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Humoral and cellular immunity against both ZIKV and poxvirus is elicited by a two-dose regimen using DNA and non-replicating vaccinia virus-based vaccine candidates

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ABSTRACT

The Zika virus (ZIKV) and poxvirus infection are considered as public health emergencies, necessitating the development of effective vaccines. Here, we report novel recombinant DNA-based and non-replicating vaccinia virus (NTV)-based vaccine candidates that express the precursor membrane-envelope (prME) or envelope (E) glycoproteins of ZIKV. After immunization of BABL/c mice with the vaccines using a homologous protocol (DNA/DNA, NTV/NTV) or heterogeneous (DNA/NTV) protocol, a similar level of anti-E IgG and neutralizing antibodies (microneutralization test) were detected in the mice. However, a significantly higher level of E-specific T cell responses was elicited in mice when a heterogeneous prime-boost protocol was used (DNA/NTV) with either the DNA-based or NTV-based vaccines. Furthermore, neutralizing antibodies and a T cell immune response against the vaccinia virus (VV) were detected in mice that were subjected to the prime-boost protocol (DNA/NTV), whereas those subjected to a homologous NTV/NTV protocol had higher levels of anti-VV IgG and neutralizing antibodies. Lastly, a novel H-2d-restricted CD8 T-cell epitope, VRSYCYEASISDMAS, was identified in the ZIKV E protein. These data demonstrate proof of concept of a bivalent vaccine candidate against ZIKV and orthopoxvirus, and support the use of DNA-prME prime and NTV-E boost protocols to protect against ZIKV and orthopoxvirus infections.

1. Introduction

Zika virus (ZIKV) was first isolated in 1947 from sentinel monkeys in the Zika forest, Uganda [1]. Sporadic infections were reported until an outbreak of ZIKV infections occurred on Yap Island, Micronesia [2]. Most cases of ZIKV infection develop symptoms of mild fever, rash, and arthralgia. Awareness of ZIKV increased further as a result of the ZIKV-induced fetal microcephaly cases that were recorded in Brazil in 2015 [3]. ZIKV has been reported to have spread to over 84 countries in the Americas and even China. In February 2016, the World Health Organization (WHO) declared ZIKV as a public health emergency due to its severe implications, such as microcephaly and Guillain-Barré syndrome [4]. ZIKV is mainly transmitted by mosquitoes, although some reports have suggested that ZIKV may be contracted through sexual intercourse and vertical transmission [5,6]. In a mouse model, ZIKV infection can cause damage to testis and lead to infertility [7]. Above all, it is urgent to develop a safe and effective vaccine and therapy [8].

The genome of ZIKV contains a single open reading frame (ORF) encoding a polyprotein that is cleaved into three structural proteins [9], namely, capsid (C), precursor membrane/membrane (prM/M), and envelope (E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) by viral and cellular proteases. The E protein is responsible for receptor binding, virus

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entry and membrane fusion, and is a major target of neutralizing antibodies [9]. The NS1 protein also constitutes a major target for antibody-mediated protection against ZIKV infection [10]. Due to their ability to induce a robust immune response, the prM proteins are the viral antigen most frequently present in ZIKV vaccines [8]. ZIKV strains can be divided into two lineages: African and Asian. Serum neutralization assays have suggested that ZIKV exists as a single serotype [11], which means one vaccine can provide protection against all of the ZIKV strains.

There are no available therapies or approved vaccines for ZIKV infection [8]. A variety of ZIKV vaccine candidates are currently being studied [12–19], including an inactivated ZIKV vaccine, a live attenuated vaccine, a DNA vaccine, a virus-vector ZIKV vaccine, a mRNA vaccine, and a Zika virus-like particle vaccine, some of which have been evaluated in clinical trials. Currently, it is assumed that a reasonable titer of neutralizing antibodies and a sufficient T-cell response suggest protective immunity. Although several new vaccines are promising, the need remains to develop other new and efficient vaccines.

The vaccinia virus (VV) Tiantan strain has been widely used for decades in China as an effective vaccine during the eradication era of smallpox before 1980 [20], and it is widely used as a vaccine vector [21,22]. Although the WHO declared in 1980 that smallpox was eradicated, the risk of its use as a biological weapon still remains. Additionally, other orthopox-related infection, such as monkeypox and cowpox, have recently been reported in certain populations [23]. Despite its eradication, the smallpox vaccine remains a public health issue due to concerns relating to bioterrorism and zoonotic orthopoxvirus outbreaks. Thus, the development of safer and more effective vaccines against smallpox and the zoonotic orthopoxvirus is a significant challenge [23]. VV can carry large foreign genes or multiple genes and can rapidly replicate [24]. A novel high-throughput VV neutralization assay revealed that there is a low prevalence of neutralizing antibodies against the VV Tiantan strain in the Chinese population, which indicates that vaccines based on VV may have low pre-existing immunity [25]. Due to the side effects of replicating VV in immunocompromised patients, attenuated and non-replicating VVs have been developed and have become increasingly popular as vaccine vectors for emerging and biothreat viruses [26,27]. Our laboratory generated a non-replicating VV Tiantan strain (NTV) vector by deleting the 26 genes between the C and K digestion fragments of *Hind* III that are associated with host range and viral virulence [22]. This vector can propagate in primary chicken embryo fibroblasts, but not in cells derived from humans. Importantly, it has reported that a NTV-based vaccine against hepatitis C induces robust immunity in mice and macaques [22].

