An inactivated recombinant rabies virus displaying the Zika virus prM-E induces protective immunity against both pathogens

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Abstract

The global spread of Zika virus (ZIKV), which caused a pandemic associated with Congenital Zika Syndrome and neuropathology in newborns and adults, prompted the pursuit of a safe and effective vaccine. Here, three kinds of recombinant rabies virus (RABV) encoding the ZIKV prM-E protein were constructed: ZI-D (prM-E), ZI-E (transmembrane domain (TM) of prM-E replaced with RABV G) and ZI-F (signal peptide and TM domain of prM-E replaced with the region of RABV G). When the TM of prM-E was replaced with the region of RABV G (termed ZI-E), it promoted ZIKV E protein localization on the cell membrane and assembly on recombinant viruses. In addition, the change in the signal peptide with RABV G (termed ZI-F) was not conducive to foreign protein expression. The immunogenicity of recombinant viruses mixed with a complex adjuvant of ISA 201 VG and poly(I:C) was tested in BALB/c mice. After immunization with ZI-E, the anti-ZIKV IgG antibody lasted for at least 10 weeks. The titers of neutralizing antibodies (NAbs) against ZIKV and RABV at week 6 were all greater than the protective titers. Moreover, ZI-E stimulated the proliferation of splenic lymphocytes and promoted the secretion of cytokines. It also promoted the production of central memory T cells (TCMs) among CD4+/CD8+ T cells and stimulated B cell activation and maturation. These results indicate that ZI-E could induce ZIKV-specific humoral and cellular immune responses, which have the potential to be developed into a promising vaccine for protection against both ZIKV and RABV infections.

Author summary

There is no approved vaccine for Zika virus (ZIKV) disease. Many research efforts are ongoing, e.g., inactivated, DNA, or viral vector vaccines. However, due to the complexity of the pathogenesis and immunology of ZIKV, new ideas about vaccine development still need to be considered. Rabies virus (RABV) vectored vaccines have been developed for...
many viruses, such as against Lassa virus, canine distemper virus, Middle East respiratory syndrome coronavirus, and filovirus, based on the advantages of the vector. In this study, three recombinant RABVs expressing ZIKV prM-E, named ZI-D, ZI-E and ZI-F, are described. Since ZI-D and ZI-E could express foreign proteins successfully, the author evaluated the immunogenicity of ZI-D and ZI-E mixed with a complex adjuvant of ISA 201 VG and poly(I:C) in BALB/c mice. The study demonstrates that ZI-E induced mice to produce NAbs against both RABV and ZIKV and elicited specific cellular immune responses. The authors believe that the ZI-E vaccine based on the RABV vector has the potential to prevent ZIKV and RABV infections. It has the potential to be used in ZIKV-RABV binary vaccines in areas where both ZIKV and RABV are threats.

Introduction

Zika virus (ZIKV), which belongs to the family *Flaviviridae* and the genus *Flavivirus*, is an arbovirus and was first isolated from a febrile rhesus macaque in Uganda in 1947 [1]. In May 2015, a large-scale outbreak of ZIKV disease occurred in Brazil, showing a trend of spread and cross-border transmission. According to a World Health Organization (WHO) report, as of July 2019, a total of 87 countries and territories had cases of infection [2]. Fever, rash, arthralgia and conjunctivitis are the main clinical symptoms, and the disease rarely causes death in adults, but it can cause a high risk of Congenital Zika Syndrome (CZS, a combination of severe neurological anomalies) in newborn babies after infection of pregnant women [3,4].

Currently, there is no approved vaccine or effective treatment for ZIKV disease. However, intensive research efforts in this field have been ongoing. Using other viruses as vectors to express the main structural proteins of ZIKV is a strategy to develop ZIKV vaccines, including the recombinant replication-deficient adenovirus [5], the rhesus adenovirus serotype 52 vectored vaccine [6], the attenuated poxvirus vectored vaccine [7], and the attenuated recombinant vesicular stomatitis virus vectored vaccine [8]. However, due to the complexity of the pathogenesis and immunology of ZIKV, there are many uncertainties associated with the development of vaccines [9,10]. New ideas and methods still need to be further considered [11]. Several important characteristics recommend rabies virus (RABV) vectors as vaccine delivery platforms. First, RABV can be replicated and transcribed with high efficiency in target cells to produce abundant virus-specific proteins that favor the packaging of the recombinant virus. Furthermore, since few people possess serum antibodies against RABV, preexisting RABV seropositivity is negligible [12]. Using reverse genetic manipulation techniques, novel RABV vectored vaccines have been developed for many viruses based on the advantages of the vector [12], such as against Lassa virus (LASV) [13], canine distemper virus (CDV) [14], Middle East respiratory syndrome coronavirus (MERS-CoV) [15] and filovirus [16].

