Detection of CD8\(^+\) T cell-mediated immune responses to bacterial infection in mice

Efficient activation of CD8\(^+\) T cells is critical for bacterial resistance and eradicating malignancy in the body. Here, we present a step-by-step protocol to use *Listeria monocytogenes* expressing OVA (LmOVA) to stimulate endogenous CD8\(^+\) T cells. We describe the steps for adoptive transfer of OT-I CD8\(^+\) T cells to CD45.1 mice and then detail the steps for detection of the antigen-specific CD8\(^+\) T cells in response to LmOVA.
Protocol
Detection of CD8\(^+\) T cell-mediated immune responses to bacterial infection in mice

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SUMMARY
Efficient activation of CD8\(^+\) T cells is critical for bacterial resistance and eradicating malignancy in the body. Here, we present a step-by-step protocol to use Listeria monocytogenes expressing OVA (LmOVA) to stimulate endogenous CD8\(^+\) T cells. We describe the steps for adoptive transfer of OT-I CD8\(^+\) T cells to CD45.1 mice and then detail the steps for detection of the antigen-specific CD8\(^+\) T cells in response to LmOVA.

For complete details on the use and execution of this protocol, please refer to Wu et al. (2021).

BEFORE YOU BEGIN
Preparation of animal maintenance and genotyping

© Timing: 60 min

1. Animal maintenance: The experimental mice are generally maintained under a 12/12 h light/dark cycle at 22°C–26°C and fed with sterile pellet food and water ad libitum. The animal facilities used in this study must be accredited by an Association for Assessment and Accreditation of Laboratory Animal Care International and Institutional Animal Care and Use Committee (IACUC). All of the mice should be maintained in pathogen-free facilities and used strictly in accordance with the protocols approved by the IACUC.

2. OT-I mice genotyping: Suggested primer sequences for genotyping of OT-I mice, the PCR reaction master mix and PCR cycling conditions are listed in the following:
   a. Primer sequences:
      OT-I mice genotyping primer forward: CAGCAGCAGGTGAGACAAAGT
      OT-I mice genotyping primer reverse: GGCTTTATAATTAGCTTGGTCC
   b. PCR reaction master mix:

| Reagent                          | Final concentration | Amount  |
|---------------------------------|---------------------|---------|
| 2X Taq Master Mix(Dye)          | n/a                 | 10 μL   |
| Template DNA                   | n/a                 | 1 μL    |
| 5’ OT-I genotyping primer (10 μm/L) | 0.5 μm/L           | 1 μL    |

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c. PCR cycling conditions:

| Steps               | Temperature | Time | Cycles |
|---------------------|-------------|------|--------|
| Initial Denaturation| 95°C        | 2 min| 1      |
| Denaturation        | 95°C        | 20 s | 30 cycles |
| Annealing           | 60°C        | 20 s |        |
| Extension           | 72°C        | 30 s |        |
| Final extension     | 72°C        | 5 min| 1      |
| Hold                | 4°C         | Forever|       |

3. CD45.1 mice genotyping: CD45.1 mice can be identified by flow cytometry analysis using anti-mouse CD45.1 antibody. We suggest to use clone A20 (Biolegend, Cat#110714), but other clones can be tried.

**Preparation of LmOVA culture, storage, counting**

 ($) Timing: 2 days

4. Culture of LmOVA: *L. monocytogenes* operation. All experiments involve the use of *L. monocytogenes* should be operated in a BSL-2 laboratory. Additionally, individuals performing this protocol require safety training on the proper handling of *L. monocytogenes*. Mice are recommended to be used at age 6–8 weeks.

   a. The LmOVA are plated on a brain heart infusion (BHI) agar plate (with 5 µg/mL erythromycin) and cultured at 37°C for 24 h.

   b. A single colony on the plate is then inoculated into BHI medium (with 5 µg/mL erythromycin) and cultured 12 h–16 h at 37°C for expansion.

