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TEMPLATE for PROTOCOL UNIT

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Characterization and purification of mouse Mucosal-associated Invariant T (MAIT) cells

Running Title: Mouse MAIT cell flow cytometric analysis

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Significance Statement

MAIT cell-mediated immunity is increasingly recognised as being important for protection against pathogens. Additionally, MAIT cell modulation of other cell types can accelerate or ameliorate immunopathology. However, mouse MAIT cell research has been hampered by the relative infrequency of these cells in naïve wildtype mice. These detailed protocols, describing the use of mouse MR1-tetramers to specifically identify mouse MAIT cells, and models developed to boost their numbers and analyse their function in mice, are provided to facilitate MAIT cell research.

ABSTRACT

This unit describes the utility of various mouse models of infection and immunization for studying MAIT cell immunity: MAIT cells can be isolated from the lungs (or from other tissues/organs), identified and characterised using MR1-tetramers in combination with a range of antibodies by flow cytometry. The response kinetics, cytokine profiles and functional differentiation of lung MAIT cells are studied following infection with bacterial pathogens *Legionella longbeachae* or *Salmonella* Typhimurium, or immunisation with synthetic MAIT cell antigen plus Toll like receptor (TLR) agonist. MAIT cells enriched or expanded during the process can be used for further studies. Step by step protocols are listed for MAIT cell sorting and adoptive transfer. Mice can then be challenged and MAIT cells tracked and further examined.

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Basic Protocol 1: DETECTION AND CHARACTERISATION OF MOUSE LUNG MAIT CELLS BY FLOW CYTOMETRIC STAINING

Alternate Protocol 2: ASSESSMENT OF MAIT CELL FUNCTIONAL CAPACITY BY INTRACELLULAR CYTOKINE STAINING (ICS) AND TRANSCRIPTION FACTOR (TF) STAINING

Support Protocol 1: PREPARATION OF MR1-TETRAMER REAGENTS

Support Protocol 2: PREPARATION OF MAIT CELLS FROM BLOOD AND OTHER ORGANS FOR FLOW CYTOMETRY

Support Protocol 3: ENRICHMENT OF MAIT CELLS FROM BLOOD AND OTHER ORGANS FOR FLOW CYTOMETRY

Basic Protocol 2: MAIT CELL EXPANSION IN THE LUNGS OF SPF-HOUSED MICE USING BACTERIAL INFECTION

Alternate Protocol 2: MAIT CELL EXPANSION USING ANTIGEN AND TLR AGONISTS

Support Protocol 4: INTRANASAL INOCULATION OF MICE

Support Protocol 5: FACS SORTING MAIT CELLS AND ADOPTIVE TRANSFER

Support Protocol 6: BLEEDING (TAIL/SUBMANDIBULAR VEIN)

Support Protocol 7: INTRAVENOUS INJECTION VIA THE TAIL VEIN (including adoptive cell transfer)

Support Protocol 8: INTRAPERITONEAL INJECTION

Keywords: mucosal-associated invariant T (MAIT) cells; MR1-tetramers; flow cytometry; mouse models; respiratory infection

INTRODUCTION

Mucosal-associated Invariant T (MAIT) cells recognize and respond to vitamin B metabolite-based antigens presented on Major Histocompatibility Complex Class I-related molecule-1 (MR1) (Kjer-Nielsen et al., 2012). Specifically, the MAIT cell antigen 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) is formed from the vitamin B2 (riboflavin) biosynthesis precursor 5-amino-6-D-ribitylaminouracil (5-A-RU) and the small cellular metabolite, methylglyoxal (Corbett et

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al., 2014; Kjer-Nielsen et al., 2012). Riboflavin synthesis is an essential metabolic pathway in many microbes, including bacteria and fungi, and MAIT cells play a protective role in the clearance of at least some infections (D'Souza, Chen, & Corbett, 2018; A. Meierovics, Yankelevich, & Cowley, 2013; Wang et al., 2018). MAIT cells are present in most mammals, and reside in mucosal and other tissues, as well as in the circulation (Boudinot et al., 2016; Treiner et al., 2005). In humans, these cells are abundant (average 5% of T cells in blood) (Ben Youssef et al., 2018; Gherardin et al., 2018; Koay et al., 2016; Rahimpour et al., 2015), and their numbers have been reported to change in a number of disease states and infections (Chiba, Murayama, & Miyake, 2018; Cosgrove et al., 2013; Rouxel & Lehuen, 2018). Methods for the analysis of MAIT cells in human samples, and further information on MAIT cells are described in a parallel Protocol Manuscript: Characterisation of Human MAIT cells (Mucosal Associated Invariant T cells).

In contrast, in naïve wild-type mice held in a specific-pathogen-free facilities MAIT cells are relatively rare (Koay et al., 2016; Rahimpour et al., 2015), making it difficult to conduct detailed analyses and hence hampering the advance of MAIT cell studies. This unit describes the utility of various mouse models of infection and immunization for studying MAIT cell immunity. Mouse MR1-tetramer-based flow cytometric detection of MAIT cells isolated from the lungs of mice is described. This can be used in conjunction with other surface phenotypic markers or intracellular cytokine and transcription factor staining. To increase MAIT cell yields, infection with bacterial pathogens *Legionella longbeachae (Wang et al., 2018)* or *Salmonella* Typhimurium (Chen et al., 2017), or an immunization scheme (TLR agonist plus the MAIT cell antigen 5-OP-RU) can be used to first expand MAIT cells to large numbers (Chen et al., 2017; D'Souza, Pediongco, et al., 2018; Wang et al., 2018). Sufficient MAIT cells can then be functionally examined or sorted for adoptive transfer via which MAIT cells are further studied in the recipient mice. These protocols create a research platform for analysis and manipulation of lung MAIT cells in homeostasis and disease.

STRATEGIC PLANNING

Here we describe the methods used to investigate the anti-bacterial functions of MAIT cells *in vivo* in murine models. These protocols include:

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1. Basic Protocol-1, describing the detection and characterization of mouse MAIT cells isolated from the lungs by flow cytometry. Alternate Protocol-1 describes intracellular staining (ICS) for cytokines and transcription factors (TF). Support protocols for preparing MR1-tetramer reagents and the isolation and enrichment of single cells from other tissues are provided.
2. Basic Protocol-2, describing the expansion of MAIT cells in the lungs of SPF-housed mice by bacterial infection. Alternate Protocol-2 describes their expansion using synthetic antigen and TLR agonists. Support protocols include inoculation of mice and sorting of MAIT cells for adoptive transfer.

These protocols can be used in combination depending on the desired analysis and the research question being addressed. For example, surface staining of MAIT cells can be combined with methods to immunize mice or prime MAIT cells; adoptive transfer of MAIT cells from cytokine deficient mice can be used to assess the role of MAIT cell-derived cytokines in the context of infection. Thus, the timing of these experiments needs to be carefully planned.

There are **biosafety** and **ethical** considerations when handling infectious organisms and working with mouse models. Although guidance is provided here (see Commentary), researchers should follow the relevant legislation and guidelines applicable to their own institutions and laboratories. Permits will be required before work can commence.

BASIC PROTOCOL 1

DETECTION AND CHARACTERISATION OF MOUSE LUNG MAIT CELLS BY FLOW CYTOMETRIC STAINING

MAIT cells can be detected by flow cytometry in single cell preparations isolated from the lungs of mice using MR1-tetramer reagents in combination with a range of antibodies specific for cell-surface markers (Chen et al., 2017; D'Souza, Pediongco, et al., 2018; Koay et al., 2016; Rahimpour et al., 2015; Wang et al., 2018). Since MAIT cells are low in number in naïve C57BL/6 mice (representing only ~1% of T cells in the lungs (Chen et al., 2017; Rahimpour et al., 2015)) these protocols are

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typically used in the setting of bacterial infection models (described in Basic-Protocol-2). The protocols were developed on the basis of a pulmonary infection mouse model with *L. longbeachae* and they can apply to alternative pulmonary bacterial infections with minor adjustments as outlined below.

Materials

Naïve or infected adult C57BL/6 mice or other suitable strains of laboratory mice. Mice are of 6-12 weeks of age, if not specified otherwise. *3-5 mice are routinely used for one experiment to satisfy statistical requirements as one mouse provides one data point.*

Mr1$^{-/-}$ mice (number, age and gender matched to C57BL/6) (Chen et al., 2017; Treiner et al., 2003) (MAIT deficient mice on a C57BL/6 genetic background) (optional)

Dissecting instruments

10 ml syringe with 23- or 26-gauge needle

Tubes (1.7 ml: Axygen, Cat#MCT-175-C; 10 ml: SARSTEDT, Cat#62.9924.284; 50 ml: FALCON, Cat#227261; FACS tube: FALCON, Cat#352008 and 352054 (sorting))

Petri dishes (Cellstar, Cat#639160)

Transfer pipettes

1 ml syringe plungers

70 μm cell strainers (Biostrategy, Cat#BDAA352350)

Scalpel blades (size 23)

Ice box

Collagenase medium: (See Reagents and Solutions)

Fluorescence activated flow cytometry (FACS) buffer: see recipe

Roswell Park Memorial Institute (RPMI) medium

Tris-based Ammonium Chloride (TAC) hypotonic Red Blood Cell (RBC) lysis buffer: see recipe

FACS Fixation buffer (FACS fix): see recipe

MR1-5-OP-RU and MR1-6-FP monomers (Corbett et al., 2014; Reantragoon et al., 2013): available from the NIH tetramer core facility, on application. These are tetramerized

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prior to use (see Support Protocol-1) or can be ordered as tetramers conjugated to PE, BV421 or APC fluorochromes (see Internet Resources).

Antibodies specific for mouse cell surface markers:

1. CD19-PerCP-Cy5.5; BD Bioscience; Cat#: 561113; clone #: ID3
2. TCRβ-APC; BD Bioscience; Cat#: 553174; clone #: H57-597
3. CD45.2-FITC; BD Bioscience; Cat#: 553772; clone #: 104
4. CD4-APCcy7; BD Bioscience; Cat#: 561830; clone #: GK1.5 (RUO)
5. CD8α-PE; BD Bioscience; Cat#: 553032; clone #: 53-6.7

To reduce background staining, anti-CD16/32 (Biburger, Trenkwald, & Nimmerjahn, 2015) (FcγRIII, 2.4G2 hybridoma) culture supernatant is used as a diluent for the antibody mix. Dead cells are gated out with staining of 7-aminoactinomycin D (7-AAD, Sigma, Cat#:A9400) or alternative live-dead marker

CO_2 chamber connected to a regulator with a flowrate reading or alternate method for euthanizing mice

Refrigerated centrifuge

Flow cytometer with capacity for ≥ 9 fluorochromes. (e.g. BD Fortessa)

Cuvettes

Spectrophotometer (Eppendorf BioPhotometer or equivalent)

Trypan blue

Hemocytometer

Inverted light microscope

Protocol Steps:

1. Euthanise mice by CO_2 asphyxiation:

   a) Take animal cage to CO_2 point in laboratory. If only some animals in a cage are to be killed, transfer these animals to a clean cage with paper towel on the floor. All steps are performed at RT.

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b) Set the CO_2 flow rate to 20% of the chamber volume per minute (acceptable range 15- 25%). This is 5 l/min for our cages.

c) Place Perspex lid over cage and allowing cage to fill with carbon dioxide. Observe the animals once CO_2 flow commences. They should not appear overtly distressed. Within 2 min the animals should become recumbent.

d) Mice must be kept in the chamber for at least 5 min or 1 min after visible cessation of breathing.

Assess mice for loss of consciousness and indicators of death immediately after removal from the chamber. A combination of methods should be used to ensure loss of consciousness including a firm toe pinch, lack of visible respiration, lack of palpable heartbeat, grey mucous membranes and loss of the corneal reflex.

2. Cut the chest cage and exposed the heart and lungs (avoid nicking lung lobes). Perfuse the mouse through the right ventricle using 10 ml cold RPMI loaded in a syringe with a 26 or 23-gauge needle. A similar protocol is available elsewhere (Lancelin & Guerrero-Plata, 2011).

A successful perfusion will result in the lungs ballooning up and appearing pale as blood is flushed out. Safety glasses should be worn.

3. Remove the lung lobes carefully (avoid the thymus) with dissection instruments and place the lungs into ice-cold 2 ml of RPMI.

A spleen or a few lymph nodes can be collected for use as staining controls. Splenocytes or lymphocytes are good sources of cells for making single colour flow cytometric compensation controls.

4. In the laboratory, prewarm collagenase medium and shaking incubator to 37 °C.
5. Chop lungs into fine pieces using a scalpel blade (size 23).

