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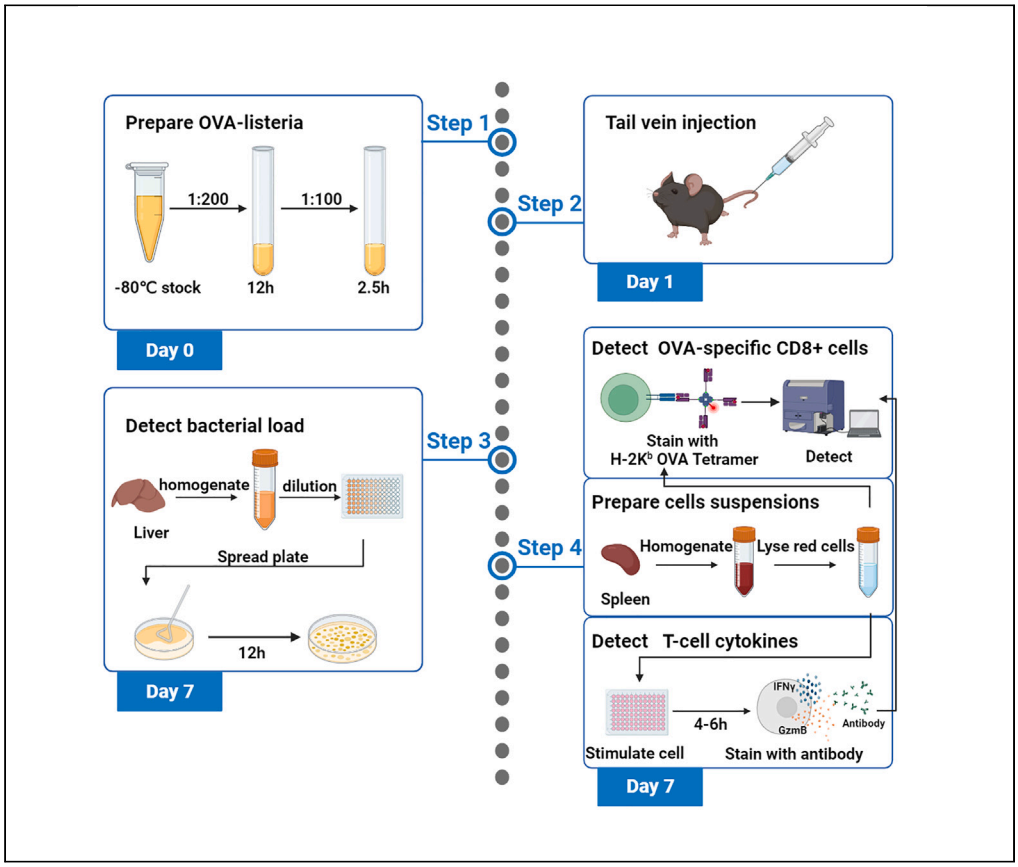
Protocol

Detection of the CD8$^+$ T cell immune response in mice infected with OVA-*Listeria monocytogenes*



T cells are able to recognize and kill pathogens that infect host cells, including bacteria, viruses, and tumor cells. Here, we present a protocol to detect T cell function and bacterial load in OVA-*Listeria monocytogenes*-infected mice. We provide a detailed description of the steps for detecting OVA-specific CD8$^+$ T cells and their cytokine expression levels in splenocytes using flow cytometry on day 7 after infecting mice with OVA-*Listeria monocytogenes*. Additionally, we describe the steps for detecting the OVA-*Listeria monocytogenes* load in the mouse liver.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Establish a mouse model of OVA-*Listeria monocytogenes* infection

Detect the OVA-*Listeria monocytogenes* load in the mouse liver

Flow cytometry analysis of OVA-specific CD8$^+$ T cells

Flow cytometry analysis of T cells cytokine levels

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Detection of the CD8$^+$ T cell immune response in mice infected with OVA-*Listeria monocytogenes*

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SUMMARY

T cells are able to recognize and kill pathogens that infect host cells, including bacteria, viruses, and tumor cells. Here, we present a protocol to detect T cell function and bacterial load in OVA-*Listeria monocytogenes*-infected mice. We provide a detailed description of the steps for detecting OVA-specific CD8$^+$ T cells and their cytokine expression levels in splenocytes using flow cytometry on day 7 after infecting mice with OVA-*Listeria monocytogenes*. Additionally, we describe the steps for detecting the OVA-*Listeria monocytogenes* load in the mouse liver.

For complete details on the use and execution of this protocol, please refer to Chen et al.[1]

BEFORE YOU BEGIN

Listeria monocytogenes is a gram-positive facultative intracellular bacterial pathogen that is responsible for listeriosis.[2] *Listeria monocytogenes* can survive, replicate and spread in host cells, posing a serious threat to human health. Wild-type mice clear infection within 7–10 days of *Listeria monocytogenes* infection, relying on expansion of antigen-specific CD8$^+$ T cells. Then, memory cells are built that are highly resistant to subsequent lethal attacks.[3]

The *Listeria monocytogenes* used in this protocol expresses OVA (OVA-*Listeria monocytogenes*). This protocol describes the step-by-step procedure for OVA-*Listeria monocytogenes* preparation, tail vein injection, bacterial load detection, OVA-specific CD8$^+$ T cell detection, and T cell cytokines detection. This method can be used to explore the effects of various genes on CD8$^+$ T-cell immune responses *in vivo*.