The E protein, a structural protein, is the only envelope glycoprotein of ZIKV and plays important roles in the molecular recognition, pathogenesis and immune process of the virus. E protein containing neutralizing epitopes has been targeted in ZIKV vaccine research [17–20]. PrM-E can be cleaved into the prM and E proteins by host cell signal peptidase [21], and prM plays a critical role in folding the E protein [18]. In this study, a recombinant RABV expressing ZIKV prM-E protein was constructed based on a RABV reverse genetic operating system [22]. Humoral and cellular immune responses were stimulated after intramuscular immunization in mice.
Materials and methods

Ethics statement

All of the mice were treated in accordance with the Chinese ethical guidelines for the welfare of laboratory animals (GB 14925–2001). The study was approved by the Animal Welfare and Ethics Committee of the Institute of Veterinary Medicine of the Military Academy of Sciences (Laboratory Animal Care and Use Committee Authorization permit number JSY-DW-2018-02).

Viruses, cells and antibodies

The recombinant viruses were grown in suspended baby hamster kidney (BHK-21) cells that were maintained in CD BHK-21 Production Medium (Thermo Fisher Scientific, Waltham, MA, USA) with 1% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) at 37°C and 120 rpm. The ZIKV ZKC2 2016 strain was cultivated in C6/36 cells (10% MEM with 10% FBS) at 27°C. BSR cells, which are a cloned cell line derived from BHK-21 cells, were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY, USA) that contained 5% FBS. NA cells were maintained in DMEM that contained 10% FBS.

A fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) against RABV N protein (800–092) was purchased from Fujirebio (Melvin, PA, USA). An anti-RABV G mAb (MAB8727) was purchased from Millipore (Billerica, MA, USA). A rabbit anti-ZIKV E polyclonal antibody (GTX133314) was purchased from GeneTex (Alton PkwyIrvine, CA, USA). TRITC-conjugated goat anti-mouse IgG (T5393) was purchased from Sigma (St. Louis, MO, USA). FITC-conjugated goat anti-rabbit IgG (ab6717), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (ab6789), HRP-conjugated goat anti-rabbit IgG (ab6721), donkey anti-mouse IgG H&L (ab39593, 10 nm gold) and donkey anti-rabbit IgG H&L (ab105296, 18 nm gold) were purchased from Abcam (Cambridge, MA, USA).

cDNA construction of vaccine vectors

The reverse genetic operating system of the vaccine vector RABV SRV9 has been described previously [22]. In this vector, an exogenous gene expression component “PE-PS-BsiWI-PmeI” was introduced, and foreign proteins could be expressed through the two restriction sites “BsiWI-PmeI”. Full-length ZIKV prM-E cDNA was retrieved from GenBank (Accession: KX601168.1). The TM or signal sequence region of ZIKV prM-E was replaced by the corresponding regions of the RABV SRV9 strain (Accession: KX601168.1). Foreign genes were optimized for mammalian cells and synthesized by Sangon Biotech (Shanghai, China), and genes were introduced into the vector RABV SRV9 using BsiWI I and PmeI I restriction digestion sites. Three kinds of full-length viral cDNA containing ZIKV prM-E were constructed, namely ZI-D (full-length prM-E), ZI-E (full-length prM-E with TM region replaced by the corresponding region of SRV9) and ZI-F (full-length prM-E with the signal sequence and TM replaced by SRV9).

The recombinant viruses were recovered as described previously [22]. Briefly, Lipofectamine 3000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) was used to cotransfect the full-length viral cDNA along with the helper plasmids (encoding the RABV N, P, G and L proteins, respectively) into BSR cells. Seven days later, the supernatants were harvested and analyzed by immunostaining for RABV N.

Immunofluorescence analysis (IFA)

For detection or titration of RABV, NA cells were seeded in 96-well plates and infected with tenfold serial dilutions of viruses (50 μl/well). Each dilution was performed in quadruplicate.
Forty-eight hours later, the cells were fixed with 80% cold acetone, and a FITC-conjugated anti-RABV N mAb (1:200) served as the detection signal for RABV. Fluorescence was observed under a fluorescence microscope (Olympus, Tokyo, Japan). The titer of RABV was calculated according to the Reed-Muench method.

