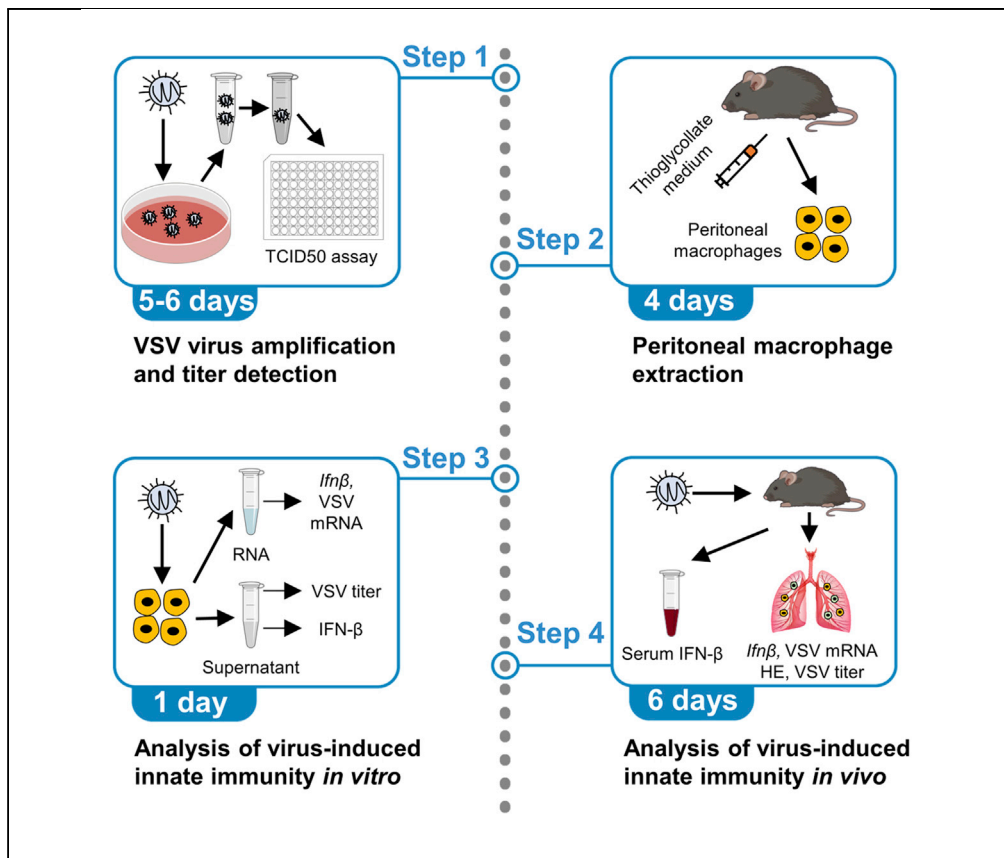


Protocol

In vitro and *in vivo* evaluation of virus-induced innate immunity in mouse



Innate immunity is the first line of host defense against viral infection. As one of the innate immune cell types, antigen-presenting cells play an important role in the process of antiviral immunity. This protocol describes the analysis of innate immunity induced by vesicular stomatitis virus infection of peritoneal macrophages *in vitro* and *in vivo* detection of IFN-β production and lung injury.

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Highlights

Detailed protocol for VSV virus amplification and titer detection

Fast and efficient collection of peritoneal macrophages from mice

Analysis of *in vitro* innate immunity induced by VSV infection of peritoneal macrophages

In vivo system to model VSV infection induced IFN-β production and lung injury

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Protocol

In vitro and *in vivo* evaluation of virus-induced innate immunity in mouse

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SUMMARY

Innate immunity is the first line of host defense against viral infection. As one of the innate immune cell types, antigen-presenting cells play an important role in the process of antiviral immunity. This protocol describes the analysis of innate immunity induced by vesicular stomatitis virus infection of peritoneal macrophages *in vitro* and *in vivo* detection of IFN- β production and lung injury. For complete details on the use and execution of this protocol, please refer to Shen et al. (2021).

BEFORE YOU BEGIN

Prepare mice

Note: Age- and sex-matched 6–12-week-old mice were used to perform the same experiment. Mice should be available in sufficient numbers before all experiments.

Mice used in this protocol can be fed ad libitum with a standard chow diet and water.

Prepare reagents and cells

⌚ Timing: 1–2 h

1. Make sure all reagents mentioned under [materials and equipment](#) are available.
2. Make sure the cultured Vero cells are in good condition before virus amplification and TCID50 assay.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
VSV	Dr. Hui Zheng	N/A

(Continued on next page)



Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
TRIzol	Life Technologies	Cat #15596018
All-in-One cDNA Synthesis SuperMix	Bimake	Cat #B24408
2× SYBR Green qPCR Master Mix	Bimake	Cat #B21203
Red Blood Cell Lysis Buffer	Solarbio	Cat #R1010
Penicillin streptomycin	Gibco	Cat #15140-122
DMEM (high glucose, pyruvate)	Gibco	Cat #11995
Fetal bovine serum	Gibco	Cat #10099158
0.05% Trypsin-EDTA	Gibco	Cat #25300-054
Thioglycollate Medium	BD	Cat #225650
Crystal violet stain solution, 0.1%	Solarbio	Cat #G1063
Paraformaldehyde, 4%	Solarbio	Cat #P1110
Hematoxylin	ZSGB-BIO	Cat #ZLI-9610
Eosin	ZSGB-BIO	Cat #ZLI-9613
Neutral balsam	Solarbio	Cat #G8590
Critical commercial assays		
Quantikine ELISA Mouse IFN-β Immunoassay	R&D Systems	Cat #MIFNB0
Experimental models: Cell lines		
Vero	ATCC	Cat #CRL-6318
Mouse primary peritoneal macrophages	This paper	N/A
Experimental models: Organisms/strains		
Mouse: C57BL/6	Beijing HFK Bioscience Co., Ltd	N/A
Oligonucleotides		
lfnb1 Fw GGGCTTGGCAATCCTTATCT	Sangon Biotech	N/A
lfnb1 Rv CCTGCAACCACCACTCATTCT	Sangon Biotech	N/A
VSV Fw ACGGCGTACTTCCAGATGG	Sangon Biotech	N/A
VSV Rv CTCGGTTCAAGATCCAGGT	Sangon Biotech	N/A
Software and algorithms		
GraphPad Prism	GraphPad	RRID: SCR_002798
Adobe Illustrator	Adobe	RRID: SCR_010279

MATERIALS AND EQUIPMENT

Cell culture medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Preheat in 37°C water bath before use.

Brewer thioglycollate medium: Suspend 40.5 g of Thioglycollate Medium in 1 L of distilled water (4.05%, w/v) and boil to dissolve the medium completely. Sterilize the dissolved medium by autoclaving at 121°C for 15 min. The prepared medium can be stored in a sterile environment away from light at 24°C–26°C for up to one year.

Harvest medium: Make 3% FBS in sterile PBS and store at 4°C for up to 3 months.

Ethanol solutions of different concentrations: To make 500 mL ethanol solutions, mix the appropriate amount of anhydrous ethanol with tap water. Store at 24°C–26°C for up to one year.

STEP-BY-STEP METHOD DETAILS

Virus amplification

⌚ **Timing:** 2 days

This step details how to amplify vesicular stomatitis virus (VSV) in preparation for infecting cells or mice and activating their innate immunity.

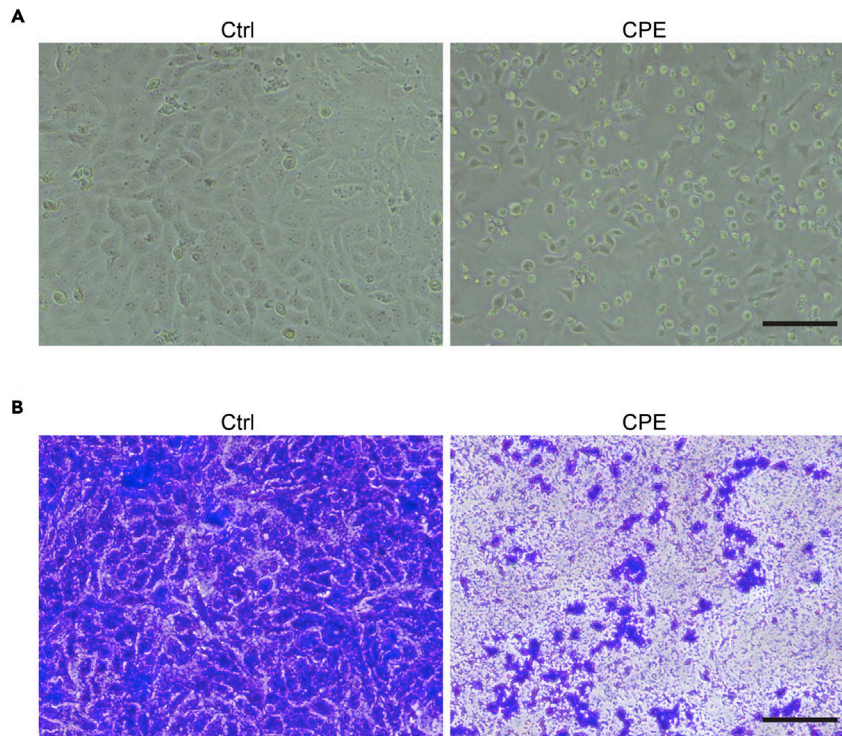


Figure 1. VSV infection-induced cytopathic effect (CPE)

(A) Representative images of normal Vero cells and cells displaying CPE (scale bar, 100 μ m).

(B) Microscopy of crystal violet-stained normal Vero cells and cells displaying CPE (scale bar, 100 μ m).

△ CRITICAL: *In vitro* viral infection should be performed in a Biosafety Level 2 laboratory. The experimenter must use personal protective equipment, and virus-related experiments should be carried out in a biosafety cabinet.

1. Plate Vero cells into a 10 cm cell culture dish and culture in cell culture medium for 10–12 h to achieve a cell density of about 80% per dish.

Alternatives: As an alternative, HEK-293T cells can also be used for VSV amplification.

2. Wash the cells once with PBS, add 10 mL of cell culture medium to each dish, and infect cells with VSV diluted with DMEM (multiplicity of infection (MOI), 0.1).

Note: $\text{MOI} = \text{virus titer (PFU/ml)} \times \text{virus volume (mL)} / \text{number of infected cells}$

3. After culturing for about 24 h at 37°C, 5% CO₂, observe the infected cells for cytopathic effect (CPE), when most of the cells display CPE, freeze the cell culture dish containing supernatant and cells directly in a –80°C freezer. A representative image of CPE is shown in [Figure 1A](#).