Experiments with mice require institutional permission. All animal experiments in this protocol were approved by and conducted under the supervision and evaluation of the Experimental Animal Ethics Committee of Jinan University (Approval NO. IACUC-20191120-06) (Guangdong Province, China).All the mice should be housed under specific-pathogen-free conditions and domesticated in an animal biosafety level 2 (ABSL-2) container at least 3 days before the experiments.

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The process of organ harvesting from mice, extraction and spreading of liver suspension needs to be performed in a sterile hood.

Prepare all reagents following the recipes in the materials and equipment section. Store them as directed.

Preparation one: Culture and preservation of OVA-*Listeria monocytogenes*

Timing: 12–14 h

Listeria monocytogenes (10403S) with engineered OVA was a gift from Dr. Hao Shen of the University of Pennsylvania and cultured in brain heart infusion (BHI) media with 5 μg/mL erythromycin. The entire operation of this bacteria was carried out in a biological safety cabinet in the BSL-2 laboratory.

1. Recovery, Culture and Preservation of OVA-*Listeria monocytogenes*.
   a. Melt the the frozen bacterial solution frozen at −80 °C at room temperature (20°C–25°C).
   b. Add 3 mL BHI medium and 1.5 μL of erythromycin into the sterile cryopreserved tube.
   c. Mix thoroughly by pipetting up and down.
   d. Add 30 μL bacterial solution.
   e. Incubate for 12 h in a shaker with a rotation speed of 220 rpm/min and a temperature of 37°C.
   f. Add 80% glycerol to the bacterial solution at a ratio of 1:1 (v/v).
   g. Mix thoroughly by pipetting up and down.
   h. Store at −80°C for long-term storage.

   Note: If the bacterial solution is stored at −80°C for more than 3 months, to ensure the activity and toxicity of the bacterial solution, fresh bacterial solution can be extracted from the liver tissue of infected mice.

Preparation two: Quantification of OVA-*Listeria monocytogenes*

Timing: 2 days

To obtain a more active bacterial solution, achieve a better infection effect, and establish a more stable mouse model, we explored the relationship between the shaking time, the OD value and the number of colonies.

2. Mesure the OD of the culture, and calculate the number of OVA-*Listeria monocytogenes*.
   a. Melt the the frozen bacterial solution frozen at −80 °C at room temperature (20°C–25°C).
   b. Add 2 mL BHI medium and 1 μL erythromycin into the sterile cryopreserved tube.
   c. Mix thoroughly by pipetting up and down.
   d. Add 10 μL bacterial solution. Incubate for 12 h in a shaker with a rotation speed of 220 rpm/min and a temperature of 37°C.
   e. Add 2 mL BHI medium containing 5 μg/mL erythromycin to 4 shaker tubes.
   f. Add 20 μL activated bacterial solution to each tube.
   g. Collect the samples at 1 h, 2 h, 2.5 h and 3.5 h.
   h. Detect the OD of the bacterial solution under the condition of absorbance at 600 nm.
   i. Perform 1:10 serial dilutions of bacterial solution in PBS to 10^{-8} dilution.
   j. Spread 100 μL of undiluted and each subsequent dilution onto BHI plates. Incubate plates at 37°C for 12 h.
   k. Count the number of colonies on each plate.
   l. Calculate the OVA-*Listeria monocytogenes* concentration of the culture at 1 h, 2 h, 2.5 h and 3.5 h.

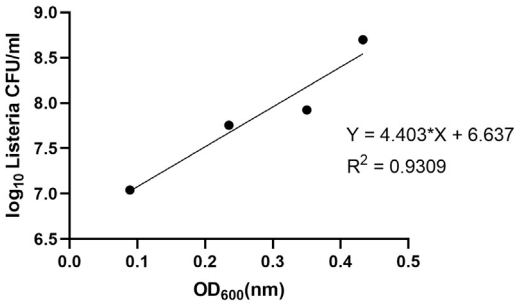

Figure 1. Standard curve of optical density (OD) versus bacterial number

The correlation between OD600 and bacterial number for OVA-*Listeria monocytogenes* was established by creating a standard curve. The OD of a BHI broth-grown OVA-*Listeria monocytogenes* culture at 37°C was measured using light of the indicated wavelengths (OD600 nm). The bacterial number was determined by performing serial dilutions and plating at various time points (1 h, 2 h, 2.5 h, and 3 h).

$$\text{Concentration (CFU / mL)} = \text{number of colonies} \times \text{dilution factor} / 0.1\ \text{mL}$$

m. Statistical relationship between CFU and optical density of OVA-*Listeria monocytogenes* (Figure 1).

