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Characterisation of Human MAIT cells (Mucosal-Associated Invariant T cells)

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Significance Statement

The biology of Mucosal-associated invariant T cells (MAIT cells) is a rapidly growing field of interest. MAIT cells are an abundant subset of unconventional T cells which play a role in anti-microbial immunity. Here we describe protocols to characterise the phenotype of human MAIT cells, using fluorescently-labelled MHC related protein-1 (MR1) tetramers, antibodies and flow cytometry. We also describe protocols to generate MAIT TCR positive reporter cell lines and antigen-presenting cell lines, which are useful for the characterisation of MAIT TCR-mediated antigen responses.

ABSTRACT

Mucosal-associated invariant T cells (MAIT cells) are a subset of unconventional T cells restricted by the Major Histocompatibility Complex (MHC) class I-like molecule, MHC related protein-1 (MR1). MAIT cells are found throughout the body and are frequent in human blood and liver. Unlike conventional T cells, which are stimulated by peptide antigens presented by MHC molecules, MAIT cells recognise metabolite antigens derived from an intermediate in the microbial biosynthesis of riboflavin. MAIT cells mediate protective immunity to infections with riboflavin producing microbes via the production of cytokines, and cytotoxicity. The discovery of stimulating MAIT cell antigens allowed for the development of an analytical tool, the MR1 tetramer, which binds specifically to the MAIT T cell receptor (TCR) and is becoming the gold standard for the identification of MAIT cells by flow cytometry. This unit describes protocols to characterise the phenotype of human MAIT cells in blood and tissues using fluorescently-labelled human MR1 tetramers alongside antibodies specific for MAIT cell markers in flow cytometry.

ALTERNATE PROTOCOL 4: Determination of the MAIT cell activation phenotype based on staining for intracellular markers
ALTERNATE PROTOCOL 5: Determination of the MAIT cell activation phenotype by measuring secreted cytokines
ALTERNATE PROTOCOL 6: Determination of the MAIT cell activation phenotype based on fluorescent dye dilution due to proliferation
ALTERNATE PROTOCOL 7: Use of plate-bound CD3/CD28 stimulation to assess the capacity of MAIT cells to produce cytokine independently of Ag
ALTERNATE PROTOCOL 8: Use of bead-immobilised CD3/CD28 stimulation to assess the capacity of MAIT cells to produce cytokine independently of Ag
ALTERNATE PROTOCOL 9: Use of MR1 blocking antibody to determine MR1-Ag dependence in MAIT cell activation
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SUPPORT PROTOCOL 5: Cloning of MAIT TCR □- and □-chain and MR1 genes for transfection of cell lines
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SUPPORT PROTOCOL 8: Generation of monocyte-derived dendritic cells
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BASIC PROTOCOL 3: Characterisation of MAIT cell TCRs by generating TCR positive reporter cell lines
ALTERNATE PROTOCOL 11: Evaluation of the MAIT TCR-mediated MR1-Ag response with TCR positive reporter cell lines
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SUPPORT PROTOCOL 10: Generation of 293T cells, transiently transfected with the TCR of interest

Keywords:

MAIT cells, MR1, tetramer, human, metabolite, antigen, riboflavin, immune system, flow cytometry

INTRODUCTION

Mucosal-associated invariant T cells (MAIT cells) are a subset of unconventional T cells, expressing an αβ T cell receptor (TCR) and restricted by the monomorphic Major Histocompatibility Complex (MHC) class I-like molecule, MHC related protein-1 (MR1) (Hashimoto, Hirai, & Kurosawa, 1995; Porcelli, Yockey, Brenner, & Balk, 1993; Tilloy et al., 1999; Treiner et al., 2003). Unlike conventional T cells, which recognise peptide antigens (Ags), or Natural Killer T cells (NKT cells), which recognise glycolipid Ags (Rossjohn, Pellicci, Patel, Gapin, & Godfrey, 2012), MAIT cells recognise metabolite Ags (Corbett et al., 2014; Kjer-Nielsen et al., 2012), and reviewed in (Eckle et al., 2015; Kjer-Nielsen et al., 2018). The most potent MAIT cell Ags, which activate all MAIT cells, originate from an intermediate in the microbial biosynthesis of riboflavin (vitamin B2), 5-amino-6-D-ribitylaminouracil (5-A-RU). 5-A-

RU combines with the abundant metabolic by-products methylglyoxal and glyoxal, forming the MAIT cell Ags 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU), respectively (Corbett et al., 2014). Interestingly, MR1 also binds folate-derived non-stimulating ligands, which act as competitive inhibitors of 5-A-RU-derived Ags. They include the naturally occurring folic acid degradation product 6-formyl pterin (6-FP) (Kjer-Nielsen et al., 2012) and its synthetic analogue acetyl-6-FP (Ac-6-FP) (Eckle et al., 2014). Riboflavin biosynthesis is absent in mammals, including humans, but is present in most bacteria and yeast, providing a microbial signature for MAIT cell surveillance. Indeed, MAIT cells are stimulated by and play a key role in protection from riboflavin producing microbes, reviewed in (Kjer-Nielsen et al., 2018). Furthermore, MAIT cells can also respond to viruses in an MR1-Ag independent manner, reviewed in (Ussher, Willberg, & Klenerman, 2018) and their role in autoimmune and immune-mediated diseases and cancer is being investigated, reviewed in (Godfrey, Koay, McCluskey, & Gherardin, 2019; Rouxel & Lehuen, 2018), including their potential role in drug allergies (Keller et al., 2017).

An invaluable tool to study T cells by flow cytometry is the MHC tetramer, which is the fluorescently-labelled tetrameric version of the soluble component of the MHC molecule in complex with the relevant Ag. MHC class-I (MHC-I) tetramers loaded with peptide Ags were originally developed by Altman *et al.* for the identification and characterisation of conventional T cells (Altman et al., 1996). Later, the same technology was applied to NKT cells, where CD1d tetramers loaded with glycolipid Ags were generated (Benlagha, Weiss, Beavis, Teyton, & Bendelac, 2000; Matsuda et al., 2000). Most recently MR1 tetramers were produced (Corbett et al., 2014; Reantragoon et al., 2013), which are becoming the gold standard for the identification of MAIT cells by flow cytometry. These are available from the National Institutes of Health (NIH) tetramer core facility, USA (see Internet resources). MR1 tetramers loaded with the non-stimulating molecule 6-FP (Kjer-Nielsen et al., 2012) are used as a control reagent for 5-OP-RU loaded MR1 tetramers (Reantragoon et al., 2013). Whilst the availability of MR1 tetramers has led to a rapid increase in understanding of MAIT cell biology, much is still to be learned about their basic function. Eventually such knowledge will guide the development of immunotherapies that exploit the functional properties of MAIT cells, with unconventional T cells emerging as an attractive target for immunotherapies (D'Souza et al., 2019; Godfrey, Le Nours, Andrews, Uldrich, & Rossjohn, 2018).

Here we describe in Basic protocol 1 how to determine the frequency, absolute numbers (Alternate protocol 1), and steady state phenotype of human MAIT cells in blood and tissues. Methods include the use of fluorescently-labelled MR1 tetramers alongside antibodies specific for surface and intracellular (transcription factors) (Alternate protocol 2) markers in flow cytometry. As a specialised aspect of the MAIT cell phenotype, we furthermore describe the method for determining the TCR usage of MAIT cells (Alternate protocol 3). Similarly, Basic protocol 2 describes how to assess the phenotype of human MAIT cells, activated by MR1-Ag, in flow cytometry based on surface and

intracellular markers (Alternate protocols 4 and 5) or fluorescent dye dilution upon proliferation (Alternate protocol 6). Whilst Alternate protocol 4 describes the method for staining for intracellular markers, Alternate protocol 5 describes the method of measuring secreted cytokines. A series of Alternate protocols then presents methods for experimental controls to assess the capacity of MAIT cells to be stimulated in an Ag-independent manner (Alternate protocols 7 and 8), as well as to determine MR1-Ag dependence of the observed MAIT cell activation (Alternate protocols 9 and 10). Extending on determining the MAIT TCR usage (Alternate protocol 3), in Support protocols 5, 6 and 10, we describe how to generate human cell lines engineered to express a MAIT TCR of interest, allowing the characterisation of MAIT TCRs in a defined system (Basic protocol 3), including their stimulatory capacity based on surface phenotype (Alternate protocol 11) or based on cytokine secretion (Alternate protocol 14). Methods for experimental controls to assess the capacity of MAIT cells to be stimulated in an Ag-independent manner and to determine MR1-Ag dependence of the observed MAIT cell activation are either identical to Alternate protocols 7 and 10 or presented as additional protocols (Alternate protocols 12-13).

Strategic Planning

Experiments with primary human MAIT cells need to be performed in a Biosafety level 2 facility if they involve infectious samples or if the experiment involves *in vitro* infections. The level of biosafety might be higher depending on the pathogen involved. Even experiments with samples from healthy blood or tissue donors are best performed in a Biosafety level 2 facility, due to the risk of pathogens not detected by routine blood screening. Experiments with human cell lines should also be performed in a Biosafety level 2 facility (given that these are immortal cell lines), or in a facility of higher Biosafety level, depending on the pathogen involved. In addition, *in vitro* experiments require sterile tissue culture technique. Whilst not mentioned in any of the methods provided, all experiments are ideally performed with triplicate samples and at least three times independently.

The frequency of the MAIT cell population is highly variable between individuals (Gherardin, Souter, et al., 2018). Further, sex-specific differences might exist and the MAIT cell frequency fluctuates with age, tissue location, disease and treatment (Gherardin, Loh, et al., 2018; Gherardin, Souter, et al., 2018; Hinks et al., 2016; Leeansyah, Loh, Nixon, & Sandberg, 2014; Loh et al., 2016). Hence consideration needs to be taken regarding the minimum number of peripheral blood mononuclear cells (PBMCs) analysed in Basic protocol 1 in order to acquire sufficient events allowing high quality flow cytometry data analysis. This can be achieved by increasing the sample size, or, if the population of interest is particularly rare, an enrichment step for MAIT cells prior to characterisation or purification can be employed (Support protocols 3 and 4).

The following outlines considerations for experimental controls. As an internal control, the phenotypes and frequencies of non-MAIT T cells alongside MAIT cells can be analysed in the same sample, e.g. all non-MAIT αβ T cells, TRAV1-2^{+} non-MAIT αβ T cells, MHC-I tetramer specific T cells and NKT cells. Depending on the research questions, sample controls are required, such as tissue vs PBMCs from the same donor, healthy vs diseased tissue from the same donor, or healthy vs patient donor matching age and sex. It may also be of interest to include PBMC samples of a previously characterised donor as an internal positive control.

BASIC PROTOCOL 1

DETERMINING THE FREQUENCY AND STEADY STATE SURFACE PHENOTYPE OF HUMAN MAIT CELLS

This protocol describes how to perform a flow cytometric analysis to identify and determine the frequency of human MAIT cells using fluorescently-labelled MR1-5-OP-RU tetramer and a TRAV1-2 TCR α-chain-specific monoclonal antibody (mAb) in human PBMCs, prepared as per Support protocol 1. We also include a basic flow cytometric MAIT cell-surface marker phenotyping panel of fluorescently-labelled mAbs. This can be expanded to include additional cell-surface markers (Table 1), such as maturation markers and chemokine receptors as well as transcription factors which requires intracellular staining of cells (Alternate protocol 2). Alternate protocol 1 provides the methods to determine absolute numbers of human MAIT cells in PBMCs, which may be useful for determining changes in MAIT cell dynamics in diseased cohorts (Gherardin, Loh, et al., 2018). Using flow cytometry, MAIT cells can also be characterised in peripheral tissues as per the Basic protocol, following the preparation of single cell suspensions, where we provide a protocol for lymph nodes as an example (Support protocol 2). For samples in which MAIT cells are rare, Support protocols 3 and 4 can be employed to enrich and isolate MAIT cells from single cell suspensions. A specialized aspect of the phenotypic characterisation of MAIT cells is determining their TCR α- and β-chain usage by performing a nested multiplex polymerase chain reaction (RT-PCR) following reverse transcription to cDNA (Alternate protocol 3) (G. C. Wang, Dash, McCullers, Doherty, & Thomas, 2012), which can be applied on isolated single MAIT cells (Support protocol 3) (Reantragoon et al., 2013).

Materials

- PBMC samples in suspension (generated as per Support protocol 1 and maintained as per Appendix)
- Reagents, equipment and their use for counting viable cells using trypan blue exclusion (see Appendix)
- 96-well v-bottom plate (Greiner Bio-One, cat. no. 651180)

- Live/dead cell discrimination stain (see Appendix for live/dead cell discrimination stains and their use)
- Cell-surface staining mixture (BV421-labelled MR1-5-OP-RU tetramer and fluorochrome-conjugated mAbs specific for the cell-surface phenotype markers of MAIT cells as per Tables 1 and 2; fluorochrome-conjugated isotype control mAbs, matching the isotype, fluorochrome and concentration of the relevant mAbs; BV421-labelled MR1-6-FP tetramer control matching the MR1-5-OP-RU tetramer concentration; see Appendix for MR1 tetramer generation and use)
- Phosphate-buffered saline (PBS, stored at 4°C)
- Flow cytometry wash buffer (flow wash) A (see Reagents and solutions)
- Flow cytometry cell fixation buffer (flow fix) (see Reagents and solutions)
- Flow cytometry acquisition tubes (Falcon, cat. no. 352008)
- 40 μm cell strainers (Miltenyi Biotec, cat. no. 130-098-458)
- Single colour control samples for flow cytometry data acquisition (see Appendix)
- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix)

- Tabletop centrifuge (Beckman Coulter Allegra X-12R)
- Flow cytometer (BD LSR Fortessa)

Protocol steps

1. Take an aliquot of each PBMC sample, and count viable cells using trypan blue exclusion (see Appendix). Resuspend PBMCs at 1 x 10^7 cells/ml in PBS and aliquot 100 μl per well of a 96-well v-bottom plate.

2. Pellet cells (450 xg, 5 min, 4°C), discard supernatants and stain cells with fixable live/dead cell discrimination stain as per Appendix, e.g. compatible with this staining panel is Live/dead Aqua stain, typically used in a 1/800 dilution of the stock, prepared as per Appendix.

3. Resuspend the cell pellets in 50 μl of cell-surface staining mixture prepared in flow wash A as per Table 2. Incubate cells for 30 min on ice in the dark.

   Note: If cells are co-stained with MR1-5-OP-RU tetramer and anti-$\alpha\beta$ TCR or anti-TRAV1-2 antibody (as the case here), anti-$\alpha\beta$ TCR or anti-TRAV1-2 antibody may be added only for the last 15 minutes of the staining incubation period. Since tetramer has a lower avidity for TCR compared to TCR specific mAbs, adding the tetramer prior to TCR specific mAb may improve co-staining. Several clones of TRAV1-2 specific antibodies are available, with 3C10 being the most commonly used clone, originally developed by Lantz and colleagues (Martin et al., 2009). Up to 5 x 10^6 cells can be stained in a volume of 50 μl without compromising fluorescence intensity. Instead of resuspending cell pellets by

pipetting, the covered 96-well v-bottom plate can be gently resuspended by firmly holding against a vortex to create a swirling motion at the maximal setting.

4. Wash cells twice by adding 150 µl of flow wash A, resuspending and pelleting cells (450 xg, 5 min, 4°C) and discarding the supernatants.
5. Fix cells by resuspending cell pellets in 100 µl of flow fix, followed by incubation on ice in the dark for 20 min.
   Note: Samples can be stored in the dark on ice or in a 4°C refrigerator for up to 72 hr without comprising fluorescence intensity.
6. Transfer samples to flow cytometry acquisition tubes and analyse on a flow cytometer, acquiring data for each fluorochrome-conjugate used and live/dead stain.
   Note: It is recommended to subject cells to a 40 µm cell strainer before flow cytometry to ensure clumped cells are removed. This will help prevent blockages in the flow cytometer.
7. Following sequential gating on live, single cells, T cells, identify MAIT cells as MR1-5-OP-RU Tet$^+$ TRAV1-2$^+$ and assess phenotypic markers within this population (Figure 1). Analyse control staining and control samples in the same manner. Flow cytometry data is typically displayed in the form of dot plots and histograms, determining the percentages of populations and the geometric mean fluorescence intensities (gMFI).
   Note: It is recommended to subject cells to a 40 µm cell strainer before flow cytometry to ensure clumped cells are removed. This will help prevent blockages in the flow cytometer.

[*Insert Figure 1 near here]

ALTERNATE PROTOCOL 1

DETERMINING THE ABSOLUTE NUMBER OF HUMAN MAIT CELLS

In settings of disease, MAIT cell frequencies as percentages of all T cells have been shown to be altered compared to healthy control groups, e.g. (Gherardin, Loh, et al., 2018; Loh et al., 2016) and reviewed in (Godfrey et al., 2019). In principle, a change of MAIT cell frequency can be due to a change in absolute numbers of MAIT cells but not of other T cells, or, due to a change in absolute numbers of other T cells but not of MAIT cells, or both. Thus, to differentiate the origin of an altered MAIT cell frequency, absolute numbers of MAIT cells and non-MAIT T cells per volume of blood are determined as described here in separate samples alongside samples prepared using Basic protocol 1. The same method can also be applied to tissue samples, when a single cell suspension of tissue at a set weight was prepared in a set volume, as per Support protocol 2.

Additional materials

- Anti-coagulated whole blood, prepared as per Support protocol 1
- BD Multitest CD3/CD16 + CD56/CD45/CD19 (BD, cat. no. 340500)
- BD Trucount beads (BD, cat. no. 340334)
- BD FACS lysing solution, 10X concentrate (BD, cat. no. 349202)
- Sterile water

Protocol steps

1. Dilute BD FACS lysing solution, 10X concentrate 1/10 in sterile water.
   Note: The prepared solution is stable for 1 month if kept in the dark in the refrigerator in a glass bottle, as per manufacturer's instructions.
2. Pipette 10 µl of BD MultiTest to the Trucount tube which contains the bead pellet, just above the stainless-steel retainer, whilst not touching the bead pellet.
3. Pipette 50 µl of well-mixed, anticoagulated whole blood onto the side of the Trucount tube, just above the stainless-steel retainer.
4. Cap the Trucount tube and vortex gently to mix. Incubate for 15 min in the dark at room temperature (RT).
5. Add 450 µl of 1XBD FACS lysing solution to the tube.
6. Cap the tube and vortex gently to mix. Incubate for 15 min in the dark at RT.
7. Acquire the sample on a flow cytometer according to the manufacturer's instructions for BD Trucount beads.

   Note: Store in the dark at RT if not acquired immediately. Acquire within 1 hr.
8. To calculate the absolute numbers of MAIT cells per µl, first determine the absolute numbers of $CD3^+$ T cells from the Trucount analysis according to the manufacturer's instructions. Multiply the *frequency* of MAIT cells of the $CD3^+$ T cell population by the *absolute* number of $CD3^+$ T cells per µl of blood to derive the absolute number of MAIT cells per µl of blood.

ALTERNATE PROTOCOL 2

DETERMINING THE TRANSCRIPTION FACTOR PHENOTYPE OF HUMAN MAIT CELLS

In addition to cell-surface phenotypic markers, MAIT cells can constitutively express several intranuclear transcription factors, which are important during their development and differentiation as well as for their function (Gherardin, Souter, et al., 2018; Koay et al., 2016; Kurioka et al., 2017). Here we describe analysis of transcription factors (listed in Table 1), including promyelocytic leukemia zinc finger (PLZF), by intranuclear staining in flow cytometry using dedicated commercial kits, compatible with surface staining as per Basic protocol 1.

The staining pattern for transcription factors is not always bimodal, rather a shift in fluorescence is observed. Thus, it is highly recommended to include in each experiment a sample that is stained with all fluorescently-labelled mAbs except with that specific for the transcription factor, referred to as fluorescence minus one (FMO) sample (Figure 2). Alternatively, samples stained with fluorochrome-conjugated isotype control mAbs, matching the isotype, fluorochrome and concentration of the relevant transcription factor specific mAbs can be included.

[*Insert Figure 2 near here]

Additional materials

- Fixation/Permeabilisation Concentrate (eBioscience, cat. no. 00-5123-43)
- Fixation/Permeabilisation Diluent (eBioscience, cat. no. 00-5223-56)
- Permeabilisation Buffer (10X) (eBioscience, cat. no. 00-8333-56)
- Sterile water
- mAbs specific for transcription factors of MAIT cells, as per Table 1; fluorochrome-conjugated isotype control mAbs, matching the isotype, fluorochrome and concentration of the relevant mAbs.

Protocol steps

1. Follow the Basic protocol up to step 4.
2. Dilute Fixation/Permeabilisation Concentrate, 1 part concentrate to 3 parts Fixation/Permeabilisation Diluent.
3. Resuspend cells in 100 µl of diluted Fixation/Permeabilisation solution. Incubate for 20 min

on ice.

4. Dilute Permeabilisation Buffer (10X), 1 part of Permeabilisation Buffer (10X) to 9 parts of sterile water. Prepare required volume of transcription factor mAb cocktail with 1X Permeabilisation Buffer.

   Note: Use 1X Permeabilisation Buffer in the mAb cocktail mix as this will ensure that cells remain permeabilised and allows for optimal transcription factor staining.

5. Add 100 µl of diluted 1X Permeabilisation Buffer directly to cells in Fixation/Permeabilisation solution, resuspend and pellet cells (450 xg, 5 min, 4°C; there is no need for incubation prior to pelleting), then discard the supernatant.

6. Resuspend the cell pellet in 50 µl of mAb cocktail and incubate for 30 min on ice.

7. Add 150 µl of diluted 1X Permeabilisation Buffer to the cells in mAb cocktail, pellet cells (450 xg, 5 min, 4°C) and discard supernatant. Repeat wash with 1X Permeabilisation Buffer.

8. Resuspend cells in 100 µl of flow wash A and proceed to step 5 of the Basic protocol.

   Note: Samples can be stored in the dark on ice or in the refrigerator for up to 72 hr without comprising fluorescence intensity.