Lungs can be weighed before being chopped if bacterial loads (CFU counts) are to be evaluated against tissue weight (grams). However, lung perfusion should not be conducted as the process would change the physiological weight of lungs. The chopping and mixing step is recommended as intranasal infection/immunisation could be patchy or only in one lobe. The chopping and mixing will avoid biased sampling and result in least individual variation in both MAIT cell enumeration and

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bacterial load estimation (CFU counts). Change scalpel blade at least between sample groups, to avoid cross-contamination.

6. Place lung tissue from each mouse into a 1.5 ml Eppendorf tube containing 0.5-1 ml/lung of prewarmed collagenase/DNAse medium. Incubate tubes on their sides in a shaking incubator at 37 °C, 100-180 rpm, for 90 min.

Half of the lung tissue from each mouse yields sufficient cell numbers for FACS analysis. This is not only to save reagents, but also to spare the other half lung for other assays (e.g. homogenisation for bacterial load or tissue cytokine profiling).

7. During the digestion, prepare single colour controls, tetramer blocking mix and tetramer-antibody staining mix (see recipes).

It is convenient for single colour staining controls to use part of a spleen or lymph nodes pushed through a 70 μm cell strainer. For spleen (not needed for lymph nodes) cells are then resuspended in 5 ml TAC (hypotonic RBC lysis buffer) for 5 min at 37 °C, then washed once with 5 ml FACS buffer. Single colour controls are prepared by staining an aliquot of splenocytes or cells from lymph nodes prepared as for MAIT cell staining, but separately, with individual antibodies, MR1-5-OP-RU tetramer-BV421 and 7-AAD in separate tubes. For small populations of cells or antibodies where a clear population of cells may not be evident, an alternative antibody (e.g. CD3) can be used with the same fluorochrome conjugate. Flow Cytometry Compensation Beads (Invitrogen, Cat#01-2222-42) can be used as an alternative.

8. After 90 min, gently push the digested tissue through a 70 μm cell strainer into a petri dish with the plunger from a 1 ml syringe. Rinse the residual sample with extra buffer for maximum cell yield.

Cells from multiple lungs (if required) can be pooled into a single 50 ml Falcon with a total of 50 ml of sterile FACS buffer.

9. Centrifuge at 500 x *g* for 5 min at 4 °C to pellet the cells. Tip off supernatant.

10. (optional) Add 2 ml prewarmed TAC to each lung, mix well and incubate in a 37 °C water bath for 5 min to lyse RBCs, then top up with 3 ml cold FACS buffer before centrifugation at 500 x *g* for 5 min.

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If lung perfusion has been successful in step 2 few RBC should remain and this step may be omitted.

11. Tip off supernatant and resuspend cells in 5 ml of FACS buffer.

12. Transfer 1–1.5x 10^6 cells per sample into a FACS tube.

Cells numbers can be determined by counting cells with a hemocytometer or estimated by measuring the $OD_{600\,nm}$ of a representative sample from each experimental group. The constant for converting an $OD_{600\,nm}$ reading to cell concentration needs to be established beforehand.

13. Centrifuge at 500 x *g* for 5 min at 4 °C to pellet the cells and remove supernatant.

14. Add 20 µl of Tetramer-antibody blocking mix (material list) and incubate for 15 min at RT, then without centrifugation, add 40 µl Tetramer-antibody staining mix (material list), mix well and incubate at RT in the dark for a further 30 min.

7-AAD will be used for gating out dead cells (see Figure 1).

[*Insert Figure 1 near here]

15. Wash cells twice with FACS buffer and resuspend cells in 150-200 µl FACS fix (to kill any remaining bacteria and maintain sample staining).

16. (optional) Estimate the total number of cells (including MAIT cells) from the assayed organ, by adding 30,000 Counting Beads (CB) (BD Bioscience SpheroTM calibration particles, 6.0–6.4 µm in size, Cat #556296) to the FACS tube containing samples ready for FACS. The actual CB concentration is calculated with a hemocytometer under a microscope prior to each experiment.

The numbers of CB and MAIT cells acquired will be shown in FlowJo software (Treestar) during data analysis via separate gating. As it is a homogenous solution, the proportion of CB and MAIT cells acquired during data acquisition will be the same. Therefore:

- *The # of CB acquired divided by 30,000 = the # of MAIT cell acquired, divided by the total # of MAIT cells in the tube.*
- *Therefore, the total # of MAIT cells in the tube = 30,000 times the # of MAIT cell acquired, divided by the # of CB acquired.*

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- *With the amount (X μl) taken for tetramer-antibody staining from the total volume (Y μl) of single cell suspension from the assayed organ, the total number of MAIT cells is estimated as: the total # of MAIT cells in the tube multiplied by Y, divided by X. In case only half the organ is used, multiply by 2 to estimate the # of MAIT cells in the whole organ.*
- *Calculation of total cell # of other subsets (e.g. $CD4^+$, or $CD8^+$ T cells as internal controls) is the same as above.*

17. Immediately before FACS, pass samples through a 100 μl nylon mesh to avoid potential blockage of flow cytometer.

To avoid using many expensive commercial cell strainers for filtering cells, it is possible to buy large sheets of 100 μm nylon mesh. Rectangles can be cut to size to cover a FACS tube for each sample.

18. Analyse samples immediately on the flow cytometer or store them overnight at 4 °C, in the dark, before analysis.

19. Refer to Figure 1 for the cell gating strategy for FACS.

Beads and lymphocytes were gated by forward versus side scatter, and single cells gated by scatter area versus height. 7-AAD^+ or FVD^+ dead cells were excluded from the $CD45^+$ population, and $CD19^+$ B cells were excluded from $TCR\beta^+$ population. Autofluorescent cells were excluded by displaying cells with BV525 versus BV585 channels, which flank BV421 in their emission spectra. Mouse MAIT cells were finally defined as $7AAD^-$, $CD45.2^+$, $CD19^-$, $TCR\beta^+$, MR1-5-OP-RU $tetramer^+$ population after exclusion of auto-fluorescence.

If available, it is useful to include one or more samples from an $Mr1^{-/-}$ mouse (MAIT cell-deficient) as a comparison. These mice should have no events in the MAIT cell gate (occasionally a few "background" events will be seen).

ALTERNATE PROTOCOL 1

ASSESSMENT OF MAIT CELL FUNCTIONAL CAPACITY BY INTRACELLULAR CYTOKINE STAINING (ICS) AND TRANSCRIPTION FACTOR (TF) STAINING

In order to study the cytokines produced or transcription factors expressed, MAIT cells are analysed for the expression of intracellular or intranuclear molecules. The antibody/tetramer staining protocols (Basic Protocol-1) are modified to achieve the best staining.

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The permeabilization/fixation steps may denature some proteins affecting the binding of some antibodies; therefore, surface staining is performed separately prior to the staining of intracellular or intranuclear molecules. It is worth noting that the extra steps for permeabilization/fixation appears to affect the tetramer staining. As such a higher concentration of tetramer may be required. It is recommended that each user titrate the MR1-tetramers. Stomach samples from naïve mice are suitable for intranuclear transcription factor staining in our hands but consistent intracellular cytokine staining of stomach samples has not yet been achieved (most likely due to some unidentified ingredients in the buffers used for separation of stomach MAIT cells). MAIT cells from other tissues (Support Protocol-2) can be used for both ICS or TF staining.

Materials

Naïve or infected adult C57BL/6 mice or other suitable strains of laboratory mice. Mice are of 6-12 weeks of age, if not specified otherwise. 3-5 mice are routinely used for one experiment to satisfy statistical requirements as one mouse provides one data point.

Mr1$^{-/-}$ mice (number, age and gender matched to C57BL/6) (Chen et al., 2017; Treiner et al., 2003) (MAIT cell-deficient mice on a C57BL/6 genetic background) (optional)

Dissecting instruments

10 ml syringe with 23- or 26-gauge needle

Tubes (1.7 ml: Axygen, Cat#MCT-175-C; 10 ml: SARSTEDT, Cat#62.9924.284; 50 ml: FALCON, Cat#227261; FACS tube: FALCON, Cat#352008 and 352054 (sorting))

Petri dishes (Cellstar, Cat#639160)

Transfer pipettes

1 ml syringe plungers

70 μm cell strainers (Biostrategy, Cat#BDAA352350)

Scalpel blades (size 23)

Ice box

Collagenase medium: (See Reagents and Solutions)

Fluorescence activated flow cytometry (FACS) buffer: see recipe

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Roswell Park Memorial Institute (RPMI) medium

Tris-based Ammonium Chloride (TAC) hypotonic Red Blood Cell (RBC) lysis buffer: see recipe

FACS Fixation buffer (FACS fix): see recipe

MR1-5-OP-RU and MR1-6-FP monomers(Corbett et al., 2014; Reantragoon et al., 2013): available from the NIH tetramer core facility, on application. These are tetramerized prior to use (see Support Protocol-1) or can be ordered as tetramers conjugated to PE, BV421 or APC fluorochromes (see Internet Resources).

Antibodies specific for mouse cell surface markers and ICS (cytokines):

1. CD19-PerCP-Cy5.5; BD Bioscience; Cat#: 561113; clone #: ID3
2. TCRβ-APC; BD Bioscience; Cat#: 553174; clone #: H57-597
3. CD45.2-FITC; BD Bioscience; Cat#: 553772; clone #: 104
    - Anti-IFNγ-PE-Cy7; BD Bioscience; Cat#: 557649; clone: XMG1.2
    - Anti-IL-17-PE; BD Bioscience; Cat# clone: 559502; TC11-18H10;

Antibodies specific for mouse cell surface markers and Transcription factors:

1. CD19-PerCP-Cy5.5; BD Bioscience; Cat#: 561113; clone #: ID3
2. TCRβ-PE; BD Bioscience; Cat#: 561081; clone #: H57-597
3. CD45.2-FITC; BD Bioscience; Cat#: 553772; clone #: 104
    - Anti-T-bet-PE-Cy7; eBioscience; Cat#: 25-5825-82; clone#: 4B10
    - RORγt-APC; eBioscience; Cat#: 17-6981-82; clone# B2D

To reduce background staining, anti-CD16/32 (Biburger et al., 2015) (FcγRIII, 2.4G2 hybridoma) culture supernatant is used as a diluent for the antibody mix. Dead cells are gated out with staining of Fixable viability dye or alternative live-dead marker

Fixation/ Permeabilization Kit (BD, Cat#555028), containing

- Fixation/permeabilization buffer
- Fixation wash buffer
- Golgi plug (Brefeldin A, can be purchased separately, BD, Cat #555029)

Fixable viability dye (FVD-e780, InvitroGen, Cat#65-0865-14) or 7-AAD (Sigma, Cat#A9400)

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Phorbol myristate acetate (PMA)

Ionomycin

CO_2 chamber connected to a regulator with a flowrate reading or alternate method for euthanizing mice

Refrigerated centrifuge

Flow cytometer with capacity for ≥ 9 fluorochromes. (e.g. BD Fortessa)

Cuvettes

Spectrophotometer (Eppendorf BioPhotometer or equivalent)

Trypan blue

Hemocytometer

Inverted light microscope

Protocol Steps:

1. Prepare single cell suspensions as described in Basic protocols-1 from step 1 to 11. For ICS, treat cells with Golgi plug (with active component brefeldin A, provided in the aforementioned kit, final dilution 1:1000) or brefeldin A (10 μg/ml) during all processing steps to inhibit transport processes leading to the accumulation of cytokines intracellularly, except steps for cell washing at 4 ^{o}C. For ICS staining, keep samples cold for all steps except for the collagenase digestion.
2. Incubate cells at 37 °C, 5% CO_2 for 3-4 hr to enrich cytokine production.

(For ICS only, not needed for TF staining): Incubate cells at 37 °C, 5% CO_2 for 3-4 hr to enrich cytokine production. Positive control samples can be stimulated with phorbol 12-myristate 13-acetate (PMA) and Ionomycin (final concentrations 20 ng/ml and 1 μg/ml, respectively) during this incubation step. Isotype controls for each cytokine-specific antibody may be included.

3. Wash cells twice with ice cold 1 ml PBS to remove medium (each wash involved adding the PBS, centrifugation at 500 x *g* for 5 min at 4 °C to pellet the cells and removing the supernatant by pouring off).
4. Resuspend cells with 0.5-1 ml fixable viability dye (FVD-e780) solution (1:1000) and incubate on ice for 40 min in the dark.

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5. Wash cells with 1 ml FACS buffer.

6. Add 20 μl tetramer-antibodies blocking mix and incubate for 15 min at RT, then add 40 μl Tetramer-antibody staining mix (containing Ab against surface markers), mix well and incubate at RT in the dark for a further 30 min.

7. Resuspend cells with 0.5-1 ml fix/permeabilization solution (BD, Cat#555028), mix well and incubate on ice for 40-60 min in the dark.

