Published in final edited form as:

Methods Mol Biol. 2020 ; 2098: 219–235. doi:10.1007/978-1-0716-0207-2_15.

Generation of MR1-Restricted T Cell Clones by Limiting Dilution Cloning of MR1 Tetramer⁺ Cells

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Abstract

Tetramers are a powerful tool for identification of T cell subsets that are restricted by specific antigen presenting molecules and their cognate antigens. The generation of T cell clones from specific T cell subsets allows for further investigation of the phenotype and function of these cells. Here, we describe a method for sorting and cloning of MR1-restricted T cells using the MR1/5-OP-RU tetramer. This protocol can be easily modified to enrich for expansion of specific or unique subsets of MR1-restricted T cell clones from any tissue to further characterize the phenotype and function of those cells.

Keywords

MAIT cells; MR1-restricted T cells; T cell cloning

1 Introduction

MR1-restricted T cells are a subset of unconventional T cells that recognize microbially derived small molecule metabolites presented on the Class I-like molecule, MR1. These cells were originally detected in the CD4⁺CD8[−] T cell compartment of mucosal tissues and defined as mucosal-associated invariant T (MAIT) cells based on this localization and the expression of an invariant T cell receptor (TCR) α chain characterized by TRAV1–2 rearranged with TRAJ33 [1]. As such, MAIT cells were initially identified based on their expression of TRAV1–2, CD161, and CD26 [2–5]. Using *Mycobacterium tuberculosis*-infected dendritic cells as a stimulus in a limiting dilution assay with purified CD8⁺ T cells, our group expanded numerous MAIT cell clones with this phenotypic definition [4].

Subsequent reports have demonstrated that the TCR repertoire is more diverse than originally thought, with expression of additional TRAJ and TCR β chains, as well as TCR α chains besides TRAV1–2 [6–10]. Many of these findings were made possible following the identification of small molecule metabolite ligands and subsequent generation of an MR1 tetramer with one of these ligands, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) [11, 12]. Indeed, the use of antigen-specific tetramers has uncovered the existence of a broader diversity of MR1-restricted T cells, even beyond TCR diversity. Here, we describe a protocol for sorting and cloning of MR1-restricted T cells with the MR1/5-OP-RU tetramer. The ability to isolate and clone from unique MR1-restricted T cell subsets, such as those that are TRAV1–2⁺ or CD4⁺, provides the opportunity for further investigation into the functional potential for these cells.

2 Materials

Prepare all media and solutions in a Class II laminar flow biosafety cabinet.

2.1 T Cell Enrichment (Optional, See Note 1)

1. Supplies: Polystyrene FACS tubes, 15- and 50-mL conical tubes, sterile media bottles, bottle-top filters for sterile filtration.
2. Equipment: Benchtop centrifuge with swinging bucket rotor, water bath, hemocytometer, light microscope, 37 °C tissue culture incubator with 5% CO₂.
3. RPMI-FBS media: 500 mL RPMI, 60 mL heat-inactivated fetal bovine serum (FBS) (*see Note 2*), 50 µg/mL gentamicin, 4 mM L-glutamine, sterile filtered.
4. DNase I: 100 mg vial of DNase I resuspended in RPMI-HuS media at 3 mg/mL, aliquoted, and stored at –80 °C.
5. RPMI-HuS media: 500 mL RPMI, 55 mL heat-inactivated human serum (HuS) (*see Note 3*), 50 µg/mL gentamicin, 4 mM L-glutamine, sterile filtered.
6. EasySep™ Human T cell enrichment kit: Negative Selection (*see Note 4*). Kit includes enrichment cocktail and magnetic particles.
7. EasySep™ buffer: Phosphate buffered saline (PBS), 2% FBS, 1 mM ethylenediaminetetraacetic acid (EDTA).
8. EasySep™ Magnetic block.

¹The EasySep T cell enrichment is optional. It can help clean up complex samples like those from the lung (e.g., bronchoalveolar lavage) and reduce sort time. It is not necessary for cloning from peripheral or cord blood samples.

²In our experience, FBS can cause background in ELISPOT and flow cytometry assays. As a result, we pretest all lots of FBS prior to use for cloning and downstream assays and select those with minimal background. FBS should be heat inactivated by incubation at 56 °C for 45 min after thawing.

