Journal of Visualized Experiments

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

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE61559R1
Full Title:	Isolation of Mouse Kidney-Resident CD8+ T cells for Flow Cytometry Analysis
Keywords:	CD8; Kidney-resident; LCMV; Flow Cytometry; mouse; intravascular labeling
Corresponding Author:	Nu Zhang University of Texas Health Science Center at San Antonio San Antonio, TX UNITED STATES
Corresponding Author's Institution:	University of Texas Health Science Center at San Antonio
Corresponding Author E-Mail:	ZhangN3@uthscsa.edu
Order of Authors:	Wei Liao
	Chaoyu Ma
	Nu Zhang
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	San Antonio, TX, USA

Dear Dr. Bajaj,

We are submitting a revised manuscript titled “Isolation of Mouse Kidney-Resident $CD8^+$ T cells for Flow Cytometry Analysis”. In this manuscript, we describe a commonly used protocol to isolate and analyze mouse kidney-resident $CD8^+$ T cells following acute viral infection.

Thank you very much,

Nu Zhang, Ph.D.
Department of Microbiology, Immunology & Molecular Genetics
Long School of Medicine
UT Health San Antonio

TITLE:

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

AUTHORS AND AFFILIATIONS:

Wei Liao[1,2], Chaoyu Ma[1], Nu Zhang[1]

[1]Department of Microbiology, Immunology and Molecular Genetics, School of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA

[2]Department of Dermatology, Hunan Key Laboratory of Medical Epigenomics, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China

Corresponding Author:

Nu Zhang (zhangn3@uthscsa.edu)

Email Addresses of Co-Authors:

Wei Liao (liaow@uthscsa.edu)

Chaoyu Ma (mac4@uthscsa.edu)

KEYWORDS:

CD8, kidney resident, LCMV, flow cytometry, mouse, intravascular labeling

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.

ABSTRACT:

Tissue-resident memory T cell (T_{RM}) is a rapidly expanding field of immunology research. Isolating 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 for 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 characterizing 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 lymphocytic choriomeningitis virus (LCMV) infection model in C57BL/6 mice, we demonstrate intravascular CD8$^+$ T cell labeling, enzymatic digestion, and density gradient centrifugation to isolate and enrich lymphocytes from mouse kidneys to make samples ready for the subsequent flow cytometry analysis.

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[1-5]. Comparing with circulating T cells, T_{RM} cells carry distinct surface markers with unique transcription programs[6-8]. Expanding our knowledge of T_{RM} biology is the key to understanding T cell responses which is 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} biology[9]. Kidney harbors many 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 kidney[10]. 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 in mice $CD8^+$ effector and memory T cells are tracked, 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 model[11]. 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} labs[12]. In brief, 3-5 minutes before euthanasia, 3 μg/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 labeled. 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 before[13]. 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. Use P14 mice at 6-12 weeks of age. Ensure that the sex of donor P14 TCR transgenic mouse

should match the sex of C57BL/6 recipient mice. Otherwise, cells such as male T cells transferring into female mice will result in immune-mediated rejection of circulating donor T cells in around 12 days[14].

1.2. Purify naïve $CD8^+$ T cells from the spleen and lymph nodes of a P14 TCR transgenic mouse using mouse $CD8^+$ T cell purification kit following the manufacturer's protocol.

1.2.1 Briefly, dissect spleen and lymph nodes, homogenize the samples to generate single cell suspension.

1.2.2 To enrich for naïve T cells, add biotin-anti-CD44 antibody during the first antibody incubation step[13].

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

1.3. Use a hemocytometer to count the cells and inject 1,000 to 10,000 purified P14 T cells in 200 µL of PBS into each sex matched C57BL/6 recipient via the tail vein.

NOTE: Detailed tail vein injection protocol is described in step 2.

1.4. On the next day, all C57BL/6 recipients receive 2 x 10^5 pfu LCMV Armstrong diluted in 200 µL of 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 (day 30 after step 1.4) is 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. Ensure each mouse receive 200 µL of PBS containing 3 µg antibody.