Two DNA vaccines that contained the coding sequence for the envelope E protein of ZIKV have been tested in clinical trials and reported to be safe and immunogenic [18]. A ZIKV vaccine based on the NTV platform may offer a more favorable safety profile than other ZIKV vaccine candidates and still be highly immunogenic to both ZIKV and poxvirus. Heterogeneous immunization using DNA-based vaccines and rVV-based vaccines seems to enhance both humoral and cell immune response [22,28]. Additionally, this approach could represent an effective vaccine platform for emerging diseases, due to the strength and longevity of the induced immunity. To further optimize this vaccination strategy and to enhance the protective immunity against ZIKV, we constructed a NTV-based ZIKV vaccine expressing the E protein and a DNA-based ZIKV vaccine expressing the prME protein. We then analyzed ZIKV- and VV-specific immune responses in BALB/c mice after homologous and heterogeneous immunization protocols. In addition, a novel H-2d-resricted CD8 T-cell epitope in ZIKV E protein was identified in BALB/c mice by ELISPOT using 15mer (overlapping by 10 amino acid) pools of overlapping peptides corresponding to the ZIKV-E protein.

2. Materials and methods

2.1. Cells, viruses, and animals

Human embryonic kidney (293 T, ATCC #CRL-11268), baby hamster kidney (BHK-21, ATCC-CCL-10), and Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, South Logan, UT, USA) and supplemented with 10% fetal bovine serum (FBS) (Gibco, NY, USA) and 1% penicillin and streptomycin (Gibco, NY, USA) at 37 °C with 5% CO_2. The Zika-SMGC-1 virus strain was originally isolated from imported patient samples from South America. The Zika-SMGC-1 virus strain was propagated in suckling mice through intracerebral inoculation and titrated by plaque assays in BHK-21 cells.

Female five- to six-week-old BABL/c mice (SPF grade) were purchased from the Beijing Vital River Laboratory Animal Technology, and subsequently housed and treated/vaccinated in a temperature-controlled, light-cycled facility in accordance with the guidelines of the Institutional Laboratory Animal Care and Use Committee (IACUC), Chinese Center for Disease Control and Prevention (China CDC).

2.2. Construction of recombinant DNA- and NTV-based vaccines expressing ZIKV structural protein

The DNA fragment encoding the ZIKV prME protein (amino acids 122-794, H/PF/2013 French Polynesian isolated strain, GenBank Accession Number KJ776791.1) was synthesized by SBS Genentech Co., Ltd. (Beijing), based on codon optimization. The signal sequence was replaced with that derived from the Japanese encephalitis virus (JEV, from JEV-GKP/0944234). The recombinant plasmid VRC-prME (DNA-based ZIKV vaccine) was generated by cloning the synthesized sequence into a pVRC-8301vector (provided by Dr. Gary Nabel, VRC, NIH) between the *Eco*RV and *BamH*I sites [29] and confirmed by sequencing (Fig. 1A). The recombinant plasmid was then amplified in *Escherichia coli* DH5α cells and purified using an Endo-Free Plasmid Maxi Kit (QIAGEN, Germany).

The construction of the NTV-based vaccine expressing the ZIKV E protein was performed as previously reported [22]. Briefly, the DNA fragment encoding the ZIKV E protein (amino acid 290–794, GenBank AHZ10358.1) was synthesized and cloned into the VV expression vector, pJSC1175-LacZ, under the control of the vaccinia specific promoter, 7.5 K, in the BamHI site. The recombinant pJSC1175-LacZ-E was subjected to homologous recombination with NTV to generate the NTV-E vaccine candidate through TKL and TKR homologous sequences (Fig. 1A). The recombinant NTV-E vaccine was selected by a blue-white selection assay, amplified, and titrated in chicken embryo fibroblasts (CEF) and stored at −80 °C.

2.3. Western blot analysis

For analysis of ZIKV E protein expression, 293 T cells were transiently transfected with the recombinant plasmid VRC-prME and the VRC-8301 empty vector (mock) using a jetPRIME kit (Polyplus) according to the manufacturer's instructions. CEF were infected with 10 MOI of NTV-E and NTV-LacZ (mock). At the indicated time post-infection, the cell pellets and supernatant were harvested. Cell pellets were processed with a lysis buffer (Beyotime, Shanghai) and boiled for 10 min. The denatured samples were separated on 4–12% SDS-PAGE gels and transferred onto nitrocellulose membranes. An anti-ZIKV E protein polyclonal antibody

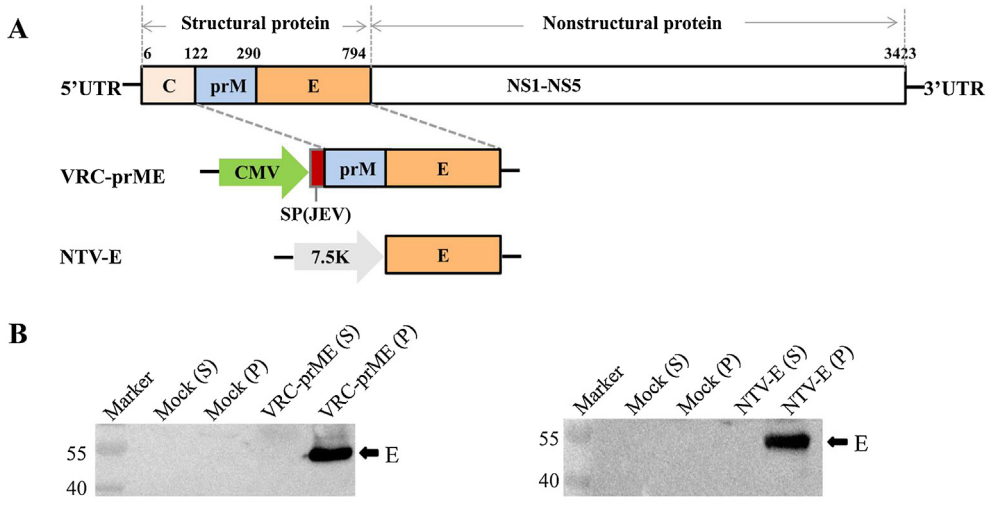


Fig. 1. Construction and antigen expression analysis of recombinant DNA-based and NTV-based ZIKV vaccine candidates (VRC-prME and NTV-E). Schematic diagrams of the recombinant DNA-based vaccine and NTV-based vaccine encoding prME and E protein respectively (A). Western blot analysis of ZIKV E protein expression in *vitro*. Cell pellets and supernatant from VRC-prME and VRC-8301(mock) transfected 293 T cells and from NTV-E and NTV-LacZ (mock) infected CEF were incubated with mouse anti-ZIKV E protein polyclonal antibody (B). CMV cytomegalovirus promoter; SP Japanese encephalitis virus signal peptides. 7.5 K vaccinia virus 7.5 K promoter. S supernatant; P pellets.

