(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 May 2019 (16.05.2019)

(10) International Publication Number
WO 2019/094801 A1

(51) International Patent Classification:
A61K 39/00 (2006.01) *C12N 15/40* (2006.01)
A61K 39/12 (2006.01) *C12N 15/63* (2006.01)
C07K 14/18 (2006.01) *C12N 15/79* (2006.01)

(21) International Application Number: PCT/US2018/060137

(22) International Filing Date: 09 November 2018 (09.11.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/584,629 10 November 2017 (10.11.2017) US

(71) Applicants: RESEARCH INSTITUTE AT NATIONWIDE CHILDREN'S HOSPITAL [US/US]; 700 Children's Drive, Columbus, Ohio 43205 (US). **OHIO STATE INNOVATION FOUNDATION** [US/US]; 1524 North High Street, Columbus, Ohio 43201 (US).

(72) Inventors: PEEPLES, Mark; 208 N. Roosevelt Avenue, Bexley, Ohio 43209 (US). **LI, Jianrong**; 5199 Brandonway Court, Dublin, Ohio 43017 (US). **BOYAKA, Prosper N.**; 4381 Lyon Drive, Columbus, Ohio 43220 (US). **LI, Anzhong**; 2209 Teardrop Avenue, Columbus, Ohio 43235 (US). **LU, Mijia**; 1297 Bunker Hill Blvd., Apt. D, Columbus, Ohio 43220 (US). **MA, Yuanmei**; 1168 Prince Andrew Court, Pittsburgh, Pennsylvania 15237 (US).

(74) Agent: WIWCHAR, Michael T. et al.; 2200 Wells Fargo Center, 90 South Seventh Street, Minneapolis, Minnesota 55402 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM,

(54) Title: RECOMBINANT VECTORS ENCODING ZIKA VIRUS PROTEIN SUBUNITS

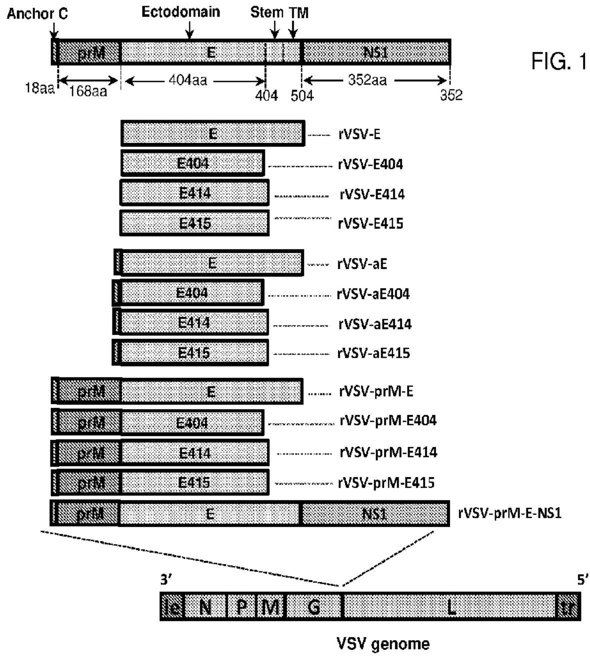

FIG. 1A

(57) Abstract: Embodiments disclosed herein provide compositions, methods, and uses for recombinant vectors encoding Zika virus (ZIKV) protein subunits, and immunogenic compositions thereof. Certain embodiments provide recombinant vectors encoding ZIKV nonstructural protein 1 (NS 1), and optionally, ZIKV envelope (E) protein and premembrane (prM) protein. Other embodiments provide expression cassettes comprising a promoter operably linked to a polynucleotide that encodes the ZIKV NS 1 protein, and optionally ZIKV E and prM proteins. In some embodiments, the disclosed expression cassettes can be incorporated into a vector to produce a recombinant vector. Also provided are immunogenic compositions comprising one or more recombinant vectors described herein, and methods for inducing an immune response against ZIKV in a subject comprising administering to the subject an immunologically effective dose of an immunogenic composition of the present disclosure.

WO 2019/094801 A1

[Continued on next page]

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

RECOMBINANT VECTORS ENCODING ZIKA VIRUS PROTEIN SUBUNITS

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[1] This invention was made with government support under Grant No. P01 AI112524 awarded by the National Institutes of Health. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[2] This PCT application claims priority to United States Provisional Patent Application No. 62/584,629, filed November 10, 2017, the disclosure of which is incorporated herein by reference in its entirety.

FIELD

[3] Embodiments disclosed herein provide for compositions, methods, and uses of recombinant vectors encoding Zika virus protein subunits and immunogenic compositions thereof. Certain embodiments provide recombinant vectors encoding Zika virus nonstructural protein 1 (NS1 protein), and optionally Zika virus envelope (E) protein and premembrane (prM) protein. In certain embodiments, the vector encoding the Zika virus protein subunits is a vesicular stomatitis virus (VSV) vector. In some embodiments, the VSV vector backbone comprises an attenuating mutation. Other embodiments provide immunogenic compositions comprising a recombinant vector of the present disclosure. In some embodiments, the immunogenic compositions can be used in methods for inducing an effective immune response against Zika virus infection in a subject.

SEQUENCE LISTING

[4] The instant application contains a Sequence Listing which has been submitted via EFS-Web in computer readable form, and which is hereby incorporated by reference in its entirety. The ASCII copy, created on November 6, 2018, is named 509892.16_SEQ_LIST_ST25.txt, and is 32,964,087 bytes in size.

BACKGROUND

[5] Zika virus (ZIKV) is a mosquito-borne flavivirus that was first identified in monkeys from the Zika Forest, near Lake Victoria, Uganda in 1947. Sporadic outbreaks of ZIKV have since been reported in Africa and Asia. Historically, people infected with Zika virus have no or mild symptoms, including fever, rash, muscle pain, red eyes, headache, and conjunctivitis. In 2015, a ZIKV pandemic began in South America, Central America, the Caribbean, and the USA, suddenly becoming a global public health

issue. ZIKV from these recent outbreaks caused microcephaly, birth defects, Guillain-Barré syndrome, and other severe neurological disorders. ZIKV is primarily transmitted through the bite of an infected *Aedes* species mosquito (*e.g.*, *Ae. Aegypti* and *Ae. albopictus*) although other transmission modes such as sexual, blood transfusion, and maternal-fetal are also possible.

[6] Currently, more than 50 countries in South America, North American, Asia, Africa, Oceania, and Micronesia have reported indigenous human ZIKV cases. The first confirmed case of ZIKV in in the U.S. was in Florida in 2016. Between that date and June of 2017 there have been 41,891 ZIKV cases confirmed in the US, including 5,296 cases in the continental U.S. and 36,595 cases in the U.S. territories of Puerto Rico, the U.S. Virgin Islands and American Samoa.

[7] ZIKV is a member of the virus family *Flaviviridae*, which also includes other globally prevalent human pathogens such as dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), and Japanese encephalitis virus (JEV). The ZIKV genome encodes a single polyprotein that is cleaved posttranslationally into three structural proteins (capsid, pre-membrane, and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The E protein is a type II fusion protein which mediates cellular attachment and membrane fusion, and is the target for most neutralizing antibodies (Abs). Flavivirus prM protein typically associates with E to form heterodimers and is important for proper folding of E. Co-expression of prM and E of several flaviviruses including ZIKV results in the secretion of virus-like particles (VLPs) termed recombinant subviral particles. The prM protein is an integral part of both virions and subviral particles, and undergoes a cleavage event during virus maturation. Therefore, prM and E proteins have been the primary targets for the rational design of subunit and recombinant flavivirus vaccines.

