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## Evaluation of Zika virus-specific T-cell responses in the immunoprivileged organs of infected *Ifnar1*<sup>-/-</sup> mice --Manuscript Draft--

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**Editor**

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**Ref.: Submission of a manuscript entitled: "Zika virus-specific T-cell response evaluation in the immune privilege organs of virus-infected and vaccine-inoculated *Ifnar1*<sup>-/-</sup> mice"**

Dear Dr. Jaydev Upponi,

I would like to thank you for the invitation and I also appreciate the opportunity to submit the our current work on the methods of Zika virus (ZIKV)-specific T-cell response evaluation in the immune privilege organs of virus-infected and vaccine-inoculated *Ifnar1*<sup>-/-</sup> mice. The recent outbreak of ZIKV has emerged as a global health concern. This virus can induce the infections of immune privilege organs, e.g. brain, testis, and lead to severe clinical outcomes. Though it was indicated that the T-cells could infiltrate into these tissues, the functions and characteristics of these tissue-infiltrated immune cells are still known. Thus, we established the protocols to evaluate the features of the antigen-specific T-cells in the spleen and also the immune privilege organs of the ZIKV-infected mice and the vaccine immunized mice. Our methods may be used to evaluate the functions of the virus-specific T-cells during the infection of the emerging viruses, such as Ebola virus and Zika virus, which can infect the immune privilege organs.

We believe our work would attract a broad spectrum of readership and fulfill the scope of your prestigious journal. We warrant that all authors have seen and approved the manuscript, contributed significantly to the work, and also that the manuscript has not been previously published nor is not being considered for publication elsewhere.

We hope you will make a favorable decision.

Looking forward to hearing from you.

All the best

Yours sincerely,

William J. Liu

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**TITLE:**

Evaluation of Zika Virus-specific T-cell Responses in Immunoprivileged Organs of Infected *Ifnar1*<sup>-/-</sup> Mice

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**KEYWORDS:**

Zika virus, antigen-specific, T cell, vaccination, immunoprivileged organs, tetramer

**SUMMARY:**

A protocol to evaluate antigen-specific T-cell responses in the immunoprivileged organs of the *Ifnar1*<sup>-/-</sup> murine model for the Zika virus (ZIKV) infection is described. This method is pivotal for investigating the cellular mechanisms of the protection and immunopathogenesis of ZIKV vaccines and is also valuable for their efficacy evaluation.

**ABSTRACT:**

The Zika virus (ZIKV) can induce inflammation in immunoprivileged organs (*e.g.*, the brain and testis), leading to the Guillain-Barré syndrome and damaging the testes. During an infection with the ZIKV, immune cells have been shown to infiltrate into the tissues. However, the cellular mechanisms that define the protection and/or immunopathogenesis of these immune cells during a ZIKV infection are still largely unknown. Herein, we describe methods to evaluate the virus-specific T-cell functionality in these immunoprivileged organs of ZIKV-infected mice. These methods include a) a ZIKV infection and vaccine inoculation in *Ifnar1*<sup>-/-</sup> mice; b) histopathology, immunofluorescence, and immunohistochemistry assays to detect the virus infection and inflammation in the brain, testes, and spleen; c) the preparation of a tetramer of ZIKV-derived T-cell epitopes; d) the detection of ZIKV-specific T cells in the monocytes isolated from the brain,

testes, and spleen. Using these approaches, it is possible to detect the antigen-specific T cells that have infiltrated into the immunoprivileged organs and to evaluate the functions of these T cells during the infection: potential immune protection *via* virus clearance and/or immunopathogenesis to exacerbate the inflammation. These findings may also help to clarify the contribution of T cells induced by the immunization against ZIKV.

## INTRODUCTION:

The ZIKV is a mosquito-borne flavivirus that was first isolated in 1947 in Uganda from a febrile rhesus macaque. Recently, the ZIKV has become a public health emergency, due to its rapid dissemination in the Americas and its unexpected link to microcephaly and Guillain-Barré syndrome<sup>1-3</sup>. From epidemiological data, the ZIKV has been suspected to be the cause of the Guillain-Barré syndrome in around 1 per 4,000 infected adults<sup>4</sup>. Moreover, a correlation between the ZIKV and testes infection/damage in the mouse model has been demonstrated, suggesting that the ZIKV infection, under certain circumstances, can bypass the blood-testis barrier and eventually lead to male infertility<sup>5</sup>. These findings highlight the importance of completely understanding the induction of protective or pathologic immune responses during a ZIKV infection.

Much remains to be learned about the cellular immune responses to the ZIKV. CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses to the capsid, envelope, and nonstructural protein 1 (NS1) have been observed in ZIKV-infected monkeys and humans<sup>6-8</sup>. In mice, several studies have indicated that CD8<sup>+</sup> T cells play a protective role in controlling the ZIKV replication<sup>9-11</sup>. Importantly, Jurado *et al.* demonstrated that a ZIKV infection results in the breakdown of the blood-brain barrier and perivascular infiltration of CD8<sup>+</sup> effector T cells within the testes of *Ifnar1*<sup>-/-</sup> mice. Furthermore, they showed that CD8<sup>+</sup> T cells instigate ZIKV-associated paralysis and appear to play a role in the neonatal brain immunopathology. In a previous study, we prepared the ZIKV-E<sub>294-302</sub> tetramer and showed that ZIKV-specific CD8<sup>+</sup> T cells exist in the brains and spinal cords of ZIKV-infected *Ifnar1*<sup>-/-</sup> mice, which may have important implications for the design and development of ZIKV vaccines<sup>10</sup>.

In response to the urgent need for vaccination to prevent ZIKV infection, several vaccines are in the preclinical stages of development, including RNA vaccines, recombinant vector-based vaccines, and purified protein subunit vaccines. The plasmid DNA vaccine is in phase 1 clinical trials<sup>12</sup>. The evaluation of safety and efficacy of ZIKV vaccines is, therefore, important. One advantage of the vaccines is their ability to elicit specific T-cell responses, which may be important for protection against the ZIKV. By using ZIKV-derived T-cell epitope-related tetramers, the T-cell immunoreactivity induced by an adenovirus-vector-based ZIKV vaccine could be detected in immunized mice, and both M and E glycoproteins were shown to induce robust antigen-specific CD8<sup>+</sup> T-cell immunoreactivity<sup>13</sup>.

During a virus infection, the recognition of peptide antigens presented by major histocompatibility complex (MHC) molecules to the T-cell receptor (TCR) is an important T-cell-mediated mechanism for protecting the host<sup>14</sup>. Based on this theory, tetramer technology is a unique tool to elucidate the roles that antigen-specific T cells play in regulating the immune

responses<sup>15</sup>. This protocol describes the establishment of the *Ifnar1*<sup>-/-</sup> mouse model for ZIKV infections, and the detection of reactive T cells in the spleen, brain, and testes of the mice by using tetramer technology. Additionally, we used the ZIKV-E<sub>294-302</sub> tetramer to detect and evaluate T-cell immunoreactivity induced by a ZIKV vaccine (AdC7-M/E) in immunized mice. This study provides guidance for investigating T-cell responses in immunoprivileged organs and provides a reference for the potential applications in the placenta or fetal brain.

## **PROTOCOL:**

Animal experiments were approved by the Institutional Animal Care and Use Committee of National Institute for Viral Disease Control and Prevention, China CDC. All experiments were performed following the Institutional Animal Care and Use Committee-approved animal protocols.

### **1. Virus Infection**

1.1. Keep adult (6- to 8-week-old) *Ifnar1*<sup>-/-</sup> (50% male and 50% female) mice in standard special pathogen-free (SPF) conditions, and allow them regular access to food and water.

1.2. Infect the *Ifnar1*<sup>-/-</sup> mice with the ZIKV *via* a retro-orbital inoculation of 100  $\mu$ L of ZIKV in a phosphate-buffered saline (PBS) buffer containing  $1 \times 10^4$  focus-forming units (FFUs) of the virus. Similarly, provide control mice with an equal volume of PBS.

CAUTION: Infect the mice under ABSL2 conditions and perform these procedures with virus-infected mice in the biosafety cabinet.

1.3. Monitor the weight and clinical signs (tremors, staggered march, bilateral flaccid hind limb) of the mice daily for 15 d.

### **2. Immunization with the ZIKV Vaccine**

2.1. Use *Ifnar1*<sup>-/-</sup> (50% male and 50% female) mice between 6 and 8 weeks of age. Keep the mice in standard SPF conditions of 18 – 29 °C and a 12-h light/dark cycle with access to food and water.

2.2. Immunize the *Ifnar1*<sup>-/-</sup> mice with the ZIKV vaccine: inject 100  $\mu$ L of AdC7-M/E [ $4 \times 10^{10}$  (virus particles)] in PBS buffer *via* an intramuscular injection (the i.m. route) using a 1-mL syringe with a 23-G needle.

2.3. Similarly, inject control mice with an equal volume of PBS.

### **3. Isolation of the Monocytes from the Spleen**

Note: The isolation of the monocytes from the spleen is described as mentioned previously<sup>16</sup>.

3.1. Anesthetize the immunized and ZIKV-infected mice 7 d post-inoculation, using a 5% isoflurane concentration in 100% oxygen (with a flow rate of 1 L/min). Euthanize the mice by cervical dislocation. Then, immediately dip the entire mouse into 75% ethanol.

CAUTION: From this step onward, all experimental procedures should be performed aseptically in a biosafety cabinet.

3.2. Using needles, fix the limbs of the (euthanized, previously infected/immunized) mice on the foam plate with the abdomen facing up.

3.3. Cut the skin along the abdominal midline to the thorax with a sterile scalpel, cut the abdominal muscles with scissors, expose the abdominal cavity, and gently remove the liver.

Note: The long, dark-red organ to the left of the stomach is the spleen.