(Zoonogen, Beijing) was used at a dilution of 1:1000, followed by horseradish peroxidase-conjugated (HRP) goat anti-mouse IgG secondary antibody at a dilution of 1:2000. The membranes were developed with a chemiluminescent substrate and analyzed using a chemiluminescence imager.

2.4. Immunizations

For animal immunization, six-week-old female BABL/c mice were divided randomly into five groups (n = 8 for each group; Fig. 2). For the DNA vaccine, mice were immunized twice with 20 μg at four-week intervals through an intracutaneous route using electroporation (i.d. + Ep) [30]. For the NTV-based vaccine, mice were immunized at a dose of 1×10^7 PFU through an intramuscular (i.m.) route. Blood and splenocytes were collected two weeks after each immunization to analyze the humoral and cellular immune responses. Animal protocols used in the present study were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the National Institute for Viral Disease Control and Prevention (NIVDC) of China.

2.5. ELISA

For detecting humoral immune response, ELISA was used as previously described [31]. Briefly, plates (Corning, Shanghai, China, Asia) were coated with 4 μg/ml of ZIKV-E protein or purified VV stock per well in a buffer (pH 9.6) at 4 °C overnight. The plates were blocked with 10% goat serum in PBS for 2 h at 37 °C. After blocking, a four-fold series of diluted mouse serum was added, and plates were incubated for 1 h at 37 °C. Then, plates were incubated with a goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at 37 °C followed by development using a 3,3′,5, 5′-tetramethylbenzidine (TMB) substrate. The reaction was read on a plate reader (Multiskan MK3) at 450 nm optical density after the reaction was stopped by adding 2 M H_2SO_4. Values 2.1-fold higher than the control group were considered to be positive.

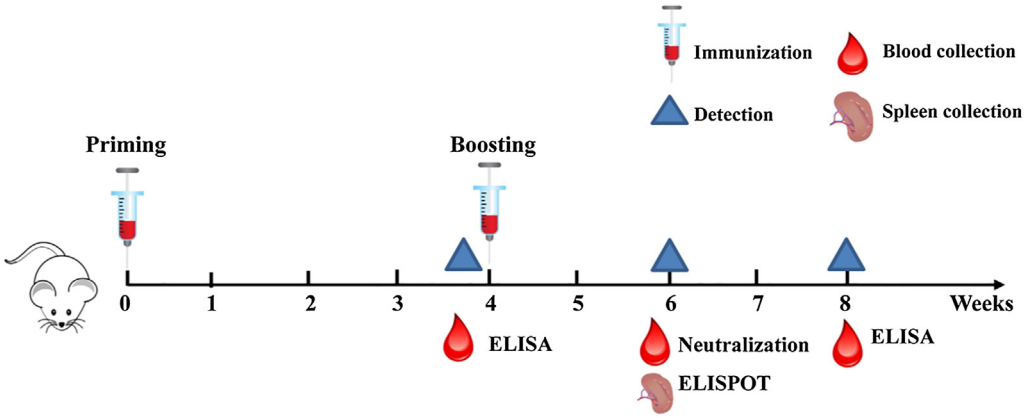
2.6. Microneutralization assay

The neutralizing activity against ZIKV in mouse serum was initially determined using a microneutralization assay as described previously [31]. Briefly, a heat-inactivated serial dilution of serums was mixed with equal volumes of ZIKV-SMGC-1 (100 PFU) and incubated at 37 °C with 5% CO_2. After 2 h, the serum-virus mixtures were added to a monolayer of Vero cells and incubated in 96-well cell culture plates at 37 °C for four days. Then, cells were fixed with a methanol-ethanol mixture after being precooled at −20 °C for 30 min. The plates were then blocked with 5% non-fat milk at room temperature (RT) after which the anti-ZIKV Z6 antibody (4 μg/mL) was added for 2 h at RT. Cells were then washed with PBST and a goat anti-human IgG-HRP dilution was added for 1 h at RT. The plates were washed and developed with TMB substrate after the reaction was stopped with 2 M H_2SO_4. Absorbance was read at 450 nm. Neutralization titers (MN50) were determined by fitting nonlinear regression curves using GraphPad Prism 7 and calculating the reciprocal of the serum dilution required for 50% neutralization of infection. MN50 titers predicted by the non-liner regression as <20 were reported as half of the limit of detection.

Neutralizing antibodies against VV in mouse serum following the NTV-based immunization protocol was evaluated using a luciferase-based assay as previously reported [25]. Briefly, a heat-inactivated serum dilution series was mixed with the recombinant vaccinia virus expressing luciferase for 2 h at 37 °C. The mixtures were then added onto a layer of BHK-21 in 96-well cell culture plates and incubated for 2 h 37 °C. DMEM containing 2% FBS was added to the cells. After 24 h of incubation, the luciferase substrate was added for 2 min and fluorescence was detected using a fluorescence analyzer. The percentage of inhibition was calculated as follows: Inhibition (%) = 100 ×{1 − (Average values of fluorescence for each dilution/Average values of fluorescence in virus control)}. GraphPad Prism software was used to perform nonlinear regression analysis of % inhibition versus a log transformation of each individual serum dilution to facilitate linear interpolation of actual MN50 titers at peak vaccination response.

