

Simultaneous Intranasal/Intravascular Antibody Labeling of CD4⁺ T Cells in Mouse Lungs

Yanqun Wang^{1, #}, Jing Sun^{1, #}, Rudragouda Channappanavar², Jingxian Zhao¹,
Stanley Perlman^{2, *} and Jincun Zhao^{1, *}

¹State Key Laboratory of Respiratory Diseases, Guangzhou Institute of Respiratory Disease, the First Affiliated Hospital of Guangzhou Medical University, Guangzhou, China; ²Departments of Microbiology, University of Iowa, Iowa City, Iowa, USA

*For correspondence: stanley-perlman@uiowa.edu; zhaojincun@gird.cn

#Contributed equally to this work

[Abstract] CD4⁺ T cell responses have been shown to be protective in many respiratory virus infections. In the respiratory tract, CD4⁺ T cells include cells in the airway and parenchyma and cells adhering to the pulmonary vasculature. Here we discuss in detail the methods that are useful for characterizing CD4⁺ T cells in different anatomic locations in mouse lungs.

Keywords: Memory CD4⁺ T cell, Lung, Bronchoalveolar lavage fluid, Intranasal/intravascular antibody labeling, Flow cytometry

[Background] To distinguish memory T cells in the circulation and tissues, a method for intravascular staining of T cells has been developed (Anderson *et al.*, 2012). This method has been widely used to define memory T cells in many organs and tissues, including lungs, spleens and intestines. However, memory T cells in the respiratory tract are located in three unique anatomic locations, *i.e.*, airway, parenchyma and pulmonary vasculature. Intravascular staining cannot distinguish cells in the airway and parenchyma, since they are both isolated from the circulation and intravascularly-administered antibodies will not stain these two populations. We designed a simultaneous intranasal/intravascular antibody labeling assay that can label and distinguish cells in all three locations using minimal amount of antibodies.

Materials and Reagents

1. 1.5 ml microtubes (Thermo Fisher Scientific, Thermo ScientificTM, catalog number: 69715)
2. 1 ml insulin syringe fitted with 28 G x ½ needle (BD, catalog number: 329420)
3. Precision glide needles (20 G x 1) (BD, catalog number: 305175)
4. 5 ml polystyrene round bottom tubes (Corning, Falcon[®], catalog number: 352054)
5. Precision glide needles (25 G x 5/8) (BD, catalog number: 305122)
6. 200 μ l tips (Thermo Fisher Scientific, InvitrogenTM, catalog number: AM12655)
7. 15 ml screw cap conical tubes (SARSTEDT, catalog number: 62.554.002)
8. 10 ml syringes (BD, catalog number: 309604)
9. 12 well cell culture plates (Corning, Costar[®], catalog number: 3513)