For confocal microscopy analysis, NA cells were seeded on confocal dishes and infected at an MOI of 1 with the different viruses. Forty-eight hours later, the cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.2% Triton X-100 or nonpermeabilized. After blocking with 1% BSA, the cells were incubated with an anti-RABV G mAb (1:100) and an anti-ZIKV E polyclonal antibody (1:100). Then, a TRITC-conjugated anti-mouse antibody (1:500) and a FITC-conjugated anti-rabbit antibody (1:300) were added to the corresponding primary antibodies. The cells were stained with 4,6-diamidino-2-phenylindole (DAPI) and imaged with a confocal microscope.

Electron microscopy analysis
Recombinant RABVs were negatively stained with uranyl acetate and then examined under an electron microscope. For immunoelectron microscopy, after attachment to copper mesh, the samples were double stained with an anti-RABV G mAb (1:50) and a rabbit anti-ZIKV E polyclonal antibody (1:50). After they were washed with PBS, the samples were labeled with donkey anti-rabbit IgG (18 nm gold, 1:20) and donkey anti-mouse IgG (10 nm gold, 1:20). The stained samples were examined under an electron microscope.

One-step growth curves
BHK-21 suspension cells in 30-ml volumes were infected with the recombinant viruses and RABV SRV9 at MOIs of 0.1, 0.5 and 1, and samples of 200 μl were harvested every 24 h. The samples harvested from 1–4 days post infection (dpi) were titrated in NA cells as described above.

Virus purification
Recombinant RABV was inoculated into BHK-21 suspension cells at an MOI of 0.5 for large-scale purification. The supernatant was collected on the 2nd and 4th days and replaced with fresh medium. After centrifugation at 3000 rpm for 30 min, the viruses in the supernatants were precipitated with zinc acetate. After resuspension, the viruses were purified by ultracentrifugation through a 10–20–30–40–55% sucrose gradient for 1.5 h at 50,200 g. The viruses that remained in 30–40% sucrose were resuspended in STE (0.15 M NaCl, 0.001 M EDTA, 0.01 M Tris-base, pH = 7.4) and inactivated using beta-propiolactone (BPL) (Serva Electrophoresis GmbH, Heidelberg, Germany) at a 1:3000 dilution. After incubation at 4˚C for 24 h, BPL was hydrolyzed at 37˚C for 2 h. The protein concentrations of the inactivated viruses were measured using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and western blot (WB)
Purified viruses were denatured in loading buffer at 100˚C for 5 min. 30 μg of denatured proteins were separated by 10% SDS-PAGE. For total protein analysis, the gels were stained with Coomassie brilliant blue. For WB, the proteins separated by SDS-PAGE were transferred from the gels to nitrocellulose (NC) membranes (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for immunoblot analysis. Polyclonal rabbit anti-ZIKV E antibodies (1:700) were used as
the primary antibodies, and HRP-conjugated goat anti-rabbit IgG (1:5000) was used as the secondary antibody. Electrochemiluminescence (ECL) Western Blotting Substrate (Pierce, IL, USA) was added, and the bands were captured using a Tanon-5200 Chemiluminescent Imaging System (Tanon, Shanghai, China).

Immunizations in mice
Female BALB/c mice (6–8 weeks) were randomly divided into 3 groups (n = 9/group) and vaccinated intramuscularly (IM) with ZI-D or ZI-E mixed with a complex adjuvant of ISA 201 VG (containing special mineral oil, Seppic, Paris, France) and poly(I:C) (Sigma, St. Louis, MO, USA). To ensure the immune effect, we chose twice the dose of references [15,16,23], i.e., 20 μg as the immune dose. Each milliliter of vaccine contains 55% volume ratio of ISA 201 VG and 200 μg of poly(I:C). Mice that received PBS were used as the controls. Nine mice from each group were boosted twice at 2-week intervals. The sera were collected at 0-, 2-, 5-, and 8-weeks post immunization (wpi) and heat inactivated at 56˚C for 30 min.

ELISA
Indirect ELISA was performed to detect the IgG titer or the ratio of IgG2a/IgG1 in serum. Purified ZIKV E protein from insect cells (MyBioSource, San Diego, CA, USA) was diluted in coating buffer (50 mM Na₂CO₃, pH 9.6) at a concentration of 0.5 μg/ml and then plated in 96-well microtiter plates (Corning-Costar, Corning, NY, USA) at a volume of 100 μl/well. The plates were incubated at 4˚C overnight. After blocking with 5% nonfat milk, the plates were incubated with twofold serial dilutions of sera in blocking buffer. Next, HRP-conjugated goat anti-mouse IgG (H+L) (1:2000, BioWorld, St. Louis, MO, USA), IgG1, or IgG2a (1:2000, Southern Biotech, Birmingham, AL, USA) was added. Then, tetramethylbenzidine (TMB, Sigma, St. Louis, MO, USA) was added as an acidic buffer, and 2 M H₂SO₄ was used to stop color development. The absorbance was read at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Neutralization titer assay
A virus neutralization assay (VNA) for ZIKV was performed as previously described [11]. Briefly, twofold serial dilutions of heat-inactivated serum were mixed with equal volumes of ZIKV (500 PFU/ml). After incubation for 1 h at 37˚C, 200 μl of the mixture was transferred to monolayer BHK cells in 12-well plates, and the cells were incubated for another 1 h. Then, the inoculum was removed and replaced with semisolid agarose medium. After incubation for four days, the cells were fixed and stained with crystal violet. The titers were determined by a standard 50% plaque reduction neutralization test (PRNT₅₀).