2.7. IFN-γ ELISPOT assay

Peptide pools consisting of 15-amino acid (aa) long peptides overlapping by 10 aa and spanning the entire length sequence of



Vaccination Group	Prime	Boost	Route/Dose	Abbreviation
Mock (NTV+NTV)	NTV-LacZ	NTV-LacZ	im., 10^7PFU/mice	Control groups
Mock (DNA+NTV)	VRC-8301	NTV-LacZ	id.+EP, 20ug/mice im., 10^7PFU/mice	
VRC-prME+VRC-prME	VRC-prME	VRC-prME	id.+EP, 20ug/mice	Homologous prime-boost groups
NTV-E+NTV-E	NTV-E	NTV-E	im., 10^7PFU/mice	
VRC-prME+NTV-E	VRC-prME	NTV-E	id.+EP, 20ug/mice im., 10^7PFU/mice	Heterologous prime-boost groups

Fig. 2. Schematic of the immunization and immune analyses. BABL/c mice were divided randomly to groups. Each mouse was immunized with one (prime) or two (prime-boost) doses of 20 μg of VRC-prME or empty vector through the intracutaneous route using electroporation (id + Ep) or 10^7 PFU of NTV-E or NTV empty vector through the intramuscular route. Blood was collected for humoral immune response detection at 4, 6, and 8 weeks by ELISA and microneutralization assay at 6 weeks. Splenocytes were collected at 6 weeks for cellular immune response detection by ELISPOT.

E protein were commercially synthesized (ZhongkeYaGuang Co., Beijing, China). All of the peptides were dissolved in DMSO at 50 mg/mL and used at 8 μg/μL in experiments. PMA and ionomycin stimulations were used as the positive control.

Two weeks after boost immunization, half of the mice from each group were selected randomly and killed. Spleens were obtained and used for IFN-γ ELISPOT as previously reported [31,32]. Briefly, 96-well ELISPOT plates were coated with a purified anti-mouse IFN-γ capture antibody (BD ELISPOT Set, USA) and incubated overnight at 4 °C. The following day, single-cell splenocyte suspensions from each group were stimulated with or without ZIKV-E peptide pools spanning the entire ZIKV-E protein (8 μg/μL), or NTV in presence of a positive control. The plates were then incubated at 37 °C in 5% CO_2 for 20–24 h. The next steps were performed according to the manufacturer's instruction. A Spot Forming Unit (SFU) represents a T cell secreting IFN-γ. The plates were then detected using an ELISPOT plate reader.

2.8. Statistical analysis

Comparisons of binding antibody and neutralizing antibody titers and T cell responses among immunization groups were conducted by one-way ANOVA. All of the statistical analyses were computed with GraphPad Prism 7.0 version and P values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Vaccine construction and ZIKV-E protein expression analysis

A codon-optimized prME gene was synthesized and inserted into a DNA vaccine vector (VRC8301) to generate a recombinant DNA vaccine candidate called VRC-prME. A naive ZIKV E gene was used to generate an NTV-E vaccine candidate (Fig. 1A). ZIKV E protein expression was detected by Western blot analysis of 293 T transfected with VRC-plasmids or of CEF infected by NTVs at 10 MOI (Fig. 1B). ZIKV E protein expression was detected as a single band of approximately 45 k Da.

3.2. Antigen-specific IgG responses induced by vaccination

To evaluate the immunogenicity of the VRC-prME and NTV-E vaccine candidates, groups of BALB/c mice were immunized with both vaccine candidates and serum samples were analyzed for antigen-specific IgGs using ELISA. The results showed that VRC-prME and NTV-E induced the production of ZIKV E-specific IgG antibodies, but there were no significant differences between homologous immunization and heterogeneous immunization (Fig. 3A). In addition, the anti-VV IgG titer induced by either NTV-E or NTV-LacZ (mock) was increased after boosting the immunization and there was no significant difference between these two groups (Fig. 3B). These results indicate that a high level of ZIKV E antigen-specific IgG was induced by either the DNA- or NTV-based ZIKV vaccines (with homologous or heterogeneous boost). Furthermore, a VV-specific IgG response was detected after homologous or heterogeneous boosting using NTV-based ZIKV vaccines.

3.3. Neutralizing antibody response elicited by VRC-prME and NTV-E.

The neutralizing antibody response in mice treated with the VRC-prME and NTV-E vaccine candidates was evaluated using a microneutralization assay. VRC-prME and/or NTV-E immunization elicited robust neutralizing activity against ZIKV, but no significant differences were observed between different immunization proto-