⚠ CRITICAL: Before bacterial plating, the plate needs to be warmed and ensured that the plate is dry.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TruStain FcX PLUS (anti-mouse CD16/32) Antibody, clone S17011E, used as 1:200 dilution	BioLegend	Cat# 156604; RRID: AB_2783138
Anti-CD8 (Mouse) mAb-FITC, clone KT15, used as 1:160 dilution	MBL Life science	Code No. K0227-4
APC anti-mouse CD8a Antibody, clone 53–6.7, used as 1:80 dilution	BioLegend	Cat# 100712; RRID: AB_312751
Alexa Fluor 488 anti-mouse CD4 Antibody, clone GK1.5, used as 1:800 dilution	BioLegend	Cat# 100423; RRID: AB_389302
PE/Cyanine7 anti-mouse/human CD44 Antibody, clone IM7, used as 1:80 dilution	BioLegend	Cat# 103030; RRID: AB_830787
Brilliant Violet 510 anti-mouse/human CD44 Antibody, clone IM7, used as 1:40 dilution	BioLegend	Cat# 103044; RRID: AB_2650923
PE/Cy7 anti-mouse IFN-γ Antibody, clone XMG1.2, used as 1:20 dilution	BioLegend	Cat# 505826; RRID: AB_2295770
PE anti-human/mouse Granzyme B Recombinant Antibody, clone QA18A28, used as 1:20 dilution	BioLegend	Cat# 396406; RRID: AB_2801075
PerCP/Cyanine5.5 anti-mouse IL-2 Antibody, clone JES6-5H4, used as 1:20 dilution	BioLegend	Cat# 503822; RRID: AB_2123676
Brilliant Violet 421 anti-mouse TNF-α Antibody, clone MP6-XT22, used as 1:80 dilution	BioLegend	Cat# 506328; RRID: AB_2562902
Bacterial and virus strains		
OVA-*Listeria monocytogenes*	University of Pennsylvania	N/A
Chemicals, peptides, and recombinant proteins		
T-Select H-2Kb OVA Tetramer-SIINFEKL-PE	MBL Life science	Code No. TS-5001-1C
7-AAD Viability Staining Solution	BioLegend	Cat# 420404
Zombie NIR Fixable Viability Kit	BioLegend	Cat# 423105
Brain heart infusion (BHI)	Oxoid	Cat# CM1135
Agar	Biosharp	Cat# BS195-1k
Cell Activation Cocktail (with Brefeldin A)	BioLegend	Cat# 423304

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
10× Intracellular Staining Perm Wash Buffer	BioLegend	Cat# 421002
Fixation buffer	BioLegend	Cat# 420801
RPMI medium 1640	Gibco	Cat# C11875500BT
PBS	Gibco	Cat# C10010500BT
0.5 M EDTA solution	Thermo Scientific	Cat# 1861274
NH_4Cl	Macklin	Cat# A801304-500g
$KHCO_3$	Macklin	Cat# P816192-500g
Glycerol	Macklin	Cat# G810575-500mL
Erythromycin	Macklin	Cat# E808820-1g
Ethanol anhydrous	Macklin	Cat# E809056-500mL
Experimental models: Organisms/strains		
Mouse: C57BL/6N mice (wild-type, female, 8 weeks)	Cyagen	N/A
Software and algorithms		
FlowJo software version 10.7	FlowJo LLC	https://www.flowjo.com/
Prism version 8	GraphPad	https://www.graphpad.com/l
Other		
Insulin syringes	Kindly Group	Cat# U-40
Test tube	Biosharp	Cat# BS-PPT-12-S
Cell spreader	Nest	Cat# 711001
Aseptic homogenizing bag	BKMAN	Cat# B-CYD1211
Petri dishes	Biosharp	Cat# BS-90-D
1.5-mL EP tube	Axygen	Cat# MCT-150-C
15-mL centrifuge tube	Corning	Cat# 430791
50-mL centrifuge tube	Corning	Cat# 430291
6-well cell culture plate	Costar	Cat# 3599
Cell strainer 40 μm	Biologix	Cat# 15-1040
Cell strainer 70 μm	Biologix	Cat# 15-1070
Tail Vein Injection Holder Platform	Yuyan Instruments	Cat# YAN-Q9G
Eppendorf centrifuge 5418 R	Eppendorf	Cat# 5401000137
Sigma refrigerated centrifuge	Sigma	Cat# 4407
Absorbance microplate reader	BioTek	Cat# EPOCH2T
Millipore Q3 Ultrapure water system	Millipore	Cat# Direct-Q 3V
EXFLOW flow cytometer	Dakewe	Cat# EXFLOW-206
Cytek Aurora	Cytek	3 Laser 16V-14B-8R
Forma Steri-Cycle CO2 Incubator	Thermo Scientific	Cat# 371
Cell culture microscope	Olympus LS	Cat# CKX53
Counting chambers with V-slash	Marienfeld	Cat# 0650030

MATERIALS AND EQUIPMENT

Red blood cell lysis buffer		
Reagent	Final concentration	Amount
NH_4Cl	155 mM	4.145 g
$KHCO_3$	10 mM	0.5 g
EDTA(0.5 M)	0.1 mM	100 μL
ddH_2O	N/A	500 mL
Total	**N/A**	**500 mL**
Adjust the pH to 7.3 with a pH meter, store at 2°C–8°C for one year.		

- BHI medium: Add 37.5 g BHI power in 1 L ddH2O, sterilize at 121°C for 20 min, keep sterile, store at 2°C–8°C for three months.

- BHI plate: Add 37.5 g BHI power and 15 g agar in 1 L ddH2O, sterilize at 121°C for 20 min. Then, add 0.5 mL erythromycin solution and pour the mixture into petri dishes. Store at 2°C–8°C for one month.
- Erythromycin solution: Dissolve 100 mg erythromycin in 10 mL absolute ethanol, store at −20°C for six months.
- 80% (v/v) glycerol solution: Add 80 mL glycerol in 20 mL ddH2O, sterilize at 121°C for 20 min, store at 2°C–8°C for one year.
- 0.2% BSA PBS: Add 1 g BSA in 500 mL PBS, keep sterile, store on ice or at 2°C–8°C.

STEP-BY-STEP METHOD DETAILS

Part one: Preparation of OVA-*Listeria monocytogenes* and tail vein injection in mice

Timing: 16–18 h

This section details how to prepare OVA-*Listeria monocytogenes* and perform tail vein injection.

Note: The previous data determined the relationship between time, OD600 and bacterial concentration. After 12 h of activation, the *Listeria monocytogenes* was transferred. The OD600 was measured to be 0.35 after 2.5 h of transfer, and the concentration of *Listeria monocytogenes* in the bacterial solution was 8.4×10^7 CFU/mL. The bacterial injection per mouse was 1.25×10^7 CFU. A total of 150 μL of bacterial solution with OD600 = 3.5 was required for each mouse.