ALTERNATE PROTOCOL 3

DETERMINING THE TCR USAGE OF HUMAN MAIT CELLS

The TCR is a heterodimer expressed at the cell surface after somatic recombination of the gene segments of the TCRα and TCRβ or the TCRγ and TCRδ chains during thymic T cell development, reviewed in (Krangel, 2009). Briefly, a rearranged TCRα/TCRγ chain is comprised of a variable (V) amino-terminal region, joining (J) region and constant (C) region, while the TCRβ- and TCRγ-chains contain additional diversity (D) regions. The human TCRα locus is present on chromosome 14, has one constant, 50 functional J, and 45 V segments, while the TCRβ locus, present on chromosome 7, has two C, two D, 13 J, and 48 V gene segments. There are 3 surface-exposed Complementarity Determining Region (CDR) loops, which are the most variable regions within a TCR heterodimer. The CDR1 and CDR2 loops are both germline-encoded in the V-region segments. The CDR3 loop is the result of recombination events between VJ (TCRα and TCRγ) or VDJ regions (TCRβ or TCRδ) and contains key determinants required for Ag recognition. Recombination and rearrangement events generate diverse TCRs, where it is estimated that there are nearly $2x10^7$ TCR $\alpha\beta$ combinations available in humans (Arstila et al., 1999).

MAIT cells were originally described to feature a nearly invariant TCR α-chain, namely a rearrangement of the *TRAV1-2* gene segment joined to the *TRAJ33* gene segment which allowed for two variable amino acids encoded at the V-J junction. Later it was found that the TCR α-chain can feature diversity beyond the canonical *TRAV1-2-TRAJ33* rearrangement, where frequently also *TRAJ12* and *20* gene segments are used (Gold et al., 2010; Lepore et al., 2014; Reantragoon et al., 2013), as well as other *TRAJ* gene segments (Gold et al., 2014). Whilst the repertoire of TCR β-chains paired with the semi-invariant α-chain appears skewed (Porcelli et al., 1993; Tilloy et al., 1999), being dominated by *TRBV6* and *TRBV20*, there is appreciable variation in the TCR β-chain usage, especially in the CDR3β loop (Lepore et al., 2014).

This protocol provides a nested multiplex PCR method for deriving the sequence of the V(D)J junction of the rearranged TCRα- and TCRβ-chains of MAIT cells at the single-cell level, which allows for determining the TCR V(D)J usage and CDR3 loop sequences (Nguyen et al., 2017; Sant et al., 2018; Valkenburg et al., 2016; G. C. Wang et al., 2012). Single MAIT cells are isolated based on phenotypic identification as per Basic protocol 1 but using fluorescence-activated cell sorting (FACS) as per Support protocol 3 instead of flow cytometric analysis. Single-cell TCR mRNA is reverse transcribed to cDNA and nested multiplex PCR performed using a mix of primers that amplify pairs of TCR α- and β-chains from the same cell. After confirming the presence of PCR product by agarose gel electrophoresis, the product is purified, sequenced and TCR usage analysed.

Additional materials

- 5 ml sterile polypropylene tubes (Falcon, cat. no. 352063)
- 96-well PCR plates (Eppendorf, cat. no. 951020401) and cap strips (Eppendorf, cat. no. 0030124839)
- 96-well PCR freezer block (Eppendorf, cat. no. 3881000023)
- 1% Triton X-100 (Sigma, cat. no T8787), diluted in nuclease free water, stored at -20°C
- RNaseZap (Invitrogen, cat. no. AM9780)
- Kimwipes (KIMTECH, cat. no. 34155)
- SuperScript VILO cDNA Synthesis Kit (Invitrogen, cat. no. 11754250)
- dNTPs (Qiagen, cat. no. 201912)
- Taq DNA polymerase (Qiagen, cat. no. 201205)
- HPLC-grade water

- Custom DNA synthesis service for purchase of DNA oligonucleotides, namely *TRAV, TRAC*, *TRBV* and *TRBC* gene segment specific oligonucleotides, see (G. C. Wang et al., 2012) for primer sequences.
- ExoSAP-IT (Affymetrix, cat. no. 78201)
- DNAse-free reagent reservoir (Costar, cat. no. 870)
- p50 multichannel pipette
- Thermocycler
- Equipment and reagents to perform agarose gel electrophoresis (not described here)
- Access to a DNA Sanger sequencing service
- Access to the following free online tool: IMGT/V-QUEST (see Internet sources)

Protocol steps

1. Preform Support protocol 3, steps 1-4.
2. Plate single-cell FACS samples directly into 96-well PCR plates. Only sort into 80 wells per plate (columns 1-10). Place each plate onto a 96-well PCR freezer block immediately after and cap the plates with cap strips.

   Note: Column 12 of each PCR plate is left empty for a no template PCR control. Consider sorting excess cells into a back-up plate.

3. Centrifuge the plates (1159 xg, 1 min, 4°C).

   Note: At this stage, plates can be stored at -80°C.

4. Prior to preparing the cDNA buffer, treat the workbench and the pipettes with RNaseZap. Gently pour RNaseZap onto a Kimwipe and wipe the workbench and pipettes. Avoid creating aerosols.
5. Make up the appropriate volume of RT-PCR reaction mix based on the number of wells sorted into. i.e. for a full plate of 80 cells plus 8 controls, make enough RT-PCR reaction mix for 100 samples as per Table 3.
6. Dispense 2.5 µl of RT reaction mix into each sample well. Cap the wells and centrifuge plate (1159 xg, 1 min, 4°C).
7. Place plate in a thermocycler and cycle under the following conditions:

25°C 10 min

42°C 120 min

85°C 5 min

16°C hold

8. Centrifuge plate (1159 xg, 1 min, 4°C).

   Note: Proceed directly to nested PCR. Alternatively, cover plate in plastic wrap and store at 4°C (overnight) or -20°C (long term).

9. Prepare a 5 pmol per µl stock dilution for each of the TCRα- and β-chain primers for the external and internal PCR round as per Table 4.

10. Prepare the mastermix for the external round PCR as per Table 5. Make up appropriate volume of external PCR mix containing external primers for both *TRAV* and *TRBV* gene segments if amplifying TCR$\alpha\beta$.

11. Pour the mastermix into a sterile DNAse-free reagent reservoir and dispense 22.5 µl directly into wells containing cDNA samples using a p50 multichannel pipet. Carefully cap the plate using the original cap strip and centrifuge (1159 xg, 1 min, 4°C). Place the plate in a thermocycler and cycle under the following conditions:

    95°C 2 min

    35 cycles of:

    95°C 20 sec

    52°C 20 sec

    72°C 45 sec

    1 cycle of:

    72°C 7 min

    16°C hold

12. Centrifuge the plate (1159 xg, 1 min, 4°C).

    Note: Proceed directly to nested PCR. Alternatively, cover the plate in plastic wrap and store at 4°C (overnight) or -20°C (long term).

13. Make up appropriate volumes of internal round PCR mastermixes, preparing separate TRAV and TRBV mixes for amplifying TCR$\alpha\beta$ as per Table 6.

14. Pour the master mixes into sterile DNAse-free reagent reservoir and dispense 22.5 μl directly into two clean 96-well PCR plates. Add 2.5 μl of external PCR product to each well. Carefully cap the plate using the original cap strip and centrifuge (1159 xg, 1 min, 4°C).
15. Place the plate in the thermocycler and cycle under the same external round conditions as in step 12.
16. Confirm the presence of PCR product by analysing 5 μl of PCR product by agarose gel electrophoresis using a 2% agarose gel (not described here).

    Note: Proceed to clean-up and sequencing or store plates wrapped in plastic wrap at -20 ℃.
17. For Sanger Sequencing of positively amplified TCRα- and β-chains, remove ExoSAP-IT from -20°C freezer and keep on ice throughout procedure.
18. Add 5 μl of positive PCR products to a new 96-well PCR plate and add 1 μl ExoSAP-IT for a combined 6 μl reaction volume.
19. Incubate at 37°C for 15 min in a thermocycler.

    Note: This step causes degradation of remaining primers and nucleotides.
20. Incubate at 80°C for 15 min in a thermocycler.

    Note: This step causes inactivation of ExoSAP-IT. Treated PCR products may be stored at -20 °C until required.
21. Subject samples to DNA Sanger sequencing.
22. Determine the TCRα- and β-chain usage from DNA sequences obtained with the online tool IMGT/V-QUEST.

SUPPORT PROTOCOL 1

PREPARATION OF HUMAN BLOOD

In human blood, MAIT cells are present in peripheral blood mononuclear cells (PBMCs), composed of lymphocytes and monocytes. Using density gradient centrifugation, PBMCs can be separated from other cells contained in blood, polymorphonuclear cells (granulocytes, neutrophils) and red blood cells, as schematically summarised in Figure 3. Applicable to Basic protocols 1 and 2, PBMCs can be used directly after preparation or cryopreserved for future use.

Note: The use of human blood requires approval by a human ethics committee.

[*Insert Figure 3 near here]

Materials

- Vacuette Heparin Tubes, 9 ml (Greiner bio-one, cat. no. 455051) (Alternatively, a 60 ml buffy pack may be used.)
- Ficoll-Paque stored at RT in the dark (GE, cat. no. 17-1440-30)
- Materials required for maintenance of cells (as per Appendix).
- Reagents, equipment and their use for counting viable cells using trypan blue exclusion (see Appendix)
- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix)
- Optional: PBS (stored at 4°C)
- Pasteur glass pipettes (Bacto, cat. no. D812), sterilised

Protocol steps

Collect blood into Vacuette Heparin Tubes. Alternatively, a 60 ml buffy pack, supplied from a blood collection centre, may be used.

1. Transfer the blood from 5 Vacuette Heparin Tubes into one 50 ml tube and top up to 30 ml with RPMI 1640 media. For a 60 ml blood pack, divide sample across three 50 ml tubes, ~20 ml per tube, and top up to 30 ml with RPMI 1640 media.

   Note: Heparin, contained in the tubes, activates antithrombins, which block the coagulation cascade and produce a whole blood/plasma sample rather than clotted blood plus serum. Buffy packs contain an anti-coagulant as specified by the blood collection centre, such as 10 % citrate phosphate dextrose. In 9 ml of blood, the range of PBMCs is ~5-10 x10^6 cells, in a buffy pack ~5 x 10^8- 10^9. Instead of RPMI media, PBS may be used.

2. Per 50 ml tube of blood/media, aliquot 15 ml of Ficoll-Paque into a separate 50 ml tube.
3. Gently overlay Ficoll-Paque with 30 ml of diluted blood.

   Note: While overlaying, the 50 ml tube should be tilted in a 45-degree angle for the first 4 ml of blood. Overlaying is done slowly to ensure that a clear interface of 2 layers forms, which prevents blood from squirting to the bottom layer of the tube before centrifugation.

4. Place 50 ml tubes in aerosol centrifuge buckets with lids attached. Centrifuge (800 xg, 20

min, RT) with the brake off (deceleration on the centrifuge is set to 0).

Note: This step separates cells based on a density gradient. It is imperative that the deceleration on the centrifuge is set to 0. Failure to do so will result in a disrupted PBMC interface. For optimal results set the centrifuge acceleration to 5 and deceleration to 0.

5. Carefully remove the tubes from the aerosol buckets, do not disturb the interface. Harvest the interface containing the PBMCs using a sterile Pasteur pipette and transfer into a new 50 ml tube. Top up the 50 ml tube contained harvested PBMCs with RPMI 1640 media. Centrifuge tube immediately (500 xg, 15 min, 4˚C), with the brake ON (Acceleration and deceleration on the centrifuge are set to Max).

   Note: PBMCs from up to 3 Ficoll-paque tubes from the same donor can be pooled into one new 50 ml tube.

6. Discard the supernatant by aspiration, resuspend in 50 ml of RPMI 1640 media. Centrifuge (500 xg, 5 min, 4˚C). Repeat.

7. Discard the supernatant by aspiration and resuspend the pellet in 10 ml of RPMI 1640 media.

   Note: To resuspend the pellet uniformly, first add a small amount of RPMI, i.e. 2 ml, to resuspend and then top up to 10 ml.

8. Mix thoroughly by resuspending and count viable cells of a 10 µl aliquot.

   Note: PBMCs can either be used fresh for downstream protocols i.e. Basic protocols 1 and 2, or cryopreserved for future use, as per Appendix, Maintenance of cells.

SUPPORT PROTOCOL 2

PREPARATION OF SINGLE CELL SUSPENSIONS FROM LYMPH NODE TISSUE

Human MAIT cells in tissues can be characterised as per Basic protocol 1 following the generation of a single cell suspension of the tissue. We provide here a method for the generation of a cell suspension of lymph nodes (LN) with enzymatic digestion as an example (Koutsakos et al., 2019; Koutsakos et al., 2018; Sant et al., 2018).

Materials

- 3-4 Human lymph nodes (LNs), stored in University of Wisconsin (UW) perfusion liquid and rinsed with PBS before use.
- Sterile dissection equipment: scalpel blade/holder, tweezers, scissors
- 70 µm nylon mesh cell strainer with filters for 50 ml tubes (Miltenyi Biotec, cat. no. 130-098-462)
- PBS (stored at 4°C)
- DNAse1 Roche 10000U (Roche, cat. no. 04536282001)
- Collagenase Type III (Worthington, cat. no. LS004182)
- 0.5 M EDTA
- EDTA-HBSS: hanks balanced salt solution (HBSS) with 2 mM EDTA, stored at 4°C
- 5 ml syringes
- Maintenance of cells (as per Appendix).
- Reagents, equipment and their use for counting viable cells using trypan blue exclusion (see Appendix)
- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix).

Protocol steps

1. Isolate 3-4 LNs from fatty tissue and place each LN into a 5 ml tube. Add 1 ml of RPMI 1640 media (at RT) and cut the tissue into small pieces with sterile surgical grade scissors.
2. Prepare a 2X digestion mixture in RPMI 1640 media, containing 2 mg/ml collagenase type III and 1 mg/ml DNase1 (as the concentrations of the 2x mixture).
   Note: Weigh collagenase type III whilst wearing a face mask as inhalation may cause sensitization. Collagenase type III is also suitable for digesting lung and spleen tissues (Pizzolla et al., 2018).
3. Add 1 ml of digestion mixture to the LN tube. Gently vortex and incubate at 37˚C for 40-45 min, gently vortexing every 15 min.
4. Add 40 µl of 0.5 M EDTA to the lymph node tube for a final concentration of 10 mM.
   Note: Addition of EDTA inhibits the enzymatic activity of collagenase and helps to prevent cells from clumping. Subsequent steps include EDTA in wash buffers.
5. Apply a 70 µm cell strainer onto a sterile 50 ml tube, add the tissue mixture with a 10 ml disposable pipette and mash the digested tissue through the strainer using the plastic top end of a sterile 5 ml syringe plug. Rinse the tissue digestion tube with EDTA-HBSS and pass through the cell strainer. Top up to 50 ml with 2 mM EDTA-HBSS.

6. Pellet cells (500 xg, 5 min, RT) and aspirate the supernatant with a Pasteur pipette.
7. Resuspend the cell pellet in 10 ml of RPMI 1640 with a 10 ml disposable pipette and count viable cells of a 10 µl aliquot.
   Note: LNs in suspension can either be used fresh for downstream protocols i.e. Basic protocols 1 and 2. Alternatively, samples can be cryopreserved for future use, as per Appendix, Maintenance of cells. It is recommended to store aliquots of 1-2 x 10^7 cells per vial to account for cell death upon thawing.

SUPPORT PROTOCOL 3

ISOLATION OF MAIT CELLS BY FACS

The bulk isolation of MAIT cells from other cells in suspension can be of interest in order to perform functional assays with MAIT cells only (Basic protocol 2). The isolation of single MAIT cells is required in order to perform single cell TCR sequencing (Alternate protocol 3) and Next-Generation Sequencing (not described as part of this article). MAIT cells can be isolated specifically by FACS using MR1-5-OP-RU tetramers in combination with fluorescently-labelled antibodies specific for TRAV1-2 and CD161. Alternatively, whilst they do not accurately identify MAIT cells (see Commentary section), surrogate markers of MAIT cells, such as TRAV1-2 and CD161, can be used in the absence of MR1-5-OP-RU tetramer (Gherardin, Souter, et al., 2018). The latter is of advantage when sorted MAIT cells are subsequently used in activation assays, to avoid potential stimulation by MR1-5-OP-RU tetramers via recycling of 5-OP-RU Ag from tetramers which might be presented on MR1 to MAIT cells or possibly via TCR cross-linking. Although, TCR cross-linking could also occur when using TRAV1-2-specific antibody.

Additional materials

- 5 ml sterile polypropylene tubes (Falcon, cat. no. 352063)
- BD Aria FACS machine

Protocol steps

1. Determine the approximate number of cells required for the assay. For example, to purify MAIT cells from PBMCs for a stimulation assay, typically, $5x10^3$ cells are required per sample. For example, for a 20-sample experiment, $1x10^5$ cells are required. Thus, from an average donor (Gherardin, Souter, et al., 2018), $9×10^6$ viable PBMCs should be prepared to ensure $1x10^5$ MAIT cells are obtained.

2. Perform Basic protocol steps 1-4 on 9×10^6 PBMCs in a tube, including staining for the markers of choice as per Basic protocol 1 (e.g. Live/dead, CD19, CD14, CD161, CD3, TRAV1-2).
3. Filter samples through 40 μm cell strainer into 5 ml polypropylene tubes
4. Sort MAIT cells on a BD Aria FACS machine, identifying MAIT cells as per the Basic protocol 1, step 17.
5. Wash purified MAIT cells, collected in a tube, using 1 ml of RF10 media (450 xg, 5 min 4°C), aspirate media and resuspended in either RF10 media for culture in a tissue culture incubator, or in flow wash for further handling.

SUPPORT PROTOCOL 4

ISOLATION OF MAIT CELLS BY MAGNETIC ENRICHMENT

Instead of using FACS to enrich MAIT cells from PBMCs (Support protocol 3), magnetic enrichment, also referred to as magnetic activated cell sorting (MACS), can be performed. MACS purifies cells based on staining for a single marker, using PE-labelled MR1-OP-RU tetramer (Gherardin et al., 2016) or PE-labelled TRAV1-2 specific mAb (Koay et al., 2016), followed by staining with magnetic anti-PE microbeads which are then captured using a magnetic column. Both tetramer and mAb-based MACS approaches significantly enrich MAIT cells within a population. MAIT cells constitute a substantial portion of TRAV1-2^+ T cells (Gherardin, Souter, et al., 2018), however the MAIT cell purity upon MACS is lower compared to FACS. For example, in an average donor (Gherardin, Souter, et al., 2018), the purity of TRAV1-2^+ cells after MACS is typically ~90%, of which 40-80% of cells co-express the MAIT cell surrogate markers (TRAV1-2^+ and CD161^+). Thus, we do not recommend using MACS instead of FACS, but in combination with MACS, whereby when dealing with samples with low MAIT cell frequencies or rare MAIT cell subsets, MAIT cells are first enriched by MACS followed by purification using FACS.

Materials

- PBMC samples (generated as per Support protocol 1)
- Reagents, equipment and their use for counting viable cells using trypan blue exclusion (see Appendix)
- PE-labelled MR1-5-OP-RU tetramer or PE-conjugated TRAV1-2 specific mAb (clone 3C10) (see Appendix for MR1 tetramer generation and use)
- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix)

- Maintenance of cells (see Appendix)
- Anti-PE MicroBeads (Miltenyi Biotec, cat. no. 130-048-801)
- Magnetic enrichment LS column (Miltenyi Biotec, cat. no. 130-042-401)
- Magnet and stand (Miltenyi Biotec, cat. no. 130-092-857)
- MACS buffer (see Reagents and solutions)

Protocol steps

1. Determine the approximate number of cells required for the assay. For example, to purify MAIT cells from PBMCs for a stimulation assay involving co-culture with a cell line, typically, $5x10^4$ cells are required per sample. For example, for a 20-sample experiment, $1x10^6$ cells are required. From an average donor (Gherardin, Souter, et al., 2018) typically $>1\times10^6$ TRAV1-2$^+$ cells are recovered from a TRAV1-2 enrichment of 10^8 PBMCs.
2. Take an aliquot of the PBMC single cell suspension and count viable cells using trypan blue exclusion (see Appendix).
3. Aliquot 10^8 viable PBMCs into a separate tube and pellet by centrifugation (450 xg, 5 min, 4°C).
4. Wash cells with 1 ml PBS and centrifuge cells (450 xg, 5 min, 4°C).
5. Aspirate the PBS from the pellet and resuspend PBMCs in 1 ml of cold MACS buffer supplemented with a 1:100 dilution of anti-TRAV1-2-PE mAb and incubate at 4°C for 30 min. Gently resuspend the cells after incubating for 15 min to prevent cells from pelleting.

   Note: Alternatively, to enrich for MAIT cells using MR1-5-OP-RU tetramer, perform step 5 using 1 μg/ml of PE-conjugated MR1-5-OP-RU tetramer and incubate at RT.
6. Wash PBMCs with 1 ml of cold MACS buffer and centrifuge cells (450 xg, 5 min, 4°C).
7. Aspirate supernatant and resuspend PBMCs in 1 ml of MACS buffer. Then add 100 μl of anti-PE microbeads and mix thoroughly. Incubate PBMCs at 4°C for 20 min. Gently resuspend cells after incubating for 10 min.

   Note: Ensure that anti-PE microbeads are vortexed well before taking an aliquot, as microbeads may form a sediment in storage.
8. Wash PBMCs using 1 ml of cold MACS buffer and centrifuge (450 xg, 5 min, 4°C).
9. Repeat step 8.

Note: It is important to thoroughly wash cells after staining with anti-PE microbeads to remove free microbeads that can contribute to non-specific enrichment.

10. Resuspend PBMCs in 3.5 ml of cold MACS buffer and filter cells into a fresh tube using a 40 μm cell strainer.
11. Prepare magnetic column by placing it into the magnetic stand and passing 3 ml of MACS buffer over the column.

    Note: The flow through from the column can be collected in a 50 ml tube and discarded.
12. After the MACS buffer has passed through the column, add the 3.5 ml of PBMC suspension onto the column and collect to the flow through in a 50 ml tube, placed below the column.
13. Repeat step 12, passing the PBMCs a second time over the column.

    Note: Flow through can be saved to assess the efficiency of the TRAV1-2 enrichment by flow cytometry.
14. Wash the column using 3 ml of cold MACS buffer.
15. Repeat step 14.
16. Pass 5 ml of cold MACS buffer over the column and quickly remove the column from the magnet. Using the plunger provided with the column, elute the enriched cells into a new 10 ml tube.
17. TRAV1-2 enriched cells are ready for further handling, e.g. as per Support protocol 3.