A VNA for RABV was conducted using a fluorescent antibody virus neutralization (FAVN) test. Briefly, 100 μl volumes of threefold serial dilutions of serum were mixed with 100 TCID₅₀ of CVS-11 stain in 96-well plates, and the mixtures were incubated for 1 h at 37˚C. Then, NA cells were added and incubated for 48 h at 37˚C. The cells were fixed with cold 80% acetone and stained with FITC-conjugated anti-RABV N antibodies (1:200). The positive/negative wells were determined under a fluorescence microscope, and the titers of RABV NAbs are expressed in IU/ml by comparison with a standard serum sample (0.5 IU/ml).

Splenocyte proliferation assay
One week after the last immunization, three mice from each group were euthanized. Mouse spleens were collected and disrupted with the plunger of a syringe. The splenocyte suspensions
were strained through a 100 μm mesh, and red blood cells were lysed by erythrocyte lysis buffer (Solarbio, Beijing, China). Then, 2.5×10^5 splenocytes (suspended in complete RPMI 1640), along with purified ZIKV E antigen (10 μg/ml), were plated in 96-well plates for culture. After 44 h, 10 μl of commercial TransDetect Cell Counting Kit-8 (CCK-8) reagent (KeyGEN Biotech, Nanjing, China) was added to the cells, and the cells were cultivated for another 4 h. Then, the absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The proliferation index (PI) was calculated as follows: (OD stimulated cultures−OD unstimulated cultures)/(OD unstimulated cultures−OD control cultures).

**Ex vivo IFN-γ and IL-4 ELISpot assay**

A total of 5×10^5 splenocytes (treated as in the splenocyte proliferation assay), along with purified ZIKV E antigen (10 μg/ml), were plated in 96-well ELISpot plates (MABTECH, Nacka, Sweden). After 24 h, the cells were incubated with a biotinylated IFN-γ or IL-4 antibody (1:1000) and later labeled with streptavidin-conjugated HRP (1:1000). TMB was used to develop spots, and SFCs were acquired using an ELISpot reader (Multispotreader Spectrum, AID, Strasberg, Germany).

**Measurement of cytokine levels in splenocyte culture supernatants**

A total of 5×10^5 splenocytes (treated as in the splenocyte proliferation assay), along with purified ZIKV E antigen (10 μg/ml), were plated in 96-well plates. After culture for 72 h, the supernatant was collected for the detection of cytokines using a Meso Scale Discovery (MSD) kit, and the data were measured with a Meso QuickPlex SQ120 (Meso Scale Diagnostics, Rockville, MD, USA).

**Cell-surface molecule staining**

A total of 5×10^5 splenocytes (treated as in the splenocyte proliferation assay) along with purified ZIKV E antigen (10 μg/ml) were plated in 96-well plates. After culture for 72 h, the cells were collected in PBS containing 2% FBS and 0.1% NaN₃. Then, the cells were stained with 0.25 μg of FITC-conjugated anti-CD4, PE-conjugated anti-CD8, APC-conjugated anti-CD19, PE/Cy7-conjugated anti-CD69, APC-conjugated anti-CD44 or PerCP-Cy5.5-conjugated anti-CD62L (BD Biosciences, Franklin, CA, USA) antibodies for 30 min at 4°C. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences, Franklin, CA, USA).

**Statistical analyses**

All of the experiments were repeated three times, and the results are expressed as the mean ± SD. All statistical analyses were performed with Student’s t-test in GraphPad software, version 8.0.2. A p value of ≤0.05 was considered statistically significant.