³Our human serum is prepared by drawing 400 cc of blood from research donors. The blood is allowed to clot for 2 h, then the serum is removed and heat inactivated at 56 °C for 45 min. The serum from the donors is pooled together (4 pools of approximately 7 donors each), divided into 30 mL aliquots and frozen at –80 °C. As with FBS, pools are tested for background prior to use. Serum from at least 2 pools is used to make RPMI-HuS. Commercial sources of human serum are available and should be tested prior to use to assess T cell expansion and background activation.

⁴Miltenyi T cell negative selection kit and columns can also be used (Pan T Cell Isolation Kit, human).

2.2 Staining and Sorting of T Cells

1. Supplies: 96-well V-bottom plate, 96-well U-bottom plates, 15 mL conical tubes, ice.
2. Equipment: Hemocytometer, light microscope, benchtop centrifuge with swinging bucket rotor, flow sorter (e.g., BD Influx), 37 °C tissue culture incubator with 5% CO₂.
3. PE-conjugated MR1 Tetramer: NIH Tetramer core (*see* Note 5).
4. Tetramer buffer: PBS with 2% FBS, sterile filtered.
5. Antibodies: *See* Table 1.
6. RPMI-HuS with antibiotics (e.g., 100 units/mL penicillin, 0.1 mg/mL streptomycin, 250 ng/mL Amphotericin B) (*see* Note 6).
7. IL-2 (stock concentration 3 µg/mL in RPMI-HuS, final concentration in media for rest 0.5 ng/mL).

2.3 Cloning by Limiting Dilution Assay

1. Supplies: 96-well U-bottom plates, 15- and 50-mL conical tubes, Mr. Frosty™ (*see* Note 7).
2. Equipment: Benchtop centrifuge with swinging bucket rotor, water bath, irradiation source (*see* Note 8), 37 °C tissue culture incubator with 5% CO₂, inverted light microscope.
3. RPMI-HuS (*see* Subheading 2.1, **item 3**).
4. Lymphoblastoid cell lines (LCL) from two donors (*see* Note 9).
5. Peripheral blood mononuclear cells (PBMC) from at least two donors (*see* Note 10).
6. Cytokines: *See* Table 2.
7. α-CD3 (clone OKT3) (*see* Table 2).

⁵Brighter conjugates such as PE or BV421 are preferable for tetramer staining. A titration should be performed on each new stock of tetramer received from the NIH tetramer core to determine the best dilution for staining. We typically use a 1:500 dilution of tetramer for sorting. The MR1-6FP control tetramer (also available from the NIH Tetramer Core) should be used on a separate sample prior to sorting to verify where positive tetramer staining appears relative to the control to determine appropriate gates for sorting.

⁶Antibiotics/antifungals are not necessary when sorting from PBMC or cord blood samples, but should be used if sorting from a sample that could result in contamination (e.g., bronchoalveolar lavage).

⁷A Mr. Frosty™ is designed to cool cells by −1 °C/min for preservation. Any other mechanism that achieves this optimal cooling rate for cell cryopreservation will also work.

⁸Irradiation is used to stop replication of the LCL and PBMC feeder cells so that they will not expand during the course of the T cell expansions. We have used both cesium irradiation and X-ray irradiation with equal success. It is important to ensure that samples are in the optimal location in the irradiation source for full dosing to prevent replication. Prior to cloning, proper irradiation methods should be confirmed by culturing irradiated cells and verifying that there is no growth.

⁹Human lymphoblastoid cell lines (LCL) can be established by infecting resting B cells from PBMC in vitro with Epstein Barr Virus (EBV) [13, 14]. LCL should be cultured in RPMI-FBS, and maintained at a cell density of 1e5–1e6 cells/mL. LCL are available for purchase commercially.

¹⁰Peripheral blood mononuclear cells (PBMC) are isolated from whole blood or apheresis product by standard protocols (e.g., density gradient centrifugation). PBMC are available for purchase commercially.

8. Freezing media: 50% FBS, 40% RPMI, 10% dimethylsulfoxide (DMSO), sterile filtered. For samples with fewer or less hearty cells, such as cord blood, pediatric PBMC, or bronchoalveolar lavage, 90% FBS with 10% DMSO can be used.