BASIC PROTOCOL 2

DETERMINING THE ACTIVATION PHENOTYPE OF HUMAN MAIT CELLS IN BLOOD

Whilst robust MAIT cell activation is observed when PBMCs are stimulated in the absence of exogenously added APCs, PBMCs can be co-incubated with APC lines (generated as per Support protocols 5-7) or autologous PBMC derived APCs, such as monocyte-derived dendritic cells (moDCs, generated as per Support protocol 8). The use of exogenously added APCs allows 'separating' Ag presentation and T cell activation, thereby avoiding MR1-Ag presentation by MAIT cells. This is, for example, required in order to formally test for MR1-Ag dependence of the observed stimulation by using a panel of APC lines expressing wild-type levels of MR1, overexpressing MR1 (Support protocols 5 and 6) (Huang et al., 2008; Lepore et al., 2017; Reantragoon et al., 2012; H. Wang et al., 2018; Young et al., 2013), or

lacking MR1 (Support protocol 7) (Laugel et al., 2016; H. Wang et al., 2018). MAIT cell activation can be measured based on expression of activation-specific surface markers (as outlined in Table 1). Alternatively, activation specific intracellular markers can be evaluated, including cytokines and markers of cytotoxicity (perforin/granzyme) (as outlined in Table 1), which can be measured by intracellular staining (Alternate protocol 4); cytokines secreted into tissue culture supernatants can also be determined using enzyme-linked immunosorbent assay (ELISA)-based techniques such as commercially available Cytometric Bead Array (CBA) from BD (Alternate protocol 5). Notably, for CBA, it is important to use a purified population of MAIT cells (generated as per Support protocols 3 and 4), to ensure secreted cytokines are derived from MAIT cells and not from other lymphocyte populations present in the cultures. Whilst cytotoxicity of MAIT cells can be determined by examining the expression of degranulation markers such as surface expressed CD107a and intracellularly expressed perforin/granzyme (as described as part of this protocol), cytotoxicity can also be measured as the killing of target APCs in a chromium release or radio-labelled DNA degradation assays (not described here), as well as the uptake of fluorescent dyes (as per Appendix for live/dead cell discrimination stains and their use). Furthermore, the ability of MAIT cells to proliferate as well as the number of divisions can be determined by fluorescent dye dilution (Alternate protocol 6).

The first paragraph of the Strategic planning section applies here as well. In addition, we provide a method for expanding primary MAIT cells *in vitro* (Support protocol 9), via CD3/CD28 stimulation, which allows analysis of samples from donors with low frequencies of MAIT cells or rare populations of MAIT cells as per Basic protocols 1 or 2.

The following outlines considerations for experimental controls. Evaluation of the activation phenotype of non-MAIT T cells, such as all non-MAIT $\alpha\beta$ T cells, TRAV1-2$^+$ non-MAIT $\alpha\beta$ T cells, MHC-I tetramer specific T cells or CD1d tetramer$^+$ NKT cells, in the same sample, act as negative controls for MAIT cell specific activation. Furthermore, Ag-independent stimulation serves as a positive control for the functional capacity of MAIT cells. This can be achieved with a combination of phorbol 12-myristate 13-acetate (PMA), which activates protein kinase C, and ionomycin, which is a calcium ionophore. PMA/Ionomycin bypasses the TCR complex leading to activation of several intracellular signalling pathways. Alternatively, CD3/CD28 stimulation can be performed, whereby plate- or bead-immobilised antibodies specific for CD3 and CD28 lead to TCR cross-linking (Alternate protocols 7 and 8). Thus, whilst CD3/CD28-mediated stimulation is dependent on surface expressed TCR, PMA/Ionomycin stimulation occurs in the absence of TCR ligation. In response to both, CD3/CD28 and PMA/Ionomycin, MAIT cells secrete TNF, IFNγ and granzyme B (Dias, Sobkowiak, Sandberg, & Leeansyah, 2016; Gherardin, Souter, et al., 2018; Kurioka et al., 2017; Slichter et al., 2016), while

minimal IL-17A production is detected (Gherardin, Souter, et al., 2018; Slichter et al., 2016). Similarly, incubation in the presence of APCs that lack MR1 (Laugel et al., 2016; H. Wang et al., 2018) (Support protocol 7), as compared to cells proficient in MR1 or overexpressing MR1 (Reantragoon et al., 2012; H. Wang et al., 2018) (Support protocols 5 and 6), or in the absence of relevant Ag but in the presence of vehicle control, media or irrelevant Ag, and/or in the presence of MR1 blocking antibody as compared to isotype control antibody (Alternate protocol 6), or in the presence of non-activating competitively inhibiting Ags (Ac-6-FP) in addition to stimulating Ag (Alternate protocol 7) serve as controls for the MR1-Ag specific reactivity in MAIT cell activation assays.

Materials

- PBMC samples in suspension (generated as per Support protocol 1 and maintained as per Appendix)
- APC lines in suspension (generated as per Support protocols 5-7 and maintained as per Appendix)
- Reagents, equipment and their use for counting viable cells using trypan blue exclusion (see Appendix)
- MAIT cell Ags (see Appendix)
- Optional: folate-free RF10 media (prepared as RF10 media per Appendix, Maintenance of cell lines, but using folate free RPMI medium, Gibco cat. no. 27016021)
- 96-well u-bottom plate (TPP, cat. no. 92697)
- Stock of PMA (Sigma, cat. no. P1585): Reconstitute stock at 0.1 mg/ml in DMSO, store at -20°C.
- Stock of Ionomycin (Sigma, cat. no. I3909): Reconstitute stock at 1 mg/ml in DMSO, store at -20°C.
- 96-well v-bottom plate (Greiner Bio-One, cat. no. 651180)
- Live/dead cell discriminator stain (see Appendix for live/dead discriminator stains and their use)
- Fluorochrome-labelled MR1-5-OP-RU tetramer and fluorochrome-conjugated mAbs specific for the cell-surface and activation phenotype markers of MAIT cells as per Tables 1 and 7; fluorochrome-conjugated isotype control mAbs, matching the isotype, fluorochrome and concentration of the relevant Abs; fluorochrome-labelled MR1-6-FP tetramer control matching the MR1-5-OP-RU tetramer concentration (see Appendix for MR1 tetramer generation and use)
- Flow cytometry wash buffer (flow wash) B: 2% fetal calf serum (FCS) (v/v) in PBS and stored at 4°C for 1 month.
- Single colour control samples for flow cytometry data acquisition (see Appendix)
- Flow cytometry cell fixation buffer (flow fix) (see Reagents and solutions)

- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix)
- Flow cytometry acquisition tubes (Falcon, cat. no. 352008)
- 40 μm cell strainers (Miltenyi Biotec, cat. no. 130-098-458)
- Tabletop centrifuge (Beckman Coulter Allegra X-12R)
- Flow cytometer (BD LSR Fortessa)

Protocol steps

1. Map out the types of samples: for example, stimulating purified MAIT cells (Support protocols 3 and 4) with MR1 deficient or MR1-overexpressing cell lines (Support protocols 5-7 as APCs.
   a) Flow cytometry single colour staining controls (Appendix).
   b) Samples per donor, e.g. the following (for different ways to stimulate MAIT cells in an Ag-MR1 dependent manner, see Appendix, MAIT cell Ags):
      - MAIT cells + Nil
      - MAIT cells + 5-OP-RU
      - MAIT cells + PMA/Ionomycin
      - MAIT cells + APC.MR1$^{-/-}$ + Nil
      - MAIT cells + APC.MR1$^{-/-}$ + 5-OP-RU
      - MAIT cells + APC.MR1 + Nil
      - MAIT cells + APC.MR1 + 5-OP-RU
2. Take an aliquot of single cell suspension from each cell population and count viable cells using trypan blue exclusion (see Appendix). Typically, 1×10^4 viable MAIT cells and $5x10^3$ APCs are needed per sample.
3. Centrifuge (450 xg, 5 min, 4°C) the required volumes of APC suspensions, aspirate the supernatants and resuspend the pellets in 100 μl of RF10 media per sample or 100 μl RF10 media supplemented with 1 nM 5-OP-RU. For example, if using a 1 μM 5-OP-RU stock, add 0.4 μl of stock to 400 μl of RF10 media.

   Note: To reduce the amount of 6-FP present in the activation assay instead of RF10 media, folate-free RF10 media can be used here and in subsequent steps. Since FCS also contains folate, dialysed FCS may be used.

4. Incubate the APCs at 37°C in a tissue culture incubator for 1 hr.
5. Wash the APCs using 1 ml of RF10 media, centrifuge cells (450 xg, 5 min, RT) and discard supernatant.
6. Repeat wash in step 5 three times.

7. Resuspend APCs in 100 µl of RF10 media per sample and distribute 100 µl per sample in wells of a 96-well plate. For samples without APCs, add 100 µl of RF10 media to the wells. For samples with PMA/Ionomycin stimulation, add a final concentration of 10 ng/ml PMA and 1 µg/ml Ionomycin and adjust the volume of RF10 media accordingly to reach a final volume of 100 µl.
8. Centrifuge (450 xg, 5 min, 4°C) the required volume of MAIT cell suspensions, aspirate the supernatants and resuspend the pellets in 100 µl of RF10 media per sample. Add 100 µl of MAIT cell suspension to wells with RF10 media (+/- PMA/Ionomycin) and APC lines, respectively.
9. Incubate samples for 7 hr in a tissue culture incubator.
   Note: It might be of interest to test several incubation periods as the expression levels of different markers peak at different timepoints. E.g. especially for PMA/Ionomycin a shorter incubation period might be preferred.
10. After the stimulation, transfer cells into a 96-well v-bottom plate or v-bottom tubes for centrifugation, antibody staining and wash steps.
11. Pellet cells (450 xg, 5 min, 4°C), discard supernatants and stain cells with fixable live/dead cell discrimination stain as per Appendix, e.g. compatible with this staining panel is live/dead Near Infrared stain, typically used in a 1/1000 dilution of the stock, prepared as per Appendix.
12. Perform staining as per Basic protocol 1, step 3 onwards, but prepare cell-surface staining mixture in flow wash B as per Table 7.

ALTERNATE PROTOCOL 4

DETERMINATION OF THE MAIT CELL ACTIVATION PHENOTYPE BASED ON STAINING FOR INTRACELLULAR MARKERS

MAIT cell activation via Ag or independently of Ag (using anti-CD3/CD28 antibodies or PMA/Ionomycin) causes the production of cytokines and cytotoxic granules, outlined in Table 1, that can be measured by intracellular cytokine staining (ICS) upon preventing protein secretion from the cell. ICS is a robust method for detecting differences in MAIT cell activation *in vitro* (Gherardin, Souter, et al., 2018). In particular, it is best suited for identifying cytokines produced by specific cells, here MAIT cells, within mixed populations of cells such as in PBMCs.

Additional Materials

- Activated MAIT cell samples generated using Basic protocol 2 through step 8
- Brefeldin A stock solution (Sigma, cat. no. B7651, prepare at 10 mg/ml in DMSO and store at -20°C)
- 1 % Saponin stock solution: reconstitute Saponin powder (Sigma, cat. no. 47036) at 1% (w/v) in H_2O, sterile filter using a 0.2 μm filter and store at 4°C.
- 2% (v/v) paraformaldehyde (PFA)/PBS, stored at -20°C and protected from light, for 1 year.
- PBS (stored at 4°C)
- Fluorochrome-conjugated mAbs specific for intracellular markers of MAIT cells as per Tables 1 and 8; fluorochrome-conjugated isotype control mAbs, matching the isotype, fluorochrome and concentration of the relevant Abs.

Note: As an alternative to Brefeldin A and saponin stocks, a Fixation/Permeabilisation kit can be used as per instructions by the manufacturer which contains the protein transport inhibitors GolgiPlug™ (BD Biosciences, cat. no. 555028) or Golgi Stop™ (BD Biosciences, cat. no. 554715) and a permeabilisation reagent.

Protocol steps

1. Perform Basic protocol 2, steps 1 to 8.
2. Incubate samples for 1 hr at 37°C in a tissue culture incubator.
3. To each 200 μl sample, add 20 μl of a solution containing 110 μg/ml Brefeldin A in RF10, warmed in a 37°C water bath, for a final concentration of 10 μg/ml Brefeldin A.
   Note: Do not resuspend cells, as this may interfere with any interactions formed between T cells and APCs.
4. Incubate cells for a further 6 hr at 37°C in a tissue culture incubator.
5. Perform Basic protocol steps 10 and 11.
6. Perform staining as per Basic protocol 1, step 3, but prepare cell-surface staining mixture in flow wash B as per Table 7.

   Note: When performing an ICS, use double the concentration of surface mAbs and MR1 tetramer as compared to the Basic protocol to ensure each fluorochrome is sufficiently bright after fixation and permeabilisation steps. Following surface stain, do not wash cells.
7. Aliquot 50 μl of 2% paraformaldehyde (PFA) to each sample directly for a final concentration of 1% PFA and resuspend. Incubate cells for 20 min at RT.
8. Add 100 μl of PBS, centrifuge cells (450 xg, 5 min, 4°C) and discard supernatant.
9. Repeat wash step using 200 μl of PBS.

10. Prepare intracellular antibody stain mixture, 50 μl per sample, in 0.3% saponin in PBS as per Table 8.
11. Resuspend cells in 50 μl per sample of intracellular antibody stain and incubate at 4°C overnight in the dark.
12. The next day, repeat the wash in step 9.
13. Centrifuge cells (450 xg, 5 min, 4°C), aspirate PBS and resuspend cells in 100 μl PBS.
    Note: Samples are already fixed so that resuspension in PBS is sufficient.
14. Continue as per Basic protocol 1, step 6.

ALTERNATE PROTOCOL 5

DETERMINATION OF THE MAIT CELL ACTIVATION PHENOTYPE BY MEASURING SECRETED CYTOKINES

Following stimulation, cytokines and other secreted molecules can be measured in the cell supernatant using commercially developed cytometric bead arrays kits (CBA), involving antibody coated capture beads in conjunction with paired fluorochrome-conjugated detection antibodies specific for the cytokine of interest. Compared to ICS (Alternate protocol 4), CBA is a non-cell based, high-throughput assay enabling the detection of many secreted molecules simultaneously. However, the detected secreted molecules cannot be assigned to a specific cell type. This can be overcome by using purified populations of MAIT cells and control cells as per Basic protocol 2.

Additional materials

- Activated MAIT cells generated using Basic protocol 2 through step 9
- Cytokine bead array kit (BD Biosciences, cat. no. 551809).

Protocol steps

1. Map out the types of samples: for example, stimulating purified MAIT cells (Support protocols 3 and 4) with MR1 deficient or MR1-overexpressing cell lines (Support protocols 5-7) as APCs.
    a. Serially diluted cytokine standards
    b. Samples per donor, e.g. the following (for different ways to stimulate MAIT cells in an Ag-MR1 dependent manner, see Appendix, MAIT cell Ags):
        - MAIT cells + Nil
        - MAIT cells + 5-OP-RU

- MAIT cells + PMA/Ionomycin
- MAIT cells + APC.MR1$^{-/-}$ + Nil
- MAIT cells + APC.MR1$^{-/-}$ + 5-OP-RU
- MAIT cells + APC.MR1 + Nil
- MAIT cells + APC.MR1 + 5-OP-RU
- Non-MAIT T cells + Nil
- Non-MAIT T cells + 5-OP-RU
- Non-MAIT T cells + PMA/Ionomycin
- Non-MAIT T cells + APC.MR1$^{-/-}$ + Nil
- Non-MAIT T cells + APC.MR1$^{-/-}$ + 5-OP-RU
- Non-MAIT T cells + APC.MR1 + Nil
- Non-MAIT T cells + APC.MR1 + 5-OP-RU
- APC.MR1$^{-/-}$ + Nil
- APC.MR1 + Nil

2. Perform a MAIT cell activation assay as per Basic protocol 2, steps 2-9.
   Note: Detection of cytokines relies on sufficient concentrations secreted into the supernatant. The sensitivity of the assay can be optimized by altering the number of cells in the culture, volume of culture, or the stimulation time to increase/decrease the concentration of secreted cytokines.
3. Prepare cytokine standards as per manufacturer's instructions. Refer to Table 1 for details regarding the relevant cytokines produced by MAIT cells.
   Note: Prepare each of the cytokine standard dilutions in duplicate or triplicate to increase the accuracy of data interpolation during analysis.
4. Transfer cells into a v-bottom 96-well plate or v-bottom tubes and centrifuge (450 xg, 5 min, 4°C) to pellet cells.
5. Carefully remove supernatant from pelleted cells and transfer to a new v-bottom plate/tubes.
   Note: It is unlikely that all of the supernatant collected will be analysed, therefore, to prevent cell contamination, leave some of the media in each well. E.g. if the cells are cultured in 200 µl per sample, collect 150 µl only. Excess supernatant can be stored frozen at -20°C, however repeated freeze/thaw cycles impact cytokine concentration measurements.
6. Perform a 1:2 dilution using 5 µl of each sample using the assay diluent provided by the manufacturer.
   Note: In some experiments, cytokine concentrations within the supernatant will exceed the sensitivity of the assay (concentrations of ~1-5 ng/ml) and will require further dilution.
7. Prepare the cytokine capture bead mastermix (the cytokines you wish to identify in the supernatant) in capture bead diluent by performing a 1:50 dilution of each capture bead into capture bead diluent. For each 5 µl sample, 5 µl of capture bead mastermix will be added. E.g. for 12 samples, prepare 70 µl of mastermix with 1.4 µl of each capture bead. Refer to the manufacturer's instructions to make sure the capture beads are compatible within the same mastermix.

Note: Ensure that the capture beads are thoroughly mixed prior to aliquoting, as the beads may sediment during storage.

8. Aliquot 5 μl of the capture bead mastermix into each of the samples, mix thoroughly and incubate for 45 min at RT in the dark.
9. Prepare the cytokine detection reagent in detection reagent diluent by performing a 1:50 dilution of each cytokine detection reagent, similarly, as described in step 7. For each 5 μl sample, add 5 μl of detection reagent mastermix.
10. Aliquot 5 μl of the detection reagent mastermix into each of the samples without washing, mix thoroughly and incubate at RT for a further 45 min in the dark.
11. Aliquot 200 μl of CBA wash buffer (provided by the manufacturer) to each well, resuspend and centrifuge (200 xg, 5 min, 4°C).
12. Aspirate wash and repeat step 11.
13. Resuspend samples in 50 μl of CBA wash buffer and transfer samples to flow cytometry acquisition tubes and analyse on a flow cytometer.
    Note: Refer to manufacturer's instructions regarding acquisition, as some flow cytometers may not be suitable for CBA assay analysis or require additional steps not described here.
14. Data can be analysed using the FCAP Array software (refer to the instructions provided by the manufacturer) or alternatively, using FlowJo (Tree Star Inc.) and a graphing software (e.g. Microsoft excel or GraphPad Prism).

ALTERNATE PROTOCOL 6

DETERMINATION OF THE MAIT CELL ACTIVATION PHENOTYPE BASED ON FLUORESCENT DYE DILUTION DUE TO PROLIFERATION

MAIT cells proliferate in response to both specific (e.g. 5-OP-RU Ag) and non-specific (e.g. CD3/CD28) stimulation during long term (> 3 days) *in vitro* activation assays. Proliferation can be measured using fluorescence dyes that bind to intracellular proteins and are progressively diluted as the cells divide. By measuring the fluorescence intensity of the dye, and counting separate fluorescent peaks, it is possible to accurately determine the number of cellular divisions that have occurred over the course of the culture, up to 5-8 divisions depending on the reagent used. Measuring proliferation of MAIT cells can be utilized to distinguish subtle differences between stimulation conditions (e.g. MAIT cell activation by low affinity Ags) that may increase over time, or in cases where early post stimulus, identification of MAIT cells is compromised due to down-regulation of TCR.

Proliferation assays might be performed in RF10 media supplemented with low doses of recombinant human IL-2 and IL-7. However, this is not required to observe robust MAIT cell proliferation in response to e.g. 5-OP-RU Ag.

Additional materials

- APCs generated using Basic protocol 2 through step 7
- PBS (stored at 4°C)
- Cell trace violet (CTV, ThermoFisher, cat. no. C34571)

  Note: Alternatively, Carboxyfluorescein succinimidyl ester (CFSE, ThermoFisher, cat. no. C34554) or a similar proliferation dye can be used.

Protocol steps

1. Map out an activation assay as per Basic protocol 2, step 1, and prepare APCs as per Basic protocol 2, steps 2-7.
2. Take an aliquot of single cell suspension from each PBMC donor and count viable cells using trypan blue exclusion (see Appendix). Typically, 1×10^6 viable cells are needed per sample to obtain a sizeable population of MAIT cells in an average donor.
3. Centrifuge (450 xg, 5 min, 4°C) the required volumes of PBMC cell suspensions, aspirate the supernatants and wash the PBMC pellets with 100 μl of warm PBS per 10^7 cells.
4. Centrifuge cells (450 xg, 5 min, RT) and repeat wash in step 3.
5. Dilute CTV stock solution (in DMSO, prepared as per manufacturer's instructions) into warm PBS at a 1:150 dilution and resuspend PBMC pellets in diluted CTV. For example, for 10^7 cells in 150 μl, add 1 μl of CTV. Incubate cells for 15 min at 37˚C in a tissue culture incubator.
6. Quench the CTV labelling of PBMCs by adding 1 ml of warm RF10 media. Centrifuge cells (450 xg, 5 min, RT), aspirate media, resuspend PBMCs in 100 μl of RF10 media per sample. Add 100 μl of labelled MAIT cell suspension to wells with RF10 media (+/- PMA/Ionomycin) and APC lines, respectively.
7. Incubate samples for 5 days in a tissue culture incubator.

   Note: It is advisable to perform a time course (e.g. 3, 5, 7 days of stimulation) to determine the optimal number of cell divisions by proliferating MAIT cells in response to new stimuli. Figure 4 displays MAIT cell proliferation after 5 days in response to 5-OP-RU stimulus.
8. Continue with Basic protocol 2, step 10.

   Note: Adjust the antibody cocktail and flow cytometry acquisition and analysis to accommodate the fluorescent dye used (CTV or CFSE).

[*Insert Figure 4]

ALTERNATE PROTOCOL 7

USE OF PLATE-BOUND CD3/CD28 STIMULATION TO ASSESS THE CAPACITY OF MAIT CELLS TO PRODUCE CYTOKINE INDEPENDENTLY OF AG

MAIT cells are activated in response to non-specific stimulation such as PMA/Ionomycin as per Basic protocol 2 which omits the need of surface receptor stimulation. Non-specific stimulation of MAIT cells can also be achieved via CD3/TCR cross-linking using plate-bound (Alternate protocol 7) or bead-immobilised (Alternate protocol 8) antibodies specific to surface-expressed CD3 and CD28.