3.4. Remove the spleen, rinse it 3x in PBS to remove any blood, and place it in 1.5 mL of ice-cold Roswell Park Memorial Institute medium (RPMI). Keep the spleen on ice.

3.5. To generate a single-cell suspension from the spleen, place the organ(s) on a sterile 40- $\mu$ m-mesh cell strainer on top of a 50-mL tube and add 2 mL of ice-cold RPMI medium containing 10% fetal bovine serum (FBS). Using the plunger of a 5-mL syringe, mechanically mash the organ(s) and add medium until the organ has been fully ground through the mesh.

3.6. Transfer the cell suspension to a 15-mL tube and centrifuge it at 600 x *g* for 5 min at 4 °C. Remove the supernatant.

3.7. Resuspend the cells with 5 mL of a red blood cell (RBC) lysis buffer (NH<sub>4</sub>Cl, Na<sub>2</sub>EDTA, and KHCO<sub>3</sub>; see the **Table of Materials**) and incubate the lysis reaction at room temperature for 5 – 6 min.

3.8. Stop the RBC lysis with 10 mL of ice-cold RPMI/FBS medium and centrifuge the tube at 600 x *g* for 5 min at 4 °C. Remove the supernatant.

3.9. Resuspend the cells with 10 mL of complete RPMI medium (RPMI with 10% vol/vol FBS and 100 U/mL penicillin-streptomycin). Transfer 10  $\mu$ L of the cell suspension to a small tube, mix it with 10  $\mu$ L of 0.4% wt/vol trypan blue, and count the number of cells using a hemocytometer.

#### 4. Isolation of Monocytes from the Brain and Testes

4.1. Anesthetize the ZIKV-infected male mice 7 d post-inoculation, using 5% isoflurane in 100% oxygen (with a flow rate of 1 L/min). Euthanize the mice by cervical dislocation. Then, immediately dip the entire mouse into 75% ethanol.

CAUTION: From this step onwards, all experimental procedures must be performed aseptically in a biosafety cabinet. The isolation of monocytes from the brain and testes is described as mentioned previously<sup>17</sup>.

4.2. Immobilize a (euthanized, previously infected) male mouse in the prone position on a cutting board.

4.3. Secure the scalp with a straight 1 x 2 teeth forceps, and use Iris scissors to make a midline incision on the scalp to expose the skull.

4.4. Clamp the two sides of the orbits with sharp tweezers and fix the brain. Place one tip of sharp Iris scissors into the *foramen magnum* and cut laterally into the skull. Repeat this step for the other side.

4.5. Use sharp Iris scissors to carefully cut from the same cavity, up the midline, toward the nose. Try to keep the end of the scissors as superficial as possible to avoid injuring the brain.

4.6. Lift the brain with forceps and use sharp Iris scissors to carefully dissect the cranial nerve fibers. Remove the brain with forceps and place it in a 15-mL tube containing 5 mL of ice-cold RPMI/10% FBS medium.

4.7. Grab the abdominal skin with forceps and use sharp Iris scissors to make a longitudinal incision through the integument and abdominal wall and expose the lowermost part of the abdomen. Push the testes up to the incision. Gently pull the fat layer with tweezers and expose a globular testis on both sides.

4.8. Use sharp Iris scissors to carefully dissect the fat layer and epididymis. Place the testes in a 15-mL tube containing 5 mL of ice-cold RPMI/10% FBS medium with forceps.

4.9. To generate a single-cell suspension from the brain or testes, place the organ on a sterile cell strainer with a 100- $\mu$ m mesh on top of a 50-mL tube and add 2 mL of ice-cold RPMI/10% FBS medium. Using the plunger of a 5-mL syringe, mash the organ and add medium until the organ has been fully ground through the mesh.

4.10. Transfer the cell suspension to a 15-mL tube and centrifuge it at 600 x *g* for 5 min at 4 °C. Remove the supernatant.

4.11. Resuspend the cells with 5 mL of 30% density gradient medium and, then, add them very slowly to 2 mL of 70% density gradient medium in a 15-mL tube.

4.12. Switch off the brake and centrifuge the tubes at 4 °C at 800 x *g* for 30 min. Obtain the lymphocytes from the middle layer.

4.13. Transfer the interphase to a fresh 15-mL tube, add 10 mL of cold RPMI/10% FBS medium,



and centrifuge the tube at 300 x *g* for 10 min at 4 °C. Remove the supernatant.

4.14. Resuspend the cells with 10 mL of ice-cold RPMI/FBS medium and centrifuge them at 600 x *g* for 5 min at 4 °C. Remove the supernatant.

4.15. Resuspend the cells with 10 mL of complete RPMI medium and count the number of cells as in step 3.9.

## 5. Tetramer Preparation

5.1. Construct plasmids expressing the extracellular domains of H-2D<sup>b</sup> with a biotinylated site tag on the C-terminus of the α3 domain and the β<sub>2</sub>m by using the pET28a vector with an NdeI and XhoI restriction site<sup>18</sup>.

5.2. Express and prepare inclusion bodies of H-2D<sup>b</sup> and β<sub>2</sub>m as described previously<sup>19</sup>.

5.3. Renature and purify the MHC/peptide complex H-2D<sup>b</sup>-E<sub>294-302</sub>.

5.3.1. Prepare 200 mL of a refolding solution [100 mM Tris-HCl (pH 8.0), 400 mM L-arginine, 2 mM EDTA-Na, 5 mM GSG, and 0.5 mM GSSG]. Add protease inhibitors: 2 mL of phenylmethylsulphonyl fluoride (PMSF, stock 100 mM), 100 μL of pepstatin (stock 2 mg/mL), and 100 μL of leupeptin (stock 2 mg/mL). Cool the buffer at 4 °C for 30 min.

5.3.2. Add H-2D<sup>b</sup>, β<sub>2</sub>m, and peptide to the refolding solution.

5.3.2.1. Inject 500 μL of β<sub>2</sub>m dissolved in a guanidine solution (30 mg/mL stock) using a syringe. For this, use a 23-G needle with a 5-mL syringe and inject the β<sub>2</sub>m into the refolding solution drop by drop. Keep the solution constantly and slowly stirring at 150 rpm at 4 °C.

5.3.2.2. After β<sub>2</sub>m has been dissolved in the refolding solution, resolve 2 mg of E<sub>294-302</sub> peptide in 200 μL of DMSO and quickly inject it into the refolding solution using a pipette. Slowly stir the solution at 150 rpm at 4 °C for 15 min.

5.3.2.3. Inject 1.5 mL of H-2D<sup>b</sup> dissolved in a guanidine solution. Keep the stir bar rotating at 150 rpm for the refolding of the H-2D<sup>b</sup> at 4 °C for 8 - 10 h.

Note: The solution was placed in a closed box in a cold room.

5.3.3. Concentrate the refolded protein in a pressurized chamber with a 10 kDa membrane. Exchange the buffer [20 mM Tris-HCl (pH8.0), 50 mM NaCl] to the chamber and concentrate it to a final volume of 30 - 50 mL.

5.3.4. Transfer the refolding solution to a centrifuge tube and spin it at 2,500 x *g* for 15 min at 4°C.

5.3.5. Carefully transfer the supernatant to a 10 kDa centrifugal filter and further concentrate it to a final volume of 500  $\mu$ L at 2,500 x *g* for 30 min.

5.3.6. Transfer the supernatant to a fresh tube and spin it at 12,000 x *g* for 15 min. Purify the protein with S200 10/300 GL gel filtration chromatography.

5.3.7. Collect the MHC complex peak and concentrate it to a final volume of 350  $\mu$ L.

5.4. To generate a 500- $\mu$ L reaction volume for biotinylation, add the reagents in the following order: 100  $\mu$ L of solution A [0.5M bicine (pH 8.3)], 100  $\mu$ L of solution B (100 mM ATP, 100 mM MgOAc, 200  $\mu$ M biotin), 100  $\mu$ L of extra d-biotin (500  $\mu$ M biotin), 20  $\mu$ L of BirA enzyme (60  $\mu$ g), 0.5  $\mu$ L of pepstatin (2 mg/mL), and 0.5  $\mu$ L of leupeptin (2 mg/mL). Incubate the reaction tube overnight at 4  $^{\circ}$ C.

CAUTION: Do not add any EDTA to the biotinylating reaction.

5.5. Purify the MHC using a S200 10/300 GL gel filtration column to remove any extra biotin.

5.6. Determine the biotinylating efficiency with a streptavidin-shift assay<sup>18</sup>.

5.6.1. Prepare three samples, A, B, and C, on ice for 30 min. Then, analyze the results by a 10% SDS-PAGE. Sample A consists of 8  $\mu$ L of biotinylated MHC molecules and 2  $\mu$ L of exchange buffer, sample B of 8  $\mu$ L of biotinylated MHC molecules and 2  $\mu$ L of streptavidin (20 mg/mL), and sample C of 2  $\mu$ L of streptavidin (20 mg/mL) and 8  $\mu$ L exchange buffer.

CAUTION: Do not boil the sample.

5.7. Form multimers of the biotinylated MHC molecules.

5.7.1. Produce tetramer by mixing the biotinylated E<sub>294-302</sub> peptide-H<sub>2</sub>D<sup>b</sup> complex with phycoerythrin-labeled streptavidin at a mole ratio of 1:5 to ensure a complete binding of all the biotinylated MHC molecules.

5.7.2. Calculate the amount of streptavidin-conjugate needed for tetramerization.

5.7.2.1. Determine the moles of the MHC/peptide complexes accounting for the protein concentration and the molar weight (example: 1.8 mg of total protein = 40 nmol).

5.7.2.2. Calculate the moles of the streptavidin-conjugate needed by dividing the moles of the MHC/peptide by 5 (example: 40/5 = 8 nmol of streptavidin-conjugate).