5. Storage of LmOVA: 500 µL of LmOVA can be diluted with 500 µL of 50% sterilized glycerol and stored at -80°C.

6. Counting of LmOVA:

   a. A frozen aliquot of LmOVA is thawed at room temperature and spun at 3000 g for 10 min.

   b. 1 mL of LmOVA precipitate is then remixed with the same volume of 1 mL of brain heart infusion (BHI) medium, which has been warmed at 25°C.

   c. The mixture is further diluted to a new BHI medium at a ratio of 1:100, 1:1000, 1:10,000, 1:100,000.

   d. Subsequently, 100 µL of the mixture is taken and spread on BHI agar plates and grown at 37°C for 24 h.

   e. The number of colonies (n) on the BHI agar plate is calculated to obtain the colony forming units (CFU) of LmOVA on the agar plate. The concentration of LmOVA (Con) can be calculated by Con (CFU/mL) = n * 10^dilution factor/volume.

△ CRITICAL: As freezing leads to partial loss of activity of LmOVA, it would be more accurate to calculate the CFU of LmOVA after freezing. Also, do not store LmOVA at -80°C for more than 6 months (If the LmOVA has been stored for a long time, it is usually necessary to recover and amplify the LmOVA again). We recommend recalculating the CFU of LmOVA each time it is used to ensure accuracy.
## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| BV421 anti-MHC class I tetramer (Kb/OVA257-264) antibody | NIH Tetramer Core Facility | Cat#53999 |
| FITC anti-human/mouse Granzyme B Antibody | BioLegend | Cat#372206, RRID:AB_2687030 |
| PE anti-mouse TNF-α Antibody | BioLegend | Cat#506306, RRID:AB_315427 |
| APC anti-mouse IFN-γ Antibody | BioLegend | Cat#505810 |
| FITC anti-mouse CD8a Antibody | BioLegend | Cat#100804, RRID:AB_312765 |
| APC/Cyanine7 anti-mouse CD8a Antibody | BioLegend | Cat#155016 |
| PE anti-mouse/human CD44 Antibody | BioLegend | Cat#103008, RRID:AB_312959 |
| FITC anti-mouse CD62L Antibody | BioLegend | Cat#104406, RRID:AB_313093 |
| APC anti-mouse CD45.1 Antibody | BioLegend | Cat#1110714, RRID:AB_313503 |
| PE anti-mouse CD45.2 Antibody | BioLegend | Cat#111103, RRID:AB_2892273 |
| Biotin anti-mouse CD8a Antibody (53-6.7) | BioLegend | Cat#100704 |
| **Critical commercial assays** |        |            |
| Intracellular Fixation & Permeabilization Buffer Set | eBioscience | Cat#88-8824-00 |
| EasySep™ Mouse Biotin Positive Selection Kit | Stem Cell | Cat#18556 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| 1X Red Cell Lysis | Tiangen | Cat#RT122-02 |
| Ovalbumin | Sigma-Aldrich | Cat#A5503 |
| PBS | Sigma-Aldrich | Cat#D5652 |
| Brain-Heart Infusion (BHI) | ABOX | Cat#02-348 |
| Brefeldin A solution (1000x) | Thermo Fisher Scientific | Cat#00-4506-51 |
| Monensin solution (1000x) | Thermo Fisher Scientific | Cat#00-4505-51 |
| Ethylorcin | Sigma-Aldrich | Cat#E5389 |
| PMA | Sigma-Aldrich | Cat#P1585 |
| Ionomycin | Sigma-Aldrich | Cat#I3909 |
| Complete Freund’s Adjuvant (CFA) | Sigma-Aldrich | Cat#F5881 |
| Glycerol | Sigma-Aldrich | Cat#G5516 |
| Agarose G10 | Biowest | Cat#111860 |
| Fetal bovine serum(FBS) | Geminibio | Cat#900-108 |
| 2X Taq Master Mix(Dye) | CWBIO | Cat#CW6062M |
| RPMI 1640 medium | Macgene | Cat#CM10041 |
| Penicillin/Streptomycin | Thermo Fisher Scientific | Cat#15140122 |
| **Experimental models: Organisms/strains** |        |            |
| CD45.1 C57BL/6 mouse (6–8 weeks old, male) | The Jackson Laboratory | JAX:002014 |
| OT-1 C57BL/6 mouse (Tg(TcraTcrb1100Mjb) (CD45.2 background) (6–8 weeks old, male) | The Jackson Laboratory | JAX:003831 |
| CS7BL/6 mice (CD45.2 background) | Vital River Laboratory Animal Technology (Beijing, China) | N/A |
| L. monocytogenes expressing the full-length ovalbumin (LmOVA) | Generated by Dr. Zhongjun Dong lab | N/A |
| **Software and algorithms** |        |            |
| FlowJo v7.6 | BD | www.flowjo.com |
| GraphPad Prism v7.0 | GraphPad Software, Inc. USA | N/A |
| FACSDiva software Version 8.0 | BD | N/A |
| **Other** |        |            |
| EasyEights™ EasySep™ Magnet | STEMCELL Technologies | Cat#18103 |
| Cell strainer 70 μM | Falcon | Cat#352350 |
| 1mL Insulin Syringe (Ultra-Fine) | BD | N/A |

