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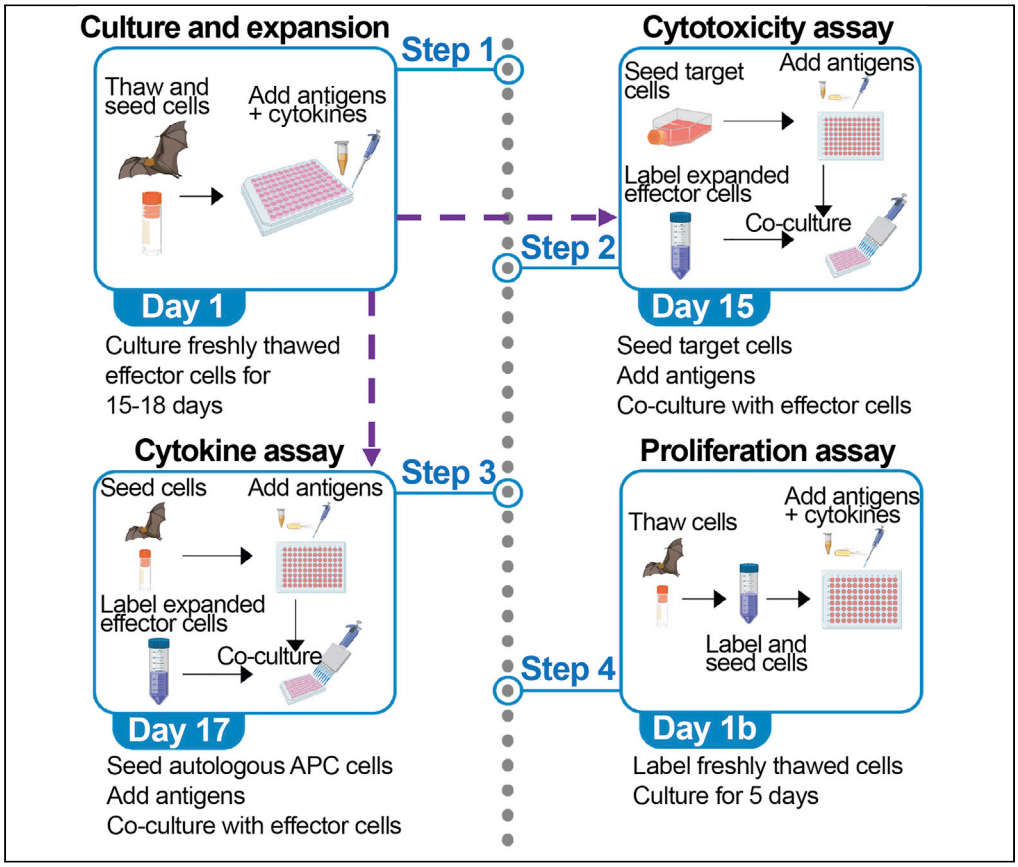
Protocol

Culture, expansion, and flow-cytometry-based functional analysis of pteropid bat MR1-restricted unconventional T cells



Bats harbor viruses of global public health significance. Understanding bat immune systems may provide intervention strategies to prevent zoonotic disease transmission and identify therapeutic targets. This protocol describes how to culture and expand pteropid bat unconventional T cells, restricted by the MHC-I-related protein 1 (MR1), an MHC-I-like protein. Using multicolor flow-cytometry-based techniques, we examine pteropid MR1T cell functionality, including proliferative capacity, cytotoxicity, and cytokine production. This protocol can be adapted to aid immunological research in other bat species.

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Highlights

Studies into bat immune systems are hampered by the lack of specific protocols

Protocol describes culture and expansion of pteropid bat MR1-restricted T cells

Performs antigen-specific stimulations of pteropid bat MR1-restricted T cells

Flow-cytometry-based analyses of proliferation, cytotoxicity, and cytokine production

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Culture, expansion, and flow-cytometry-based functional analysis of pteropid bat MR1-restricted unconventional T cells

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SUMMARY

Bats harbor viruses of global public health significance. Understanding bat immune systems may provide intervention strategies to prevent zoonotic disease transmission and identify therapeutic targets. This protocol describes how to culture and expand pteropid bat unconventional T cells, restricted by the MHC-I-related protein 1 (MR1), an MHC-I-like protein. Using multicolor flow-cytometry-based techniques, we examine pteropid MR1T cell functionality, including proliferative capacity, cytotoxicity, and cytokine production. This protocol can be adapted to aid immunological research in other bat species.

For complete details on the use and execution of this protocol, please refer to Leeansyah et al. (2020b)

BEFORE YOU BEGIN

The main steps of the protocol include expansion of primary pteropid bat cell culture and their functional characterization through cytotoxicity and cytokine assay. This protocol describes the reagents used to identify and characterize pteropid MR1-restricted T (MR1T) cells, and has been adapted from our protocols for human mucosal-associated invariant T (MAIT) cell work (Boulouis et al., 2020; Dias et al., 2017b; Dias et al., 2016; Sia et al., 2020). In this work, pteropid MR1T cells are defined as T cells that are restricted by MR1 and recognize ligands bound and presented by MR1. These cells are identified by positive staining with MR1 tetramers loaded with the prototypical MR1 ligand 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU).

⚠ CRITICAL: Before working on bat cells, we highly recommend researchers to have rabies and tetanus vaccinations, at the least. Hepatitis B vaccination should also be done if working with human blood. Other recommended vaccinations would be dependent on the

country and the research institute. Hence, work with institutional safety team for proper laboratory safety and risk assessments and documentations before commencing work. Always work in a NSF-49 certified BSL-II hood. Wear gloves, lab coat, and safety glasses. Decontaminate waste into fresh 10% bleach for treatment overnight, and conduct proper disposal into a biohazard bin.

Reagents discussed below can be prepared and stored before beginning experiments.

Media and reagent preparation

Timing: ~1 h

1. T cell culture and expansion media
   a. ImmunoCult™-XF T Cell Expansion Medium + CTS™ Immune Cell Serum Replacement (CTS) + antibiotics (50 μg/mL gentamicin + 100 μg/mL Normocin) (IC culture medium) (See Materials and Equipment section).
   b. IC Culture Media + 10 ng/mL recombinant human (rh) interleukin (IL)-2 + 20 ng/mL IL-7 (IC culture medium + 2× cytokines) (See Materials and Equipment section).
2. Cell line culture media and assay media
   a. For bat cell lines: Dulbecco's Modified Eagle Medium (DMEM) + 10% low-endotoxin fetal bovine serum (FBS) (D10 medium) (See Materials and Equipment section).
   b. For human cell lines: RPMI-1640 medium + 10% FBS + antibiotics + 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (R10 medium) (See Materials and Equipment section).
3. Dissection and freezing media
   a. Dissection harvest media: D10 medium (See Materials and Equipment section).
   b. Freezing media: 90% FBS + 10% dimethyl sulfoxide (DMSO) (See Materials and Equipment section).
4. MR1 ligands 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and acetyl-6-formylpterin (Ac-6-FP) dissolved in DMSO (Corbett et al., 2014; Eckle et al., 2014; Mak et al., 2017) and in culture media (See Materials and Equipment section).

⚠ CRITICAL: When handling MR1 ligands, always work in the dark and quickly. Prepare aliquot stocks and freeze at −80°C immediately. 5-OP-RU is stable in DMSO, but exposure time to aqueous media should be minimized as much as possible to maximize activity, as 5-OP-RU converts rapidly to a much less active lumazine in water (Mak et al., 2017). All 5-OP-RU and Ac-6-FP working solutions were diluted from the DMSO stocks with appropriate culture media immediately (within 2 min) prior to any functional assay.

Cryopreserved bat cells

Timing: ~1 day

In this Protocol, we used the pteropodid bat model, the frugivorous black flying fox, Pteropus alecto (Pa). Primary bat cells were obtained from euthanized bats with irreparable physical damage from the Queensland bat rescue center, Queensland, Australia. Bone marrow and spleen were processed for cryopreservation, shipped to Duke-National University of Singapore (NUS) Medical School, Singapore, and stored in −80°C for later work. More information on the processing can be referred to (Irving et al., 2020).

5. Bone marrow
   a. Obtain rib bone (nearest to body) and trim away excess flesh.
   b. Fill syringes with cold (2°C–8°C) D10 medium (DMEM+10% FBS) and attach 18-gauge needle.
   c. Cut away both caps of the bone.
   d. Portion the bone into 2 halves.

e. Flush the bone marrow with cold media, by inserting the needle to the middle of the marrow.
f. Flush from both ends and collect cells into new 50 mL tube.
g. Wash recovered cells twice with cold D10 medium.
h. Lyse red blood cells with 1× red blood cell (RBC) lysis buffer (See Materials and Equipment section) for 10 min at 23°C–25°C.
i. Wash cells with cold cell-culture grade PBS (1×).
j. Freeze cells at 10^7 cells per vial in 1 mL with freezing media.

6. Spleen
   a. Obtain spleen and keep tissue in cold D10 medium until ready.
   b. Cut spleen tissue into tiny pieces with a surgical scissors.
   c. Strain the spleen tissue through a 100 μm cell strainer into a 50 mL falcon tube or petri dish with 5 mL of D10 medium (See Materials and Equipment section).
   d. Push the remaining tissue through the cell strainer with the flat end of a plunger from a new syringe.
   e. Flush the cell strainer with cold media.
   f. Collect cells into a new 50 mL falcon tube.
   g. Wash the recovered cells twice with cold D10 medium to improve viability during extraction.
   h. Lyse red blood cells with 1× RBC lysis buffer for 10 min at 23°C–25°C.
   i. Wash cells with cold cell culture-grade PBS.
   j. Freeze cells at 10^7 cells per vial in 1 mL with freezing media.

⚠ CRITICAL: Bat cells have a tendency of poor viability. Work fast, keep everything on ice, and keep working centrifuge temperature cold (4°C).

Cultured cell lines

Timing: ~2 weeks

7. Bat cell lines – *P. alecto* kidney cells (PaKi) and *P. alecto* lung (PaLu) cell lines.
8. Human cell line- 293T cells stably-expressing human MR1 (293T-hMR1) and HeLa cell lines.