8. Wash cells twice with 1 ml Permeabilization wash buffer.

9. Resuspend cells with 100 μl Permeabilization wash buffer containing antibodies with appropriate conjugates (e.g. ICS: anti-IFNγ-APC-Cy7 (BD, Cat#581479); anti-IL-17-PE (BD, Cat#559502), or TF staining: anti-T-bet (eBioscience, Cat#25-5825-82); anti-RORγt (eBioscience, Cat#17-5981-82).

10. Stain the cells for at least 1 hr or overnight at 4 ^{o}C in the dark.

11. Wash cells twice with 1 ml Permeabilization wash buffer.

12. Resuspend pellets with 150-200 μl FACS buffer, and samples are ready for FACS analysis.

Steps 6-12 are modified from an ICS commercial kit manual (FOXP3/Transcriptional Factor staining buffer set, BD (InvitroGen, Cat#00-5523-00).

At this stage, you should have the following samples:

- *Single colour controls (cells from spleen or lymph node)*
    - *Unstained cells from spleen or lymph node, to be used for setting voltage, side/forward scatter values.*
    - *Cells stained separately with each individual antibody used to identify MAIT cells. In case of intracellular staining, an alternative antibody specific to CD4 or CD8, conjugated with the same fluorochrome can be used. These controls will be used for compensation set up of the flow cytometer.*
    - *Infected lung cells stained with MR1-5-OP-RU tetramer-BV421, for BV421 channel compensation.*

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- *7-AAD stained cells, for 7-AAD channel compensation to gate out dead cells. 7-AAD excited at 488 nm and emits at a maximum wavelength of 647 nm. Avoiding using antibody-conjugate fluorochrome with an emission close to 647 nm.*

- *Negative controls:*
  - *Cells without stimulation, only minimal background staining is expected for the cytokine stains.*
  - *Isotype controls for each cytokine-specific antibody. Minimal staining from antibody stickiness expected.*
- *Positive controls: stimulated with PMA and Ionomycin. Clear T cell populations from both MAIT and conventional T cells, stained with cytokine-specific antibody are expected, as the stimulation activates all T cells indiscriminately.*
- *Test samples:*
  - *Naïve samples: Compared with isotype controls, one may expect proportionally (i.e. %) more MAIT cells than conventional T cells to be stained with cytokine-specific antibodies, as MAIT cells are innate-like T cells which may have small amounts of premade cytokines (e.g IL-17) stored intracellularly, while the majority of conventional T cells do not.*
  - *Infected samples: In the case of Salmonella infection, one may expect more T cells than naïve T cells to be stained with cytokine-specific antibodies,*

SUPPORT PROTOCOL 1

PREPARATION OF MR1-TETRAMER REAGENTS

The NIH tetramer core facility provides mouse 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. Alternatively, the NIH tetramer core facility provides biotinylated MR1 monomers which can be conjugated to commercially available Streptavidin-Fluorochrome. See Internet Resources for details.

Materials

MR1-5-OP-RU and MR1-6-FP biotinylated monomers

Streptavidin (Bioscientific, Cat#CLPR0791)

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Tris-buffered saline (TBS): see recipe

Streptavidin-PE (BD, Cat#554061 0.5 mg/ml) or Streptavidin-BV421 (Biolegend, Cat#405225 0.5 mg/ml)

Pipettes with 20 µl and 10 µl tips

Ice box

Protocol Steps:

1. Expand a 5 µg aliquot of biotinylated MR1-5-OP-RU monomer or MR1-6-FP monomer to 18 µl volume in TBS. Dilute 6.8 µl of commercially available streptavidin-PE (at 0.5 mg/ml) or unlabelled streptavidin to 17 µl final volume. In case the monomers are already diluted, adjust the amount of streptavidin TBS diluent accordingly.
2. Add 1/10th of the streptavidin-PE solution (1.7 µl) to the monomer solution and pipette to mix. Place the remaining streptavidin-PE solution on ice.

This is done to ensure maximal production of tetramers and minimal production of dimers or trimmers. There is an excessive ratio (>4:1) of MR1-monomers particularly during the early steps, which warrants every streptavidin-PE molecule to conjugate with four MR1-monomers, therefore most final products are tetramers. The volumes can be scaled up for larger aliquots.

3. Incubate at room temperature for 10 min in the dark.
4. Repeat until all the streptavidin-PE solution has been added. This will give a final volume of 35 µl containing 0.143 µg/µl of tetramer.

We commonly use Streptavidin-Fluorochrome conjugates to PE or BV421 which are very bright, but other fluorochrome conjugates can also be used.

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 (~46 kDa) to Streptavidin-Fluorochrome (~57 kDa for Streptavidin). We typically find

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the optional ratio is 1:8-1:10 (Streptavidin-Fluorochrome: MR1-monomer), rather than the theoretical ratio of 1:4.

MR1-5-OP-RU or MR1-6-FP monomers should be stored at -80℃ until ready for use, at which point small aliquots can be tetramerized and stored at 4 ℃ and protected from light for at least 1 month.

It is recommended to perform a titration experiment to calculate the optimal final concentration of the freshly made tetramers prior to use: Lung cells are stained with MR1-5-OP-RU in serial dilutions (1:200, 1:400, 1:600, 1:800, 1:1000, 1:1600). The optimal final concentration is defined with the highest ratio of tetramer staining (MFI) of MAIT (specific staining) over conventional T cells (background). Typically, a dilution of 1:200-1:800 of a stock with a concentration of 0.143 μg/μl can be used. Higher concentrations may be needed in experiments that involve intracellular cytokine staining.

SUPPORT PROTOCOL 2

PREPARATION OF MAIT CELLS FROM BLOOD AND OTHER ORGANS FOR FLOW CYTOMETRY

Materials

Naïve or infected adult C57BL/6 mice or other suitable strains of laboratory mice. Mice are of 6-12 weeks of age, if not specified otherwise. 3-5 mice are routinely used for one experiment to satisfy statistical requirements as one mouse provides one data point. In case of preparing MAIT cells for adoptive transfer, multiple same organs can be pooled and processed together.

Dissecting instruments

Dissecting board and pins

80% v/v ethanol

10 ml syringe with 21- or 23-gauge needle

Tubes (1.7 ml: Axygen, Cat#MCT-175-C; 10 ml: SARSTEDT, Cat#62.9924.284; 50 ml: FALCON, Cat#227261; FACS tube: FALCON, Cat#352008 and 352054 (sorting))

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Petri dishes (Cellstar, Cat#639160)

Transfer pipettes

1 ml syringe plungers

70 μm cell strainers (Biostrategy, Cat#BDAA352350)

Scalpel blades (size 23)

Ice box

Collagenase medium: (See Reagents and Solutions)

Fluorescence activated flow cytometry (FACS) buffer: see recipe

Roswell Park Memorial Institute (RPMI) medium

Tris-based Ammonium Chloride (TAC) hypotonic Red Blood Cell (RBC) lysis buffer: see recipe

FACS Fixation buffer (FACS fix): see recipe

MR1-5-OP-RU and MR1-6-FP monomers (Corbett et al., 2014; Reantragoon et al., 2013): available from the NIH tetramer core facility, on application. These are tetramerized prior to use (see Support Protocol-1) or can be ordered as tetramers conjugated to PE, BV421 or APC fluorochromes (see Internet Resources).

Antibodies specific for mouse cell surface markers:

1. CD19-PerCP-Cy5.5; BD Bioscience; Cat#: 561113; clone #: ID3
2. TCRβ-APC; BD Bioscience; Cat#: 553174; clone #: H57-597
3. CD45.2-FITC; BD Bioscience; Cat#: 553772; clone #: 104
4. CD4-APCcy7; BD Bioscience; Cat#: 561830; clone #: GK1.5 (RUO)
5. CD8a-PE; BD Bioscience; Cat#: 553032; clone #: 53-6.7.

To reduce background staining, anti-CD16/32 (Biburger et al., 2015) (FcγRIII, 2.4G2 hybridoma) culture supernatant is used as a diluent for the antibody mix. Dead cells are gated out with staining of 7-aminoactinomycin D (7-AAD, Sigma, Cat#:A9400) or alternative live-dead marker.

CO_2 chamber connected to a regulator with a flowrate reading or alternate method for euthanizing mice

Refrigerated centrifuge

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Flow cytometer with capacity for ≥ 9 fluorochromes. (e.g. BD Fortessa)

Cuvettes

Spectrophotometer (Eppendorf BioPhotometer or equivalent)

Trypan blue

Hemocytometer

Inverted light microscope

Percoll gradients: 33.75% v/v, 40% v/v and 70% v/v solutions: see recipe (for liver and kidney only)

Steel mesh strainers (for liver and kidney only)

Stomach digestion buffer (for stomach only): see recipe

Protocol Steps:

1. Perform CO_2 euthanasia *(as described in Basic protocol-1)*
2. Following euthanasia, place the mouse on a dissecting board and douse with 80% v/v ethanol. Lift the abdominal skin and make a small incision with scissors at the base of the sternum. With your fingers, peel the skin over the forelimbs, hindlimbs and neck of the mouse.

Liver:

1. Expose liver by cutting the abdomen wall with scissors and forceps. Gently push the intestine to the left side. Locate the portal vein by gently flipping up the liver lobes. Perform liver perfusion by injecting 10 ml of ice-cold PBS or RPMI via the portal vein, with a 26-gauge needle. Successful perfusion will lead a colour change of the liver lobes: dark red to pale (sand coloured). Gallbladder removed before livers are harvested and kept in cold RPMI medium for transport to the laboratory.
2. Gently push the liver through a stainless-steel mesh and wash cells twice with 50 ml ice cold PBS.
3. Enrich liver lymphocytes with Percoll gradient centrifugation:

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a. Resuspend cells in 20 ml 33.75% Percoll. Underneath this layer, use a transfer pipette to layer 20 ml 70% v/v Percoll.
b. Centrifuge this gradient at 900 x *g* 20 °C for 20 min at room temperature with the centrifuge brake OFF. Lymphocytes and other immune cells will form a visible interphase thin layer between 33.75% and 70% v/v Percoll post centrifugation.
c. During this spin prepare single-colour controls *(staining an aliquot of splenocytes or cells from lymph nodes prepared separately, the same way as for MAIT cell staining, but with individual antibodies in separate tubes)* and tetramer/antibody mix.
d. Collect the interphase between 33.75% and 70% v/v Percoll into a fresh 50 ml Falcon and top up with FACS buffer.
e. Continue from step 9 in Basic Protocol-1 to finish staining.

Kidney:

1. Expose kidneys by cutting the abdomen wall with scissors and forceps, and harvest the kidneys by gentle dissection.
2. Preparation is the same as for liver except a 40/70% v/v Percoll gradient is used. Perfusion prior to harvesting the kidneys is not necessary.

Stomach:

3. Harvest the stomach by cutting the oesophagus and duodenum (the beginning of small intestine). Keep the upper forestomach intact.
4. Cut open the stomach along the midline using a scalpel, remove content (food), rinse then keep the stomach in RPMI.
5. Transfer the stomach to a petri dish and perfuse with stomach digestion buffer by inserting a needle (27G) near the limiting ridge and flushing with buffer until the stomach inflates (~8 ml buffer per stomach).

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6. Transfer the inflated stomach and buffer into a 15 ml tube and incubate at 37 ^{o}C for 15 min in a shaking incubator.

7. Roughly chop into small pieces and pass fluid through a 70 μm cell strainer.

8. Collect cells by centrifuging at 1400 rpm for 5 min at 4^{o}C.

9. Wash cells with FACS buffer and use for further staining as per Basic Protocol-1.

Stomach lymphocyte preparation protocols are also described in detail elsewhere (D'Souza, Pediongco, et al., 2018; Ng & Sutton, 2015).

10. At this stage ensure that you should have the following samples:

- *Single colour controls (cells from spleen or lymph node)*
    - *Unstained cells from spleen or lymph node, to be used for setting voltage, side/forward scatter values.*
    - *Cells stained with individual antibodies used to identify MAIT cells. In case of intracellular staining, an alternative antibody specific to CD4 or CD8, conjugated with the same fluorochrome, will be used. These controls will be used for compensation.*
    - *Lung cells stained with MR1-5-OP-RU tetramer-BV421, for BV421 channel compensation.*
    - *7-AAD stained cell, for 7-AAD channel compensation to gate out dead cells. It is excited at 488 nm and emitting at a maximum wavelength of 647 nm. Avoiding using antibody-conjugate fluorochrome with an emission close to 647nm.*
- *Negative controls:*
    - *Cells without stimulation, only background staining expected.*
    - *Isotype controls for each cytokine-specific antibody. Minimal staining from antibody stickiness expected.*
- *Positive controls: stimulated with PMA and Ionomycin. Clear T cell populations from both MAIT and conventional T cells, stained with cytokine-specific antibody expected, as the stimulation activates all T cells indiscriminately.*
- *Test samples:*
    - *Naïve samples: Compared with isotype controls, one may expect proportionally (e.g. %) more MAIT cells than conventional T cells stained with cytokine-specific antibody,*

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as MAIT cells are all innate-like T cells and might have small amount of premade cytokines stored intracellularly, while majority of conventional T cells do not.