2.4 Phenotypic and Functional Analysis of Buttons

1. Supplies: 96-well V-bottom plates, 15- and 50-mL conical tubes, ELISPOT Plates (mixed cellulose ester member *see* Note 11), syringe, and 0.22 μ m syringe filter.
2. Equipment: Benchtop centrifuge with swinging bucket rotor, water bath, light microscope, hemocytometer, ELISPOT reader (*see* Note 12), and flow cytometer (*see* Note 13).
3. Tetramer buffer (*see* Subheading 2.2, **item 4**).
4. PBMC or MR1 clone control for staining (e.g., ref. 9).
5. Antibodies: *See* Table 3.
6. 1% paraformaldehyde in PBS.
7. ELISPOT Buffers (EB): EB I: Prepare 0.1 M NaHCO₃ (4.2 g NaHCO₃ in 500 mL diH₂O). Prepare 0.1 M Na₂CO₃ (5.3 g Na₂CO₃ in 500 mL diH₂O). Add the NaHCO₃ solution to the Na₂CO₃ solution until the pH is 9.6, then sterile filter. EB II: Add 5 g of bovine serum albumin (BSA) to 1 L PBS. Warm at 37 °C to dissolve the BSA. When BSA is in solution, add 500 μ L Tween-20 and sterile filter.
8. PBS, PBS-Tw: 19.2 g of PBS powder, 1 mL Tween20, 2 L diH₂O, stir for 1 h.
9. ELISPOT antibodies: e.g., Anti-human IFN γ coating antibody (clone 1_D1K), anti-human IFN γ ALP-conjugated detection antibody (clone 7-B6-1) (Mabtech) (*see* Note 14).
10. 5-Bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP-NBT) solution.
11. A549 cell line (ATCC CCL-185) and the A549-MR1^{-/-} cell line [9] (*see* Note 15).
12. RPMI-HuS (*see* Subheading 2.1, **item 3**).

¹¹PVDF membranes can also be used but require additional steps to activate the membrane. We have had the best success in our assays with the MultiScreen_{HTS} plates; however, any mixed cellulose or PVDF membrane and plate that is sterile can be used for this assay.

¹²This protocol describes the ELISPOT method to assess the functional capacity of putative MAIT cell clones (buttons), which requires an ELISPOT reader and software. Testing for function of the buttons could also be achieved through different flow cytometry-based methods if an ELISPOT reader is not available (e.g., refs. 9, 10).

¹³The flow cytometer should have the capacity to detect a minimum of 6 parameters to analyze live/dead, CD3, CD4, CD8, TRAV1-2, and the MR1 tetramer to confirm that the expanded button is a MAIT cell. Examples include the BD LSR, Fortessa, or Symphony.

¹⁴Examples are given for IFN γ coating and secondary antibodies and the related developing reagent. ELISPOT assays can also be done to analyze markers such as TNF α or Granzyme B. There is also the option to use an HRP-conjugated secondary or to use biotin-conjugated secondary antibodies followed by streptavidin-conjugated HRP or ALP. See the Millipore or Mabtech websites for alternative ELISPOT antibodies.

¹⁵As an alternate to the A549-MR1^{-/-} cell line, blocking with the α -MR1 26.5 antibody clone can be used to demonstrate MR1 restriction as described by Gold et al. [4].

13. *M. smegmatis* or other MAIT cell bacterial antigen (*see* Note 16).
14. Phytohemagglutinin (PHA): 5 mg/mL stock in RPMI-HuS, 10 µg/mL final concentration.

2.5 Rapid Expansion of MAIT Cell Clones from Buttons

1. Supplies: T-25 tissue culture flasks, 15- and 50-mL conical tubes, Mr. Frosty™.
2. Equipment: Benchtop centrifuge with swinging bucket rotor, water bath, irradiation source, hemocytometer, light microscope.
3. RPMI-HuS (*see* Subheading 2.1, **item 3**).
4. LCL, PBMC (*see* Subheading 2.3, **items 4 and 5**).
5. α-CD3 (*see* Subheading 2.3, **item 7**, Table 2).
6. IL-2 (*see* Table 2).
7. Freezing media (*see* Subheading 2.3, **item 8**).

3 Methods

All tissue culture procedures should be carried out in a Class II laminar flow biosafety cabinet. All incubations should be done in a 37 °C incubator with 5% CO₂.