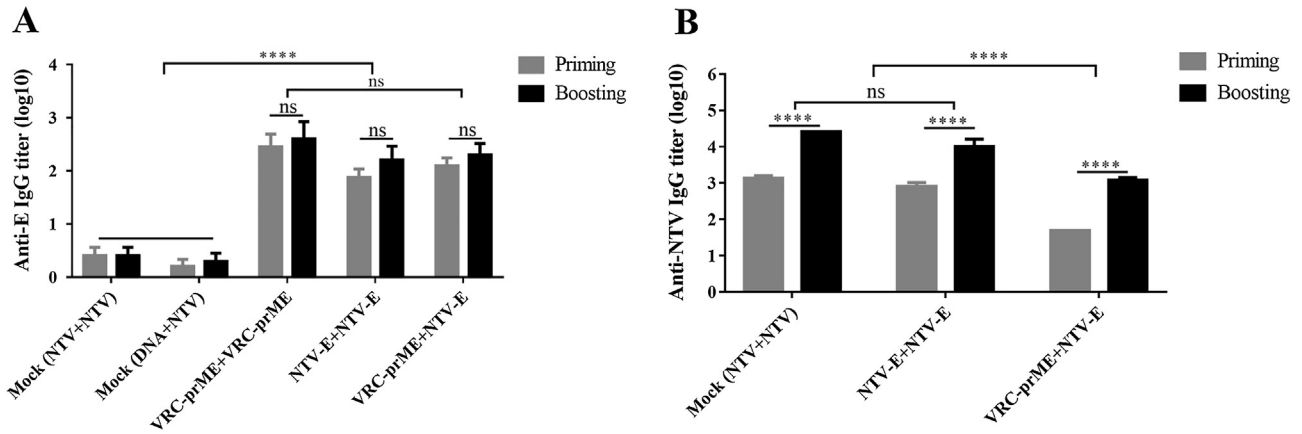


Fig. 3. Immunogenicity evaluation of VRC-prME or/and NTV-E vaccines. Serum samples were collected at 4 weeks (priming) and 8 weeks (boosting). Anti-ZIKV E protein antibody levels in the serum of immunized mice were detected by ELISA using purified ZIKV E protein as the capture antigen and shown as end-point dilution titers (A). Serum samples were collected from NTV-E and NTV empty vector immunization groups at four weeks and eight weeks. Anti-VV antibody levels in serum were detected by ELISA with VV as the detection antigen and shown as end-point titer (B). Data are shown as the mean ± SEM and were analyzed by one-way ANOVA (n.s. $P > 0.05$; $^{****}P < 0.0001$).

cols (Fig. 4A–D). Representative data from the ZIKV neutralizing assay are shown in Fig. 4B. Additionally, we observed significantly higher levels of neutralizing antibodies against VV when the NTV-based vaccine immunization was used (Fig. 5A). The neutralizing antibody titer elicited by two doses of the NTV-E was significantly higher than that elicited by the VRC-prME-priming and NTV-E-boosting immunizations, which in turn was significantly higher than that achieved following two doses of VRC-prME (Fig. 5B). These results show that a significant level of neutralizing antibodies against both ZIKV and VV was generated in mice primed with VRC-prME and boosted with NTV-E vaccine.

3.4. Screening and mapping of H-2d-restricted CD8 T-cell epitopes in ZIKV E protein

To the best of our knowledge, the H-2d-restricted CD8 T-cell immunodominant epitopes in ZIKV E have not been reported. Here, we conducted an E-specific immunodominant epitope-screening

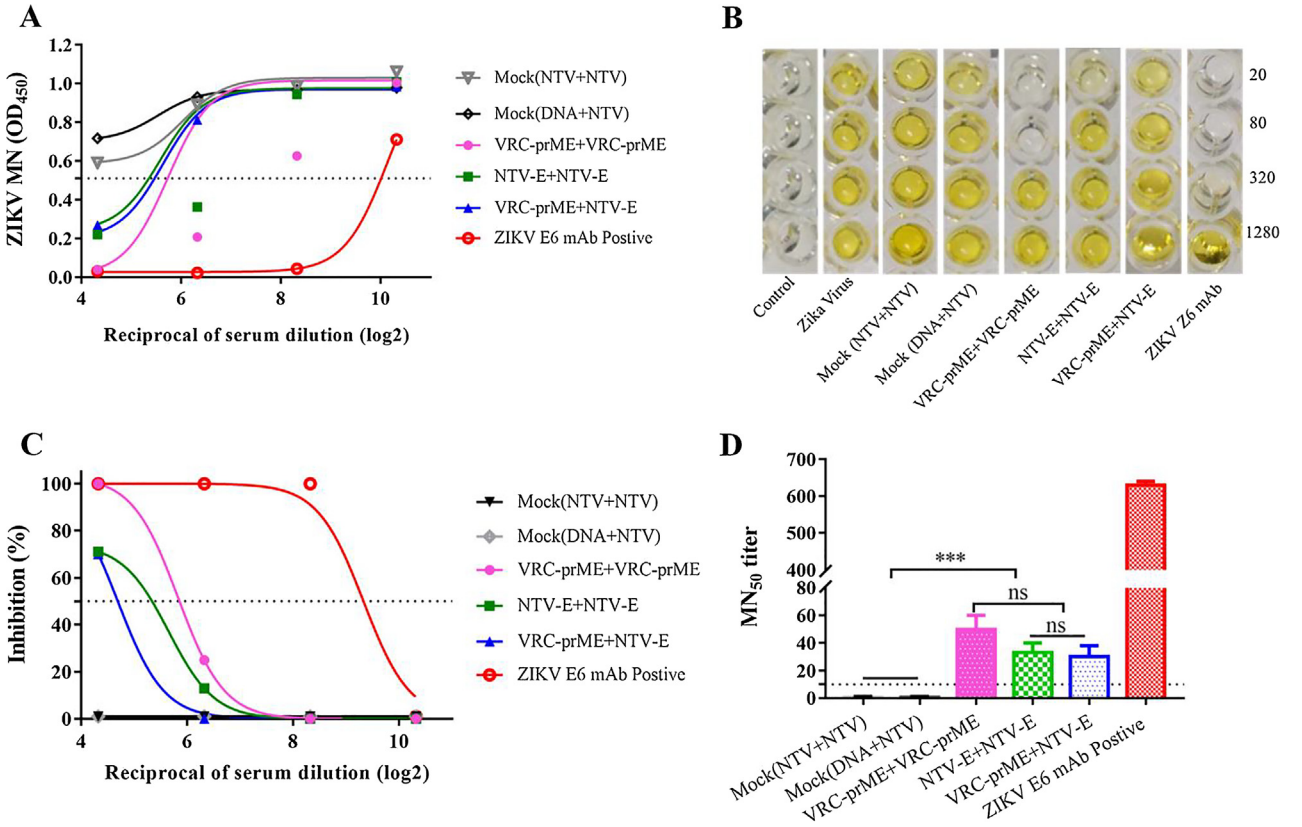


Fig. 4. Neutralizing antibody against ZIKV induced by VRC-prME and NTV-E. Serum samples were collected at two weeks after boost immunization and mixed with an equal volume of ZIKV-SMGC-1(100PFU). Anti-ZIKV neutralizing antibody levels in serum were detected using a microneutralization assay shown as optical density at 450 nm (A), inhibition rates (C), and MN50 titer (D). One representative graph from each group of animals is shown (B). Yellow represents non-neutralized ZIKV. White represents neutralized ZIKV. ZIKV Z6, human ZIKV monoclonal antibody. Data are shown as the mean ± SEM and were analyzed by one-way ANOVA (n.s. $P > 0.05$; $^{***}P < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