**Results**

**Construction of recombinant virus with three different strategies**

To express the prM-E proteins of ZIKV, we generated recombinant RABV based on the SRV9 strain [22] with three strategies (Fig 1). ZI-D (codon-optimized ZIKV prM-E, Fig 1A), ZI-E (in which the transmembrane (TM) domain of prM-E (amino acids 451–504) was replaced with the TM-cytoplasmic domain (TM-CD) of RABV, Fig 1B) and ZI-F (in which the signal peptide and TM domain of prM-E were replaced with the signal peptide and TM-CD region of RABV, Fig 1C) were cloned into the RABV SRV9 vector using two unique restriction sites (BsiWI and PmeI) that flank a RABV transcription start/stop signal between the RABV P and
M genes. All three recombinant viruses were successfully recovered. To verify the expression of foreign proteins, NA cells were infected at a multiplicity of infection (MOI) of 1 and immunostained with antibodies against RABV G and flavivirus E. The results showed that both RABV G and ZIKV E could be detected in ZI-D and ZI-E after the cells were permeabilized. However, in ZI-F, only RABV-G was expressed, and ZIKV E was not detected (Fig 2A). Moreover, to detect whether ZIKV E protein could be detected on the cell surface, nonpermeabilized cells were analyzed by confocal immunofluorescence. The results indicated that the ZIKV E protein was present on the surface of ZI-E-infected cells, while there was little ZIKV E protein present on the surface of ZI-D-infected cells (Fig 2B). No ZIKV E protein was detected in ZI-F-infected cells.

Fig 1. Schematic diagram for cDNA construction of vaccine vectors. Based on the reverse genetic operating system of the vaccine vector RABV SRV9, which contains the exogenous gene expression component, foreign genes were cloned between the P and M genes of full-length SRV9 using BsiWI and PmeI restriction digestion sites. (A) ZI-D: full-length prM-E gene of ZIKV. (B) ZI-E: full-length prM-E of ZIKV with the TM domain replaced by the TM-CD of RABV-G. (C) ZI-F: full-length prM-E of ZIKV with the signal sequence and TM domain replaced by the corresponding region of RABV.

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To detect the incorporation of foreign protein in the recombinant viruses, sucrose-purified virions from infected BHK cells were analyzed by SDS-PAGE and western blotting (WB). SDS-PAGE of purified ZI-D, ZI-E and ZI-F showed similar migration of proteins of the expected size for the RABV proteins compared with the RABV SRV9 controls (Fig 2C). However, ZIKV E protein (55 kDa) comigrated with RABV N protein (57 kDa) and was difficult to confirm. Therefore, incorporation of ZIKV E protein was detected by WB analysis. The results (Fig 2D) indicated that ZI-D and ZI-E incorporated ZIKV E protein in recombinant viruses with correct protein cleavage and that ZI-E was expressed at higher levels than ZI-D. There was no target band for ZI-F.

To detect whether the presence of foreign proteins affected the structure of the virus, the recombinant viruses were analyzed by electron microscopy. Fig 2E shows that typical-sized, bullet-shaped RABV particles could be seen in ZI-D, ZI-E and ZI-F. Furthermore, the viruses were analyzed by dual-label immunogold electron microscopy with anti-RABV G (10 nm gold particles) and anti-flavivirus E antibodies. Both ZI-D and ZI-E were found to react with anti-RABV G and anti-flavivirus E antibodies, while ZI-F was labeled only by anti-RABV G.
G antibodies (Fig 2E). The immunogold electron microscopy results were consistent with the fluorescence staining and WB results.

Growth kinetics of the recombinant viruses

To identify whether foreign proteins would affect RABV growth kinetics, one-step growth curves of ZI-D and ZI-E were analyzed using BHK-21 suspension cells (Fig 3). The titers of the recombinant viruses all reached $10^8$ TCID$_{50}$/ml, and the viral titer increased with increasing MOI (Fig 3A and 3B). Moreover, at an MOI = 0.5, there were no significant differences in growth abilities among recombinant viruses and RABV SRV9 (Fig 3C). The results indicated that the growth kinetics were not affected by foreign protein expression and that the recombinant viruses could be cultured in suspension, conducive to large-scale virus production.

Recombinant viruses induce ZIKV-specific IgGs and neutralizing antibodies (NAbs)

Based on the above identification results, we chose ZI-D/ZI-E as an immunogen. BALB/c mice were intramuscularly immunized with 20 μg of beta-propiolactone (BPL)-inactivated ZI-D or ZI-E, following a three-inoculation vaccination schedule (Fig 4A). Serum was collected, and the humoral immune response was analyzed periodically until week 10. Analysis of the levels of IgG against ZIKV E by enzyme-linked immunosorbent assay (ELISA) indicated that both inactivated ZI-D and ZI-E achieved appreciable IgG responses against ZIKV E (Fig 4B). Then, we examined the quality of this humoral response by IgG2a and IgG1 subtype-specific ZIKV E ELISA at week 6 (Fig 4C). IgG2a/IgG1 ratios less than 1.0 indicated that ZI-D/ZI-E induced a T-helper (Th)2-biased response.