[8] Recently, several ZIKV vaccine candidates have been reported, including nucleic acid (DNA and mRNA), inactivated virus, subunit, VLP, vectored vaccines, and live attenuated vaccines. These vaccine candidates triggered various degrees of humoral and cellular immunity and protection in rodent and/or nonhuman primate models. Among these candidates, DNA vaccine, subunit vaccine, and inactivated vaccine have been initiated for clinical trials. Currently, all ZIKV subunit, DNA, and mRNA vaccines undergoing preclinical or clinical trial have been targeted on the E or prM-E antigen. Despite these efforts, currently, there is no FDA-approved vaccine or antiviral drug for ZIKV.

SUMMARY

[9] The present disclosure provides recombinant vectors capable of expressing Zika virus protein subunits and eliciting an immune response against Zika virus when introduced into a subject.

[10] In a first aspect, the present disclosure provides recombinant vectors comprising a polynucleotide sequence encoding a Zika virus nonstructural protein 1 (NS1 protein). In some embodiments, the Zika

virus NS1 protein has at least 90% amino acid sequence identity with SEQ ID NO: 14. In some embodiments, the Zika virus NS1 protein comprises an amino acid sequence according to SEQ ID NO: 14.

[11] In some embodiments, the recombinant vector further comprises one or more polynucleotide sequences encoding a Zika virus envelope (E) protein or truncation mutant thereof, and a Zika virus premembrane (prM) protein.

[12] In some embodiments, the Zika virus E protein has at least 90% amino acid sequence identity with SEQ ID NO: 4; the Zika virus E protein truncation mutant has at least 90% amino acid sequence identity with one of SEQ ID NO 10 (E404), SEQ ID NO: 8 (E414), or SEQ ID NO: 6 (E415); and the Zika virus prM protein has at least 90% amino acid sequence identity with SEQ ID NO: 12.

[13] In some embodiments, the recombinant vector encodes the Zika virus NS1 protein, the Zika virus E protein or truncation mutant thereof, and the Zika virus prM protein.

[14] In some embodiments, the recombinant vector comprises a DNA plasmid vector or an RNA viral vector. In some embodiments, the viral vector is selected from the group comprising adenovirus, adeno-associated virus (AAV), retrovirus, lentivirus, vaccinia virus, cytomegalovirus, Sendai virus, modified vaccinia Ankara virus, and vesicular stomatitis virus (VSV).

[15] In some embodiments, the recombinant vector comprises a VSV vector.

[16] In some embodiments, VSV vector comprises at least one mutation in a methyltransferase-encoding region of an L protein of the VSV vector.

[17] In some embodiments, the at least one mutation is a nucleic acid mutation that results in an amino acid mutation at a position in the VSV vector selected from the group of K1651, G1670, D1762, K1795, and E1833.

[18] In some embodiments, the at least one mutation is a nucleic acid mutation that results in a G1670A mutation in the VSV vector.

[19] In some embodiments, the VSV vector comprises a nucleic acid sequence having at least 90% sequence identity to SEQ ID NO: 16.

[20] In some embodiments, the VSV vector comprises a nucleic acid sequence according to SEQ ID NO: 16, or SEQ ID NO: 16 encoding a G→A mutation at amino acid position 1670 of VSV L protein.

[21] In a second aspect, the present disclosure immunogenic compositions comprising at least one recombinant vector according of the present disclosure and a pharmaceutically acceptable excipient. In some embodiments, the immunogenic composition comprises an adjuvant.

[22] In a third aspect, the present disclosure provides methods for inducing an effective immune response against Zika virus in a subject, the method comprising administering to the subject an immunologically effective dose of an immunogenic composition of the present disclosure.

[23] In some embodiments, the subject is human. In some embodiments, the subject is pregnant, may be pregnant, or is trying to get pregnant.

[24] In some embodiments, the immunogenic composition is administered to the subject via a route selected from intranasal administration, subcutaneous administration, intramuscular administration, intradermal administration, and oral administration.

[25] In some embodiments, at least one subsequent immunologically effective dose of the immunogenic composition is administered to the subject.

[26] In a fourth aspect, the present disclosure provides methods for inducing an effective immune response against Zika virus in a subject, the method comprising expressing a Zika virus nonstructural protein 1 (NS1 protein) in cells of the subject. In some embodiments, the Zika virus NS1 protein has at least 90% amino acid sequence identity with SEQ ID NO: 14. In some embodiments, the Zika virus NS1 protein comprises an amino acid sequence according to SEQ ID NO: 14.

[27] In some embodiments, methods for inducing an effective immune response against Zika virus in a subject further comprise co-expressing a Zika virus envelope (E) protein or a truncation mutant thereof, and a Zika virus premembrane (prM) protein.

[28] In some embodiments, the Zika virus E protein has at least 90% amino acid sequence identity with SEQ ID NO: 4; the Zika virus E protein truncation mutant has at least 90% amino acid sequence identity with one of SEQ ID NO 10 (E404), SEQ ID NO: 8 (E414), or SEQ ID NO: 6 (E415); and the Zika virus prM protein has at least 90% amino acid sequence identity with SEQ ID NO: 12.

[29] In some embodiments, the Zika virus protein(s) are expressed from a recombinant vesicular stomatitis virus (VSV) vector.

[30] In a fifth aspect, the present disclosure provides expression cassettes comprising a promoter operably linked to a polynucleotide encoding a Zika virus nonstructural protein 1 (NS1 protein).

[31] In some embodiments, the polynucleotide encoding the Zika virus NS1 protein further encodes a Zika virus envelope (E) protein or a truncation mutant thereof, and a Zika virus premembrane (prM) protein. In some embodiments, the Zika virus NS1 protein has at least 90% amino acid sequence identity with SEQ ID NO: 14, the Zika virus E protein has at least 90% amino acid sequence identity with SEQ ID NO: 4, the Zika virus E protein truncation mutant has at least 90% sequence identity with one of SEQ ID NO 10 (E404), SEQ ID NO: 8 (E414), or SEQ ID NO: 6 (E415), and the Zika virus prM protein has at least 90% amino acid sequence identity with SEQ ID NO: 12.

BRIEF DESCRIPTION OF THE DRAWINGS

[32] **FIG. 1A** is an illustration of the Zika virus genome and the strategy employed to construct recombinant VSV expressing ZIKV antigens.

[33] **FIG. 1B** is a series of representative photographs depicting the plaque morphology of recombinant VSVs (rVSVs) expressing ZIKV antigens.

[34] **FIG. 2** is a representative line graph illustrating the single-step growth curves of the indicated rVSV vectors.

[35] **FIG. 3** is a representative photograph of an SDA-PAGE blot illustrating detection of ZIKV E protein expression from the indicated rVSV vectors using [^{35}S]methionine labelling.

[36] **FIGS. 4A-3C** are representative digital photographs of Western blots illustrating expression of ZIKV E protein truncations by VSV vector (FIG. 3A), expression of full-length ZIKV E protein by VSV vector (FIG. 3B), and expression of ZIKV NS1 protein by VSV vector (FIG. 3C).

[37] **FIG. 4D** is a series of representative digital photographs of Western blots illustrating the kinetics of ZIKV E protein expression by VSV vectors encoding the indicated ZIKV protein subunits.

[38] **FIG. 4E** is a series of representative electron micrographs depicting ZIKV virus-like particles (VLPs) resulting from expression of ZIKV protein subunits from the indicated recombinant VSV vectors.