⚠ CRITICAL: Allow cell lines to recover and grow for at least 2 weeks before using for assay. Always monitor for significant morphological changes. Discard if cells looking abnormal. Not recommended to use cell lines passage number (P) beyond P40 for PaKi cells, P20 for PaLu cells, P17 for 293T-hMR1 cells, and P20 for HeLa cells.

Preparation of bacterial cultures

Timing: ~1 h

9. Formaldehyde fixation of frozen bacteria stock (Dias et al., 2017b; Dias et al., 2016).
   a. Frozen vials of cultured bacteria stock with known numbers (CFU/mL).
      i. Any riboflavin-synthesis competent *Escherichia coli* strains, incl. the *RibA*$^+$ 1100-2 strain.
      ii. Any riboflavin-synthesis incompetent *E. coli* strains, incl. the *RibA*$^-$ BSV18 strain.
   b. 1% formaldehyde for fixing bacteria
      i. Formaldehyde diluted in PBS.

Flow-cytometry reagents

Timing: ~5 min

10. Fluorescence-activated cell sorting (FACS) buffer: PBS + 2 mM ethylenediaminetetraacetic acid (EDTA) + 2% FBS) (FACS buffer) (See Materials and Equipment section).

11. MR1-Tetramers conjugated with fluorochromes
    a. hMR1-5-OP-RU
    b. hMR1-6-FP

⚠ CRITICAL: Light and temperature sensitive reagents.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies/tetramers		
Human MR1-5-OP-RU tetramer PE (dilution: 1/800; final: 1.625 µg/mL)	The NIH Tetramer Core Facility, Emory University NIH	N/A
Human MR1-6-FP tetramer BV421, AF680 (dilution: 1/400; final: 4 µg/mL for BV421; 1/200; final: 8 µg/mL for AF680)		N/A
Anti-human CD3ε-FITC (dilution: 1/100; final: 10 µg/mL)	Bio-Rad	Clone: CD3-12; Cat#: MCA1477F; RRID: AB_566708
Anti-human Perforin PF647 (dilution: 1/50; final: 20 µg/mL)	Mabtech	Clone: Pf-344; Cat#: 3465-72-100T; RRID:AB_2888642
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation	Invitrogen	N/A
Anti-human active Caspase 3 BV650 (dilution: 1/40; final: n/a)	BD Biosciences	Clone: C92-605; Cat#: 564096; RRID: AB_2738589
Anti-human T-bet PE-Cy7 (dilution: 1/200; final: 2 µg/mL)	Invitrogen/Thermo Fischer	Clone: eBio4B10; Cat#: 25-5825-82; RRID: AB_11042699
Anti-human Eomes PE-610 (dilution: 1/100; final: 2.5 µg/mL)	Invitrogen	Clone: WD1928; Cat#: 61-4877-42; RRID: AB_2574616
Anti-human PLZF APC (dilution: 1/100; final: n/a)	R&D Systems	Clone: 6318100; Cat#: IC2944A; RRID: AB_10730709
Anti-human RORγt BV650 (Dilution: 1/50; final: n/a)	BD Biosciences	Clone: Q21-559; Cat#: 563424; RRID: AB_2738197
Anti-human IL17A BV786 (dilution: 1/50; final: 2 µg/mL)	BioLegend	Clone: BL168; Cat#: 512338; RRID: AB_2566765
Anti-human TNF BV711 (dilution: 1/50; final: 2 µg/mL)	BioLegend	Clone: Mab11; Cat#: 502939; RRID: AB_2562740
Bacterial and virus strains		
E. coli EC120S	Singapore General Hospital archived bacteria repository (Lim et al., 2018)	N/A
E. coli 1100-2	Coli Genetic Stock Center, Yale University	*E. coli* laboratory strain *RibA*$^+$, riboflavin-synthesis competent
E. coli BSV18	Coli Genetic Stock Center, Yale University	*E. coli* laboratory strain *RibA*-deficient, riboflavin-synthesis incompetent congenic strain of *E. coli* 1100-2
Biological samples		
P. alecto bone marrow	Duke-NUS Medical School (Singapore) & Australian Animal Health Laboratory (AAHL), CSIRO (Victoria, Australia).	N/A
P. alecto spleen	Duke-NUS Medical School (Singapore) & Australian Animal Health Laboratory (AAHL), CSIRO (Victoria, Australia)	N/A
Chemicals, peptides, and recombinant proteins		
5-(2-Oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU)	Institute for Molecular Bioscience, University of Queensland, Australia	N/A
Acetyl-6-formylpterin (Ac-6-FP)	Schircks Laboratories	Product# 11.418
CellTrace™ Violet Cell Proliferation Kit	Invitrogen	Cat#: C34557

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BD Transcription Factor Staining Kit	BD Biosciences	Cat#: 562574
Recombinant human IL-2	PeproTech	Cat#: 200-02
Recombinant human IL-7	R&D Systems	Cat#: 207-IL
Normocin	InvivoGen	Cat#: ant-nr-1
Gentamicin	Gibco	Cat#: 15750060
Formaldehyde solution	Sigma-Aldrich	Cat#: 252549-100ML
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#: D2438
BD GolgiStop™Protein Transport Inhibitor (containing monensin)	BD Biosciences	Cat#: 554724
Leukocyte Activation Cocktail, with BD GolgiPlug™ (PMA)	BD Biosciences	Cat#: 550583
Ficoll-Paque PLUS density gradient media	GE Healthcare	Cat#: 17144003
ImmunoCult™-XF T Cell Expansion Medium	STEMCELL Technologies	Cat#: 10981
CTS™ Immune Cell Serum Replacement	Thermo Fisher	Cat#: A2596101
HEPES (1M)	Gibco	Cat#: 15630080
UltraPure™ 0.5M EDTA, pH 8.0	Invitrogen	Cat#: 15575020
HyClone Dulbecco's Phosphate Buffered Saline Without Calcium, Magnesium, Phenol Red (PBS)	HyClone	Cat#: SH30028.02
Fetal Bovine Serum, qualified, Brazil (FBS)	Gibco	Cat#: 10270106
Fetal Bovine Serum (FBS) South America, Ultra-low Endotoxin	Biowest	Cat#: S1860
RPMI-1640 Medium, no glutamine	Gibco	Cat#: 21870076
DMEM, high glucose	Gibco	Cat#: 11965092
DMEM, high glucose	Nacalai Tesque	Cat#: 08459-64
Trypsin-EDTA (0.25%), phenol red	Gibco	Cat#: 25200056
L-Glutamine (200 mM)	Gibco	Cat#: 25030081
BD™ CompBead Anti-Mouse Ig, κ/Negative Control Compensation Particles Set	BD Biosciences	Cat#: 552843
UltraComp eBeads Compensation Beads	Invitrogen	Cat#: 01-2222-42
eBioscience™ 10× RBC Lysis Buffer (Multi-species)	Invitrogen	Cat#: 00-4300-54
Experimental models: cell lines		
Bat: PaKi and PaLu cells	CSIRO Livestock Industries, Australian Animal Health Laboratory, Geelong, Australia, Australian Biosecurity Cooperative Research Centre for Emerging Infectious Diseases, Brisbane, Australia (Crameri et al., 2009) OR Duke-NUS Medical School (Singapore) & Australian Animal Health Laboratory (AAHL), CSIRO (Victoria, Australia)	N/A
Human: 293T-hMR1 cells	Washington University at St. Louis, MO, United States	N/A
Human: HeLa cells (ATCC CCL-2)	ATCC	Cat#: CCL-2
Experimental models: organisms/strains		
Pteropus alecto	Duke-NUS Medical School (Singapore) & Australian Animal Health Laboratory (AAHL), CSIRO (Victoria, Australia)	N/A
Software and algorithms		
FlowJo v. 9.9 or 10	FlowJo	https://www.flowjo.com/
GraphPad Prism v. 9.0	GraphPad	https://www.graphpad.com/
BD FACS Diva™ Software v8.0	BD Biosciences	N/A

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
96-Well V-bottom plate	Thermo Scientific	Cat#: 249662
96-Well U-bottom plate	Corning, Costar	Cat#: 3799
96-Well flat-bottom plate	Corning, Costar	Cat#: 3596
50 mL Centrifuge tubes	Greiner Bio-One	Cat#: 227261
15 mL Centrifuge tubes	Corning, Falcon	Cat#: 352096
1.5 mL Microcentrifuge tubes	Axygen	Cat#: MCT-150-C
1.2 mL Cluster tubes	Corning, Costar	Cat#: 4401
Sorvall ST40R Centrifuge	Thermo Scientific	Cat#: 75004524
Eppendorf® Centrifuge 5424/ 5424 R	Eppendorf	Cat#: Z722960
BD LSR Fortessa™ Flow Cytometer	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

Growth media

IC culture medium		
Reagent	Final concentration	Amount
ImmunoCult™-XF T Cell Expansion Medium	n/a	46 mL
CTS™ Immune Cell SR	8%	4 mL
Normocin	100 μg/mL	0.1 mL
Gentamicin	50 μg/mL	0.05 mL
Total	**n/a**	**50 mL**
Store at 4°C for a maximum of 30 days.		

IC expansion medium (2×)		
Reagent	Final concentration	Amount
ImmunoCult™-XF T Cell Expansion Medium	n/a	46 mL
CTS™ Immune Cell SR	8%	4 mL
Normocin	100 μg /mL	0.1 mL
Gentamicin	50 μg /mL	0.05 mL
rh-IL-2 (100 μg/mL)	10 ng/mL	0.005 mL
rh-IL-7 (50 μg/mL)	20 ng/mL	0.02 mL
Total	**n/a**	**50 mL**
Store at 4°C for a maximum of 30 days.		

Note: Prepare 2× IC expansion media for routine media change.

Note: Dilute to 1× with IC culture media during the initial culture set-up.

D10 medium		
Reagent	Final concentration	Amount
DMEM	n/a	45 mL
FBS-Low endotoxin	10%	5 mL
Total	**n/a**	**50 mL**
Store at 4°C for a maximum of 30 days.		

RPMI medium stock without FBS		
Reagent	Final concentration	Amount
RPMI	n/a	500 mL
L-glutamine	2 mM	5 mL
Normocin	100 μg/mL	1 mL
Gentamicin	50 μg/mL	0.5 mL
HEPES	25 mM	12.5 mL
Total	**n/a**	**519 mL**
Store at 4°C. Add L-glutamine to the same final concentration if the stock medium remains after 30 days.		

