Isolation of Mouse Kidney-Resident CD8⁺ T cells for Flow Cytometry Analysis

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Abstract

Tissue-resident memory T cell (T_{RM}) is a rapidly expanding field of immunology research. To isolate T cells from various non-lymphoid tissues is one of the key steps to investigate T_{RM}s. There are slight variations in lymphocyte isolation protocols in different organs. Kidney is an essential non-lymphoid organ with numerous immune cell infiltration especially after pathogen exposure or autoimmune activation. In recent years, multiple labs including our own have started to characterize kidney-resident CD8⁺ T cells in various physiological and pathological settings in both mouse and human. Due to the abundance of T lymphocytes, kidney represents an attractive model organ to study T_{RM}s in non-mucosal or non-barrier tissues. Here, we will describe a protocol commonly used in T_{RM}-focused labs to isolate CD8⁺ T cells from mouse kidneys following systemic viral infection. Briefly, using an acute LCMV (lymphocytic choriomeningitis virus) infection model in C57BL/6 mice, we will demonstrate intravascular CD8⁺ T cell labeling, enzymatic digestion and density gradient centrifugation to isolate and enrich lymphocytes from mouse kidneys. The resulted samples will be ready for subsequent flow cytometry analysis.

SUMMARY:

Following viral infection, kidney harbors a relatively large number of CD8⁺ T cells and offers an opportunity to study non-mucosal T_{RM} cells. Here, we describe a protocol to isolate mouse kidney lymphocytes for flow cytometry analysis.

Keywords

CD8; Kidney-resident; LCMV; flow cytometry; mouse; intravascular labeling

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DISCLOSURES:

The authors have no relevant financial disclosures.

INTRODUCTION:

Tissue-resident memory (T_{RM}) T cells represent one of the most abundant of T cell populations in adult human and infected mice. T_{RM} cells provide the first line of immune defense and are critically involved in various physiological and pathological processes^{1, 2, 3, 4, 5}. Comparing with circulating T cells, T_{RM} cells carry distinct surface markers with unique transcription programs^{6, 7, 8}. Expanding our knowledge of T_{RM} biology is the key to understand T cell responses and essential for future development of T cell-based vaccines and immunotherapies.

In addition to commonly shared molecular markers of T_{RM} s across all non-lymphoid tissues, accumulating evidence suggest that tissue-specific features are a central component of T_{RM} biology⁹. Kidney harbors a large number of immune cells including T_{RM} cells after infection and offers a great opportunity to study T_{RM} cell biology in a non-mucosal tissue. Acute LCMV (lymphocytic choriomeningitis virus) infection via intraperitoneal route is a well-established systemic infection model to study antigen-specific T cell responses in mice. The infection is usually resolved in 7-10 days in wild type mice and leave a large number of LCMV-specific memory T cells in a variety of tissues, including the kidney¹⁰. P14 TCR (T cell receptor) transgenic mice carry $CD8^+$ T cells recognize one of the immune-dominant epitopes of LCMV presented by MHC-I (class I major histocompatibility complex) molecule H2-D^b in C57BL/6 mice. Combining congenically marked P14 T cell adoptive transfer and LCMV infection is a widely used mouse model to track $CD8^+$ effector and memory T cells, including T_{RM} differentiation and homeostasis.

In some barrier tissues, such as intestinal intraepithelial lymphocytes (IEL) compartment and salivary glands, established lymphocyte isolation procedure yields high percentage of T_{RM} cells with minimal blood borne T cell contamination in mouse LCMV model¹¹. However, in non-barrier tissues, such as the kidney, dense vascular network contains a large number of circulating $CD8^+$ T cells. It has been well documented that even successful perfusion cannot efficiently remove all circulating $CD8^+$ T cells. To overcome this technical hurdle, intravascular antibody staining has been established as one of the most commonly used techniques in T_{RM} labs¹². In brief, 3-5 minutes before euthanasia, 3 μ g/mouse anti- $CD8$ antibody (to label $CD8^+$ T cells) is delivered intravenously. Intact blood vessel wall prevents rapid diffusion of the antibody within this short period of time (i.e., 3-5 minutes) and only intravascular cells are labelled. Following standard lymphocyte isolation protocol, intravascular versus extravascular cells can be easily distinguished using flow cytometry. Here, we will describe a standard protocol commonly used in T_{RM} labs to perform intravascular labeling, lymphocyte isolation and flow cytometry analysis of kidney $CD8^+$ T cells using a C57BL/6 mouse that has received $CD45.1^+$ P14 T cells and LCMV infection 30 days before¹³. Same protocol can be used to study both effector and memory T cells in the kidney.