1. Preparation of OVA-*Listeria monocytogenes*.
   a. Add 10 μL bacterial solution (frozen at −80°C) to 2 mL BHI medium containing erythromycin. Incubate in a shaker at 220 rpm/min for 12 h at 37°C.
   b. Add 20 μL activated bacterial solution to 2 mL BHI medium containing erythromycin. Incubate in a shaker at 220 rpm/min for 2.5 h at 37°C.

   Note: If more mice need to be injected, the culture system can be scaled up. The culture volume used here was 2 mL.

   ⚠ CRITICAL: After 2.5 h of bacterial transfer culture, the logarithmic growth phase was reached, and the growth rate of *Listeria monocytogenes* was very fast. The incubation time needs to be strictly controlled. It should be stopped immediately after 2.5 h.

   c. Collect the bacterial solution into 1.5 EP tubes, centrifuge at 2100 × *g* for 10 min at 4°C, discard the supernatant.
   d. Add 1 mL of PBS to resuspend *Listeria monocytogenes*, centrifuge at 2100 × *g* for 10 min at 4°C, discard the supernatant.
   e. Repeat step d. three times.
   f. Resuspend the bacterial solution in PBS to a concentration of 1.25×10^8 CFU/mL.
2. Tail vein injection.
   a. Immobilization of the mouse with a mouse immobilizer.
   b. Wipe the mouse tail with an alcohol swab to make the veins more visible.
   c. Inject 100 μL of bacterial suspension into mice from the tail vein with a syringe.

   ⚠ CRITICAL: When injecting into the tail vein of mice, be careful not to make mistakes and apply pressure to the wound with cotton after withdrawing the syringe to avoid fluid leakage.

   d. Keep the mice with free access to water and food after the injection of OVA-*Listeria monocytogenes*. Observe the mouse's status every day.

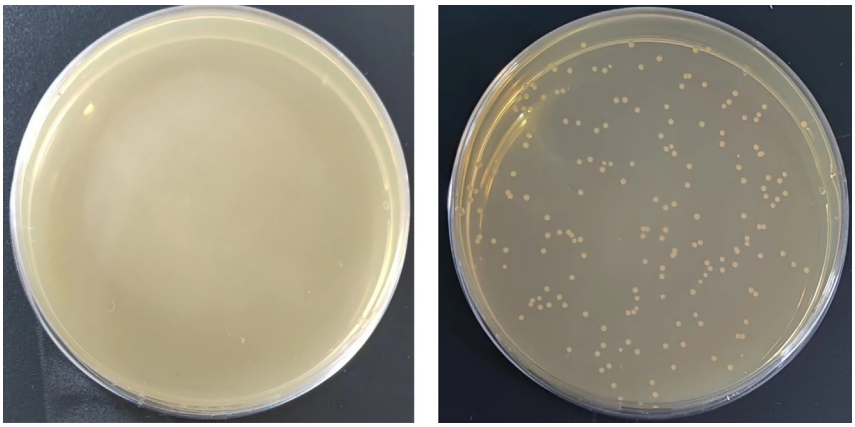

Figure 2. Liver homogenate from OVA-*Listeria monocytogenes*-infected and uninfected mice cultured on BHI plates

Livers were collected from OVA-*Listeria monocytogenes*-infected and uninfected mice at 7 days post-infection. Tissue homogenate was prepared and spread on plates. The plates were then incubated at 37°C 12 h. Plate spread of liver homogenate from OVA-*Listeria monocytogenes*-uninfected mice (left) and infected mice (right).

Part two: Detection of bacterial load in the liver (on day 7 after infection)

Timing: 3–5 h

This section details the detection of mouse liver bacterial load. Includes liver processing, extraction, and quantification of OVA-*Listeria monocytogenes*.

Note: This experimental result reflects the ability of $CD8^+$ T cells to clear OVA-*Listeria monocytogenes* in mice.

3. Prepare BHI plates in advance, preheat and dry them in a 37°C incubator for 1 h.
4. Preparation of Liver Tissue Grinding Suspension.
   a. Prepare and label homogenization bags.
   b. Remove and weigh the whole livers from mice. Place the whole liver into a homogenization bag.
   c. Seal the homogenization bag and use a cylindrical tool to roll the surface of the homogenization bag to thoroughly homogenize the liver.
   d. Add 5 mL of PBS.
   e. Filter through a 70 μm sieve.
5. Diluted liver suspension.
   a. Transfer 200 μL of liver suspension to a 96-well plate.
   b. Perform 1:10 serial dilutions of liver suspension in PBS to 10^{-5} dilution within the wells of the 96-well plate.
6. Spread Liver Suspension.
   a. Spread 100 μL of each dilution onto a predried BHI plate using a spreader. Incubate plates at 37°C for 12 h.
   b. Count the number of CFU for each dilution, and calculate the CFU per tissue (Figure 2).

$$\text{CFU / tissue} = (\text{CFU / 0.1 mL} \times \text{dilution factor} \times 5\ \text{mL}) \text{ /liver weight}$$

⚠ CRITICAL: Be careful to grind the liver thoroughly and mix well before diluting the liver suspension.

⚠ CRITICAL: Make sure the BHI plate is dry before coating it. Additionally, ensure that it is completely dry before finishing the coating process. Two control samples need to be set up. The first control is the coating plate of liver suspension from uninjected mice to rule out non-specificity and cross-contamination. The second control is the coating plate of the serially diluted suspension of known OVA-*Listeria monocytogenes* quantity to ensure the quality control of the agar plate.