[39] **FIG. 4F** is a representative digital photograph of Western blots illustrating the kinetics of ZIKV NS1-specific antibody induced by mtdVSV expressing ZIKV antigen.

[40] **FIG. 5** is a representative line graph illustrating the dynamics of mouse body weight following inoculation with the indicated rVSV vectors expressing ZIKV protein subunits.

[41] **FIG. 6** is a representative line graph depicting the kinetics of ZIKV-specific ELISA antibody induced by the indicated recombinant VSV vectors expressing ZIKV protein subunits.

[42] **FIG. 7A** is a series of representative photographs depicting the plaque morphology of MTase-defective recombinant VSV (mtdVSV) vector expressing the indicated ZIKV protein subunits.

[43] **FIG. 7B** is a representative line graph depicting the single-step growth curve of mtdVSVs expressing the indicated ZIKV protein subunits.

[44] **FIG. 7C** are representative digital photographs of Western blots illustrating expression of ZIKV E protein by the indicated mtdVSV vectors.

[45] **FIG. 7D** is a series of representative digital photographs of Western blots illustrating the kinetics of ZIKV E protein expression from the indicated mtdVSV vectors.

[46] **FIG. 7E** is a representative digital photograph of a Western blot illustrating the release of ZIKV NS1 protein into cell culture supernatant.

[47] **FIG. 8A** is a representative line graph illustrating the dynamics of mouse body weight following inoculation with the indicated mtdVSV vector.

[48] **FIG. 8B** is a representative line graph illustrating the kinetics of ZIKV-specific antibody induced by the indicated mtdVSV vectors expressing ZIKV protein subunits.

[49] **FIGS. 8C & 8D** are representative dot plots illustrating levels of ZIKV-specific neutralizing

antibody titer at week 5 post-inoculation with the indicated mtdVSV vector (FIG. 8C) or ZIKV NS1-specific antibody detected by ELISA following inoculation with the indicated mtdVSV vector (FIG. 8D).

[50] **FIGS. 9A-9E** are representative bar graphs illustrating that MTase-defective rVSV-based vectors induce ZIKV-specific T helper cell responses. The representative bar graphs illustrate proliferation of CD4+ T cells (CD4^{+}CD3^{+}) (FIG. 9A), and the frequencies of ZIKV-specific Th1 cells (IFN-γ^{+}CD4^{+} and TNF-α^{+}CD4^{+}) (FIG. 9B), Th2 cells (IL-4^{+}CD4^{+}, IL-5^{+}CD4^{+}, IL-10^{+}CD4^{+}) (FIG. 9C), Th17 cells (IL-17A^{+} CD4^{+}) (FIG. 9D), and Tfh cells (IL-21^{+} CD4^{+}) (FIG. 9E). Data are expressed as mean % positive cells (the mean of 15 samples: 3 wells × 5 mice) ± SD. *indicates that the group was statistically different with unstimulated and DMEM groups ($p \leq 0.05$). P value in from left to right for each panel: (A) **** P = 3.55 × 10-9, **** P = 4.10 × 10-6, **** P = 4.21 × 10-7. (B) ** P = 0.00676, ** P = 0.00394, **** P = 7.58 × 10-6, **** P = 3.32 × 10-5. (C) * P = 0.0243, ** P = 0.00180, * P = 0.0304, * P = 0.0149, *** P = 0.000409, **** P = 7.72× 10-6, * P = 0.0102. (D) ** P = 0.00749, **** P = 2.52× 10-6, *** P = 0.000907. (E) *** P = 0.000313, *** P = 0.000162.

[51] **FIG. 10** is a representative line graph illustrating the dynamics of body weight change of BALB/c mice after immunization with the indicated mtdVSV vectors.

[52] **FIG. 11** is a representative line graph illustrating the dynamic of body weight change of BALB/c mice inoculated with the indicated mtdVSV vector, after challenge with ZIKV. The average body weights of ten mice are shown. No significant difference in body weight was observed among groups ($P>0.05$).

[53] **FIG. 12A** is a representative line graph illustrating the dynamic of viremia in unimmunized mice following challenge with ZIKV Cambodian strain. Data were analyzed using one-way multiple comparisons and compared to the placebo DMEM group (****$p < 0.0001$; N.S. indicates not significant).

[54] **FIG. 12B** is a representative dot plot illustrating the protective effects of MTase-defective rVSV vectors expressing the indicated ZIKV protein subunits in BALB/c mice as measured by viremia.

[55] **FIG. 13** is a representative line graph illustrating the dynamics of body weight changes of A129 mice following inoculation with the indicated vectors.

[56] **FIGS. 14A-14G** are representative dot plots illustrating the ability of MTase-defective rVSV vectors expressing the indicated ZIKV protein subunits to induce ZIKV-specific antibody in A129 mice. ZIKV E protein-specific antibody was measured by ELISA at week 1 (FIG. 14A) and at week 3 (FIG. 14B) post-immunization. ZIKV-specific neutralizing Ab was measured at week 1 (FIG. 14C) and at week 3 (FIG. 14D) post-immunization. ZIKV NS1 protein-specific Ab was measured by ELISA at week 1 (FIG. 14E) and at week 3 (FIG. 14F) post-immunization. Exact P value in each panel: (A) **** P = 1.36 × 10-6; (C) **** P = 5.44 × 10-5; (F) **** P = 6.70 × 10-5; (G) **** P = 4.32 × 10-6, N.S. = not significant.

[57] **FIG. 15A** is a representative dot plot illustrating the clinical scores of A129 mice inoculated with

the indicated vector, after ZIKV challenge.

[58] **FIG. 15B** is a representative line graph illustrating weight change in A129 mice inoculated with the indicated vector, after ZIKV challenge.

[59] **FIG. 15C** is a representative line graph illustrating the body weight change for individual A129 mice inoculated with the pCI-NS1 vector.

[60] **FIG. 15D** is a representative photograph of a Western blot illustrating the expression of ZIKV NS1 protein by rVSV-G1670A and pCI vectors in BSRT7 cells.

[61] **FIG. 15E** is a line graph illustrating the NS1-specific antibody response in BALB/c mice. P value from top to bottom: **** $P = 3.95 \times 10-5$, **** $P = 3.48 \times 10-5$, **** $P = 1.51 \times 10-5$, ** $P = 0.00183$.

[62] **FIG. 15F** is a line graph illustrating the E-specific antibody response in BALB/c mice.

[63] **FIG. 15G** is a dot plot illustrating that NS1 alone provided partial protection against viremia in BALB/c mice at day 3 post-challenge. P value from top to bottom: **** $P = 4.15 \times 10-6$, *** $P = 0.000161$, *** $P = 0.000767$, *** $P = 0.000187$, * $P = 0.0250$.

[64] **FIG. 15H** is a dot plot illustrating that NS1 alone provided protection against viremia in BALB/c mice at day 7 post-challenge. P value from top to bottom: ** $P = 0.00510$, *** $P = 0.000114$, ** $P = 0.00277$, * $P = 0.0308$

[65] **FIGS. 16A-16F** are representative dot plots illustrating the ability of MTase-defective rVSV vectors expressing the indicated ZIKV protein subunits to protect A129 mice from viremia following ZIKV challenge and prevent ZIKV replication *in vivo*. Mice were inoculated with the indicated vectors and subjected to ZIKV challenge. After ZIKV challenge, the level of viremia was measured at day 3 (FIG. 16A) and at day 7 (FIG. 16B) post-challenge. At day 7 post-challenge, all mice were terminated, brain (FIG. 16C), lung (FIG. 16D), spleen (FIG. 16E), and uterus/ovary (FIG. 16F) tissues were harvested and analyzed for ZIKV RNA. Data were analyzed using one-way multiple comparisons and compared to the placebo DMEM group or the pCI group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; N.S., not significant).