Note: CPE refers to the phenomenon that the virus proliferates in large quantities in the host cells, resulting in cytopathic changes such as roundness of infected cells, growth inhibition and even cell death. Therefore, CPE-positive cells showed less crystal violet staining ([Figure 1B](#)).

4. Defrost the frozen cells at 24°C–26°C, and transfer the cell medium to a 15 mL centrifuge tube.

5. Centrifuge the cell medium at $3000 \times g$, 20 min at 4°C .
6. Collect the supernatant, remove about 100 μL for virus titer determination, and store the remainder in a -80°C freezer.

▮▮ Pause point: Sample can be stored at -80°C for up to one year at this point or processed as described below.

TCID50 assay

⌚ **Timing:** 4 days

This step details how to determine the virus titer.

7. Twenty-four hours before the experiment, add 100 μL Vero cell suspension (about 1×10^3 cells) to each well of the flat-bottom 96-well plate.
8. Prepare 10 sterile 1.5-mL tubes for serial dilution. Fill the first tube with 990 μL DMEM and the remaining 9 tubes with 900 μL DMEM.
9. Dilution of the virus to be tested: add 10 μL supernatant to the first tube containing 990 μL DMEM for 1:100 dilution (10^{-2}). Mix, transfer 100 μL to the second tube containing 900 μL DMEM for a 10-fold dilution (10^{-3}), and repeat until the dilution is 10^{-11} (Figure 2A).
10. Remove the 96-well plate from the incubator and check that the cells are in good condition. Add virus dilutions from 10^{-4} to 10^{-11} successively to the plate, with one row for each dilution, 10 replicates in each row, and 90 μL to each well. Add 90 μL DMEM without virus to the 11th and 12th wells of each row as control.
11. Place the 96-well plate in a 37°C , 5% CO_2 cell incubator for further culture.
12. After 3 days, discard the medium in the wells, and add 50 μL 0.1% crystal violet stain solution to each well.
13. After staining for 30 min at 37°C , observe the cells for CPE and count CPE-positive wells. Calculate the positive rate for each row, and determine the virus titer by the Spearman–Karber method (Figure 2B) (Yuan et al., 2020).

Collection of peritoneal macrophages

⌚ **Timing:** 4 days

This step details how to isolate peritoneal macrophages from mice (Figure 3A).

Note: Peritoneal macrophages and bone marrow-derived macrophages can both be used to detect antiviral innate immunity (Li et al., 2016a; Wang et al., 2017). Here we focus on peritoneal macrophage isolation. Minimize unnecessary operations in the extraction process to maximize cell yield and avoid contamination.

14. Inject each age- and sex-matched 6–12-week-old mice intraperitoneally with 0.7 mL of Brewer thioglycollate medium (Layoun et al., 2015; Ray and Dittel, 2010). The inflammatory response lasts for 4 days, and the mice are euthanized on day 4 by an appropriate method approved by the supervising institution.

Note: Macrophages will have been greatly enriched two days after injection of Brewer thioglycollate medium, and peritoneal macrophages can be extracted within 2–4 days after injection.

15. Soak the mice in 70% alcohol for 5 min. Cut the extraperitoneal skin with sterile scissors and forceps to expose the inner skin lining the peritoneal cavity.