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**MATERIALS AND EQUIPMENT**

### Eythromycin stock

| Reagent              | Final concentration | Amount |
|----------------------|---------------------|--------|
| Eythromycin          | 50 mg/mL            | 50 mg  |
| Ethanol              | n/a                 | 1 mL   |
| **Total**            | n/a                 | 1 mL   |

**Note:** Store the erythromycin stock solution at −20°C. The stock solution can be stored at −20°C for 6 months.

### BHI medium

| Reagent              | Final concentration | Amount |
|----------------------|---------------------|--------|
| BHI powder           | 37 g/L              | 37 g   |
| ddH2O                | n/a                 | 1000 mL|
| Eythromycin stock (50 mg/mL) | 5 μg/mL | 100 µL |
| **Total**            | n/a                 | 1000 mL|

**Note:** The medium can be stored at 4°C for 1 month.

△ CRITICAL: Autoclave the medium for at least 15 minutes at 121°C on a slow exhaust cycle. When the medium is cooled to below 50°C, 100 µL of erythromycin solution (50 mg/mL) is added to 1000 mL BHI medium.

### BHI agar plate

| Reagent              | Final concentration | Amount |
|----------------------|---------------------|--------|
| BHI powder           | 37 g/L              | 37 g   |
| Agarose              | 15 g/L              | 15g    |
| ddH2O                | n/a                 | 1000 mL|
| Eythromycin stock (50 mg/mL) | 5 μg/mL | 100 µL |
| **Total**            | n/a                 | 1000 mL|

**Note:** BHI agar plates can be stored at 4°C for 1 month.
△CRITICAL: When the medium is cooled to approximately 50°C, add 100 μL of erythromycin (50 mg/mL) to 1000 mL of BHI agar solution and pour 10 mL of BHI agar solution into a 100 mm dish. BHI agar plates can be cooled and solidified at room temperature (25°C).

Flow cytometer
We use BD LSRFortessa SORP with 4 lasers including following configurations: 488 nm, 640 nm, 561 nm, 405 nm.

Alternatives: Other types of flow cytometers with different lasers can be selected for analysis. The laser depends on the fluorescence of the selected staining antibody.

STEP-BY-STEP METHOD DETAILS
Evaluation of endogenous antigen-specific CD8+ T cell production and expansion in vivo by LmOVA

© Timing: 7 days

Bacteria Listeria monocytogenes (L. monocytogenes) is a gram-positive intracellular pathogen whose clearance requires CD8+ T cell immunity. Recombinant L. monocytogenes expressing a full length of ovalbumin protein are named LmOVA. Through T cell receptor-specific recognition of ovalbumin peptide residues 257–264 (OVA257-264), LmOVA is able to induce the conversion of a fraction of CD8+ T cells into antigen-specific cells (Yang et al., 2016).

Here, mice are immunized with LmOVA for 7 days. Splenocytes are isolated and stained with antibody to detect the CD8+ T cells which specific response to OVA and the cytokine production. The Scheme of this experiment is shown in Figure 1A.