Part three: Detection of OVA-specific $CD8^+$ T cells in the spleen (on day 7 after infection)

Timing: 4–6 h

This section details the detection of OVA-specific $CD8^+$ T-cell immune responses in mice.

7. Prepare mouse splenocytes.
   a. Remove the whole spleens from mice.
   b. Cut the whole spleen in half with scissors and place on a 40 μm sieve.
   c. House the sieve in a 50 mL centrifuge tube. Wet the mesh and tissue with 0.2% BSA PBS.
   d. Grind the tissue gently to dissociate cells with the core of a 2.5 mL syringe. Add 0.2% BSA PBS buffer while grinding.
   e. Centrifugation at 400 × *g* for 5 min at room temperature (20°C–25°C), and discard the supernatant.
8. Lyse red blood cells.
   a. Add 2 mL erythrocyte lysate and gently resuspend the cells by pipetting up and down. Let stand at room temperature (20°C–25°C) for 2 min.
   b. Add 8 mL 0.2% BSA PBS buffer. Centrifugation at 400 × *g* for 5 min at room temperature (20°C–25°C), and discard the supernatant.
   c. Add 8 mL 0.2% BSA PBS buffer, repeat centrifugation as in step b.
   d. Discard the supernatant and add 2 mL 0.2% BSA PBS buffer to resuspend the cells.
9. Cell count.
   a. Count viable cells and adjust to 1×10^7 cells/mL with 0.2% BSA PBS buffer.
   b. Distribute the cell suspension into 1.5 mL microcentrifuge tubes with 100 μL/tube (1×10^6 cells/tube).
10. Block Fc receptors.

Note: Fc receptors bind antibody-antigen immune complexes and mediate adaptive immune responses. TruStain FcX PLUS is specific to the common epitope of CD16/CD32. It is useful for blocking nonspecific binding of immunoglobulin to Fc receptors.

   a. Add 0.5 μL TruStain FcX PLUS (anti-mouse CD16/32) Antibody to each sample tube.
   b. Mix well by vortexing. Place on ice for 10 min.
11. Cell surface staining with PE H-$2K^b$ OVA tetramer.
   a. Add 10 μL H-2Kb OVA Tetramer-PE to each test tube and mixed well.
   b. Incubate for 1 h at room temperature (20°C–25°C) in the dark.
12. Cell surface staining with FITC anti-mouse CD8 and PE/Cy7 anti-mouse CD44 antibodies.

Note: CD44 is a marker of T-cell activation.[4] Colabeling of mouse spleen cells with CD8 antibody and CD44 antibody enables precise analysis of OVA-specific $CD8^+$ T cells.

   a. Add 0.625 μL FITC anti-mouse CD8 antibody and 1.25 μL PE/Cy7 anti-mouse CD44 antibody to each test tube and mixed well. Incubate for 20 min on ice in the dark.
   b. Add 1.5 mL 0.2% BSA PBS buffer, and centrifuge at 400 × *g* for 5 min. Discard the supernatant carefully.
   c. Repeat step b twice.

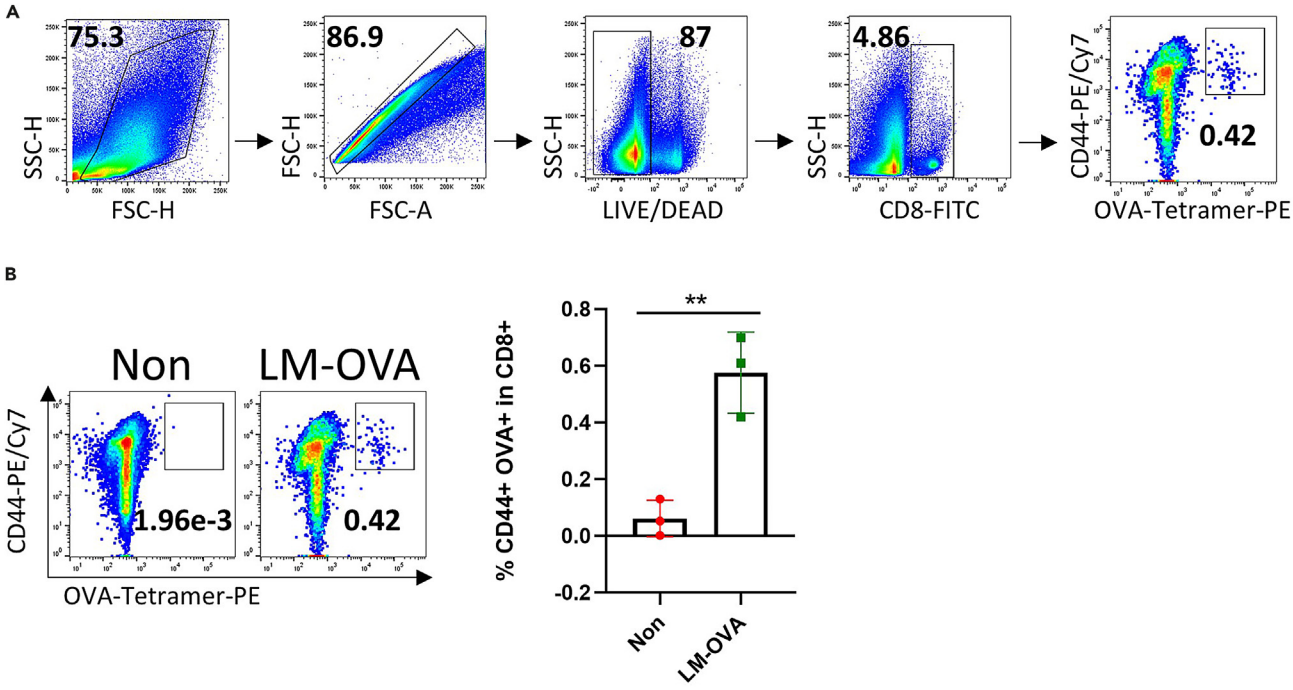

Figure 3. OVA-specific CD8$^+$ T cells from the spleen before and after OVA-*Listeria monocytogenes* infection