10. 3 ml syringes (BD, catalog number: 309657)
11. 50 ml screw cap conical tubes (SARSTEDT, catalog number: 62.547.004)
12. Cell strainers (70 μ m nylon) (Corning, Falcon[®], catalog number: 352350)
13. 60 x 15 mm tissue culture dish (Corning, Costar[®], catalog number: 353802)
14. 10 ml stripettes (Corning, Costar[®], catalog number: 3548)
15. Gauze sponges (4 x 4 inch) (Pro Advantage by NDC, catalog number: P157118)
16. 0.2 μ m filter (EMD Millipore, catalog numbers: SCGPU11RE and SLGP033RS)
17. 2.5 ml graduate transfer pipettes (RPI, catalog number: 147501-1S)
18. Absorbent pads (COVIDIEN, catalog number: 949)
19. Mouse
20. CD45-brilliant violet 510 (BV510) (Clone: 30-F11) (BioLegend, catalog number: 103138)
21. 1x Dulbecco's phosphate buffered saline (DPBS) (Thermo Fisher Scientific, GibcoTM, catalog number: 14190-144)
22. CD90.2-APC-eFluor 780 (Clone: 53-2.1) (Affymetrix, eBioscience, catalog number: 47-0902)
23. Isoflurane (USP inhalation vapour, liquid) (Drugs, catalog number: 57319-559-06)
24. Ethanol (Sigma-Aldrich, catalog number: 459836)
25. Trypan blue solution (Thermo Fisher Scientific, GibcoTM, catalog number: 15250061)
26. CD16/32-PerCP/Cy5.5 (Clone: 93) (BioLegend, catalog number: 101324)
27. CD4-FITC (Clone: RM4-5) (BioLegend, catalog number: 100510)
28. 2,2,2-tribromoethanol (Sigma-Aldrich, catalog number: T48402)
29. 2-methyl-2-butanol (Sigma-Aldrich, catalog number: 152463)
30. Nano-pure water (Thermo Fisher Scientific, InvitrogenTM, catalog number: 10977015)
31. RPMI medium 1640 (Thermo Fisher Scientific, GibcoTM, catalog number: 11875093)
32. HEPES (1 M) (Thermo Fisher Scientific, GibcoTM, catalog number: 15630080)
33. L-glutamine (200 mM) 100x (Thermo Fisher Scientific, GibcoTM, catalog number: 25030081)
34. Fetal bovine serum (FBS) (Atlanta Biologicals, catalog number: S11150)
35. MEM non-essential amino acids solution 100x (Thermo Fisher Scientific, GibcoTM, catalog number: 11140050)
36. Sodium pyruvate 100x (Thermo Fisher Scientific, GibcoTM, catalog number: 11360070)
37. Penicillin/streptomycin 100x (Thermo Fisher Scientific, GibcoTM, catalog number: 15140122)
38. 2-mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
39. Collagenase D (Roche Diagnostics, catalog number: 11088882001)
40. DNase I (Roche Diagnostics, catalog number: 10104159001)
41. Hank's balanced salt solution (HBSS) (Thermo Fisher Scientific, GibcoTM, catalog number: 14025)
42. Sodium azide, NaN₃ (AMRESCO, catalog number: 0639)
43. Potassium bicarbonate, KHCO₃ (Sigma-Aldrich, catalog number: 237205)
44. Ammonium chloride, NH₄Cl (Sigma-Aldrich, catalog number: A9434)
45. EDTA-Na₂ (Sigma-Aldrich, catalog number: E9884)

46. BD Cytofix fixation solution (BD, catalog number: 554655)
47. Avertin (see Recipes)
48. Complete RPMI 1640 medium (see Recipes)
49. Digestion buffer (see Recipes)
50. FACS buffer (see Recipes)
51. ACK lysis buffer (see Recipes)

Equipment

1. Desiccator (SP Scienceware - Bel-Art Products - H-B Instrument, model: Space Saver Vacuum Desiccators)
2. Fume hood (LABSCAPE)
3. Pipetman P10 (Eppendorf, model: Research plus)
4. Pipetman P200 (Eppendorf, model: Research plus)
5. Pipetman P1000 (Eppendorf, model: Research plus)
6. Pipet aid (Eppendorf, model: Eppendorf Easypet 3)
7. Heat lamp (Whitehead Industrial, catalog number: 30715)
8. Mouse restrainer (Braintree Scientific, catalog number: TV-150 STD)
9. Spray bottle with 70% ethanol (SKS Science Products, catalog number: 0185-11)
10. Surgical scissors (Sklar Surgical Instrument, catalog number: 47-1246)
11. Tweezers (Sklar Surgical Instrument, catalog number: 66-7644)
12. Polystyrene foam (Regular polystyrene box top)
13. Rocker (Labnet, catalog number: S0500)
14. Refrigerated tabletop centrifuge (Beckman Coulter, model: Allegra 6R)
15. Hemocytometer (Thermo Fisher Scientific, catalog number: 99503)
16. Flow cytometer (BD, model: FACSVerse)