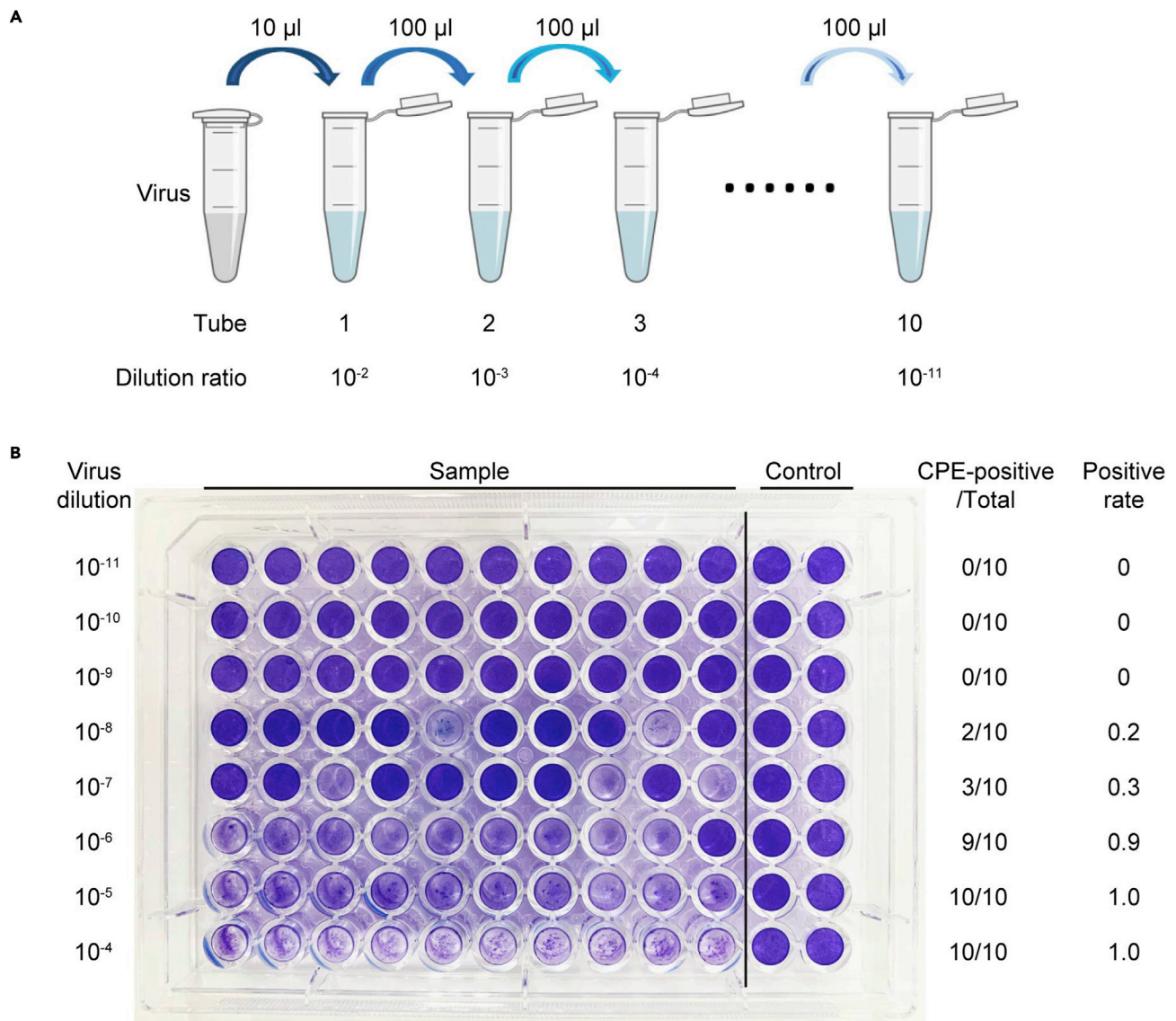


Figure 2. Detection of virus titer by TCID50 assay

(A) Illustrative overview of virus dilution.

(B) Example of TCID50 assay and virus titer calculation.

16. Inject 8 mL of cold harvest medium into the peritoneal cavity using a 10-mL syringe with a 26G needle. Be careful not to prick any organs.
17. Gently massage the abdomen of the mice for 10–20 s.
18. Collect peritoneal fluid containing macrophages using a 2-mL syringe and transfer to a sterile centrifuge tube on ice.
19. Centrifuge the peritoneal exudate cells at 400 × g for 8 min at 4°C (Figure 3B, the left panel), and discard the supernatant.

Optional: To remove erythrocytes, resuspend the cells with 1–2 mL red blood cell (RBC) lysis buffer per mouse, and leave them at 24°C–26°C for 5 min. Add 5 mL cell culture medium and centrifuge the suspension at 400 × g for 8 min at 4°C, and discard the supernatant.