5.7.2.3. Calculate the amount of streptavidin needed (in  $\mu$ g) depending on the streptavidin-conjugate (example: streptavidin-PE [300,000 g/M]  $\rightarrow$  8 nmol needed = 2,400 g).

5.7.3. Divide streptavidin-phycoerythrin into 10 samples. Add each sample to a tube containing the biotinylated E<sub>294-302</sub> peptide-H<sub>2</sub>D<sup>b</sup> complex, at an interval of 20 min. After loading the last sample, incubate the reaction tube at 4 °C overnight in dark.

5.8. Apply the multimerized reagents into a 100 kDa spin tube and concentrate it by centrifugation at 2,000 x *g* at 4 °C to a volume of < 100 µL.

5.9. Dilute the sample in the spin tube to 4 mL using PBS (pH 8.0) and concentrate it again to < 100 µL.

5.10. Repeat the buffer exchange step in PBS (pH 8.0) 4x.

5.11. Fill up the total volume again to 500 µL using PBS (pH 8.0). Concentrate the sample to an estimated concentration of 2 - 2.5 mg/mL at 2,000 x *g* at 4 °C. Store the sample in the dark at 4 °C.

## 6. Flow Cytometry

6.1. Incubate the cell suspensions (from step 3.9 and/or step 4.15) at 4 °C with 0.1 µL of anti-murine CD16/CD32 Fc-Receptor blocking reagent per 20 µL (dilution factor 1:200) for 10 min, to prevent any unspecific binding.

6.2. Centrifuge the tetramer tube at 20,000 x *g* for at least 15 min at 4 °C.

6.3. Add 20 µL of the cell suspension to a 96-well plate containing 1 x 10<sup>6</sup> cells each well.

6.4. Prepare a sufficient volume of the E<sub>294-302</sub> tetramer mix to stain all experimental tubes. Prepare an excess of 15% of the total volume of this mix to account for pipetting errors. Dilute the E<sub>294-302</sub> tetramers (2 mg/mL, 1 µL/test) in FACS buffer (PBS, 0.5% FBS) so that 20 µL of the E<sub>294-302</sub> tetramer mix is added to each test.

6.5. Add 20 µL of the E<sub>294-302</sub> tetramer mix to the 96-well plate. By the end of this step, the final volume in each well should be 40 µL. Incubate the plate in the dark at room temp for 30 min.

6.6. Add primary antibodies (FITC-conjugated or APC-conjugated anti-CD3 (0.2 mg/mL), PerCP-conjugated anti-CD8 (0.2 mg/mL)) at 1 µL/test to the cell suspension and, then, incubate it at 4 °C for 30 min in the dark.

6.7. Wash the cells 2x with 2 mL of FACS buffer and centrifuge them at 600 x *g* for 5 min. Carefully aspirate the supernatant and resuspend the cells with 200 µL of FACS buffer.

6.8. Store the samples temporarily at 4 °C in the dark until the flow cytometric analysis.

6.9. Use the following gating strategy for the flow cytometric analysis.

6.9.1. Create a gate on diagonally clustered singlets by plotting a forward scatter (FSC) *versus* side scatter (SSC) area.

6.9.2. Then, gate on CD3<sup>+</sup> cells by side scatter (SSC) *versus* CD3; next, gate on CD3<sup>+</sup> CD8<sup>+</sup> cells; finally, outline CD8<sup>+</sup> tetramer<sup>+</sup> cells.

## **7. Histopathology, Immunofluorescence, and Immunohistochemistry Assay**

### **7.1. Histopathology assay**

7.1.1. Collect the brain and testis tissues of the ZIKV-infected *Ifnar1*<sup>-/-</sup> mice 7 d post-inoculation and fix them in 4% neutral-buffered formaldehyde.

CAUTION: Paraformaldehyde is toxic; wear appropriate personal protective equipment.

7.1.2. Embed the tissue in paraffin.

7.1.3. Section the tissue at 5 mm using a vibratome.

7.1.4. Stain the tissue with hematoxylin and eosin (H&E).

### **7.2. Immunohistochemistry assay**

7.2.1. Deparaffinize, rehydrate, and antigen-retrieve the tissue sections, based on procedures described previously<sup>20</sup>.

7.2.2. Treat the tissue sections with 3% H<sub>2</sub>O<sub>2</sub> in PBS (pH 7.6) for 10 min and block them with 1% bovine serum albumin (BSA) for 10 min.

7.2.3. Incubate the tissue sections with rat anti-mouse CD3 antibody (dilution: 1/1,000) for 8 h at room temperature and, then, incubate them at 4 °C overnight.

7.2.4. Rinse the tissues with PBS and, then, incubate them with 3 drops of biotinylated secondary antibody (dilution: 1/1,000) for 2 h at room temperature, followed by 3 drops of avidin-biotin-peroxidase (dilution: 1/200) at room temperature for 30 min.

7.2.5. Bind, with 3 drops of 30, 30-diaminobenzidine tetrahydrochloride (dilution: 1/1,000), as described previously<sup>21</sup>.

7.2.6. Counterstain the tissue sections with Mayer's hematoxylin.

### **7.3. Immunofluorescence assay**

7.3.1. Air-dry the frozen testis sections (6 mm) for 10 min at room temperature.

7.3.2. Fix them with ice-cold acetone for 10 min.

7.3.3. Wash the sections with PBS for 3x and block them with a blocking buffer (1% BSA, 0.3% Triton, 1x PBS) at 37 °C for 30 min.

7.3.4. Incubate the tissue sections with primary antibody (Z6) (20 µg/mL) at 4 °C overnight.

7.3.5. Rinse the tissues with PBS and apply the secondary antibody (dilution factor: 1/200) for 1 h at 37 °C.

7.3.6. Wash the tissue sections with PBS and counterstain them for nuclei using 4', 6-diamidino-2-phenylindole (DAPI) (dilution factor: 1/1,000), following the manufacturer's instructions.

#### REPRESENTATIVE RESULTS:

Following these methods, we have developed a murine model for ZIKV infections. *lfnar1*<sup>-/-</sup> mice at ~6 - 8 weeks of age were infected with 1 x 10<sup>4</sup> focus-forming units (FFU) of the ZIKV by retroorbital injection. Pathological symptoms and signs (**Figure 1A**), as well as weight changes (**Figure 1B**), were observed in the *lfnar1*<sup>-/-</sup> mice after an infection with the ZIKV. The murine brains showed obvious edema and hyperemia (**Figure 1C**). Meanwhile, the testes shrank gradually (**Figure 1D**). Furthermore, pathological changes and the destruction of tissue were found in the brain and testes (**Figure 2A**). We performed an immunofluorescence assay to detect the ZIKV in the brain and testes (**Figure 2B**). High viral loads were detected in the brain and spleen by immunostaining (**Figure 2B**). Immunohistochemistry showed a robust infiltration of CD3<sup>+</sup> T cells into the mice brain after the infection with the ZIKV (**Figure 2C**).

To detect and evaluate ZIKV-specific T-cell responses, we prepared a mouse MHC-I H-2D<sup>b</sup>-E<sub>294-302</sub> tetramer. The peptide E<sub>294-302</sub> can help the H-2D<sup>b</sup> renature properly and yield a high amount of the soluble MHC-I (**Figure 3A**). In the shift assay, a high efficiency in biotinylation could be observed (**Figure 3B**). Subsequently, three streptavidin fluorescence (APC, PE, and BV421)-tagged pMHC-I tetramers were produced to detect ZIKV-specific T cells (**Figure 3C**). The PE-labeled tetramer had a higher efficacy to detect the specific CD8<sup>+</sup> T cell compared to the APC- and BV421-labeled tetramers, though the difference was not statistically significant.

Using the E<sub>294-302</sub> tetramer, we detected ZIKV-specific T lymphocytes in the spleen of the infected mice by flow cytometry at 7 d post-inoculation of the ZIKV (3.49 ± 0.4508%). Also, similar to the method described in section 3 of this protocol, lymphocytes from 4 weeks post-immunization were isolated with the AdC7-M/E vaccine and, then, ZIKV-specific T lymphocytes were detected in the spleen (6.89 ± 1.36%) (**Figure 4**).

Furthermore, we detected the lymphocytes infiltrated into the immunoprivileged organs, such as the brain and testes, after the ZIKV infection. The gates were set to select for CD3<sup>+</sup>CD8<sup>+</sup> T cells

in total lymphocytes of the brain and testes. A high ratio of the E<sub>294-302</sub> tetramer-specific T cells could be detected in the brain (42.2% in CD3<sup>+</sup>CD8<sup>+</sup> T cells) and the testicular (26.4% in CD3<sup>+</sup>CD8<sup>+</sup> T cells) lymphocytes (Figure 5).

#### FIGURE LEGENDS:

**Figure 1: Characterization of the ZIKV infection in *Ifnar1*<sup>-/-</sup> mice.** *Ifnar1*<sup>-/-</sup> mice at 6 - 8 weeks of age were infected with 10<sup>4</sup> focus-forming units (FFU) of the ZIKV by retroorbital injection, and mice injected with PBS were used as uninfected controls. The (A) morphology and (B) weight changes of infected *Ifnar1*<sup>-/-</sup> mice were monitored. The red arrows indicate *Ifnar1*<sup>-/-</sup> mice presented with myeloparalysis and motor paraparesis after the infection. (C) Brains at 7 d post-inoculation and (D) testes at 0, 7, 14, and 30 d post-inoculation were harvested. Representative images of the brain and testes from the mice are shown with a ruler to indicate the sizes. The error bars represent the mean ± SEM. *n* = 10 mice per group per experiment.