Software

1. Flowjo software (Version 10.0.7)

Procedure

- A. Simultaneous intranasal/intravascular antibody labeling (see Videos 1 and 2)
 1. Dilute 0.25 µg CD45-BV510 antibody in 100 µl DPBS in a 1.5 ml microtube (for intranasal labelling). Dilute 0.5 µg CD90.2-APC-eFuor 780 antibody in 300 µl DPBS in another 1.5 ml microtube and load the antibody into a 1 ml insulin syringe with a 28 G x ½ needle (for intravenous labelling). Place the microtube and the syringe on ice until use. (see Note 1)
 2. Put a plastic desiccator in a fume hood. Place some gauze in the bottom chamber followed by

5 ml of isoflurane. (see Note 2)

3. Place a mouse on the grate inside the desiccator (see Note 3).
4. Load a P200 micropipette with 100 μ l of CD45-BV510 antibody.
5. Immediately after the rate of respiration slows, pick up the mouse, hold it vertically and slowly deliver 100 μ l of CD45-BV510 antibody onto the mouse nostrils. Hold the mouse upright so that the liquid on the nostrils is completely aspirated into the lungs.
6. Place the mouse back in the cage. After 2 min, turn on the heat lamp over the cage and warm up the mouse for 1 min.
7. Remove the mouse from the cage, put it in a mouse restrainer and inject 0.5 μ g CD90.2-APC-eFluor 780 antibody in 300 μ l DPBS through the tail vein with the 1 ml insulin syringe prepared in point 1.
8. Release the mouse from the restrainer and immediately anesthetize the mouse by intraperitoneal injection of 300 μ l of avertin solution. Secure the mouse in the palm of the hand and hold it horizontally, then insert the needle at a shallow angle tangential to the mouse and slowly dispense avertin solution into the peritoneal cavity.

Video 1. Intranasal administration of antibodies



Video 2. Intravenous administration of antibodies



B. Harvest cells from airway (see Video 3)

Video 3. Harvest of bronchoalveolar fluid, to obtain airway resident cells



1. Prepare a 1 ml syringe with 20 G x 1 needle. Carefully pass about 1.25 inches 20 G tubing onto the needle. Sharpen the end of the tubing with scissors. Load the syringe with 1 ml complete RPMI 1640 medium and put it on ice until use (Figure 1).

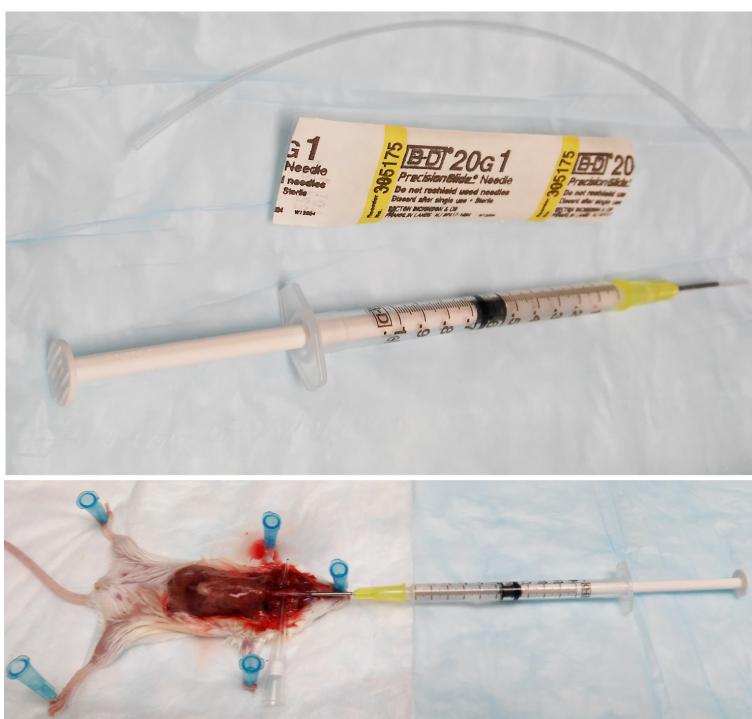


Figure 1. Representative pictures of syringe preparation and surgery for BALF lavage

2. When the mouse is fully anesthetized, typically within 2 min, immobilize it on a piece of polystyrene foam with absorbent pad on top by pinning each limb with a 25 G x 5/8 needle.
3. Use a spray bottle filled with 70% ethanol to wet the fur of the mouse.