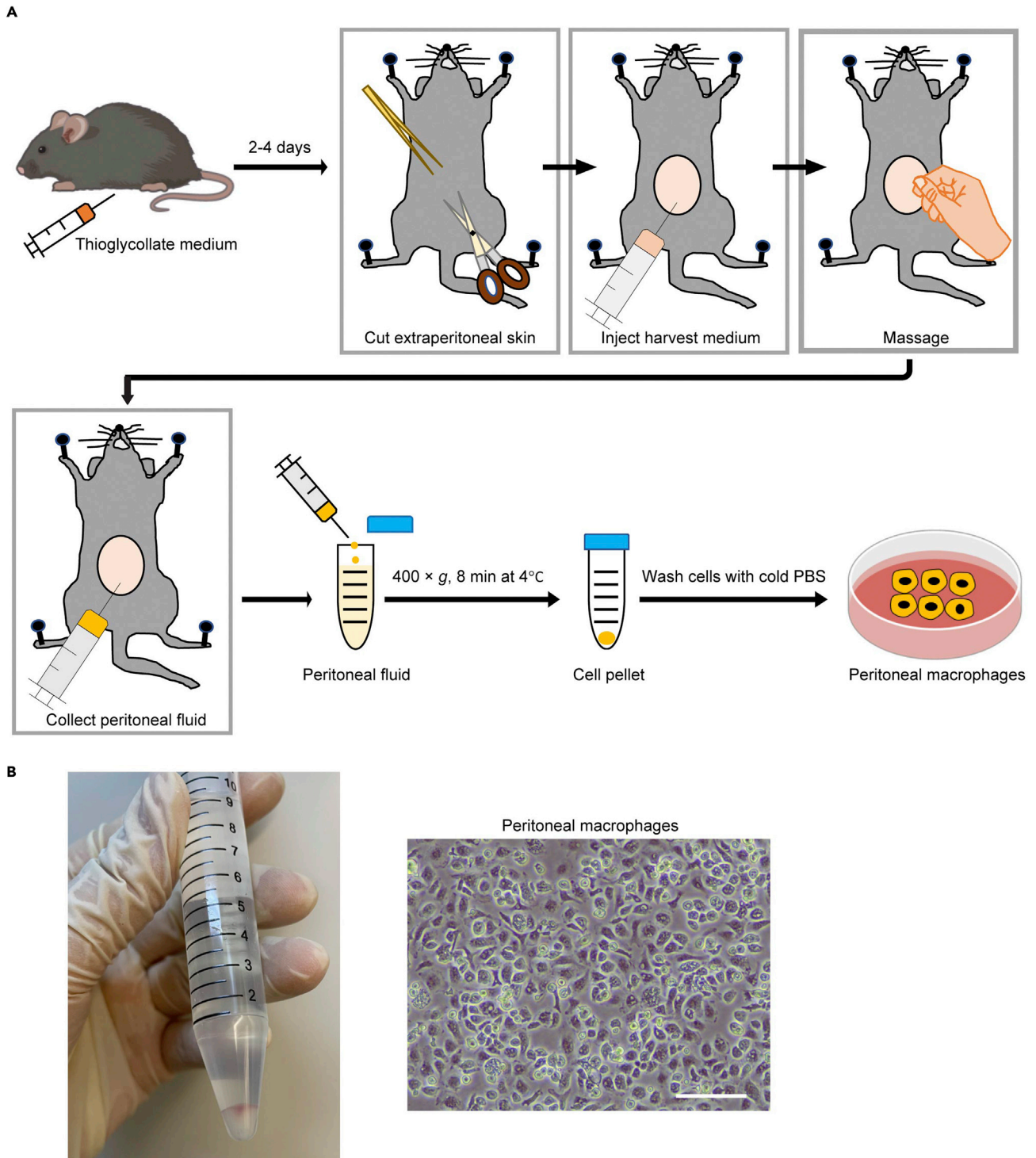


Figure 3. Collection of peritoneal macrophages

(A) Illustration describing mouse peritoneal macrophage collection.

(B) Representative photographs of extracted peritoneal macrophages (left) and their morphology (right) (scale bar, 100 μ m).

20. Wash cells with cold PBS and centrifuge at $400 \times g$ for 8 min at 4°C , discard PBS, and resuspend the peritoneal macrophages (approx. 1×10^7 cells per mouse) in 1 mL cell culture medium.
21. Count the cells and adjust the concentration to 1×10^6 cells/mL in cell culture medium. Add the cell suspension to wells (1 mL/well for a 12-well plate).
22. Culture at 37°C , 5% CO_2 for 1–2 h (Layoun et al., 2015; Pineda-Torra et al., 2015). Remove non-adherent cells by gentle washing with PBS. Observe the phenotype of peritoneal macrophages by microscope (Figure 3B, the right panel). The macrophages can then be used for further experiments.

⚠ **CRITICAL:** Peritoneal macrophages are best used the same day or the next day after the extraction, as their condition will deteriorate upon prolonged culture.

Virus infection *in vitro*

⌚ **Timing:** 8–20 h

This section describes the steps for peritoneal macrophage infection and innate immunity detection *in vitro*.

⚠ **CRITICAL:** VSV needs to be stored at -80°C and thawed on ice before use. Cell experiments should be carried out in a biosafety cabinet.

23. Wash the cultured macrophages twice with PBS. Culture the cells in DMEM and infect them with VSV, diluted with DMEM (MOI, 0.1), for 1 h at 37°C , 5% CO_2 .
24. Replace the medium with cell culture medium and culture for the required times at 37°C , 5% CO_2 (Zhang et al., 2017).
25. Extract mRNA from cultured cells and reverse-transcribe with a reverse transcription system. Detect the expression of *Irfn1* and VSV mRNA by qPCR (Figures 4A and 4B).

Note: To detect the antiviral ability of peritoneal macrophages, VSV infection is generally limited to 4–12 h.

26. For determining the secretion of IFN- β and the VSV titer, collect the supernatant by centrifugation at $400 \times g$ for 8 min at 4°C . Assay immediately, or aliquot and keep at -80°C until analysis. Avoid repeated freeze-thaw cycles.

⏸ **Pause point:** Collected supernatant can be stored at -80°C for up to one year at this point.

27. The secretion of IFN- β into the cell culture supernatant was measured using a mouse IFN- β ELISA kit (R&D Systems) (Figure 4C) according to the manufacturer's protocol (https://resources.rndsystems.com/pdfs/datasheets/mifnb0.pdf?v=20210426&_ga=2.132074575.1128226102.1619430566-905862661.1619430565).
28. Determine the VSV titer in the supernatant by TCID50 assay (Figure 4D).

Virus infection *in vivo*

⌚ **Timing:** 2 days

This section describes the steps for constructing a virus infection model and detecting innate immunity *in vivo*.

⚠ **CRITICAL:** *In vivo* viral infection should be performed in Animal Biosafety Level 2 facilities.

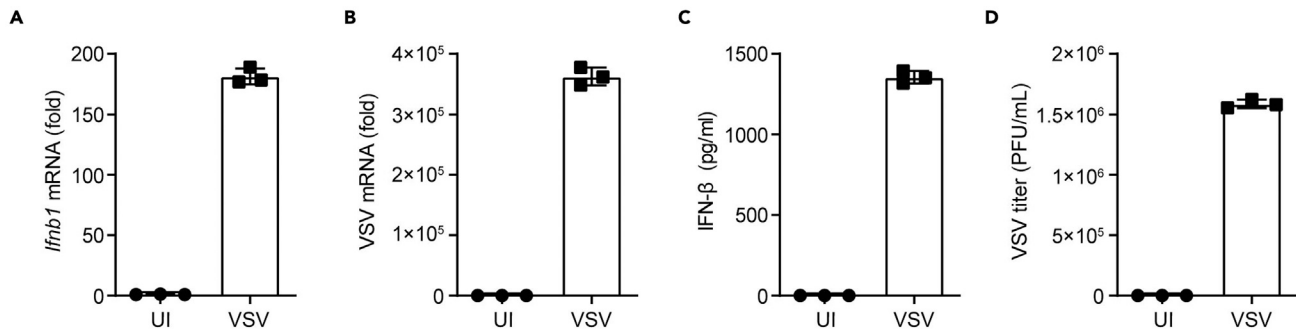


Figure 4. Virus infection in vitro

Mouse peritoneal macrophages were infected with VSV (MOI, 0.1) for 8 h. (A and B) qPCR detection of *Ifnb1* (A) and VSV (B) mRNA in uninfected (UI) and VSV-infected groups. (C) Secreted IFN-β in supernatants was detected by ELISA. (D) VSV titers in supernatants were determined by TCID50 assay.