**Figure 2: Detection of the ZIKV and lymphocytes in the brain and testis of ZIKV-infected *Ifnar1*<sup>-/-</sup> mice.** (A) This panel shows histopathological changes in the brain and testes from ZIKV-infected mice compared with negative controls at 7 d post-inoculation. The scale bar is 25 μm (left panel) and 50 μm (right panel). (B) An immunofluorescence assay was performed with the anti-ZIKV Z6 antibody (green). All tissue samples were collected from ZIKV-infected mice at 7 d post-inoculation. The nuclei were stained with DAPI (blue). The scale bar is 50 μm. (C) Immunohistochemistry shows a robust infiltration of CD3<sup>+</sup> T cells into the brain and testis. The scale bar is 25 μm (left panel) and 50 μm (right panel). Purple indicates hematoxylin, brown represents the CD3 antibody.

**Figure 3: Preparation of ZIKV-specific pMHC-I tetramers.** (A) The binding of E<sub>294-302</sub> to H-2D<sup>b</sup> is elucidated by an *in vitro* refolding. Blue indicates the H-2D<sup>b</sup>-E<sub>294-302</sub> protein. Orange represents the negative control without peptide. (B) This panel shows the H-2D<sup>b</sup>-E<sub>294-302</sub> shift assay. (C) The mock represents a PBS control. Three streptavidin fluorescence (APC, PE, and BV421)-tagged pMHC-I tetramers were used to detect ZIKV-specific T cells.

**Figure 4: Flow cytometric analysis of virus-specific CD8<sup>+</sup> T cells in the spleen of ZIKV-infected and vaccine-immunized mice.** *Ifnar1*<sup>-/-</sup> mice at 6 weeks of age were either infected with 10<sup>4</sup> focus-forming units (FFU) of ZIKV or received a single-dose of 4 x 10<sup>10</sup> viral particles of AdC7-M/E or PBS as a negative control. After 7 days post-infection or 4 weeks post-vaccination, mouse splenocytes were harvested and analyzed by flow cytometry. The data are shown as mean ± SD. Statistical analysis was performed using one-way ANOVA (not significant: *P* > 0.05; \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; \*\*\*\**P* < 0.0001).

**Figure 5: Gating strategy and representative results of virus-specific CD8<sup>+</sup> T cells in the brain and testes of ZIKV-infected mice.** Representative plots are shown for the infiltrated lymphocytes in (A) the brain and (B) the testis. A succession of gates is set to select lymphoid-scatter<sup>+</sup> and CD3<sup>+</sup> events. Of these, CD8<sup>+</sup> events are gated for the analysis of epitope-specific T cells.

## DISCUSSION:

Immunogenic T-cell epitope plays a significant role in cellular immunity against pathogens<sup>22</sup>. Thus, the detection of ZIKV-specific T cells in immunoprivileged organs is a critical methodology to understand T-cell responses against the natural ZIKV infection. Meanwhile, T-cell response detection is an excellent tool to investigate the efficacy of the viral vaccine. Here, we show a comprehensive protocol to visualize the experiments, which include the isolation of lymphocytes from the spleen, brain, and testes of ZIKV-infected mice, the preparation of the immunodominant epitope E<sub>294-302</sub> tetramer, and the recognition of ZIKV-specific CD8<sup>+</sup> T cells in immunoprivileged organs of ZIKV-infected mice.

A previous study showed that a live ZIKV or its RNA can be detected in the semen of male patients, which indicates that the ZIKV can bypass the blood-testis barrier and replicate itself in the reproductive system<sup>23</sup>. Previously, we also showed that the ZIKV can cause testes damage and lead to male infertility in mice<sup>24</sup>. ZIKV infection can lead to viremia in rhesus monkeys, and the viral RNA can be detected in the central nervous system (CNS), as well as in the visceral organs. Immunohistochemistry revealed that ZIKV-specific antigens were presented in the CNS and the multiple peripheral organs<sup>25</sup>. Also, in murine models, ZIKV infection can induce a robust antiviral CD8<sup>+</sup> T-cell response in the spleen and CNS<sup>25</sup>.

Compared to previous work, this study establishes systematic methods to detect ZIKV-specific CD8<sup>+</sup> T-cell responses in the brain and testes, which are immunoprivileged sites. It is important to assess the functionality of virus-specific T cells in the immunoprivileged organs of the ZIKV-infected mice. The usage of tetramers to detect ZIKV-specific CD8<sup>+</sup> T-cell responses in immunoprivileged organs would greatly enhance our understanding of ZIKV infections and their host immune responses. Using E<sub>294-302</sub> tetramer, virus-specific T cells in brain and testis can be isolated by flow cytometry, to investigate the cellular mechanisms of the protection and immunopathogenesis during a ZIKV infection. Meanwhile, it is helpful for researchers to investigate further the functions of the CD8<sup>+</sup> T cells to control the ZIKV, or to enhance the immunopathogenesis in these organs during ZIKV infection.

To analyze the antigen-specific murine CD8<sup>+</sup> T-cell responses in the immunoprivileged organs, we prepared H-2D<sup>b</sup>-E<sub>294-302</sub> tetramer and detected the CD8<sup>+</sup> T cells by flow cytometry. Tetramers are a powerful tool to detect antigen-specific T cells. Here, three types of fluorochrome-conjugated streptavidin (APC, PE, and BV421) were generated. Although there are no statistically significant differences in the APC-, BV421-, and PE-labeled tetramers for detecting antigen-specific T cells, PE-labeled pMHC-I tetramers yielded the best results. Hence, the PE-labeled tetramer was used throughout this study. Interestingly, based on the PE-labeled H-2D<sup>b</sup>-E<sub>294-302</sub> tetramer, we detected high ratios of antigen-specific T cells in both the brain and testes, which indicate the migration ability of the virus-specific T cells from the blood to immunoprivileged organs.

However, there are some limitations to the protocol. The H-2D<sup>b</sup>-E<sub>294-302</sub> tetramer is not useful for human T-cell detection, because tetramer detection is dependent on MHC restriction. The screening of immunodominant HLA-restricted peptides is still needed. Besides, retro-orbital infection is effective for a ZIKV infection but might be not a convenient operation for some

investigators. Thus, other routes of infection, including peritoneal, subcutaneous, or intravenous, are also recommended.

In the protocol described here, a critical step is the isolation of monocytes from brain and testis. It is important to acquire high-quality lymphocytes; thus, it is important to pay attention to, for example, the centrifugal speed, the strength of the grinding tissue, and the dissection of the brain and testis tissue. Besides, for tetramer preparation, protease inhibitors (PMSF, pepstatin, leupeptin) are helpful when protecting a protein from being degraded. Therefore, it makes sense to add a protease inhibitor to the refolding buffer and exchange the buffer during the process of the tetramer preparation.

In conclusion, we present the methods of detecting antigen-specific T-cell responses in the immunoprivileged organs of the *Ifnar1<sup>-/-</sup>* mouse model for a ZIKV infection. This platform can be widely applied to investigate the immune mechanisms of emerging and re-emerging viruses which can bypass the barriers between the blood and the immunoprivileged organs. Moreover, this study may pave the way for the future development of candidate vaccines and immunotherapies.

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The authors have nothing to declare.