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

2.2. Properly heat the tail vein of 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 µL of prediluted anti-CD8α antibody mix into a 100 U 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 µL of anti-CD8α antibody via the tail vein.

NOTE: If there is a resistance or a white area is seen above the needle on the tail, the needle is not inside the vein. Start the 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 CO_2 inhalation will take up to 5 min 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 of 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 of the 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 **Table 1** for the recipes for all solution/buffer used in the current protocol.

3.2. Add 300 μL of digestion solution into the 1.5 mL 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 1.5 mm in diameters. No decapsulation step is required.

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

NOTE: 6 well plates are used for convenience only 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, homogenize the tissue with the plunger flange of a 3 mL syringe directly inside the dish, and transfer into 15 mL conical tubes.

NOTE: Usually filtration through a cell strainer is not required at this step because density gradient centrifugation is performed next to purify live lymphocytes.

3.5. Spin down the samples at 500 *x g* for 5 min at 4 °C.

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

4. Density gradient centrifugation to enrich lymphocytes from the digested kidney

4.1. Spin down the sample at 500 *x g* for 5 min at 4 °C.

4.2. Remove the supernatant and resuspend the cell pellet with 5 mL of 44% density gradient medium/RPMI mix (44% density gradient medium + 56% RPMI without FBS).

4.3. Put the tip of a 3 mL pipette containing 3 mL of 67% density gradient medium/PBS (67% density gradient medium + 33% PBS) directly to the bottom of each tube, and slowly release the solution so that the heavy solution (67% density gradient medium /PBS) forms a distinct layer at the bottom and the light solution (44% density gradient medium/RPMI) is raised up. Together, cocktail layers will form.

NOTE: For newly arrived density gradient medium from the vendor, add 1/10 volume of sterile 10x PBS to the bottle, mix well and consider the PBS-balanced density gradient medium as 100% solution.

4.4. Spin the samples at 900 *x 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: A clear lymphocyte layer is identified 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 4-6x slowly to ensure sufficient mixing.

NOTE: This step is important to wash out any residual density gradient medium.

4.8. Spin down the samples at 500 *x g* for 5 min at 4 °C.

4.9. Remove the supernatant and re-suspend the cell pellet with 500 µL of complete RPMI medium. 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 1 x 10^6 cells for each FACS staining.

NOTE: Use a U-bottom 96 well plate for FACS staining. However, if only a limited number of samples are involved, 5 mL FACS tubes can also be used.

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

5.3. Resuspend each sample in 50 µL of FACS buffer containing 10 µg/mL of 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 50 µL of surface staining antibody mixture for each sample so that the final staining volume is 100 µL/each sample. Incubate on ice for 30 min in the dark.

NOTE: Include fluorescently labeled streptavidin (2 µg/mL or follow manufacturer's recommendation) in the antibody mixture to label CD8α^+ intravascular CD8$^+$ T cells and use fluorescent labeled anti-CD8β antibody to label all CD8 T cells. Antibody mixture is made by

adding desired amounts of interested antibodies into the 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 500 *x g* for 5 min at 4 °C and discard the supernatant.

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

NOTE: Even with density gradient medium spin, enzymatic digestion step often induces significant cell death in tissue-resident T cells due to ARTC2.2/P2RX7 signaling[15]. Live/death dye staining is highly recommended before fixation. Anti-ARTC2 nanobody 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 500 *x g* for 5 min at 4 °C and discard the supernatant.

5.8. Resuspend the cells in 100 μL/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 *x g* for 5 min at 4 °C and discard the supernatant.

5.10. Resuspend the cells in 250 μL/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 μM nylon mesh to remove possible cell clusters that may clog the FACS machine.