Spleens were harvested from OVA-*Listeria monocytogenes*-infected and uninfected mice at 7 days post-infection. A single-cell suspension was prepared and stained with anti-mouse CD8-FITC, CD44-PE/Cy7 antibody and OVA-Tetramer-PE.

(A) Gating strategy for OVA-specific CD8$^+$ T cells in mouse spleens.

(B) Representative flow cytometry plots (left) and percentages (right) of OVA-specific CD8$^+$ T cells from the spleen before and after OVA-*Listeria monocytogenes* infection. Data are representative of three independent experiments (B). Unpaired t test for the measurements between the two groups(B):**$p < 0.01$.

d. Suspend the cells in 500 μL of 0.2% BSA PBS buffer.
e. Resuspend cell pellet in 0.5 mL of 0.2% BSA PBS buffer and add 5 μL of 7-AAD per million cells and incubate for 5–10 min in the dark before analysis.
f. Perform flow cytometric analysis. Representative data and a gating strategy are shown in (Figure 3).

Note: If the samples cannot be analyzed in time after processing, they can be stored at 4°C–8°C degrees in the dark but should not exceed 4 h.

⚠ CRITICAL: Mouse cells are fragile, so dissociating mouse spleen cells needs to be gentle and always moist.

⚠ CRITICAL: When lysing mouse spleen erythrocytes with erythrocyte lysing solution, it is important to strictly control the time and not exceed the recommended duration. This is to avoid any potential damage to other cells present in the sample.

Part four: Detection of functional subsets of spleen T cells (on day 7 after infection)

Timing: 8–10 h

This section describes the detection of functional T-cell immune responses in mice. Here, we assessed the function of T cells by detecting the expression of intracellular IFNγ, GzmB, TNFα and IL2.

Note: The sample preparation steps are the same as steps 7, 8, and 9 in Part three.

13. Prepare mouse splenocytes.

    The operation is the same as Step 7 in Part three.

14. Lyse red blood cells.

    The operation is the same as Step 8 in Part three.

15. Cell count

    The operation is the same as Step 9 in Part three.

16. Cell Activation
    a. Aspirate a total of 1×10^6 cells and centrifuge at 400 × *g* for 5 min at room temperature (20°C–25°C) to remove the supernatant.
    b. Add 200 μL of 1640 medium to resuspend the cells, and then add them to 96-well plates.
    c. Add 0.4 μL of Cell Activation Cocktail (with Brefeldin A) to each well.
    d. The cells were incubated at 37°C in a CO_2 incubator for 4 h.

⚠ CRITICAL: The Cell Activation Cocktail stimulates cells for between 4–6 h. Do not exceed 6 h.

17. Block Fc receptors.
    a. Harvest the activated cells and centrifuge them at 400 × *g* for 5 min. Discard the supernatant.
    b. Add 1.5 mL of PBS buffer to the cell pellet, mix by vortexing or pipetting.
    c. Centrifuge the harvested activated cells at 400 × *g* for 5 min. Discard the supernatant.
    d. Resuspend cells in diluted 100 μL Zombie NIR solution.
    e. Incubate the cells at room temperature (20°C–25°C), in the dark, for 30 min.
    f. Add 1.5 mL of 0.2% BSA PBS buffer to the cell pellet, mix by vortexing or pipetting.
    g. Centrifuge the sample at 400 × *g* for 5 min. Discard the supernatant.
    h. Repeat step f and g.
    i. Resuspend cells in 100 μL of 0.2% BSA PBS buffer.
    j. Add 0.5 μL TruStain FcX PLUS (anti-mouse CD16/32) Antibody to each sample tube and mix well. Incubate on ice for 10 min.
18. Cell surface staining with APC anti-mouse CD8, Alexa Fluor 488 anti-mouse CD4 and BV510 anti-mouse CD44 antibodies.
    a. Add 1.25 μL of APC anti-mouse CD8, 0.12 μL of Alexa Fluor 488 anti-mouse CD4 and 2.5 μL of BV510 anti-mouse CD44 antibody to each sample tube. Mix well. Incubate on ice for 20 min in the dark.
    b. Add 1 mL 0.2% BSA PBS buffer and centrifuge at 400 × *g* for 5 min at 4°C.
    c. Discard the supernatant carefully. Vortex the tube and resuspend the cell pellet with the residual liquid.

⚠ CRITICAL: When staining with Zombie NIR, it is important to ensure that the PBS used does not contain any other proteins such as BSA or FBS.

⚠ CRITICAL: Please vortex thoroughly to ensure uniform mixing of the cells and to prevent uneven fixation in later stages.

19. Intracellular staining with PE/Cy7 anti-mouse IFNγ, PE anti-mouse GzmB, BV421 anti-mouse TNFα, PerCP/Cy5.5 anti-mouse IL2.
    a. Dilute 10× Intracellular Staining Perm Wash Buffer to 1× in DI water.