R10 medium		
Reagent	Final concentration	Amount
RPMI medium stock without FBS	n/a	45 mL
FBS	10%	5 mL
Total	**n/a**	**50 mL**
Store at 4°C for a maximum of 30 days.		

1× RBC Lysis Buffer		
Reagent	Final concentration	Amount
10× RBC lysis buffer	1×	5 mL
dH_2O	n/a	45 mL
Total	**n/a**	**50 mL**
Store at 4°C for a maximum of 30 days.		

Freezing Media		
Reagent	Final concentration	Amount
FBS	90%	n/a
DMSO	10%	n/a
Total	**n/a**	**n/a**

Note: Prepare freezing media fresh on day of freezing and keep on ice until use.

5-OP-RU		
Reagent	Final concentration	Amount
5-OP-RU	10 μM	n/a
DMSO	n/a	n/a
Total	**n/a**	n/a
Store main stock at −80°C and working aliquots at −20°C.		

⚠ CRITICAL: 5-OP-RU is sensitive to water and degrades easily. Only add into culture media immediately before the intended experiments (within 2 min). 5-OP-RU working stocks diluted in DMSO can be stored at −20°C and should not be subjected to more than three freeze-thaw cycles. Store main aliquots at −80°C.

Ac-6-FP		
Reagent	Final concentration	Amount
Ac-6-FP	50 mM	n/a

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Continued		
Reagent	Final concentration	Amount
DMSO	n/a	n/a
Total	**n/a**	**n/a**
Aliquot and store at −20°C.		

Formaldehyde (1% in PBS)		
Reagent	Final concentration	Amount
10% Formaldehyde in PBS	1%	1 mL
PBS	n/a	9 mL
Total	**n/a**	**10 mL**
Store at 23°C–25°C in the dark.		

FACS Buffer		
Reagent	Final concentration	Amount
PBS	n/a	488 mL
EDTA	2 mM	2 mL
FBS	2%	10 mL
Total	**n/a**	**500 mL**
Store at 4°C. Working aliquots are recommended. Keep stock bottle sterile.		

CellTrace™ Violet Cell Proliferation Kit		
Reagent	Final concentration	Amount
CellTrace™ Violet dye	n/a	1 tube
DMSO	n/a	20 μL
Total	**n/a**	**20 μL**
Store reconstituted working vials at 4°C for a maximum of 3 months.		

⚠ CRITICAL: Handle with minimal light exposure.

Live-Dead Fixable Viability Dye		
Reagent	Final concentration	Amount
LIVE/DEAD™ Fixable Near-IR Stain	n/a	1 tube
DMSO	n/a	50 μL
Total	**n/a**	**50 μL**
Store reconstituted working vial at 4°C for a maximum of 3 months.		

⚠ CRITICAL: Handle with minimal light exposure.

BD Transcription Factor Staining Kit – fixative (BD TF Fix buffer)		
Reagent	Final concentration	Amount
4× BD Fix/perm buffer	1×	n/a
Diluent	n/a	n/a
Total	**n/a**	**n/a**
Prepare 1× buffer fresh, store main stock solutions at 4°C.		

Alternatives: eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Cat. No 00-5523-00)

BD Transcription Factor Staining Kit - perm wash (BD TF perm wash)		
Reagent	Final concentration	Amount
5× BD Perm Wash buffer	1×	n/a
dH_2O	n/a	n/a
Total	**n/a**	**n/a**
Store at 4°C.		

Alternatives: eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Cat. No 00-5523-00)

STEP-BY-STEP METHOD DETAILS

Part I: Pteropid bat MR1T cell culture and expansion

Timing: ~14 days

The cultured pteropid MR1T cells can be used for functional assessment, including cytotoxicity and cytokine production. This expansion protocol enables higher cell numbers and a highly enriched population of MR1T cells at the end of culture, overcoming the challenge of resource limitations, including the need for large amounts of primary bat immune cells.

⚠ CRITICAL: Viability of bat cells tends to be low. Thaw a few vials from multiple bats as a backup.

Note: This protocol is adapted from a human MAIT cell expansion protocol (Boulouis et al., 2020). The overall timeline representation of Part I is depicted in Figures 1 and 2.

1. Initiation of primary culture from cryopreserved bat cells
   a. Thawing of primary bat cells
      i. Thaw cryovials in a water bath set at 37°C until a small piece of ice is left.
      ii. Transport cryovials into biosafety cabinet class-II (BSC-II) on wet ice.

⚠ CRITICAL: Practice aseptic technique, keep everything sterile and work in a BSC-II from this step onwards.

      iii. In BSC-II, transfer cells into a sterile 50 mL falcon tube.
      iv. Add cold (2°C–8°C) R10 medium dropwise onto cells. Swirl tube to mix as media is being added. Mix well.

Note: This thawing step describes thawing of 10^7 cells/mL. For every 1 mL of cells, add 10 mL of media. Scale proportion of media and buffers accordingly.

      v. Centrifuge tube at 390 *x g* for 5 min at 4°C.
      vi. Discard supernatant into diluted (10%) bleach. Resuspend cells with 10 mL of cold PBS. Centrifuge tube at 390 *x g* for 5 min at 4°C. Repeat step twice for a total of 3× PBS washes.
      vii. Resuspend pellet in 1 mL cold R10 medium and allow cells to recover at 23°C–25°C for 30 min.
      viii. Perform cell count by Trypan Blue exclusion. (Troubleshooting - Problem 1)

⚠ CRITICAL: Keep reagents on ice and centrifuge cold at 4°C. Wash out DMSO thoroughly to minimize DMSO toxicity on bat immune cells. This recovery step is critical before culturing.

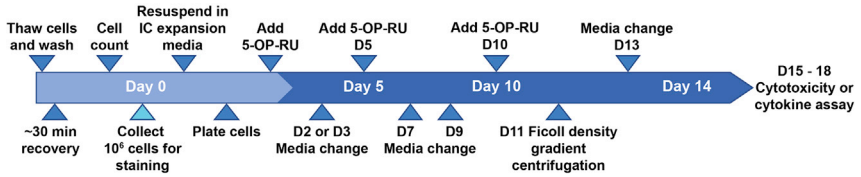

Figure 1. Timeline of Pa MR1T cell expansion culture

b. Culturing primary bat MR1T cells
   i. Wash the cells by adding 10 mL PBS to cells, mix well and centrifuge tube at 390 *x g* for 5 min at 4°C.
   ii. Discard supernatant by inverting the tube and pipette out any excess residual liquid. Re-suspend cells in complete expansion media (IC expansion medium (2× cytokines) diluted with base IC culture medium at 1:1 ratio), at 5 × 10^6 cells/mL.
   iii. Add 5-OP-RU to final concentration of 10 nM (x1000 dilution). Mix well and plate cells onto sterile 96-well U-bottom plate, at 200 μL per well (10^6 cells/well).
   iv. Incubate cells at 37°C in the 5% CO_2 incubator.
   v. Replace half volume of media with IC expansion medium (2×cytokines), every 2 days by pipetting out half volume of media and add back the equivalent volume. Mix well. If cells are accidentally disturbed during the media change, centrifuge supernatant and resuspend pellet with equivalent volume of fresh media before adding back to the wells.
   vi. Add 5-OP-RU at final concentration of 10 nM to cells on day 5 and 10.
   vii. Recover viable cells with Ficoll density gradient centrifugation as described (Dias et al., 2017b) on day 11 .(Troubleshooting - Problem 2).

⚠ CRITICAL: 5-OP-RU degrades rapidly upon exposure to aqueous solution. Only add into culture media immediately before the intended experiments (within 2 min). Preferably work in a darkened BSC-II. We recommend titrating 5-OP-RU to determine the optimal concentrations for your experimental set-up.

2. Staining cells and flow-cytometry-based analysis on day 0, 10, and 14 post-culture

Note: This staining step is for routine monitoring of expanded culture.

Note: This is the most basic staining cocktail for Pa MR1T cell identification.

a. Transfer 10^6 cells into a 96-well V-bottom plate.
b. Wash once with 200 μL FACS buffer. Spin at 390 × *g*, 5 min at 4°C. Discard supernatant by pipetting.
c. (Blocking) Add 50 μL of 10% FCS or if available, 10% bat serum diluted in FACS buffer to block cells for 30 min at 4°C. Additionally, pre-block cells with human (h)MR1-6-FP fluorescent-conjugated tetramer diluted in FACS buffer for 40 min at 4°C.

Note: Initial pre-blocking experiments are recommended to determine the binding specificity of hMR1-5-OP-RU tetramer to pteropid MR1T cells (Leeansyah et al., 2020b). Once

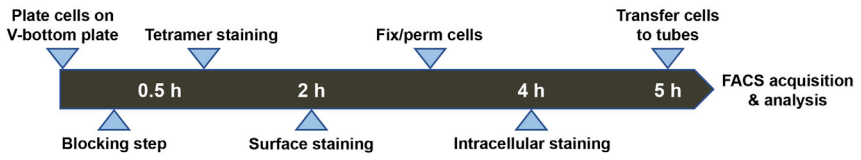

Figure 2. Timeline of the staining process for flow-cytometry-based analysis

this has been determined in your experimental set-up, the pre-blocking step may be omitted. We previously showed that hMR1-5-OP-RU tetramer binds with high specificity to Pa MR1T cells (Leeansyah et al., 2020b). By contrast, through pre-blocking experiments with hMR1-5-OP-RU tetramer, we showed that hMR1-6-FP tetramer bind weakly and with low specificity to Pa MR1T cells (Leeansyah et al., 2020b).