Additional materials

- Mouse anti-human CD3 (clone UCHT1, BD Pharmingen cat. no. 555329) and mouse anti-human CD28 (clone CD28.2, BD Pharmingen cat. no. 555725) mAbs
- PBS (stored at 4°C)
- MAIT cells or non-MAIT T cells (generated from following Basic protocol 2, steps 2 and 8)

Protocol steps

1. The day before setting up a stimulation assay, coat wells of a 96-well flat-bottom plate with anti-CD3/CD28 mAb mixture in 50 µl of PBS, containing 10 µg/ml anti-CD3 and 2 µg/ml anti-CD28 mAbs and store plate overnight at 4°C.

   Note: Coated plates can also be prepared on the day however should be incubated for 4 hr at 37°C in a tissue culture incubator.

2. Immediately prior to adding cells (MAIT cells or non-MAIT T cells), remove unbound anti-CD3 and anti-CD28 mAbs from the 96-well plate by washing 3x with 200 µl of PBS.

   Note: Whilst it is essential to remove all supernatant during washing, avoid letting the well dry out.

3. Add $5x10^3$ MAIT cells/non-MAIT T cells, prepared as per Basic protocol 2, steps 2 and 8, in 200 µl RF10 media.
4. Continue incubation as per Basic protocol 2, step 9.
   Note: This protocol can also be used when performing Alternate protocol 6.

ALTERNATE PROTOCOL 8

USE OF BEAD-IMMOBILISED CD3/CD28 STIMULATION TO ASSESS THE CAPACITY OF MAIT CELLS TO PRODUCE CYTOKINE INDEPENDENTLY OF AG

Additional materials

- Dynabeads Human T-Activator CD3/CD28 (ThermoFisher, cat. no. 11131D).
- MAIT cells or non-MAIT T cells (generated from following Basic protocol 2, steps 2 and 8)

Protocol steps

1. Vortex anti-CD3/CD28 beads for >30 sec.
2. Aliquot the required volume of anti-CD3/CD28 beads (typically used at a 1:1 ratio of beads to T cells, refer to manufacturer for details) into a 1.5 ml tube and wash with 200 μl of RF10 media.

   Note: Alternatively, beads can be washed using a magnetic stand available from the manufacturer.
3. Centrifuge anti-CD3/CD28 beads (450 xg, 4°C, 5 min), aspirate media and resuspend in 20 μl of RF10 media per sample.
4. Aliquot washed anti-CD3/CD28 beads onto PBMCs, prepared as per Basic protocol 2, steps 2 and 8, in a total volume of 200 μl of RF10 media.
5. Continue incubation as per Basic protocol 2, step 9.

ALTERNATE PROTOCOL 9

USE OF MR1 BLOCKING ANTIBODY TO DETERMINE MR1-AG DEPENDENCE IN MAIT CELL ACTIVATION

APC lines knocked out for MR1 represent the ideal tool for determining MR1-Ag dependence of MAIT cell activation (Laugel et al., 2016; H. Wang et al., 2018). Alternatively, MR1 blocking Abs, such as the 26.5 (Huang et al., 2005) or 8F2.F9 (Chua et al., 2011), as compared to isotype control Abs can be used in combination with APCs expressing wild-type levels of MR1 or overexpressing MR1, as

previously shown by our teams (Corbett et al., 2014; Eckle et al., 2014; Gherardin et al., 2016; Keller et al., 2017; Kjer-Nielsen et al., 2012; Patel et al., 2013; Reantragoon et al., 2013; Reantragoon et al., 2012) and others (Chua et al., 2011; Gold et al., 2013; Huang et al., 2009; Kurioka et al., 2017; Lepore et al., 2017; Salio et al., 2017; Young et al., 2013).

Additional materials

- Anti-MR1 mAb 26.5 (isotype: mouse IgG2a, Biolegend, cat. no. 361103) and a suitable isotype control antibody (e.g. clone MOPC-173 Biolegend, cat. no. 400223).

Protocol steps

1. Perform Basic protocol 2, steps 1-6.
2. Incubate APCs with 26.5 blocking mAb or relevant isotype control mAb at 20 μg/ml final concentration in 100 μl of RF10 media for 1 hr in a tissue culture incubator.

   Note: Titrating amounts of MR1 blocking antibody may be explored for dose-dependent blocking.
3. Continue as per Basic protocol, step 8.

   Note: At the end of the stimulation, cells must be washed at least 3x with 200 μl of flow wash B, prior to staining with MR1 tetramers to prevent free anti-MR1 mAb from binding to MR1 tetramers which reduces/prevents MR1 tetramer staining.

ALTERNATE PROTOCOL 10

USE OF AC-6-FP COMPETITIVE INHIBITION TO DETERMINE MR1-AG DEPENDENCE IN MAIT CELL ACTIVATION

Acetyl-6-formylpterin (Ac-6-FP) acts as a strong competitive inhibitor of stimulating MAIT cell Ags (Eckle et al., 2014; Keller et al., 2017). Thus, a reduced MAIT cell response to stimulating Ag in the presence of Ac-6-FP represents an alternate method to determine the Ag-specificity of a MAIT TCR positive reporter cell line.

Additional materials

- Samples collected from Basic protocol 2, step 2

- Ac-6-FP and vehicle control (see Appendix, MAIT cell Ags)

Protocol steps

1. Perform Basic protocol 2, steps 1-2.
2. Immediately prior to Ag sensitisation of APCs as per Basic protocol 2 step 3, 100 µM of Ac-6-FP (in respect to the total incubation volume of 200 µl), or vehicle control, are added in 10 µl per well of 100 µl APC cells in suspension.

   Note: 100 µM of Ac-6-FP typically causes complete inhibition of MAIT cell activation by 5-OP-RU (Eckle et al., 2014; Keller et al., 2017). 6-FP can also be used as a competitive inhibitor (Kjer-Nielsen et al., 2012), but is a weaker competitive inhibitor as compared to Ac-6-FP (Eckle et al., 2014; Keller et al., 2017). Titrating amounts of competitive inhibitors yield dose-dependent competitive inhibition which in turn allows for the determination of the half-maximum inhibition by inhibitory compounds (IC_{50} values). To determine IC_{50} values, background activation levels are subtracted, data normalized, inhibitor concentrations transformed to log and nonlinear regression of log (inhibitor concentration) versus the normalized response.
3. Continue incubation as per Basic protocol 2, step 4. In step 8, reduce the volume in which 1×10^4 viable MAIT cells are added per well from 100 to 90 µl for samples containing Ac-6-FP.

SUPPORT PROTOCOL 5

CLONING OF MAIT TCR α- AND β-CHAIN AND MR1 GENES FOR TRANSDUCTION OF CELL LINES

We describe here cloning of MR1 (as relevant to Basic protocols 2 and 3) as well as of TCR α- and β-chain genes of interest (as relevant to Basic protocol 3 and as determined in Alternate protocol 3) into a murine stem cell virus (MSCV) vector, MSCV-IRES-GFP II (pMIG II), which is part of a retroviral transduction system, established by the laboratory of Dr. Dario Vignali (Holst et al., 2006; Szymczak et al., 2004). The full-length TCR α- and β-chains are cloned in a bicistronic fashion into the pMIG II vector, whilst a single TCR chain or MR1 are cloned in a monocistronic fashion. pMIG II vector gene expression is driven by the MSCV-long terminal repeat (LTR), allowing for high level constitutive expression of the target gene. In a bicistronic pMIG.TCR vector, the N-terminal TCR α-chain cistron lacking a STOP codon and the C-terminal TCR β-chain cistron with a STOP codon are interrupted by the 2A peptide (P2A) of porcine teschovirus-1 causing co-translational cleavage between the N- and C-terminal cistrons through a ribosomal skip mechanism for stoichiometric co-expression of the two cistrons. The P2A peptide remains attached to the N-terminal cistron; no effect on protein

function/antigenicity has been observed (Holst et al., 2006). The GlySerGly spacer positioned N-terminal to the P2A peptide ensures complete co-translational cleavage. The pMIG II plasmid contains coding sequences for the green fluorescent protein (GFP) gene, directly co-translated via an internal ribosomal entry site (IRES), used as reporter for transfection/transduction efficiency. A schematic overview of cloning of a monocistronic or a bicistronic fragment into pMIG II is displayed in Figure 5.

[*Insert Figure 5 near here]

Materials

- pMSCV-IRES-GFP II (pMIG II) plasmid (Addgene, cat. no. 52107) (Holst et al., 2006)
- Gene synthesis service for purchase of TCR gene of interest or MR1 gene
- Equipment and reagents for molecular cloning (not described here)
- An endonuclease free plasmid preparation kit for large scale plasmid preparation, such as the EndoFree Plasmid Maxi Kit (Qiagen, cat. no. 12362)
- Access to the following free online database: IMGT®, the international ImMunoGeneTics information system® (see Internet sources)

Protocol steps

1. Design full length TCR DNA sequences for the TCR α- and β-chains with the TCR usage as determined based on Alternate protocol 3 using the IMGT database (-> IMGT Repertoire (IG and TR) -> 1. Locus and Genes -> 7. Gene tables). Similarly, the sequence of the MR1 gene is available on gene databases.

2. Perform the gene design as follows: The TCR α- and β-chain sequences are connected to form a bicistronic fragment, interrupted by the GSG-spacer and P2A peptide, and unique restriction sites for cloning into pMIG II are added: EcoRI at the N-terminus and XhoI at the C-terminus. The Stop codon from the TCR α-chain is removed but present for the TCR β-chain. Similarly, for cloning of MR1 or a single TCR chain, unique restriction sites for cloning into pMIG II are added: EcoRI at the N-terminus and XhoI at the C-terminus.

3. Obtain the pMIG II DNA insert commercially in the form of plasmid DNA or a gene string.
4. Clone the bicistronic or monocistronic DNA fragment into the pMIG II vector using standard molecular cloning techniques (not described here).
5. Prepare a plasmid stock, using an endonuclease free plasmid kit for large scale plasmid preparation as per the manufacturer instructions.

SUPPORT PROTOCOL 6

GENERATION OF A CELL LINE STABLY TRANSFECTED WITH GENE OF INTEREST

To generate APC lines stably transduced with MR1, or TCR reporter cell lines stably transfected with a TCR of interest, whereby DNA is integrated into the genomic DNA, we describe here the use of the murine stem cell virus (MSCV) retroviral transduction system, established by the laboratory of Dr Dario Vignali (Holst et al., 2006; Szymczak et al., 2004). Notably, retroviruses produced using this protocol are replication incompetent but are able to enter human cells and integrate into genomic DNA. Various APC lines are available. Similarly, various TCR deficient human T cell lines are available for TCR transduction. Table 9 and 10 list the APC and T cell lines we and others have routinely transduced with MR1 and TCR, including relevant details for each of these cell lines. Whilst T cell lines described in Table 10 are proficient in CD3, the functional capacity of the lines can be boosted by sequentially transducing or co-transducing T cell lines with genes encoding full-length CD3 (CD3 γ-, δ-, ζ- and ε-chains), cloned into pMIG, as originally demonstrated for murine CD3 (Szymczak et al., 2004) and as previously performed with human CD3 (Gherardin et al., 2016), and TCR. For example, first a T cell clone overexpressing CD3 can be generated which is then transduced with the TCR of interest. T cell lines deficient in β_2m or MR1 can also be generated using CRISPR/Cas9. Such lines are useful in cell line activation assays (Alternate protocol 11) by preventing T cell lines from presenting Ags via MR1 to each other during stimulation.

Note: When aiming to generate a set of T cell lines that will be compared alongside each other in downstream assays, we typically generate the lines at the same time, this way achieving similar transduction levels.

Materials

- Target APC lines (e.g. as listed in Table 9) or target T cell lines (e.g. as listed in Table 10) of choice.
- 293T cells, as described in Table 9. 293T cells are used as a packaging cell line for the generation of retroviral vectors. A cell suspension can be generated by trypsinisation (as per Appendix, Maintenance of cells).
- 10 cm tissue culture dishes (Falcon, cat. no. 353003)
- Reagents and equipment required for maintenance of cell lines (as per Appendix)
- Reagents, equipment and their use for counting viable cells using trypan blue exclusion (see Appendix)
- Endonuclease-free plasmid large scale preparation of TCR of choice cloned into pMIG II (pMIG.TCR), generated as per Support protocol 5.
- Endonuclease-free plasmid large scale preparation of the following helper plasmids which encode for ampicillin resistance and are available on request from Dr. Dario

Vignali; materials transfer agreement from St. Jude Children's Research Hospital required:
    - packaging plasmid pEQ.PAM(-E) (Szymczak et al., 2004)
    - pVSV-G, which encodes the vesicular stomatitis virus G envelope protein under control of the CMV promoter (Burns, Friedmann, Driever, Burrascano, & Yee, 1993)
- Fugene6 transfection reagent, stored at 4˚C (Roche, cat. no. 11814443001 or Promega cat. no. E2691)
- Hexadimethrine bromide (polybrene) (6 mg/ml in H_2O, stored at -20˚C, Sigma, cat. no. H9268)
- Opti-MEM media (Invitrogen cat. no. 31985070)
- PBS (stored at 4°C)
- Minisart syringe filter with a pore size of 0.45 µm (Sartorius, cat. no. 16555)
- Single colour control samples for flow cytometry data acquisition (see Appendix).
- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix).

Protocol steps

1. Map out the types of transduction samples. For example, to generate a TCR positive reporter cell line, use:
   SKW-3 cells + MAIT TCR
   SKW-3 cells + control MAIT TCR
   SKW-3 cells + control non-MAIT TCR

2. Day 1: Per transduction sample, plate out $1x10^6$ 293T cells in 10 ml DMEM10 in a 10 cm tissue culture dish. Incubate in tissue culture incubator overnight.

3. Day 2: Prepare transfection mixtures:

   a) In a 1.5 ml tube per sample, add 470 µl of Opti-MEM media (equilibrated to RT). Without touching the walls of the tube, add 30 µl Fugene6 reagent directly to the medium. Gently tap tube to mix. Incubate at RT for 5 min.

   b) In a separate tube combine DNA (no more than 10-12 µg in total): 4 µg pMIG.TCR, 4 µg pEQ.PAM(-E), 2 µg pVSV-G.

   Dropwise add Fugene6/Opti-MEM media mixture to DNA, gently tap to mix and incubate for 15 min at RT.

4. Replace DMEM10 media on 293T cells to remove any cells that might not have adhered and dropwise add Fugene6/Opti-MEM media/DNA mixture to the 293T cells, whilst gently swirling the dish. Incubate cells in a tissue culture incubator overnight.
5. Day 3: In the morning replace virus containing media on 293T cells with fresh DMEM10 media.
6. In the evening pellet an aliquot of 10^5 cells of the target cell line in a 10 ml tube (335 xg, 5 min, RT) and discard supernatant. Pull up virus containing media from 293T cells with a 10 ml syringe and filter through a 0.45 µm filter onto the target cell pellet. Add 10 µl of polybrene, resuspend the pellet gently with a transfer pipette and transfer the cell suspension to a 10 cm tissue culture dish. Carefully replace DMEM10 media on 293T cells without disrupting the cell lawn. Incubate both, virus producing 293T cells and target cells, in the tissue culture incubator overnight.

   Note: Target cell lines are typically cultured in RF10 media. In our experience, target cell lines can be readily grown in DMEM10 media. However, it might be preferred to first adapt the target cell line over several days to DMEM10 media prior to transduction.
7. Days 4-6: In the morning and evening repeat transfer of 293T cell supernatant onto the same pelleted target cells, followed by addition of 10 µl of polybrene and replenishing of DMEM10 media on 293T cells as outlined in step 6.
   Note: If supernatant on 293T cells appears acidic, as judged by intense yellow colour, either stop the procedure early and move to step 7 or transfer a fraction of 293T cell virus containing supernatant and make up to 10 ml with fresh DMEM10 media.
8. Day 7: Pellet target cells (335 xg, 5 min, RT), discard supernatant and replenish with fresh RF10 media and incubate for a few days to recover potential loss in numbers upon transduction.
9. To assess the transduction efficiency of the 293T cells, the level of GFP fluorescence in transduced versus untransduced cells can be determined by flow cytometry in an aliquot of cells. For this purpose, 293T cells are dislodged by trypsinisation (Appendix, Maintenance of cells) and resuspended in 10 ml of flow wash B. A 500 µl aliquot is washed twice with 1 ml of flow wash B (450 xg, 5 min, 4°C) and fixed with 200 µl flow fix.
10. To assess the transduction efficiency of the newly generated target cell line, an aliquot of the cell line can be assessed in flow cytometry, e.g. determining GFP and CD3 expression of a TCR positive reporter cell line as per Basic protocol 3.
    Note: Whilst transduction can be highly efficient, with most cells appearing positive for GFP and surface expression of the gene of interest, it might be preferred to isolate cells characterised by high or intermediate levels of gene expression. If this is the case, proceed to next step.
11. Use FACS as per Support protocol 3, to isolate single cells (yielding in the generation of a clone) or bulk cells (yielding in the generation of a cell line). Briefly, stain 1-2x10^6 cells

with mAbs specific for surface markers in 200 μl, wash twice with 2 ml flow wash B (450 xg, 5 min, 4°C), resuspend in 400 μl of 10% FCS/PBS (sterile filtered) and subject to a 40 μm cell strainer prior to sorting.

Note: When generating a set of cell lines it is recommended to isolate cells based on similar level of GFP/surface marker expression, allowing side-by-side comparisons in experiments.

12. Wash isolated cells once in 1 ml of RF10 media and culture until cells have expanded sufficiently to generate several samples for liquid nitrogen storage and to perform experiments.

Note: In the case of transduced cell lines as compared to clones, it is recommended to freeze several aliquots early post isolation, since during culture, untransduced cells or cells expressing low levels of the marker gene of interest might grow faster and outcompete cells expressing high levels of the gene of interest. In this case, prior to each experiment a fresh set of frozen cell samples is thawed.

SUPPORT PROTOCOL 7

GENERATION OF A STABLE MR1 KNOCKOUT ANTIGEN-PRESENTING CELL LINE

The addition of Ags generally leads to an upregulation of MR1 on all nucleated cells, including MAIT cells (Corbett et al., 2014; Eckle et al., 2014; Kjer-Nielsen et al., 2012; McWilliam et al., 2016). When performing a MAIT cell activation assay with an APC line (Basic protocol 2, Alternate protocol 11), it is beneficial to include an MR1 knockout APC line as a negative control. MR1-deficient APC lines can also be transduced with defined amounts of wild type MR1 or with mutant versions of MR1. MR1 knockout cell lines are generated using the endonuclease Cas9, which when associated with a gene specific short guide RNA (sg-RNA) silences the gene of interest (CRISPR/Cas9). This is generally done by transduction of cells with a lentiviral plasmid, established in the Zhang lab (Sanjana, Shalem, & Zhang, 2014). This plasmid contains two expression cassettes, one for the codon optimized Cas9 and the other for the chimeric sg-RNA.

Materials

- Target APC lines (e.g. as listed in Table 9) of choice.
- 293T cells, as described in Table 9. 293T cells are used as a packaging cell line for the generation of retroviral vectors. A cell suspension can be generated by trypsinisation (as per Appendix, Maintenance of cells).
- Reagents and equipment required for maintenance of cell lines (as per Appendix).

- Access to a free online service to design sg-RNAs, such as Benchling (see internet sources)
- Custom RNA/DNA synthesis service for purchase of sgRNA and DNA oligonucleotides
- T4 polynucleotide kinase (NEB, cat. no. M0201S)
- Nuclease free water
- Restriction enzyme BsmBI (NEB, cat. no. R0580)
- Equipment and reagents for molecular cloning (not described here)
- Lenticrispr v2 plasmid either encoding for the puromycin resistance gene (Addgene, cat. no. 52961) or encoding for GFP (Addgene, cat. no. 82416) (Walter et al., 2017)
- Endonuclease free plasmid large scale preparation of the following lentivirus packaging plasmids (Dull et al., 1998), which encode for the ampicillin resistance gene
    - pRSV-rev (Addgene, cat. no.12253), encodes for HIV1gp6
    - pMDLg/pRRE (Addgene, cat. no. 12251), encodes for HIV1 GAG/POL
    - pMD2.G (Addgene, cat. no. 12259), encodes for the vesicular stomatitis virus G envelope protein
- Puromycin dihydrochloride (Gibco, cat. no. A1113803)
- Fugene6 transfection reagent, stored at 4˚C (Roche, cat. no. 11814443001 or Promega cat. no. E2691)
- PE-labelled anti-MR1 mAb 26.5 (isotype: mouse IgG2a, Biolegend, cat. no. 361105) and suitable isotype control antibody (e.g. clone MOPC-173 Biolegend cat. no. 400213).
- A plasmid preparation kit for small scale plasmid preparation, such as the PureYield Plasmid Miniprep System (Promega, cat. no. A1223)
- Reagents, equipment and their use for counting viable cells using trypan blue exclusion (see Appendix)
- PBS (stored at 4°C)
- Live/dead cell discrimination stain (see Appendix for live/dead cell discrimination stains and their use)
- Complete FcR block (Miltenyi Biotec, cat. no. 130-059-901)
- Flow cytometry wash buffer (flow wash) B: 2%FCS (v/v) in PBS and stored at 4°C for 1 month.
- Hexadimethrine bromide (polybrene) (6 mg/ml in H_2O, stored at -20˚C, Sigma, cat. no. H9268)
- Opti-MEM media (Invitrogen cat. no. 31985070)
- Minisart syringe filter with a pore size of 0.45 µm (Sartorius, cat. no. 16555)
- Single colour control samples for flow cytometry data acquisition (see Appendix).
- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix).
- Materials for genomic DNA sequencing of the knockout cell line (not described here)
- Thermocycler

Protocol steps

1. Perform the *in silico* design of a 20-mer sg-RNA specific for MR1 using a free online service such as Benchling (see internet sources) and purchase from a custom synthesis service.

   Note: For the MR1 gene, we suggest that the sg-RNA targets the first three exons, owing to the existence of MR1 isoforms which may be expressed if the guide is specific for DNA after exon 3.

2. Modify the lenticrispr v2 plasmid as follows:
   Purchase the following 2 DNA oligonucleotides, which allow for cloning of the 20mer sg-RNA and its complementary sequence via BsmBI restriction enzyme sites, included as overhangs as part of the oligonucleotides.