REPRESENTATIVE RESULTS:

The protocol described here is summarized in a flow chart (**Figure 1A**). At day 30 post LCMV infection, we performed intravascular labeling of $CD8^+$ T cells. 5 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 **Figure 1B**, even after density centrifugation-mediated lymphocyte enrichment, it was very common to see a large portion of non-lymphocytes in the final product. However, after gating on live lymphocytes, it was easy to identify $CD8^+$ T lymphocytes. We could distinguish intravascular vs extravascular $CD8^+$ T cells. Due to highly correlative expression pattern of CD8α and CD8β on $CD8^+$ T cells, it is expected that intravascular $CD8^+$ T cells exhibit a diagonal pattern on CD8α-CD8β FACS profile. As expected, only extravascular $CD8^+$ T cell efficiently acquired T_{RM} phenotypes, such as upregulation of CD69 and downregulation of Ly6C (**Figure 1B**). In our experiments, we were 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 were labeled with i.v.CD8α antibody and, therefore, belonged to the intravascular compartment (**Figure 1C**).

FIGURE AND TABLE LEGENDS:

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 1: List of recipes for all solution and buffer used in current protocol.

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 death[15], lymphocytes yield from the current protocol is best suited for immediate phenotypic analysis. However, with established protocol to inhibit P2RX7 induced cell death[16], 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. Consistent with published results[17], there is almost no extravascular $CD8^+$ T cells in these "clean" mice (**Figure 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.

The gold standard to definitively identify T_{RM} cells is to perform parabiosis experiment. Although the protocol described here provides a convenient way to identify kidney-resident T cells, the results from this protocol only prove that at the time when the mice are euthanized, the T cells

are residing outside the blood vessels in the kidney. This protocol alone does not provide any information about the migratory history of T cells.

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 can be studied using a similar protocol. Further, as described before[12], 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.

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).

DISCLOSURES:

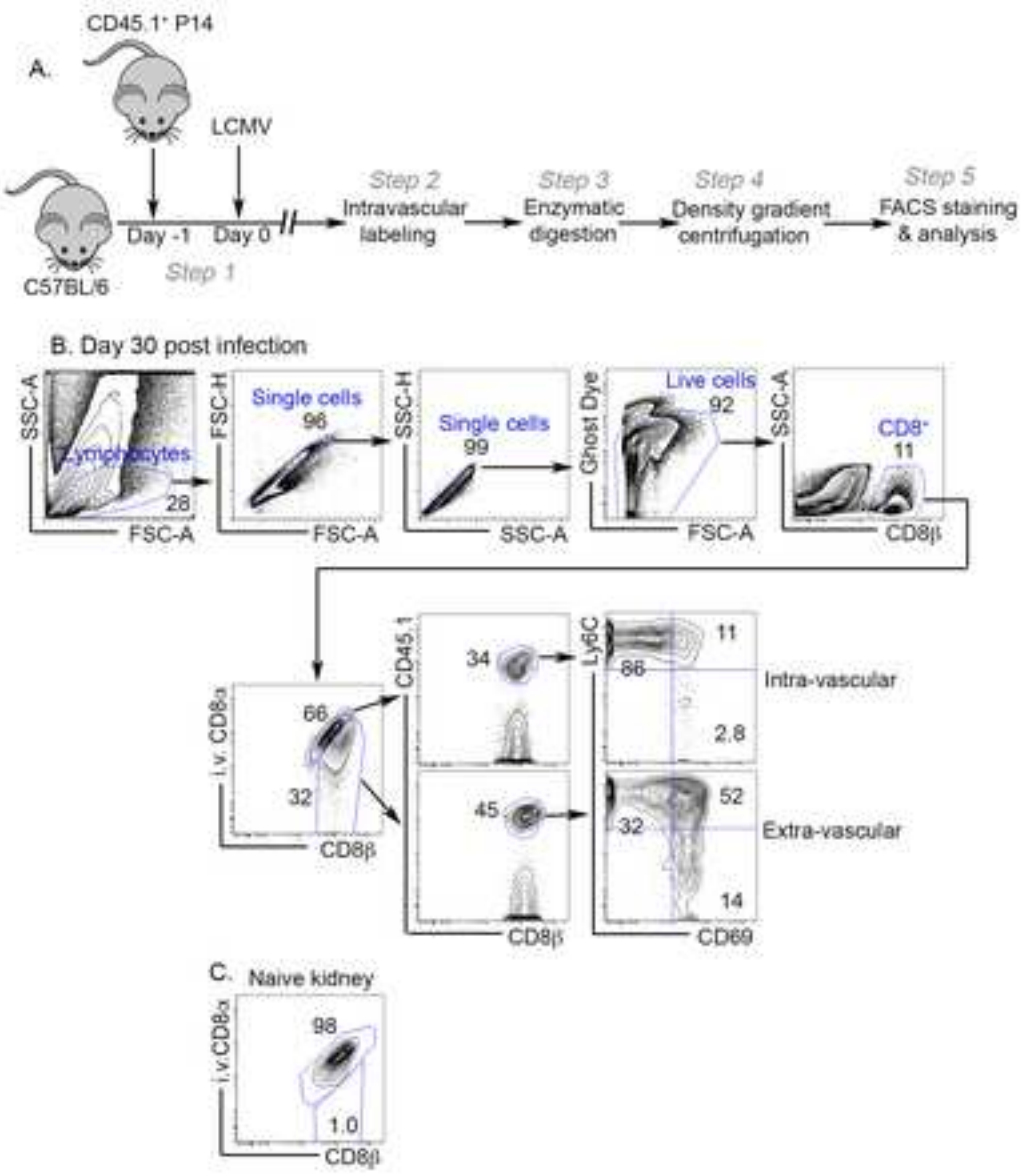
The authors have no relevant financial disclosures.