29. Infect age- and sex-matched 6–12-week-old mice with VSV (2×10^8 PFU/mouse) by intraperitoneal injection.
30. Euthanize the mice 24 h later by an appropriate method approved by the supervising institution.
31. To measure the IFN-β level in the serum, allow the blood samples to clot for 2 h at 24°C–26°C and then centrifuge for 20 min at $2000 \times g$ at 4°C. Collect serum and assay immediately, or aliquot and store samples at –80°C. Avoid repeated freeze-thaw cycles.
32. Collect mouse lung, spleen, and liver and cut into small pieces for further analysis.

▣▣ Pause point: Fresh tissue pieces are placed in 1.5-mL tubes and frozen quickly with liquid nitrogen. The samples can be stored at –80°C for up to 6 months.

33. To detect gene expression and measure VSV titer in mouse tissues, homogenize lung, spleen, and liver in TRIzol or DMEM, and collect the fluid by centrifugation at $400 \times g$ for 8 min at 4°C.

Note: We usually homogenize 1 g of tissue in 1 mL of liquid, although the proportions can be varied.

34. After extracting lung RNA and converting it to cDNA with a reverse transcription system, the expression of *Ifnb1* and VSV mRNA was analyzed by qPCR (Figures 5A and 5B).
35. Determine VSV titer in homogenized lung tissue supernatant by TCID50 assay (Figure 5C).
36. The secretion of IFN-β in mouse serum was measured using a mouse IFN-β ELISA kit (Figure 5D).

Lung injury caused by viral infection

⌚ Timing: 4 days

This section details how to detect lung injury after VSV infection *in vivo*.

Note: Virus infection usually causes a variety of organ damage in mice, whereas VSV infection usually leads to lung injury, including alveolar collapse and immune cell infiltration.

37. Place lung tissues in tissue processing embedding cassettes and embed them in paraffin via the following sequence of steps (Figure 6A):
 - a. 48 h in 4% paraformaldehyde
 - b. 1 h in 70% ethanol (I)
 - c. 2 h in 70% ethanol (II)

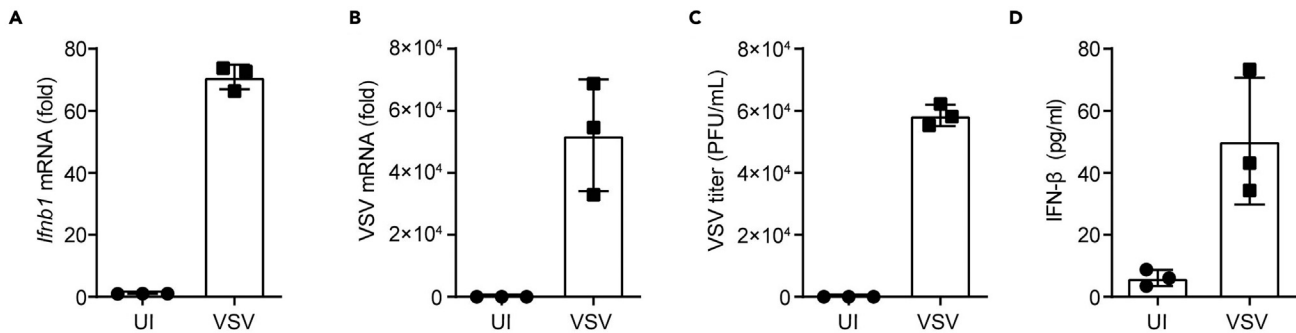


Figure 5. Virus infection in vivo

Wild-type mice were intraperitoneally injected with PBS or VSV (2×10^8 PFU per mouse) and were sacrificed 24 h after infection. (A and B) qPCR analysis of *Ifnb1* (A) and VSV (B) mRNA in uninfected (UI) and VSV-infected mouse lungs. (C) VSV titers in mouse lungs were determined by TCID50 assay. (D) Serum IFN- β concentrations were measured by ELISA.

- d. 30 min in 80% ethanol
- e. 30 min in 90% ethanol
- f. 30 min in 95% ethanol (I)
- g. 2 h in 95% ethanol (II)
- h. 30 min in anhydrous ethanol (I)
- i. 40 min in anhydrous ethanol (II)
- j. 20 min in xylene (I)
- k. 35 min in xylene (II)
- l. 12 h in paraffin

△ CRITICAL: All steps using organic solvents should be carried out in a laboratory fume hood (the same below). Paraffin embedding needs to be performed using an embedding machine. To protect the experimenter, harmful gas must be prevented from spreading during the experiment.

▣ Pause point: The embedded paraffin tissue can be stored at 24°C–26°C for up to one year at this point or processed as described below.

38. Cut 5- μ m lung tissue sections using a microtome, and detect lung injury by hematoxylin/eosin (HE) staining.
39. Place slides containing paraffin sections in a slide holder (Figure 6B), deparaffinize and rehydrate the sections as follows:
 - a. 2×10 min in xylene (blot excess xylene before transferring to ethanol)
 - b. 2×5 min in anhydrous ethanol
 - c. 10 min in 95% ethanol
 - d. 10 min in 80% ethanol
40. Rinse in distilled water for 5 min.
41. Apply hematoxylin to the lung tissue with a pipette and stain for 2–3 min.
42. Rinse in running tap water for 5 min.
43. Differentiate with 0.5% acid alcohol for 1–3 s.
44. Rinse well in tap water for 5 min.
45. Rinse in deionized water for 5 min.
46. Counterstain in eosin for 4–5 min.

