by gating on live/dead marker-negative cells. Next, CD3+CD8+ T cells are selected, and MAIT cells are identified as CD161+ +Vα7.2+ cells. Finally, the activation profile of MAIT cells is determined by analyzing the expression of IFNγ, TNFα, and GzmB

3.3.2 Gating Strategy to Identify MAIT Cells and Analyze Their Activation

1. Exclude doublets by gating on singlets using the parameters FSC-A and FSC-H (Fig. 8).
2. Identify lymphocytes by size (FSC-A) and granularity (SSC-A).
3. Exclude dead cells by gating on live/dead marker-negative cells (*see* **Note 15**).
4. Gate on CD3+CD8+ T cells. Optional: If the staining panel includes CD4, CD4+ T cells can be excluded to identify CD3+CD4-CD8+ T cells for further analysis. Depending on the experimental question, additional cell populations can be identified and analyzed, including CD3+CD4+CD8−, CD3+CD4−CD8−, and CD3+CD4+CD8+ T cells (*see* **Note 17**).
5. Plot CD161 vs. Vα7.2 TCR. In the CD3+CD8+ T cell gate, two CD161+ populations can be distinguished, a CD161 intermediate population (CD161+) and a CD161 bright population (CD161++). MAIT cells are identified as CD161++Vα7.2+ T cells (*see* **Note 18**).
6. Within the MAIT cell gate, the expression of cytokines, activation markers, or other readouts can be analyzed, for example, IFNγ, TNFα, GzmB, or CD69 expression, depending on the antibody staining panel.

4 Notes

1. The manufacturer recommends using 100 µL of anti-CD3-Biotin, 100 µL anti-CD28-Biotin, and 100 µL of anti-CD2-Biotin to load the Anti-Biotin MACSiBead Particles. For better comparability with other assays and work from other groups, we load the beads only with anti-CD3 and anti-CD28 antibodies and substitute the anti-CD2 antibody with 100 µL MACS Buffer. This approach successfully activates T cells including MAIT cells. If your needs and experimental setup require, 100 µL MACS Buffer can be replaced with 100 µL anti-CD2-Biotin.
2. Cell numbers can be adapted depending on the needs and experimental setting. We recommend culturing 0.25–2 million (PBMCs) or 0.1–1 million (CD8+ T cells) per well of a 96-well U-bottom cell culture plate, depending on required cell numbers for analysis and on assay duration. For stimulations of >5 days, we recommend not to exceed 0.25 million cells per well. For an overnight stimulation, cell numbers of up to 2 million cells per well can be used. If the experimental design allows, high cell densities can be accommodated for a longer time period by replacing the media and any stimuli periodically.
3. We usually culture cells in a final volume of 200 µL per well of a 96-well U-bottom cell culture plate. If any additional reagents are added, make sure to adapt the cited volumes of cell

suspension and reagents to obtain a final culture medium volume of 200 µL.

4. Increasing concentrations of PMA and Ionomycin correlates with increased cell death of MAIT cells, most likely due to activation-induced cell death.

5. Incubation time can be varied depending on the experimental question. Time courses from 0 h up to 110 h can be performed. Nutrient deprivation and accumulation of harmful metabolites can compromise long-term experiments of >5 days. If the experimental question allows, the media (and if necessary, any stimuli) should be replaced periodically from day 5. As mentioned in **Notes 2**, **7**, and **8**, it is important to carefully consider cell numbers.

6. In our standard protocol we use anti-CD3 at a concentration of 2.5 µg/mL in combination with soluble anti-CD28 at a concentration of 1 µg/mL. Depending on the experimental question, the efficacy of other antibody concentrations should be explored. For example, co-stimulation with other antibodies, cytokines, or other stimuli could require suboptimal stimulation with antibody concentrations as low as 0.1–0.5 µg/mL. Stronger responses could possibly be reached using higher antibody concentrations with up to 10 µg/mL. Please note that overstimulation can lead to increased cell death most likely due to activation-induced cell death. In addition, high anti-CD3/anti-CD28 antibody concentrations can lead to down-regulation of the TCR which can make subsequent analysis via flow cytometry difficult when staining for TCR-associated antigens.

7. When using a plate-bound approach of anti-CD3 antibodies with soluble CD28 antibodies to stimulate MAIT cells, we recommend a cell density of 0.1–0.5 million PBMCs or CD8+ T cells per well of a 96-well flat-bottom cell culture plate. Efficacy of lower and higher cell densities should be titrated for particular experimental questions. Very low cell densities could compromise experiments due to insufficient cell-to-cell contact. Higher cell densities could compromise experiments due to insufficient contact of the cells with anti-CD3 adsorbed to the well surface.

8. When culturing CD8+ T cells in the presence of THP-1cells, we recommend cell densities of 0.2 million CD8+ T cells and 0.1 million THP-1 cells per well (CD8+ T cell to THP-1 ratio of 2:1). Other cell densities and ratios can be tested for particular experimental needs. Depending on the duration of the experiment, we recommend not to use less than 0.1 million CD8+ T cells and 0.05 million THP-1 cells per well or exceed 2 million total cells per well in overnight assays.