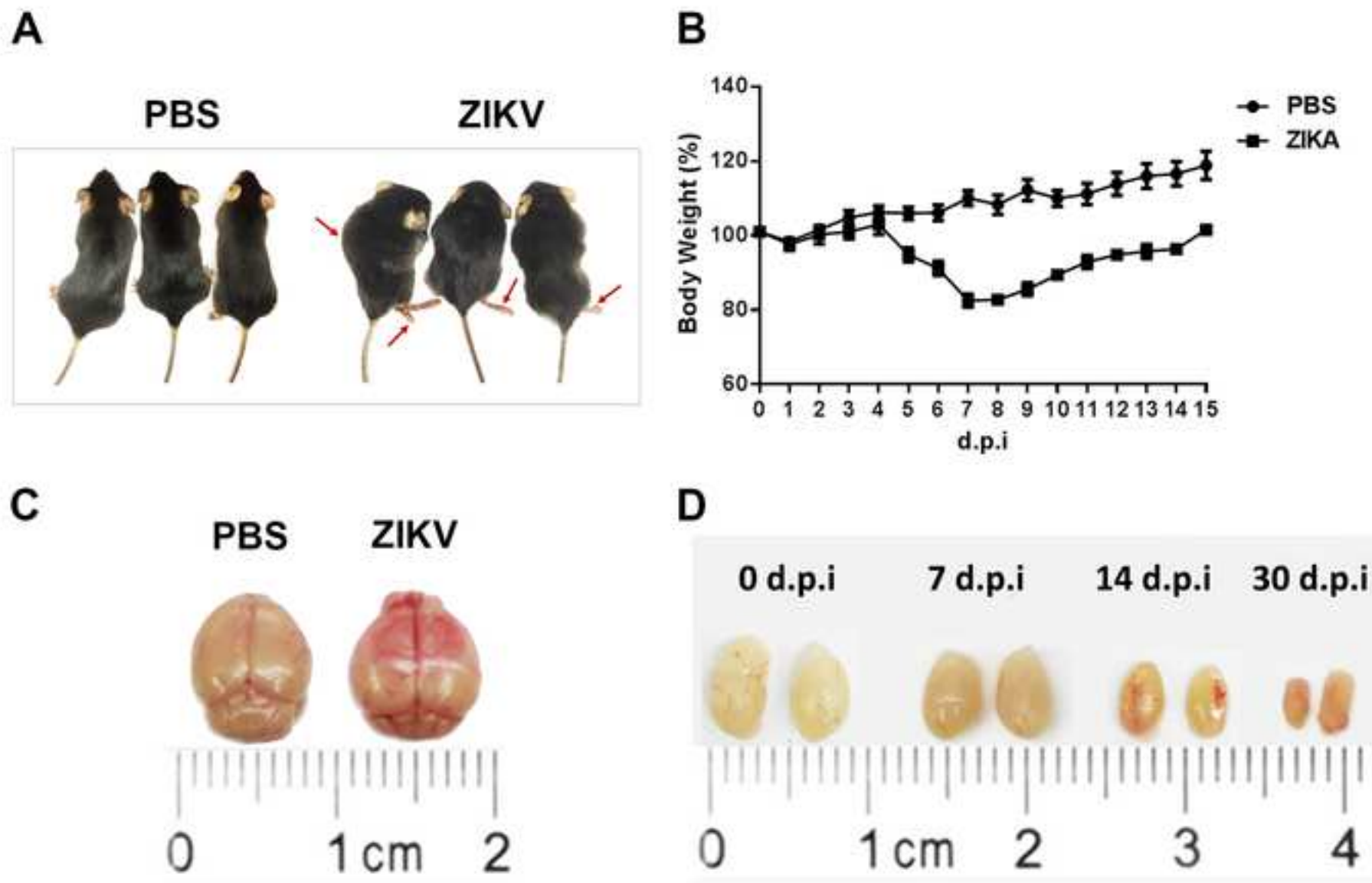
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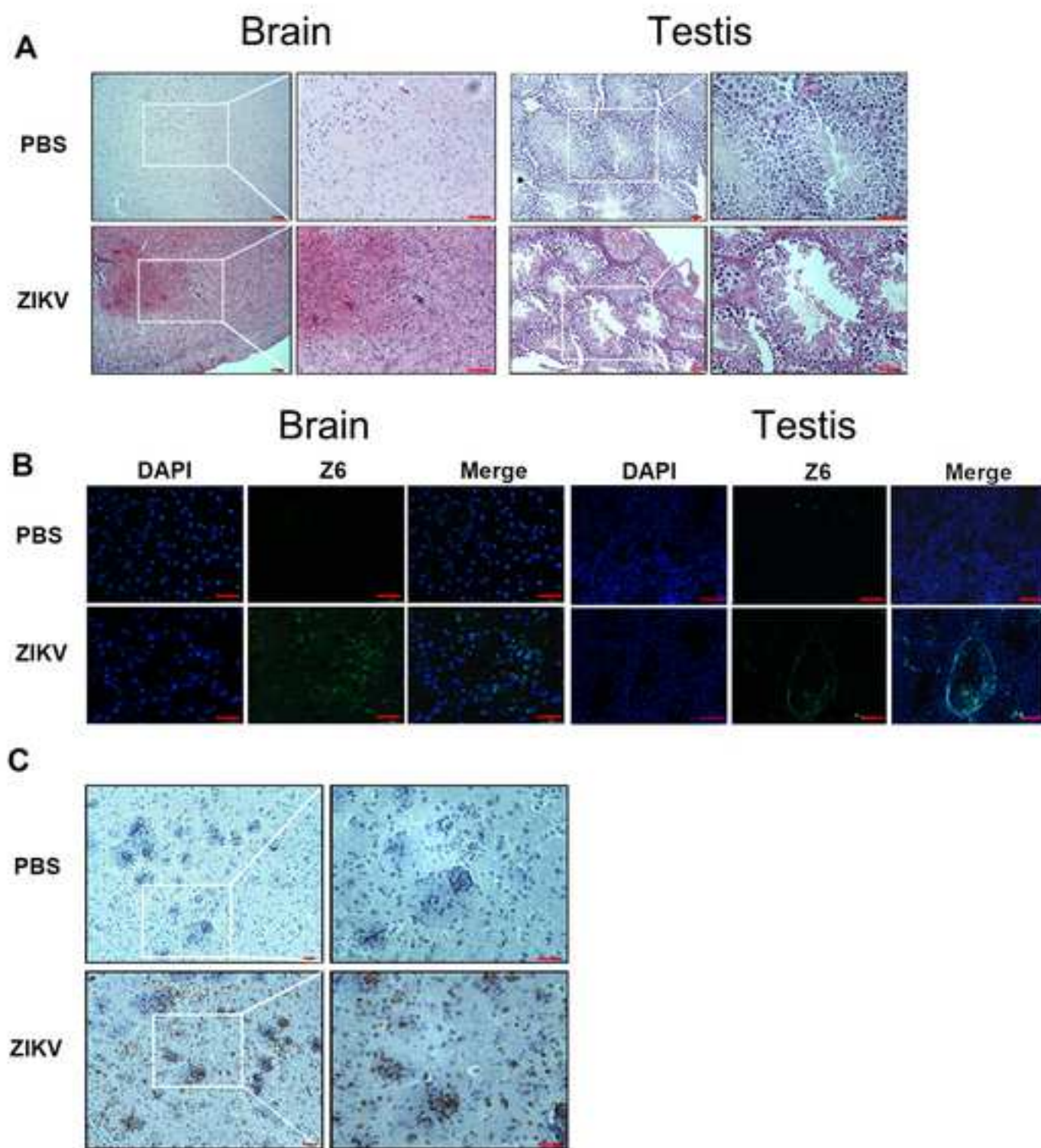
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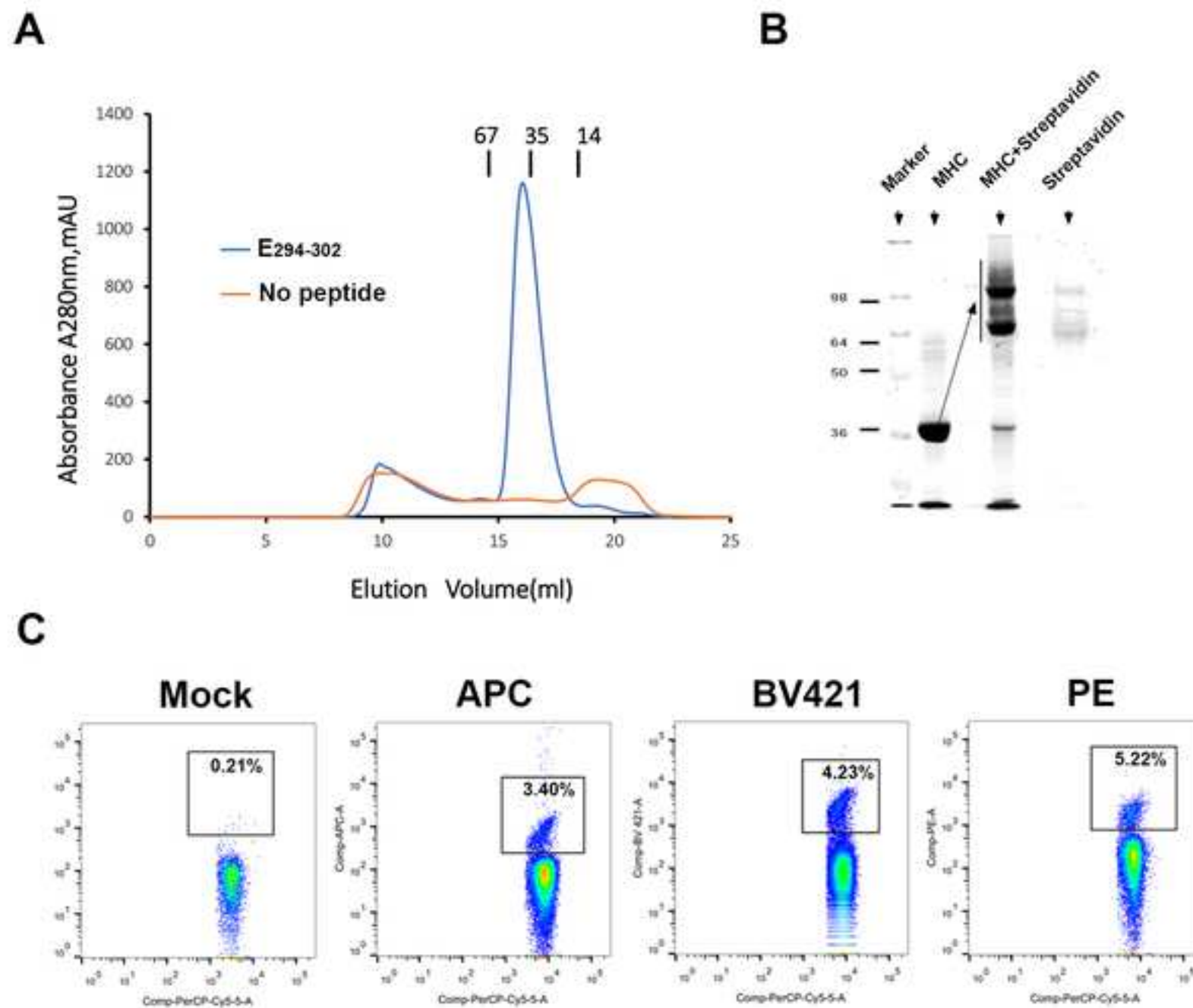
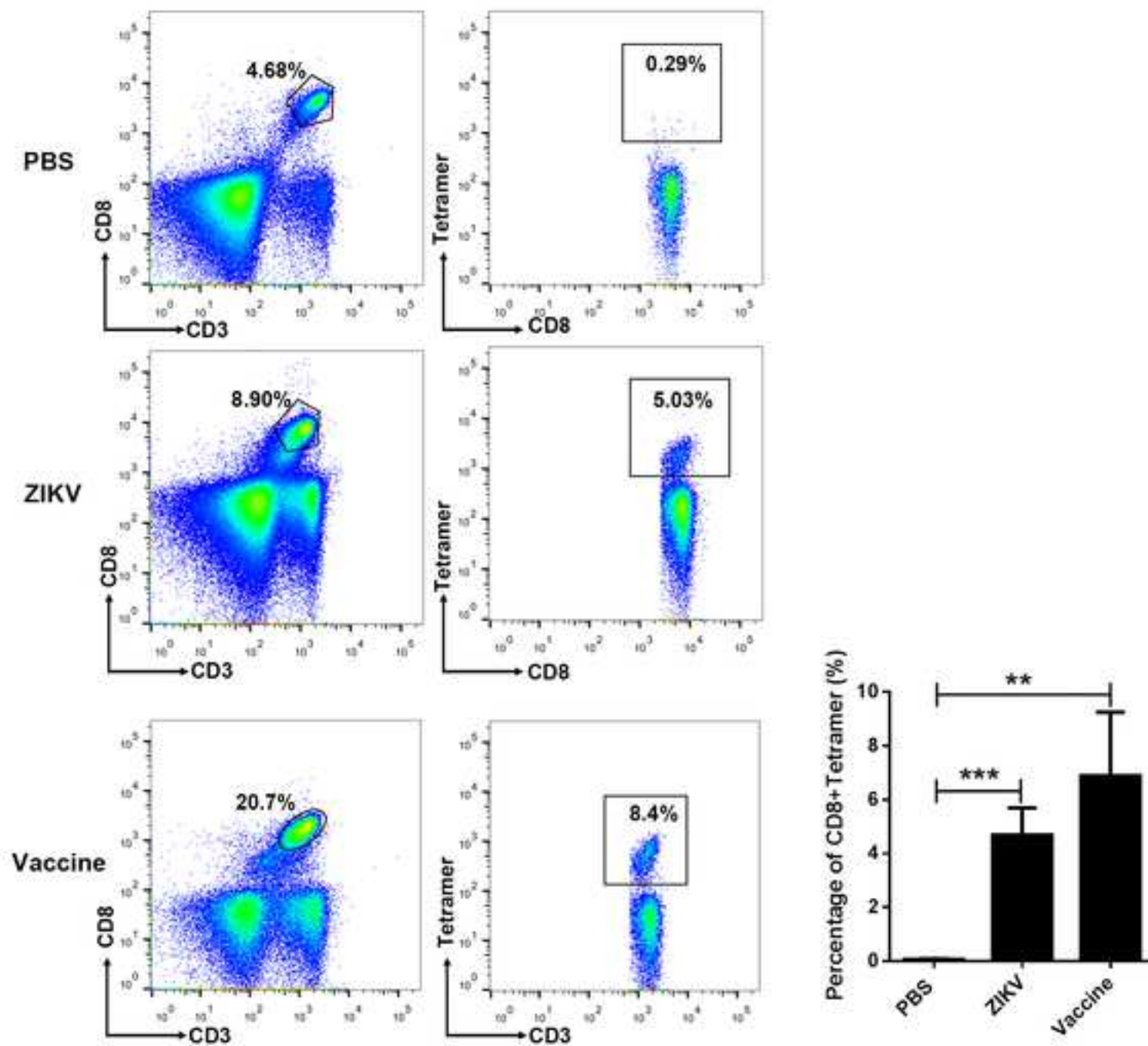
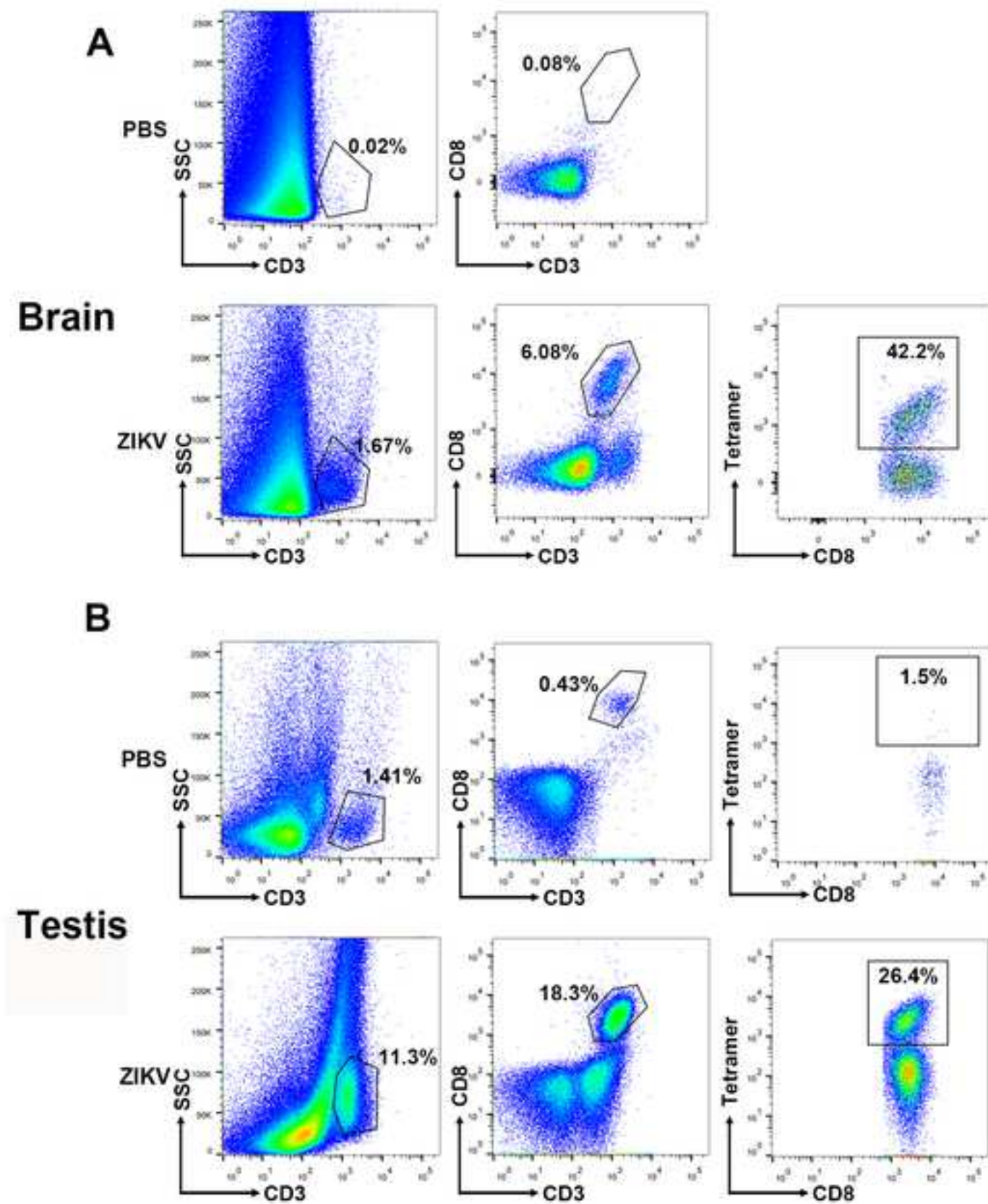