To predict protection against ZIKV, the development of neutralizing antibodies (NAbs) (Fig 4D) was quantified in the serum of mice at week 6. The results showed that most of the immunized mice generated protective titers (>10). Moreover, the titer of ZI-E was slightly higher than that of ZI-D, which might have been due to increased expression of the target protein of ZIKV.

In addition, RABV NAbs were evaluated using the World Organization for Animal Health (OIE) standard, which considers values $>0.5$ international unit (IU)/ml to be protective against RABV. By week 6, the concentrations in the immunized groups had all reached at least 23.38 IU/ml, much higher than 0.5 IU/ml (Fig 4C), indicating that the addition of ZIKV E to the RABV backbone did not compromise the ability of the backbone to generate protective RABV NAbs.

**Fig 3. Growth curves of the recombinant virus.** (A-B) BHK suspension cells were infected with ZI-D or ZI-E at an MOI of 0.1, 0.5 or 1. On days 1, 2, 3 and 4 after infection, samples were obtained, and the RABV titers were determined in NA cells. (C) RABV titer comparison of the recombinant virus with RABV SRV9 at an MOI of 0.5. The tests were repeated three times.

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Fig 4. Immunization evaluation. (A) Immunization strategy. BALB/c mice (n = 9) were immunized IM with ZI-D or ZI-E (20 μg) mixed with a complex adjuvant of ISA 201 VG and poly(I:C). The mice were boosted twice at 2-week intervals. Mice that received PBS were used as controls. Blood samples (n = 6/group) were collected on days 0, 14, 28, 42 and 70. On the 35th day, mouse spleens (n = 3/group) were collected. (B) ZIKV E-specific IgG titers were assessed by indirect ELISA with the purified E protein and are displayed as the end-point dilution titers. (C) IgG2a/IgG1 ratios at week 6 as assessed by indirect ELISA with the purified E protein. (D) ZIKV NAb titer at week 6 as determined by a standard PRNT50. (E) RABV NAb titer at week 6 as determined by a FAVN test.

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Splenocyte proliferation and cytokine secretion by ex vivo restimulation

One week after the last immunization with ZI-D or ZI-E, an ex vivo splenocyte proliferation assay was performed to evaluate the effects of the vaccines on splenocyte proliferation responses (Fig 5A). After stimulation with the ZIKV E protein, splenocytes from mice in the immunized groups proliferated more efficiently than those from mice in the PBS control group.

Moreover, the capacity of splenocytes to produce interferon (IFN-γ) and interleukin (IL-4) in response to antigens was quantified in vitro by enzyme-linked immunosorbent spot (ELISpot) assay (Fig 5B). Significantly elevated numbers of spot-forming cells (SFCs) for IFN-γ and IL-4 responses were detected in immunized mouse splenocytes, and ZI-E induced more robust IL-4 responses than ZI-D.

Next, we tested whether the immunized groups exhibited elevated secretion of cytokines. The levels of cytokines were detected in the supernatant of stimulated splenocytes from all of

Fig 5. Splenocyte proliferation. One week after the last immunization, mouse spleens (n = 3/group) were collected, and splenocytes were restimulated with purified ZIKV E protein. (A) The proliferative index was detected using a CCK-8 assay. (B) The levels of IFN-γ and IL-4 secreted by splenocytes were quantified by ELISpot assay. (C) Cytokine levels in splenocyte culture supernatants were measured by MSD. Each sample was repeated three times. The data are expressed as the mean ± SD for each group. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

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Next, we tested whether the immunized groups exhibited elevated secretion of cytokines. The levels of cytokines were detected in the supernatant of stimulated splenocytes from all of
the groups (Fig 5C). The results showed that the levels of Th1 cytokines (IL-2, IFN-γ and TNF-α) and Th2 cytokines (IL-4, IL-5, IL-6 and IL-10) in the immunized groups were significantly higher than those in the PBS control group; in addition, the levels of these cytokines produced by ZI-E were higher than those produced by ZI-D. Moreover, the trends in the cytokines IFN-γ and IL-4 were in accordance with the findings of the ELISpot assay. Overall, the data above indicated that ZI-D and ZI-E could induce the secretion of a balance of Th1 and Th2 cytokines.

These data suggested that ZI-D and ZI-E evoked immune cell proliferation and elicited potent antigen-specific cellular immune responses in mice. Moreover, ZI-E had greater potential to induce cytokine production than ZI-D.