PROTOCOL:

All animal experiments performed following this protocol must be approved by the respective institutional Animal Care and Use Committee (IACUC). All procedures described here have been approved by IACUC UT Health San Antonio.

1. Adoptive transfer of P14 T cells into C57BL/6 recipients and LCMV infection

1.1 Purify naïve CD8⁺ T cells from the spleen and lymph nodes of a P14 TCR transgenic mouse using mouse Mojo CD8⁺ T cell purification kit (Biolegend) following manufactory protocol. Briefly, dissect spleen and lymph nodes, mash the samples to generate single cell suspension. To enrich for naïve T cells, add biotin-anti-CD44 antibody during the first antibody incubation step¹³.

NOTE: Negative selection Mojo kit contains two major components for two steps of incubation (i.e., biotin-antibody mixture incubation and streptavidin-magnetic bead incubation).

NOTE: P14 mice at 6-12 weeks of age are suitable for this purpose. The sex of donor P14 TCR transgenic mouse should match the sex of C57BL/6 recipient mice. Otherwise, such as male T cells transferring into female mice will result in immune-mediated rejection of circulating donor T cells in around 12 days¹⁴.

1.2 Use a hemocytometer to count the cells and inject 1000 to 10,000 purified P14 T cells in 200 µl PBS into each sex matched C57BL/6 recipient via the tail vein.

NOTE: Detailed tail vein injection protocol will be described in Step 2.

1.3 On the next day, all C57BL/6 recipients receive 2x10⁵ pfu LCMV Armstrong diluted in 200 µl PBS via an intraperitoneal route.

2. Intravascular labeling of CD8⁺ T cells

NOTE: Depending on the research interests, different time points can be chosen following infection. An example of day 30 post infection will be demonstrated, which is often considered as an early memory time point for CD8 T cell response.

2.1. Dilute biotin-anti-CD8α antibody to 15 µg/ml in PBS. Each mouse will receive 200 µl PBS containing 3 µg antibody;

NOTE: We use biotin-anti-CD8 antibody so that it will be more flexible to design the final FACS staining panel. Fluorescent dye-labelled antibody can be used directly. However, it is important to use different clones of monoclonal antibodies for intravenous labeling vs FACS staining.

2.2. Properly heat the mice with an overhead heat lamp for 5-10 min to dilate the veins;

NOTE: Extra care must be taken to prevent overheating the animals. Reduce the heat or remove the heat lamp if the mice stopped to move around.

2.3. Draw 200 μ L of prediluted anti-CD8 α antibody mix into a 100U insulin syringe (28G) and remove any air bubbles by moving the piston up and down. Bend the needle to create a 150° angle between the needle and syringe, bevel up, so the needle will be parallel to the vein;

2.4. Restrain the mouse with a rodent restrainer of appropriate size and spray the tail with 70% ethanol to make the vein clearly visible;

NOTE: The duration of the restraint should be kept to a minimum.

2.5. Hold the tail at the distal end with thumb and middle fingers of the non-dominant hand. Place the index finger underneath the site where the needle will be inserted;

2.6. Hold the syringe with the dominant hand and insert the needle into the vein in parallel towards the direction of the heart;

NOTE: When placing correctly, the needle should move smoothly into the vein.

2.7. Slowly Inject 200 μ L bio-anti-CD8 α antibody via the tail vein;

NOTE: If there is resistance or a white area shows above the needle on the tail, the needle is not inside the vein. Start initial injection close to the tip of the tail. When necessary, move forward towards the bottom of the tail for subsequent injections .

2.8. Remove the needle and gently apply compression until bleeding stops;

2.9. Return the mouse to the cage, and euthanize the mouse after 3-5 min;

NOTE: The mice should be euthanized via isoflurane chamber followed by cervical dislocation. Other methods, such as CO₂ inhalation will take up to 5 minutes and may introduce unnecessary variation of intravenous antibody labeling time.