9. When stimulating MAIT cells by using 5-OP-RU in the presence of APCs, we recommend using a 5-OP-RU concentration of 10 nM for optimal stimulation. Certain experimental questions can require other 5-OP-RU concentrations, for example, for suboptimal stimulation. Therefore, a range of concentrations (e.g., 1 pM–1 μM) should be tested and titrated for their efficacy before use (Fig. 3).
10. We recommend adding one bacterial colony per 10 mL LB Broth medium. The total volume of bacterial culture can be scaled up or down depending on the final number of bacteria required. Remember to adjust Erlenmeyer flask size to the culture volume to allow optimal oxygen supply to the bacteria. We recommend an Erlenmeyer flask capacity to LB Broth volume ratio of 5:1.
11. MAIT cells can be stimulated in an MR1-dependent manner using PFA-fixed *E. coli* in a model with PBMCs (model 1) or with isolated CD8+ T cells in combination with THP-1 cells (model 2). In model 1, PFA-fixed *E. coli* are processed by APCs contained within the PBMCs. MAIT cells become activated by *E. coli*-derived riboflavin metabolites presented by MR1 on APCs, in combination with cytokines, such as IL-12 + IL-18, produced by the activated APCs. In model 2, PFA-fixed *E. coli* are processed by THP-1 cells that activate MAIT cells within isolated CD8+ T cells via MR1 and cytokines. THP-1 cells are more efficient APCs compared to those contained within PBMCs, and frequencies of activated MAIT cells are usually higher in model 2 compared with model 1.
12. We recommend performing a pilot experiment with each fresh batch of PFA-fixed *E. coli* to test their potency to activate MAIT cells. The dose–response should be investigated at a range of 0–100 CFU per cell in steps of 10 CFU and smaller steps of 5 CFU at <20 CFU. The CFU required for maximal activation (we recommend staining for intracellular IFNγ expression) should be fairly consistent and is in our hands around 10 CFU per PBMC and 30 CFU per THP-1 cell. As the PFA-fixed *E. coli* degrade over time, their potency to activate MAIT cells decreases. Therefore, we recommend producing new batches frequently. The fixed bacteria should be reasonably stable for up to 8 weeks at 2–8 °C.
13. When stimulating MAIT cells with the cytokines IL-12 and IL-18, we recommend a concentration of 50 ng/mL each for optimal stimulation. Certain experimental questions require simulation with suboptimal IL-12 + IL-18 concentrations as low as 1–10 ng/mL. Excessively high cytokine concentrations greater than 50 ng/mL can lead to increased cell death through overstimulation. The efficacy of other concentrations

should be tested and titrated for their specific purpose before use (Fig. 7).

14. We recommend a TLR4 agonist (*E. coli* K12 LPS) or TLR8 agonist (ssRNA40/Lyovec) concentration of 1 μg/mL to stimulate MAIT cells indirectly within CD8+ T cells plus THP-1 cells or within PBMCs, respectively. Depending on your experimental needs, TLR agonist concentration can be adapted and further titrated. The manufacturer InvivoGen recommends a range of 10 ng–10 μg/mL for *E. coli* K12 LPS, and 0.25–10 μg/mL for ssRNA40/Lyovec.
15. The antibody staining panel can be extended by using the channel of the viability dye as a dump channel. The dump channel includes cells which are not of interest and could contaminate downstream analysis, for example, pan-γδTCR or CD14. By adding additional markers of the same color as the viability marker to the antibody staining mix, those cell populations can be merged in one channel. During analysis, dead cells and unwanted cell populations can be excluded together by gating on dump channel fluorochrome-negative cells.
16. Strong MAIT cell activation can lead to downregulation of CD3 and Vα7.2 TCR expression, which makes subsequent analysis difficult. In such cases, intracellular staining of CD3 and Vα7.2 TCR can rescue some of the CD3 and Vα7.2 TCR staining intensity. To stain for those markers intracellularly, include the CD3 and Vα7.2 TCR antibodies in the antibody staining mix at **step 15** instead of **step 7**.
17. Most MAIT cells are CD3+CD4−CD8+, but subsets have been identified within other CD3+ T cell populations including CD4-CD8-, CD4+CD8−, and CD4+CD8+ [9].
18. In flow cytometric analysis, MAIT cells are usually defined by high expression of the surrogate marker CD161 in combination with Vα7.2 TCR. Alternative surrogate markers can be used for MAIT cell identification, including CD26 and IL18Rα, but are less reliable than CD161 [9]. MR1-Ag tetramers are now freely available from the NIH for accurate MAIT cell identification. Use of the MR1 tetramer is particularly important in populations where MAIT cell frequency is low or where staining with surrogate markers is not very reliable, e.g., CD4+ T cells [9].

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