d. Wash cells with 150 μL FACS buffer. Spin at 390 × *g*, 5 min at 4°C. Discard supernatant by pipetting.
e. (hMR1-5-OP-RU tetramer staining) Stain cells with 50 μL diluted fluorochrome conjugated -hMR1-5-OP-RU and hMR1-6-FP tetramer for 40 min at 23°C–25°C, in the dark. (Trouble-shooting – Problem 5)
f. Wash cells with 150 μL FACS buffer. Spin at 390 × *g*, 5 min at 4°C. Discard supernatant.
g. (Surface staining) Stain cells with 50 μL surface staining cocktail with viability dye for 30 min at 4°C, in the dark.
h. Wash cells with 150 μL FACS buffer. Spin at 390 × *g*, 5 min at 4°C. Discard supernatant.
i. (Fixation) Fix cells with 100 μL 1× BD TF Fix buffer for 40 min at 4°C, in the dark.
j. Change centrifuge speed to 757 × *g*.
k. Add 100 μL 1× BD TF Perm Wash. Spin at 757 × *g*, 5 min at 4°C. Discard supernatant.
l. Add 200 μL 1× BD TF perm wash to remove the remaining fixative buffer. Spin at 757 × *g*, 5 min at 4°C. Discard supernatant by pipetting.
m. Spin again at 757 × *g*, 5 min at 4°C.
n. Pipette out and remove remaining excess residual liquid to avoid diluting the staining cocktail.
o. (Intracellular staining) Stain cells with 50 μL intracellular staining cocktail containing anti-CD3ϵ and anti-Perforin in 1× BD TF perm wash for 40 min at 4°C, in the dark.
p. Wash cells with 150 μL 1× BD TF perm wash. Spin at 757× *g*, 5 min at 4°C. Discard supernatant by pipetting.
q. Resuspend cells in 100 μL FACS buffer.
r. Transfer cells to FACS tubes. Store tubes in the dark at 4°C until acquisition.
s. Acquire cells on a flow cytometer. (Refer to Materials and Equipment)
t. Export files as Flow Cytometry Standard (FCS) 3.0.
u. Analyze files using flow-cytometry-based analysis software such as FlowJo.

Part II: Functional analysis—cytotoxicity

Timing: ~3 days

Note: This part is the continuation of Part I and should be planned accordingly. The overall timeline representation of Part II is depicted in Figure 3. The suggested experimental layout is outlined in Figure 4.

The second part of this protocol is used to examine the cytotoxic potential of the cultured MR1T cells by measuring apoptosis or cell death of cognate antigen-pulsed target cells.

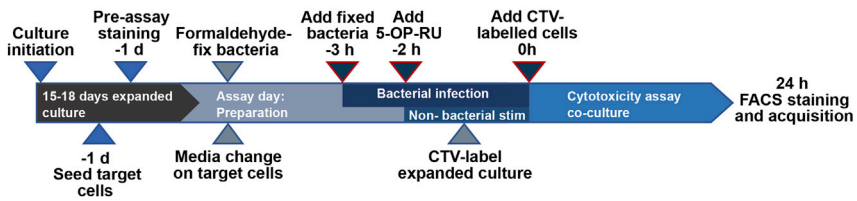

Figure 3. Timeline of the Pa MR1T cell cytotoxicity assay

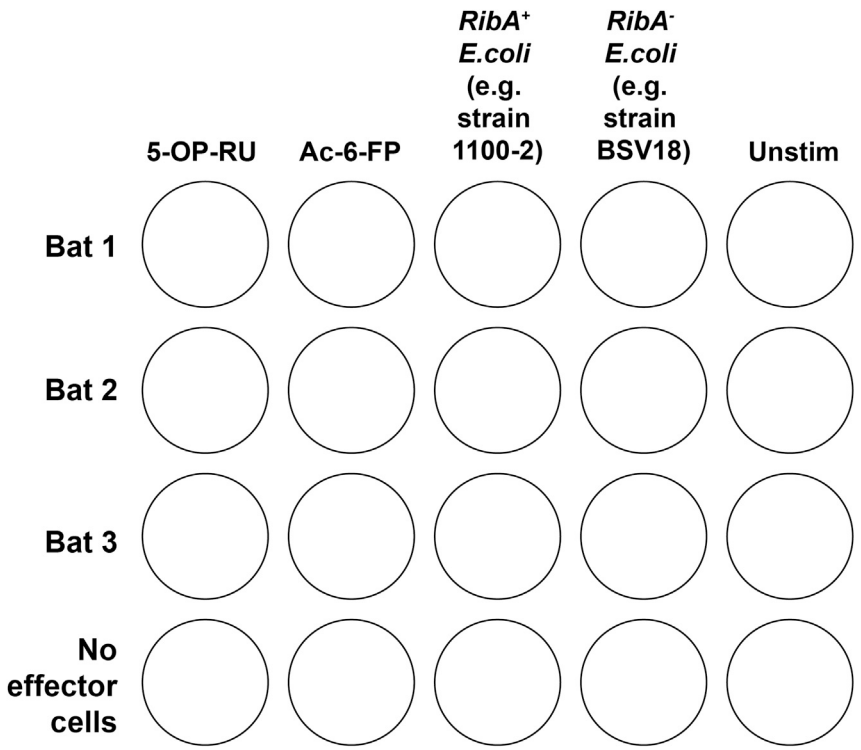

Figure 4. Plate layout for the Pa MR1T cell cytotoxicity assay

Target cell lines are pulsed with synthetic MR1 ligand 5-OP-RU or riboflavin biosynthesis proficient *E. coli*, producing these MR1 ligands. Target cells presenting cognate MAIT cell antigens on the MR1 molecule are recognized and killed by the expanded MR1T cells as effector cells, via the release of cytolytic granules such as perforin (Leeansyah et al., 2020b). Because MR1 is highly conserved in eutherian mammals, functional cross-reactivity has been observed between pteropid MR1T cells to human MR1 (Leeansyah et al., 2020b). Thus, cell lines of both pteropid bat and human origins can be used to assess pteropid MR1T cell cytotoxic capacity.

3. MR1 antigen/bacterial-pulsing of target cell lines (PaKi, HEK293T-MR1, HeLa) (-3.5 h)
   a. (~18 h before experiment) Seed cells at 5×10^4 cells per well in 200 µL cell culture media (Media and reagents preparation, point 2), into a 96-well flat bottom plate 18 h before assay.
   b. On the day of experiment: harvest cells from three representative wells and count cells. Use the average cell numbers to determine the bacterial dose required and the number of effector cells at a pre-determined effector:target cell (E:T) ratio. You can use the table below as a guide.

Target cell number (α = number of samples or experimental conditions)	$\alpha \times 10^6$
Bacterial cell number (e.g., bacterial dose=30)	$30 \times (\alpha \times 10^6)$
Effector cell number (e.g., E:T=10)	$10 \times (\alpha \times 10^6)$

   c. Remove media and add back 90 µL fresh pre-warm R10 medium.

Note: Pre-warm R10 medium at 37°C.

   d. Prepare formaldehyde-fixed bacteria (−3.5 h) (Dias et al., 2017b; Dias et al., 2016):

i. Thaw stock tube on ice, and add 5×10^8 bacteria into 1 mL PBS in a 1.5 mL tube. Spin at 5000 × *g* for 3 min at 4°C.
ii. Resuspend bacteria in 500 μL of 1% formaldehyde.
iii. Set timer to 3 min - vortex at maximum speed for the first 60 s, and then again during the final 30 s.
iv. Add 500 μL PBS to wash and spin at 5000 × *g* for 3 min at 4°C.
v. Discard supernatant by pipetting.
vi. Resuspend cells in 1 mL PBS to wash. Spin at 5000 × *g* for 3 min at 4°C. Discard supernatant.
vii. Repeat PBS wash.
viii. Resuspend bacteria in 1 mL cold (2°C–8°C) R10 medium.
ix. Adjust bacteria stock for a bacterial dose of 30 CFU per target cell in 10 μL R10 media with appropriate number of samples.

Note: You can use the table below as a guide for calculation. Bacterial numbers for the bacteria stock should be enumerated before freezing. Refer to previously published protocols for step-by-step guidelines how to enumerate bacterial numbers, which are expressed as colony-forming units (CFU)/mL (Boulouis et al., 2020; Sia et al., 2020).

	Per sample	Total samples
Target cell number (α = number of samples)	$\alpha\times10^6$	e.g., 50
Bacterial cell number (e.g., bacterial dose=30)	30 × ($\alpha\times10^6$) in 10 μL	50 × 30 × ($\alpha\times10^6$) in 500 μL

x. Add 10 μL of adjusted bacteria stock to target cells.

Note: Refer to Figure 4 for a plate layout illustration.
xi. Spin at 58× *g* for 2 min with minimal brake at 23°C–25°C.
xii. Incubate at 37°C in the 5% CO_2 incubator for 3 h.

e. After 1 h of incubation with bacteria, prepare and add 2 nM 5-OP-RU or 25 μM Ac-6-FP to corresponding wells and incubate for 2 h.

4. Preparation of effector cells (−2 h)

Note: Start preparing the effector cells after the addition of 5-OP-RU and Ac-6-FP to the target cells. Determine purity of effector (expanded MR1T) cells one day before assay by flow-cytometry-based analysis (Point 2).

a. Collect expanded day 15–18 MR1T cells into a sterile 50 mL falcon tube.
b. Spin at 390 × *g*, 5 min at 23°C–25°C. Decant supernatant.
c. Add 10 mL PBS to wash and perform cell count by Trypan Blue exclusion.
d. Spin at 390 × *g*, 5 min at 23°C–25°C. Discard supernatant by pipetting.
e. Resuspend cells at 5×10^6 cells/mL with PBS.
f. Dilute CellTrace™ Violet (CTV) stocks 1:50 with PBS, in the dark.

Note: Work in a darkened BSC-II when using CTV.

g. Add diluted CTV to cells at a 1:100 dilution (final 1:5000), in the dark.
h. Mix well by gentle vortexing to achieve uniform labeling of effector cells and wrap the tube with aluminum foil.
i. Incubate tube at 37°C for 30 min in a 5% CO_2 incubator.
j. Add 20 mL of pre-warmed R10 medium to quench reaction.
k. Incubate for a further 15 min at 37°C in a 5% CO_2 incubator.

l. Spin cells at 390 × *g*, 5 min at 23°C–25°C to wash.
m. Wash cells twice with 15 mL pre-warmed (37°C) PBS. Spin cells at 390 × *g*, 5 min at 23°C–25°C.
n. Resuspend cells in R10 medium at a determined cell concentration for adjusted E:T ratio, purity, and number of samples per 100 μL.

Note: You can use the table below as a guide for calculation.