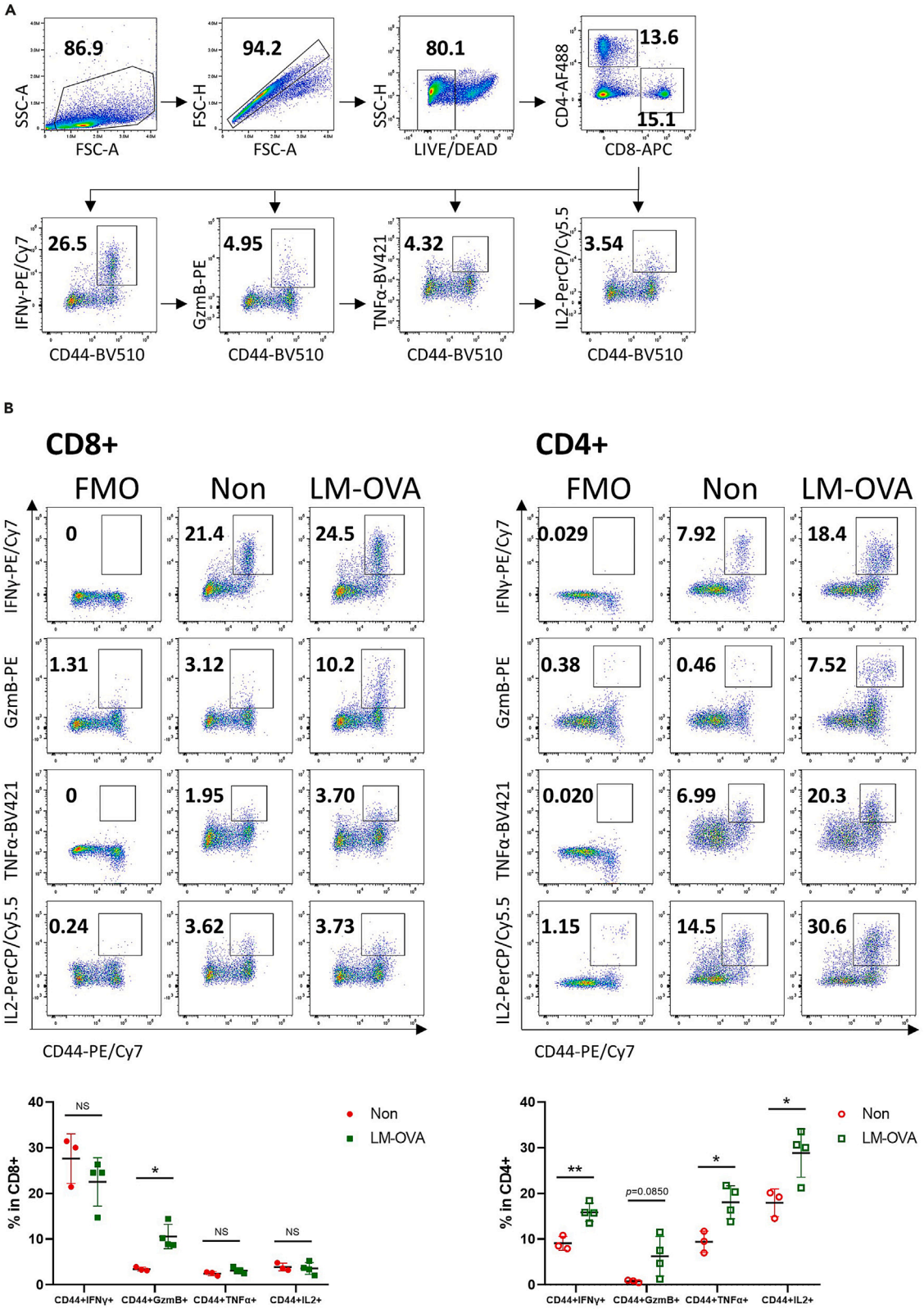
A
86.9
SSC-A
FSC-A
94.2
FSC-H
FSC-A
80.1
SSC-H
LIVE/DEAD
13.6
15.1
CD4-AF488
CD8-APC
26.5
IFNγ-PE/Cy7
CD44-BV510
4.95
GzmB-PE
CD44-BV510
4.32
TNFα-BV421
CD44-BV510
3.54
IL2-PerCP/Cy5.5
CD44-BV510
B
CD8+
FMO
Non
LM-OVA
0
21.4
24.5
1.31
3.12
10.2
0
1.95
3.70
0.24
3.62
3.73
IFNγ-PE/Cy7
GzmB-PE
TNFα-BV421
IL2-PerCP/Cy5.5
CD44-PE/Cy7
CD4+
FMO
Non
LM-OVA
0.029
7.92
18.4
0.38
0.46
7.52
0.020
6.99
20.3
1.15
14.5
30.6
IFNγ-PE/Cy7
GzmB-PE
TNFα-BV421
IL2-PerCP/Cy5.5
CD44-PE/Cy7
% in CD8+
40
30
20
10
0
NS
*
NS
NS
Non
LM-OVA
CD44+IFNγ+
CD44+GzmB+
CD44+TNFα+
CD44+IL2+
% in CD4+
40
30
20
10
0
**
p=0.0850
*
*
Non
LM-OVA
CD44+IFNγ+
CD44+GzmB+
CD44+TNFα+
CD44+IL2+

Figure 4. Plot of Ifn-γ, Granzyme-B (Gzmb), Tnfα and Il2 in T cells from OVA-*Listeria monocytogenes*-infected and uninfected mice

Spleens were harvested from OVA-*Listeria monocytogenes*-infected and uninfected mice at 7 days post-infection. A single-cell suspension was prepared, stimulated with Cell Activation Cocktail (with Brefeldin A) for 4 h and stained with anti-mouse CD8-APC, CD4- Alexa Fluor 488, CD44-BV510, IFNγ-PE/Cy7, Gzmb-PE, TNFα-BV421 and IL2-PerCP/Cy5.5 antibodies.