- *Infected samples: In case of Salmonella infection, one should expect significantly more MAIT cells than naïve MAIT cells to be stained with cytokine-specific antibody*

SUPPORT PROTOCOL 3

ENRICHMENT OF MAIT CELLS FROM BLOOD AND OTHER ORGANS FOR FLOW CYTOMETRY

In order to study rare MAIT cells in naïve wild-type mouse organs, a tetramer-associated magnetic enrichment (TAME) can be employed to enhance detection. Here we describe the TAME method using naïve C57BL/6 thymus where MAIT cells represent only ~0.05% of T cells (Koay et al., 2016; Rahimpour et al., 2015). This protocol describes the use of PE-conjugated MR1-5-OP-RU tetramer enrichment using anti-PE microbeads, but can be adapted to use with the multiple fluorochrome options available via MACS technology. It is highly recommended that researchers familiarise themselves with detailed tools, resources and datasheets from MACS (See Internet Resources) to determine the most suitable enrichment strategy. Expected results are shown in Figure 2.

[*Insert Figure 2 near here]

Materials

Naïve or infected adult C57BL/6 mice or other suitable strains of laboratory mice. Mice are of 6-12 weeks of age, if not specified otherwise. 3-5 mice are routinely used for one experiment to satisfy statistical requirements as one mouse provides one data point.

Mr1$^{-/-}$ mice (number, age and gender matched to C57BL/6) (Chen et al., 2017; Treiner et al., 2003) (MAIT cell-deficient mice on a C57BL/6 genetic background) (optional)

Dissecting instruments

10 ml syringe with 21- or 23-gauge needle

Tubes (1.7 ml: Axygen, Cat#MCT-175-C; 10 ml: SARSTEDT, Cat#62.9924.284; 50 ml: FALCON, Cat#227261; FACS tube: FALCON, Cat#352008 and 352054 (sorting))

Petri dishes (Cellstar, Cat#639160)

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Transfer pipettes

1 ml syringe plungers

70 μm cell strainers (Biostrategy, Cat#BDAA352350)

Scalpel blades (size 23)

Ice box

Collagenase medium: (See Reagents and Solutions)

Fluorescence activated flow cytometry (FACS) buffer: see recipe

Roswell Park Memorial Institute (RPMI) medium

Tris-based Ammonium Chloride (TAC) hypotonic Red Blood Cell (RBC) lysis buffer: see recipe

FACS Fixation buffer (FACS fix): see recipe

MR1-5-OP-RU and MR1-6-FP monomers (Corbett et al., 2014; Reantragoon et al., 2013): available from the NIH tetramer core facility, on application. These are tetramerized prior to use (see Support Protocol-1) or can be ordered as tetramers conjugated to PE, BV421 or APC fluorochromes (see Internet Resources).

Antibodies specific for mouse cell surface markers:

1. CD19-PerCP-Cy5.5; BD Bioscience; Cat#: 561113; clone #: ID3
2. TCRβ-APC; BD Bioscience; Cat#: 553174; clone #: H57-597
3. CD45.2-FITC; BD Bioscience; Cat#: 553772; clone #: 104
4. CD4-APCcy7; BD Bioscience; Cat#: 561830; clone #: GK1.5 (RUO)
5. CD8a-PE; BD Bioscience; Cat#: 553032; clone #: 53-6.7.

To reduce background staining, anti-CD16/32(Biburger et al., 2015) (FcγRIII, 2.4G2 hybridoma) culture supernatant is used as a diluent for the antibody mix. Dead cells are gated out with staining of 7-aminoactinomycin D (7-AAD) or alternative live-dead marker

CO_2 chamber connected to a regulator with a flowrate reading or alternate method for euthanizing mice

Refrigerated centrifuge

Flow cytometer with capacity for ≥ 9 fluorochromes. (e.g. BD Fortessa)

Cuvettes

Spectrophotometer (Eppendorf BioPhotometer or equivalent)

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Trypan blue

Hemocytometer

Inverted light microscope

Magnetic-activated cell sorting (MACS) buffer: phosphate buffered saline (PBS), 0.5% bovine serum albumin (BSA) and 2 mM EDTA.

Anti-PE MACS® MicroBeads for magnetic labelling of cells (Miltenyi Biotec, Cat#: 130-048-801)

MACS® LS Columns (Miltenyi Biotec, Cat#: 130-042-401)

MACS® Separator with LS column adaptor (Miltenyi Biotec, Cat#: 130-091-051)

Protocol Steps:

1. Dissect the thymus from the mouse, and gently push the tissue through a 70 μm cell strainer into a 10 ml tube with a syringe plunger to create a single cell suspension. Rinse the strainer with FACS buffer for maximum cell yield.

Although a thymus from a 6-8 week old C57BL/6 mouse will generally yield approximately 1–1.5x 10^8 cells, thymii sizes and cell yields vary widely with mouse age and genotype. Multiple thymii can be pooled to increase yield.

2. Centrifuge 500 x *g* for 5 min to pellet the cells. Pour off supernatant and resuspend cells in FACS buffer.

3. Set 2 x 10^6 cells aside as a pre-enriched sample.

4. Resuspend the rest of the thymus in 10^7 cells per ml of FACS buffer, add PE-conjugated MR1-tetramer (see Support Protocol-1), mix well and incubate for 30 min at RT.

The amount used is dependent on the optimal final concentration determined by serial dilution (Support Protocol-1), and is scaled relative to total ml of FACS buffer (i.e. if staining 5x10^7 in 5 ml, and tetramer titration was determined to be 1:600, 8.33 μl of tetramer will be needed).

5. Wash cells twice with 5 ml FACS buffer (each wash involves adding buffer, centrifugation at 500 x *g* for 5 min at 4 °C and pouring off supernatant) and resuspend cells in 80 μl of MACS buffer per 10^7 total cells.

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6. Add 20 µl of Anti-PE MACS® MicroBeads per 10^7 total cells by following manufacturer's instructions, mix well and incubate for 30 min at 4 °C.

7. Wash cells twice with MACS buffer and resuspend up to 10^8 cells in 5 ml of MACS buffer.

8. Prepare rinsed LS columns as per manufacturer's instructions and place a flow-through collection tube under the LS column.

9. Apply cell suspension onto the column.

10. Wash column with 3 ml of MACS buffer after the unlabelled cells have passed into the flow-through. Perform washing steps by adding 3 ml of MACS buffer three times, adding new buffer only when the column reservoir is empty.

11. Remove LS column from the separator and place it on a new 10 ml collection tube.

12. Apply 5 ml of MACS buffer, immediately flush out magnetically labelled cells by firmly pushing the LS plunger into the LS column.

13. Centrifuge 500 x *g* for 5 min to pellet the cells. Suction off the supernatant and resuspend cells in FACS buffer for flow cytometry without further staining with PE-conjugated MR1 tetramer. Cells are stored on ice prior to flow cytometry.

It is difficult to estimate an accurate number of MAIT cells you will detect. Nevertheless, one should expect a clear MR1-5-OP-RU tetramer$^+$ population detectable by FACS, with an estimated 100 to 300-fold increase in percentage compared to cellular fractions not subjected to tetramer enrichment.

BASIC PROTOCOL 2

MAIT CELL EXPANSION IN THE LUNGS OF SPF-HOUSED MICE USING BACTERIAL INFECTION

MAIT cells are relatively rare in naïve SPF-housed C57BL/6 mice (Rahimpour et al., 2015), typically comprising about 10^4 recoverable pulmonary MAIT cells in a naïve adult C57BL/6 mouse(Wang et al., 2018). For some applications it is desirable to boost the number of MAIT cells prior to analysis or for harvesting sufficient numbers of MAIT cells for adoptive transfer experiments. A number of methods have been developed to boost their numbers (Chen et al., 2017; D'Souza, Pediongco, et al., 2018;

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Wang et al., 2018) (bacterial infections or administration of MAIT cell Ag (5-OP-RU) in combination with an adjuvant: live/dead bacteria, TLR agonists and cytokines), allowing their isolation in large numbers from mouse lungs, suitable for different purposes. Here we present the MAIT expansion protocols with bacterial infections (*Salmonella* Typhimurium BRD 509 or *Legionella longbeachae* NSW150) and immunisation with CpG plus 5-OP-RU (Alternate Protocol 2).

Materials

BCYE medium agar plates: see recipe

BYE broth: see recipe

Streptomycin (50 mg/ml, premade aliquots stored at -20 ^{o}C)

Bacteria:

- *L. longbeachae* (NSW150, *Strep^{r}*) (Wang et al., 2018)
- *Salmonella enterica* var Typhimurium BRD509, attenuated vaccine strain from the wild type strain SL1344, Streptomycin resistant (SL1344, *ΔaroA ΔaroD, Strep^{r}*) (Chen et al., 2017)

BSC II biosafety cabinet

Spectrophotometer (Eppendorf BioPhotometer or equivalent)

Bacteria culture orbital shaker and incubator

Isoflurane

Isofluorane anaesthesia machine, with controls for the flow rate of oxygen and isoflurane

Mice could be wild type C57BL/6 (Ly5.1) or C57BL/6 (Ly5.2), or other genetically modified strains (e.g. IL-17 KO mice).

1. Five days prior to infecting mice, streak out *L. longbeachae* on a BCYE agar plate containing 50 μg/ml streptomycin and incubate plate at 37°C. Workable size white colonies should appear over 4 days of incubation.

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L. longbeachae was originally from a clinical isolate (NSW150) and has acquired streptomycin resistance during laboratory propagation. Growth media are liquid medium: buffered yeast extract (BYE) broth supplemented with cysteine or solid medium: buffered charcoal yeast extract (BCYE) agar, with streptomycin (30-50 μg/ml). Glycerol stocks are made by resuspending bacteria in BYE broth with 50% v/v glycerol (ChemSupply Cat#GA010-P) and stored at -80℃.

2. The day before infection, pick a single colony and inoculate 10 ml BYE containing 50 μg/ml streptomycin and incubate overnight at 37 ^{o}C, shaking at 180 rpm. Prewarm 4 more 10 ml tubes of BYE broth (with streptomycin) to 37 ^{o}C without shaking.
3. On the day of infection, measure the optical density $(OD)_{600\ nm}$ of the overnight culture and re-inoculate fresh 10 ml pre-warmed medium. Calculated the amount of *L. longbeachae* used for re-inoculation by measuring the $OD_{600\ nm}$ of the overnight culture so that the re-inoculated culture (prior to incubation) is about 0.2 $OD_{600\ nm}$. Make 3 more re-inoculations with each time having 25% less of the amount of overnight culture. As such you will have 4 re-inoculations with fresh medium with a starting $OD_{600\ nm}$ of approximately 0.2, 0.15, 0.1 and 0.08. This is to ensure you will have an optimal log-phase culture to work with from the next 3 hours and throughout the rest of the day. The reinoculated cultures are incubated with shaking at 37 ^{o}C for >2 hr to ensure the bacteria will replicate at least once to reach an $OD_{600\ nm}$ between 0.2-0.6 from one 10 ml culture. The doubling time for *L. longbeachae* is about 2 hr.
4. Measure the $OD_{600\ nm}$ of the re-inoculated cultures at regular intervals during the incubation until an $OD_{600\ nm}$ between 0.2-0.6 is reached.
5. Calculate the required CFU of bacteria by estimating 1 O.D. = $8x10^8$ CFU (this constant needs to be established by individual researchers).
6. Dilute with 5% v/v BYE in PBS to a final concentration of $4x10^5$ CFU/ml, allowing 50 µl inoculum/mouse i.e. $2x10^4$ CFU/50 µl/mouse.

The viability of L. longbeachae is much higher in 5% BYE buffer than in PBS. It is particularly important for experiments with a large number of mice to be infected and the infection process takes over 2 hours.

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7. Infect mice intranasally with 2x10^4 CFU *L. longbeachae* in 50 µl 5% BYE (in PBS) under isofluorane anaesthesia (see Support Protocol-4).
8. Allow mice to recover and monitor mice for 7 days to allow the infection to take its course. During this time MAIT cells expand dramatically from 10^4 to up to 2.5 x 10^6 MAIT cells, or from 1% to 20-30% of all αβ-T cells (Chen et al., 2017; Wang et al., 2018).