REFERENCES:

1. Mueller, S. N., Gebhardt, T., Carbone, F. R., Heath, W.R. Memory T cell subsets, migration patterns, and tissue residence. *Annual Review of Immunology.* **31**, 137-161 (2013).
2. Mueller, S. N., Mackay, L. K. Tissue-resident memory T cells: local specialists in immune defence. *Nature Reviews: Immunology.* **16**, 79-89 (2016).
3. Park, C. O., Kupper, T. S. The emerging role of resident memory T cells in protective immunity and inflammatory disease. *Nature Medicine.* **21**, 688-697 (2015).
4. Schenkel, J. M. et al. T cell memory. Resident memory CD8 T cells trigger protective innate and adaptive immune responses. *Science.* **346**, 98-101 (2014).
5. Thome, J. J., Farber, D. L. Emerging concepts in tissue-resident T cells: lessons from humans. *Trends in Immunology.* **36**, 428-435 (2015).
6. Mackay, L. K. et al. Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. *Science.* **352**, 459-463 (2016).
7. Mackay, L. K. et al. T-box Transcription Factors Combine with the Cytokines TGF-beta and IL-15 to Control Tissue-Resident Memory T Cell Fate. *Immunity.* **43**, 1101-1111 (2015).
8. Milner, J. J. et al. Runx3 programs CD8(+) T cell residency in non-lymphoid tissues and tumours. *Nature.* **552**, 253-257 (2017).
9. Liu, Y., Ma, C., Zhang, N. Tissue-Specific Control of Tissue-Resident Memory T Cells. *Critical Reviews in Immunology.* **38**, 79-103 (2018).
10. Steinert, E. M. et al. Quantifying Memory CD8 T Cells Reveals Regionalization of

Immunosurveillance. *Cell.* **161**, 737-749 (2015).
11. Qiu, Z., Sheridan, B. S. Isolating Lymphocytes from the Mouse Small Intestinal Immune System. *Journal of Visualized Experiments.* (132) 57281 (2018).
12. Anderson, K. G. et al. Intravascular staining for discrimination of vascular and tissue leukocytes. *Nature Protocols.* **9**, 209-222 (2014).
13. Ma, C., Mishra, S., Demel, E. L., Liu, Y., Zhang, N. TGF-beta Controls the Formation of Kidney-Resident T Cells via Promoting Effector T Cell Extravasation. *Journal of Immunology.* **198**, 749-756 (2017).
14. Gebhardt, T. et al. Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature.* **477**, 216-219 (2011).
15. Borges da Silva, H., Wang, H., Qian, L. J., Hogquist, K. A., Jameson, S.C. ARTC2.2/P2RX7 Signaling during Cell Isolation Distorts Function and Quantification of Tissue-Resident CD8(+) T Cell and Invariant NKT Subsets. *Journal of Immunology.* **202**, 2153-2163 (2019).
16. Fernandez-Ruiz, D. et al. Liver-Resident Memory CD8(+) T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection. *Immunity.* **45**, 889-902 (2016).
17. Beura, L. K. et al. Normalizing the environment recapitulates adult human immune traits in laboratory mice. *Nature.* **532**, 512-516 (2016).