3.1 T Cell Enrichment (Optional, See Note 1)

1. Begin with cryopreserved samples containing an appropriate number of cells from tissue of choice (e.g., PBMC, single cell suspensions from lung, cord blood, etc.) (*see* Note 17). Thaw frozen cell sample(s) in 37 °C water bath and immediately transfer to RPMI-FBS containing 100 µL DNase I. Centrifuge at room temperature for 10 min at 300 × *g*.
2. Resuspend cell pellet in pre-warmed RPMI-HuS containing 100 µL DNase I and count using a hemocytometer.
3. Centrifuge cells again and resuspend at 50e6 cells/mL in EasySep buffer.
4. Transfer sample to polystyrene FACS tube and add Enrichment cocktail at 50 µL/50e6 cells, mix gently with a pipette, and incubate at room temperature for 10 min.
5. Just before the end of the incubation, prepare magnetic particles by vortexing for 30 s. Add 150 µL magnetic particles per 50e6 cells and incubate for 5 min.
6. Add 2.5 mL EasySep buffer to the samples and mix gently with a pipette.

¹⁶. *E. coli* or another microbial antigen can also be used as an antigen for the ELISPOT assay. A functional titration should be done ahead of time for any bacterial antigen stock to determine the appropriate concentration to use in the ELISPOT assay.

¹⁷. The minimum number of cells to use will depend on the expected MR1T cell frequency. We have achieved successful clones starting with as few as 3.5e6 live PBMC per cloning sample.

7. Incubate tubes upright in the EasySep magnetic block for 5 min. After incubation, lift magnet and pour contents into a fresh tube (*see* Note 18). Proceed with Subheading 3.2 to stain and sort T cells.

3.2 Staining and Sorting of T Cells

1. Determine the number of samples required for staining. Samples should consist of unstained cells, single stain controls for all markers, and cells stained with the full panel (*see* Note 19).
2. From the optional T cell enrichment in Subheading 3.1, **step 7**, or from freshly thawed PBMC if T cell enrichment is not necessary, remove a sample for counting, then centrifuge cells at $300 \times g$ for 10 min.
3. After centrifugation, resuspend samples in 25 μ L of tetramer buffer for every 2e6 cells.
4. Transfer each staining sample (consisting of 2e6 cells) to a 96-well V-bottom plate and centrifuge at $300 \times g$ for 5 min.
5. Resuspend each sample in a total of 25 μ L tetramer buffer containing the appropriate MR1 tetramer dilution, or no tetramer (for controls) and incubate in the dark for 45 min at room temperature.
6. While incubating, prepare the antibody master mixes in tetramer buffer (for controls and full panel), with a final volume of 25 μ L per sample. *See* Table 1 for an example sorting panel.
7. Add the 25 μ L antibody mixture including live/dead directly to the 25 μ L tetramer mixture already in the wells and mix gently. Incubate at 4 °C for 30 min, then wash at 4 °C twice with tetramer buffer.
8. Transfer cells on ice to a flow sorter. After using single stained controls to appropriately set up voltage parameters and gates, sort tetramer⁺ cells of interest into a 15-mL conical tube containing 5 mL of RPMI-HuS with a low dose of IL-2 (0.5 ng/mL). Figure 1 demonstrates an example of sort plots with proper staining and gating.
9. Wash cells twice in RPMI-HuS media (containing PSF, depending on the source of the cells (*see* Note 6)).
10. Rest cells overnight in a 96-well U-bottom plate in 200 μ L of RPMI-HuS with a low dose of IL-2 (0.5 ng/mL) at 15e6 cells/well (*see* Note 20).

¹⁸.Ensure that liquid contents are retained following magnetic bead selection. When inverting the magnet, do so in one continuous motion, and pipet drips from the tube lip to maximize yield.

¹⁹.Markers used in sorting panel will be determined by the desired cloning outcome. For example, TRAV1–2 should be included in the panel if TRAV1–2⁺ or TRAV1–2[–] clones are specifically desired.

²⁰.Cells should be placed in wells near center of plate to minimize evaporation. Assume that approximately half of the cells will die overnight.

3.3 Cloning by Limiting Dilution Assay (LDA)

Use RPMI-HuS for all steps of the LDA.