1. Preparation of LmOVA before mice immunization
   a. Spin down 5 × 10⁵ CFU of LmOVA bacteria (per mouse) at 3000 g for 10 min at 25°C.
   b. Wash LmOVA twice with 1 mL of phosphate-buffered saline (PBS) and centrifuge at 3000 g for 10 min at 25°C.
   c. Discard the supernatant.
   d. Resuspend the LmOVA precipitate with 100 μL PBS. Intravenous injection (i.v.) of LmOVA into mice (Figure 1B).

2. Obtain organs
   On day 7, use CO₂ for mice euthanasia and collect spleens and livers (in general, bigger spleens are observed if the corresponding mice are infected with LmOVA (Figure 1E). The surgical procedure to harvest the organs is as follows.
   a. Mice treatment with CO₂ for euthanasia.
   b. Mice are sprayed with 75% ethanol. This step will reduce the probability of the cells being contaminated by bacteria.
   c. Spleen collection (Figure 1C):
      i. Place the mouse on its side with one of its limbs facing outward.
      ii. Use forceps and surgical scissors to cut a small incision in the outer epidermis of the mouse, as shown in Figure 1C.
      iii. Use the new forceps and surgical scissors to make a small incision in the inner epidermis of the mouse.
      iv. Cut gastro-splenic ligament with surgical scissors and forceps to separate the spleen from the stomach.
      v. Put the spleen in a 6-well cell culture dish with 2 mL PBS for next step.
   d. Liver collection (Figure 1D):
      i. Place the mouse on ventral surface as shown in Figure 1D and secure limbs using needles.
Figure 1. Priming endogenous CD8+ T cells in vivo by tail vein injection of LmOVA

(A) Scheme of the experiment.
(B) Demonstration of LmOVA tail vein injection operation.
(C) The surgical procedure scheme for harvesting spleen.
(D) The surgical procedure scheme for harvesting liver.
(E) Spleens of PBS-treated (-) mice or LmOVA infected mice for 7 days. Top panel: Spleens of mice infected with LmOVA. Bottom panel: Spleens of mice treated with PBS (-).
ii. Use forceps and surgical scissors to cut abdominal outer epidermis of mouse.
iii. Use the new forceps and surgical scissors to make an incision in the diaphragm of the mouse.
iv. Carefully separate the liver from the diaphragm.
v. Put the liver in a 6-well cell culture dish with 2 mL PBS for next step.

3. Preparation of the splenocytes

**Timing:** 25 min

a. Place a 70 μm cell strainer on a 50 mL tube.
b. Put a mouse spleen on the 70-μm cell strainer and homogenize using the rubber end of a syringe with 2 mL PBS.
c. Flush and wash the strainer with another 2 mL PBS.
d. Spin down the spleen homogenate at 400 g at 4°C for 10 min.
e. Resuspend the precipitate with 1 mL Red Cell Lysis at 25°C for 5 min to eliminate red cells.
f. Add 9 mL PBS into the tube to end the red cell lysis process, and spin down splenocytes at 400 g for 10 min at 4°C.
g. Resuspend the cell precipitate using 1 mL PBS and put on ice for next step.

4. Antigen-specific CD8⁺ T cell staining

**Timing:** 50 min

Antigen-specific CD8⁺ T cell staining (MHC class I tetramer (Kb/OVA257-264) staining) using FITC anti-mouse CD8a Antibody, PE anti-mouse/human CD44 Antibody, and BV421 anti-MHC class I tetramer (Kb/OVA257-264) antibody.

a. Take 20 μL splenocytes into a 1.5 mL EP tube with 500 μL PBS and spin down at 1200 g for 5 min at 4°C.
b. Mix the staining antibodies with PBS+3% Fetal Bovine Serum(FBS). (Final concentration: BV421 anti-MHC class I tetramer (Kb/OVA257-264) antibody 2.5 μg/mL; PE anti-mouse/human CD44 Antibody 1 μg/mL; FITC anti-mouse CD8a Antibody 2.5 μg/mL).
c. Add 50 μL volume of antibody cocktail to each sample and keep on ice for 40 min avoid from light.
d. Wash the cells twice with 600 μL PBS.
e. Finally, resuspend the cell pellet with 400 μL PBS, which are ready for FACS assay.
f. Gate the OVA-TET⁺ CD8⁺ T cell group (Figure 2A).