Figure 4







Name of Material/ Equipment	Company	Catalog Number
Z6	our lab	
CD3	Santa Cruz	sc-1127; RRID: AB_631128
Fluorescein-Conjuated Affinipure	ZSGB-BIO	
Goat anti-human IgG(H+L)		ZF-0308
Rabbit Anti-Goat IgG, FITC	CWBIO	
Conjugated		CW0115
FITC anti-mouse CD3	Biolegend	17A2
APC anti-mouse CD3	Biolegend	100236
PerCP anti-mouse CD8a	Biolegend	100732
Percoll	GE Healthcare	P8370
PMSF	Ameresco	0754-5G
Streptadivin	BD	S4762-FZ
Pepstatin	Sigma	P4265-5MG
Leupeptin	Sigma	L8511
Peptides	Scilight-peptide	
RPMI 1640	Hyclone	SH30809.01
DMSO	MP	219605590
APC-Streptadivin	BD	554067
FITC-Streptadivin	BD	554060
PE-Streptadivin	BD	554061
BV421-Streptadivin	BD	563259
PageRuler Unstained Protein	Thermo Fisher	
Ladder	Scientific	26614
Cell Strainers	BF	BF10-5040
Amicon Ultra 100kDa	Millipore	UFC510024
Ultracentrifuge	Thermo Fisher	
	Scientific	
FACS Cantol		



Superdex 200 Increase 10/300 GL	GE healthcare	
		28990944
AKTA PURE	GE healthcare	
red blood cell lysis buffer	Solarbio	R1010

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infertility in mice. Cell, 2016, 167: 1511-1524 e1510

Toxic and corrosive reagent. Handle with care

Gives a clear solution at 10mg/ml in PBS and stored at -20 °C

5 mg in 2.5 ml DMSO, aliquots stored at -20°C

5 mg in 2.5 ml dH 2 O, aliquots stored at - 20°C

Must be resuspended in DMSO and stored at -20 °C

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Store at room temperature.

Must be stored and maintained at 4°C. Do not freeze.

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1. The manuscript will benefit from thorough language revision (especially in the Protocol section) as there are a number of grammatical errors. Please thoroughly review the manuscript and edit any errors.

**Reply:** We recheck the manuscript to edit grammatical errors.

2. Please reduce this to 50 words.

**Reply:** A protocol to evaluate antigen-specific T-cell responses in the immunoprivileged organs of the *Ifnar1*<sup>-/-</sup> murine model for Zika virus (ZIKV) infection is described. This method is pivotal for investigating the cellular mechanisms of the protection and immunopathogenesis, this work is also valuable for efficacy evaluation of ZIKV vaccines.

3. Are the animals allowed regular access to food and water?

**Reply:** Keep adult (6-8) weeks *Ifnar1*<sup>-/-</sup> (50% male and 50% female) mice allowed regular access to food and water in standard special pathogen free (SPF) conditions.

4. What is the mouse age?

**Reply:** Keep adult (6-8) weeks *Ifnar1*<sup>-/-</sup> (50% male and 50% female) mice allowed regular access to food and water in standard special pathogen free (SPF) conditions.