[66] **FIG. 17** is a series of photographs illustrating the ability of the indicated vectors to prevent ZIKV-induced encephalitis in A129 mice.

[67] **FIGS. 18A & 18B** are representative digital photographs of Western blots illustrating a comparison of ZIKV E protein expression by pCI-prM-E and rVSV-G1670A-prM-E (FIG. 18A), and the expression of ZIKV NS1 protein by pCI-NS1 (FIG. 18B).

[68] **FIG. 19A** is a line graph illustrating the dynamics of viremia in unimmunized mice after challenge with ZIKV.

[69] **FIG. 19B** is a dot plot illustrating the quantification of VSV RNA in the brains of BALB/c mice. Data were expressed together with the GMT of 10 mice (black bars). P value from top to bottom: **** P

= 4.25 × 10-7, *** P = 0.000710, *** P = 0.000371.

[70] **FIG. 19C** is a line graph illustrating the kinetics of ZIKV E-specific antibody induced by mtdVSV expressing ZIKV antigen. Data are expressed as the GMT of five mice □ standard deviation.

[71] **FIG. 19D** is a line graph illustrating the kinetics of ZIKV NS1-specific antibody induced by mtdVSV expressing ZIKV antigen.

[72] **FIG. 19E** is a dot plot illustrating the ability of mtdVSV-based vaccine to protect BALB/c mice from viremia at day 3 post-challenge. P value from top to bottom: **** P = 1.02 × 10-5, **** P = 6.06 × 10-5, ** P = 0.00345, *** P = 0.00310

[73] **FIG. 19F** is a dot plot illustrating the ability of mtdVSV-based vaccine to protect BALB/c mice from viremia at day 7 post-challenge. P value from top to bottom: *** P = 3.89 × 10-5, * P = 0.0201. Significance was calculated using t-test. N.S. indicates not significant.

[74] **FIG. 20A** is a line graph illustrating weight loss in A129 mice immunized intramuscularly with rVSV-prM-E-NS1.

[75] **FIG. 20B** is a dot plot illustrating ZIKV E-specific antibody in A129 at week 4 post immunization with rVSV-prM-E-NS1 in A129 mice.

[76] **FIG. 20B** is a dot plot illustrating ZIKV NS1-specific antibody in A129 at week 4 post immunization with rVSV-prM-E-NS1 in A129 mice.

[77] **FIG. 20D** is a line graph illustrating weight loss following challenge with ZIKV in A129 mice immunized intramuscularly with rVSV-prM-E-NS1.

[78] **FIG. 20E** is a line graph illustrating viremia following challenge with ZIKV in A129 mice immunized intramuscularly with rVSV-prM-E-NS1.

[79] **FIG. 21** is an illustration of the VSV genome and the strategy employed to construct G1670A MTase-defective rVSV (mtd-rVSV) expressing ZIKV antigens.

[80] **FIG**. **22** is a line graph illustrating the dynamics of body weight change of BALB/c mice after immunization with mtdVSV-based vaccine candidates.

[81] **FIG. 23** is a line graph illustrating the dynamics of body weight change of immunized BALB/c mice after challenge with ZIKV.

[82] **FIG**. **24** is an illustration of the VSV genome and the strategy employed to construct D1762 MTase-defective rVSV (mtd-rVSV) expressing ZIKV NS1.

[83] **FIG. 25A** is a dot plot illustrating viremia (RNA copies) in mice on day 3 post-challenge.

[84] **FIG. 25B** is a dot plot illustrating viremia (RNA copies) in mice on day 7 post-challenge.

[85] **FIG. 25C** is a dot plot illustrating viremia (PFU) in mice on day 3 post-challenge.

[86] **FIG. 25D** is a dot plot illustrating viremia (PFU) in mice on day 7 post-challenge.

[87] **FIG. 26A** is a line graph illustrating the dynamics of mouse body weight change after vaccination

with rVSV-D1762A-483-NS1.

[88] **FIG. 26B** is a line graph illustrating the kinetics of ZIKV NS1-specific antibody induced by rVSV-D1762A-483-NS1.

[89] **FIG. 26C** is a line graph illustrating body weight changes after ZIKV challenge.

[90] **FIG. 26D** is a line graph illustrating body weight change of each mouse after ZIKV challenge.

[91] **FIG. 26E** is a survival curve of A129 mice after ZIKV challenge.

[92] **FIG. 26F** is a dot plot illustrating viremia in A129 mice on day 3 after challenge with ZIKV.

DEFINITIONS

[93] An "immunogenic composition" refers to any mixture, aqueous solution, non-aqueous solution, suspension, emulsion, gel, or the like, containing a recombinant vector provided by the present disclosure and at least one other component. Other components can be, for example, one or more pharmaceutical agents, carriers, vehicles, excipients, or a combination thereof. Generally, immunogenic compositions can be prepared by uniformly combining the recombinant vector with a liquid carrier, vehicle, or excipient, or a finely divided solid carrier, vehicle, or excipient, or combination thereof. An immunogenic composition includes enough recombinant vector to produce an effective immune response. Accordingly, the immunogenic compositions described herein encompass any composition made by admixing a compound of recombinant vector described herein and a pharmaceutically acceptable carrier, vehicle, or excipient. By "pharmaceutically acceptable" it is meant that the carrier, vehicle, or excipient is approved, or approvable, by a regulatory body such as the FDA and/or is capable of being incorporated into a human pharmaceutical therapeutic.

[94] As used herein, the term "effective immune response" refers to an immune response that confers immunity against an infection, reduces the probability of infection recurrence, confers maternal immunity to an offspring, or prevents development of disease resulting from an infection. For instance, an immune response is considered to be an "effective immune response" if it is sufficient to prevent a subject or an offspring of a subject from developing a Zika virus infection after administration of or exposure to a challenge dose of Zika virus. An effective immune response can include a cell mediated immune response, and/or a humoral immune response. An immune response is also considered to be an "effective immune response" if it is sufficient to prevent Zika disease in a subject infected with Zika virus; although Zika infection may present, an effective immune response prevents development of Zika disease in the subject.

[95] The term "immunologically effective dose" refers to an amount of a vaccine or immunogenic composition provided by the present disclosure sufficient to cause an effective immune response. The immunologically effective dose can be administered in one or more administrations. The precise

determination of what would be considered an immunologically effective dose can be based on factors individual to each subject, including but not limited to the subject's age, size, and route of administration, as well as the judgment of the prescribing physician.

DETAILED DESCRIPTION

[96] In the following sections, various compositions and methods are described in order to detail various embodiments. Practicing the various embodiments does not require the employment of all of the specific details outlined herein, but rather concentrations, time, and other specific details may be modified. In some cases, well known methods or components have not been included in the description

[97] It is described herein for the first time that inoculation of a subject with a Zika virus (ZIKV) nonstructural protein 1 (NS1 protein) antigen provides partial protection against ZIKV challenge. Embodiments disclosed herein provide compositions, methods, and uses for recombinant vectors encoding ZIKV protein subunits, and immunogenic compositions thereof. Certain embodiments provide recombinant vectors encoding ZIKV NS1 protein, or variants thereof, and optionally, ZIKV envelope (E) protein, or variants thereof, and/or premembrane (prM) protein, or variants thereof. Other embodiments provide expression cassettes comprising a promoter operably linked to a polynucleotide that encodes the ZIKV NS1 protein, and optionally ZIKV E and prM proteins. In some embodiments, the disclosed expression cassettes can be incorporated into a vector to produce a recombinant vector. Also provided are immunogenic compositions comprising one or more recombinant vectors described herein, and methods for inducing an immune response against ZIKV in a subject comprising administering to the subject an immunologically effective dose of an immunogenic composition of the present disclosure.