5. OVA TET⁺ CD8⁺ T cell counting (Figure 2B).

**Timing:** 15 min

The total number of cells in the spleen (n) is counted using a Neubauer chamber or an automated cell counter (e.g., BIO-RAD TC20). And the number of OVA-TET⁺ CD8⁺ T cells (N) can be calculated as N=n*percentage of OVA-TET⁺ CD8⁺ T cells (cell counting can also be performed with FACS beads).

6. FACS analysis of cytokine production by endogenous antigen-specific CD8⁺ T cells

**Timing:** 20 h

Staining strategy: cells are stained with APC-Cy7 anti-mouse CD8a Antibody, APC anti-mouse IFN-γ Antibody and FITC anti-human/mouse Granzyme B Antibody accordingly.

a. Take 50 μL splenocyte suspension and put into a 48-well.
b. Add 500 μL 1640 medium containing 10% FBS and antibiotics (100 Units/mL penicillin and 100 μg/mL streptomycin), and stimulate the cells with 50 ng/mL PMA and 1 μM ionomycin for 4 h in the presence of Brefeldin A (3 μg/mL) and Monensin (2 μM/L).

c. Collect the cells and wash twice with 500 μL PBS buffer (1200 g, 5 min).

d. Stain the splenocytes with APC-Cy7 anti-mouse CD8a Antibody on ice for 40 min avoid from light.

e. Fix and permeabilize the cells using an Intrace llular Fixation & Permeabilization Buffer Set (eBioscience, Cat#88-8824-00), and the corresponding procedure is described below (Wu et al., 2021):

   i. Collect the cells and wash with 500 μL PBS buffer (1200 g, 5 min).
   ii. Prepare single cell suspension for each sample in a tube with 100 μL PBS .
   iii. Add 100 μL IC Fixing Buffer from the Intracellular Fixation & Permeabilization Buffer Set (eBioscience, Cat#88-8824-00) into each tube and mix gently. The mixture is then incubated at room temperature (25°C) for 20–30 min avoid exposure from light.
   iv. Add 800 μL 1 x Permeabilization Buffer (diluted from the 10 x Permeabilization Buffer with ddH2O) into each tube and centrifuge for 5 min at 700 g. Discard supernatant.
   v. Repeat the step “iv”.

f. Stain the cells with a fluorescent dye mixture (APC anti-mouse IFN-γ and FITC anti-human/ mouse Granzyme B Antibody diluted in 1 x Permeabilization Buffer at 1:200, 50 μL/per sample) at 4°C 12 h–16 h.

g. Wash the cells with PBS twice and resuspend them with 400 μL PBS for FACS analysis.

h. Gate the IFN-γ+ CD8+ T cells and Granzyme B+ CD8+ T cells using Flowjo software (Figure 3A).

i. Calculate the population and the number of IFN-γ+ CD8+ T cells or Granzyme B+ CD8+ T cells (Figure 3B).

△ CRITICAL: Membrane receptor(s) (e.g., CD8 and CD44) should be stained before fixation, because fixation may cause poor staining of a range of proteins on the membrane. All staining processes require light avoidance.
Figure 3. Cytokine staining of endogenous CD8+ T cells infected with LmOVA

Priming endogenous CD8+ T cells in vivo by tail vein injection of LmOVA (Figure 1A). 7 Days later, collect the splenocytes and restimulate them with PMA/Ionomycin and stain the cytokine.

(A) FACS gating strategy of cytokine producing CD8+ T cells.