   5′-CACCG[NNNNNNNNNNNNNNNNNNNN]-3′

   3′-C[NNNNNNNNNNNNNNNNNNNN]CAAA-5′

   Anneal and phosphorylate the oligonucleotides by combining 1 µl of each oligonucleotide, 1 µl 10 x T4 polynucleotide kinase reaction buffer, 0.5 µl T4 polynucleotide kinase and 7.5 µl nuclease free water and perform the following thermocycler protocol:
   1. 37°C for 30 min
   2. 95°C for 5 min
   3. 25°C for 45 min
3. Clone the sg-RNA into the modified lenticrispr v2 plasmid using the restriction enzyme BsmBI and standard molecular cloning techniques (not described here).
4. Prepare plasmid stocks using a small-scale plasmid preparation kit according to manufacturer instructions.
5. If you are using the lenticrispr v2 plasmid containing the puromycin resistance gene, determine the optimal dose of puromycin required to kill the target cell line. For this purpose, titrate the puromycin concentration at doubling dilutions starting at 1 mg/ml in a small-scale cell culture of the target cell line (e.g. a 24-well tissue culture plate); about 15 dilutions are needed. Check for cell death 3 and 7 days later to determine the lowest dose which leads to complete loss of cells.

   Note: Generally, a puromycin dose between 1 µg/ml and 0.1 µg/ml is sufficient, however this is cell dependent. For adherent cells, loss of adherence is a good indicator of cell death; for other cells, a trypan blue stain is the simplest method.

6. Perform transduction with lenticrisprV2-MR1 as per Support protocol 6, steps 2-8, but in step 3, the transfection mixture b) is composed of 5µg lenticrisprV2-MR1, 2.5µg pMDLg, 1.5 µg pMD2g and 1.25µg pRSV-Rev.

   Note: We find that with the increased viral titre of the lenticrisprV2, one round of transduction is adequate for producing the necessary results. Additional rounds may be done if titres are low.

7. Continue to culture cells in RF10 media if transduction was performed with the lenticrispr v2-GFP plasmid. If transduction was performed with the lenticrispr v2-puromycin plasmid, once

cells have reached 60% confluency, wash cells in 10 ml of RF10 media (450 xg, 5 min, RT) and resuspend cells in RF10 media supplemented with the predetermined concentration of puromycin.

8. Optional but recommended: In preparation for sorting cells knocked-out for MR1 based on a lack of MR1 expression, incubate ~5 x10^6 transduced cells in 500 µl RF10 media, supplemented with 10 µM of Ac-6-FP for 4 hr in a tissue culture incubator.

   Note: Ac-6-FP allows for the most robust MR1 surface expression. 5-OP-RU and 6-FP can also be used, however MR1 upregulation is comparably lower and less long sustained (Eckle et al., 2014).

9. Wash 5 x 10^6 cells in 5 ml of PBS and pellet (450 g, 4°C, 5 min), discard supernatants and stain cells with fixable live/dead cell discrimination stains, as per Appendix.

10. Stain 5x10^6 cells in 45 µl of flow wash B plus 5 µl of complete FcR block for 10 min at RT. Then add 50 µl of PE-labelled anti-MR1 26.5 mAb or isotype control mAb at a final concentration of 4 µg/ml and incubate for a further 20 min at RT.
    Note: Due to the high background staining by 26.5 mAb, mediated by Fc receptors (FcR), it is especially important to block with FcR block prior to staining. We highly recommend the one offered by Miltenyi Biotec; other commercial FcR blockers do not appear to be as effective. If a commercial FcR block is not available, blocking with 40% normal mouse serum can also improve staining. It is also vital that a second sample is stained with the isotype control mAb, which will be used to set the correct gates during sorting.
11. Wash cells twice by adding 1 ml of flow wash B, resuspending and pelleting cells (450 xg, 5 min, 4°C) and discarding the supernatants.
12. Filter samples through 40 µm cell strainer into 5 ml polypropylene tubes.
13. Bulk-sort cells on a FACS machine for cells that are negative for MR1 expression and, in the case of transduction with the lenticrisprV2-GFP plasmid, those which are also GFP positive.
    Note: In the first round of sorting it is unlikely that all cells are MR1 knock outs.
14. Collect the MR1 negative, or MR1 negative GFP positive cells in a tube, and wash these cells using 1 ml of RF10 media. Centrifuge the samples (450 xg, 5 min, 4°C), aspirate media and resuspend in RF10 media for expansion as per step 7.
15. Repeat steps 8 to 14, this time sort a bulk sample as well as single clones onto a u-bottom plate containing 200 µl of RF10 media per well.

    Note: Single cell clones of the knockout cell lines are ultimately needed for MAIT cell activation assays (Basic protocols 2 and 3). The bulk sorted cells can be expanded and stored long-term as a back-up to generate more clones.

16. Expand the single sorted cells and store aliquots long-term in liquid nitrogen (see Appendix, Maintenance of Cell Culture).

    Note: Single sorted cells expand slowly. Once a pellet is visible in a well, transfer step-wise to larger wells for expansion.

17. Verify MR1 knockout cell lines by measuring MR1 surface expression (as per steps 8-10) and assessing MR1 presentation in stimulation assays (Basic protocol 3). Also perform genomic sequencing at the sg-RNA site to ensure that a cut was made and that additional nucleotides have been inserted (not described here).

SUPPORT PROTOCOL 8

GENERATION OF MONOCYTE-DERIVED DENDRITIC CELLS

To avoid alloreactivity, it is recommended to generate autologous monocyte-derived dendritic cells (moDCs), that is moDCs from the same donor whose PBMCs are used in activation assays. We find that the yield of moDCs generated from PBMCs varies between PBMC donors, typically about 2 x 10^6 moDCs are generated from 2 x 10^7 PBMCs. The purity of moDCs generated this way has been estimated to be > 70 % (Figueroa et al., 2016).

Materials

- PBMCs prepared as per Support protocol 1, freshly prepared samples or samples stored in liquid nitrogen.
- Materials and reagents required for maintenance of cells (as per Appendix)
- Reagents, equipment and their use for counting viable cells using trypan blue exclusion (see Appendix)
- Human GM-CSF (e.g. Miltenyi Biotec, cat. no. 130-095-372), prepare a stock solution of 1.5 x 10^5 U/ml as per instructions by the manufacturer.
- Human IL-4 (e.g. Miltenyi Biotec, cat. no. 130-094-117), prepare a stock solution of 5 x 10^4 U/ml as per instructions by the manufacturer.
- 6-well cell culture plates (Greiner, cell star cat. no. 657160)
- moDC wash (see Reagents and solutions)
- Live/dead cell discrimination stain (see Appendix for live/dead cell discrimination stains and their use)
- Fluorochrome-conjugated mAbs specific for CD80 (e.g. clone 2D10), CD86 (e.g. clone IT2.2), CD54 (e.g. clone HA58), HLA-DR (e.g. clone LN3), MHC-I (e.g. clone w6/32); fluorochrome-conjugated isotype control mAbs, matching the isotype, fluorochrome and concentration of each mAb.

- Flow cytometry wash buffer (flow wash) B: 2% FCS (v/v) in PBS and stored at 4°C for 1 month.
- Flow cytometry cell fixation buffer (flow fix) (see Reagents and solutions)
- Single colour control samples for flow cytometry data acquisition (see Appendix)
- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix)

Protocol steps

1. Resuspend $8x10^6$ PBMCs in 36 ml of RF10 media and distribute 6 ml per well in a 6-well plate. Incubate for 2 hr in a tissue culture incubator.

   Note: This allows for monocytes to adhere to the plate, whilst other PBMCs are in the supernatant. Alternatively, monocytes can be purified from PBMCs based on CD14 expression, using MACS enrichment (as per Support protocol 3) with human CD14 MicroBeads (Miltenyi Biotec, cat. no. 130-050-201).

2. Gently swirl plate and use a transfer pipette to remove supernatant from each well, which can be collected in a tube.

3. Wash each well twice with RF10 media and collect supernatant as in step 2.

   Note: Cells contained in combined supernatant (PBMCs minus monocytes) can be prepared for liquid nitrogen storage (as per Appendix, Maintenance of cells) and used in activation or other PBMC assays as per Basic protocols 1 and 2.

4. To each well with adherent cells add 5 ml of RF10 media, supplemented with 300 U/ml hGM-CSF and 100 U/ml hIL-4 cytokines, i.e. 10 µl of each cytokine stock per well.

   Note: This causes the cells to differentiate into moDCs.

5. Incubate for 5 days in tissue culture incubator.

6. Observe under microscope moDCs, which show dendrites. Some moDCs might have detached, some might be adherent. For appearance of moDCs by light microscopy and flow cytometric characterisation, refer to the literature, e.g. (Sallusto & Lanzavecchia, 1994).

7. Using a transfer pipette, from each well collect supernatant containing detached moDCs and place into a tube, leaving behind about 1 ml.

8. Use a cell scraper to scrape off adherent cells from each well and transfer into the same tube as the tube containing detached moDCs.

9. Add 1 ml moDC wash (warmed in a 37°C water bath) per well, scrape off adherent cells, collect into the same tube as before, repeat one more time (or until no cells remain on the plate, as observed under the microscope).
10. Pellet moDCs (930 xg, 5 min, RT).
11. Resuspend moDCs in RF10 media and count viable cells using trypan blue exclusion (see Appendix). moDCs can be used in an activation assay (e.g. 100,000 moDCs and 500,000 PBMCs (minus monocytes), see Basic protocol 2) or prepared for liquid nitrogen storage (as per Appendix, Maintenance of cells). moDCs can be characterised phenotypically based on downregulation of CD14 as compared to expression on monocytes, expression of CD80 and CD86, CD54, HLA-DR and MHC-I by flow cytometry as per Basic protocol 3, using fluorochrome-conjugated antibodies specific for the listed markers.

SUPPORT PROTOCOL 9

EXPANSION OF PRIMARY MAIT CELLS *IN VITRO*

When analysing samples from donors with low MAIT cell frequencies or rare MAIT cell subsets, small numbers of MAIT cells can be expanded after isolation using FACS (Support protocol 3) and/or MACS (Support protocol 4). Purified MAIT cells are stimulated using anti-CD3 and anti-CD28 mAbs that are bound to the surface of the culture-well to enhance cross-linking. In addition, phytohaemagglutinin (PHA) is added to the culture to further stimulate cell proliferation. After 2 days, activated MAIT cells are removed from anti-CD3/anti-CD28 stimulation and allowed to proliferate for 12 days. Notably, prolonged *in vitro* expansion may alter T cell-surface marker expression and lead to preferential expansion of certain subsets, as shown previously for non-MAIT T cells, e.g. (Hasan et al., 2000) . Thus, the surface phenotype of expanded T cells may not be truly reflective of the phenotype seen *in vivo*.

Materials

- Anti-CD3 (clone OKT3) and anti-CD28 (clone L293) mAbs
- Recombinant human IL-2 (rh-IL-2) (Peprotech, cat. no. 200-02), IL-7 (rh-IL-7) (Peprotech, cat. no. 200-07) and IL-15 (rh-IL-15) (Peprotech, cat. no. 200-15)
- AIM-V media (Gibco, cat. no. 12055091)
- PHA (Sigma, cat. no. 526511)
- PBS

- Purified MAIT cells in suspension (generated as per Support protocols 3 and 4 and maintained as per Appendix)
- Optional: Irradiated (not described here) PBMCs (labelled with fluorescent proliferation dye, as per Alternate protocol 6, steps 2-6)
- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix)

Protocol steps

1. The day before expansion, coat a flat-bottom 96-well plate with 10 μg/ml anti-CD3 and 5 μg/ml anti-CD28 in 50 μl of PBS per sample and incubate plate overnight at 4°C.

   Note: The culture plate can also be prepared on the day, however should be incubated for 4 hr at 37°C in a tissue culture incubator.

2. On the day of expansion, prepare the T cell culture media (220 μl per sample), by mixing a 1:1 ratio of RF10 media with AIM-V media and supplementing with 200 U/ml rh-IL-2, 50 ng/ml rh-IL-7, 25 ng/ml rh-IL-15 and 3 μg/ml of PHA and warming to 37°C.

   Note: We advise preparing a larger stock of T cell culture media without PHA that will be used throughout the expansion process (can be stored for 1 month at 4°C) and supplementing an aliquot of media with PHA for the initial stimulation with anti-CD3/anti-CD28 mAbs.

3. Immediately prior to transfer of cells, remove unbound anti-CD3 and anti-CD28 mAbs from the 96-well plate by washing 3x with 200 μl of PBS.

4. Purify MAIT cells as per Support protocols 3 and/or 4 and centrifuge (450 xg, 5 min, 4°C) to pellet cells.

   Note: If the number of purified MAIT cells is low (<10^4 cells), mix the cells with irradiated PBMCs to improve cell pelleting and 'feed' the purified MAIT cells during expansion.

5. Resuspend cells in 220 μl of T cell media containing PHA and aliquot into the 96-well plate coated with anti-CD3 and anti-CD28 mAbs. Fill the wells immediately surrounding wells containing cells with 200 μl of sterile PBS to minimize evaporation of T cell media from the cells during culture. Incubate for 2 days at 37°C in a tissue culture incubator.

6. After 2 days, gently aspirate most of the media from each well and resuspend cells in the residual media, typically around 40 μl.

7. Transfer the activated cells to a u-bottom 96-well plate, add 200 µl of T cell media (no added PHA) to each of the wells and fill the wells immediately surrounding wells containing cells with 200 µl of sterile PBS, as described in step 5. Incubate cells for 12 days at 37°C in a tissue culture incubator.

   Note: Periodically examine the cells under magnification using a light microscope to assess whether the cells are expanding. Small clusters of cells should become visible as the cells expand.

8. As the cells proliferate, replace the media as described in step 7 without transferring cells to a new plate. Once the number of expanding cells exceeds the size of the well (>2x10^5 cells per well), resuspend the cells and divide the total volume between multiple new wells, topping up the wells with fresh T cell media.

   Note: It may be necessary to transfer expanding cells into a plate with a larger well size (e.g. 48- or 28-well plate), however, ensure that the cells are not seeded at a density that prevents cell-cell contact.

9. After 14 days, examine the expanded cells by flow cytometry to assess the efficiency of expansion, viability and cell phenotype as per Basic protocol 1.

   Note: Remaining irradiated PBMC 'feeder' cells can be gated out during flow cytometry using the fluorescent proliferation dye.

10. Expanded MAIT cells can be used immediately for functional assays or frozen and stored in liquid nitrogen (see Appendix).

BASIC PROTOCOL 3

CHARACTERISATION OF MAIT CELL TCRS BY GENERATING TCR POSITIVE REPORTER CELL LINES

To characterise the function of a MAIT TCR in an isolated manner, MAIT TCR α- and β-chain genes of a primary MAIT cell clone (e.g. from human PBMCs) are sequenced (Alternate protocol 3), cloned into a vector for transfection (Support protocol 5), and a MAIT TCR positive reporter cell line is generated (Support protocols 6 and 10), which can then be characterised by flow cytometry (Eckle et al., 2014; Gherardin et al., 2016; Keller et al., 2017; Reantragoon et al., 2013; Reantragoon et al., 2012). Evaluation of the TCR surface expression allows the determination of TCR α- and β-chains forming a cell-surface expressed heterodimer. Generally, during T cell development TCR α- and β-chains are subject to allelic exclusion so that a single αβ TCR is expressed by a given T cell clone, which is also referred to as clonal distribution. However, up to 30% of mature human T cells express two different TCR α-chains (Padovan et al., 1993) and a small number (~1%) of mature human T cells

express two different TCR β-chains (Padovan et al., 1995). Thus, by assessing the surface expression of the possible combinations of TCR α- and β-chain genes sequenced from a single MAIT cell clone, the relevant αβ TCR combination can be determined. Furthermore, when stained with MR1 tetramer or evaluated in a MAIT TCR positive reporter cell line activation assay, it can be tested if the TCR is reactive to MR1 tetramer and can mediate an MR1-Ag response, respectively (Corbett et al., 2014; Eckle et al., 2014; Gherardin et al., 2016; Keller et al., 2017; Kjer-Nielsen et al., 2012; Patel et al., 2013; Reantragoon et al., 2013; Reantragoon et al., 2012). The generation of a TCR-deficient T cell line (e.g. Jurkat or SKW-3), stably transduced with the TCR of interest (Support protocol 6) (Corbett et al., 2014; Eckle et al., 2014; Gherardin et al., 2016; Keller et al., 2017; Kjer-Nielsen et al., 2012; Patel et al., 2013; Reantragoon et al., 2013; Reantragoon et al., 2012), allows for determining TCR surface expression, MR1 tetramer reactivity (both described as part of this Basic protocol 3) and TCR-mediated MR1-Ag response (Alternate protocol 11). If only TCR surface expression and MR1 tetramer reactivity are of interest, transient transfection of a cell line (e.g. 293T) with the TCR of interest, is sufficient (Support protocol 10) (Gherardin et al., 2016). A panel of MAIT TCR positive reporter cell lines, expressing similar levels of MAIT TCRs, allows for a relative comparison of MR1 tetramer reactivity and MR1-Ag responsiveness amongst the lines which directly relates to the combined affinity and kinetics of the interaction between the MAIT TCR and MR1-Ag (Eckle et al., 2014; Gherardin et al., 2016; Reantragoon et al., 2012). Furthermore, cell lines with MAIT TCRs that bear mutations of individual residues in their complementarity determining region (CDR) loops can be used to probe the contribution of individual MAIT TCR residues to the MR1-Ag recognition when compared to cell lines expressing similar levels of the wild-type TCR (Eckle et al., 2014; Gherardin et al., 2016; Patel et al., 2013; Reantragoon et al., 2012). For example, the conserved MAIT TCR α-chain residue Tyr95 when mutated to Alanine or Phenylalanine completely abrogates MR1 tetramer binding and MAIT cell activation (Corbett et al., 2014; Patel et al., 2013).

The following outlines considerations for experimental controls. Generation of TCR positive reporter cell lines with a previously characterised MAIT TCR and a non-MAIT TCR, in parallel to cell lines with newly identified TCRs of interest, serve as a positive and negative controls, respectively, for both the generation of new MAIT TCR positive reporter cell lines as well as their functional characterisation. For example the A-F7 MAIT TCR (Eckle et al., 2014; Reantragoon et al., 2012), originally identified by Tilloy *et al.* (Tilloy et al., 1999), can serve as a positive control. The LC13 TCR, isolated from a conventional $CD8^+$ T cell clone and specific for the Epstein-Barr virus nuclear Ag 3A peptide (FLRGRAYGL), presented by HLA-B*08:01 (Burrows, Sculley, Misko, Schmidt, & Moss, 1990; Gras et al., 2010; Kjer-Nielsen et al., 2003), can serve as a negative control. Furthermore, Ag-independent stimulation serves as a positive control for the functional capacity of MAIT cells. This can be achieved with PMA/Ionomycin or bead-immobilised CD3/CD28 specific mAbs (Alternate protocol 12). Similarly, incubation in the presence of APCs that lack MR1 (Laugel et al., 2016; H. Wang et al., 2018) (Support protocol 7), as compared to cells proficient in MR1 or overexpressing MR1 (Reantragoon et al., 2012; H. Wang et al., 2018) (Support protocols 5 and 6), or in the absence of relevant Ag but in the presence of vehicle control, media or irrelevant Ag, and/or in the presence of MR1 blocking

antibody as compared to isotype control antibody (Alternate protocol 13), or in the presence of non-activating competitively inhibiting Ags (Ac-6-FP) in addition to stimulating Ag (as per Alternate protocol 10) serve as controls for the MR1-Ag specific reactivity in MAIT cell activation assays.

Whilst Alternate protocol 11 describes the evaluation of MAIT cell activation based on CD69 surface upregulation in flow cytometry, one of the T cell lines we commonly use (Jurkat76) is also capable of producing IL-2 upon activation, which can be measured in ELISA (Alternate protocol 14).

Materials

- Cell lines in suspension (generated as per Support protocols 6 or 10 and maintained as per Appendix)
- Reagents, equipment and their use for counting viable cells using trypan blue exclusion (see Appendix)
- Fluorochrome-conjugated monoclonal mAbs specific for CD3 (e.g. anti-CD3ε, clone OKT3 or SK7) and/or specific for TCR αβ (e.g. clone T10B9) and/or specific for TRAV1-2 (clone 3C10) and/or MR1-5-OP-RU tetramer; fluorochrome-conjugated isotype control mAbs, matching the isotype, fluorochrome and concentration of the CD3 and/or TCR αβ and or TRAV1-2 specific Abs; MR1-6-FP tetramer control matching the MR1-5-OP-RU tetramer concentration (see Appendix for MR1 tetramer generation and use)
- Live/dead cell discrimination stain (see Appendix for live/dead cell discrimination stains and their use)
- Flow cytometry wash buffer (flow wash) B: 2% FCS (v/v) in PBS and stored at 4°C for 1 month.
- Flow cytometry cell fixation buffer (flow fix) (see Reagents and solutions)
- Single colour control samples for flow cytometry data acquisition (see Appendix)
- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix)

- Tabletop centrifuge (Beckman Coulter Allegra X-12R)
- Flow cytometer (BD LSR Fortessa)

Protocol steps

1. Map out the types of samples:
   a) Flow cytometry single colour staining controls (Appendix).
   b) Types of TCR positive reporter cell lines, e.g. 1 new MAIT TCR positive reporter cell line, 1 positive control (MAIT TCR positive reporter cell line), 1 negative control (non-MAIT TCR positive reporter cell line)

c) Samples per T cell line, e.g. the following:
   - Unstained
   - Stained with live/dead stain, anti-CD3 and anti-αβ TCR antibodies
   - Stained with live/dead stain, anti-CD3 and anti-TRAV1-2 antibodies
   - Stained with live/dead stain, anti-CD3 antibody and MR1-5-OP-RU tetramer
   - Stained with live/dead stain, isotype controls of anti-CD3 and anti-αβ TCR antibodies
   - Stained with live/dead stain, isotype controls of anti-CD3 and anti-TRAV1-2 antibodies
   - Stained with live/dead stain, isotype control of anti-CD3 antibody and MR1-6-FP tetramer

Note: CD3-specific staining serves as an indirect mean to determine TCR expression, since CD3 is only surface-expressed in conjunction with an $\alpha\beta$ TCR heterodimer, whereby the level of CD3 expression correlates with the level of $\alpha\beta$ TCR expression. Staining specific for $\alpha\beta$ TCR or TRAV1-2 directly evaluates $\alpha\beta$ TCR heterodimer expression and expression of an $\alpha\beta$ TCR heterodimer that bears the MAIT TCR α-chain segment TRAV1-2, respectively. MR1-5-OP-RU tetramer staining evaluates simultaneously the expression of an $\alpha\beta$ TCR heterodimer and the capacity of the expressed $\alpha\beta$ TCR to react with the tetramer. Thus, MR1-5-OP-RU tetramer staining is most informative if compared to TCR expression determined via CD3 and/or αβ TCR and/or TRAV1-2-specific staining. Since MR1-5-OP-RU tetramer and anti- $\alpha\beta$ TCR or anti-TRAV1-2 antibodies compete for binding to TCR, it is recommended to prepare separate staining samples as listed above. Alternatively, a two-step staining is recommended as described in step 5.