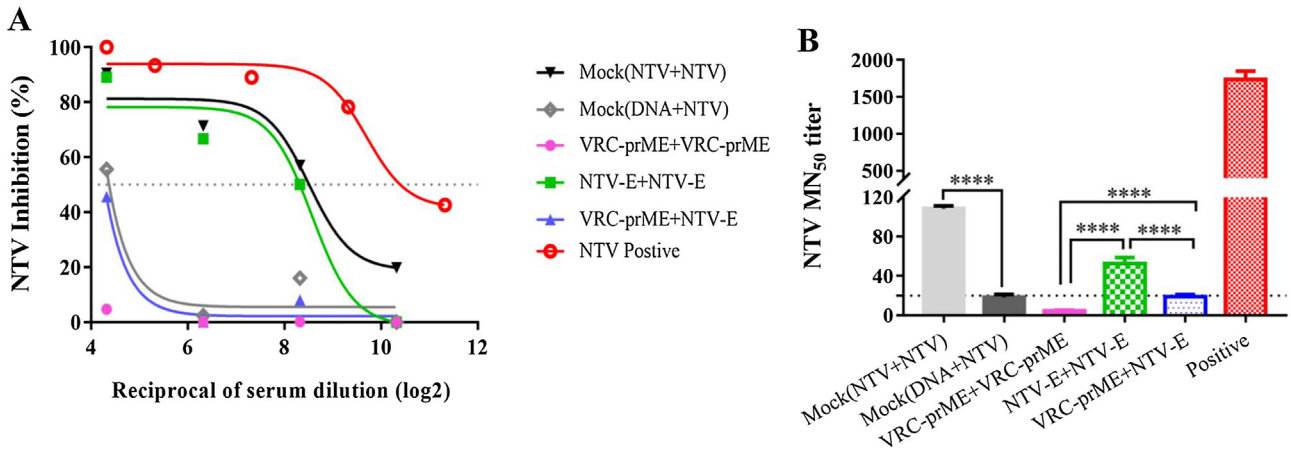
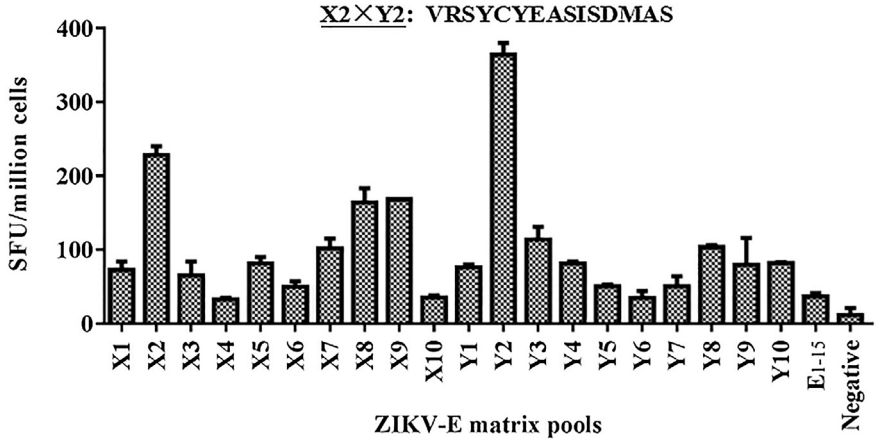


Fig. 5. Neutralizing antibody against VV induced by VRC-prME and NTV-E. Serum samples were collected at two weeks after boost immunization and mixed with VV. Anti-VV neutralizing antibody levels shown as inhibition (A) and MN50 (B). Data are shown as the mean ± SEM and were analyzed by one-way ANOVA ($^{****}P < 0.0001$).

assay in BABL/c mice via ELISPOT using 15mer (overlapping by 10 amino acid) pools of overlapping peptides corresponding to the ZIKV-E protein. Splenocytes were harvested from the mice two weeks after boost immunization with VRC-prME, which was followed by stimulation with ZIKV-E peptide pools (Fig. 6). There were several peptides that elicited a strong T cell response. Of these peptides, the epitope E_{56-70} "VRSYCYEASISDMAS", which was confirmed to contain a H2-Kd restricted immunodominance epitope, induced the strongest IFN-γ response (Fig. 6). In addition, the E_{1-15} peptide induced a weaker T cell immune response. Previous reports have shown that E_{4-12} is an immunodominant epitope in C57BL/6 mice (H-2b-restricted), so we also set E_{1-15} peptide alone as the control in this study. However, our results suggest that E_{4-12} is not a dominant T-cell epitope in BALB/c mice (H-2d-restricted) because the E_{1-15} peptide only induced a weak T cell response.

3.5. T cell response induced by VRC-prME and NTV-E

To analyze T cell immune responses induced by VRC-prME and NTV-E, splenocytes from mice were stimulated with either the H-2d-restricted T-cell immunodominant peptide (E_{56-70}) from the



Peptide pools	Y1	Y2	Y3	Y4	Y5	Y6	Y7	Y8	Y9	Y10
X1	1	2	3	4	5	6	7	8	9	10
X2	11	12	13	14	15	16	17	18	19	20
X3	21	22	23	24	25	26	27	28	29	30
X4	31	32	33	34	35	36	37	38	39	40
X5	41	42	43	44	45	46	47	48	49	50
X6	51	52	53	54	55	56	57	58	59	60
X7	61	62	63	64	65	66	67	68	69	70
X8	71	72	73	74	75	76	77	78	79	80
X9	81	82	83	84	85	86	87	88	89	90
X10	91	92	93	94	95	96	97	98	99	

Fig. 6. Screen of ZIKV E-specific peptides. The ZIKV-E peptide pools consisting of 15mers (overlapping 10 amino acids) spinning the entire ZIKV-E protein are shown as 10 × 10 matrix pools (under). VRC-prME immunized splenocytes were collected to detect the dominant epitope which elicited the strongest T cell response at two weeks after boost immunization and stimulated with or without ZIKV-E peptide pools. The dot represents a splenocyte secreting IFN-γ and is shown as Spot Forming Unit (SFU) (upper). A specific dominant peptide (VRSYCYEASISDMAS) was found through cross analysis of peptide pools. Data are shown as the mean ± SEM.