Boosting MAIT cells with S. *Typhimurium BRD509 (follows the same procedure as for L. longbeachae) except for the following:*

- *The medium for* S. *Typhimurium BRD509 is Luria broth or agar with streptomycin (50-100 µg/ml final). It takes 2 days for colony formation on agar plates streaked from a glycerol stock.*

Salmonella enterica var Typhimurium BRD509, attenuated vaccine strain from the wild type strain SL1344, Streptomycin resistant (SL1344, ΔaroA ΔaroD, Strep^r) (Chen et al., 2017). Growth media are Luria-Bertani (LB) broth and Luria agar plates, with streptomycin (30-50 µg/ml). Glycerol stocks are made by resuspending bacteria in LB broth with 50% v/v glycerol and stored at -80 °C.

- *The starting culture from a single colony can be grown with shaking or static (without shaking). However, reinoculated culture for preparation of the bacterial inoculum is carried out static in order to increase the infection efficiency.*
- *The doubling time is approximately 30 min.*
- *Infect mice with* S. *Typhimurium BRD509 at 1-2 x 10^6 CFU in 50 µl PBS intranasally.*
- S. *Typhimurium BRD509 is less virulent and requires less frequent mouse monitoring (subject to approval from the relevant animal ethics committee).*
- *Complete clearance to undetectable levels in wild-type C57BL/6 mice takes approximately 10-12 weeks for* S. *Typhimurium BRD509 compared to 3-4 weeks for* L. *longbeachae.*
- *MAIT cell yield estimation: one* S. *Typhimurium BRD509 (1-2x10^6 CFU) infected mouse will yield 1-2x10^6 sorted MAIT cells, which are enough for 10-20 recipient mice (10^5 MAIT cells per*

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recipient mouse). L. longbeachae (2x10^4 CFU) infection is less efficient at expanding MAIT cells, with about 60% yield compared to S. *Typhimurium BRD509 infection.*

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ALTERNATE PROTOCOL 2

MAIT CELL EXPANSION USING ANTIGEN AND TLR AGONISTS

In addition to their rapid expansion in the lungs in response to infection, MAIT cells can be expanded in the lungs by inoculation of mice with synthetic 5-OP-RU antigen in combination with various TLR agonists (Chen et al., 2017; D'Souza, Pediongco, et al., 2018). These sterile methods of MAIT cell expansion may be preferable for some experimental settings. Firstly, the activation signals are defined (TLR agonists and 5-OP-RU). As such only MAIT cells, but not other T cell subsets are enriched. This is particularly important for priming mice in vaccination experiments (Chen et al., 2017; D'Souza, Pediongco, et al., 2018; Wang et al., 2018) since the data interpretation after challenge will not be complicated by other immune components stimulated by bacterial infection. Secondly, the sorted MAIT cells will be free of any possible contamination with bacteria. This is critical for transfer experiments using severely immune-compromised mice as recipients, particularly when following MAIT cells for a prolonged period of time.

Materials

Mice could be wild type C57BL/6 (Ly5.1) or C57BL/6 (Ly5.2), or other genetically modified strains (e.g. IL-17 KO mice). Mice are of age 6-12 weeks of age, unless specified otherwise.

Spectrophotometer (Eppendorf BioPhotometer or equivalent)

Isoflurane

Isofluorane anaesthesia machine, with controls for the flow rate of oxygen and isoflurane

Synthetic MAIT cell antigen: 5-OP-RU (see reagents and solutions)

TLR agonists (CpG, Poly I:C) (e.g. InVivoGen, Catalog #tlrl-kit1mw)

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1. Plan experiment with an expected number of MAIT cells to determine the number of mice to be immunised and have mice organised beforehand.

The MAIT cell yield via sterile immunization (5-OP-RU with TLR agonists) is less than by bacterial infections: One S. *Typhimurium BRD509 (1-2x10^6 CFU) infected mouse will yield ~1-2x10^6 sorted MAIT cells, which are enough for 10-20 recipient mice (10^5 MAIT cells per recipient mouse). Immunization (5-OP-RU with TLR agonists) and L. longbeachae (2x10^4 CFU) infection give about 40% and 60% yield respectively compared to* S. *Typhimurium BRD509 infection.*

2. Day 0: Immunise mice intranasally (see Support Protocol-4) with 5-OP-RU (76 nmol) in combination with TLR agonist: 20 μg CpG1688 or 50 μg poly I:C prepared in 50 μl PBS or saline per mouse.

3. Deliver 5-OP-RU only, intranasally again on days 1, 2 and 4.

4. Mice are killed for assaying MAIT cells on day 7 (see Basic Protocol-1).

SUPPORT PROTOCOL 4

INTRANASAL INOCULATION OF MICE

Intranasal inoculation with bacteria, or with TLR agonist in combination of 5-OP-RU, is performed on mice that have been anesthetized by isoflurane inhalation (Chen et al., 2017). Intranasal delivery of an inoculum is considered to be simple and straightforward to perform. However accurate delivery of the whole 50 μl inoculum is tricky and requires sufficient practice. This is particularly important when seeking replicable CFU data, and variation from individual mouse data points by this readout can be critical to assess a potentially significant role of MAIT cells (Wang et al., 2018).

Materials

Naïve or infected adult C57BL/6 mice or other suitable strains of laboratory mice. Mice are of 6-12 weeks of age, if not specified otherwise. 3-5 mice are routinely used for one experiment to satisfy statistical requirements as one mouse provides one data point.

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Mr1$^{-/-}$ mice (number, age and gender matched to C57BL/6) (Chen et al., 2017; Treiner et al., 2003) (MAIT deficient mice on a C57BL/6 genetic background) (optional)

Ice box

Roswell Park Memorial Institute (RPMI) medium or PBS

Refrigerated centrifuge

Flow cytometer with capacity for $\geq$ 9 fluorochromes. (e.g. BD Fortessa)

Cuvettes

Spectrophotometer (Eppendorf BioPhotometer or equivalent)

Pippete and tips.

Isoflurane

Isofluorane anaesthesia machine, with controls for the flow rate of oxygen and isoflurane

Synthetic MAIT cell antigen: 5-OP-RU

TLR agonists (CpG, Poly I:C) (e.g. InVivoGen, Catalog #tlrl-kit1mw)

BCYE medium agar plates: see recipe

BYE broth: see recipe

Streptomycin (50 mg/ml, premade aliquots stored at -20 ^{o}C)

Bacteria:

- *L. longbeachae* (NSW150, *Strep*^{r}) (Wang et al., 2018)
- *Salmonella enterica var Typhimurium BRD509, attenuated vaccine strain from the wild type* strain SL1344, Streptomycin resistant (SL1344, *ΔaroA ΔaroD, Strep*^{r}) (Chen et al., 2017)

BSC II biosafety cabinet

Bacteria culture orbital shaker and incubator

Protocol Steps:

1. Plan experiment to determine the number of mice needed. One mouse provides one data point. At each time point 3-5 mice are necessary to satisfy statistical requirements.

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2. Prepare bacterial inoculum or sterile inoculum of a TLR agonist in combination with synthetic MAIT cell antigen 5-OP-RU as required. Also refer to basic protocol 2 and alternate protocol 2.
3. Anaesthetise mice, one at a time, by placing in a chamber filled with 1-5% Isoflurane in O_2 at a constant flow rate of 2 l/min.

The breathing rhythm is essential for successful nasal instillation. The correct breathing rhythm (about 3 counts for every 2 seconds) must be determined during practice by individual researchers. Too shallow or deep anaesthesia will result in mice not inhaling the inoculum properly.

4. Remove mouse from the chamber and administer 50 μl of the inoculum (2 x 10^4 CFU) drop-wise with a pipette onto the nasal region, ensuring that each drop is inhaled before continuing with the next drop.

All procedures involving infection of mice with Risk Group 2 microorganisms should be performed in a Class II biosafety cabinet.

The inoculum preparation should be kept at RT and resuspended by gently vortexing or flicking the tube between each mouse to ensure consistent dosing. Cold inoculum may cause mice to sneeze, resulting in poor inoculation efficiency.

5. Return the mouse to a box, placing it on paper towel and watch until it fully recovers from the anaesthesia.

Mice are in a state of light anaesthesia and should regain consciousness within 1-2 min.

6. Monitor mice (until culling for the experimental end-point) according to protocols established for each infection or immunisation by the local institutional governing animal ethics committee.

Bacterial infections cause illness in mice. Individual mice must be monitored for weight change and their appearance and activity. The peak of infection is normally ~day 3-6 post infection. Mice recover slowly and appear normal within about 7-14 days post infection. Sterile immunisation with TLR agonist will cause transient adverse symptoms (weight loss <20% and ruffled fur within the first 3 days) and mice recover fully within one week. Each immunisation with various doses needs be tailored with a specific monitoring scheme which should be established during trial experiments. Every procedure on mice must first be approved by the local governing animal ethics committee.

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SUPPORT PROTOCOL 5

FACS SORTING MAIT CELLS AND ADOPTIVE TRANSFER

Since MAIT cells are rare in wild-type C57BL/6 mice, for adoptive transfer experiments or *in vitro* activation assays, the MAIT cell population can first be expanded to sufficient numbers using intranasal bacterial infection or immunization (5-OP-RU with TLR agonists) (Chen et al., 2017; Wang et al., 2018). When planning the adoptive transfer experiment, first estimate the numbers of MAIT cells needed and determine the number of donor mice required. On average, one *S.* Typhimurium BRD509 (1-2x10^6 CFU) infected mouse will yield 1-2x10^6 sorted MAIT cells, sufficient for 10-20 recipient mice (10^5 MAIT cells per recipient mouse in this case). *L. longbeachae* (2x10^4 CFU) infection and sterile immunization (5-OP-RU with TLR agonists) are less efficient at expanding MAIT cells, with about 60% and 40% yield, respectively, compared to *S.* Typhimurium BRD509 infection. Donor mice are typically infected or immunized 7 days earlier than the adoptive transfer.

Materials

Naïve or infected adult C57BL/6 mice or other suitable strains of laboratory mice. Mice are of 6-12 weeks of age, if not specified otherwise. 3-5 mice are routinely used for one experiment to satisfy statistical requirements as one mouse provides one data point.

Dissecting instruments

10 ml syringe with 23 or 26 gauge needle

Tubes (1.7 ml: Axygen, Cat#MCT-175-C; 10 ml: SARSTEDT, Cat#62.9924.284; 50 ml: FALCON, Cat#227261; FACS tube: FALCON, Cat#352008 and 352054 (sorting))

Petri dishes (Cellstar, Cat#639160)

Transfer pipettes

1 ml syringe plungers

70 μm cell strainers (Biostrategy, Cat#BDAA352350)

Scalpel blades (size 23)

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Ice box

Collagenase medium: (See Reagents and Solutions)

Fluorescence activated flow cytometry (FACS) buffer: see recipe

Roswell Park Memorial Institute (RPMI) medium

Tris-based Ammonium Chloride (TAC) hypotonic Red Blood Cell (RBC) lysis buffer: see recipe

MR1-5-OP-RU and MR1-6-FP monomers (Corbett et al., 2014; Reantragoon et al., 2013): available from the NIH tetramer core facility, on application. These are tetramerized prior to use (see Support Protocol-1) or can be ordered as tetramers conjugated to PE, BV421 or APC fluorochromes (see Internet Resources).

Antibodies specific for mouse cell surface markers:

1. CD19-PerCP-Cy5.5; BD Bioscience; Cat#: 561113; clone #: ID3
2. TCRβ-APC; BD Bioscience; Cat#: 553174; clone #: H57-597
3. CD45.2-FITC; BD Bioscience; Cat#: 553772; clone #: 104
4. CD4-APCcy7; BD Bioscience; Cat#: 561830; clone #: GK1.5 (RUO)
5. CD8a-PE; BD Bioscience; Cat#: 553032; clone #: 53-6.7.

To reduce background staining, anti-CD16/32 (Biburger et al., 2015) (FcγRIII, 2.4G2 hybridoma) culture supernatant is used as a diluent for the antibody mix. Dead cells are gated out with staining of 7-aminoactinomycin D (7-AAD) or alternative live-dead marker

CO_2 chamber connected to a regulator with a flowrate reading or alternate method for euthanizing mice

Refrigerated centrifuge

Flow cytometer with capacity for $\geq$ 9 fluorochromes. (e.g. BD FACSAria III Cell Sorter)

Cuvettes

Spectrophotometer (Eppendorf BioPhotometer or equivalent)

Trypan blue

Hemocytometer

Inverted light microscope

Flow sorter (e.g. BD Aria)

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FACS tubes for sorting (FALCON Cat#352054)

Cell strainers (30 μm, Miltenyi Biotec, Ct#130-098-458)

MAIT cells will be transferred to mice therefore all reagents should be sterile, and all steps should be carried out in a BSC II Biosafety cabinet. Buffers should not contain toxic chemicals, such as azide, that affect cell viability.