Note: Eosin is highly soluble in water. Excess stain is removed by washing in running water.

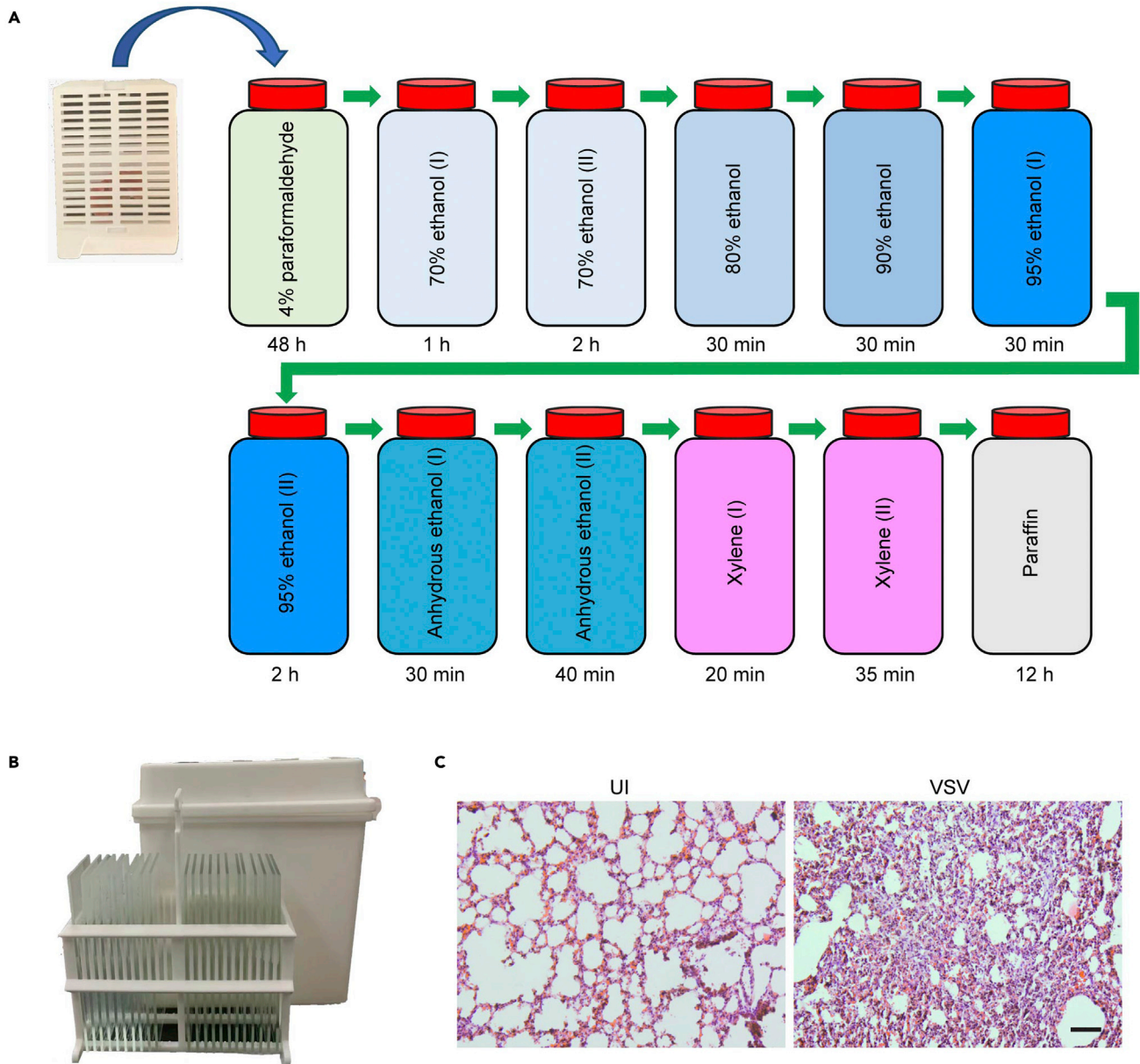


Figure 6. Detection of lung injury induced by VSV infection

(A) Steps of lung tissue dehydration and embedding.

(B) Slide holder and slides containing paraffin sections.

(C) Microscopy of HE-stained lung sections from mice uninfected (UI) or infected with VSV (2×10^8 PFU per mouse) for 24 h (scale bar, 100 μ m).

47. Dehydrate:

- a. 3 s in 80% ethanol
- b. 3 s in 95% ethanol
- c. 2×10 min in anhydrous ethanol (blot excess ethanol before transferring to xylene)
- d. 2×10 min in xylene

48. Place a coverslip on the slide using neutral balsam. Apply a drop of neutral balsam to the slide, angle the coverslip, and let it fall gently onto the slide, taking care to leave no bubbles. Allow the neutral balsam to spread beneath the coverslip, covering the entire tissue.

49. Observe and photograph with a light microscope, as illustrated in Figure 6C.

EXPECTED OUTCOMES

From each of three age- and sex-matched 6–12-week-old mice, $(1.1 \pm 0.2) \times 10^7$ peritoneal macrophages were isolated. Cells were allowed to adhere to the 12-well plate (Figure 3B, the right panel) and were then infected with VSV for 8 h. Examples of gene expression, IFN- β secretion, and VSV titer analyses using qRT-PCR, ELISA, and TCID50 assay, respectively, are shown in Figure 4.