5. Can you provide some examples of signs to check for?

**Reply:** tremors, stagger march start/stop, bilateral flaccid hind limb.

6. Unclear what these mice are used for after this section. Are the lymphocytes isolated from these mice in the same manner as described in Protocol Sections 2-3? If so, section 4 should be moved to before section 2

**Reply:** In previous section 4, the lymphocytes isolated from these mice in the same manner, so we move to before section 2.

7. What is the total injection volume?

**Reply:** inject 100  $\mu$ L

8. Mention needle gauge.

**Reply:** 23 G needle.

Immunize the *Ifnar1*<sup>-/-</sup> mice with ZIKV vaccine: inject 100  $\mu$ L AdC7-M/E ( $4 \times 10^{10}$  virus particle v. p) in PBS buffer via the i. m. route (intramuscular injection) using a 1 ml syringe with 23 G needle.

9. In oxygen? If so, mention flow rate.

**Reply:** in 100% oxygen (flow rate:1 L/min)

10. Mention the site of the initial incision. How large is the incision? How deep?

**Reply:** Cut the skin along the abdominal midline to the thorax with a sterile scalpel.

11. What is the FBS %?

**Reply:**10%

2 mL ice-cold RPMI containing 10% fetal bovine serum (FBS).

12. What is the buffer composition? Please add it to the table of materials.

**Reply:** red blood cell (RBC) lysis buffer ( $\text{NH}_4\text{Cl}$ ,  $\text{Na}_2\text{EDTA}$ ,  $\text{KHCO}_3$ )

complete RPMI medium (10% vol/vol FBS, 100 U/ml penicillin-streptomycin)

13. How? Mention counting method used.

**Reply:** Transfer 10  $\mu\text{L}$  of the cell suspension to a small tube, mix with 10  $\mu\text{L}$  of 0.4% w/v trypan blue and count the number of cells using a hemocytometer.

14. 1 mouse at a time? Also, please specify that this is relevant only to male mice.

**Reply:** Immobilize a male mouse in the prone position on a cutting board.

15. How? Mention any tools used.

**Reply:** Remove brain with forceps and place it in 15 mL tube containing 5 mL ice-cold RPMI/10%FBS medium.

16. Please mention all surgery steps in detail. Mention tools used.

**Reply:** Grab abdominal skin with forceps and use sharp iris scissors to make a longitudinal incision through the integument and abdominal wall and expose the lowermost part of abdomen. Push the testis up to the incision. Gently pull the fat layer with tweezers and expose a globular testis on both sides.

4.8 Use sharp iris scissors to carefully dissect the fat layer and epididymis. Place the testis in a 15 mL tube containing 5 mL ice-cold RPMI/FBS medium with forceps.

17. Plasmid construction needs a reference.

**Reply:** we insert a reference.

18. What is the source? Is it a lyophilized powder? If so, unclear how you can “inject” it using a syringe.

**Reply:** 5.3.2 Inject and dilute H-2Db and  $\beta 2\text{m}$  in-guanidine solution (stock 30 mg/mL) and peptide to the refolding solution following the order from step 5.3.2.1 to 5.3.2.3.

19. How do you inject the dry powder using a syringe? Something is missing here,

**Reply:** 5.3.2.1 Inject 500  $\mu\text{L}$   $\beta 2\text{m}$  in-guanidine solution in syringe. For this, change the needle of 5 mL syringe to a 23 G needle from 1 mL syringe and inject into refolding solution drop by drop. Keep constantly and slowly stirring at 150× g at 4°C.

What is the source? Unclear what peptide is used.



5.3.2.2 Inject 200  $\mu$ L E294-302 peptide solution. After  $\beta$ 2m has been dissolved in refolding solution, resolve 2 mg peptide in 200  $\mu$ L DMSO and directly inject into the refolding solution quickly using pipette. Slowly stir at 150 $\times$  g at 4  $^{\circ}$ C for 15 min.

20. Needle gauge? Unclear what is meant by “needle from a 1 mL syringe”

**Reply:** 5.3.2.1 Inject 500  $\mu$ L  $\beta$ 2m in-guanidine solution in syringe. For this, change the needle of 5 mL syringe to a 23 G needle from 1 mL syringe and inject into refolding solution drop by drop. Keep constantly and slowly stirring at 150 $\times$  g at 4 $^{\circ}$ C.

21. Unclear where this came from. Please check your steps for continuity. Is the refolding solution being continuously stirred? If so, mention stirring speed. How is the buffer temperature maintained?

**Reply:** 5.3.2.2 Inject 200  $\mu$ L E294-302 peptide solution. After  $\beta$ 2m has been dissolved in refolding solution, resolve 2 mg peptide in 200  $\mu$ L DMSO and directly inject into the refolding solution quickly using pipette. Slowly stir at 150 $\times$  g at 4  $^{\circ}$ C for 15 min.

22. What is the source? Unclear what peptide is used.

**Reply:** 5.3.2.3 Inject 1.5 mL H-2Db in-guanidine solution in syringe. Keep the stir bar rotating at 150 $\times$  g for the refolding of the H-2Db at 4  $^{\circ}$ C for 8-10 h. We place the solution in a closed box in a cold room.

23. What is the stirring speed? How is the buffer temperature maintained?

**Reply:** Keep the stir bar rotating at 150 $\times$  g for the refolding of the H-2Db at 4  $^{\circ}$ C for 8-10 h. We place the solution in a closed box in a cold room.

24. How is the temperature maintained? Is this done in a cold room?

**Reply:** We place the solution in a closed box in a cold room.

25. Units?

**Reply:** 5.3.3 Concentrate refolded protein in a pressurized chamber with a 10 kDa membrane.

26. Mention centrifugation speed (in g) and duration.

**Reply:** 5.3.5 Carefully transfer supernatant to a 10 kDa centrifugal filter and further concentrate to a final volume of 500  $\mu$ L at 2,500  $\times$  g for 30 min.

27. Please replace the commercial name with a generic alternative.

**Reply:** we replace the commercial name.

28. How is this done? Please provide a reference or describe the steps.

**Reply:** we cited a reference.

29. What are the compositions (and concentrations) of solutions A and B?

**Reply:** 5.4 To generate a 500  $\mu\text{L}$  reaction volume for biotinylation, add the reagents in order: 100  $\mu\text{L}$  solution A (0.5M bicine PH8.3), 100 $\mu\text{L}$  solution B (100 mM ATP,100mM MgOAc,200 $\mu\text{M}$  biotin), 100  $\mu\text{L}$  extra d-biotin (500 $\mu\text{M}$  biotin), 20  $\mu\text{L}$  BirA enzyme (60 $\mu\text{g}$ ), 0.5  $\mu\text{L}$  pepstatin (2 mg/mL) and 0.5  $\mu\text{L}$  leupeptin (2 mg/mL). Incubate the reaction tube overnight at 4°C.

30. What is the concentration?

**Reply:** 5.4 To generate a 500  $\mu\text{L}$  reaction volume for biotinylation, add the reagents in order: 100  $\mu\text{L}$  solution A (0.5M bicine PH8.3), 100 $\mu\text{L}$  solution B (100 mM ATP,100mM MgOAc,200 $\mu\text{M}$  biotin), 100  $\mu\text{L}$  extra d-biotin (500 $\mu\text{M}$  biotin), 20  $\mu\text{L}$  BirA enzyme (60 $\mu\text{g}$ ), 0.5  $\mu\text{L}$  pepstatin (2 mg/mL) and 0.5  $\mu\text{L}$  leupeptin (2 mg/mL). Incubate the reaction tube overnight at 4°C.

31. Which buffer?

**Reply:** 5.6.1 Prepare three samples A, B and C on ice for 30 min. Then analyze the results by a 10% SDS-PAGE. A: 8  $\mu\text{L}$  biotinylated MHC molecules + 2  $\mu\text{L}$  exchange buffer; B: 8  $\mu\text{L}$  biotinylated MHC molecules + 2  $\mu\text{L}$  streptavidin (20 mg/mL); C: 2  $\mu\text{L}$  streptavidin (20 mg/mL) + 8  $\mu\text{L}$  exchange buffer.

32. Unclear what you are doing here because multiple steps are missing. Please describe all steps for multimerization.

**Reply:**5.7 Multimerization of biotinylated MHC molecules.

5.7.1 To produce tetramer by mixing the biotinylated E294-302 peptide-H2Db complex with phycoerythrin-labeled streptavidin at mole ratio of 1:5 to ensure a complete binding of all the biotinylated MHC molecules.

5.7.2 Calculation of the amount of streptavidin-conjugate needed for tetramerization.

5.7.2.1 Determine moles MHC/ peptide complexes accounting for the protein concentration and the MW (example: 1.8 mg total protein = 40 nmoles).

5.7.2.2 Calculate moles streptavidin-conjugate needed by dividing moles MHC/ peptide by 5 (example: 40: 5 => 8 nmoles streptavidin-conjugate).

5.7.2.3 Calculate the amount of streptavidin needed (in  $\mu\text{g}$ ) depending on the streptavidin-conjugate (example: streptavidin-PE [ 300,000 g/ M] ->8 nmoles needed => 2400 g).

5.7.3 Divide streptavidin-phycoerythrin into 10 samples. Add each sample to a brown tube containing biotinylated E294-302 peptide-H2Db complex at an interval of 20 min. After loading the last sample, incubate the reaction brown tube at 4°C overnight in dark.

33. How?

**Reply:** Fill up total volume again to 500  $\mu\text{L}$  using PBS (pH 8.0). Concentrate to an estimated concentration of 2-2.5 mg/mL at 2000 x g at 4°C. Store in the dark at 4°C.