	Per sample	Total samples
Target cell number (α = number of samples)	$\alpha \times 10^6$	e.g., 50
Effector cell number (e.g., E:T=10)	10 x ($\alpha \times 10^6$)	e.g., 50
Effector cell purity (e.g., 60%)	10 × (100 ÷ 60) × ($\alpha \times 10^6$) in 100 μL	50 × 10 × (100 ÷ 60) x ($\alpha \times 10^6$) in 5000 μL

5. Cytotoxicity assay (at 0 h)
   a. Adjust effector cells to final volume of 100 μL with an effector to target cell (E:T) ratio of 10:1.
   b. Add 100 μL of effector cells to target.

Note: Work in a darkened BSC-II. Refer to Figure 4 for a plate layout illustration.

   c. Mix gently and spin at 58× *g* for 2 min with minimal brake at 23°C–25°C to allow cell-to-cell contact.
   d. Incubate for 24 h at 37°C in a 5% CO_2 incubator.
   e. After 24 h, mix wells of the assay-plate and transfer to a 96-well V-bottom plate to collect floating cells. Spin 96-well V-bottom plate at 390 × *g*, 5 min at 23°C–25°C. Discard supernatant by pipetting.
   f. Resuspend wells of assay plate with 200 μL PBS and transfer PBS wash to the same 96-well V-bottom plate to collect remaining floating cells. Spin V-bottom plate at 390 × *g*, 5 min at 23°C–25°C. Discard supernatant by pipetting.
   g. Add 30 μL of Trypsin-EDTA to wells of assay-plate and treat cells for 5 min, at 37°C in a 5% CO_2 incubator.

Note: PaKi cells can be very adherent to plate. The cells can be trypsinized further but no longer than 10 min. (Troubleshooting – Problem 4)

   h. Add 100 μL of R10 to wells to stop reaction.
   i. Mix and transfer cells from assay-plate to the same 96-well V-bottom plate. Spin V-bottom plate at 390 × *g*, 5 min at 23°C–25°C. Discard supernatant.
   j. Wash cells on the V-bottom plate with 200 μL cold (2°C- 8°C) FACS buffer. Spin V-bottom plate at 390× *g*, 5 min at 23°C–25°C. Discard supernatant by pipetting.
   k. Proceed to staining for flow-cytometry-based analysis.

Note: Refer to Part I, point 2 above for staining. Intracellular staining cocktail for cytotoxicity assay is composed of anti-CD3ϵ, anti-Perforin and anti-active Caspase 3 diluted in 1× BD TF perm wash.

Note: We recommend titrating the optimal concentration of MR1 ligands and bacterial dose to be used in this experimental set-up. A time-kinetic study is also recommended to identify the best timing for capturing maximum target cell killing, e.g., 3, 6, 12 h. If human cell lines are used as target cells, MR1-dependency of MR1T cell cytotoxicity can be determined by using a blocking monoclonal antibody (mAb) against human MR1 (Leeansyah et al., 2020b), clone 26.5 (Biolegend, catalog No. 361103) (Huang et al., 2005). No blocking mAb against pteropid MR1 is commercially available at present.

Pause point: Effector cells for 0-h control can be kept at 4°C and stained together at the assay's endpoint. Fixed bacteria can be kept on ice for up to 2 hours if cells are not ready. Cytotoxicity assay can be done with expanded cells between day 15–18. Stain expanded culture 1 day before the assay to determine purity of effector cells, which may guide adjustment of the required E:T ratio (Dias et al., 2016). Flow-cytometry-based acquisition of stained cells should be done immediately if possible, or the samples can be stored protected from light at 4^0C for up to 24 h. Due to the cell number limitations, we recommend users to acquire as many cells as possible.

Part III: Functional analysis—cytokine production

Timing: ~2 days

Note: This part is the continuation of Part I and should be planned accordingly. The overall timeline representation of Part III is depicted in Figure 5. Suggested experimental layout is outlined in Figure 6.

The third part of the protocol explores the cytokine produced by the expanded MR1T cells upon recognition of specific antigens or following phorbol esters and calcium ionophore stimulations. The assay may give insights on how pteropid MR1T cells would orchestrate downstream immune responses *via* the production of cytokines.

Freshly-thawed autologous bat immune cells will need to be supplied as antigen-presenting cells (APCs) as the expansion procedure depletes the APCs originally contained at the beginning of the expansion culture (Leeansyah et al., 2020b). These added autologous APCs will take up the cognate antigens and present them to the expanded Pa MR1T cells. Due to the lack of pteropid bat-specific reagents, we were able to verify only two cytokines that can be readily detected by using the reagents listed in this Protocol: tumor necrosis factor (TNF) and IL-17.

This assay also allows examination of IL-17 and TNF production by freshly-thawed (non-expanded) bat primary immune cells.

6. Preparation of autologous APCs (−4.5 h)
   a. Thaw cells as described above and determine cell number (Refer to Part I, point 1a).
   b. Seed cells at 5×10^4 cells per well in 90 μL into a sterile 96-well U-bottom plate in R10 medium. Add PBS to empty wells to minimize evaporation.

Note: Refer to Figure 6 for a plate layout illustration.

   c. Prepare fixed bacteria (−3.5 h) (Refer to Part II, Point 3d)
      i. Adjust fixed bacteria stock for a bacterial dose of 3 CFU per 0.05×10^6 autologous target cell in 10 μL and with number of reactions.

Note: See also Part II, point 3d-ix for guide to calculation. Refer to Figure 6 for a plate layout illustration.

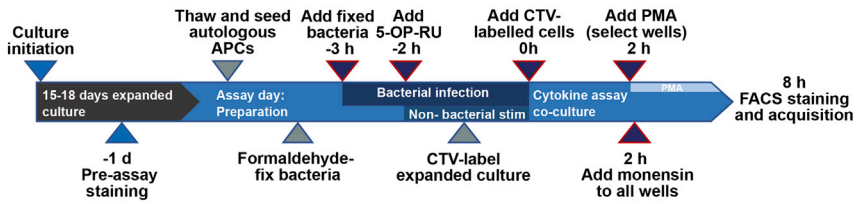

Figure 5. Timeline of the Pa MR1T cell cytokine production assay

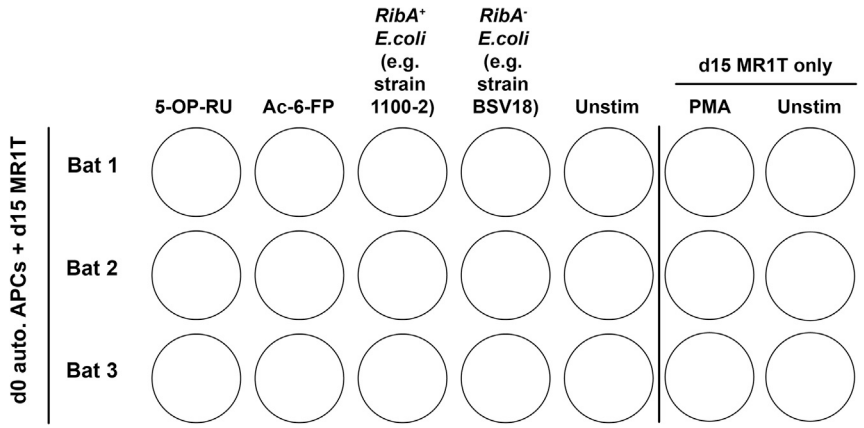

Figure 6. Plate layout for the Pa MR1T cell cytokine production assay

ii. Incubate for 3 h at 37°C in a 5% CO_2 incubator.

d. Prepare and add 2 nM 5-OP-RU or 25 μM Ac-6-FP and incubate for 2 h at 37°C in 5% CO_2 incubator (−2 h).

Note: Add diluted 5-OP-RU and Ac-6-FP immediately. Work in a darkened BSC-II. Refer to Figure 6 for a plate layout illustration.

7. Preparation of expanded Pa MR1T cells (−2 h)

Note: Start preparing the effector cells after the addition of 5-OP-RU and Ac-6-FP into the target cells. Determine the purity of expanded MR1T cells one day before assay by flow-cytometry-based analysis (Point 2). Refer to Part II, point 4 for preparation and CTV labelling of the effector cells. Work in a darkened BSC-II when using CTV.

a. Resuspend CTV-labeled effector cells in R10 medium at adjusted 2.5 × 10^5 per 100 μL.

8. Co-culture assay for cytokine detection (start of time = 0 h)

a. Add 100 μL of effector (Pa MR1T cells) to target cells (autologous APCs) (E:T ratio of 5:1).

Note: Work in a darkened BSC-II. Refer to Figure 6 for a plate layout illustration.

b. Mix gently at surface. Incubate for at least 2 h at 37°C in a 5% CO_2 incubator.

c. During the last 6 hours of the assay, add BD Leukocyte Activation Cocktail, with BD GolgiPlug™ (PMA and ionomycin with Brefeldin A) in 1:1000 dilution to PMA control wells. Add BD GolgiStop™ Protein Transport Inhibitor (containing monensin) in 1:1000 dilution to all wells.

Note: Work in a darkened BSC-II. Refer to Figure 6 for a plate layout illustration.

d. At the experimental endpoint, mix and transfer cells to a 96-well V-bottom plate. Spin the V-bottom plate at 390 × *g*, 5 min at 23°C–25°C. Discard supernatant.

e. Wash assay plate once with 200 μL of PBS and transfer the PBS wash to the same V-bottom plate. Spin V-bottom plate at 390 × *g*, 5 min at 23°C–25°C. Discard supernatant.

f. Proceed to staining for flow-cytometry-based analysis.

Note: Refer to Part I, point 2 above for staining. Intracellular cocktail for cytokine assay staining would be anti-CD3ϵ, anti-TNF, and anti-IL17 diluted in 1× BD TF perm wash.

Note: We recommend titrating the optimal concentrations of MR1 ligands and bacterial dose. A time-kinetic study is also recommended to identify the best timing for capturing maximum cytokine production: e.g., 8, 10, 12, 24, 48, and 72 h of co-culture.

Pause point: This assay can be done with expanded cells between day 15–18. Stain expanded culture 1 day before the assay to determine purity of effector cells, which may guide adjustment of the required E:T ratio (Dias et al., 2016). Flow-cytometry-based acquisition of stained cells should be done immediately if possible, or the samples can be stored protected from light at 4^0C for up to 24 h.