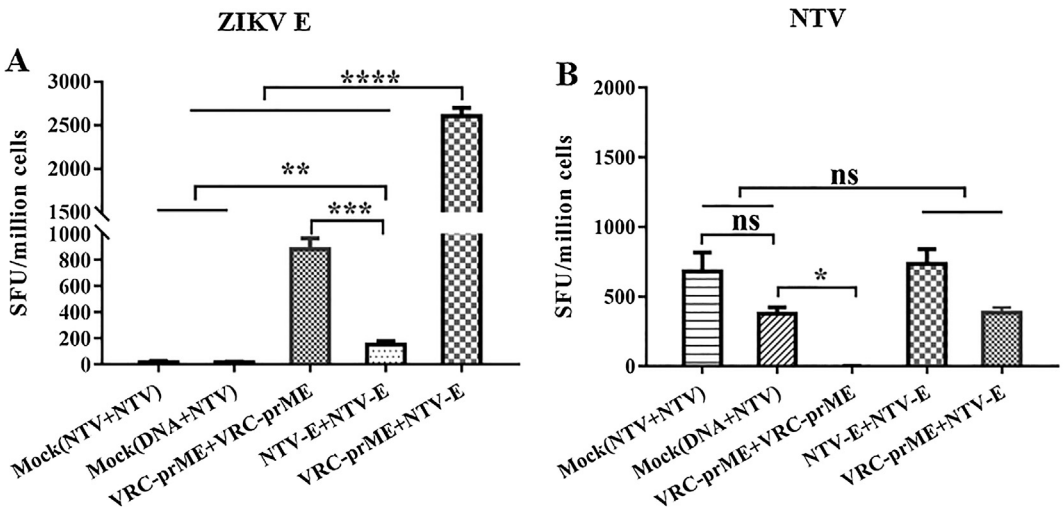


Fig. 7. Characterization of the cellular immune response of VRC-prME and NTV-E vaccines. The ZIKV-E specific IFN-γ secreting splenocytes were analyzed using a commercial ELISPOT kit. Splenocytes from each group were harvested and stimulated with or without dominant peptides (A) or VV (B). Data are shown as mean ± SEM and analyzed by one-way ANOVA (B) (n.s. $P > 0.05$, $^{*}P < 0.05$; $^{****}P < 0.0001$).

ZIKV-E protein or purified NTV, and ELISPOT was used to detect their ability to secrete IFN-γ (Fig. 7). The results revealed that VRC-prME/VRC-prME homologous immunization lead to a significantly greater level of T cell response (about 1000 SFU/million splenocytes) than that achieved by NTV-E homologous immunization (about 150 SFU/million splenocytes). Notably, VRC-prME/NTV-E heterogeneous immunization elicited the most robust T cell response (about 2800 SFU/million splenocytes), suggesting that priming with DNA-based vaccines and boosting with NTV-based vaccines may yield a synergistic effect (Fig. 7A). We also analyzed T cell immune responses against NTV in mouse splenocytes at two weeks post-boosting immunization by ELISPOT using NTV as a stimulant. The results show that a single or double dose of NTV induced a significant T cell response (Fig. 7B). These results suggest that a significant T cell response against both ZIKV and VV was induced in mice primed with VRC-prME and boosted with the NTV-E vaccine.

4. Discussion

Over the last two years, vaccines against ZIKV have made great progress, but there are still no licensed vaccines or drugs for ZIKV infection [19]. Thus, it is still urgent to develop safe and immunogenic vaccine candidates. One of the promising new candidates is a DNA-rVV prime-boost vaccination protocol that has been demonstrated in preclinical studies to be effective against several infectious diseases [21,24,28]. DNA vaccines against ZIKV were amongst the first platforms and reported by several groups, some of which have undergone human clinical trials [18,33]. Here, we generated recombinant DNA-based and NTV-based Zika virus vaccine candidates expressing the prME and E structural proteins (VRC-prME, NTV-E), and evaluated the responses induced by homologous (DNA/DNA, NTV-E/NTV-E) immunization and heterogeneous (DNA-prime/NTV-boost) immunization protocols. After immunization of the BALB/c mice, similar levels of anti-E of ZIKV IgG and neutralizing antibodies were detected using both protocols. However, a significantly higher level of ZIKV E-specific T cell response was elicited in mice when a heterogeneous prime-boost protocol (DNA/NTV) was used. Furthermore, a significant neutralizing antibody and T cell immune response against VV was detected in mice subjected to a prime-boost protocol (DNA/NTV), despite inducing lower levels of anti-VV IgG and neutralizing antibodies than those elicited by a homologous NTV/NTV protocol. Additionally, a novel H-2d-restricted CD8 T-cell epitope in the ZIKV E protein was identified: VRSYCYEASISDMAS. These data characterize a proof of concept bivalent vaccine candidate against ZIKV and orthopoxvirus, and support the consideration of DNA-prME priming and NTV-E boosting to prevent ZIKV and orthopoxvirus infections.