1. Determine the number of wells that will be used for cloning, based on the estimated number of T cells remaining after sorting and rest. Ideally, T cells should be seeded in 1 plate each of 1, 3, 10, and 30 cells/well (*see* Note 21).
2. Centrifuge LCL to wash, resuspend in media, and count. Resuspend at a final concentration of $3 \times 10^4/50 \mu\text{L/well}$ ($6 \times 10^5/\text{mL}$).
3. Thaw and wash allogeneic PBMC, resuspend in media, and count. Resuspend at a final concentration of $1.5 \times 10^5/\text{well}$ in $50 \mu\text{L/well}$ ($3 \times 10^6/\text{mL}$).
4. Irradiate the LCL with a total dose of 6000 cGy and the PBMC with a total dose of 3000 cGy.
5. Combine cytokines (IL-2, IL-12, IL-7, and IL-15) and $\alpha\text{-CD3}$ at the concentrations indicated in Table 2 into a total volume of $50 \mu\text{L/well}$ (*see* Note 22).
6. Add the LCL ($50 \mu\text{L}$), PBMC ($50 \mu\text{L}$), and cytokine/ $\alpha\text{-CD3}$ ($50 \mu\text{L}$) mixtures to each well of the 96-well U-bottom plate (s) for a total volume of $150 \mu\text{L/well}$.
7. Finally, add the T cells at 1, 3, 10, or 30 T cells/well in $50 \mu\text{L}$ volumes for a final total volume of $200 \mu\text{L/well}$.
8. Cover the plates loosely with foil and incubate for 7–14 days at 37°C .
9. On day 5, the $\alpha\text{-CD3}$ should be washed from the wells as follows. Visually observe the plate to confirm that the cells are settled at the bottom of the wells. Carefully remove $100 \mu\text{L}$ of media from each well without disturbing the cells at the bottom of the well. Replace with $100 \mu\text{L}$ of fresh media supplemented with all cytokines being used. Repeat this wash every 2–3 days until buttons are screened.
10. At day 7, check the plates for growth of buttons by visual inspection of the wells (*see* Fig. 2). If buttons are not observed check the plates again at days 10–14, until buttons are clearly observed. Plates with fewer than 30 buttons are preferred as these are more likely to be clonal.
11. Visually screen a second time using an inverted microscope and select the best buttons to carry forward. The best buttons will be nearly perfectly round, with a well-defined edge. The cells should be densely cluttered and free from visible contamination with other cell types.

²¹The number of cells per well and the number of plates may need to be adjusted depending on the starting sample. For example, if no or few buttons grow in plates that are seeded with 1 or 3 cells/well, it may be wise to increase plates with higher numbers of cells per well. However, too many cells per well can often result in buttons that are not clonal. If the number of cells obtained after sorting are limiting, we would recommend starting with plates containing 3 and 10 cells/well, with incomplete plates of both cell numbers if necessary.

²²IL-2 is required for the LDA, while the other cytokines are recommended, particularly for samples that are more challenging to clone from (e.g., bronchoalveolar lavage or cord blood). Ensure that concentration calculations are based on the final volume of $200 \mu\text{L/well}$.

12. Transfer all buttons to be tested to a new 96-well U-bottom plate. Due to expected evaporation, the original 200 μ L volume will be closer to 170 μ L.
13. Gently mix and remove 45 μ L from each well and transfer to a new 96-well U-bottom plate to stain as described in Subheading 3.4.
14. 30 μ L of the remaining button will be used for ELISPOT assay as described in Subheading 3.5.
15. Bring the volume of the remaining button up to a total of 200 μ L of RPMI-HuS with a low dose of IL-2 (0.5 ng/mL) and incubate overnight. If the button has the appropriate phenotype and function, this will be used for rapid expansion as described in Subheading 3.6 (*see Note 23*).

3.4 Flow Cytometry Staining of Buttons

1. Staining volumes for screening are reduced to account for the reduction in cell numbers from those described in Subheading 3.2. *See Table 3* for an example staining panel for screening the buttons. As with sorting, use an existing MAIT cell clone or PBMC sample for single stain controls and as a control for the full staining panel.
2. Centrifuge cells transferred to the 96-well U-bottom plate in Subheading 3.3, **step 13** and wash once with tetramer buffer. Resuspend the cells in 25 μ L of total volume tetramer buffer that contains MR1 tetramer. Incubate at room temperature for 45 min.
3. During the incubation, prepare surface stains and live/dead master mix in 25 μ L tetramer buffer per well. Staining panel should include MR1 tetramer, TRAV1–2, CD3, CD8, CD4, and live/dead, as well as CD26, CD161, or other markers as desired.
4. After 45 min, add 25 μ L of the antibody master mix to each well on top of the 25 μ L tetramer already in the well. Incubate an additional 30 min at 4 °C. Centrifuge the plate and wash the pellet twice with tetramer buffer.
5. Resuspend pellets in 1% PFA in PBS overnight to fix.
6. Acquire samples on a flow cytometer within 24 h of staining. Figure 3 shows examples of MAIT cell buttons likely to be clonal versus those that are not.