4. Cut open the skin covering the abdominal and thoracic cavities through to the lower jaw. Use scissors to remove the tissue from the neck and expose the trachea.
5. Use tweezers to separate the trachea from the underlying tissue and place a 200 μ l tip under the trachea.
6. Use a razor to cut through $\frac{1}{2}$ of the trachea. Insert the 1 ml syringe with 20 G x 1 needle into the incision. Lavage for 4 times with complete RPMI 1640 medium. Collect the bronchoalveolar lavage fluid (BALF) in a 15 ml conical tube on ice. Typically 3-3.5 ml of BALF is recovered (Figure 1).
7. Spin down cells for 5 min at 400 $\times g$ at 4 °C in a bucket tabletop centrifuge and resuspend the cells in 1 ml of ice cold FACS buffer (see Note 5).

C. Sacrifice the mouse and harvest cells from lung (see Video 4)

Video 4. Perfusing and harvesting lungs for cellular analysis



1. After collection of BALF, cutting open the abdominal cavity to expose the underside of the diaphragm. Cut through the diaphragm with scissors and then remove the ribcage to fully expose the heart and lung.
2. Fill a 10 ml syringe with ice cold sterile DPBS and attach a 25 G x 5/8 needle. Insert the needle into the left ventricle of the heart and smoothly dispense 5 ml of DPBS into the heart. In the meantime, use tweezers to break the right atria to allow blood to drain from circulation. Remove the needle from the left ventricle and insert it into the right ventricle to directly perfuse the lung with the remaining 5 ml of DPBS. (see Note 6)
3. Cut the heart away from the lung and then remove the lung from the thoracic cavity after cutting the trachea and any remaining connective tissue.
4. Place the lung into the well of a 12 well tissue culture plate filled with 2.5 ml of DPBS on ice.
5. Rinse the lung with DPBS and transfer it into another well without DPBS. Mince the lungs into very fine pieces using scissors.
6. Transfer minced lung with a 2.5 ml transfer pipette to 5 ml of digestion buffer in a 15 ml conical

tube.

7. Place tubes on a rocker and gently rotate at room temperature for 30 min in the dark.
8. Place a 70 μ m cell strainer into a 60 x 15 mm tissue culture dish.
9. Transfer lung tissue in digestion buffer to the cell strainer using a 2.5 ml transfer pipette. Gently press and dissociate tissue through strainer with the flat end of a 3 ml syringe plunger. Process tissues until there is only connective tissue remaining on the strainer and rinse the strainer with complete RPMI 1640 medium. Transfer the resulting suspension to a 50 ml conical tube.
10. Spin down lung cells for 5 min at 400 x g at 4 °C in a bucket tabletop centrifuge.
11. Pour off supernatant and resuspend the cells in 3 ml of ACK buffer for 1 min to lyse the remaining red blood cells. Neutralize the ACK buffer with 30 ml of ice cold DPBS.
12. Spin down the cells for 5 min at 400 x g at 4 °C and resuspend the cells in 5 ml of ice cold FACS buffer.