Figure 1



Supplemental Table 1.

Reagent	Recipe
100% Percoll	9 volumes of Percoll + 1 volume of 10xPBS
44% Percoll/RPMI	44% Vol of 100% Percoll + 56% Vol serum free RPMI
67% Percoll/PBS	67% Vol of 100% Percoll + 33% Vol PBS
Digestion buffer	RPMI + 2% FCS + 1mg/ml collagenase B
PBS/5% FCS washing buffer	PBS + 5% FCS
FACS buffer	PBS + 2% FCS + 0.02% NaN_3
Fixing buffer	PBS + 2% formaldehyde

NOTE
Made fresh from 100% Percoll
Made fresh from 100% Percoll
Made fresh from collagenase stock
Stored at 4°C
Stored at RT and protected from light

Name of Material/ Equipment	Company
1.5 ml microcentrifuge tubes	Fisherbrand
15 ml Conical Tubes	Corning
3 ml syringe	BD
37C incubator	VWR
Biotin α-CD8α antibody(Clone 53-6.7)	Tonbo Biosciences
Calf Serum	GE Healthcare Life Sciences
Collegenase B	Millipore Sigma
Disposable Graduated Transfer Pipettes	Fisherbrand
Insulin Syringe	BD
Micro Dissecting Spring Scissors	Roboz Surgical
Mojosort Mouse CD8 Naïve T Cells Isolation Kit	Biolegend
overhead heat lamp	Amazon
PBS	
Percoll	GE Healthcare Life Sciences
rocker	VWR
RPMI	GE Healthcare Life Sciences
Solid Brass Mouse Restrainer	Braintree Scientific, Inc
Swing Bucket Centrifuge with refrigerator	Thermofisher
Tissue Culture 6-well plate	Corning

Catalog Number

05-408-130
431470
309657

30-0081-U025
SH30073.03
11088807001
13-711-9AM
329424
RS 5692
480043
B00333PZZG

17089101

SH30096.01
SAI-MBR

3516

We thank the editor and reviewers for their suggestions and comments.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
OK

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points
Ok

3. Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol.
Ok

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
Ok

5. The Protocol should contain only action items that direct the reader to do something.
Ok

6. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.
Ok

7. Please ensure you answer the “how” question, i.e., how is the step performed?
Ok

8. 1.1: Please explain briefly how this is done.
We have included detailed description.

9. 1.2: How do you count the cells?
We have added requested information.

10. 2.9: How is the euthanasia performed in your experiment?
We have added requested information.

11. 2.10: How do you perform kidney dissection?
Kidney is an organ can be easily identified for anyone with basic biology knowledge. There is no specific technique required to dissect a kidney. Will be shown in the video.

12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Ok

13. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.
Ok

14. Please include data from control experiment as well.
The results from a non-infected naïve mouse were added.

15. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

The figure is un-published. No copyright information is required.

16. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Ok

17. Please do not abbreviate the journal titles in the references section.
Ok

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This paper describes a technique for isolating CD8+ T cells from the kidney and identifying and differentiating the intravascular and extravascular cells amongst that population. Data are shown 30 days after LCMV infection in a mouse carrying LCMV-specific TCR-transgenic T cells. The technical aspects of the paper are clear and easy to follow.
Thank you!

Major Concerns:
The data shown are derived solely from infected mice. It might serve as a useful comparison to show data from a non-infected mouse. This would provide the readership an idea as to the rarity of these cells under basal conditions.
The results from an uninfected naïve mouse were added.

In 2.9, presumably the mode of euthanasia is important and should be explicitly defined. Is cervical dislocation the optimal technique? Should CO2 inhalation be excluded because presumably it takes longer for the mouse's heart to stop beating and therefore has the potential to introduce variability in the results due to differences in the time the antibody is in the circulation prior to death?
Yes. The reviewer is correct. This step is clarified.