**Lymphocyte activation assays**

CD69 is a surface antigen present after T cell activation; therefore, we detected the proportion of CD69+ lymphocytes in the spleen by flow cytometry to analyze the activation of CD4+ and CD8+ T cells and B cells. The results showed that the proportions of CD4+CD69+ T cells (Fig 6A and 6B), CD8+CD69+ T cells (Fig 6C and 6D) and CD19+CD69+ B cells (Fig 6E and 6F) in the ZI-E group were all significantly higher than those in the ZI-D and PBS control groups, indicating that ZI-E with a complex adjuvant of ISA 201 VG and poly(I:C) could promote T cell and B cell activation.

Moreover, we detected the proportion of central memory T cells (TCMs, CD44+CD62L+). As shown in Fig 7, the TCM proportion among CD4+ T cells (Fig 7A and 7B) or CD8+ T cells (Fig 7C and 7D) in the ZI-E group was significantly different from that in the ZI-D and PBS control groups. This result indicated that ZI-E could promote the production of TCMs by CD4+ and CD8+ T cells.

**Discussion**

On November 18, 2016, the WHO announced that ZIKV was no longer a public health emergency of international concern, but it is still a significant and sustained public health problem. The WHO has emphasized that ZIKV remains a major threat to some areas and has stated that it will fight the virus with a long-term strategy. Moreover, ZIKV disease was listed as a "Disease X" by the WHO in the "Research and Development (R&D) Blueprint for Action to Prevent Epidemics" [24]. In February 2018, the WHO listed ZIKV disease as one of the priority study diseases because of the possibility of repeated outbreaks and the lack of specific drugs and vaccines. Therefore, research on the pathogenesis of ZIKV and the development of a safe and effective ZIKV vaccine are still critical for disease prevention and control.

Among the structural proteins of ZIKV, the E protein can induce the host to produce NAbs and mount protective responses. Researchers have used different strategies to construct vaccines, including incorporation of the whole prM-E gene, various truncation mutants of the prM-E gene or introduction of the corresponding Japanese encephalitis virus (JEV) sequence [19,20], which have had different effects. Larocca [20] produced ZIKV DNA vaccines and found that the prM-E DNA vaccine elicited higher E-specific antibody titers than the DNA vaccine with only the E gene. Therefore, in this research, prM-E of ZIKV was chosen as the target protein to construct a RABV vectored vaccine that could induce cellular and humoral immune responses against ZIKV. Although the prM protein was not evaluated, the E protein was detected by WB using anti-ZIKV E antibodies, and the target band of approximately 55 kDa was consistent with other reports [8,25]. This result indicated that prM-E was cleaved to the E protein correctly.
The RABV SRV9 strain was derived from the SAD strain by plaque purification in BHK cells, and it is nonpathogenic to dogs, rats, guinea pigs, deer, and 3-week-old mice [22]. The vector RABV SRV9, which can highly express foreign proteins, was constructed in our lab [22] and has been used in vaccine development [26,27]. Therefore, we chose the SRV9 strain as the vector to construct three recombinant viruses expressing ZIKV prM-E: ZI-D, ZI-E and ZI-F. Compared with ZI-D, ZI-E replaced the TM region of prM-E with the region of RABV G. Confocal immunofluorescence results showed that the localization of ZIKV E protein on the cell membrane infected with ZI-E was more than that of ZI-D. This phenomenon was consistent with the WB results, in which the ZIKV E protein of ZI-E detected was higher than that of ZI-D. These results indicated that TM region replacement with RABV G could contribute to foreign proteins locating on the cell membrane and being packaged on recombinant viruses. The different immune effects might be due to the different proportions of ZIKV E protein on the ZI-D and ZI-E viruses. In addition, compared with ZI-E, ZI-F replaced the signal peptide.

Fig 6. Lymphocyte activation. One week after the last immunization, mouse splenocytes (n = 3/group) were collected and restimulated with purified ZIKV E protein. The activation of lymphocytes, including CD4+ T cells (A-B), CD8+ T cells (C-D) and B cells (E-F), was evaluated by flow cytometry. (A, C, E) Representative flow cytometric plots of lymphocytes from each group. (B, D, F) Percentages of the indicated lymphocytes. The data are expressed as the mean ± SD for each group. * p<0.05; ** p<0.01; *** p<0.001.

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of prM-E with that of RABV G. Confocal immunofluorescence, WB and immunogold electron microscopy results showed that no ZIKV E protein expressed. This result indicated that signal peptide replacement might influence ZIKV E protein expression. In addition, the recombinant viruses were obtained by suspension culture of BHK cells, and expression of the exogenous protein did not affect the titer of RABV, making this system convenient for large-scale culture of the virus and the production of antigens.