2. Take a 10 μl aliquot of each cell line culture, and count viable cells using trypan blue exclusion (see Appendix). Per sample 1×10^5 (at least 2×10^4) viable cells are needed.
3. Pellet the required volumes of cell suspensions (450 xg, 5 min, 4°C), discard supernatants and resuspend pellets in 200 μl flow wash B per sample and aliquot each into a v-shaped tube or a well of a v-bottom plate.
4. Pellet cells (450 xg, 5 min, 4°C), discard supernatants and stain cells with fixable live/dead cell discrimination stains as per Appendix.
5. Stain the cells by resuspending the cell pellets in 40 μl of staining mixture prepared in flow wash B. Incubate samples for 30 min on ice in the dark.
   Note: If cells are co-stained with MR1-5-OP-RU tetramer and anti- $\alpha\beta$ TCR or anti-TRAV1-2 antibody, anti- $\alpha\beta$ TCR or anti-TRAV1-2 antibody may be added only for the last 15 minutes of the staining incubation period. Since tetramer has a lower avidity for TCR compared to TCR specific mAbs, adding the tetramer prior to TCR specific mAb improves co-staining.
6. Wash cells twice with 200 μl flow wash B (450 xg, 5 min, 4°C).
7. Fix cells by resuspending pellets in 70 μl flow fix and incubate for 20 min in the dark on ice.
   Note: Samples can be stored in the dark on ice or in the refrigerator for up to 72 hr without compromising fluorescence intensity.

8. Transfer cells to flow cytometry acquisition tubes and analyse on a flow cytometer, acquiring data for each fluorochrome-conjugate used, as well as GFP and live/dead stain.
9. Following sequential gating on live, single cells, display flow cytometry data in the form of histograms, comparing the geometric mean fluorescence intensities (gMFI) of CD3-, TCR-, TRAV1-2-specific antibody versus isotype control staining and MR1-5-OP-RU versus MR1-6-FP tetramer staining (Figure 6). To compare TCR expression levels between cell lines, in each line, gate cells for a similar level of GFP expression and compare CD3 and/or TCR and/or TRAV1-2 expression levels (gMFI). Similarly, to compare MR1-5-OP-RU tetramer reactivities between cell lines, in each line, gate cells for a similar level of CD3 and/or TCR and/or TRAV1-2 expression and compare MR1-5-OP-RU tetramer staining levels (gMFI).

[*Insert Figure 6 near here]

ALTERNATE PROTOCOL 11

EVALUATION OF THE MAIT TCR-MEDIATED MR1-AG RESPONSE WITH TCR POSITIVE REPORTER CELL LINES

This protocol describes the evaluation of the functional response of MAIT TCR positive reporter cell lines to MR1-Ag presenting APC lines (generated as per Support protocols 5-7), using CD69 upregulation as a marker of T cell activation in flow cytometry. Alternatively, IL-2 cytokine production can be measured (Alternate protocol 14). This protocol involves the use of PMA/Ionomycin stimulation to assess Ag independent stimulatory capacity of the MAIT TCR positive reporter cell line, in contrast to Alternate protocols 7 and 12, describing the use of CD3/CD28 stimulation. To determine MR1-Ag dependence, this protocol describes the use of MR1 deficient cell lines, in contrast to Alternate Protocol 13 which uses MR1 blocking mAb or Alternate protocol 6 which uses competitively inhibiting Ag.

Materials

- TCR positive reporter cell lines in suspension (generated as per Support protocol 5 and 6 and maintained as per Appendix)
- APC lines in suspension (generated as per Support protocols 5-7 and maintained as per Appendix)
- Optional: folate-free RF10 media (prepared as RF10 media per Appendix, Maintenance pf cell lines, but using folate free RPMI medium, Gibco cat. no. 27016021)
- Reagents, equipment and their use for counting viable cells using trypan blue exclusion (see Appendix)
- MAIT cell Ags (see Appendix)

- Stock of PMA (Sigma, cat. no. P1585): Reconstitute stock at 0.1 mg/ml in DMSO, store at -20°C.

- Stock of Ionomycin (Sigma, cat. no. I3909): Reconstitute stock at 1 mg/ml in DMSO, store at -20°C.

- Fluorochrome-conjugated mAbs specific for CD3 (e.g. anti-CD3ε, clone OKT3 or SK7) and CD69 (e.g. clone FN50); fluorochrome-conjugated isotype control mAbs, matching the isotype, fluorochrome and concentration of the CD69-specific antibody. Optional: Fluorochrome-conjugated mAb expressed by the APC but not the TCR expressing reporter cell line, e.g. specific for CD19 (e.g. clone SJ25C1) expressed on C1R cells.
  Note: Being able to specifically gate on the TCR expressing reporter cell line to assess CD69 upregulation, increases the CD69 signal to background ratio. In some cases, the TCR expressing reporter cell line and APC line can be distinguished based on GFP level. Alternatively, fluorochrome-conjugated mAbs can be used to e.g. gate out CD19 expressing APCs (e.g. C1R cells). Identifying activated T cells based on CD3 or TCR staining is suboptimal, due to downregulation of the TCR complex upon T cell stimulation.
- Live/dead cell discrimination stain (see Appendix for live/dead cell discrimination stains and their use)
- Flow cytometry wash buffer (flow wash) B: 2% FCS (v/v) in PBS and stored at 4°C for 1 month.
- Flow cytometry cell fixation buffer (flow fix) (see Reagents and solutions)
- Single colour control samples for flow cytometry data acquisition (see Appendix)
- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix)

Protocol steps

1. Map out the types of samples:
   a) Flow cytometry single colour staining controls (Appendix).
   d) Types of TCR positive reporter cell lines, e.g. 1 new MAIT TCR positive reporter cell line, 1 positive control (MAIT TCR positive reporter cell line), 1 negative control (non-MAIT TCR positive reporter cell line)
   b) Samples per T cell line, e.g. the following (for different ways to stimulate MAIT cells in an Ag-MR1 dependent manner, see Appendix, MAIT cell Ags):
      - TCR positive reporter cell line
      - TCR positive reporter cell line stimulated with PMA/Ionomycin
      - TCR positive reporter cell line + APC + Nil
      - TCR positive reporter cell line + APC.MR1 + Nil
      - TCR positive reporter cell line + APC.MR1$^{-/-}$ + Nil
      - TCR positive reporter cell line + APC + 5-OP-RU

- TCR positive reporter cell line + APC.MR1 + 5-OP-RU
- TCR positive reporter cell line + APC.MR1$^{-/-}$ + 5-OP-RU
- TCR positive reporter cell line + APC + vehicle of 5-OP-RU
- TCR positive reporter cell line + APC.MR1 + vehicle of 5-OP-RU
- TCR positive reporter cell line + APC.MR1$^{-/-}$ + vehicle of 5-OP-RU
- TCR positive reporter cell line + APC + Ac-6-FP
- TCR positive reporter cell line + APC.MR1 + Ac-6-FP
- TCR positive reporter cell line + APC.MR1$^{-/-}$ + Ac-6-FP
- TCR positive reporter cell line + APC + vehicle of Ac-6-FP
- TCR positive reporter cell line + APC.MR1 + vehicle of Ac-6-FP
- TCR positive reporter cell line + APC.MR1$^{-/-}$ + vehicle of Ac-6-FP

2. Take a 10 µl aliquot of each T cell line and APC cell line culture, and count viable cells using trypan blue exclusion (see Appendix). Per sample 1 x 10^5 viable cells of each, the TCR positive reporter cell line and the APC line, are needed.

3. Perform Basic protocol 2, steps 3-7.

   Note: If continued presence and presentation of Ag is desired, co-incubate APCs, Ag and T cells without washing. Unless TCR positive reporter cell lines deficient in β_2m or MR1 are used (see Support protocol 6), the continued presence of Ag causes self-presentation of Ag by MAIT TCR positive reporter cell line-expressed MR1, which might be OK in many experimental scenarios, such as when comparing Ag potencies or TCR positive reporter cell lines that solely differ in their TCR usage.

4. Centrifuge (450 xg, 5 min, 4°C) the required volume of MAIT TCR positive reporter cell line suspensions, aspirate the supernatants and resuspend the pellets in 100 µl of RF10 media per sample. Add 100 µl of MAIT cell suspension to wells with RF10 media (+/- PMA/Ionomycin) and APC lines, respectively.
5. Incubate cells overnight for 16 hr (at least for 8 hr) in a tissue culture incubator.
6. Perform Basic protocol 3, steps 6-9.
7. Following sequential gating on live, single cells, T cells (e.g. based on GFP or by gating out CD19$^+$ APCs) display flow cytometry data in the form of histograms, comparing the gMFI of CD69 staining on all T cells, or a selection based on GFP expression, in order to maximise the CD69 signal which is highest within the highest TCR expressing cells within the T cell population. To compare CD69 expression levels between cell lines, in each line, gate cells for a similar level of GFP expression and compare CD69 expression levels (gMFI) relative to background.

ALTERNATE PROTOCOL 12

USE OF BEAD-IMMOBILISED CD3/CD28 STIMULATION TO ASSESS THE CAPACITY OF TCR POSITIVE REPORTER CELL LINES TO PRODUCE CYTOKINE INDEPENDENTLY OF AG

Additional materials (as compared to Alternate protocol 11)

- Dynabeads Human T-Activator CD3/CD28 (ThermoFisher, cat. no. 11131D).

Protocol steps

1. Vortex anti-CD3/CD28 beads for >30 sec.
2. Aliquot the required volume of anti-CD3/CD28 beads (typically used at a ratio of 2.5:1 beads to T cells) into a 1.5 ml tube and wash with 200 µl of RF10 media.

   Note: Alternatively, beads can be washed using a magnetic stand available from the manufacturer.
3. Centrifuge anti-CD3/CD28 beads (450 xg, 5 min, 4°C), aspirate media and resuspend in 20 µl of RF10 media per sample.
4. Aliquot washed anti-CD3/CD28 beads onto T cells in a total volume of 200 µl of RF10.
5. Continue incubation as per Alternate protocol 11, step 5.

ALTERNATE PROTOCOL 13

USE OF MR1 BLOCKING ANTIBODY TO DETERMINE MR1-AG DEPENDENCE IN TCR POSITIVE REPORTER CELL LINE ACTIVATION

See Alternate protocol 9 for background information.

Additional materials (as compared to Alternate protocol 11)

- Anti-MR1 mAb 26.5 (isotype: mouse IgG2a, Biolegend, cat. no. 361103) and suitable isotype control antibody.

Protocol steps

1. Perform Alternate protocol 11, steps 1-3.

2. Incubate APCs with 26.5 blocking mAb or relevant isotype control mAb at 20 μg/ml final concentration in 100 μl of RF10 media for 1 hr in a tissue culture incubator.

   Note: Titrating amounts of MR1 blocking antibody may be explored for dose-dependent blocking. Continue Alternate protocol 11, step 3.

ALTERNATE PROTOCOL 14

EVALUATION OF THE MAIT TCR-MEDIATED MR1-AG RESPONSE WITH TCR POSITIVE REPORTER CELL LINES: ANALYSIS OF IL-2 SECRETION AS A MARKER OF ACTIVATION

Whilst SKW-3 T cells, Jurkat RT3-T3.5 and Jurkat76 cells express CD69 as a marker of activation (Table 10), Jurkat76 cells can also produce IL-2 (Heemskerk et al., 2003), which can be measured in ELISA, allowing the evaluation of MAIT cell activation in a more high-throughput fashion, e.g. when screening the activation of a TCR positive reporter cell line by several doses of various Ags (Eckle et al., 2014; Keller et al., 2017). Whilst in flow cytometry, the activation of cells expressing similar levels of TCR, based on gating for CD3 expression levels, can be compared, this is not possible in ELISA, unless T cell clones expressing identical levels of TCR were generated (see Support protocol 3).

Additional materials (as compared to Alternate protocol 11)

- Jurkat76 T cells (generated as per Support protocols 5 and 6 and maintained as per Appendix)
- BD OptEIA Human IL-2 ELISA Set (cat. no. 555190)
- Kit and plate-reader instrument to measure substrate conversion by horse radish peroxidase (HRP)

Protocol steps

1. The experiment is set up as per Alternate protocol 11, steps 1-4, but using Jurkat76 T cells.

2. Incubate cells overnight in a tissue culture incubator for 21 hr.

3. Pellets cells (450 xg, 5 min, 4°C) and harvest supernatants, ideally avoiding cells (e.g. harvest 150 µl) and freeze at -80°C to kill any cells that might have been carried over.
4. Thaw frozen supernatants in tissue culture incubator and determine IL-2 concentration in 100 µl of supernatant by ELISA using the BD OptEIA kit as per instructions of the manufacturer. In brief, IL-2 is assayed with biotinylated anti-IL-2 mAb and substrate conversion by HRP-Streptavidin detected at 492 nm emission using a kit of choice.

SUPPORT PROTOCOL 10

GENERATION OF 293T CELLS, TRANSIENTLY TRANSFECTED WITH THE TCR OF INTEREST

To generate 293T cells transiently transfected with a TCR of interest, whereby DNA is not integrated into the genomic DNA, we use the same vector (pMIG II), as used as part of the murine stem cell virus (MSCV) retroviral transduction system, established by the laboratory of Dr. Dario Vignali (Holst et al., 2006; Szymczak et al., 2004) and a simplified version of Support protocol 6.

Materials

- 293T cells (see Table 9 for more information)
- Maintenance of cell lines (as per Appendix).
- Reagents, equipment and their use for counting viable cells using trypan blue exclusion (see Appendix)
- Endonuclease free plasmid large scale preparation of TCR of choice cloned into pMIG II (pMIG.TCR), as per Support protocol 5.
- Endonuclease free plasmid large scale preparation of full length human CD3 cloned into pMIG II (see Support protocol 5 for more information).
  Note: Since 293T cells are not a T cell line, they lack CD3. Thus, to allow for TCR surface expression, co-transfection with CD3 is required.
- Fugene6 transfection reagent (Roche, cat. no. 11814443001 or Promega cat. no. E2691)
- Opti-MEM media (Invitrogen cat. no. 31985070)
- Single colour control samples for flow cytometry data acquisition (see Appendix).
- Consumables and equipment for general tissue culture and flow cytometry experiment (see Appendix).

Protocol steps

1. Map out the types of transfection samples, for example:
   293T cells + MAIT TCR
   293T cells + control MAIT TCR
   293T cells + control non-MAIT TCR
2. Perform Support protocol 6, steps 2-5, but in step 3, the transfection mixture b) is composed of 4 µg pMIG.TCR and 4 µg pMIG.CD3.
3. Day 4: 48 hr following transfection, cells are ready for flow cytometric analysis as per Basic protocol 3, following dislodgement by trypsinisation (Appendix).

REAGENTS AND SOLUTIONS

- Flow cytometry wash buffer (flow wash) A: Add BSA to a concentration of 0.5% (w/v) and EDTA pH8 to a concentration of 2 mM to PBS and store the solution at 4°C for 1 month.
- MACS buffer: Add FCS to a concentration of 0.5 % (v/v) and EDTA pH8 to a concentration of 2.5mM EDTA to PBS. Store the solution at -20°C.
- Flow cytometry cell fixation buffer (flow fix): Add glucose to a concentration of 3.2% (w/v) and paraformaldehyde to a concentration of 1% (v/v) to PBS and store the solution at 4°C and protected from light for 1 month.

  Note: Fixation covalently crosslinks molecules on cells and stabilises expression of antibody-bound surface proteins.
- moDC wash: Add FCS to a concentration of 1% (v/v) and EDTA pH8 to a concentration of 5 mM to PBS. Sterile filter using a 0.2 µm filter and store the solution at 4°C for 1 month.

APPENDIX

Live/dead cell discrimination stains and their use

Live/dead discrimination stains allow for the exclusion of dead cell populations that may non-specifically stain with fluorophore-conjugated Abs during flow cytometry analysis. Fixable live/dead stains are commercially available in a number of dyes excited by the UV, 405, 488, 532, 561, or 633

nm lasers, and are compatible with downstream flow cytometric protocols which involve fixation or fixation and permeabilisation. Live/dead fixable dyes bind to amine groups of proteins. Whilst dyes cannot penetrate the membranes of live cells, resulting in dim staining based on dye binding to cell-surface proteins, dyes penetrate compromised membranes of dead cells, resulting in a more intense staining based on dye binding to intracellular and cell-surface proteins. Given these properties, live/dead staining is optimally performed in protein-free solutions such as PBS.

Reconstitute the live/dead discrimination dye (e.g. Fixable Aqua Dead Cell Stain Kit, ThermoFisher, cat. no. L34957, or Fixable Near Infrared Dead Cell Stain Kit, ThermoFisher, cat. no. L10119) as per instructions by the manufacturer in 50 µl of DMSO (provided by the manufacturer), store in 5 µl aliquots in -20°C freezer; exposure to moisture abrogates the sensitivity of live/dead dyes, avoid freeze-thawing. Prior to cell-surface staining, stain up to 5 x10^6 cells in a 96-well v-bottom plate in 50 µl of a 1:800-1:1000 dilution of live/dead stain. Incubate the plate in the dark for 10 min at RT. Resuspend the cells in 150 µl of PBS, centrifuge (450 xg, 5 min, 4°C) and discard the supernatant.

Single colour control samples for flow cytometry data acquisition

Multiparameter flow cytometric analyses requires the acquisition of single stained controls to correct for fluorescence spillover. Generally single colour compensation controls should be as bright or brighter than the fluorescence of the experimental samples, allowing for an accurate compensation matrix calculation. Single colour control samples can be prepared using cells stained with single fluorochrome-labelled mAbs as per Basic protocol 1. However, we highly recommend the use of compensation control beads, especially when the Ag of interest is rare, as they provide robust fluorescence signals. Compensation control beads are commercially available (BD, anti-mouse Ig κ, cat. no. 552843) and can bind a single or several Ab isotypes e.g. anti-rat, -mouse, -hamster.

To prepare single stain compensation controls select the appropriate beads with the correct Ab isotype. Vortex anti-mouse Ig κ positive and negative control bead vials thoroughly in a 1.5 ml tube, add three drops of positive and negative beads, and top up with 500 µl of flow wash A. Pipette 50 µl into each well of a v-bottom plate for the required number of compensation controls and include an unstained control. Add 1 µl of each fluorophore required to the respective well and pipette up and down. *Note: For accurate compensation matrix calculations, it is important to use the exact fluorophore as in the experimental panel, furthermore for tandem dyes, lot numbers can vary so use of the same antibody as the experimental panel is also required.* Incubate the plate in the dark for 10 min at RT. Resuspend in 150 µl of flow wash A and centrifuge (935 xg, 5 min, 4°C). Discard the supernatant and resuspend the beads in 150 µl of flow fix and transfer to flow cytometry acquisition tubes. *Notes: Store compensation beads in the dark at 4°C for up to 1-2 days, some dyes may remain stable beyond this time. During acquisition if the compensation control appears off scale, prepare*

another compensation control with a dilution of the original antibody e.g. 1/10, rather than lowering the photo multiplier voltage as this will affect the resolution of the experimental sample.

Maintenance of cells

All cells are cultured in a humidified incubator at 37°C with 5% CO_2, referred to here as a tissue culture incubator. Cells are grown in RPMI1640 medium (Gibco, cat. no. 21870) or DMEM medium (Gibco, cat. no. 11960) supplemented with 10% fetal calf serum (FCS, heat inactivated for 20 min at 56°C in a water bath) and serum complement (SC, see Table 11 for ingredients of a stock solution). Throughout this article, this media is referred to as RF10 and DMEM10 medium, respectively. SC stock solutions are sterile filtered using Stericup and Steritop filters (Millipore, cat. no. SCGPT05RE) and 30 ml aliquots stored at -20°C. RF10 and DMEM10 are stored at 4°C and warmed in a 37°C water bath prior to adding to cells. 293T cells are adherent and are maintained at 0.1-1.0x10^6 cells/ml in DMEM10 media. To generate a cell suspension, media is aspirated and the adherent cells are washed twice with PBS warmed in a 37°C water bath. Adherent cells are then incubated with a trypsinisation solution of choice at 37°C in a tissue culture incubator until cells dislodge. Cells are then resuspended and washed with DMEM10 media or flow wash. T cell lines and APC lines are cultured in suspension in RF10 media at 0.3-1.0 x 10^6 cells/ml.

For long-term storage, pellet cells in suspension in sterile 50 ml or 10 ml tubes (335 xg, 5 min, 4°C), discard the supernatant, and resuspend cells in sterile freezing media (10% dimethyl sulfoxide (DMSO, Sigma, cat. no. D2650)/FCS, pre-chilled on ice and stored at -20˚C) at approximately 5x10^6 (cell lines) or 0.5-2x10^7 (primary cells) cells per ml. Transfer 1 ml aliquots of the cell suspension to cryovials (Nunc, cat. no. based on desired size) and freeze immediately at a controlled rate in a freezing unit (for example CoolCell, Biocision, cat. no FTS30) at -80°C before transfer to liquid nitrogen storage on the next day. Pre-chill the freezing units at 4˚C to ensure optimal decrease in temperature gradient prior to transfer to -80˚C. For thawing of liquid nitrogen stored cell samples, wash samples once in 10 ml RF10 media (335 xg, 5 min, RT) to remove DMSO.

Reagents, equipment and their use for counting viable cells using trypan blue exclusion

For cell counts, mix 10 µl of cells in suspension thoroughly with 10 µl of Trypan Blue (0.2% (w/v) Trypan Blue, 0.02% (w/v) azide in PBS, stored at RT), which stains dead cells blue. Transfer 10 µl of the cell/Trypan Blue mix into a Neubauer type counting chamber, covered with a cover glass, thus creating a chamber that is 0.1 mm high. Using a light microscope, count live cells (not stained in blue) within 4 small squares, including cells lying on 2 of the 4 edges of each square of the Neubauer counting chamber. Calculate the actual cell concentration in cells/ml by multiplying with 10^4/2. If

more than 100 cells are counted, dilute cells in PBS prior to counting and account for the dilution factor when calculating the concentration.