(A) Gating strategy for IFNγ, Gzmb, TNFα and IL2 positive $CD8^+$ T or $CD4^+$ cells.

(B) Representative flow cytometry plots and percentages of IFN-γ, Gzmb, TNFα and IL2 in $CD8^+$ T or $CD4^+$ cells from OVA-*Listeria monocytogenes*-infected and uninfected mice. Data are representative of three independent experiments (B). Unpaired t test for the measurements between the two groups (B):*$p < 0.05$; **$p < 0.01$.

b. Fix the cells in 0.5 mL/tube fixation buffer in the dark for 20 min at room temperature (20°C–25°C).
c. Centrifuge the sample at 400 × *g* for 5 min at room temperature (20°C–25°C). Discard the supernatant.
d. Resuspend the fixed cells in Intracellular Staining Perm Wash Buffer and centrifuge at 400 × *g* for 5 min at room temperature (20°C–25°C).
e. Repeat step d twice.
f. Resuspend fixed/permeabilized cells in 100 μL of intracellular staining Perm wash buffer.
g. Add 5 μL of PE/Cy7 anti-mouse IFN-γ, 5 μL of PE anti-mouse GzmB, 1.25 μL of BV421 anti-mouse TNFα and 5 μL of PerCP/Cy5.5 anti-mouse IL2 to each sample tube and mixed well. Incubate for 20 min in the dark at room temperature (20°C–25°C).
h. Wash twice with 1 mL of Intracellular Staining Perm Wash Buffer and centrifuge at 400 × *g* for 5 min at room temperature (20°C–25°C).
i. Resuspend fixed and intracellularly labeled cells in 400 μL 0.2% BSA PBS buffer.
j. Analyze prepared samples using flow cytometry. Representative data and a gating strategy are shown in (Figure 4).

EXPECTED OUTCOMES

T cells play a critical role in clearing *Listeria monocytogenes in vivo*. In this protocol, we induced infection in mice by injecting OVA-*Listeria monocytogenes*. We quantified *Listeria monocytogenes* levels by plating liver tissue samples from both uninjected and OVA-*Listeria monocytogenes*-injected mice. Additionally, we assessed the proportion and number of OVA-specific $CD8^+$ T cells, as well as the expression of cytokines, in response to OVA-*Listeria monocytogenes* infection. We observed an increase in OVA-specific $CD8^+$ T cells in the spleen following OVA-*Listeria monocytogenes* infection (Figure 3). Furthermore, the expression of GzmB, which is responsible for eradicating OVA-*Listeria monocytogenes*, was significantly elevated in $CD8^+$ T cells. However, there were no significant differences in the expression of IFNγ, TNFα, and IL2 (Figure 4). In parallel, OVA-*Listeria monocytogenes* stimulation resulted in increased cytokine secretion by $CD4^+$ T cells, including IFNγ, GzmB, TNFα, and IL2 (Figure 4). These findings indicate that $CD8^+$ T cells primarily eliminate target cells through GzmB, with assistance from CD4+ T cells, following OVA-*Listeria monocytogenes* infection in mice. This model provides a valuable tool for studying the immune response of $CD8^+$ T cells involving various genes.

LIMITATIONS

The main limitation of this protocol is that mice should be kept in the SPF-level environment before injection of listiria to avoid contact with external pathogens. Otherwise, they may become infected by germs, which can affect the reliability and repeatability of the experimental results.

TROUBLESHOOTING

Problem 1

The effect of different doses of *Listeria monocytogenes* on mice varies. A dose that is too low cannot elicit a strong immune response, while a dose that is too high can cause animal death (Step 1).

Potential solution

Please conduct a preliminary experiment before the formal experiment to confirm the minimum lethal dose.

Problem 2

Different generations and freezing times will affect the viability of bacteria (Step 1).

Potential solution

The sublethal dose of *Listeria monocytogenes* to mice should be determined before the experiment begins. The relationship between the OD value and CFU should be redetermined for different batches of bacteria.

Problem 3

When there are too many clones after liver cell suspension is plated, it can be difficult to quantify, while too few clones can lead to inaccurate quantification (Step 6).

Potential solution

Please perform gradient dilution of the liver cell suspension before plating and select plates with a range of 30–300 clones for counting and quantification.

Problem 4

After performing red blood cell lysis on mouse spleen samples, there may be some larger substances that cannot be dispersed and can easily clog the instrument when analyzing the samples using a flow cytometer (Step 8).

Potential solution

It is necessary to pass the samples through a 40 μm mesh sieve again before analyzing them with a flow cytometer.

Problem 5

The proportion of OVA-specific $CD8^+$ T cells in mice is very low (Step 12f).

Potential solution

A sufficient number of cells were taken for antibody incubation, and at least 600,000 cells were collected when testing on the machine.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and regents should be directed to and will be fulfilled by the lead contact, Guobing Chen (guobingchen@jnu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

No new code or data were generated as part of this study.

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AUTHOR CONTRIBUTIONS

G.B.C. conceived and wrote the protocol. Y.D.X., Z.J.W., and W.G. performed the experiments. H.Y.W., Z.X.W., and C.X.T. assisted with experiments. Y.D.X. and G.B.C. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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