[98] Current efforts to develop ZIKV subunit vaccines have been exclusively focused on prM and E proteins which rely on generating high levels of neutralizing Ab. However, it has been reported that Abs generated against one flavivirus can cross-react with other species of flavivirus without neutralizing them, which may facilitate infection by the second flavivirus in cells expressing Fc receptors. This process is called Antibody Dependent Enhancement (ADE). Additional ZIKV subunit vaccines are needed.

[99] Certain embodiments provide recombinant vectors encoding ZIKV protein subunits. Similarly to all other flaviviruses, such as dengue virus, yellow fever virus, West Nile virus, Japanese encephalitis virus, and tick-borne encephalitis virus, the ZIKV genome encodes a single polyprotein that is cleaved posttranslationally by host and viral proteases into three structural proteins (capsid (C), premembrane (prM), and envelope (E)) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). In some embodiments, a recombinant vector encodes ZIKV NS1. In some embodiments, the recombinant vector comprises a polynucleotide sequence that encodes the ZIKV NS1 protein. In certain embodiments, the recombinant vector encoding ZIKV NS1 can further encode ZIKV E and prM proteins.

In such embodiments, a continuous polynucleotide sequence can encode all three of the ZIKV E, prM, and NS1 proteins, or each of the proteins can be encoded by a distinct polynucleotide.

[100] The E protein is a type II fusion protein, which mediates cellular attachment and membrane fusion, and is the target for most neutralizing antibodies (Abs). Flavivirus prM protein typically associates with E protein to form heterodimers and is important for proper folding of E protein. Co-expression of prM and E of several flaviviruses including ZIKV results in the secretion of virus-like particles (VLPs) termed recombinant subviral particles, which have structural and functional features of the virion envelope. Flavivirus NS1 has been implicated in various functions, including genome replication and immune evasion.

[101] In some embodiments, the E protein encoded by the recombinant vector can be a full-length E protein (504 amino acids; SEQ ID NO: 4), or a truncated mutant thereof. In some embodiments, the truncated mutant can be a protein having the N-terminal 404 amino acids of the full-length E protein (SEQ ID NO: 10), the N-terminal 414 amino acids of the full-length E protein (SEQ ID NO: 8), or the N-terminal 415 amino acids of the full-length E protein (SEQ ID NO: 6).

[102] In certain embodiments, the SN1 protein encoded by the recombinant vector has an amino acid sequence having at least 90% sequence identity with the SN1 protein of ZIKV Cambodian strain (FSS13025) (SEQ ID NO: 14). In some embodiments, the ZIKV E protein encoded by the recombinant vector has an amino acid sequence having at least 90% sequence identity with the E protein of ZIKV Cambodian strain (FSS13025) (SEQ ID NO: 4). In those embodiments where the recombinant vector encodes a truncated E protein mutant, the truncated protein can have at least 90% sequence identity with the N-terminal 404 amino acids of the full-length E protein (SEQ ID NO: 10), the N-terminal 414 amino acids of the full-length E protein (SEQ ID NO: 8), or the N-terminal 415 amino acids of the full-length E protein (SEQ ID NO: 6). In some embodiments, the ZIKV prM protein encoded by the recombinant vector has an amino acid sequence having at least 90% sequence identity with the prM protein of ZIKV Cambodian strain (FSS13025) (SEQ ID NO: 12). While the sequences of the ZIKV Cambodian strain (FSS13025) are provided here, the sequences of other ZIKV strains and their NS1, E, and prM proteins are known and can be similarly used to construct a recombinant vector, or construct, as described herein. The full nucleic acid sequence of ZIKV Cambodian strain (FSS13025) is provided by SEQ ID NO: 1, and the amino acid sequence is provided by SEQ ID NO: 2.

[103] In some embodiments, where the recombinant vector encodes only the ZIKV NS1 protein, expression of NS1 alone can provide at least partial protection against ZIKV challenge, without inducing neutralizing antibody. In other embodiments, where the recombinant vector encodes ZIKV NS1, E, and prM proteins, the three ZIKV proteins can be expressed from the recombinant vector as a single polyprotein – e.g., as a prM-E-NS1 polyprotein. This prM-E-NS1 polyprotein can be cleaved

posttranslationally by host and viral proteases into the three distinct proteins: prM, E, and NS1. When expressed together, the ZIKV E, prM, and NS1 proteins can provide complete protection against ZIKV challenge.

[104] Other embodiments provide expression cassettes comprising an open reading frame (ORF) polynucleotide sequence encoding a ZIKV NS1 protein. In some embodiments, the ORF polynucleotide sequence also encodes ZIKV E protein and ZIKV prM protein.

[105] In some embodiments, the ORF polynucleotide sequence of the expression cassette is operably linked to one or more control elements compatible with expression in a selected vector. In some embodiments, the expression cassette comprises a polyadenylation site. In certain embodiments, the expression cassette can be inserted into a vector and can be expressed therefrom. In some embodiments, the one or more control elements comprise at least one promoter.

[106] Vectors useful as backbones for the recombinant vectors described herein can be any vector suitable for expression in a chosen system. For example, where it is an aim to express ZIKV protein subunits *in vitro*, an appropriate plasmid, viral vector, bacterial vector, insect vector, baculovirus expression vector, yeast vector, mammalian cell vector, or the like, can be selected. Suitable vectors can be identified by the skilled artisan taking into consideration the characteristics for expressing the ZIKV protein subunits under the desired conditions.

[107] Where it is an aim to express the ZIKV protein subunits *in vivo* in a subject, for example in order to generate an effective immune response against a ZIKV antigen, elicit protective immunity against ZIKV, and/or prevent development of Zika disease in an infected subject, vectors that are safe for use and suitable for expression in that subject should be chosen. In some embodiments, the vector is selected from adenovirus, adeno-associated virus (AAV), retrovirus, lentivirus, vaccinia virus, cytomegalovirus, Sendai virus, modified vaccinia Ankara virus, and vesicular stomatitis virus (VSV). Such viruses, when used as expression vectors, can be innately non-pathogenic in the target subjects, or can be modified to render them non-pathogenic in the target subjects.

[108] In some embodiments, the vector is vesicular stomatitis virus (VSV). VSV is a prototypical nonsegmented negative-sense (NNS) RNA virus that belongs to the *Rhabdoviridae* family. VSV is a natural pathogen of livestock such as cattle and swine. As such, there is no pre-existing immunity against VSV in the human population. VSV is an RNA virus and it does not undergo either recombination or integration into host cell DNA. VSV also grows to a high titer in a wide range of mammalian cells, an important feature for vaccine manufacturing. Thus, VSV is an excellent platform for vaccine development. VSV can accommodate multiple foreign genes, and antigens are highly expressed in both cell culture and animals by VSV, enabling the generation of strong systemic immune responses.

[109] In response to the sudden outbreaks of Ebola virus in Africa in 2013, a VSV-based Ebola virus

vaccine was tested in human clinical trials. The VSV-based Ebola virus vaccine was shown to be highly efficacious in protecting against Ebola virus infection in humans. Currently, at least 15 independent human clinical trials are ongoing worldwide to test the efficacy of VSV-based vaccine candidates and to utilize VSV as an oncolytic agent for cancer therapy. In general, VSV is safe in humans, although high doses of VSV can cause side effects in some people including joint and muscle pain.