Consumables and equipment for general tissue culture and flow cytometry experiment

General consumables include sterile tubes and vials (volumes 1.5 ml, 10 ml, 50 ml), flat-bottom 96-well plates (TPP, cat. no. 92696), u-bottom 96-well plates (TPP, cat. no. 92697), v-bottom 96-well plates (Greiner Bio-One, cat. no. 651180), reagent reservoirs (Corning, cat. no. 4870), flow cytometry acquisition tubes (Falcon, cat. no. 352008), 75 cm^2 cell culture flasks (Corning, cat. no. 430641K) and 25 cm^2 cell culture flasks (Corning, cat. no. 430639).

General pieces of equipment include a water bath, a tissue culture incubator, a light microscope, a tube vortex, a refrigerator and freezer and a class II biological safety cabinet.

Flow cytometry analysis software such as FlowJo and data analysis/graphing software such as GraphPad Prism are needed for the analysis of flow cytometry data.

MR1 tetramer generation and use

The NIH tetramer core facility (see Internet resources) provides MR1 tetramer loaded with 5-OP-RU or, as a control reagent, 6-FP, in a range of streptavidin fluorochrome conjugates, including PE, APC, BV421, Fluorescein, Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647, Alexa Fluor 680. Alternatively, the NIH tetramer facility provides biotinylated MR1 monomers which can be conjugated to commercially available Streptavidin-Fluorochrome, where we commonly use Streptavidin-Fluorochrome conjugates PE (BD Bioscience, cat. no. 554061) or BV421 (Biolegend, cat. no. 405225) which are very bright. To optimize for best staining, biotinylated MR1 monomers can be conjugated in various molar ratios with Streptavidin-Fluorochrome, e.g. testing an 8-fold, 6-fold, 4-fold molar excess of biotinylated MR1 monomer (~44 kDa) to Streptavidin-Fluorochrome (~57 kDa for Streptavidin). Aliquots of Streptavidin-Fluorochrome are added sequentially to biotinylated MR1 monomer to ensure saturation of biotin binding sites, e.g. adding $1/10^{th}$ of the solution every 10 minutes at RT, followed by resuspension via pipetting each time. MR1 tetramers can be stored in the refrigerator and protected from light for at least 1 month. We recommend titrating the tetramer prior to use on PBMCs, aiming for an optimal signal-to-noise ratio. We typically use a dilution of 1:200-1:800 of a stock with a concentration of 0.14 µg/µl, whereby we use higher concentrations in experiments that involve steps of intracellular staining. Similarly, the NIH recommends a 1:500 to 1:1000 dilution for bright fluorochrome conjugates (PE, APC, BV421) and 1:500 dilution of other fluorochrome conjugates of the 200 µg stocks of MR1 protein provided. MR1 tetramer staining can

be performed on ice, at room temperature or at 37°C. Whilst the staining intensity may increase with an increased temperature, also background staining may increase.

MAIT cell antigens

Stimulating MAIT cell Ags are currently not commercially available. However, protocols for the generation of synthetic 5-OP-RU in DMSO (Mak et al., 2017) and 5-A-RU in water (Bown, Keller, Floss, Sedlmaier, & Bacher, 1986; Mak et al., 2017) are published. The stability of 5-A-RU derived Ags has been characterised (Mak et al., 2017). 5-OP-RU is very stable in DMSO but rapidly degrades in water (Mak et al., 2017). In the absence of synthetic 5-OP-RU, 5-A-RU can be mixed with methylglyoxal solution (MG, 40 % in water, Sigma, aliquots stored in a -80°C freezer) in water or PBS for 1 hr at 4°C on a roller-mixer in the dark (Corbett et al., 2014). The reaction of 5-A-RU and MG in water does however not only generate 5-OP-RU but also other uracil and lumazine derivatives and degradation products (Mak et al., 2017). A molar ratio of 5-A-RU and MG in the range of 1:3-3.3 yields optimal generation of 5-OP-RU, which rapidly degrades with time (Mak et al., 2017). Maximum stimulation of primary MAIT cells, measured based on activation surface or intracellular marker expression, as well as proliferation, is achieved with about 0.1-5 nM of 5-OP-RU. Maximum stimulation of MAIT TCR positive reporter cell lines, measured based on activation surface marker expression, is achieved with about 0.1-1 nM of 5-OP-RU.

Non-stimulating MAIT cell Ags include 6-FP and Ac-6-FP which are commercially available as powder (Schircks). Both can be dissolved at 5 mM in water, supplemented with 17 mM NaOH. Ac-6-FP can also be dissolved at 500 mM in DMSO followed by further dilutions into water. 6-FP and Ac-6-FP are sensitive to light.

Generally, Ags are kept in the dark and as much as possible in a -80°C freezer or on dry ice. Ag stocks are not refrozen but rather aliquots prepared and used per assay. Immediately prior to their use in a cellular assay Ag stocks are freshly prepared or defrosted and diluted in PBS or RF10 media to the required concentration. In each cellular experiment, vehicle controls are incorporated matching each Ag stock preparation.

Rather than using synthetic Ags in MAIT cell activation assays, APCs infected with microbes or supernatant or lysate of microbial cultures can be used, with protocols specific to each microbe and not described here. Relevant controls include the use of microbes knocked out for production of genes that allow for the generation of 5-A-RU, microbes deficient in the riboflavin pathway, microbial culture media or lysate buffer.

ABBREVIATIONS

5-A-RU – 5-amino-6-D-ribitylaminouracil

5-OE-RU – 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil

5-OP-RU – 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil

Ab – antibody

Ag – antigen

APC – Ag-presenting cell

CBA – cytometric bead array

CDR – complementarity determining region

DNA – Deoxyribonucleic acid

ELISA – enzyme linked immunosorbent assay

FACS – fluorescence activated cell sorting

FMO – fluorescence minus one

GFP – green fluorescent protein

gMFI – geometric MFI

HBSS – hanks balanced salt solution

HRP – horse radish peroxidase

IL – interleukin

IRES – internal ribosomal entry site

LN – lymph node

LTR – long terminal repeat

PCR – polymerase chain reaction

PMA – phorbol 12-myristate 13-acetate

mAb – monoclonal Ab

MACS – magnetic activated cell sorting

moDC – monocyte-derived dendritic cell

MAIT – Mucosal-associated invariant T cell

MHC – Major Histocompatibility Complex

MFI – mean fluorescence intensity

MR1 – MHC related protein-1

mRNA – messenger Ribonucleic acid

MSCV – murine stem cell virus

NKT – Natural Killer T cell

PBS – phosphate buffered saline

PHA – phytohaemagglutinin

pMIG – MSCV-IRES-GFP

RT – room temperature

RT-PCR – reverse-transcription polymerase chain reaction

TCR – T cell receptor

COMMENTARY

Background Information

MAIT cells were originally described in peripheral blood and in the intestinal lamina propria (Porcelli et al., 1993; Treiner et al., 2003), coining the mucosal distribution of this innate-like subset (Treiner et al., 2003). Further assessments of the tissue distribution of MAIT cells have found their localization in peripheral tissues, including in the lungs (Hinks et al., 2016), liver (Dusseaux et al.,

2011), oral mucosa (Sobkowiak et al., 2019), endometrium (Dias et al., 2018) and skin (Li et al., 2017). MAIT cells have also been found in the lymphoid tissues, including thymus (Koay et al., 2016) and lymph nodes (Dusseaux et al., 2011). MAIT cells are found in high frequencies in human blood (0.5-6% of T cells) (Gherardin, Souter, et al., 2018) and in tissues such as in the liver (20-50% of T cells) (Dusseaux et al., 2011; Jeffery et al., 2016; Tang et al., 2013) and at some anatomical sites of the gastrointestinal tract (e.g. 10% in the colon) (Cosgrove et al., 2013) and in the lungs (2-4%) (Hinks et al., 2016), reviewed in (Kurioka, Walker, Klenerman, & Willberg, 2016).

MAIT cells play a key role in protective immunity to infections with riboflavin producing microbes, as demonstrated for *Mycobacterium bovis* BCG, *Klebsiella pneumoniae, Escherichia coli, Francisella tularensis* and *Legionella longbeachae,* e.g. (Chua et al., 2012; Cui et al., 2015; Georgel, Radosavljevic, Macquin, & Bahram, 2011; Meierovics, Yankelevich, & Cowley, 2013; H. Wang et al., 2018) and reviewed in (Godfrey et al., 2019; Kjer-Nielsen et al., 2018). MAIT cell immune function includes the production of proinflammatory cytokines TNF, IFNγ, IL-17A (Dusseaux et al., 2011; Le Bourhis et al., 2013) as well as cytotoxicity (Kurioka et al., 2015; Le Bourhis et al., 2013).

Prior to the MR1 tetramer being widely available, MAIT cells were identified based on surrogate markers, with most studies using a combination of TCR α-chain TRAV1-2 and C-type lectin CD161^{high} expression, sometimes including the IL-18Rα CD218 or the ectopeptidase CD26. Whilst these surrogate markers, as well as others, generally encompass MAIT cells in most individuals, for some subsets (especially CD4$^+$ CD8$\alpha\beta^+$ DP and CD4$^+$ CD8$^-$), not all MAIT cells are accurately captured, with some MAIT cells not included and some non-MAIT T cells included mistakenly, as comprehensively assessed by Gherardin *et al.* (Gherardin, Souter, et al., 2018). For example, the gene segment TRAV1-2 is not exclusive to MAIT cells, with some MHC-I-restricted T cells (Tynan et al., 2005; Tynan et al., 2007) and CD1b-restricted CD4$^+$, germline-encoded mycolyl lipid-reactive (GEM) T cells also using this TRAV gene segment (Van Rhijn et al., 2013). CD161 expression by MAIT cells has been observed to be downregulated in diseases such as rheumatoid arthritis (Koppejan et al., 2019) and HIV (Freeman, Morris, & Lederman, 2017; Leeansyah et al., 2013). It appears to be reduced in response to stimulation, however this may be caused by rapid division of activated MAIT cells that is seen in HIV-infected patients and after bacterial stimulation *in vitro* (Freeman et al., 2017; Leeansyah et al., 2013). Moreover, it is unclear to which extent surrogate markers characterise MAIT cells in tissues, as careful comparisons between MR1 tetramer and surrogate marker identification of MAIT cells have only been performed in PBMCs. Notably, very few thymic MAIT cells express CD161 and some are not TRAV1-2$^+$ (Koay et al., 2016).

Protocols described here focus on the characterisation of human MAIT cells using flow cytometry. The use of MR1-tetramers to detect MAIT cells by immunofluorescence in tissue sections is also possible (H. Wang et al., 2018), but is technically challenging, particularly when aiming to quantify

MAIT cells. Given the overlap between MAIT cells identified with MR1-tetramer versus antibodies specific for surrogate markers TRAV1-2 and CD161 in blood (as discussed above), TRAV1-2 and CD161 specific mAbs can be used in principle in immunofluorescence. However, it is unclear to which extent TRAV1-2 and CD161 identify MAIT cells in tissues (as discussed above). To date, the most reliable method of characterising MAIT cells in both humans and mice, is by flow cytometric analysis of single cell preparations.

Due to the high conservation of MR1 across species (Boudinot et al., 2016; Hashimoto et al., 1995; Riegert, Wanner, & Bahram, 1998), there is appreciable cross-reactivity for the MAIT TCR-MR1 axis between certain species such as human, non-human primates, bovine, rat and mouse (Greene et al., 2017; Huang et al., 2009; Lopez-Sagaseta, Dulberger, Crooks, et al., 2013; Lopez-Sagaseta, Dulberger, McFedries, et al., 2013). Thus, it is reasonable to assume that human MR1 tetramers can be used to characterise MAIT cells in other species. Nonetheless, at least for mouse (Chen et al., 2017; Rahimpour et al., 2015) and macaque (Greene et al., 2017; Juno et al., 2019), species-specific MR1 tetramers have been developed, which represent superior reagents for the analysis of MAIT cells in the respective species. Techniques described in this unit are in part also transferrable to studying macaque MAIT cells (Juno et al., 2019). Mouse models of disease have proven valuable to definitively assess the role of MAIT cells in disease, described as part of (Reference: Mouse MAIT cell characterisation protocol).

Another aspect of the characterisation of MAIT cells, which has been extensively studied but is not described here, includes the use of X-ray crystallography and biophysical assays, such as surface plasmon resonance, to determine the molecular mechanisms of the interaction between the MAIT cell TCR and MR1-Ag (Corbett et al., 2014; Eckle et al., 2014; Gherardin et al., 2016; Kjer-Nielsen et al., 2012; Patel et al., 2013).

Critical Parameters

As with T cell activation in general, MAIT cells also downregulate the TCR complex (TCR and CD3) upon stimulation. This causes a reduction in staining with fluorescently-labelled CD3 and TCR-specific mAbs and even more so with fluorescently-labelled MR1 tetramer, which binds to MAIT TCRs with a lower avidity as compared to mAbs to TCR and CD3. These phenomena are most relevant to MAIT cell activation assays, when activation is measured shortly after the stimulus (Basic protocol 2), whilst for example TCR surface expression is restored 5 days post stimulation when MAIT cell proliferation by fluorescent dye dilution is determined (Alternate protocol 6).

Furthermore, they might apply to samples even in the absence of *ex vivo* stimulation, for example samples from donors with an acute infection. In essence, those MAIT cells that have been activated the most might not be identified as MAIT cells in flow cytometry and instead are potentially identified as non-MAIT cells. Whilst this phenomenon cannot be entirely avoided, it can in part be mitigated, by (i) reducing the duration of the stimulus, (ii) reducing the Ag dose if applicable, (iii) improving the Ab and tetramer staining signals by using a fluorophore with a high staining index or by using secondary mAbs (e.g. anti-fluorochrome mAbs), as described by others (Dolton et al., 2015), or by using protein kinase inhibitors (PKIs) such as dasatinib , or (iv) by staining intracellularly with TCR specific mAbs. Whilst PKIs such as dasatinib have been shown to dramatically improve detection of T cells with tetramers (Lissina et al., 2009; Wooldridge et al., 2009), including atypical MR1 restricted T cells (Gherardin et al., 2016), at high concentrations, PKIs can inhibit T cell function (Weichsel et al., 2008) and therefore should be used sparingly in functional assays.

An inherent complication of using human blood and tissue is the inter-donor variation as well as the 'quality' of the samples, influenced by storage and processing of the samples, evident for example based on the presence of many dead cells, cell lysis and low lymphocyte counts. Discussing preferred storage with clinicians and minimizing time prior to processing of the samples are key. As with any experiment, using appropriate control samples, as outlined for each protocol, is imperative. Control samples allow to put a 'negative result', e.g. lack of tetramer staining, lack of cytokine production, into context.

Understanding Results

MAIT cell biology is an emerging field of research. By now a few characteristics of MAIT cells in blood have been described in several studies by several laboratories and seem to apply to many individuals (Table 1). At the same time there are some conflicting studies on MAIT cell frequencies and phenotype in diseases, warranting further investigation. Furthermore there are studies that allude to the existence of heterogenous populations of MR1 restricted T cells that differ from MAIT cells, named atypical MR1 restricted T cells or MR1T cells (Gherardin et al., 2016; Harriff et al., 2018; Koay et al., 2019; Lepore et al., 2017; Meermeier et al., 2016), respectively, and reviewed in (Godfrey et al., 2019). Some of these T cells are identified using the MR1 tetramer, they can differ from MAIT cells in their MR1-Ag reactivity pattern, including their reactivity to MR1-6-FP tetramer, can feature atypical MAIT TCR α-chain usage, transcription factor profile and phenotypic markers. Whilst they are much less frequent than MAIT cells, it is possible that in some donors they are more frequent. Thus, characterisation of MR1 tetramer positive cells that also includes assessment of surface markers, transcription factors, the TCR usage and most importantly the MR1-Ag reactivity, allows the identity of the cells to be deciphered.

Time Considerations

For a reasonable sample size and provided that single cell suspensions have been generated, the characterisation of the surface phenotype of MAIT cells takes about 1 day, the characterisation of the intracellular phenotype by flow cytometry takes about 2 days, measuring cytokine production by ELISA takes about 3 days and assessing MAIT cell proliferation takes about 5 days. Determining the MAIT TCR usage takes about 2 weeks. The generation of MAIT TCR positive reporter cell lines takes 1 week plus another 1-2 weeks for their recovery prior to bulk FACS. Depending on the numbers of cells sorted, cells might have expanded to sufficient numbers to generate samples for liquid nitrogen storage and to perform experiments 1 to 2 weeks post FACS. Cell line experiments take about 2 days. The generation of single clone MR1 knockout cell lines take about 6-8 weeks.

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INTERNET RESOURCES

- IMGT®, the international ImMunoGeneTics information system®, a free online database http://www.imgt.org (founder and director: Marie-Paule Lefranc, Montpellier, France) (Lefranc et al., 2015)
- IMGT/V-QUEST, the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis, a free online tool http://www.imgt.org/IMGT_vquest/vquest?livret=0&Option=humanTcR (Brochet, Lefranc, & Giudicelli, 2008)
- Benchling, a free online service for the *in silico* design of sgRNAs benching.com/crispr. https://benchling.com/crispr
- NIH tetramer core facility to request MR1 tetramers: http://tetramer.yerkes.emory.edu

FIGURE LEGENDS

Figure 1. Example MAIT cell gating strategy on PBMC samples.

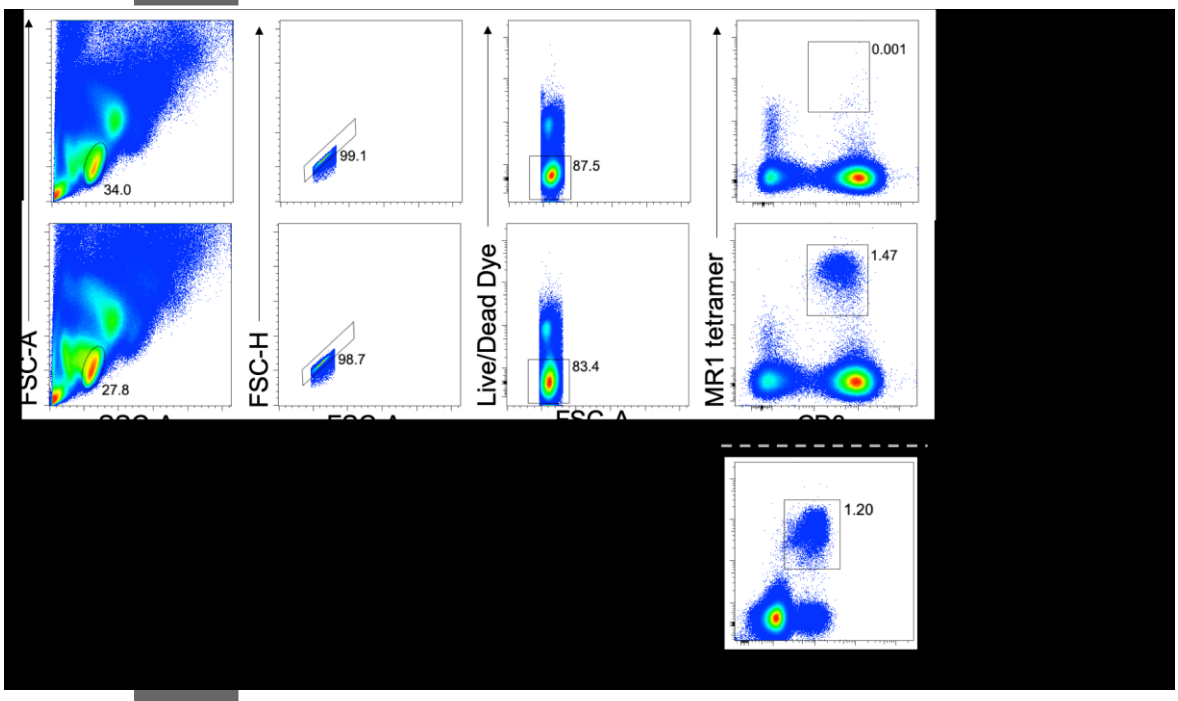


Figure 2. PLZF staining gated on MAIT cells as part of a complete staining cocktail (green histogram) as compared to the fluorescence minus one (FMO) control as a negative control (grey filled histogram), where anti-PLZF was excluded from the staining cocktail.

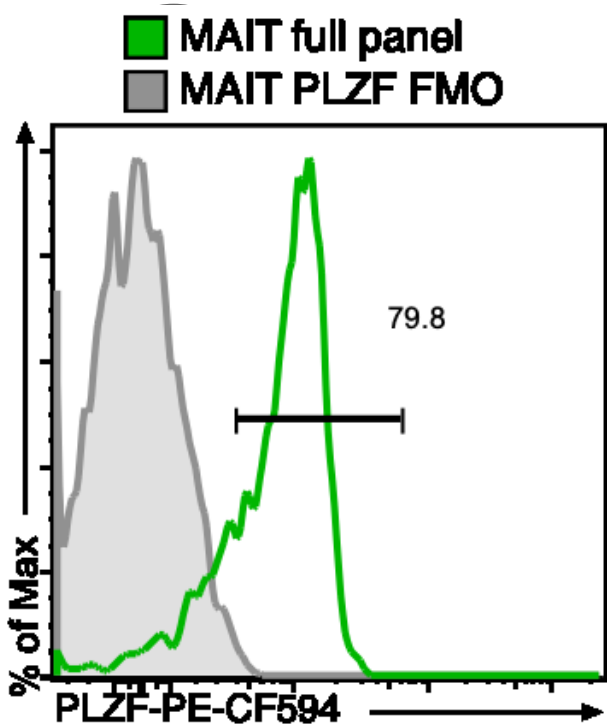


Figure 3. Schematic of PBMC isolation from blood.

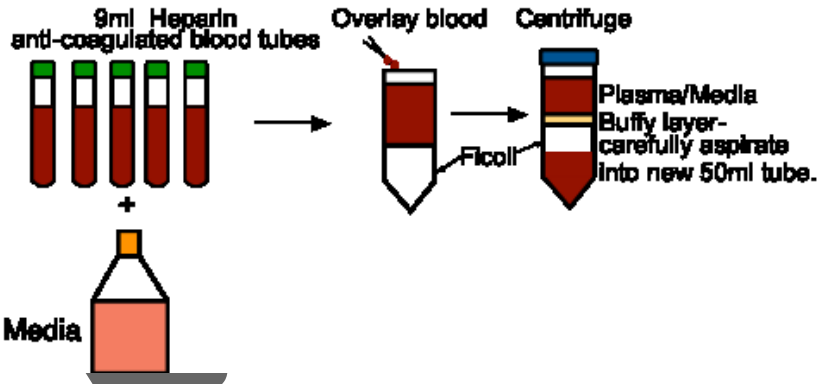


Figure 4. MAIT cell proliferation-based fluorescent dye dilution after 5-OP-RU stimulation of PBMCs. (a) THP1 cell lines over-expressing MR1 (THP1.MR1^{+}) or knocked out for MR1 (THP1.MR1^{-}) were incubated with 5-OP-RU (10 nM) prior to co-culture with cell trace violet (CTV) labelled PBMCs at a ratio of 1:5. (b) Cells were cultured for 5 days and then MAIT cell proliferation cell division peaks were derived by gating on live MR1-5-OP-RU-Tet^{+}TRAV1-2^{+} cells.