Numerous platforms and viral antigens designs have been reported as ZIKV vaccine candidates with a range of favorable attributes [19]. Nonetheless, deployment of these vaccines in humans, along with confirmation of their efficacy and safety, still remains a major challenge. Both the VRC backbone and rNTV vector, which were both used for DNA vaccine constructs and the recombinant viral vector-based vaccine in this study, have been used in prior studies in humans with excellent safety profiles [18,20]. A VV vector expressing both ZIKV prME and CHKV structural proteins induced the production of neutralizing antibodies to both viruses and conferred protection against ZIKV in mice [15]. Our data demonstrate that both the VRC-prME and NTV-E vaccine candidates are capable of eliciting a significant level of humoral immunity (IgG and neutralizing antibody) and cell-mediated immunity (CMI) (IFN-γ secreting splenocytes by ELISpot) after two doses regardless of whether prime-boost homologous or heterogeneous immunization was used. In addition, both the NTV-E/NTV-E and VRC-prME/NTV-E immunization induced a certain level of humoral immunity (IgG and neutralizing antibody) and CMI against VV.

Among ZIKV vaccine candidates, E and prM are major antigen targets [9,19]. A single immunization of C57BL/6 mice or non-human primates (NHPs) with an mRNA encoding prM-E with a JEV signal sequence elicited robust neutralization titers and conferred protection against congenital diseases [16]. A DNA vaccine including prME and the full-length E sequence elicited higher E-specific antibodies than for E sequences only, or for the △TM deletion mutant [8]. However, an adenoviral vector vaccine containing prM and E without a transmembrane domain (△TM) induced a higher level E-specific antibody response and provided 100% protection relative to that of a vaccine including prM and full-length E sequence in BABL/c mice [14]. Our results show that a similar level of anti-ZIKV E-specific IgGs and neutralizing antibodies were induced by two doses of either VRC-prME or NTV-E vaccines. This difference in immunogenicity, induced by various antigens or the same antigen in various vectors, may be further examined and optimized in the future.

It has been shown that the dengue virus E protein has a predicted N-terminal E signal peptide that is downstream of the clas-

sical prM transmembrane signal peptide, and that the maintenance of this peptide is important for the protein immunogenic potential when it is expressed by a poxvirus vector, i.e., the secretion of the poxvirus-expressed DENV E protein seems to be essential for the generation of immune responses in mice when compared to the non-secreted E protein [34] and for protection against the DENV challenge [35]. Such signal peptides have not been described for ZIKV [36]. Two DNA vaccines have been reported, and VRC 5283 (a DNA vaccine) has shown a superior neutralizing activity compared to VRC 5288 in clinical trials [18,33]. In both vaccines, the prM signal sequence in the Zika virus coding sequence was exchanged with an analogous Japanese encephalitis virus region to improve particle secretion [37]. As a matter of fact, our DNA construct (VRC-prME) also employed a similar strategy as that of VRC 5283. Our data have shown that both VRC-prME and NTV-E expressed ZIKV E protein that were not secreted by cells and have no apparent proteins that were detected in the cell supernatant (Fig. 1B). However, robust neutralizing antibodies against ZIKV-E was elicited in mice using a two dose regimen involving VRC-prME in this study (Fig. 4).

It is highly desirable for an ideal vaccine against ZIKV to induce both humoral immunity and CMI, both of which play roles in protecting adults from ZIKV infection. Huang et al. evaluated CD8+T cell immune responses in immunocompetent mice and found that robust functional CD8+T cells can be elicited during ZIKV infection, as evidenced by the finding that the adoptive transfer of ZIKV-specific CD8+T cells into naive mice prevented CNS disease and cross-protected against the Dengue virus (DENV) infection [32]. The DENV-immune serum did not protect against ZIKV infection, but the DENV-active CD8+T cells can cross-protect against secondary ZIKV infections. Our results show that immunization with VRC-prME induced a higher E-specific T cell response than that elicited by NTV-E immunization, although similar levels of humoral immunity against ZIKV E was observed for both vaccines. Furthermore, there was a significantly higher level of E-specific T cell response when the VRC-prME/NTV-E heterogeneous immunization protocol was used. These results suggest that VRC-prME/NTV-E heterogeneous immunization can enhance immunogenicity through a synergistic effect. NTV-E also induced T cell immune response against VV, which revealed that the VRC-prME-prime/NTV-E-boost protocol induced bivalent immune potency against ZIKV and VV. Additionally, we found a new peptide epitope (VRSYCYEASISDMAS) in E proteins that elicit a strong T cell response in BALB/c mice. The $E_{4\text{-}12}$ epitope, which was recognized by a majority of T cells in C57BL/6 mice during the ZIKV infection [31], only induced a very weak T cell response in our experiment (Fig. 5A).

Several limitations of the current study need to be examined. There are two kinds of animal models for vaccine protection efficacy [38]: immunocompetent mice, which simulate natural infection, and immunocompromised mice, which are extremely susceptible to ZIKV infection. Future studies will further explore the protection efficacy and related mechanisms of DNA-based and NTV-based vaccine candidates in both mouse models. Second, ZIKV NS1 protein has been confirmed to be important for modulating the ZIKV-specific immune response in the absence of detected neutralizing antibodies [39]. Since the ZIKV NS1 protein has been found to play an important role in protection against ZIKV infection, additional studies should be carried out on the immune response and protective efficacy of ZIKV vaccines expressing the NS1 protein. We also would like to continue to develop NTV-prME vaccines to see the performance of the heterologous vaccine when both vectors express the same gene products in the future. Lastly, previous reports indicated that pre-immunity against DNEV enhanced subsequent ZIKV infection by the passive transfer of DENV-immune plasma into ZIKV-susceptible mice [40–42]. We will test whether such processes can be caused by prime-boost regimens with DNA and NTV vaccines against ZIKV.

In conclusion, we evaluated the immune response of BALB/c mice to a novel replication deficient VV-based ZIKV vaccine candidate and compared the response to that elicited by a DNA-based vaccine candidate using either a homologous or heterogeneous vaccination protocol. Our results suggest that priming with DNA-prME and boosting with NTV-E constitutes a potential vaccine candidate protocol against ZIKV and VV infections.

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Conflict of interest

The authors have no competing interests to declare.

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