The adjuvant used in the immunization was ISA 201 VG + poly(I:C); these compounds are more effective when used in combination than when used separately [28]. After immunization, the anti-ZIKV IgG antibody lasted for at least 10 weeks. Upon detection of the NAb at week 6, the PRNT\textsubscript{50} value was greater than 10. There is only one serotype of ZIKV [29], and an E-specific IgG antibody log titer \( > 2.35–3.2 \) and an NAb titer \( > 10 \) are considered protective against ZIKV infection [7,17,20]. The ZI-E designed here could induce the production of sufficient antibodies to be protective against ZIKV. Moreover, the IgG2a/IgG1 ratio is usually used in studies to assess the antibody subtype [28,30–32], and the ZIKV-antibody subtype analysis in this study showed that the antibody was Th2 biased (IgG1 biased). The Th2-biased immune response could be advantageous for protection against some viruses. A Th2-biased subtype was also seen with an Ebola virus (EBOV)-RABV vaccine [33], a MERS-CoV BLP vaccine [28] and a recombinant Rift Valley fever virus (RVFV)-RABV vaccine [26]. Flavivirus-immune sera and mAbs have been shown to be capable of markedly increasing antibody-dependent enhancement (ADE) of infections [34]. Using replication-deficient chimpanzee adenovirus vector (ChAdOx1), Lopez-Camacho [35] constructed ChAdOx1 prME \( \Delta \)TM vaccine (encoding prM-E without TM). The results demonstrated that anti-ZIKV antibodies induced by
vaccines did not induce ADE to dengue virus-2 (DENV2). In this article, when ZI-E was constructed, the TM region of prM-E was replaced with the region of RABV-G. Whether the antibodies induced by ZI-E can enhance ADE against DENV will be evaluated in future studies. Moreover, after immunization with the RABV vaccine, humoral immunity against RABV is dominant. The presence of VNA in serum is considered a reliable indicator, and the WHO and OIE recommend a RABV-VNA titer of ≥0.5 IU/ml as adequate to prevent RABV [36,37]. This standard has been applied in many reports [38,39]. Therefore, in this article, only RABV-VNA was detected, and RABV-T cell responses were not assessed. The titer of RABV NAb was greater than 23.38 IU/ml 6 weeks after the first immunization, much higher than the protective limit of 0.5 IU/ml, indicating that they had protective ability against RABV.

It has been indicated that both antibody- and cell-mediated immune responses play roles in protecting adult mice from ZIKV infection [40]. In this study, ZI-E stimulated the proliferation of spleen lymphocytes and promoted the secretion of cytokines. IFN-γ and IL-4 secretion was significantly induced in splenocytes from immunized mice on day 7 post immunization. Other cytokines with adaptive immunomodulatory roles, including IL2, TNF-α, IL-5, IL-6 and IL-10, were also observed in immunized mice, suggesting that both Th1 and Th2 responses were elicited. The Th1/Th2 immune response is important in vaccination. Th1 cells are crucial for cell-mediated immunity and are characterized by the production of IFN-γ, while Th2 cells can promote humoral immunity and secrete IL-4, IL-5 and IL-6 [41]. The secreted cytokines play important roles in the immune response. If an antigen binding to a B cell receptor is not synergistic with the membrane molecules and cytokines expressed by Th cells, an effective immune response cannot be induced [42]. Meanwhile, although the precise role of T cells in ZIKV infection is not very clear, CD8+ T cells have been determined to play a key role in clearing ZIKV from the central nervous system (CNS) [7], and CD4+ T cells could contribute to protection through cytokine production and support maturation of the antibody response [34]. One of the important purposes of vaccine immunization is to activate immune cells and produce immunologic memory. ZI-E enhanced the activation of antigen-specific CD4+/CD8+ T cells (Fig 6A–6D), and it also promoted the production of TCMs among CD4+ and CD8+ T cells (Fig 7). TCMs have the ability to proliferate and produce IL-2, and they play an important role in immune protection [43]. In addition, TCMs have a strong proliferation ability and long life cycles, so they can provide long-term protection [44]. In addition, ZI-E enhanced the activation and maturation of B cells (Fig 6E and 6F), which could further promote the secretion of cytokines and increase antibody levels.

In one modeling study combining multiple factors, researchers determined that many countries across Africa and the Asia-Pacific region are vulnerable to ZIKV disease [45]. Rabies is also a threat to human health in many regions [46,47], including India and China. Therefore, the recombinant viruses designed in this study could also potentially be used as ZIKV-RABV binary vaccines in areas where both ZIKV and RABV are threats.

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models global infectious disease threats. MIC, MG, SB, and AGW have received employment income from BlueDot, and IIB has consulted to BlueDot. All other authors declare no competing interests.

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