Part IV: Functional analysis—antigen-specific MR1T cell proliferation

Timing: ~Recommended up to 3–10 days

This part examines the antigen-specific requirements of Pa MR1T cell proliferation. The overall timeline representation of Part IV is depicted in Figure 7. Suggested experimental layout is outlined in Figure 8.

Note: CTV is light-sensitive reagent. Work with minimal light exposure.

9. Labeling and culturing cells – refer to thawing step above (point 1a)
   a. Thaw and count cells as above (Refer to Part I, point 1a).
   b. Resuspend cells in 5 × 10^6 cells/mL of PBS.
   c. Collect and store 10^4 cells at 4°C for non-CTV control.

   Note: Store cells at 4°C until staining step point 10. If possible, also store 10^6 unlabeled cells for in-culture compensation control.
   d. Prepare a diluted stock of CTV in 1:40 with PBS.
   e. Add diluted CTV to cells at 1:100 (final concentration 1:4000) and proceed with CTV-labeling of cells as described in Part II, point 4.

   Note: Important to mix well with gentle vortexing. Wrap tube with foil. Always work in a darkened BSC-II when using CTV.
   f. Resuspend CTV-labeled cells at 10^6 cells per 190 μL with expansion media (1× IC expansion media).

   Note: Work in a darkened BSC-II to preserve CTV signal.
   g. Take an aliquot of 10^6 CTV-labeled cells for day 0 baseline staining. Also take an aliquot of 10^4 CTV-labeled cells as a CTV compensation control; this won't be stained). Store cells for staining.

   Note: Store cells in dark at 4°C until staining step, point 10.
   h. Plate cells into sterile 96-well U-bottom plate at 190 μL per well.

   Note: Refer to Figure 8 for experimental plating layout.

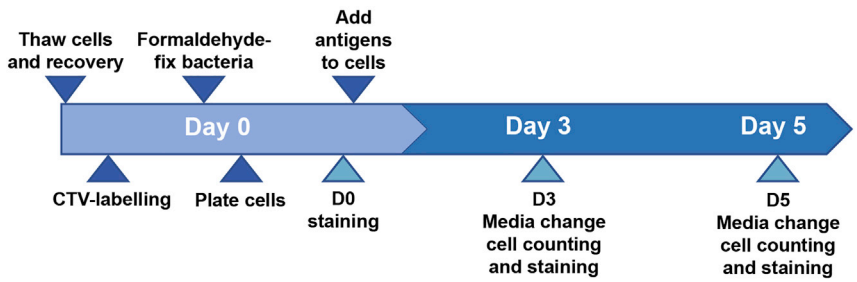

Figure 7. Timeline of Pa MR1T cell antigen-specific proliferation assay

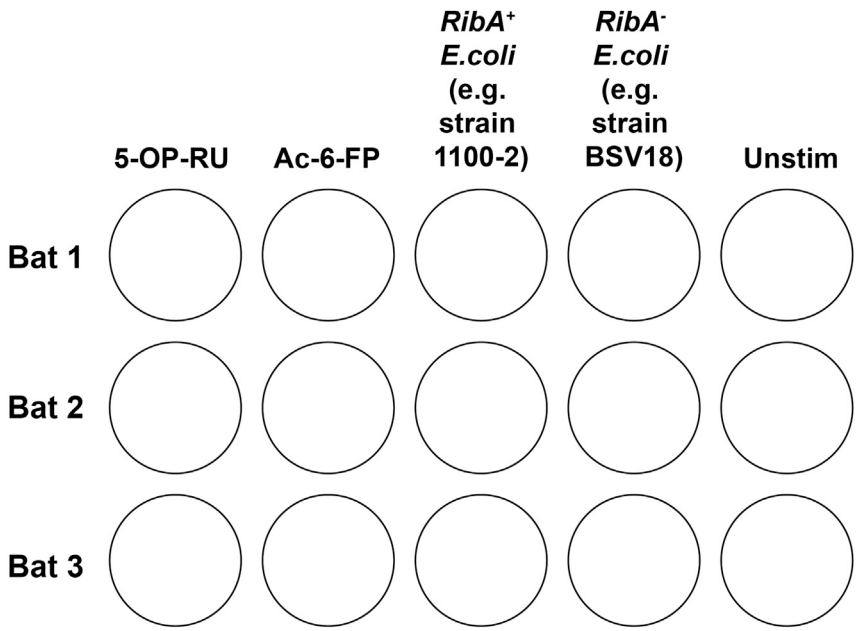

Figure 8. Plate layout for Pa MR1T cell antigen-specific proliferation assay

i. Prepare formaldehyde-fixed *E. coli* (Refer to Part II, point 3d):
   i. Resuspend fixed bacteria in 1 mL IC culture media.

j. Adjust and prepare MR1 ligands: 10 nM 5-OP-RU, or 25 μM Ac-6-FP, or formaldehyde-fixed *E. coli at* bacterial dose of 0.1 CFU/cell, diluted in IC expansion media. Add to plated cells in 10 μL to a final culture volume of 200 μL per well.

Note: Add diluted 5-OP-RU and Ac-6-FP immediately to the cells.

k. Add 200 μL PBS to remaining empty wells to reduce evaporation of culture media.

l. Culture plate at 37°C in a 5% CO_2 incubator.

m. Replace half of the media every 2 days with IC expansion media containing 2× IL-2 and IL-7 cytokines.

Note: Work in a darkened BSC-II to preserve CTV signal.

n. Perform cell count and stain cells on day 3, 5, 7 and 10 to determine expansion based on cell numbers CTV dilution. (Troubleshooting – Problem 3)

10. Staining cells for flow-cytometry-based analysis.
    a. Transfer the collected 10^6 CTV-labeled cells (Part IV, point 9 g) into a 96-well V-bottom plate.

    Note: We recommend a time-kinetic study to identify the best timing for capturing the most discernable CTV dilution peaks. We recommend day 3, 5, 7, and 10 post-stimulation. Take 50 μL of culture from well for staining. Work in a darkened BSC-II.

    b. Wash once with 200 μL FACS buffer. Spin at 390 × *g*, 5 min at 4°C. Discard supernatant.

    c. (Blocking) Add 50 μL of 10% FCS or if available, 10% bat serum diluted in FACS buffer to block cells for 30 min at 4°C.

    d. Wash cells with 150 μL FACS buffer. Spin at 390 × *g*, 5 min at 4°C. Discard supernatant.

    e. (hMR1-5-OP-RU tetramer staining) Stain cells with 50 μL diluted fluorochrome conjugated - hMR1-5-OP-RU and hMR1-6-FP tetramer for 40 min at 23°C–25°C, in dark.

    f. Wash cells with 150 μL FACS buffer. Spin at 390 × *g*, 5 min at 4°C. Discard supernatant.

g. (Surface staining) Stain cells with 50 μL surface staining cocktail with viability dye for 30 min at 4°C, in dark.
h. Transfer previously prepared aliquots of CTV-labeled cells for compensation control (point 9c and 9 g) to the empty wells on the V-bottom plate.

Note: CTV compensation control cells (point 9c and 9g) can be fixed separately or done together on the same staining plate. Compensation set-up has been thoroughly described in our previous publication (Dias et al., 2017b).

i. Wash cells with 150 μL FACS buffer. Spin at 390 × *g*, 5 min at 4°C. Discard supernatant by pipetting.
j. (Fixation) Fix cells with 100 μL 1× BD TF Fix buffer for 40 min at 4°C, in dark.
k. Add 100 μL 1× BD TF Perm Wash. Spin at 757 × *g*, 5 min at 4°C.
l. Discard supernatant by pipetting. Wash with 200 μL 1×BD TF perm wash to remove remaining fixative. Spin at 757 × *g*, 5 min at 4°C.
m. Discard supernatant by pipetting. Spin at 757× *g*, 5 min at 4°C.
n. Remove remaining excess residual liquid by pipetting.
o. (Intracellular/intranuclear staining) Stain cells with 50 μL intracellular staining cocktail containing anti-CD3ϵ, and (optional) anti-T-bet, anti-Eomes, anti-PLZF, and anti-RORγt antibodies in 1× BD TF perm wash for 40 min at 4°C, in dark.
p. Wash cells with 150 μL 1× BD TF perm wash. Spin at 757× *g*, 5 min at 4°C. Discard supernatant.
q. Resuspend cells in 100 μL FACS buffer.
r. Transfer cells to FACS tubes. Store tubes in dark at 4°C until acquisition.
s. Acquire cells on a flow cytometer. (Refer to Materials and Equipment).

⚠ CRITICAL: 5-OP-RU degrades rapidly upon exposure to aqueous solution and Ac-6-FP may be sensitive to light. Only add 5-OP-RU into culture media immediately before the intended experiments (within 2 min). Preferably work in a darkened BSC-II. We recommend titrating 5-OP-RU, Ac-6-FP, and the bacterial dose (i.e., CFU/cell) to determine the optimal concentrations for your experimental set-up. Minimize light exposure when working with CTV-labelled cells. If possible, also set apart ~10^6 unlabeled and CTV-labeled cells and culture in IC culture media throughout the culturing experiment; they can be used to set up compensation matrix and as a fluorescence minus one (FMO) control on the day of the assay.

Pause point: Flow-cytometry-based acquisition of stained cells should be done immediately if possible, or the samples can be stored protected from light at 4^0C for up to 24 h.

EXPECTED OUTCOMES

In this Protocol, we have developed methodologies to identify and culture Pa MR1T cells and the different cellular assays to examine functionality of these unconventional T cells. We provide protocols with step-by-step guidance and describe potential pitfalls for Pa MR1T cell identification, culture, and expansion in Part I, cytotoxicity in Part II, cytokine production in Part III, and antigen-specific proliferation in Part IV (Figure 9). Here, we discuss in detail strategies how to analyze the functional properties of Pa MR1T cells.