In 2.10, is the kidney put in the tube whole, or is it cut up to any extent beforehand?
We have clarified the information.

3.4 - mash not smash
Thanks! It has been corrected.

4.3 - What is meant by 'cocktail' layers? Clarify how the 67% Percoll is passed through the lower density layer. Does this relate to the positioning of the pipette tip?
We have clarified the information.

5.2 - Can something be added to the preparation to limit this type of cell death?
A note was added.

In Figure 1B, a substantial proportion of the cells are shown as being non-Trms. What are these cells? Are the Tems? Naïve cells? This data is potentially at odds with the statement from the Steinert paper (ref 10) that "Most Memory CD8 T Cells in non-lymphoid tissue (which includes the kidney) Are TRM". The authors should examine a bit more closely how their data fit or don't fit with other key manuscripts in the field. In particular in that paper the authors state that flow cytometry underestimates Trms as opposed to Tem. The authors should provide a comment on this, addressing the potential limitations of their technique particularly from a quantitative perspective.
We have expanded the discussion part. Basically, the Cell paper by Masopust group used parabiosis and microscope to show the majority of CD8 T cells in the kidney are TRM and their time point is day 90 after infection. In our hands, it is very common to identify CD69- cells in the kidney around d30-50 after LCMV infection in the kidney. It is possible that different time points lead to the difference. OR enzymatic digestion plus FACS analysis may enrich for non-Trm cells.

There is a fair amount of editorial work to be done to get this manuscript up to publication quality, but I will leave that up to the editorial team.

Minor Concerns:
The supplier and exact details on the Mojo CD8 T cell purification kit should be provided.
Provided in the Material Table.

Reviewer #2:
Manuscript Summary:
The manuscript by Ma et al, describes T cell isolation from kidney- a visceral organ of significant importance. The authors succinctly describes various steps of the isolation protocol, with special emphasis on intravascular labelling, tissue digestion and enrichment of lymphocytes via density gradient centrifugation. All the critical steps are well elaborated and will allow successful execution of the protocol by non-experts. I do not have any major concerns, but have suggested some minor edits/additions that will be helpful.

Thank you so much!

Major Concerns:
No major concerns.

Minor Concerns:
1. The use of percoll solution to enrich leucocytes should be termed as "density gradient centrifugation" instead of just density centrifugation, as it employs a discontinuous gradient to allow appropriate separation of cells.
Thanks and it has been corrected.

2. Regarding the first statement in introduction section- although TRM are a significant fraction of T cells in mice and humans, there has not been enough evidence that they represent the single most abundant T cell population at the organismal level. The authors' sentence is mostly true for non-lymphoid organs.
Thanks and it has been corrected.

3. In line-71, Pg.1, "infusion" should be replaced with "perfusion"- the correct descriptor for removing blood and associated cells from organs.
Thanks and it has been corrected.

4. In line-71, Pg.2, veil should be changed to vein.
Thanks and it has been corrected.

5. In section-3, enzymatic digestion, the authors should mention if they have tested other enzymes and found collagenease-B to be the ideal enzyme. If there is a speed associated with the gentle rocking motion, that should be mentioned.
We have not vigorously compared different collagenase. We have used collagenase B and C without noticing a dramatic difference.

Reviewer #3:
Manuscript Summary:
This manuscript describes the isolation of tissue-resident lymphocytes from the kidney and downstream analysis using flow cytometry. Many of the lymphocytes in the intact kidney reside in the vasculature, and intravenous antibody injection is used to differentiate tissue-resident lymphocytes from those in the vasculature. The protocol also includes detailed methods for digestion of the tissue and further lymphocyte purification. The protocol and discussion highlighted the concern over cell death in the isolation of Trm cells and provided additional suggestions for increasing cell yields.
Overall, I think this protocol will be of interest to many, as the use of the kidney as a source of Trm cells for comparative studies is becoming more common and there is a growing interest in the tissue-specific factors that influence Trm differentiation in the kidney and other organs. I just had a few minor concerns, detailed below.
Thanks!
Major Concerns:
No major concerns.