2.10. Dissect the kidney using scissors, transfer the whole kidney to 1.5 ml microcentrifuge tubes and leave the samples on ice until further processing.

NOTE: Intact whole kidney can be safely stored on ice for a few hours before subsequent processing and digestion.

3. Enzymatic digestion of the kidney

3.1. Prepare 6-well plates containing 3ml digestion solution (RPMI/2% FCS/1 mg/ml Collagenase B) for each mouse, store on ice;

NOTE: Stock collagenase B solution at higher concentration (e.g., 20X) can be prepared and stored at or below -20°C. Working solution should be prepared fresh. Please see Supplemental Table 1 for the recipes for all solution/buffer used in the current protocol.

3.2. Add 300 μ L of digestion solution into the 1.5ml microcentrifuge sample tubes, and mince the kidney samples with a straight spring scissor;

NOTE: The kidney tissue should be minced into even pieces with sizes no larger than 1.5mm in diameters. No decapsulation step is required.

3.2. Transfer minced kidney samples to 6-well plates containing the digestion solution;

NOTE: 6-well plates are used for the only purpose of convenience when there are multiple samples to be processed.

3.3. Incubate the samples at 37 °C with gentle rocking (around 60 rpm) for 45 min;

3.4. After digestion, mash the tissue with the plunger flange of a 3 ml syringe directly inside the dish, and transfer into 15 ml conical tubes;

NOTE: Usually we do not filter the samples through a cell strainer at this step because we will perform density gradient centrifugation next to purify live lymphocytes.

3.5. Spin down at 500X g for 5 min at 4 °C;

3.6. Resuspend the pellet in 3 ml RPMI/10% FCS.

4. Density gradient centrifugation to enrich lymphocytes from digested kidney

4.1. Spin down at 500X g for 5 min at 4 °C;

4.2. Remove the supernatant and resuspend the kidney cell pellet with 5ml 44% Percoll/RPMI mix (44% Percoll + 56% RPMI without FBS);

4.3. Put the opening of a 3ml pipet containing 3 ml 67% Percoll/PBS (67% Percoll + 33% PBS) directly to the bottom of each tube, and slowly release the solution so that the heavy solution (67% Percoll/PBS) will form a distinct layer at the bottom and the light solution (44% Percoll/RPMI) will be raised up. Together, cocktail layers will form.

NOTE: For newly arrived Percoll from the vendor, we will add 1/10 volume of sterile 10xPBS to the Percoll bottle, mix well and use the PBS-balanced Percoll as 100% Percoll for making all Percoll-containing buffers.

4.4. Spin at 900X g for 20 min at room temperature with reduced accelerator and brake setting;

NOTE: For centrifuges with accelerator and brake settings (on a 1-9 scale (1 means minimum and 9 means maximum)), use 6 for accelerator and 4 for brake.

4.5. Carefully remove the tubes from the centrifuge without disturbing the layers;

NOTE: Now you should be able to clearly identify a lymphocyte layer between pink color RPMI layer and colorless PBS layer.

4.6. Remove the top layer with a transfer pipette without touching the lymphocyte layer;

4.7. Transfer the lymphocyte layer to a new 15 ml tube, fill the tube with PBS/5% FCS and mix by inverting the tube 4-6 times slowly to ensure sufficient mixing.

NOTE: This step is important to wash out any residual Percoll.

4.8. Spin down at 500X g for 5 min at 4 °C;

4.9. Remove the supernatant and re-suspend the cell pellet with 500 µl complete RPMI medium and the cells are ready for flow cytometry staining.

5. Flow cytometry staining and analysis

NOTE: Please follow standard flow cytometry staining protocol for T lymphocytes.

5.1. Take around 10^6 cells for each FACS staining.

NOTE: We usually use a U-bottom 96-well plate for FACS staining. However, if only a limited number of samples are involved, you can use 5ml FACS tubes for FACS staining.

5.2. Spin down the cells at 500xg for 5min at 4°C. Discard the supernatant.

5.3. Resuspend each sample in 50µl FACS buffer containing 10µg/ml 2.4G2 FcR blocker (an anti-CD16/32 monoclonal antibody to block the interaction between added FACS antibody with FcR). Incubate on ice for 15 min.