[110] In certain embodiments, a polynucleotide sequence encoding ZIKV NS1 protein can be incorporated into VSV, thereby producing a recombinant VSV (rVSV) that encodes ZIKV NS1 (rVSV-NS1). Of course, other ZIKV protein encoding rVSVs can also be constructed. As described in Example 1, thirteen recombinant viruses using the wild-type VSV genome as the backbone were constructed. As depicted in the top panel of Fig. 1, the positive-sense genome of ZIKV encodes a polyprotein which is proteolytically cleaved into 10 viral proteins including capsid (C), pre-membrane (prM), envelope (E), nonstructural protein 1 (NS1), NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The middle panel of FIG. 1 illustrates expression cassettes comprising full-length ZIKV E, E truncations (E404, E414, and E415) lacking transmembrane domain, prM-E, prM-E truncations (prM-E404, prM-E414, and prM-E415), and prM-E-NS1. These genes were amplified from an infectious cDNA clone of ZIKV Cambodian strain by PCR, digested by XhoI and SmaI, and inserted into the same sites at the gene junction between G and L proteins in the VSV genome. The organization of the nonsegmented negative –sense VSV genome is depicted in the bottom panel of FIG1A (le = VSV leader sequence; N = nucleocapsid gene; P = phosphoprotein gene; M = matrix protein gene; G = glycoprotein gene; L = large polymerase gene; tr = VSV trailer sequence).

[111] In some embodiments, the VSV vector is pVSV1(+) (SEQ ID NO: 15) or pVSV1(+)-GxxL (SEQ ID NO: 16).

[112] In certain embodiments, the recombinant VSV vector encodes ZIKV E, prM, and NS1 proteins (rVSV-E-prM-NS1). As depicted in Fig. 2, rVSV-E-prM-NS1, along with the other recombinant vectors tested, grew to high titer in cell culture and had similar, but slightly lower, virus replication kinetics compared to parental rVSV.

[113] The ZIKV E, prM, and NS1 proteins expressed from rVSV-E-prM-NS1 produced virus-like particles (VLPs) that are structurally similar to native ZIKV virions (*see*, Fig. 4E and Example 3).

[114] In certain embodiments, inclusion of one or more ZIKV protein subunit-encoding polynucleotide sequences can attenuate the host viral vector. As described in Example 4, rVSVs co-expressing prM and E protein, or truncated E protein mutants, were more attenuated in mice that rVSV expressing E protein or truncated E protein mutants alone. Recombinant rVSV-E-prM-NS1 was the most attenuated virus of those tested. Mice inoculated with rVSV-E-prM-NS1 experienced little or no weight loss and did not display any other clinical signs. A single dose inoculation of mice with rVSV-E-prM-NS1 resulted in

high levels of serum antibody response as early as 1 to 2 weeks post-inoculation (*see* Example 4).

[115] In certain embodiments, and as described above, ZIKV NS1 can be expressed from any appropriate vector. The vector backbone need not be VSV. In some embodiments, the vector can be a DNA vector. For example, a polynucleotide encoding ZIKV NS1 can be incorporated into a DNA vector such as pCI. As described in Example 10, A129 mice inoculated with a pCI-NS1 recombinant vector were partially protected from ZIKV challenge, even in the absence of detectable ZIKV neutralizing antibody (*see* Example 10). In some embodiments, a non-VSV recombinant vector encodes ZIKV NS1, and optionally, ZIKV E and prM.

[116] In certain embodiments, partial protection is an effective immune response. In embodiments where NS1 is presented alone, i.e., in the absence of prM and/or E protein (or truncations thereof), an effective immune response may prevent development of Zika disease in a subject, despite the subject being infected with Zika virus. An immunogenic composition comprising NS1 alone or expressing NS1 alone would avoid Antibody Dependent Enhancement issues experienced by ZIKV subunit vaccines expressing ZIKV prM and/or E protein.

[117] Certain embodiments provide a modified, highly attenuated VSV backbone. In some embodiments, the VSV large (L) polymerase protein comprises at least one mutation in the S-Adenosyl methionine (SAM) binding site in the methyltransferase-encoding region. In some embodiments, the VSV L protein is mutated at at least one amino acid position selected from K1651, G1670, D1762, K1795, and E1833. In some embodiments, the VSV L protein is mutated at G1670, G1762, or both G1670 and G1762. The mutation(s) can be any mutation(s) that result(s) in defective mRNA cap guanine-N-7 methylation. In certain embodiments, the mutation is a G1670A mutation, a G1762A mutation, or both G1670A and G1762A mutations.

[118] In some embodiments, the ZIKV protein subunit-expressing recombinant VSVs described herein comprise at least one attenuating mutation in the VSV L protein. In some embodiments, the at least one attenuating mutation comprises G1670A and/or G1762A. As described throughout the Examples, rVSV-G1670A and rVSV-G1762A vectors were highly attenuated in both BALB/c and A129 mice. ZIKV proteins, including ZIKV E and NS1 are highly expressed by the MTase-defective rVSV-G1670A (*e.g.*, from rVSV-G1670A- prM-E and rVSV-G1670A-prM-E-NS1). Single doses of the highly attenuated rVSV-G1670A-prM-E-NS1 were observed to protect BALB/C and A129 mice from ZIKV viremia (*see* Examples 8 and 9). Similar results were observed with rVSV-G1762A serving as the VSV backbone (*see* Example 13).

[119] In other embodiments, the recombinant vectors described herein can be formulated into an immunogenic composition against ZIKV. In some embodiments, the immunogenic composition against ZIKV can be a pharmaceutical composition, such as a vaccine.

[120] In certain embodiments, the immunogenic composition against ZIKV can include one or more pharmaceutically acceptable carriers, vehicles, excipients, or any combination thereof. Suitable pharmaceutical carriers, vehicles, and excipients for formulating a pharmaceutically acceptable immunogenic compound, including vaccines, are known in the art. In some embodiments, the immunogenic composition can include at least one adjuvant for further induction of the immune system in a subject when administered. In some embodiments, the immunogenic composition can include a nanoparticle delivery system.

[121] Other embodiments provide methods for inducing an effective immune response against ZIKV in a subject. In some embodiments, the methods can include administering an immunologically effective dose of an immunogenic composition against ZIKV described herein to the subject. In other embodiments, the methods can include expressing a ZIKV NS1 protein described herein in cells of a subject. In some embodiments, the methods can further comprise co-expressing ZIKV E and prM proteins in the subject. In some embodiments, the ZIKV protein subunits can be expressed from a recombinant vector described herein.

[122] In certain embodiments, the subject is human. In some embodiments, the subject is human subject that is pregnant, may be pregnant, or is trying to get pregnant. The immunogenic composition against ZIKV can be administered to a subject at risk of acquiring a ZIKV infection, or a subject having a ZIKV infection. Accordingly, certain embodiments provide methods for preventing a ZIKV infection is such a subject comprising administering an immunogenic composition described herein.

[123] In some embodiments, an immunogenic composition against ZIKV can be administered to a patient post-infection, thereby protecting them from subsequent ZIKV infections and/or ameliorating the symptoms from subsequent infections.

[124] In some embodiments, a subject is administered at least one immunologically effective dose subsequent to an initial dose. The immunogenic composition against ZIKV can be administered to the subject once, or can be administered a plurality of times, e.g., one, two, three, four, or five times.