34. How? Mention centrifugation speed and duration.

**Reply:** Concentrate to an estimated concentration of 2-2.5 mg/mL at 2000 x g at 4°C. Store in the dark at 4°C.

35. Unclear what the various “tests” are, they were not described previously.

**Reply:** in section 1-4, we isolate lymphocyte from virus-infected mice, in section 5, we prepare E<sub>294</sub> tetramer, therefore, we want to detect virus-specific T lymphocyte by flow cytometry. In the section, we use the lymphocyte from section 3-4 and tetramer from section 5. Considering that the requirement of the protocol video, we highlight the section.

36. Which one? From 2.9? from 3.14?

**Reply:** 6.1 Incubate the cell suspension from step 3.9 and step 4.15 at 4 °C with 0.1 µL anti-murine CD16/CD32 Fc-Receptor blocking reagent (dilution factor 1:200) per 20 µL for 10 min to prevent unspecific binding.

37. How much blocking reagent?

**Reply:** 6.1 Incubate the cell suspension from step 3.9 and step 4.15 at 4 °C with 0.1 µL anti-murine CD16/CD32 Fc-Receptor blocking reagent (dilution factor 1:200) per 20 µL for 10 min to prevent unspecific binding.

38. Mention all the missing steps. Where is the test performed (e.g in a well plate)?

How much cell suspension is used per test?

**Reply:** 6.4 Prepare enough the E<sub>294</sub>-302 tetramer mix to stain all experimental tubes. Prepare an excess of 15% of the total volume of this mix to account for pipetting error. Dilute E<sub>294</sub>-302 tetramers (2 mg/mL ,1 µL /test) in FACS buffer(PBS,0.5%FBS) so that 20 µL of the E<sub>294</sub>-302 tetramer mix are added to each test.

6.5 Add 20 µL of the E<sub>294</sub>-302 tetramer mix to the 96 well plate. By the end of this step, the final volume in each well should be 40 µL. Incubate in the dark at room temp for 30 min.

39. What is the final tetramer concentration?

**Reply:** Dilute E<sub>294</sub>-302 tetramers (2 mg/mL ,1 µL /test) in FACS buffer(PBS,0.5%FBS) so that 20 µL of the E<sub>294</sub>-302 tetramer mix are added to each test.

40. But later you say 25 uL per test. Please double check for consistency.

**Reply: we recheck the mistake to make consistency, thank you.**

41. What are the antibody concentrations?

**Reply:** 6.6 Add primary antibodies FITC-conjugated or APC-conjugated anti-CD3(0.2 mg/mL), PerCP-conjugated anti-CD8(0.2 mg/mL) at 1 µL /test to the cell suspension, then incubate at 4 °C for 30 min in the dark.

42. Please add a step to mention how the analysis is performed. Describe the gating

strategy used.

**Reply:** 6.9 Gating strategy for flow cytometric analysis.

6.9.1 Create a gate on diagonally clustered singlets by plotting forward scatter (FSC) versus side scatter (SSC) area.

6.9.2 Then gate on CD3<sup>+</sup> cells by side scatter (SSC) versus CD3, next gate on CD3<sup>+</sup> CD8<sup>+</sup> cell, finally, outline CD8<sup>+</sup>tetramer<sup>+</sup> cell.

43. Which tissues? From which mouse? Infected mice or immunized mice? When are the tissues collected?

**Reply:** 7.1.1 Collect brain and testis tissues of infected the *Ifnar1*<sup>-/-</sup> mice with ZIKV at 7 days and fix in 4% neutral-buffered formaldehyde.

44. Needs a note of caution for use

**Reply:** CAUTION: Paraformaldehyde is toxic; wear appropriate personal protective equipment.

45. How? Using a vibratome?

**Reply:** 7.1.3 Section the tissue at 5 mm using a vibratome.

46. How are these steps performed? Each needs to be described or a reference must be cited.

**Reply:** we cited a reference.

47. Antibody concentration?

**Reply:** 7.2.3 Incubate the tissue sections with rat anti-mouse CD3 antibody (dilute factor:1/1000) for 8h at room temperature, then incubate at 4°C overnight.

48. Concentration and volume? Incubate for how long and at what temperature?

**Reply:** 7.2.4 Rinse with PBS, then incubate with 3 drops of biotinylated secondary antibody (dilute factor:1/1000) for 2h at room temperature followed by 3 drops of avidin-biotin-peroxidase (dilute factor:1/200) at room temperature for 30min.

49. Concentration and volume? Incubate for how long and at what temperature?

**Reply:** 7.2.4 Rinse with PBS, then incubate with 3 drops of biotinylated secondary antibody (dilute factor:1/1000) for 2h at room temperature followed by 3 drops of avidin-biotin-peroxidase (dilute factor:1/200) at room temperature for 30min.

50. Please describe the steps. Mention concentration and volume of 3, 3'-diaminobenzidine tetrahydrochloride

**Reply:** 7.2.5 Bind with 3 drops of 3, 3'-diaminobenzidine tetrahydrochloride (dilute factor:1/1000), which was performed based on a procedure described previously.

51. Concentration and volume?

**Reply:** 7.3.4 Incubate with 3 drops of the primary antibody (Z6) (20 µg/ml) at 4°C overnight.

52. Define. Unclear what this is. Please ensure that the steps to do this are described in the protocol.

**Reply:** Plaque Assay. we detect the virus titer using Plaque Assay. Because it is not the key section in our protocol, we delete the relevant result.

53. How and when is this test performed? Please ensure that is was described in the protocol.

**Reply:** we use E294-302 tetramer from section 5 to stain the lymphocyte from section 3, then we detect ZIKV-specific T-lymphocytes by flow cytometry in section 6.

54. Lymphocytes in the splenocytes? Do you mean “in the spleen”?

**Reply:** we correct the mistake. Using the E294-302-tetramer, we detected ZIKV-specific T-lymphocytes in the spleen of the infected mice by flow cytometry at the 7d. p. i of ZIKV ( $3.49 \pm 0.4508\%$ ). Also, we use the same manner as described in Protocol Sections 3 to isolate lymphocytes from 4 weeks post-immunization with the AdC7-M/E vaccine, then we detected ZIKV-specific T-lymphocytes in the spleen ( $6.89 \pm 1.36\%$ ) (Figure 4).

55. Are the lymphocytes isolated from these mice in the same manner as described in Protocol Sections 2-3?

**Reply:** Yes, we isolate the lymphocytes in the same manner as described in Protocol Sections 3-4.

56. Please add a space between Numeral and “day” e.g. “0 days”, “7 days” etc.

**Reply:** we correct in figure1.

57. Mention what was stained for and the color coding.

**Reply:** Immunohistochemistry shows robust infiltration of CD3+ T-cells into brain and testis. Scale bar: 25 µm (left) and 50 µm (right). Purple indicates hematoxylin, brown represents CD3 antibody.

58. Define pfu/g. how was this calculated? Please add these details to the protocol.

**Reply:** we detect the virus titer using Plaque Assay. Because it is not the key section in our protocol, we delete the relevant result.

59. What does “mock” represent here?

**Reply:** we use tetramer to stain the lymphocyte from the mouse in PBS group. The mock represents PBS group.

60. Please expand the discussion to cover the following in detail: modifications and

troubleshooting, limitations of the technique, critical steps within the protocol.

**Reply:** modifications and troubleshooting

Compared to previous work, in our study, we established systematic methods to detect ZIKV-specific CD8<sup>+</sup> T cell responses in the brain and testes, which are immunoprivileged sites. It is important to assess the functionality of virus-specific T-cells in the immunoprivileged organs of the ZIKV-infected mice. The usage of tetramers to detect ZIKV-specific CD8<sup>+</sup> T-cell responses in immunoprivileged organs would greatly enhance our understanding of ZIKV infection and host immune responses. Using E294-302 tetramer, we can isolate virus-specific T-cells in brain and testis by flow cytometry to investigate the cellular mechanisms of the protection and immunopathogenesis during ZIKA infection. Meanwhile, it is helpful for researchers to furtherly investigate the functions of the CD8<sup>+</sup> T-cells to control ZIKV, or to enhance the immunopathogenesis in these organs during ZIKV infection.

To analyze the antigen-specific murine CD8<sup>+</sup> T-cell responses in the immunoprivileged organs, we prepared H-2Db-E294-302 tetramer and detected the CD8<sup>+</sup> T cells by flow cytometry. Tetramer is a powerful tool to detect antigen-specific T-cell. Here, we generated three types of fluorochrome-conjugated streptavidin (APC, PE and BV421). We found that although there is no statistically significant differences in APC-, BV421-and PE-labeled tetramers for detecting antigen specific-T cells, PE-labeled pMHC-I tetramers yielded the best results in our hands. Hence, we chose to use the PE-labeled tetramer throughout the study. Interestingly, based on the PE-labeled H-2Db-E294-302 tetramer, we detected high ratios of antigen-specific T-cells in both the brain and testes, which indicate the migration ability of the virus-specific T-cells from the blood to immunoprivileged organs.

limitations of the technique

However, there exist some limitations in the protocol. The H-2Db-E294-302 tetramer is not special for human T-cell detection, because tetramer detection is dependent on MHC restriction. Screening of immunodominant HLA-restricted peptides is still needed. Besides, retro-orbital infection is effective for ZIKV infection but might be not a convenient operation for some investigators, other routes of infection including peritoneal, subcutaneous or intravenous are also recommended.

critical steps within the protocol

In our protocol, the critical steps are about isolation of monocytes from brain and testis, it is vital important to acquire high quality lymphocytes. For example, centrifugal speed, the strength of the grinding tissue and the dissection of brain and testis tissue. Besides, for tetramer preparation, protease inhibitor (PMSF, pepstatin, leupeptin) is helpful to protect protein from being degraded, therefore, we should add protease inhibitor to refolding buffer and exchange buffer during the process of tetramer preparation.