5.4. Without further centrifugation, add 50µl surface staining antibody mixture for each sample so that the final staining volume is 100µl/each sample. Incubate on ice for 30 min in the dark.

NOTE: Include fluorescent labelled streptavidin (2µg/ml or follow manufacturer's recommendation) in the antibody mixture to label CD8α⁺ intravascular CD8⁺ T cells and use fluorescent labelled anti-CD8β antibody to label all CD8 T cells. Antibody mixture was made by adding desired amounts of interested antibodies into FACS buffer.

5.5. Wash the samples with PBS twice. For each wash, fill the wells of 96-well plate with cold PBS, spin at 500xg for 5 min at 4°C and discard the supernatant.

5.6. Resuspend the cells in 100µl/well of diluted live/death dye. Incubate on ice for 30 min in the dark.

NOTE: Even with Percoll spin, enzymatic digestion step often induces significant cell death in tissue-resident T cells due to ARTC2.2/P2RX7 signaling¹⁵. Live/death dye staining is highly recommended before fixation.

NOTE: Anti-ARTC2 nanobody (Treg-Protector™, Biolegend) can be administrated into the mice before euthanasia to prevent cell isolation-induced cell death.

5.7. Wash the samples with FACS buffer twice. For each wash, fill the wells of 96-well plate with cold FACS buffer, spin at 500xg for 5 min at 4°C and discard the supernatant.

5.8. Resuspend the cells in 100 μ l/well fixing buffer. Incubate at 37°C for 10 min.

NOTE: Fixing buffer is made freshly by diluting formaldehyde with PBS to a final concentration of 2% formaldehyde.

5.9. Wash the samples with FACS buffer twice. For each wash, fill the wells of 96-well plate with cold FACS buffer, spin at 500 $\times$ g for 5 min at 4°C and discard the supernatant.

5.10. Resuspend the cells in 250 μ l/well FACS buffer. Seal the plate, store at 4°C and protect from light until FACS analysis.

NOTE: Before FACS analysis, filter the samples through a 70 μ M nylon mesh to remove possible cell clusters that may clog the FACS machine.

REPRESENTATIVE RESULTS:

The protocol described here was summarized in a flow chart (Fig. 1A). At day 30 post LCMV infection, we performed intravascular labeling of CD8⁺ T cells. Five minutes later, both kidneys of the animal were dissected, minced and subjected to collagenase digestion. Lymphocytes were further purified from the digested samples via Percoll centrifugation and analyzed by flow cytometry. As shown in Fig. 1B, even after density centrifugation-mediated lymphocyte enrichment, it is very common to see a large portion of non-lymphocytes in the final product. However, after gating on live lymphocytes, it is usually easy to identify CD8⁺ T lymphocytes. We can distinguish intravascular vs extravascular CD8⁺ T cells. Due to highly correlative expression pattern of CD8 α and CD8 β on CD8⁺ T cells, it is expected that intravascular CD8⁺ T cells exhibit a diagonal pattern on CD8 α -CD8 β FACS profile. As expected, only extravascular CD8⁺ T cell efficiently acquire T_{RM} phenotypes, such as upregulation of CD69 and downregulation of Ly6C (Fig. 1B). In our experiments, we are interested in donor P14 T cells with congenic marker CD45.1. Using the same protocol, endogenous host-derived CD8⁺ T cells can be examined as well. In contrast to infected mice, the vast majority of CD8⁺ T cells isolated from young naïve mice housed in SPF facility are labelled with i.v.CD8 α antibody and therefore belong to the intravascular compartment (Fig. 1C).

DISCUSSION:

As tissue specific immunity is a rapid expanding area of research, accumulating evidence suggest that immune cells, especially lymphocytes population can be identified in almost all organs in adult human or infected or immunized mice. LCMV mouse infection model is a well-established model to study antigen-specific T cell response, effector and memory T cell differentiation including T_{RM} biology across multiple tissues. Here, we described a protocol to analyze CD8⁺ T cells in the kidney. This protocol is largely adapted from publications focused on T_{RM}¹². Presumably due to high level P2RX7 expression and enzymatic digestion induced cell death¹⁵, lymphocytes yield from the current protocol is best suited for immediate phenotypic analysis. However, with established protocol to inhibit P2RX7 induced cell death¹⁶, it is conceivable that the current protocol can be easily modified (e.g., with the addition of inhibitors to block P2RX7 signaling) to increase the cell

survival and be suited for other long-term functional assays. Proper control groups should be included to ensure that P2RX7 inhibitors do not interfere with interested functional assays.