Identification of Pa MR1T cells

We begin by electronically plotting the time vs. side scatter area (SSC-A) in order to check how stable the sample acquisition was. Electronically gate only the flow cytometric events where acquisition was stable. Next, cellular aggregates were excluded by tight linear gating of cells at an approximately 45° angle within the forward scatter area (FSC-A) and height (FSC-H) plot. Viable cells were selected next by gating on dead-cell marker (DCM)-negative population, followed by lymphocyte selection

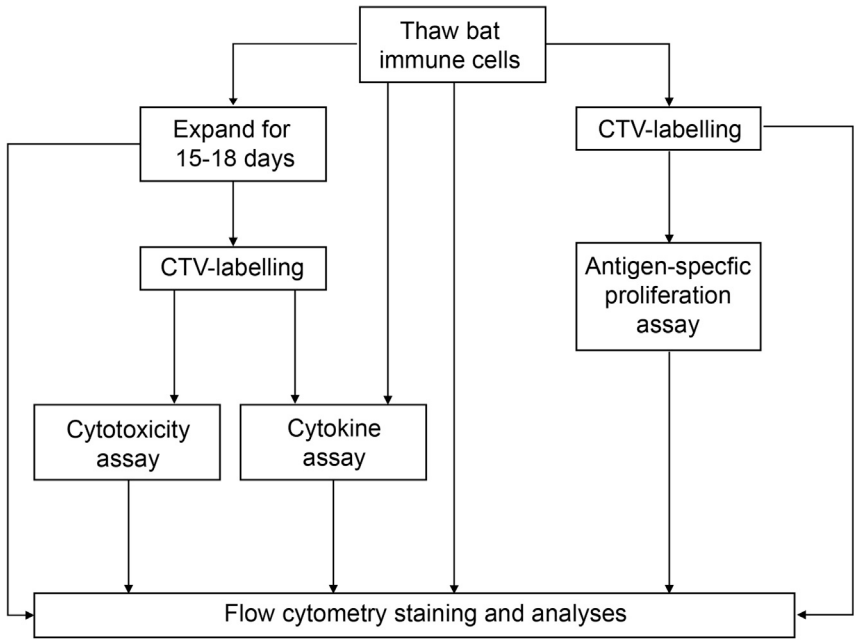

Figure 9. The overall flowchart of methodologies described in this protocol

based on their FSC and SSC properties. Finally, Pa MR1T cells were identified by their concomitant staining with anti-CD3 antibody and hMR1-5-OP-RU tetramer (Figures 10A and 10B).

Pa MR1T cell expansion

By visual inspection of the culture plate on day 5, cell expansion can be observed as gradual yellowing of the culture media and obvious cell clumps. The cells would also display pleiotropic morphologies when examined under the microscope, with larger size and higher granularity reflected by increased FSC and SSC profiles, respectively, on the flow cytometry plots. From a typical expansion culture, Pa MR1T cells achieve a purity of 60%–90% by the day of experiment (Figure 10C). Cell viability on day 10–11 of expansion is 40%–60% as detected by flow-cytometry-based analysis.

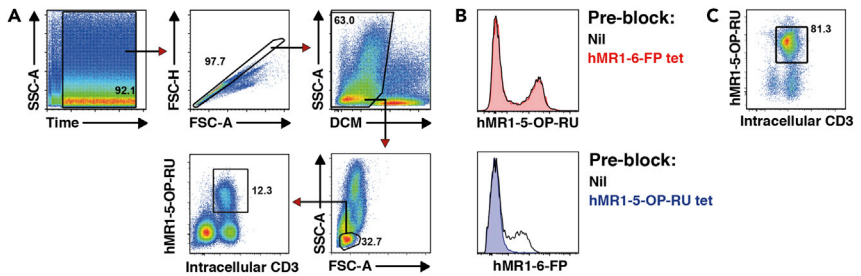

Figure 10. Identification of Pa MR1T cells by flow-cytometry-based analysis

(A) Representative FACS plots on the gating strategy for identification of Pa MR1T cells. Viable lymphocytes were selected based on dead cell exclusion (DCM^{neg}), and forward (FSC) and side scatter (SSC) parameters. Population positive for both CD3 and hMR1-5-OP-RU was selected, and Pa MR1T cells were identified as $CD3^{+}$hMR1-5-OP-RU^{+} cells.

(B) Representative histograms comparing the difference in (top) hMR1-5-OP-RU tetramer intensity with and without pre-blocking with 10 μg/mL hMR1-6-FP tetramer, and the difference in (bottom) hMR1-6-FP tetramer intensity with and without pre-blocking with 10 μg/mL hMR-5-OP-RU tetramer.

(C) Representative Pa MR1T cell purity as determined by positive staining for both CD3 and hMR-5-OP-RU tetramer 14 days post-stimulation with 5-OP-RU and rhIL-2 + IL-7.

We recommend removing dead cells by Ficoll density gradient centrifugation on day 10–11 of expansion. This will allow higher viability of expanded Pa MR1T cells on the day of experiment (typically >75%), as we previously described for human MAIT cell expansion cultures (Boulouis et al., 2020). Removing dead cells will also improve the downstream flow cytometric analyses for the MR1T cell cytotoxicity assay. By removing as many dead cells as possible before the assay, the false-positive apoptotic rates on the target cells will be substantially reduced. The described MR1T cell expansion culture should yield sufficient cells to proceed with functional assays outlined below.

Pa MR1T cell cytotoxic capacity

Because the vast majority of MR1T cells recognize riboflavin-related metabolites and mediate MR1-dependent effector function, including cytotoxicity, the cytotoxic capacity of Pa MR1T cells can be assessed using a co-culture experiment with riboflavin-producing bacteria (such as *E. coli*)- or synthetic MR1 ligand-pulsed target cells. In the co-culture experiment, target cells pulsed with 5-OP-RU or *RibA*$^{+}$ *E. coli*, triggered the killing function of effector Pa MR1T cells. By using the gating strategy shown in Figure 11 Pa MR1T cells can be distinguished from the target cells using the cell tracing dye CTV. Due to the lack of pteropid bat immune cell-specific or cross-reactive mAbs against activation markers, such as CD25, CD69, or CD107a, Pa MR1T cell activation can be assessed through the downregulation of their T cell receptor (TCR) (Dias et al., 2017a; Leeansyah et al., 2020b), as reflected by the diminished hMR1-5-OP-RU tetramer staining intensity (Figure 11). Killing of target

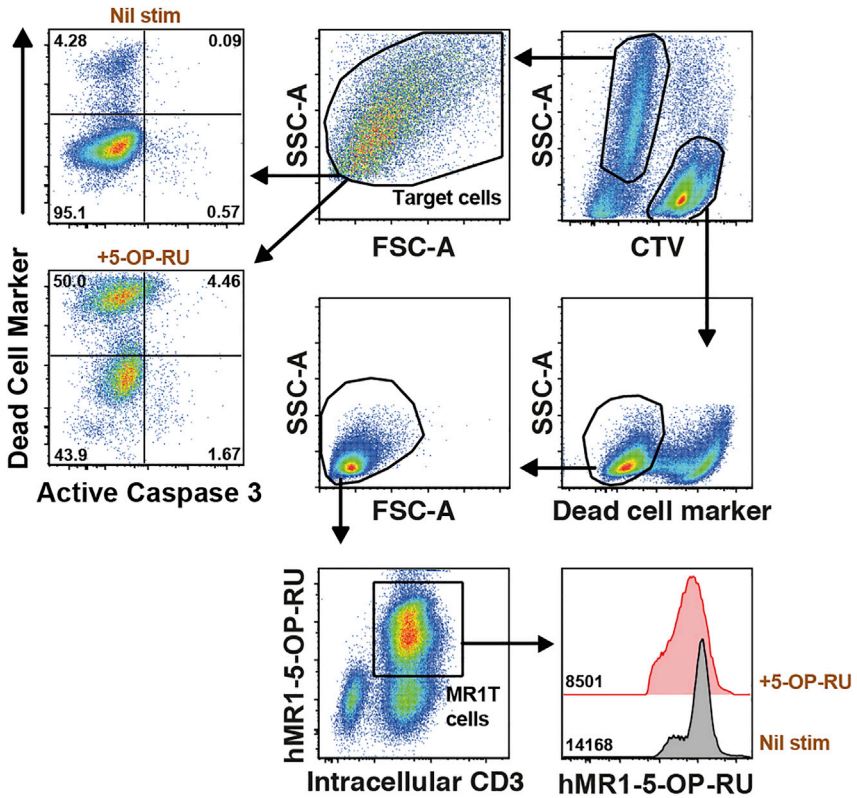

Figure 11. Pa MR1T cell cytotoxicity assay

Representative FACS plots showing gating strategy for Pa MR1T cell cytotoxicity co-culture assay with and without 5-OP-RU treatment. CD3^{+}hMR1-5-OP-RU^{+} effector cells were gated from CTV^{+}DCM^{-} cells and assessed for activation based on hMR1-5-OP-RU tetramer staining intensity. Representative histograms showing hMR1-5-OP-RU tetramer staining intensity for 5-OP-RU-treated co-culture (red) and negative control (black). CTV-negative target cells were assessed for their frequencies of DCM^{+} and Caspase 3^{+} cell population. Figure adapted and reprinted with permission from Leeansyah et al., 2020b.

cells by Pa MR1T cells is demonstrated by the high proportion of apoptosis (Caspase 3+ and/or DCM+) of target cells pulsed with the agonistic MR1 ligand 5-OP-RU (Figure 11). The MR1 dependence of Pa MR1T cells in recognizing and killing target cells can be assessed using a combinatorial approach using agonist (5-OP-RU) and non-agonist (Ac-6-FP) MR1 ligands and riboflavin-producing (*RibA*$^{+}$) and riboflavin-deficient (*RibA*$^{-}$) *E. coli* strains presented herein (Part II, point 3d and e), as well as through MR1-specific blocking mAb (Leeansyah et al., 2020b).