3.5 Functional Testing of Buttons by ELISPOT Assay

1. On the day before the buttons are to be stained (Subheading 3.4), coat 3 ELISPOT plates as follows: Per plate, add 50 μ L α -IFN γ coating antibody to 5 mL EB1. Add 50 μ L antibody mixture to each well of the ELISPOT plate and tap

²³Ideally, the flow cytometry and ELISPOT assay will be performed on the day the buttons are ready, so that rapid expansions can be done the following day. Cells from buttons can be cryopreserved if there is not enough time to do both screening methods, or if the buttons will not be screened right away (e.g., buttons from plates where many buttons expanded can be cryopreserved as a backup). It should be noted, however, that the number of cells is relatively small, and will be further reduced by cryopreservation or longer incubations, making subsequent screening and rapid expansion more challenging.

gently to completely coat the entire well. Incubate overnight at 4 °C. If more than 95 buttons will be screened, additional plates will be required.

2. Remove the coating antibody from the plates by flicking. Take care to limit the exposure of the open plate outside of the biosafety cabinet. Add 200 µL PBS to each well and incubate for 15 min at room temperature. Repeat for a total of three PBS washes.
3. Add 100 µL of RPMI-HuS to each well and incubate at room temperature for 1 h to block.
4. While blocking, prepare the antigen presenting cells (APC). Harvest A549 and A549-MR1^{-/-} cells from flasks (*see* Note 24). Resuspend pellet in RPMI-HuS and count cells. Add RPMI-HuS for a final concentration of 1e5 cells/mL.
5. After the plates have blocked for 1 h, flick the media from the wells. Then add APC to the ELISPOT wells at 100 µL (1e4 cell) per well, such that there are two plates with A549 cells and one plate with A549-MR1^{-/-} cells. Figure 4 shows a sample ELISPOT plate set up.
6. Dilute bacterial antigen in RPMI-HuS and add 50 µL antigen mixture to the wells of one A549 plate and one A549-MR1^{-/-} plate (*see* Note 16).
7. Incubate the plates for 1 h at 37 °C.
8. Gently mix the buttons and pipet 10 µL of each button into the appropriate wells (using 30 µL total of each button). Incubate for 18 h at 37 °C.
9. The following day prepare the detection antibody by adding 10 µL of anti-IFN γ -ALP to 10 mL of EBII.
10. Remove all media and cells from wells by flicking the plate into a biohazard waste bag.
11. Wash plate six times with PBS-Tw. Tap out excess wash onto paper towels prior to adding secondary antibody.
12. Add 100 µL of EBII/antibody mixture to each well and incubate at room temperature for 2 h.
13. Remove EBII/antibody mixture from plate by flicking.
14. Wash plate six times with PBS-Tw. Incubate with sixth wash for 15 min.
15. Wash plate three times with PBS, taking care to fill each well completely with PBS to remove excess PBS-Tw buffer.
16. During the 15-min incubation, prepare 10 mL BCIP/NBT solution by passing it through a 0.22-µm filter. Ensure that BCIP/NBT solution comes to room temperature before adding to the plate.

²⁴. A549 cells should be cultured and harvested based on the methods described by the ATCC CCL-185.

17. Add 100 μ L BCIP/NBT solution to each well and incubate in the dark for 10–15 min until purplish-blue spots appear in the wells. At this time, flick BCIP/NBT solution from wells and wash well with deionized water. Remove ELISPOT plate backing during washing.
18. Allow plate to dry and assess button responses with an ELISPOT reader program.