(B) Production and number of IFN-γ+ or GzmB+ CD8+ T cells from mice treated with PBS (-) (with PMA/Ionomycin), or infected with LmOVA with or without PMA/Ionomycin as indicated. All data are the mean ± s.e.m.; n = 5 independent wells per experiment. Two-tailed Student’s t test; ***p < 0.001, ****p < 0.0001.
7. Measure *L. monocytogenes* titer in liver

**Timing:** 25 h

a. Weigh the isolated liver (g).
b. Place a 70-μm cell strainer on a 50 mL tube. Put a mouse liver on the 70-μm cell strainer with 5 mL PBS and homogenize using the rubber end of a syringe.
c. Flush and wash the strainer with another 5 mL PBS.
d. Plate 100 μL organ homogenates on BHI agar plates followed by cultured at 37°C for 24 h to determine the CFU of LmOVA on agar plates (n) (Figure 4).
e. The titer of *L. monocytogenes* in liver (T) can be calculated by T = n*100/g.

**Adoptive transfer of OT-I CD8⁺ T cells to CD45.1 mice followed by immunization with LmOVA**

We use an OT-I and CD45.1 mice adoptive transfer model to evaluate OT-I CD8⁺ T cell immune responses to LmOVA challenge. OT-I mice have transgenic inserts for mouse Tcra-V2 and Tcrb-V5 genes designed to recognize the OVA257–264 peptide. These mice are commonly used as a model immunological system for studying CD8⁺ T cell response to antigen (Clarke et al., 2000; Hogquist et al., 1994). CD45.1 mice are C57BL/6 congenic strain and widely used in adoptive transfer studies to track the donor and recipient cells. These mice carry the differential leukocyte marker commonly known as CD45.1, which are distinguish with C57BL/6(CD45.2) mice (Geiger et al., 2016).

Purified OT-1 CD8⁺ T cells are adoptively transferred to CD45.1 mice and then the recipient mice are infected with LmOVA. The Scheme of this experiment is shown in Figure 5.

8. OT-I CD8⁺ T cell isolation

**Timing:** 3 h

a. Collect spleens of OT-I transgenic mice aged 6–8 weeks.
b. Enrich CD8⁺ T cells by using EasySep™ Mouse Biotin Positive Selection Kit with EasySep Magnet (Other commercial isolation kits are available, e.g., Miltenyi biotec, Cat#130-104-075; and Biolegend, Cat#480035) as described below:
   i. Put a 70 μm-cell strainer on a 50 mL centrifuge tube.
   ii. Place the spleen on the 70 μm-cell strainer and add 2 mL PBS.
   iii. Use a syringe plunger to mash the spleen on the cell strainer and wash the strainer twice with 2 mL PBS.
iv. Collect the spleen homogenate by spinning at 400 g for 5 min at 4 °C. Discard the supernatant.

v. Resuspend cells with 1 mL 1× Red Cell Lysis and incubate at 25 °C for 5 min to eliminate red cells.

vi. Stop the process by adding 9 mL PBS, followed by centrifuge at 400 g for 5 min.

vii. Discard the supernatant.

viii. Resuspend the splenocytes with 300 mL PBS + 3% FBS. Add 5 μL Biotin-CD8a (0.5 mg/mL) antibody into the tube and incubate at 25 °C for 30 min.

ix. Add 20 μL Selection cocktail from the EasySep™ Mouse Biotin Positive Selection Kit (Stem Cell, Cat#18103) to the tube and incubate at 25 °C for 30 min.

x. Prepare the RapidSphere microbeads by Vortex.

xi. Add 10 μL of the microbeads from the EasySep™ Mouse Biotin Positive Selection Kit (Stem Cell, Cat#18103) into sample in the tube and incubate at 25 °C for 30 min.

xii. Transfer the mixture to a 5 mL Falcon tube and place the tube on EasyEights™ EasySep™ Magnet (Stem Cell, Cat#18103) for 15 min.

xiii. Pick up the magnet, and in one continuous motion invert the magnet and tube, pouring off the supernatant. Add PBS + 3% FBS to top up the sample to 2.5 mL. Mix by gently pipetting up and down 2–3 times, and incubate for 15 min.

xiv. Repeat “xi” twice.

xv. Pick up the magnet, and in one continuous motion invert the magnet and tube, pouring off the supernatant.

xvi. Resuspend the cells (OT-I CD8+ T cells) with a suitable volume of PBS.