[125] In certain embodiments, immunogenic compositions against ZIKV can be administered to a subject in any manner, including for example, subcutaneously, intravenously, by oral administration, inhalation, intradermally, by transdermal application, intravaginal application, topical application, intranasally, intramuscularly, or by rectal administration. In one embodiment, an immunologically effective dose of an immunogenic composition against ZIKV is administered to a human intranasally. In other embodiments, the route of administration can be intradermal administration or intramuscular administration.

[126] In some embodiments, an immunogenic composition can be administered to a subject in an appropriate pharmaceutically acceptable carrier or diluent, co-administered with enzyme inhibitors, or in

an appropriate carrier such as liposomes. As used herein, the term "pharmaceutically acceptable carrier" includes diluents such as saline and aqueous buffer solutions. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms or other stabilizing formulation.

[127] Pharmaceutical compositions suitable for injectable use can be administered by means known in the art. For example, sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion can be used. In all cases, the composition can be sterile and can be fluid to the extent that easy syringability exists. It can be stable under the conditions of manufacture and storage and can be preserved against the contaminating action of microorganisms such as bacteria and fungi. The pharmaceutically acceptable carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion, and by the use of surfactants. Prevention of microorganisms can be achieved by heating, exposing the agent to detergent, irradiation or adding various antibacterial or antifungal agents.

[128] Sterile injectable solutions can be prepared by incorporating a vector provided by the present disclosure or a compound comprising the same (e.g. a compound that induces an immune response to ZIKV) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization.

[129] Upon formulation, sterile injectable solutions will be administered in a manner compatible with the dosage formulation and in such amount as is immunologically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above. It is contemplated that compositions are especially suitable for intramuscular, subcutaneous, intradermal, intranasal and intraperitoneal administration.

[130] In another embodiment, nasal solutions or sprays, aerosols or inhalants can be used to deliver the immunogenic composition of interest. Additional formulations that are suitable for other modes of administration include suppositories and pessaries.

[131] Certain formulations can include excipients, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like.

[132] A pharmaceutical composition can be prepared with carriers that protect active ingredients against rapid elimination from the body, such as time-release formulations or coatings. Such carriers include controlled release formulations, such as, but not limited to, microencapsulated delivery systems,

and biodegradable, biocompatible polymers, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, polyorthoesters, polylactic acid and others are known.

EXAMPLES

[133] The materials, methods, and embodiments described herein are further defined in the following Examples. Certain embodiments are defined in the Examples herein. It should be understood that these Examples, while indicating certain embodiments, are given by way of illustration only. From the disclosure herein and these Examples, one skilled in the art can ascertain the essential characteristics of this disclosure, and without departing from the spirit and scope thereof, can make various changes and modifications to the subject matter provided by this disclosure to adapt it to various usages and conditions.

[134] The following examples describe the development of a methyltransferase (MTase)-defective rVSV (mtdVSV)-based ZIKV vaccine platform. A panel of rVSV expressing ZIKV prM-E-NS1, prM-E, E, E truncation mutants, and NS1 was recovered. These mtdVSV-based ZIKV vaccine candidates were highly attenuated but remained effective in triggering ZIKV-specific antibody and T cell immunity in mice, and provided complete protection against ZIKV challenge in immunocompetent BALB/c and type 1 interferon receptor-deficient A129 mice. In addition, the examples demonstrate that NS1 alone can confer partial protection from ZIKV infection.

Example 1 – Recovery of Recombinant VSVs Expressing ZIKV Protein Subunits

[135] In one illustrative method, thirteen recombinant viruses were constructed using the wild-type VSV genome as the backbone (Fig. 1A). These constructs allowed for the comparison of the immunogenicity of various combinations of ZIKV wild-type and mutant proteins, all including the E protein, since it is known in other flaviviruses to be the main target for neutralizing antibody (Ab). ZIKV E protein consists of two principal domains, the N-terminal ectodomain containing the major neutralizing epitopes and the C-terminal transmembrane (TM) domain (Fig. 1A). The major neutralizing epitopes are located in the ectodomain of the E protein.

[136] The rVSV-E recombinant virus was constructed first, which expressed the full-length E protein (504 amino acids; SEQ ID NO: 4). Because the exact boundary between the ectodomain and the TM domain is unclear, three E truncation mutants lacking the predicted TM domain were generated and incorporated in three recombinant viruses, rVSV-E404, rVSV-E414, and rVSV-E415 which express the N-terminal 404 (SEQ ID NO: 10), 414 (SEQ ID NO: 8), and 415 (SEQ ID NO: 6) amino acids of the E protein, respectively (Fig. 1A). Four recombinant viruses were constructed that would co-express prM with E, and each of the same E deletions, respectively, as a polyprotein with anchor C (signal peptide) (Fig. 1A). These recombinant viruses were named rVSV-aE, rVSV-aE404, rVSV-aE414, and rVSV-

aE415. Also constructed were four recombinant viruses that co-expressed anchor C-prM with E, or the same E deletions, as a polyprotein (Fig. 1A). The recombinant rVSV-prM-E-NS1, which expresses prM, E, and NS1 as a polyprotein, was also constructed. (Fig. 1A)

[137] Plaques formed by rVSV were 3.5 ± 0.8 mm (mean ± standard deviation) in diameter, while plaques formed by rVSV expressing ZIKV protein subunits ranged from 2.6 ± 0.6 mm to 3.0 ± 0.6 mm in diameter (Fig. 1B), indicating that expression of these ZIKV proteins reduced the replication and/or cell-to-cell spread of rVSV. Replication kinetics of rVSV, rVSV-E, rVSV-prM-E, and rVSV-prM-E-NS1 were compared in culture. All recombinant viruses grew to high titer in cell culture and had similar but slightly lower virus replication kinetics compared to parental rVSV (Fig. 2), except for rVSV-prM-E-NS1, which had a significant delay in replication kinetics compared to parental rVSV ($P<0.05$) (Fig. 2).

[138] Recombinant viruses were constructed as described in Example 12.

Example 2 – High-Level Expression of ZIKV Proteins by the VSV Vector

[139] In an illustrative method, the expression levels of VSV-vectored ZIKV E protein and its truncations were assessed in infected cells. A 54 kDa full-length E protein was detected in cells infected with rVSV-prM-E and rVSV-prM-E-NS1 but not with rVSV-E (Figs. 3A and 3B). The NS1 protein was only detected in rVSV-prM-E-NS1 infected cells (Fig. 3C), as expected. A smaller E protein was detected in cells expressing the truncated E protein, consistent with the shorter C-terminal domain (Fig. 3A). Quantitative analysis of three independent experiments indicated that rVSVs co-expressing prM with E or E truncations had approximately 5 times greater E protein expression compared to rVSVs expressing E or E truncations without prM. At 48 h post-inoculation, cell culture supernatants were harvested and subjected to Western blot. Results demonstrated that all rVSVs co-expressing prM and E/E truncations released enough E/E truncation proteins into the supernatant to be easily detectable without the need for concentration. NS1 protein was also secreted into cell culture medium. However, no E/E truncation was detectable in cell medium from rVSVs expressing E/E truncations alone. The expression level of E/E truncations with or without anchor C signal peptide by VSV vector were compared. As indicated in Fig. 4F, rVSV constructs with anchor C had more abundant expression of E/E truncations compared to rVSV constructs without anchor C. E truncations were also detected in the supernatants of rVSV constructs with anchor C. However, full-length E protein was still not detectable by rVSV-aE, even though the anchor was fused with E. Thus, co-expression of anchor C and prM with the E/E truncations significantly increased their E expression and/or stability. These results also indicate that the prM, E, and NS1 proteins were proteolytically cleaved from the polyprotein and secreted into cell culture supernatants when expressed from a VSV vector.