3.6 Rapid Expansion of MAIT Clones from Buttons

1. After phenotypic analysis by flow cytometry and functional analysis by ELISPOT (or other assay), select desired buttons for rapid expansion (*see* Note 23).
2. Prepare LCL and PBMC in RPMI-HuS as described in Subheading 3.3, **steps 1** and **2**. Each rapid expansion flask will require 5e6 LCL and 25e6 PBMC in 5 mL each.
3. In an upright T25 flask combine the following: LCL (5e6 in 5 mL), PBMC (25e6 in 5 mL), 30 ng/mL α -CD3, the remaining T cell button, and RPMI-HuS to bring the total volume to 30 mL. Incubate overnight at 37 C. Cells should settle at the bottom of the flask.
4. Add IL-2 to a final concentration of 2 ng/mL after 24 h (Day “1” of the expansion).
5. Day 5: Wash α -CD3 from the cells as follows. Mix cells and transfer to a conical to centrifuge. Remove all excess media containing α -CD3 from the flasks by aspiration during the centrifugation. Resuspend the cell pellet in 30 mL fresh RPMI-HuS with 2 ng/mL IL-2 and return to the flask.
6. Incubate an additional 5 days, adding 2 ng/mL IL-2 every 2–3 days.
7. Culture can be tested for phenotype and function as described in Subheadings 3.3 and 3.4 starting on Day 10. PHA should be used as a control for T cell responses when testing rapid expansions.
8. Fresh IL-2 should continue to be added every 2–3 days. If pH of media drops, remove and replace 10–15 mL of media from the top of the culture with fresh media (do not disturb cells at bottom of flask). Cells will be functional from Day 10–24, depending on the clone (*see* Note 25).
9. Generate a frozen stock of the rapid expansion between Day 10–14. Pellet and resuspend 1e6 cells for every 1 mL freezing media. Place cells in a -80°C freezer using a Mr. FrostyTM, then transfer cells to a liquid nitrogen freezer for long-term storage.
10. Flow cytometry can be used as previously described to determine whether a rapid expansion is likely to be clonal.

²⁵It is ideal to initially test and cryopreserve expanded clones during day 10–14 of the expansion protocol; however, most MR1T cell clones will maintain their functional capacity out to day 21, and in some cases longer.

11. MAIT cells can be re-expanded by repeating the **steps 2 and 8** by using 5e5 freshly expanded cells (ideally during days 10–14), or by using 5e5 of the cryopreserved T cell rapid expansion (**step 9**).

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Fig. 1.

Sample staining and gating strategy for sort. All samples should be gated for live, single, lymphocytes that do not express CD14 or CD19. From there, optional dump channel markers include $\gamma\delta$ TCR or CD4. Optional markers for tetramer sorting could include TRAV1–2, CD4, CD8, or any other marker that could define a subset of tetramer⁺ MR1-restricted T cells

Fig. 2.

Analysis of button growth. Shown is the view from the bottom of a cloning plate without magnification. The majority of wells should show no growth. More than 30 buttons on a single plate could indicate that too many cells/well were used for cloning and the buttons may not be clonal. Prioritize buttons that have intermediate growth as shown above, adding in buttons with more or less growth, depending on how many will be screened. When viewed using an inverted microscope, good buttons will be nearly perfectly round, with a well-defined edge. The cells should be densely cluttered and free from visible contamination with other cell types

Fig. 3.

Flow cytometry analysis of buttons. Initially, cells should be gated on live, singlet, CD3+ lymphocytes. Of this population, cells should be further analyzed for MR1 tetramer staining and other markers of interest, depending on the original sort parameters. Shown here are cells from buttons that were initially sorted on CD8 and tetramer staining. If the whole population does not express these markers, it is not clonal, or not the cell subset that you were trying to expand. Populations that appear clonal should be further verified by TCR sequencing after rapid expansion

Fig. 4.

Layout for ELISPOT screening of buttons. Responses are only expected in plate 2 (center). Buttons that exhibit this phenotype are good candidates for further expansion. Responses in plate 1 (top) would indicate background responses from cells in the button. No responses in plate 2 (center) would indicate that the cells from the button do not recognize bacterial antigen. Responses in plate 3 (bottom) would indicate that the cells from the button are not responding to the bacterial antigen in an MR1-dependent manner. Depending on the desired outcome of the cloning, these may not be good candidates for expansion