Note: The purity of these cells can reach 90%–95%. Purity can be checked by FACS when using the isolation kit for the first time. In general, approximately 2–5×106 CD8+ T cells can be obtained from the spleen of a 6–8 week old male mouse.

△ CRITICAL: All procedures below are carried out in sterile conditions.

9. OT-I CD8+ T cell transfer

© Timing: 7 days

a. Transplant OT-I CD8+ T cells (CD45.2+; 5×10⁵ cells in 100 μL PBS/per mouse) into CD45.1+ mice by tail-vein injection.

b. The next day, intravenously inject 5×10⁵ CFU LmOVA into each CD45.1 mouse (Figure 5).

c. 7 days later, collect the CD45.1 mouse spleen.

d. Place a 70 μm-cell strainer on a 50 mL centrifuge tube. Put the spleen on the cell strainer and with 2 mL PBS.

e. Homogenize the spleen on the cell strainer using the rubber end of a syringe.
f. Flush and wash the strainer with another 2 mL PBS.
g. Spin down the spleen homogenate at 400 g at 4°C/C14°C for 10 min.
h. Discard the supernatant.
i. Lyse red cells in the suspension as we described previously.
j. Resuspend the supernatant with 1 mL PBS.
k. Stain the cells with FITC anti-mouse CD8a Antibody, APC anti-mouse CD45.1 Antibody and PE anti-mouse CD45.2 Antibody avoid exposure from light. The proportion and number of CD45.2+ CD8+ T cells can be calculated as described above (Figure 6).

10. Cytokine staining:

© Timing: 20 h

a. Take 50 μL splenocyte suspension and put into a 48-well plate.
b. Add 500 μL 1640 medium with 10% FBS and antibiotics (100 Units/mL penicillin and 100 μg/mL streptomycin) per well and stimulate the cells with 50 ng/mL PMA and 1 μM ionomycin for 4 h in the presence of Brefeldin A (3 μg/mL) and Monensin (2 μM/L).
c. Stain the cells with CD8a FITC anti-mouse CD8a Antibody.
d. Then, fix and permeabilize the cells using an Intracellular Fixation & Permeabilization Buffer Set (eBioscience, Cat#88-8824-00) as we described in step 6(e).
e. Stain the cells with a fluorescent dye mixture avoid exposure from light (APC anti-mouse IFN-γ and FITC anti-human/mouse Granzyme B Antibody diluted in 1× Permeabilization Buffer at 1:200, 50 μL/per sample) at 4°C at 12 h–16 h.
f. Wash the cells with PBS twice and resuspend them with 400 μL PBS for FACS analysis.
g. Gate the IFN-γ+CD8+ T cells and Granzyme B+CD8+ T cells using Flowjo software (Figure 7A).
Figure 7. Cytokine staining of CD45.2+ CD8+ T cells of adoptive transfer model
Purified OT-1 CD8+ cells are adoptively transferred to CD45.1 mice and then the recipient mice are infected with LmOVA (Figure 5). 7 Days later, collect the splenocytes and restimulate them with PMA/Ionomycin and stain the cytokine.
(A) FACS gating strategy of cytokine producing CD45.2+ CD8+ T cells.
h. Calculate the population and the number of IFN-γ+ CD8+ T cells or Granzyme B+ CD8+ T cells (Figure 7B).

EXPECTED OUTCOMES
In this protocol, we described two distinct procedures to immunize mice with LmOVA or PBS as a control to assessing the CD8+ T cell response to LmOVA bacterial infection. In the first section, by immunizing mice directly with LmOVA (Figure 1), we found a significant increase in the expansion of the endogenous antigen-specific CD8+ T cells (OVA-TET+CD8+CD44+ T cells) in spleens (Figure 2B). Moreover, a remarkable enhancement of production of cytokines including IFN-γ and Granzyme B can also be observed, indicating the activation of the CD8+ T cells in this model (Figure 3B).