For the *in vivo* experiment, three age- and sex-matched 6–12-week-old mice were infected with VSV (2×10^8 PFU/mouse) and sacrificed 24 h later. Serum IFN- β was detected by ELISA, and VSV mRNA and VSV titer in the lung were also analyzed using qRT-PCR and TCID50 assay, respectively (Figure 5). HE staining showed lung injury after virus infection (Figure 6C) (Li et al., 2016b).

QUANTIFICATION AND STATISTICAL ANALYSIS

Spearman–Karber method formula:

$$\text{Virus titer} = 10^{(x + 0.8)} \text{ (PFU/mL)}$$

x is the sum of CPE-positive rates at dilutions from 10^{-1} to 10^{-11} .

Note: Conditions of use of the formula: There was no CPE or growth inhibition in the negative control. CPE occurred in all wells in the row defined as the minimum dilution.

As shown in Figure 2C, CPE occurred in all wells at dilution of 10^{-5} , so the sum of CPE-positive rates at dilutions from 10^{-1} to 10^{-5} is 5, and the sum of CPE-positive rates at dilutions from 10^{-1} to 10^{-11} is:

$$x = 5 + 0.9 + 0.3 + 0.2 = 6.4$$

$$\text{Virus titer} = 10^{(6.4 + 0.8)} = 10^{7.2} \approx 1.6 \times 10^7 \text{ (PFU/mL)}$$

LIMITATIONS

It takes 2–4 days for peritoneal macrophages to accumulate, and the number of peritoneal macrophages extracted from each mouse is limited. The obtained macrophages barely proliferate and should be used without passage *in vitro*.

Virus infectivity is very important for the success of the experiment. Improper preservation of the virus, such as repeated freezing and thawing, will reduce the titer of the virus. Careful attention should be therefore paid to the proper preparation and storage of the virus. While this protocol used the RNA virus VSV, the effects of other viruses, such as the DNA virus HSV, on immune regulation should also be measurable by a similar method.

TROUBLESHOOTING

Problem 1

(Linked to 'step-by-step method details 6')

The titer of the amplified virus was too low.

Potential solution

To increase the titer of the amplified virus, the original virus with high virulence should be selected for amplification, and the Vero cells used to amplify the virus should be in good condition. In addition, the supernatant collected at step 6 can be further concentrated by mixing with $5 \times$ PEG8000 (suspend 50 g PEG8000 and 8.766 g NaCl in 200 mL of distilled water, sterilize by autoclaving at 121°C for 30 min and store at 4°C), and placed at 4°C for 24 h. Centrifuge the medium at

5000 × g for 40 min at 4°C, collect the precipitate, and resuspend with it in a small amount of pre-cooled PBS to obtain the concentrated virus.

Problem 2

(Linked to 'step-by-step method details 14')

Low yield and low purity of peritoneal macrophages.

Potential solution

First, make sure that the thioglycollate medium used in the experiment is fully dissolved, sterile, and stored in a sterile environment away from light at 24°C–26°C. Then, the number of mice required for the extraction of peritoneal macrophages should be estimated according to the experimental needs. Since the macrophages cannot be passaged during the experiment, the number of cells needed should be calculated before the experiment. The extracted cells should be cultured directly on the plates that are to be used later. In addition, some mouse models may yield fewer peritoneal macrophages than others, in which case more mice will be needed to obtain the same number of macrophages as wild-type mice.

Problem 3

(Linked to 'step-by-step method details 23')

The immune response fails to be activated during *in vitro* experiments

Potential solution

Ensure that the virus used is stored in a –80°C freezer and thawed on ice. Avoid repeated freezing and thawing to ensure its high virulence. The extracted peritoneal macrophages should be used within 24 h to ensure good cell activity. The optimum conditions for immune activation can also be explored by adjusting the duration of viral infection.

Problem 4

(Linked to 'step-by-step method details 29')

The immune response fails to be activated during *in vivo* experiments

Potential solution

Due to factors such as the strain and feeding environment of mice, the immune capacity of mice will differ, and any loss of virus infectivity will also weaken the activation of the immune system after infection. If necessary, the innate immune response of mice can be strengthened by either increasing the amount of virus used to infect the mice (2×10^8 – 1×10^9 PFU/mouse) or extending the duration of infection (1–3 days).

Problem 5

(Linked to 'step-by-step method details 38')

Uneven HE staining and poor color contrast.

Potential solution

Staining time should be appropriately shortened or prolonged according to the dye's color intensity and the room temperature. When the room temperature is high, the staining time can be shorter; otherwise, the time can be extended, or the samples can be stained in an incubator. Eosin mainly stains the cytoplasm, and the staining intensity should match that of the nucleus: if the nucleus is strongly stained, the cytoplasm should also be strongly stained to obtain a sharp contrast. Conversely, if the nucleus is lightly stained, the cytoplasm should also be lightly stained.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Qiujing Yu (yuqijing2018@tmu.edu.cn).

Materials availability

This protocol does not include unique materials.

Data and code availability

This protocol does not include data sets.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

L.S., X.S., P.H., Y.Z., Z.J., L.N., and Y.Y. conducted the experiments; L.S. interpreted the data and wrote the paper; Q.Y., T.W., and H.Z. supervised the study and polished the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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