D. Cell staining

1. Count the cells from BALF and lung using a hemocytometer in the presence of trypan blue.
2. Spin down lung cells for 5 min at 400 x g at 4 °C and resuspend the cells in FACS buffer at 1 million cells per 50 μ l.
3. Spin down cells in the BALF for 5 min at 400 x g at 4 °C. Since much fewer cells are recovered from airway, resuspend 1-5 x 10⁵ cells per 50 μ l FACS buffer as needed.
4. Dilute 0.25 μ g CD4-FITC and 0.1 μ g CD16/32 antibodies in 50 μ l FACS buffer (see Note 4).
5. Gently mix 50 μ l cells and 50 μ l antibodies together in a FACS tube.
6. Incubate the cells in the dark for 15 min at 4 °C.
7. Wash the cells once with 2 ml of FACS buffer at 400 x g for 5 min at 4 °C.
8. Remove the supernatant and resuspend the cells in 100 μ l FACS buffer.
9. Pass the cells through a 70 μ m cell strainer into new FACS tubes using a pipetman. Acquire FACS data using a flow cytometer and analyze data using Flowjo software (Figure 2). (see Note 7)
10. Flow cytometry gating strategy

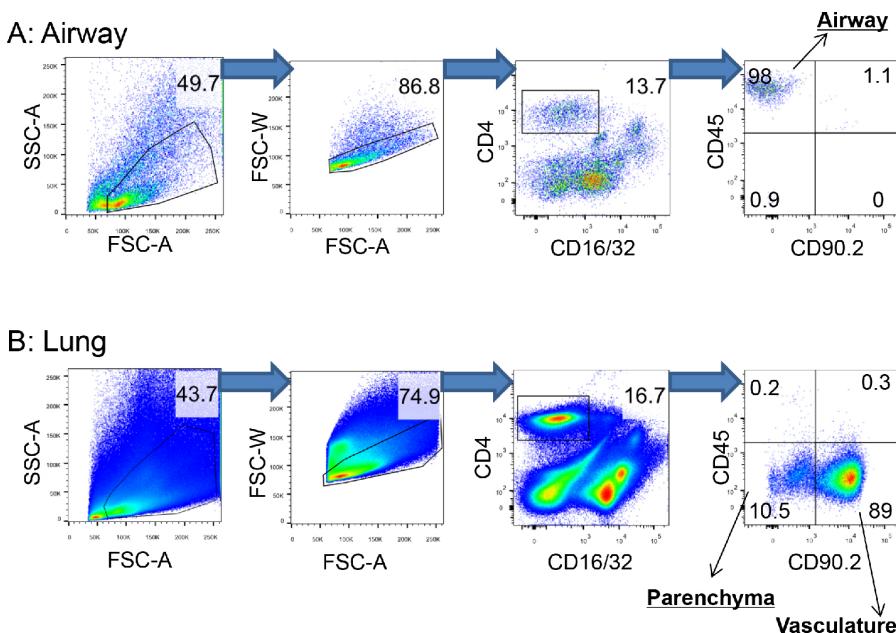


Figure 2. Gating strategy to distinguish CD4⁺ T cells in the airway, parenchyma and pulmonary vasculature. To localize memory CD4⁺ T cells in the respiratory tract, 0.25 μ g of fluorochrome-conjugated CD45 and 0.5 μ g of fluorochrome-conjugated CD90.2 antibody were injected into a SARS-CoV nucleocapsid protein-experienced mouse by i.n. and i.v. routes, respectively. Cells in the airway and lung were then harvested, stained and collected as described in Experimental Procedures and analysis using Flowjo software. A. CD4⁺ T cells in the airway: CD4⁺CD45⁺CD90.2⁻. B. CD4⁺ T cells in the parenchyma: CD4⁺CD45⁺CD90.2⁻. CD4⁺ T cells in the vasculature: CD4⁺CD45⁺CD90.2⁺. Data are representative of 10 independent experiments. (Zhao et al., 2016)