Protocol Steps:

1. Infect or immunize sufficient donor mice as described in Basic Protocol-2.

The choice of donor mice must be considered when planning the experiment: a) MHC matched unless for GVHD (graft versus host disease) model experiment; b) male MAIT cells may be rejected in recipient female mice due to the expression of Y-antigen; c) strains differing in congenic markers (CD45.1 and CD45.2) can be used when transferred MAIT cells are to be tracked.

2. Pool lungs from infected or immunized mice of the same group together and process the organs as described in step 1 to step-19 in Basic Protocol-1, with scaled-up volume of buffers and reagents.
3. Remove epithelial cells and debris by Percoll gradient centrifugation: Resuspend lung cells in 20 ml 40% Percoll and underlay carefully with 20 ml of 70% Percoll. Centrifugation is carried out at 2000 rpm at RT for 20 min with brake off.
4. Immune cells form a visible layer between the 40%: 70% Percoll and are collected by careful pipetting to another tube.
5. Cells are washed with PBS once.
6. Carried out non-specific tetramer binding blocking and tetramer/antibody staining the same way as described in Basic Protocol-1, with sufficient amount: e.g. 200 μl blocking mix and 400 μl tetramer/ antibody staining mix per lung.
7. Resuspend lung cells in PBS containing 2% FCS and filter with sterile 40 μm cell strainers into FACS tubes for cell sorting. Prepare collection tubes by adding 0.5-1 ml of sterile FCS or RPMI containing 10% FCS, ensuring the side of the tube is wet.
8. Using a similar gating strategy (Figure 1) sort MAIT cells into collection tubes.

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It is often desirable to set gates more tightly than for analysis to minimise contamination from non-gated cells. An aliquot of 10 μl sorted cells can be run through the flow sorter or analyser to confirm cell purity. A purity of ~98% can be expected.

9. Wash sorted MAIT cells with RPMI and resuspend in a volume that gives a cell concentration of 10^5 cells per 200 μl (i.e. $5x10^5$/ml).
10. Adoptively transfer cells into recipient ice by i.v. injection (see Support Protocol-7).
11. (Optional: for experiments using immunocompromised mouse strains). If the recipient mice are severely immunocompromised strains (e.g. RAG2$^{-/-}\gamma C^{-/-}$), the transferred T cells, including MAIT cells, will undergo a significant spontaneous expansion for undefined reasons. Mice should be treated twice in the first week post transfer with 0.1 mg each purified monoclonal antibodies (anti-mCD4 and anti-mCD8) to control overgrowth of any contaminating conventional T cells (see Critical Parameters and Troubleshooting: Control of contaminating conventional T cells in sorted MAIT cells). Note that MAIT cells expressing high levels of CD4 or CD8 will also be depleted.

Note that when MAIT cells are sorted from bacterially-infected mice, they may be contaminated with a few bacteria (<10 CFU/10^5 MAIT cells). This may be a potential problem when the recipient mice are severely immunocompromised (e.g. RAG2$^{-/-}\gamma C^{-/-}$ mice), particularly if the MAIT cells are also sorted from another immunocompromised line (e.g. IFN$\gamma^{-/-}$, TNF$^{-/-}$), as this combination may not be able to control the bacterial growth, which could complicate future data interpretation. Appropriate mouse monitoring is required.

SUPPORT PROTOCOL 6

Tail or Submandibular vein BLEEDING

[*Query author to include summary paragraph (minimum 2 sentence explaining what/how/results of this protocol here]

Materials

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Naïve or infected adult C57BL/6 mice or other suitable strains of laboratory mice. Mice are of 6-12 weeks of age, if not specified otherwise.

Light box (to warm mice).

Needles (18-23 gauge)

Mouse restrainer

Blood collection tubes, containing 10 µl heparin

Tail vein bleeding

1. Warm mice on a heat box and place in a restrainer.
2. About halfway down the length of the tail make a small superficial incision across the lateral vein of the tail (perpendicular to the vein) using an 18-23G needle.
3. Collect flowing blood in a labelled heparin-containing tube.

Sub-mandibular bleeding

1. Restrain mouse using a full body scruff.

For the best results the mouse needs to be tightly restrained. The eyes will bulge slightly. Your grip needs to ensure that the vein is not occluded.

2. Identify the freckle first and the *Sub-mandibular vein is just above it.* Use a 23G needle to puncture the vein.
3. Collect flowing blood in a labelled heparin-containing tube.

Up to 0.5% body weight should be taken per bleed, ie. 100 µl for a 20 g mouse. Mice should be rested for at least one week between bleeds.

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Mice recover very well from blood sampling. From our experience, this procedure will cause minor, transient pain and distress to mice. Mice should be monitored immediately after blood sampling to ensure that the bleeding has stopped and for signs of normal behaviour and movement.

SUPPORT PROTOCOL 7

INTRAVENOUS INJECTION VIA THE TAIL VEIN (including adoptive cell transfer)

[*Query author to include summary paragraph (minimum 2 sentence explaining what/how/results of this protocol here]

Materials

Naïve or pretreated adult C57BL/6 mice or other suitable strains of laboratory mice. Mice are of 6-12 weeks of age, if not specified otherwise.

Light box (to warm mice)

Syringes and needles (26-27 gauge)

Mouse restrainer

Reagents to be injected

1. Warm mice on a heat box. This normally takes about 5 min. During this time load the syringe and fix the needle.

A thermo-senser should be used to control the temperature. If the box becomes too hot, mice will start to jump. The box could be placed 1/5 off the light box, creating a cooler spot for mice to rest. If a brass mouse restrainer is to be used, it can be warmed with warm water. Use a separate mouse box to create a temporary warm water bath. The syringe with needle is always placed away from the working area to avoid accidental injuries.

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2. Position the mouse into a mouse restrainer so the lateral vein faces up. Either side of the vein can be injected. Wet the tail with 80 % ethanol to make the vein more visible.

Note, it is easier to see the tail vein of mouse strains with white fur. For C57BL/6 mice young female's tail veins are more obvious than males.

3. Perform the injection, starting half-way along the tail.

In case the first injection does not go well, further injections can be tried upward along the tail. The other side lateral vein can be used too. Mouse may need to be warmed up once more. Successful i.v injection requires training and a lot of practice.

4. Return mice to cage and record procedures and monitoring.

Most i.v injections use a relatively large volume (100-200 µl) to avoid clotting of cells and for a more accurate dose delivery. It is normal to see a small amount of bleeding post injection, which should stop within a couple of minutes. Monitor mice and make sure any bleeding has stopped.

SUPPORT PROTOCOL 8

INTRAPERITONEAL INJECTION

[*Query author to include summary paragraph (minimum 2 sentence explaining what/how/results of this protocol here]

Materials

Naïve or pretreated adult C57BL/6 mice or other suitable strains of laboratory mice. Mice are of 6-12 weeks of age, if not specified otherwise.

Syringes and Needles (26-27 gauge)

Reagents to be injected.

1. Load the syringe and place it away from the work area to avoid accidental injuries.

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2. Restrain mouse manually using a standard neck scruff hold.

3. Perform injection in the lower half of the abdomen to avoid hitting key organs. Use, a 26-29 G needle, 0.5-1 ml syringe and a routine injection 200 μl volume. When multiple injections are required, the site of injection will be rotated each time – from side to side and in two different locations on each side (lower abdomen) – to prevent bruising.

4. Return mice to shelf and fill in record of procedures and monitoring sheet.

Mice may be given a treat (e.g. sunflower seeds or rice bubbles) after each injection as positive reinforcement and to alleviate potential distress.

REAGENTS AND SOLUTIONS

5-OP-RU is 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. For further information refer to Protocol Unit: Characterisation of Human MAIT cells (Mucosal Associated Invariant T cells).

[*Copy Editor: We're co publishing the mouse and human manuscripts together – is it possible to ensure the above statement references this paper]

7-aminoactinomycin D (7-AAD, Sigma, Cat#:A9400-1mg). Dilute 1 mg powder in 1 ml H_2O or PBS to make the working stock (1 mg/ml) and store aliquots (25 μl) at -20 °C. use at 1:500 dilution in the final cell staining buffer (final concentration: 2 μg/ml).

BYE broth and BCYE medium (per 1 L):

1. To 950 ml Milli-Q H_2O, add 10 g Yeast extract (BD, Cat#90000-722), 2 g Charcoal activated (Sigma, Cat#C5510), 10 g N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES, powder, Sigma, Cat#A9758), 1 g Ketoglutarate monopotassium salt (Sigma, Cat#2000) and 17 g Agar (Oxoid, Cat#LP0011).

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2. The above solution is autoclaved, then cooled in 42 ^{o}C water bath.

3. 10 ml L-Cysteine HCl (Sigma, Cat#C7880, pre-made, filter-sterilised 40 mg/ml working stock).

4. 10 ml Ferric Pyrophosphate (Sigma, Cat#P6526, pre-made, filter-sterilised 25 mg/ml working stock).

5. Add Streptomycin (pre-made, filter-sterilised 5 mg/ml) to a final concentration of 50 μg/ml.

6. Top up to 1 Litre with autoclaved H_2O, mix well, then pour 18-20 ml per petri dish (CellStar, Cat#639160) in a hood.

7. Omit Charcoal and Agar when BYE broth is made.

Collagenase medium: Collagenase III (Worthington, Cat#LS004182), 3 mg/ml with DNAse, 5 μg/ml in Roswell Park Memorial Institute medium (RPMI, Gibco, Cat#21870-076) with 2% foetal calf serum (FCS). Filter sterilized and aliquots stored at -20 °C.

Fluorescence activated flow cytometry (FACS) buffer: phosphate buffered saline (PBS), 2% bovine serum albumin (BSA) and 0.02% azide (from 10% stock of NaN_3, Ajax FineChem, Cat#1222-1009). Do not add azide (toxic to the cells) if cells need to be kept alive for further experiments.

FACS fixation buffer: 1% formaldehyde (Ajax FineChem, Cat#809-2.5LPL), 2% glucose in PBS. Fully dissolved solution is kept cold (fridge) and dark (aluminum foil wrapped) as formaldehyde is sensitive to light.

Luria-Bertani (LB) medium: to make a litre LB, dissolve 5 g yeast extract (Oxoid, Cat#LP0021), 10 g bacto-tryptone (Oxoid, Cat#LP0037) and 10 g NaCl in a litre milli-Q water. The solution is autoclaved. Streptomycin (30-50 μg/ml final) added to broth before use. To make LB agar plate 17 g agar (Oxoid, Cat#LP0011) is added before autoclaving. For agar plates, the immediately autoclaved broth is

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cooled to 42 °C, add streptomycin (30-50 μg/ml final, Sigma, Cat#S6501) before pouring into petri dishes.

Percoll (Density 1.13 g/ml) gradients: Percoll stock (GE healthcare Cat#17-0891-01) is warmed to RT before use. To make 33.75%, 40% or 70% Percoll gradients, refer to Table 1.

Stomach digestion buffer: Hanks Balanced Salt Solution (HBSS) without calcium and magnesium, 5% FCS, 5 mM EDTA, 1 mM Dithithreitol (DTT) (add fresh).

TAC RBC lysis buffer: 0.14 M NH_4Cl (Sigma, Cat#A9434), 0.017 mM Tris (pH7.5, Sigma, Cat#T1503), then adjust pH to 7.2 with HCl (2 M). The solution is filter (0.22 μm) sterilized and kept at room temperature.

Tetramer blocking mix: This is to block non-specific tetramer binding. Dilute (1:100) blocking MR1-6-FP-tetramer (without fluorescent conjugate) with cell culture supernatant (SN) of hybridoma 2.4G2 (anti CD16/32 (Biburger et al., 2015)).

Tetramer-antibody staining mix: Dilute MR1-5-OP-RU tetramer (1:200-1:800) and other fluorochrome conjugated monoclonal antibodies: anti-CD19-PerCPCy5.5 (1:200, BD, Cat#551001), anti-CD45-FITC (1:200, BD, Cat#553772), anti-TCRβ-APC (1:200, Biolegend, Cat#100412), anti-CD4-PE (optional, BD, Cat#552051), anti-CD8-PECy7 (optional, eBioScience, Cat#12-0081-83), and 7-AAD (2 μg/ml final or 1:500 dilution of stock, Sigma, Cat#:A9400) in FACS buffer, making enough mix for all samples, plus a little excess.

TBS: 10 mM Tris, pH8, 150 mM NaCl.