Table 1Sample sorting panel to isolate CD4⁺ tetramer⁺ MR1T cells

Marker ^a	Fluorophore	Clone	Manufacturer	Cat. #	Volume/2e6 cells (μL) ^b
CD4 ^c	PeCy7	SK3	BD Biosciences	348789	2
CD14	PeCy7	M5E2	Biologend	301814	2
CD19	PeCy7	HIB19	Biologend	302216	2
γδTCR ^d (optional)	FITC	5A6.E9	ThermoFisher Scientific	TCR2061	2
Live/dead ^e	Aqua	N/A	ThermoFisher Scientific	L34957	0.25
MR1 tetramer	PE	N/A	NIH Tetramer core	N/A	25 ^f

^aMarkers can be added or removed depending on desired MR1T cell population for cloning and sorter capacity. In this case, CD4, CD14, CD19, and γδTCR are all included for “dump channels” to remove these cells from the tetramer⁺ population

^bThese antibody amounts have been titrated with each new lot and for our sorters (BD Influx and FACSARIA). As such, this is a recommended starting volume, and all antibodies should be titrated for use at a new sorter facility

^cThe volume of CD4 indicated is for adult samples. CD4 is often dimmer on cord blood and pediatric samples. Increasing the stain amount will improve population separation

^dThis is an optional marker to include in the sort panel. It is not included in the example shown in Fig. 1; however, we have used it in other sorts to gate on the γδTCR⁺ cells after gating on the CD4–CD14–CD19– dump channel

^eLive/dead is supplied in a desiccated form. It should be resuspended in 50 μL DMSO prior to use

^fThe MR1 tetramer should be titrated prior to use. We generally dilute 1:500 in tetramer buffer, then use 25 μL of the diluted tetramer/2e6 cells

Table 2

Cytokines and antibodies used for limiting dilution assay

Cytokine ^a /antibody	Source	Stock concentration ^b	Working concentration
IL-2	Pharmacy ^c	3 µg/mL	2 ng/mL
IL-12	Biologend	10 µg/mL	0.5 ng/mL
IL-7	Biologend	3 µg/mL	0.5 ng/mL
IL-15	Biologend	20 µg/mL	0.5 ng/mL
α-CD3	eBioscience OKT3 ^d	1 mg/mL	0.03 µg/mL

^a IL-2 and α-CD3 are required for cloning. Other cytokines are used when cloning from challenging samples such as cord blood or bronchoalveolar lavage. They are not necessary when cloning from PBMC

^b All stocks are made in RPMI-HuS and stored in aliquots at –80 °C

^c IL-2 is obtained from our University in-patient pharmacy under the name Proleukin

^d Other α-CD3 antibody clones have not performed as well in cloning as OKT3. The functional grade OKT3 from eBioscience is preferred

Table 3

Sample phenotyping panel for button analysis

Marker ^a	Fluorophore	Clone	Company	Cat. #	Volume per test (μL) ^b
CD3	BV650	OKT3	Biolegend	317324	0.5
CD4 ^c	BV421	OKT4	Biolegend	317434	0.5
CD8	APC-Cy7	SK1	Biolegend	344714	0.025
CD26	FITC	BA5b	Biolegend	302704	0.25
CD161	PE-Cy7	HP-3G10	Biolegend	339918	0.5
TRAV1–2	APC	3C-10	Biolegend	351807	0.25
Live/dead ^d	Aqua	N/A	ThermoFisher Scientific	L34957	0.05
MR1 tetramer ^e	PE	N/A	NIH tetramer core	N/A	25

^aMarkers can be added or removed depending on desired MR1T cell buttons and flow cytometer capacity

^bThese antibody amounts have been titrated with each new lot and for our cytometers (BD LSR, Fortessa, and Symphony). As such, this is a recommended starting volume, and all antibodies should be titrated for use at a new flow cytometry facility. Additionally, these volumes have been reduced for screening buttons. If performing flow cytometry on MR1T cells from rapid expansion flasks, increase each antibody volume by a factor of 4

^cVolume of CD4 is for adult samples. CD4 is often dimmer on cord blood and pediatric samples. Increasing the stain amount will improve population separation

^dLive/dead is supplied in a desiccated form. It should be resuspended in 50 μL of DMSO prior to use

^eThe MR1 tetramer should be titrated prior to use. For screening of buttons, we generally dilute the tetramer 1:2000 (due to the low cell numbers in a button), then use 25 μL of this dilution for each sample. If performing flow cytometry on MR1T cells from rapid expansion flasks, dilute the tetramer 1:500 instead