Notes

1. CD45 and CD90 are expressed by all CD4⁺ T cells. Both CD45 and CD90 antibodies can be used to stain CD4⁺ T cells in the lung and circulation. Phycoerythrin (PE), APC, BV (or their derivatives)-conjugated antibodies are preferentially chosen since these molecules have large molecular weights that will minimize airway/vascular leakage during *in vivo* labeling.
2. Make sure to tape over the center hole in the plastic desiccator grate because small mice are sometimes able to squeeze through this hole and jump into the bottom, isoflurane-filled chamber.
3. Here we used an antigen-experienced mouse that had been immunized with a construct expressing the SARS-CoV nucleocapsid protein (Zhao et al., 2016). This protocol can be applied to study memory and effector as well as naïve CD4⁺ T cells in mouse lungs.
4. Cells can also be stained with other phenotypic (surface) antibodies or cultured for intracellular cytokine staining as previously described (Fett et al., 2014).
5. For phenotypic staining, cells will be resuspended in FACS buffer. For intracellular staining, cell will be resuspended in complete RPMI 1640 medium.

6. The exterior surface of the lung will turn white after a good perfusion. Generally 5 ml PBS is sufficient.
7. This step is necessary to prevent clots formation in the flow cytometer. Other flow cytometers, such as LSR, Fortessa... can also be used to acquire cells.

Recipes

1. Avertin (250 ml)
5 g 2,2,2-tribromoethanol
5 ml 2-methyl-2-butanol
225 ml hot nano-pure water (95 °C)
Stir for 2 h in a fume hood and adjust volume to 250 ml
Filter through a 0.2 µm filter, aliquot and store at -20 °C
2. Complete RPMI 1640 medium (500 ml)
417 ml RPMI 1640 medium
12.5 ml 1 M HEPES
5 ml 100x L-glutamine
50 ml FBS
5 ml 100x MEM non-essential amino acids
5 ml 100x sodium pyruvate
5 ml 100x penicillin/streptomycin
0.5 ml 2-mercaptoethanol (50 mM in nano-pure H₂O, 0.2 µm filtered)
Store at 4 °C
3. Digestion buffer (100 ml)
100 mg collagenase D
10 mg DNase I
2 ml FBS
1 ml 100x glutamine
2.5 ml HEPES (1 M)
1 ml 100x penicillin/streptomycin
Adjust volume to 100 ml with HBSS
Aliquot and store at -20 °C
4. FACS buffer (500 ml)
1.7 ml sodium azide (from 30% stock made in nano-pure H₂O)
15 ml FBS
483.3 ml DPBS
Store at 4 °C
5. ACK lysis buffer
1 g KHCO₃

8.3 g NH₄Cl

37.2 mg EDTA-Na₂

Adjust volume to 1,000 ml with nano-pure H₂O and filter through a 0.2 µm filter

Store at room temperature

Acknowledgments

This work was supported by the Thousand Talents Plan Award of China 2015 (J.Z.), the Municipal Healthcare Joint-Innovation Major Project of Guangzhou (201604020011) (J.Z.) and the N.I.H. (USA) (PO1 AI060699, S.P.). The protocol described herein was based on the following manuscript: Zhao *et al.* *Immunity*. 2016 Jun 21; 44(6):1379-91.

References

1. Anderson, K. G., Sung, H., Skon, C. N., Lefrancois, L., Deisinger, A., Vezys, V. and Masopust, D. (2012). [Cutting edge: intravascular staining redefines lung CD8 T cell responses](#). *J Immunol* 189(6): 2702-2706.
2. Fett, C., Zhao, J. and Perlman, S. (2014). [Measurement of CD8 and CD4 T cell responses in mouse lungs](#). *Bio Protoc* 4(6).
3. Zhao, J., Zhao, J., Mangalam, A. K., Channappanavar, R., Fett, C., Meyerholz, D. K., Agnihothram, S., Baric, R. S., David, C. S. and Perlman, S. (2016). [Airway memory CD4⁺ T cells mediate protective immunity against emerging respiratory coronaviruses](#). *Immunity* 44(6): 1379-1391.