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.

1. Please reduce this to 50 words.

**Reply:** A protocol to evaluate antigen-specific T-cell responses in the immunoprivileged organs of the Ifnar1-/- 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.

Are the animals allowed regular access to food and water?

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

What is the mouse age?

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

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

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

1. 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.

What is the total injection volume?

**Reply:** inject 100 µL

Mention needle gauge.

**Reply:** 23 G needle.

Immunize the Ifnar1-/- mice with ZIKV vaccine: inject 100 µL AdC7-M/E (4 x 1010 virus particle v. p) in PBS buffer via the i. m. route (intramuscular injection) using a 1 ml syringe with 23 G needle.

In oxygen? If so, mention flow rate.

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

1. 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.

What is the FBS %?

**Reply:**10%

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

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

**Reply:** red blood cell (RBC) lysis buffer (NH4Cl, Na2EDTA, KHCO3)

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

1. How? Mention counting method used.

**Reply:** Transfer 10 µL of the cell suspension to a small tube, mix with 10 µL of 0.4% w/v trypan blue and count the number of cells using a hemocytometer.

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

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

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.

1. 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.

Plasmid construction needs a reference.

**Reply:** we insert a reference.

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 β2m 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.

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

**Reply:** 5.3.2.1 Inject 500 µL β2m 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 µL E294-302 peptide solution. After β2m has been dissolved in refolding solution, resolve 2 mg peptide in 200 µL DMSO and directly inject into the refolding solution quickly using pipette. Slowly stir at 150× g at 4 °C for 15 min.

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

**Reply:** 5.3.2.1 Inject 500 µL β2m 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.

1. Unclear where this came from. Please check you 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 µL E294-302 peptide solution. After β2m has been dissolved in refolding solution, resolve 2 mg peptide in 200 µL DMSO and directly inject into the refolding solution quickly using pipette. Slowly stir at 150× g at 4 °C for 15 min.

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× g for the refolding of the H-2Db at 4 °C for 8-10 h. We place the solution in a closed box in a cold room.

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

**Reply:** Keep the stir bar rotating at 150× g for the refolding of the H-2Db at 4 °C for 8-10 h. We place the solution in a closed box in a cold room.

1. How is he temperature maintained? Is this done in a cold room?

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

1. Units?

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

1. 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 µL at 2,500 x g for 30min.

Please replace the commercial name with a generic alternative.

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

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

**Reply:** we cited a reference.

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

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

What is the concentration?

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

1. 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 µL biotinylated MHC molecules + 2 µL exchange buffer; B: 8 µL biotinylated MHC molecules + 2 µL streptavidin (20 mg/mL); C: 2 µL streptavidin (20 mg/mL) + 8 µL exchange buffer.

1. 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 µg) 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.

1. How?

**Reply:** Fill up total volume again to 500 µL 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.

1. 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.

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

**Reply:** in section1-4, we isolate lymphocyte from virus-infected mice, in section 5, we prepare E294 tetramer, therefore, we want to detect virus-specific T lymphocyte by flow cytometry. In the section, we use the lymphocyte from section3-4 and tetramer from section5. Considering that the requirement of the protocol video, we highlight the section.

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 20µl for 10 min to prevent unspecific binding.

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 20µl for 10 min to prevent unspecific binding.

1. 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 E294-302 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 E294-302 tetramers (2 mg/mL ,1 µL /test) in FACS buffer(PBS,0.5%FBS) so that 20 µl of the E294-302 tetramer mix are added to each test.

6.5 Add 20 µl of the E294-302 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.

What is the final tetramer concentration?

**Reply:** Dilute E294-302 tetramers (2 mg/mL ,1 µL /test) in FACS buffer(PBS,0.5%FBS) so that 20 µl of the E294-302 tetramer mix are added to each test.

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

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

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.

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+ cells by side scatter (SSC) versus CD3, next gate on CD3+ CD8+ cell, finally, outline CD8+tetramer+ cell.

Which tissues? From which mouse? Infected mice of immunized mice? When are the tissues collected?

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

Needs a note of caution for use

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

How? Using a vibratome?

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

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

**Reply:** we cited a reference.

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.

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.

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.

Please describe the steps. Mention concentration and volume of 30, 30-diaminobenzidine tetrahydrochloride

**Reply:**7.2.5 Bind with 3 drops of 30, 30-diaminobenzidine tetrahydrochloride (dilute factor:1/1000), which was performed based on a procedure described previously.

Concentration and volume?

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

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.

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.

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± 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 ± 1.36%) (Figure 4).

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.

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

**Reply:** we correct in figure1.

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.

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.

What does “mock” represent here?

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

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+ 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+ 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+ T-cells to control ZIKV, or to enhance the immunopathogenesis in these organs during ZIKV infection.

To analyze the antigen-specific murine CD8+ T-cell responses in the immunoprivileged organs, we prepared H-2Db-E294-302 tetramer and detected the CD8+ 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.