COMMENTARY

Background Information

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MAIT cells are relatively rare in mice, ranging from ~0.1% of all αβ-T cells in the liver to ~1% in the lungs (Chen et al., 2017; Rahimpour et al., 2015), which hinders MAIT cell studies. Previously MAIT cells were defined with antibodies ($CD3^+$, $CD4^-$ and $CD8^-$) and RT-PCR of the invariant TCRα chain (Vα19i) (Martin et al., 2009; A. Meierovics et al., 2013; A. I. Meierovics & Cowley, 2016). MR1 tetramers were first developed in 2013 using a K43A mutant MR1 (Reantragoon et al., 2013). Following the identification of the MAIT cell antigens and the elucidation of how they are formed (Corbett et al., 2014; Kjer-Nielsen et al., 2012), wild-type MR1 tetramers could be developed, and are now produced by the NIH Tetramer Core Facility as permitted to be distributed by the University of Melbourne. The tetramer technology in combination with recently established infection models (Rahimpour et al., 2015; Wang et al., 2018) makes it possible to expand and characterise MAIT cells with precision. This manuscript describes practical step by step protocols and should provide a technical platform for the MAIT cell research community.

Legionella is a pathogenic group of Gram-negative bacteria, when inhaled, cause a spectrum of disease from self-limiting Pontiac fever to severe, necrotic pneumonia: Legionnaires' disease (Winn & Myerowitz, 1981). There are two main species: *L. pneumophila* and *L. longbeachae.* In Australasia and Thailand more than 50% of cases are caused by *L. longbeachae* (Gobin, Susa, Begic, Hartland, & Doric, 2009), whereas elsewhere, the predominant pathogen is *L. pneumophila*. The incidence of Legionnaires' disease has nearly trebled since 2000, with >5000 cases per year in the United States, inflicting a 10% mortality despite best treatment (Garrison et al., 2016).

Salmonellae are a group of Gram-negative, facultative anaerobic pathogenic bacteria which cost millions of lives across the world every year (Garai, Gnanadhas, & Chakravortty, 2012). Although they infect a range of hosts via the oral-gastric route and cause gastroenteritis, *S.* Typhimurium is essentially a systemic pathogen and capable of infecting many tissues and organs including the lungs (Chen et al., 2017).

One of the major pathways for innate immune response to microbes is through Toll-like receptors' (TLRs) recognition of the highly conserved structural motifs only expressed by microbial pathogens, called pathogen-associated microbial patterns (PAMPs) (Beutler, 2009). PAMPs include various

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bacterial cell wall components such as lipopolysaccharides (LPS), peptidoglycans and lipopeptides, as well as flagellin, bacterial DNA (unmethylated DNA such as CpG) and viral double-stranded RNA (Kawasaki & Kawai, 2014). Stimulation of TLRs by PAMPs initiates a signalling cascade which leads to the secretion of pro-inflammatory cytokines and effector cytokines that direct an immune response (Ozato, Tsujimura, & Tamura, 2002).

MAIT cells show highest abundance in the lungs of mice (1% of all $\alpha\beta$-T cell) (Chen et al., 2017; Rahimpour et al., 2015). To date, lung MAIT cells are the best studied in mice, whilst most human studies have used blood samples (Chiba et al., 2017; Hegde et al., 2018; Hinks et al., 2016; Khaitan et al., 2016; van Wilgenburg et al., 2016).

Ethical considerations:

All procedures involving mice should be performed only after approval from the governing institutional Animal Ethics Committee.

Safety considerations:

A biosafety committee may be involved for assessment of potential risks to human and mice from the above-mentioned pathogens. *L. longbeachae* and S. Typhimurium are both classified as Risk Group 2 pathogens. Waste at each step should be treated appropriately according to regulations and following an individual risk assessment. For Risk Group 2 pathogens, all waste is autoclaved or treated with 1 % Virkon prior to being discarded. A useful reference is the online resource: https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/pathogen-safety-data-sheets-risk-assessment.html#f. However, this should be only used as a guide and each laboratory or institute should conduct their own risk assessment and develop safe operating standards for each procedure. Safety regulations of the governing body in the local jurisdiction should be adhered to.

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L. longbeachae are Risk Group 2 pathogens that are infective primarily by the respiratory route in humans. Laboratory-acquired infections by *L. longbeachae* can potentially occur through aerosol inhalation, ingestion, or wound contact. Infections with high doses can be fatal for both humans and mice, if untreated. Any handling of *L. longbeachae* or infected tissues should be performed within a Class II Biosafety cabinet since aerosols may be generated. Care must be taken when handling culture, inoculum, and infected tissues or organs, since these may contain high concentration of bacteria. It is essential to use PPE including long-sleeved lab gowns, gloves and safety glasses. Homogenization of infected organs will generate aerosols, and therefore must be performed in a Class II Biosafety cabinet.

S. enterica Typhimurium are gram-negative enteric bacteria. They are classified as Risk Group 2 pathogens and are associated with diarrheal illness in humans. Salmonellosis is a hazard to laboratory personnel. The most common route of infection is by ingestion. *Salmonella* may be present in all mouse organs, blood and faeces from infected mice. Strict BCL-2 practices should be observed when culturing *S.* Typhimurium and when handling materials from handling infected mice.

Mice can bite and scratch, which may result in infection. Mouse fur or waste dust can cause allergy in some individuals. Health monitoring may be advised for personnel.

Azide is toxic. Handle azide-containing items with gloves.

Flow cytometers contain Class III lasers which can be harmful to human vision if used incorrectly.

Regulated use of Genetically modified organisms:

In addition to biosafety considerations, *S.* Typhimurium BRD509 strain is classified as a Genetically Modified Organism (GMO) and is subject to restricted use. In Australia, GMO dealings are regulated by the Office of Gene Technology Regulator (OGTR). No use without permission is allowed and

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conditions of approval must be strictly followed to avoid unintentional release of GMOs. For further information refer to the appropriate regulations governing the institute where work is to be conducted.

Mouse strains, including *Mr1*$^{-/-}$ mice and immune-compromised strains used to study the role of MAIT cells in infection, are classed as GMOs and may also be subject to regulated use.

Critical Parameters and Troubleshooting

FACS gating strategy. Murine MAIT cells are a relatively rare T cell subset in many organs. Any analysis needs to be performed on accurately identified cells. Thus, flow cytometric gating and correct use of control tetramer is imperative. A MAIT cells gating strategy (using the lungs as an example) is shown in Figure 1. Note that when MAIT cells are sorted it is sometimes useful to shift the gate further from other cell populations to avoid any carry-over of non-MAIT T cells.

Inoculum buffer: Saline or PBS are most commonly used for preparing bacterial inoculum and can remain viable for a few hours. However, some bacteria such as *L. longbeachae* are sensitive to the buffer used. We found that addition of culturing medium BYE (5%) in PBS helps keep the bacteria viable for at least 3 hr in the inoculum, during which time the mice can be inoculated.

Intranasal instillation: Although intranasal inoculation is a relatively simple and quick procedure, it should only be performed by trained personnel. To acquire accurate quantitative data (e.g. recovering bacterial load CFU counts from infected lungs), accurate intranasal inoculation to each mouse is essential. Successful intranasal inoculation enables a consistent number of bacteria to be delivered deep to the lungs. The maximum volume (50 μl) is used, as there is some dead volume in the nasal track/cavity and thus some of the inoculum never reaches the lungs. Inhaling the whole inoculum (50 μl) in one go (without stops) is also essential for accurate delivery of bacteria. It is common that one or two mice may be removed at the start of the experiment in case it is noted that the intranasal inoculation does not go well (e.g. inoculum is not inhaled completely before waking

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up, and a noticeable proportion of inoculum goes to the mouth or bubbles out from nares), and this is far preferable to removing data points retrospectively. Practising the procedure on many mice (>20) may be necessary prior to performing experiments. To minimise any potential individual variations, the experimental group mice (e.g. C57BL/6 vs MR1$^{-/-}$) are alternated during intranasal inoculation, especially for experiments which involve a large number of mice.

Age and gender matched mice are essential for acquisition of high-quality data sets. Female mice have very short oestrous cycles, during which reproductive hormone levels fluctuate and this may affect the infection kinetics, especially when bacteria are delivered by certain routes. In this case, male mice are preferred for consistent results.

Control of contaminating conventional T cells in sorted MAIT cells: If the recipients are RAG2$^{-/-}\gamma C^{-/-}$ mice, any transferred T cells, including MAIT cells, will spontaneously expand ("homeostatic proliferation"). We have observed that a few contaminating non-MAIT T cells (<1%) could reach up to 10-15% of all transferred T cells after two weeks, suggesting different rates of proliferation (Wang et al., 2018). This will impact on the recipients' immune capacity and may produce unexpected results. Therefore, the initial residual number of contaminating T cells must be depleted. This is achieved using two doses of monoclonal Ab to CD4 and CD8 (0.1 mg each in 100 μl volume) injected intraperitoneally (i.p.) on the 2nd and 4th/5th day post transfer.

Impact of residual bacterial contamination of the sorted MAIT cells: If MAIT cells are sorted from donor mice infected with *S.* Typhimurium BRD509, a few bacteria may be carried over. This is because *S.* Typhimurium BRD509 infection can remain unresolved for up to 12 weeks even in wild type C57BL/6 mice (Kupz et al., 2013). These few bacteria will not cause obvious effects with most experiments. However, when the donor (e.g. IFN$\gamma^{-/-}$) and recipient (e.g. RAG2$^{-/-}\gamma C^{-/-}$) mice are of immunocompromised strains, even small numbers of bacteria (e.g. 10 *S.* Typhimurium BRD509) will replicate and eventually kill the recipient mice within approximately 6 months (unpublished observation). Alternative methods to boost MAIT cells, including *L. longbeachae* infection, which is cleared completely within 3-4 weeks in wildtype mice, or sterile MAIT cell boosting (antigen plus TLR

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agonists), may be considered. In the latter case, more donor mice are required as the recovery efficiency of MAIT cells is less than that achieved by bacterial infections.

Statistical Analyses

By consulting with a bioinformatician, the most appropriate statistical analysis can be chosen. In general Student-t test is used for comparison of datasets from two groups, with normal distribution. One-way ANOVA test (possibly with multiple corrections) will be chosen for comparing datasets from multiple groups, with normal distribution. Mann-Whiney U test should be used for analysing datasets with non-normal distribution.

Understanding Results

If inoculation of mice is successful, MAIT cells should be enumerated at 20-40% of all αβ-T cells by day 7 post infection with *S.* Typhimurium BRD509 or TLR agonist in combination of 5-OP-RU(Chen et al., 2017). *Legionella* is less potent and elicits an expansion of MAIT cells to 20-30% of all αβ-T cells by day 7 post infection (Wang et al., 2018). When stained well the main cell subset populations will be well separated on flow cytometry plots, which in turn enables high quality MAIT cell sorting or other downstream MAIT cell functional assays.

Time Considerations

Basic Protocol-1. Detection and characterisation of mouse lung MAIT cells by flow cytometric staining. It is essential to perform a trial experiment including all steps – this will allow each operator to judge the time taken and plan accordingly. Ensure that when multiple organs are to be assayed, when large numbers of mice are involved, that extra time is allowed. In some cases, over 50 sample tubes can be handled in one day. Labelling all tubes ahead of time is helpful.

Harvesting organs: It takes about 3-5 min to harvest lungs per mouse. This includes killing humanely by CO_2 asphyxiation, perfusion and dissection to remove the lungs. In case 5 or more

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organs are to be assayed, the estimated time required should be doubled. Up to 20 mice can be handled by one experienced researcher for one experiment.

Preparing single cell suspensions: It takes about 2-3 min to chop the lungs of a mouse or mash a mouse liver through a steel sieve. Collagenase digestion (90 min) and Percoll gradient centrifugation (1 hr) are the most time-consuming processes. When over 20 mice or multiple organs from each mouse are to be processed, the time required will increase significantly. Allow half a day for preparing the single cell suspensions for a typical experiment.

Staining: This takes about 1-1.5 hr. The addition of counting beads (optional) does not add significantly to the time taken.

Flow cytometric analysis: Data acquisition by flow cytometry can be performed the following day. The time required depends upon sample number and number of events to be acquired. Each sample can take 1 to 5 min due to the frequency of MAIT cells. Setting up the flow cytometer takes at least 0.5 hr (including single colour compensation) and data transfer and shutting down the machine takes about 10 min. Data analysis using FlowJo software takes many hours.

Alternate Protocol-1. Assessment of MAIT cell functional capacity by ICS and TF staining. The procedures are similar to those described in Basic Protocol-1. For both ICS and TF staining cell surface staining is performed first, followed by intracellular staining, thus adding ~2 hr to the staining protocol. For ICS there is an additional 4 hr incubation with Golgiplug or Brefeldin treatment prior to staining.

Support Protocol-1. Preparation of MR1 tetramer reagents. This protocol takes approximately 2 hr and involves a small amount of work at 10 min intervals. Alternatively, the MR1-tetramers can be ordered as tetramers already conjugated to fluorochromes.

Basic Protocol-2. MAIT cell expansion in the lungs of SPF-housed mice using bacterial infection.