Kidney CD8⁺ T_{RM} cells are largely generated in response to infection or environmental antigens. We have use intravascular labeling protocol to directly analyze kidney CD8⁺ T cells isolated from 5-6-week-old naïve C57BL/6 mice recently shipped from Jax Lab. Consistent with published results¹⁷, there is almost no extravascular CD8⁺ T cells in these “clean” mice (Fig. 1C). An elegant study has demonstrated that the majority of memory CD8⁺ T cells identified in non-lymphoid tissues are T_{RM}¹⁰. In contrast, using a similar infection system, we often detect a significant population of CD69⁺ cells (most likely represent T_{EM} cells or CD69⁺ T_{RM} cells) even in the extravascular compartment. The discrepancy may be due to the facts that 1) different techniques are used, i.e., microscope vs flow cytometry; 2) early vs late memory time points are focused and 3) CD69 is not a perfect marker to identify T_{RM}. Enzymatic digestion and flow cytometry will significantly under-estimate the total number of T_{RM} cells. However, as a convenient and non-labor-intensive protocol, it is still commonly accepted in most T_{RM} studies.

In addition to infections, any kidney targeting immune responses, including autoimmune responses may induce the differentiation of a significant subset of kidney-resident T cells, which can be studied using a similar protocol. Further, as described before¹², this protocol can be easily modified to use anti-CD45 antibody (to label all hematopoietic cells) to study other kidney-resident immune cells. Together, we demonstrated a relatively convenient way to isolate and analyze CD8⁺ T cells from the kidney, which can be adapted to various models including infection and autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS:

This work is supported by NIH grants AI125701 and AI139721, Cancer Research Institute CLIP program and American Cancer Society grant RSG-18-222-01-LIB to N.Z. We thank Karla Gorena and Sebastian Montagnino from Flow Cytometry Facility. Data generated in the Flow Cytometry Shared Resource Facility were supported by the University of Texas Health Science Center at San Antonio (UTHSCSA), NIH/NCI grant P30 CA054174-20 (Clinical and Translational Research Center [CTRC] at UTHSCSA), and UL1 TR001120 (Clinical and Translational Science Award).

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Figure 1. Representative results.

(A) Flowchart of the protocol. (B) At day 30 post infection, kidney lymphocytes were analyzed by flow cytometry. Representative FACS profiles and gating strategy are shown. The numbers indicate the percentage of gated subsets in their parental population. (C) Representative FACS profile of kidney CD8⁺ T cells isolated from a naïve mouse.

Table of Materials.

List of materials used in current protocol with detailed order information.

Name of Material/ Equipment	Company	Catalog Number
1.5 ml microcentrifuge tubes	Fisherbrand	05-408-130
15 ml Conical Tubes	Corning	431470
3 ml syringe	BD	309657
37C incubator	VWR	
Biotin α -CD8 α antibody(Clone 53-6.7)	Tonbo Biosciences	30-0081-U025
Calf Serum	GE Healthcare Life Sciences	SH30073.03
Collagenase B	Millipore Sigma	11088807001
Disposable Graduated Transfer Pipettes	Fisherbrand	13-711-9AM
Insulin Syringe	BD	329424
Micro Dissecting Spring Scissors	Roboz Surgical	RS 5692
Mojosort Mouse CD8 Naïve T Cells Isolation Kit	Biolegend	480043
overhead heat lamp	Amazon	B00333PZZG
PBS		
Percoll	GE Healthcare Life Sciences	17089101
rocker	VWR	
RPMI	GE Healthcare Life Sciences	SH30096.01
Solid Brass Mouse Restrainer	Braintree Scientific, Inc	SAI-MBR
Swing Bucket Centrifuge with refrigerator	Thermofisher	
Tissue Culture 6-well plate	Corning	3516