Minor Concerns:
Line 71: should this be perfusion not infusion
Thanks and it has been corrected.

Protocol 2.3: The statement 'Bend the needle to create a 30° angle between the needle and syringe' seems wrong to

me. Is it a 30 degree bend from the original position making it 150 degrees from the syringe? I'm sure this will be cleared up by the video but it was a bit confusing.
Sorry for the confusion. It has been corrected.

Protocol 3.4: Do you 'smash the tissue with the plunger flange of a 3 ml syringe' directly in the plate, or is this through a filter of some type?
It has been clarified.

Protocol 5.1: It would be nice to know the concentration of the fluorescently labelled streptavidin that is used for staining.
The information is added.

Line 256: I liked the suggestion that inhibition of P2X7 signaling could be incorporated to prevent cell death to increase yield and for subsequent functional assays, but is it really clear whether inhibiting P2X7 works solely to prevent cell death? Can it alter the downstream functional potential of these cells as well?
Thanks for the insightful comments! The discussion is expanded.

Reviewer #4:
This manuscript provides a timely protocol for isolating TRMs from kidney, which will serve as an important reference for the fast-growing TRM research community. The procedures were clearly described with adequate details, and representative results were clearly laid out. I only have suggestions for minor edits:

Line67-68: The authors should briefly describe the "standard lymphocyte isolation procedure", as isolating lymphocytes from mucosal tissues, such as the gut, is not straightforward or "standard". Consider citing protocols such as the 2018 JOVE paper describing lymphocyte isolation from the small intestine (PMID:29553537).
Thanks for the suggestion! We have revised the description as suggested.

Line141-142: "re-inserted above the first site" is not clear, consider revising: Start initial injection close to the tip of the tail. When necessary, move forward towards the bottom of the tail for subsequent injections.
Thanks! We have revised the description as suggested.

Line146: Is there a preferred method (CO_2, cervical dislocation) for euthanizing mice for this procedure?
We have provided our method for euthanizing mice (isoflurane plus cervical dislocation)

Line146: Consider replacing "5 min" with either "3-5 min" (to be consistent with line73) or "exactly 5 min".
We have revised the description as suggested.

Line153-154: Does the Collagenase B need to be added fresh (the same day of the experiment) or the digestion solution can be premade?
We have clarified the information.

Line200-204: consider revising: "…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 for washing out any residual Percoll."
Thank you so much! We have revised the description as suggested.

Consider adding a section/table for reagent recipes.
Thanks for the suggestion. A recipe table was added.

Reviewer #5:
This protocol described how to isolate resident CD8 T cells from the kidney. It delineates essential steps but there is room for improvement to make it easier to follow.
The below suggestions are being made:
1. The authors suggest that this could also be done for CD45 instead of CD8, however, for neither suggested antibody clones are provided (IP injection or tissue labeling), and the protocol is specific for lymphocyte isolation not immune cells as a whole.
Revised as suggested. i.v. CD8a antibody clone number was provided in the Material Table.

2. For Step 1 it should be made clear if there is a specific age of mouse that is best suited for splenic harvest of T cells. Further, the adoptive transfer is not truly part of the "CD8 T cell isolation" protocol and could be considered to be removed.
Age information is included. To provide a more complete information, the initial steps of the whole experiment is included in the text part. However, these initial steps will not be included in the video part.

3. In Step 2 it should be stated that it is not necessary to use a biotin labeled Ab for IV injection, but instead that it is simply important to use two different clones for IV labeling and flow cytometry analysis.
Thanks! We have revised the manuscript accordingly.

4. In Step 2 it is not clearly stated whether it is required to decapsulate the kidney or not.
We have clarified the description.

5. Step 2 should provide some information on how long the kidney can be stored on ice prior processing.
We have added requested information.

6. In Step 3 it is unclear why a 6-well plate is needed.
We have added a NOTE to explain it.

7. Step 5 should be expanded on to provide a detailed fixing/staining protocol.
We have expanded the protocol for FACS staining and fixing.

8. Figure 1B should describe what is gated out in each plot.
We added the information in the figure.

9. In the table the column for descriptions should be removed as no detail is provided.
Removed.