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Growth of bacterial inoculums: It takes days to grow colonies depending on the bacteria to be used, as they replicate at various speeds: 4 days for *L. longbeachae* and 2 days for *S.* Typhimurium from a frozen glycerol stock. Preparation of infectious bacterial inoculum takes about half a day, as it involves re-inoculation of overnight culture to produce a log-phase culture.

Inoculation of mice: Intranasal inoculation takes about 2-3 min per mouse. When many mice are to be inoculated (e.g >50 mice), allow 2-3 hr. Intravenous inoculation is similar as it involves warming mouse and careful injection. Intraperitoneal inoculation is quick (~30 sec per mouse).

Monitoring of infected mice: Mouse monitoring may take between 5-30 min each time depending on the number of mice in one experiment. At the doses listed in this protocol mice are expected to survive the infection and clear the bacteria. Infected mice may display mild to moderate signs of illness, in particular from day 4-6 post infection when the infection peaks. The symptoms include ruffled hair, reduced activity, losing <20% of starting weight. Daily monitoring is essential for the first few experiments to establish the expected symptoms and relevant intervention criteria by individual labs. Immunisation with TLR agonist plus 5-OP-RU only renders mice with mild symptoms and only require daily monitoring in the first 3 days post procedure.

Timing until peak of MAIT cell expansion or until bacteria are cleared (for donor mice): The bacterial load (in the lungs) for *L. longbeachae* NSW150-infected mice peaks at day 3 post infection, then declines rapidly within 2 weeks. Most mice should clear the bacteria by 3 weeks. The bacterial load (in the lungs) for *S.* Typhimurium BRD509-infected mice decreases slowly following infection and there should be no detectable bacteria by 10-12 weeks post infection.

Alternate Protocol-2. MAIT cell expansion using antigen and TLR agonists.

Timing is similar as for Basic Protocol-2: MAIT cells can be harvested for adoptive transfer experiments after as little as 7 days.

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INTERNET RESOURCES

http://tetramer.yerkes.emory.edu/reagents/mr1

This site provides details and ordering information for MR1-5-OP-RU and MR1-6-FP tetramer reagents from the NIH Core Tetramer Facility, USA. A Material Transfer Agreement is required.

https://www.canada.ca/en/public-health/services/laboratory-biosafety-biosecurity/pathogen-safety-data-sheets-risk-assessment.html#f

This site provided useful information and Pathogen Safety Data Sheets on Risk Group 2 pathogens. Note that country-specific regulations will apply, and that this site should be used only as a guide.

https://www.miltenyibiotec.com/AU-en/products/macs-cell-separation.html

This site provides information on the use of MACS separation protocols (See Support Protocol-3).

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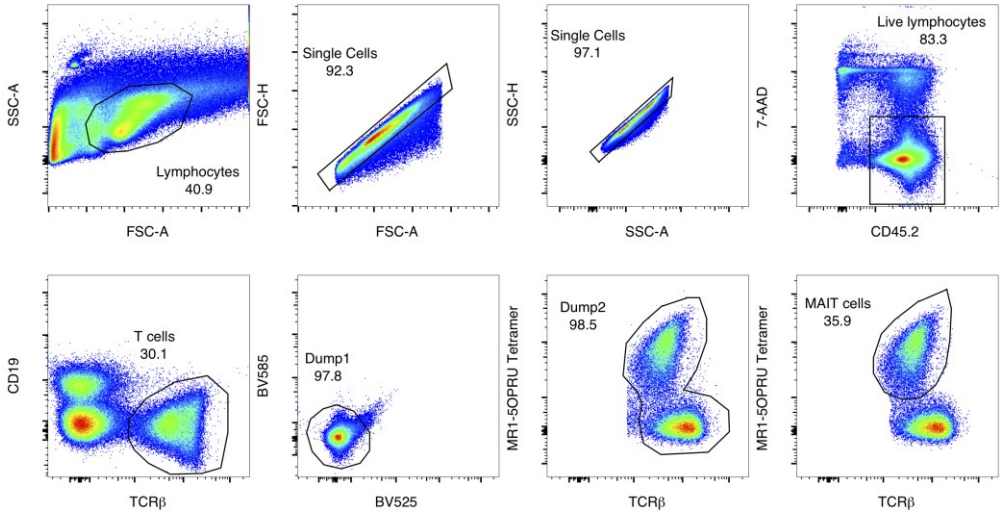


Figure 1. FACS gating strategy. Murine MAIT cells are identified by gating on the lymphocyte population and excluding doublets using forward scatter / side scatter properties; 7AAD$^-$ CD45$^+$ live lymphocytes are gated and further selected as CD19$^-$ TCR-β$^+$; autofluorescent cells are excluded using fluorescence in BV525 and BV585 channels and TCR-β$^+$ T cells or TCR-β$^+$MR1-5-OP-RU tetramer$^+$ MAIT cells are then gated as shown.

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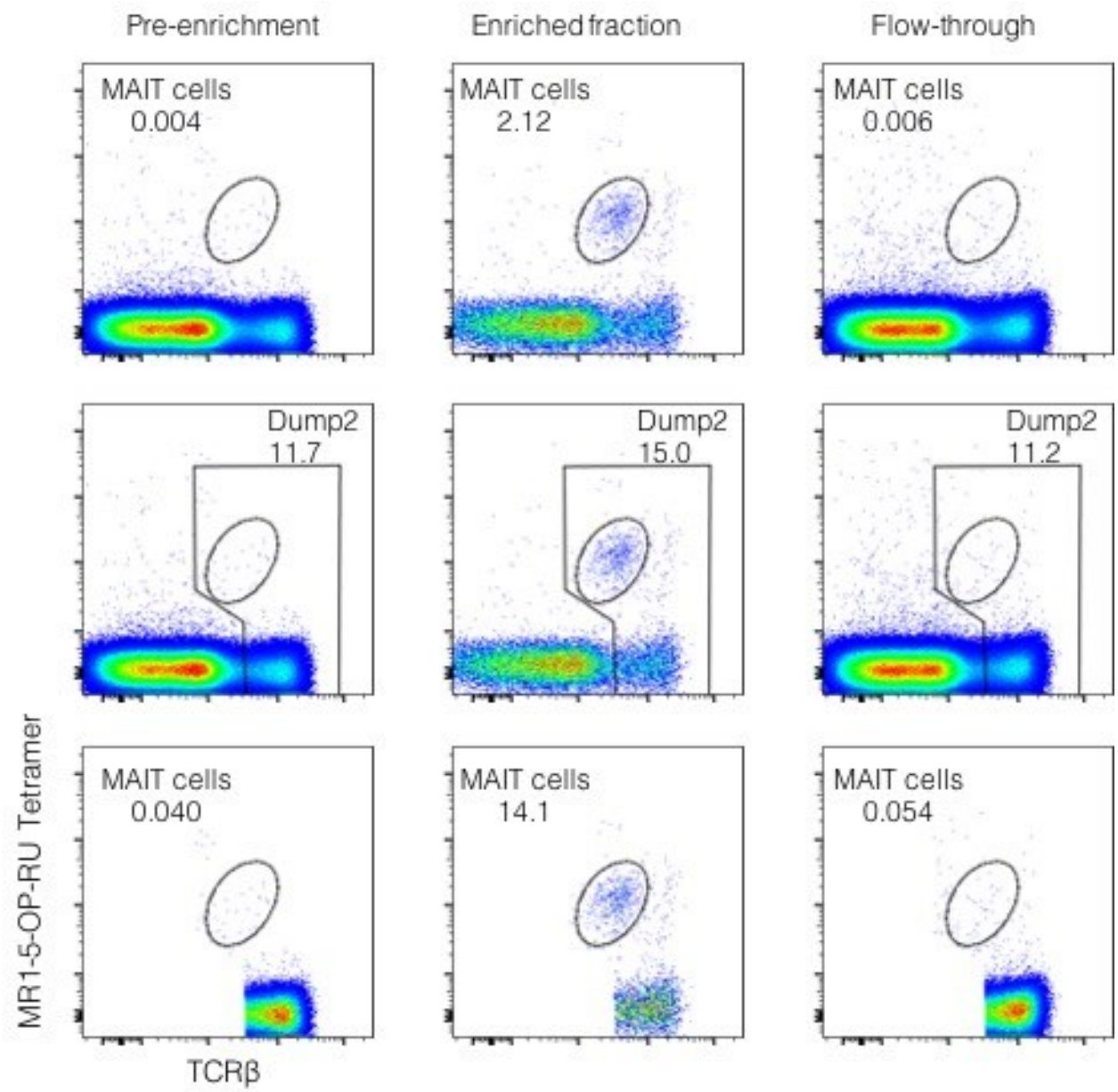


Figure 2. Expected results with Tetramer-enrichment (Support Protocol-3). Plots show pre enrichment, enriched fraction and MACS flow through stained according to Basic Protocol-1, with top plots showing live, $CD19^-$ cells after autofluorescence is excluded, and further gating for $TCR\beta^+$ and MR1-$tetramer^+$ cells shown in middle and lower plots.

TABLES

Table 1. Preparation of Percoll gradient working stocks

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33.75% Percoll stock

Desired volume (ml)	20	25	30	40	50	60	75	80	100	125	150	175
1xPBS (ml)	12.5	15.6	18.8	25	31.3	37.5	46.9	50	62.5	78.1	93.8	109.4
100% Percoll (ml)	6.8	8.4	10.1	13.5	16.9	20.3	25.3	27	33.8	42.2	50.6	59.1
10xPBS (ml)	0.8	0.9	1.1	1.5	1.9	2.3	2.8	3	3.8	4.7	5.6	6.6

40% Percoll stock

Desired volume (ml)	20	25	30	40	50	60	75	80	100	125	150	175
1xPBS (ml)	11.1	13.9	16.7	22.2	27.8	33.3	41.7	44.4	55.6	69.4	83.3	97.2
100% Percoll (ml)	8	10	12	16	20	24	30	32	40	50	60	70
10xPBS (ml)	0.9	1.1	1.3	1.8	2.2	2.7	3.3	3.6	4.4	5.6	6.7	7.8

70% Percoll stock

Desired volume (ml)	20	25	30	40	50	60	75	80	100	125	150	175
1xPBS (ml)	4.4	5.6	6.7	8.9	11.1	13.3	16.7	17.8	22.2	27.8	33.3	38.9
100% Percoll (ml)	14	17.5	21	28	35	42	52.5	56	70	87.5	105	122.5
10xPBS (ml)	1.6	1.9	2.3	3.1	3.9	4.7	5.8	6.2	7.8	9.7	11.7	13.6

ABBREVIATIONS

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

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

7-AAD – 7-aminoactinomycin D

Ab – antibody

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Ag – antigen

ANOVA – Analysis of Variance

APC – Ag presenting cell

BSCII – biological safety cabinet, class II type

BYE– buffered yeast extract medium

BCYE - buffered charcoal yeast extract medium

BSA – bovine serum albumin

CBA – cytometric bead array

CD – cluster of differentiation

CFU – colony counting unit

DTT - dithiothreitol

ELISA – enzyme linked immunosorbent assay

EDTA – Ethylenediaminetetraacetic acid

FACS – fluorescence activated cell sorting

FCS – foetal calf serum

Fluorescent conjugates for antibodies:

- APC – Allophycocyanin
- BV421 – Brilliant Violet 421
- FITC – Fluorescein
- PE – Phycoerythrin
- PerCP – Peridinin-Chlorophyll-protein

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FcγRIII (CD16) – Fc: Fragment crystallizable region; γRIII: gamma receptor III.

GMO – genetically modified organism

HBSS – hanks balanced salt solution

ICS – intracellular staining

IFNγ – interferon γ

IL – interleukin

LB – Luria Bertani

LN – lymph node

LPS – lipopolysaccharide

PMA – phorbol 12-myristate 13-acetate

mAb – monoclonal Ab

MAIT – Mucosal-Associated Invariant T cell

MHC – Major Histocompatibility Complex

MFI – mean fluorescence intensity

MR1 – MHC related protein-1

NaCl – Sodium chloride

NKT – Natural Killer T cell

OD – optical density

OGTR – office of gene technology regulation

PAMPs – Pathogen-associated molecular pattern molecules

PBS – phosphate buffered saline

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RAG-/-γC-/- – mice deficient in RAG (recombination-activation gene) and γC (the common gamma chain gene, also known as interleukin-2 receptor subunit gamma or IL-2RG)

RBC – red blood cells

RORγt – the second isoform of RAR-related orphan receptor gamma

RPM – revolutions per minute

RPMI – Roswell Park Memorial Institute medium

RT – room temperature

Strep^{r} – streptomycin resistance

TAC – Tris-based Ammonium Chloride (TAC) hypotonic Red Blood Cell (RBC) lysis buffer

T-bet– The transcription factor encoded by TBX21

TBS – Tris based saline

TCR – T cell receptor

TF – transcription factors

TLR – Toll-like receptor

TNF – tumor necrosis factor

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