Pa MR1T cell cytokine production

In this assay, autologous, freshly-thawed Pa cells are used as APCs. Activation of expanded Pa MR1T cells with 5-OP-RU− or *RibA*$^{+}$ *E.coli*−pulsed targets cells is reflected in the higher frequencies of hMR1-5-OP-RU^{+} TNF^{+} and hMR1-5-OP-RU^{+} IL17^{+} T cells (Figure 12). By contrast, this was not seen on the remaining non-MR1T cells, indicating the specificity of Pa MR1T cells for riboflavin biosynthesis derived antigen (Figure 12). MR1 ligand-dependence of Pa MR1T cells in producing TNF and IL-17 can be assessed using a combinatorial approach using 5-OP-RU, Ac-6-FP, and *RibA*$^{+}$ and *RibA*$^{-}$ *E. coli* strains presented herein (Part III, point 6c and 6d). Moreover, stimulations using phorbol esters and calcium ionophores (e.g., BD Leukocyte Activator) serve as a positive control in assessing the capability of bulk Pa T cells (incl. MR1T cells) in producing these cytokines.

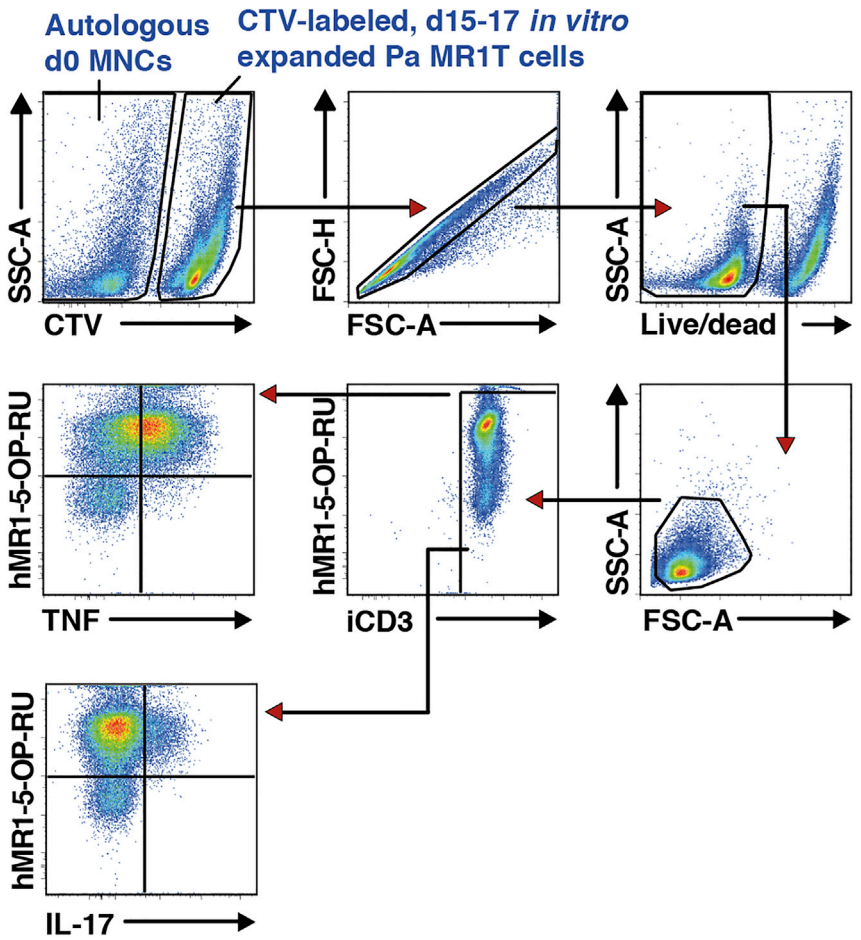

Figure 12. Pa MR1T cell cytokine production assay

Gating strategy for the cytokine assay of expanded MR1T cells co-cultured with autologous Pa freshly-thawed immune cells for assessment of TNF and IL17 production. Expanded Pa MR1T cells were identified as CTV-positive cells, and viable singlets were gated for TNF and IL17 against hMR1-5-OP-RU on total T cells. Data from a representative co-culture setup in presence of 5-OP-RU is shown. Figure reprinted with permission from Leeansyah et al., 2020b.

Pa MR1T cell antigen-specific proliferation

Antigen-specific proliferation of Pa MR1T cells can be assessed using a modified expansion protocol presented in Part I. Here, through the principle of dilution of the proliferation tracing dye, CTV, we can detect Pa MR1T cell proliferation in response to multiple antigenic stimuli by flow-cytometry-based analysis and determine proliferation-associated parameters using software-based analysis (e.g., FlowJo), including expansion and proliferation indices as reported in our recent publication (Leeansyah et al., 2020b). Due to the continuous dilution of the proliferation tracing dye CTV, the time-frame in which we are able to detect and analyze proliferation-associated parameters must first be determined. Too early after antigenic stimulation, there will be only limited proliferation, whereas too late after stimulation there will be a substantial proportion of Pa MR1T cells that has proliferated to an extent that CTV dilution peaks would be difficult to discern and analyze. In this system, we found 5 days to be suitable for assessing Pa MR1T cell proliferation (Figure 13), in agreement with our previous results with human MAIT cells (Dias et al., 2016). This experimental strategy also allows evaluation of the effect of active proliferation on expression levels of the cytolytic protein Perforin and various TFs (Figure 13).

LIMITATIONS

This protocol relies mostly on the cross-reactivity of human reagents on bat due to the lack of bat-specific reagents. This includes the identification of Pa MR1T cells using human MR1 tetramers. Although we have confirmed the binding specificity of hMR1-5-OP-RU tetramer to Pa MR1T cells through pre-blocking experiments (Leeansyah et al., 2020b), future studies ideally develop and use species-specific MR1 tetramer. Cross-reactive antibodies are not available for all markers of interest, hindering further in-depth studies. Moreover, expansion of Pa MR1T cells derived from bat primary immune cells is subject to donor-to-donor variability, leading to variable yields that may restrict the number of functional experiments that can be performed. Nevertheless, our Protocol

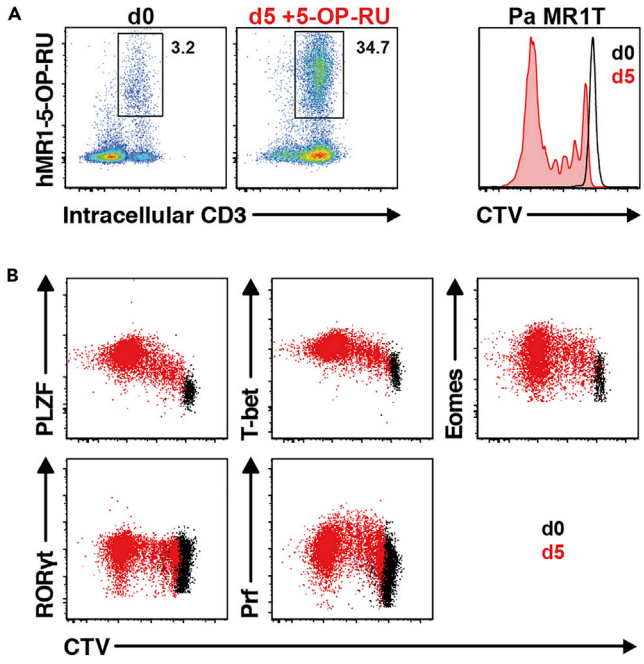

Figure 13. Pa MR1T cell antigen-specific proliferation assay

(A) Representative FACS plots showing frequencies of Pa MR1T cells (defined as $CD3^{+}$hMR1-5-OP-RU^{+}) and CTV dilution on day 0 and day 5 of culture with 5-OP-RU from a single Pa donor.

(B) Representative FACS plots showing the expression levels of different transcription factors (PLZF, T-bet, Eomes, and RORγt) and Perforin against CTV at the two different timepoints. Figure adapted and reprinted with permission from (Leeansyah et al., 2020b).

has provided significant advances in the *in vitro* culture and expansion of pteropid primary immune cells, incl. MR1T cells, and potentially also that of conventional T cells, which may also be adapted to other bat species. Given the increasing importance of MR1-restricted MAIT cells in controlling various bacterial and viral pathogens (Leeansyah et al., 2020a; Provine and Klenerman, 2020), understanding MR1-restricted T cell immunobiology in bats may provide us with novel insights on preventing zoonotic disease transmission and development of new treatment strategies (Irving et al., 2021).

TROUBLESHOOTING

Problem 1

Low viability of bat immune cells after thawing.

Potential solution

Pre-chill the centrifuge and reagents before working. Always keep cells, media, and PBS on ice. In addition, we recommend preparing experiment with a few bat donors as a back-up.

In some circumstances, cell numbers can be reduced, and adjustments to volumes and amounts of ligands or bacteria should be proportioned accordingly.

Problem 2

Inadequate Pa MR1T cell numbers following expansion of bat immune cells.

Potential solution

The viability of thawed bat immune cells at the initiation phase can affect the expansion status of the culture. Accordingly see Problem 1.

Ultimately, low cell numbers or the presence of unviable cells at the end of expansion culture could affect the setting up of the cytotoxicity assay and cytokine assay. To remove dead cells, Ficoll density gradient centrifugation at d11 is recommended, although this step may reduce yield. Purity adjustment during the experimental assays is also recommended. We recommend initiating the culture with about 7 – 10 $\times 10^6$ cells if possible.

Problem 3

The proliferation tracing dye CTV peaks are indiscernible during antigen-specific proliferation assay.

Potential solution

Shorten proliferation period. Perform analyses daily between d3-5 if cell numbers allow.

Problem 4

Cell lines not detaching after 5 minutes of Trypsin-EDTA treatment.

Potential solution

Trypsin-EDTA treatment can be continued up to 10 minutes. Alternatively, collect detached cells with culture media, and perform another round of trypsinization. We recommend maintaining cells to about 85%–90% confluency and keeping to a regular passaging schedule.

Problem 5

MR1 tetramer staining is weakening.

Potential solution

Use new batch of fluorochrome-conjugated MR1 tetramer or redo titration. In parallel, perform MR1 tetramer staining on human PBMC as a positive control.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Edwin Leeansyah (edwin.leeansyah@sz.tsinghua.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets and codes.

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AUTHOR CONTRIBUTIONS

W.R.S. and Y.Y.H. performed the experiments. W.R.S. and E.L. analyzed the data. W.R.S., Y.Y.H., and E.L. designed the experiments. Y.Y.H., R.F., and L.-F.W. provided critical reagents. E.L. conceived the study and supervised the experiments. L.-F.W. and E.L. managed the study. W.R.S. and E.L. wrote the paper, which was critically reviewed, edited, and approved by all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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