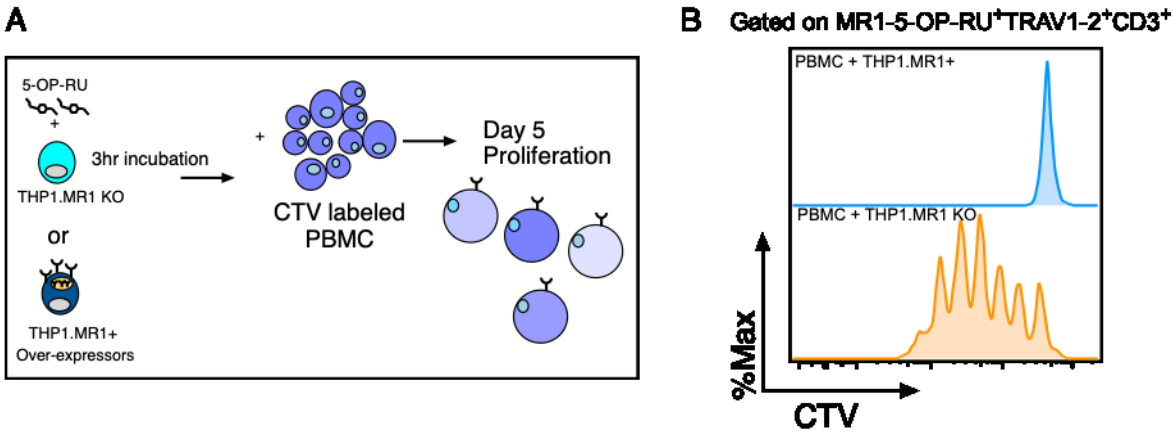


Figure 5. Schematic overview of the cloning of a monocistronic or a bicistronic fragment into the pMIG II vector for transfection of cell lines with genes of interest.

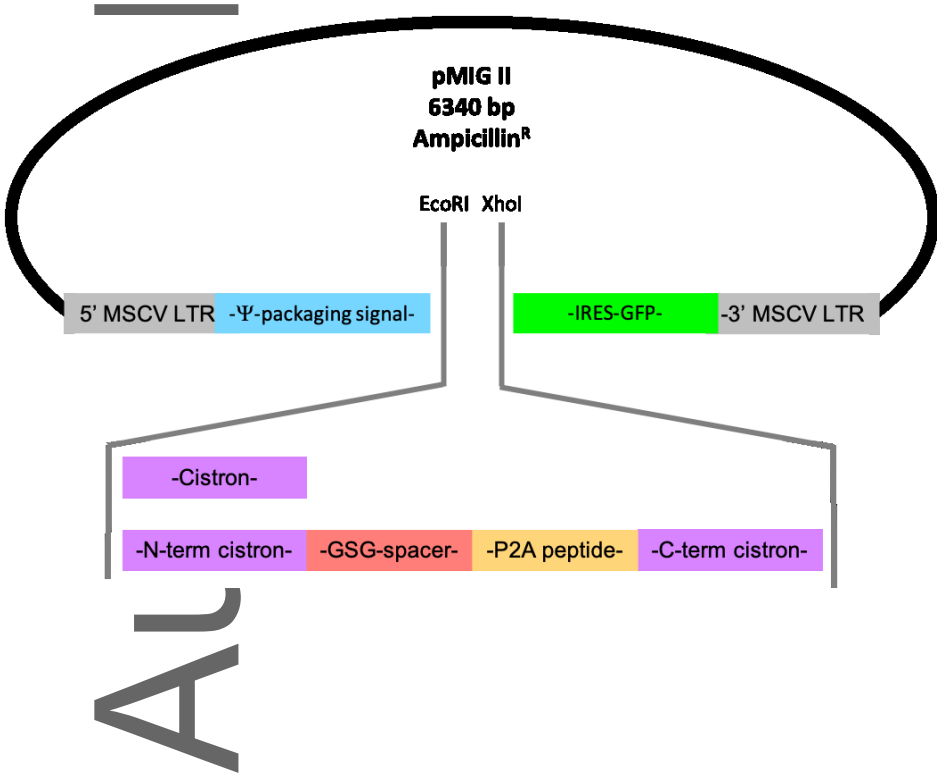
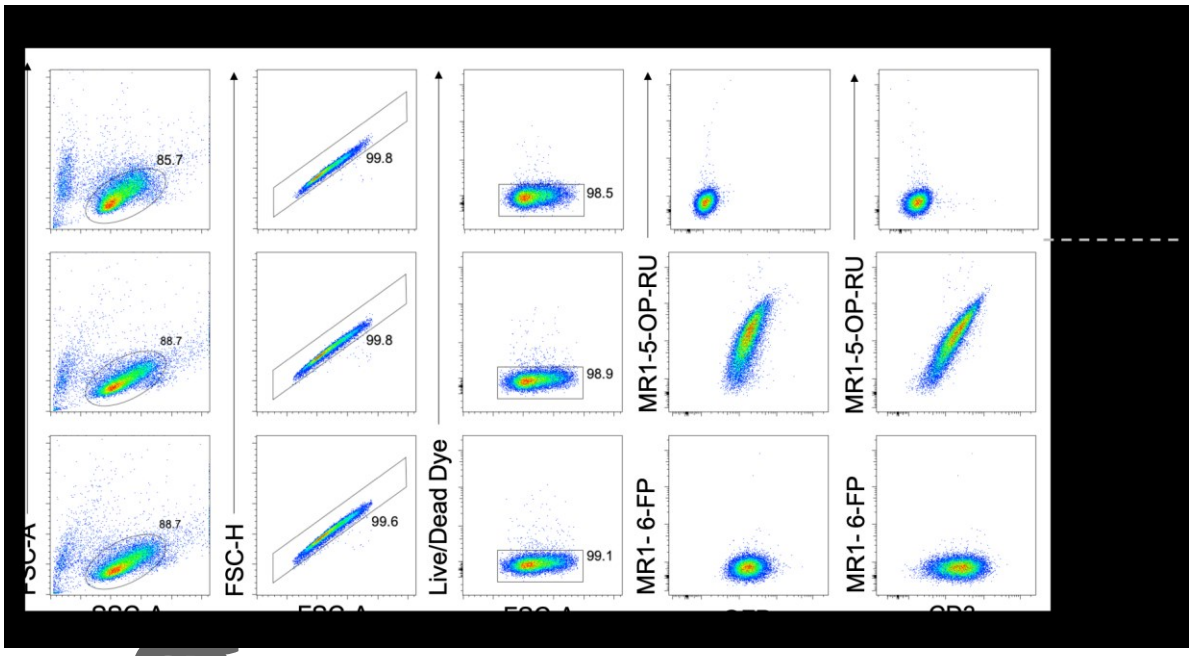


Figure 6. Example gating strategy on a clonal human MAIT TCR positive reporter cell line.



TABLES

Table 1 MAIT cell markers in human healthy adult blood

Marker	Defined by CD161^{++} TRAV1-2^{+}	Defined by MR1-5-OP-RU Tet^{+} TRAV1-2^{+}	Expression in healthy adults – steady state	Expression in healthy adults – activation	Reference
TRAV1-2			+++		(Dias et al., 2018; Gherardin, Souter, et al., 2018)
CD161			++/+++		(Gherardin, Souter, et al., 2018)
CD69			+	+++	(Gherardin, Loh, et al., 2018; Kurioka et al., 2017; Loh et al., 2016)
IL-18R			+++		(Dias et al., 2018; Gherardin, Souter, et al., 2018; Kurioka et al., 2017)
CD8αα			++		(Gherardin, Souter, et al., 2018)
CD8aβ			++		(Gherardin, Souter, et al., 2018)
CD4			+/-		(Dias et al., 2018; Gherardin, Souter, et al., 2018; Kurioka et al., 2017)
CD2			++		(Dias et al., 2018)
CD9			+		(Dias et al., 2018)
CD25			+/-	++	(Dias et al., 2018; Dias et al., 2016; Dusseaux et al., 2011; Kurioka et al., 2017; Le Bourhis et al., 2013; Leeansyah et al., 2014; Magalhaes et al., 2015)

CD26			++/+++		(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018; Sharma et al., 2015)
CD27			++/+++		(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018)
CD28			++/+++		(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018)
CD45RO			+		(Kurioka et al., 2017)
CD45RA			-		(Gherardin, Souter, et al., 2018)
α4β7			++		(Dias et al., 2018)
CCR2			+		(Kurioka et al., 2017)
CCR3			+		(Kurioka et al., 2017)
CXCR3			+		(Dusseaux et al., 2011; Kurioka et al., 2017)
CXCR4			+/++		(Dusseaux et al., 2011; Kurioka et al., 2017)
CCR4			+/-		(Kurioka et al., 2017)
CCR5			+		(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018; Kurioka et al., 2017)
CCR6			+/++		(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018; Kurioka et al., 2017)
CXCR6			+		(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018)
CCR7			+/-		(Gherardin, Souter, et al., 2018; Kurioka et al., 2017)
CCR9			+/-		(Dusseaux et al., 2011; Kurioka et al., 2017)
CD122			+		(Dusseaux et al., 2011)
CD127			++/+++		(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018; Kurioka et al., 2017)

CD150			+++		(Dusseaux et al., 2011)
CD244			++		(Dusseaux et al., 2011)
CD62L			+/-		(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018; Kurioka et al., 2017)
KIR2DL1			-		(Gherardin, Souter, et al., 2018)
KIR2DL2/3			-		(Gherardin, Souter, et al., 2018)
KIR2DL/S5			-		(Gherardin, Souter, et al., 2018)
NKp30			-		(Gherardin, Souter, et al., 2018)
NKp46			-		(Gherardin, Souter, et al., 2018)
NKp80			+		(Dusseaux et al., 2011)
CD56			+/++		(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018; Kurioka et al., 2017)
NKG2A			+/++		(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018; Kurioka et al., 2017)
NKG2D			++		(Dias et al., 2018; Dusseaux et al., 2011; Gherardin, Souter, et al., 2018)
CD38			+/-		(Kurioka et al., 2017)
CD94			+		(Dias et al., 2018; Kurioka et al., 2017)
CD95			+++		(Dusseaux et al., 2011)
PD-1			+		(Dias et al., 2018)
ICOS			-		(Dusseaux et al., 2011)

CD40L			-	++	(Salio et al., 2017)
CD137			-	++	(Salio et al., 2017)
CD107a			-	++	(Kurioka et al., 2015)
CD101			+/-		(Dias et al., 2018)
PLZF			++		(Dias et al., 2018; Gherardin, Souter, et al., 2018; Kurioka et al., 2017)
Tbet			+/++		(Dias et al., 2018; Gherardin, Souter, et al., 2018; Kurioka et al., 2017)
ThPOK			++		(Kurioka et al., 2017)
Eomes			++		(Dias et al., 2018; Kurioka et al., 2017)
GATA3			-		(Gherardin, Souter, et al., 2018)
RORγT			+		(Dias et al., 2018) (Dias et al., 2018; Kurioka et al., 2017)
Perforin			+	++/+++	(Dias et al., 2018; Kurioka et al., 2017; Kurioka et al., 2015)
GzmK			+++	++	(Kurioka et al., 2017; Kurioka et al., 2015)
GzmA			+++	++/+++	(Dias et al., 2018; Kurioka et al., 2015)
IFNγ			-	+/+++	(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018; Kurioka et al., 2017; Loh et al., 2016)
TNF			-	+/++	(Gherardin, Souter, et al., 2018)
IL-17A*			-	+/++	(Dias et al., 2018; Dusseaux et al., 2011; Gherardin, Souter, et al., 2018); Dusseaux *et al.**
IL-2*			-	++	(Dusseaux et al., 2011; Gherardin, Souter, et al., 2018)
IL-13			-	+/-	(Kurioka et al., 2017)

Gzm B			-	++/+ ++	(Kurioka et al., 2017; Kurioka et al., 2015; Loh et al., 2016)
IL-4			-	-	(Kurioka et al., 2017)
Gran ulysi n			+		(Dias et al., 2018)

Surface markers are highlighted in yellow in the first column, intracellular markers in orange and intranuclear markers in brown. A blank cell indicates that the marker has not been assessed during the preparation of this protocol. References provided are not exhaustive. *Detected in culture supernatant by cytometric bead array.

Table 2. Example fluorochrome labelled mAb/MR1 tetramer panel for determining MAIT cell steady state surface phenotype.

Marker	**Fluorophore**	**Laser**	**Clone**	**Dilution**	**Volume in µl (50 µl sample)**
MR1-5-OP-RU Tet	SA-BV421	Violet		1/200-1/800	0.25-0.0625
CD19	APC-H7	Red	HIB19	1/100	0.5
CD14	APC-H7	Red	MϕP9	1/100	0.5
CD8α	PerCP-Cy5.5	Blue	SK1	1/50	1.0
CD4	BV650	Violet	OKT4	1/200	0.3
CD161	BV605	Violet	HP-3G10	1/50	1.0
CD3	PE-CF594	Yellow/Green	UCHT1	1/200	0.3
TRAV1-2	PE	Yellow/Green	3C10	1/200	0.3

Table 3. Reverse Transcription PCR reaction mixture

	Vol (μl) per sample	**Vol (μl) for 100 samples**
5x VILO reaction mix (provided with kit)	0.5	50
10x Superscript RT (provided with kit)	0.25	25
1% Triton X-100 (Reagents and solutions)	0.275	27.5
nuclease-free water (provided with kit)	1.5	150
Total volume	2.525	252.5

Table 4. Preparation of TCR α- and β-chain external and internal primers for multiplex PCR

TCRα (TRAV) and TCRβ (TRBV)	**Primer(s)**	**To prepare:**
External forward	TRAV 1-41 Ext	For 1000 µl of 5 pmol/µl master mix stock, add 16.6 µl of each 300 pmol/µl primer stock and adjust with 333.3 µl water E.g. 16.6 µl x 40 primers= 666.7 µl + 333.3 µl = 1000 µl
External reverse	TRAC Ext	Dilute 300 pmol/µl stock $^{1}/_{60}$ to 5 pmol/µl 16.6 µl of 300 pmol/µl stock plus 983.4 µl water
Internal forward	TRAV 1-41 Int	For 1000 µl 5 pmol/µl master mix stock, add 16.6 µl of each 300 pmol/µl primer stock and adjust with 333.3 µl water As above for Ext
Internal reverse	TRAC Int	Dilute 300 pmol/µl stock $^{1}/_{60}$ to 5pmol/µl (As above for Ext)
External forward	TRBV 2-30 Ext	For 1000 µl of 5 pmol/ µl master mix stock, add 16.6 µl of each 300 pmol/µl primer stock and adjust with 550 µl water E.g. 16.6 µl x 28 primers= 464.8 µl + 535.2 µl = 1000 µl
External reverse	TRBV Ext	Dilute 300 pmol/µl stock $^{1}/_{60}$ to 5 pmol/µl 16.6 µl of 300 pmol/ul stock plus 983.4 µl water
Internal forward	TRBV 2-30 Int	For 1000 µl of 5pmol/µl master mix stock, add 16.6 µl of each 300 pmol/µl primer stock and adjust with 535.2 µl water As above for Ext
Internal reverse	TRBV Int	Dilute 300 pmol/µl stock $^{1}/_{60}$ to 5 pmol/µl (As above for Ext)

Table 5. TCR$\alpha\beta$ External Round PCR mastermix

	Vol (µl) per sample	**Vol (µl) for 100 samples**
10 x PCR buffer (contains $MgCl_2$)	2.5	250
dNTP mix (10mM)	0.5	50
TRAV Ext primers	0.5	50
TRAC Ext primer	0.5	50
TRBV Ext primers	0.5	50
TRBC Ext primer	0.5	50
Taq DNA Polymerase	0.15	15
HPLC water	17.35	1735
Total volume	22.5	2250

Table 6. TCR$\alpha\beta$ Internal Round PCR mastermix

	TRAV mix		**TRBV mix**	
	Vol (µl) per sample	**Vol (µl) for 100 samples**	**Vol (µl) per sample**	**Vol (µl) for 100 samples**
10 x CL Buffer (contains $MgCl_2$)	2.5	250	2.5	250
dNTP mix (10 mM)	0.5	50	0.5	50
TRAV Int primers	0.5	50	-	-
TRAC Int primer	0.5	50	-	-
TRBV Int primers	-	-	0.5	50
TRBC Int primer	-	-	0.5	50
Taq DNA Polymerase	0.15	15	0.15	15
HPLC water	18.35	1835	18.35	1835
Total volume	22.5	2250	22.5	2250

Table 7. Example fluorochrome-labelled mAb/MR1 tetramer panel for determining MAIT cell-surface activation phenotype.

Marker	**Fluorophore**	**Laser**	**Clone**	**Dilution**	**X1 (50 µl volume)**
MR1-5-OP-RU Tet	SA-PE	Yellow/Green		1/200-1/800	0.25-0.0625
CD19	APC-H7	Red	HIB19	1/100	0.5
CD14	APC-H7	Red	MϕP9	1/100	0.5
CD8α	BUV805	Ultraviolet	SK1	1/200	0.25
CD4	BUV496	Ultraviolet	OKT4	1/100	0.5
CD161	PE-Cy7	Yellow/Green	HP-3G10	1/100	0.5
CD3	BUV395	Ultraviolet	UCHT1	1/100	0.5
TRAV1-2	FITC	Blue	3C10	1/50	1
CD25	APC	Red	M-A251	1/50	1

Table 8. Example fluorochrome-labelled mAb/MR1 tetramer panel for determining MAIT cell intracellular activation phenotype.

Marker	**Fluorophore**	**Laser**	**Clone**	**Dilution**	**X1 (50 µl volume)**
TNF	BV421	Violet	Mab11	1/200	0.25
IFNγ	BV650	Violet	XMG1.2	1/200	0.25

Table 9. Antigen-presenting cell lines

	C1R (HMy2.CIR)	THP-1	293T (293tsA1609neo)
Access	ATCC, # CRL-1993	ATCC, # T1B-202	ATCC, # CRL-3216
Reference	(Storkus, Alexander, Payne, Dawson, & Cresswell, 1989)	(Tsuchiya et al., 1982)	(DuBridge et al., 1987; Pear, Nolan, Scott, & Baltimore, 1993)
Origin	Established from the peripheral blood of a female patient with plasma cell leukemia (ATCC, # CRL-1621), irradiated and selected for loss of MHC-I expression.	Established from the peripheral blood of a male infant with acute monocytic leukaemia (AML).	A variant of human embryonic kidney (HEK) 293T cells (ATCC, # CRL-1573), stably transfected with the SV40 T Ag. HEK293T were originally established from the embryonic kidney of a human fetus.
Characteristics	Lymphoblastoid cell line (LCL) that is fast growing in suspension.	Monocytic cell line that grows in suspension, is phagocytic, secreted lysozyme and can undergo differentiation into macrophage like cells.	Epithelial cell line that is adherent and readily transfected.
MR1 status	MR1 expressed at the cell surface when sensitized with Ag (Corbett et al., 2014; Eckle et al., 2014; Kjer-Nielsen et al., 2012; McWilliam et al., 2016).	MR1 expressed at the cell surface when sensitized with *E. coli* (Ussher et al., 2016; H. Wang et al., 2018).	MR1 present in the ER and associated with β_2m (Aldemir, 2008).

Table 10. TCR deficient T cell lines

	SKW-3	Jurkat RT3-T3.5	Jurkat76
Access	DSMZ, #ACC 53	ATCC, # T1B-153	Laboratory of Dr Mirjam Heemskerk
Reference	(Drexler, Gaedicke, & Minowada, 1985; Hirano et al., 1979)	(Ohashi et al., 1985)	(Heemskerk et al., 2003)
Origin	Established from the peripheral blood of a male patient with T cell chronic lymphocytic leukemia (CLL) and cross-contaminated with the cell line KE-37, established from a male patient with acute lymphoblastic leukemia (ALL).*	Mutant T cell line derived from the E6-1 clone of the Jurkat cell line (ATCC, # T1B-152), which was established from the peripheral blood of a male patient with acute T cell leukemia.	N/A.
TCR status	Deficient in TCR α- and β-chains.	Deficient in TCRβ mRNA, preventing TCR or CD3 surface expression (Ohashi et al., 1985). The presence of a TCR β-chain can lead to TCR heterodimer formation with the endogenous TCR α-chain, restoring TCR surface expression (Chung & Strominger, 1995; Ohashi et al.,	Deficient in TCR α- and β-chains.

		1985).**	
Co-receptor status	$CD8\alpha^+$ (low levels) and $CD4^+$ (low levels).	$CD8^-$ and $CD4^+$ (low levels).	No co-receptor surface expression detected.

* While SKW-3 cells are listed on the database of cross-contaminated or misidentified cell lines, where they are described as being contaminated with the KE-37 line, we have transfected specific TCR-encoding genes into these cells, which we then recloned and/or enriched by FACS for TCR expression. Following these steps, the cells were then validated for Ag recognition of cognate Ags in cell activation experiments.

** Since Jurkat RT3-T3.5 cells express an endogenous TCR β-chain that might in principle pair with the TCR α-chain following transduction with a TCR α- and β-chain, it is recommended to generate a control cell line, only transduced with the TCR α-chain and to evaluate this line alongside the cell lines transduced with both chains when assessing TCR surface expression, MR1 tetramer reactivity and functional capacity.

Table 11. Composition of SC for RF10 and DMEM10 media

SC ingredient	Ingredient amount added to 400 ml media	
	RPMI1640	DMEM
MEM non-essential Amino Acids Solution 10 mM (Gibco, cat. no. 11140-076)	100 ml	100 ml
Streptomycin	1 g	1 g
Benzylpenicillin	600 mg	600 mg
HEPES	11.91 g	11.91 g
L-glutamine	3 g	5.85 g
2-mercaptoethanol	35 µl	35 µl