Additionally, in the second section, we also provide a detailed procedure for an adoptive transfer model. If CD45.1 mice transplanted with exogenous OT-I (CD45.2) CD8+ T cells are further immunized with PBS or LmOVA as indicated in Figure 5, a strong elevation of OT-I (CD45.2) CD8+ T cell population in spleens can be expected upon LmOVA challenge (Figure 6B). And the remarkable enhancement of production of cytokines including IFN-γ and Granzyme B can also be observed (Figure 7B).

LIMITATIONS
One major limitation of this protocol concerns with the inconsistency of the effect of LmOVA treatment. LmOVA are usually stored in -80°C refrigerator for a long time, the time of freezing and fluctuations in refrigerator temperature can affect the activity of LmOVA. Moreover, the quality of LmOVA injected into mice is directly influenced by the operation of tail vein injection. Therefore, it is quite normal to see differences in the effect of LmOVA activation of CD8+ T cells in different time experiments. In order to address this issue, one needs to recalculate the CFU after each LmOVA recovery. The CFU needs to be re-measured at the time of each experiment, no matter how short the interval between the preceding and following experiments. Again, ensure consistency of each tail vein injection operation.

In addition, we feel it is important to emphasize that experimental waste needs to be disposed of properly. Although the LmOVA we use are attenuated, LmOVA are also somewhat pathogenic. All waste generated during these experiments must be collected in biohazard bags that can be autoclaved and disinfected before being sent to a biohazard waste treatment plant.

TROUBLESHOOTING
Problem 1
The red cells cannot be lysed sufficiently when prepare splenocyte in step 3(g) or step 8(xvi).

Potential solution
The grinding of spleen may affect the red cell lysis. Grind the spleen more adequately until there are no visible red tissue masses.

And increase the lysis time of erythrocytes if needed.

Problem 2
Low purity of isolated CD8+ T cells, or low number of isolated CD8+ T cells are harvested in step 8(xvi).

Potential solution
Increase the amount of the isolation cocktail and microbeads. Increase the microbeads wash times on EasySep Magnet.
Extend the incubation time of cocktails and microbeads.

In addition, other commercial isolation kits such as Miltenyi biotec (Cat# 130-104-075) or Biolegend (Cat# 480035) can be tried.

**Problem 3**
Intracellular cytokine staining is undetectable in step 6(h) or step 10(g).

**Potential solution**
Prolong the incubation time of Intracellular Fixation & Permeabilization Buffer solution to 1h.

Additionally, one can also use an BD CytoFix/CytoPerm kit (Cat#554722) as an alternative approach.

**Problem 4**
Tail vein injection is unsuccessful when immunized with LmOVA or transfer cells in step 1(d) or step 9(a).

**Potential solution**
We suggest to buy the mice tail vein viewable immobilizer described in key resources table to assist tail vein injection.

Alternatively, orbital intravenous injection can be tried instead of caudal intravenous injection.

**Problem 5**
FACS data are high noise and background in step 4(f) or 6(h) or 9(k) or 10(g).

**Potential solution**
High noise and background may be caused by spectral overlap between fluorophores or dead cell autofluorescence or non-specific antibody binding.

Control compensation can be performed with a new set of single-stained and unstained controls to adjust the voltage of FACS.

Use a fixable live/dead marker, such as Zombie Dyes (Biolegend, Cat# 423108), to exclude dead cells from the analysis.

Adjust the concentration of the antibody. Increase the blocking concentration of FBS when staining. Perhaps stain cells with PBS + 10% FBS. Increasing the blocking step of the Fc receptor during staining could also be tried.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for reagents and resources will be fulfilled by the lead contact, Peng Jiang (pengjiang@tsinghua.edu.cn).

**Technical contact**
Further information and technical question can contact the technical contact, Jun Wu, quinnwu017@163.com; Gen Li, genli0539@126.com.

**Materials availability**
This study did not generate new unique reagents.
Data and code availability
This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS
J.W. and G.L. performed all experiments and analyzed the data. D.L., J.Z., and Z.D. provided technical support. J.W., G.L., and P.J. wrote the paper. All authors approved the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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