[140] The kinetics of E protein expression in virus-infected cells were evaluated (Fig. 3D). E protein was detectable in rVSV-prM-E at 12 h post-infection, reached the highest expression level at 24 h post-

infection, and declined by 36 h primarily because cells were lysed by this time point, as indicated by the reduction in β-actin. Recombinant rVSV-prM-E-NS1 had a significant delay in E protein expression. E protein was detectable at 24 h and reached its highest level at 36 h post-infection, without cell death as indicated by a continued high level of β-actin. E protein was not detectable in cells infected by rVSV-E even at the time when most cells were lysed. The expression of E protein by rVSV-prM-E and rVSV-prM-E-NS1, but not by rVSV-E in virus-infected cells was confirmed by [^{35}S] methionine-cysteine metabolic labeling (Fig. 3).

Example 3 – Expression of prM-E or prM-E-NS1 by rVSV Generates Virus-Like Particles

[141] In one illustrative method, cells were infected with rVSV-E, rVSV-prM-E or rVSV-prM-E-NS1 and the cell culture medium was harvested at 24-48 h post-infection. Two types of particles, VSV and ZIKV virus-like particles (VLPs), were detected by negative-staining and electron microscopy (Fig. 4E). After separation by CsCl isopycnic gradient centrifugation, a large number of low density ZIKV VLPs were recovered from rVSV-prM-E and rVSV-prM-E-NS1 infected cells (Fig. 4E). The ZIKV VLPs expressed by VSV had a diameter of 30-40 nm, which are relatively smaller than native ZIKV virions (40-50 nm). No VLPs were detected in cell culture media from cells infected with rVSV-E (Fig. 4E. These results confirm that expression of prM-E and prM-E-NS1 but not E alone by the VSV vector resulted in the assembly of VLPs that are structurally similar to native ZIKV virions.

Example 4 – rVSV-prM-E/E Truncations Induce More Antibody Than rVSV-E/E Truncations

[142] In one illustrative method, BALB/c mice were inoculated intranasally with a single dose (10^6 PFU) of each of nine recombinant virus. A DNA vaccine (pCI plasmid encoding ZIKV prM and E; pCI-prM-E) was used as a control. Mice were intramuscularly injected with 50 µg of pCI-prM-E and were boosted with same dose of pCI-prM-E two weeks later. Mice infected with rVSV exhibited severe clinical signs, including ataxia, hyperexcitability, and paralysis. At 7 days post-inoculation, two of the five mice were dead, and the remaining three mice were dead at day 10 post-inoculation.

[143] rVSVs expressing ZIKV protein subunits showed various degrees of attenuation. Mice inoculated with these recombinant viruses had mild clinical signs (such as a ruffled coat) and experienced body weight losses for 1 week, but started to gain weight by 10 days (Fig. 5). Overall, rVSVs co-expressing prM and E/E truncation mutants were more attenuated in mice than rVSV expressing E/E truncation alone. rVSV-prM-E and rVSV-prM-E414 had significantly less body weight loss compared to rVSV-E ($P = 0.021$) and rVSV-E414 ($P = 0.045$) respectively at day 7 post-inoculation.

[144] Recombinant rVSV-prM-E-NS1 was the most attenuated virus (Fig. 5). Mice inoculated with this virus experienced little or no weight loss and did not display any other clinical signs. This experiment demonstrated that rVSV expressing ZIKV antigens, particularly rVSV-prM-E-NS1, were significantly attenuated in mice compared to the parental rVSV.

[145] The dynamics of ZIKV E-specific Ab production following vaccination, determined by ELISA, is summarized in Fig. 6. At 1-week post-inoculation, most (3 or 4 out of 5) mice inoculated with rVSV co-expressing prM-E/E truncation mutants had high levels of serum IgG against ZIKV E protein. At week 2 post-inoculation, all mice in these groups had developed IgG Ab. Ab titers further increased and remained at a high level through week 5. In contrast, none of the mice vaccinated with rVSV expressing E/E truncation mutants without prM had detectable ZIKV-specific antibody by week 1. The same was true for rVSV-prM-E-NS1. At week 2, Ab was observed in these groups and increased through week 5. However, the Ab titers in these groups were significantly lower than those of the viruses co-expressing prM-E/E truncation mutants only ($P < 0.05$).

[146] Ab was not detectable in the DNA vaccine group until week 5, despite the fact that these mice had been given two doses. The Ab detected in the DNA vaccine group at week 5 post-immunization was also lower than that induced by the rVSV-prM-E/E truncations. These results demonstrate that a single-dose inoculation of mice with rVSV co-expressing prM-E/E truncations or prM-E-NS1 triggered high levels of serum antibody response as early as 1 to 2 weeks post-inoculation.

Example 5 – Attenuation of Recombinant VSV Expressing ZIKV Protein Subunits

[147] In one illustrative method, VSV was further attenuated to enhance VSV as a vector by a specific mutation that inhibits its mRNA cap methyltransferase (MTase) activity. A single point mutation (G16870) in the S-Adenosyl methionine (SAM) binding site in the MTase region of the large (L) polymerase protein was previously demonstrated to result in a recombinant virus (rVSV-G1670A) that was defective in mRNA cap guanine-N-7 methylation but not ribose 2'-O methylation (*see* Li et al., 2006. Proceedings of the National Academy of Sciences of the United States of America 103:8493-8498). Compared to rVSV, this recombinant virus was highly attenuated in cell culture as well as in mice.

[148] The G1670A mutation was introduced into rVSV-E, rVSV-aE, rVSV-prM-E, and rVSV-prM-E-NS1 to generate rVSV-G1670A-E, rVSV-G1670A-aE, rVSV-G1670A-prM-E, and rVSV-G1670A-prM-E-NS1, respectively (Fig. 7A). The plaque diameters of rVSV-G1670A-E, rVSV-G1670A-aE, rVSV-G1670A-prM-E, and rVSV-G1670A-prM-E-NS1 were 1.70 ± 0.20, 1.67 ± 0.111.71 ± 0.15, and 1.05 ± 0.12 mm respectively; significantly smaller than the recombinant viruses derived from the wild-type VSV backbone (*cf.* Fig. 7A and Fig. 1B). Single-step replication curves indicated that rVSV-G1670A-E, rVSV-G1670A-aE, and rVSV-G1670A-prM-E had replication kinetics similar to rVSV-G1670A, whereas rVSV-G1670A-prM-E-NS1 had a significant delay ($P = 5.82 \times 10^{9}$ and 0.0021 at time points 12 and 24 h, respectively; Fig. 7B). At 24 h post-infection, the expression of E can be ranked rVSV-G1670A-prM-E > rVSV-G1670A-prM-E-NS1 > rVSV-G1670A-aE(Fig. 7C). No E protein expression was detected from rVSV-G1670A or rVSV-G1670A-E, but NS1 protein expression was detected from rVSV-G1670A-prM-E-NS1 (Fig. 7C).

[149] In a kinetic experiment, E protein expression was maximal from rVSV-G1670A-aE and rVSV-G1670A-prM-E at 12 and 36 h, respectively, but was delayed in rVSV-G1670A-prM-E-NS1-infected cells (Fig. 7D).

[150] Similarly, E and NS1 proteins were secreted into cell culture medium in virus-infected cells (Figs. 7D and 7E). Compared to the parental rVSV vector, E protein expression was delayed from the rVSV-G1670A vector (Fig.2D), suggesting that the rVSV-G1670A vector was more attenuated. A large number of ZIKV VLPs were found in cell culture medium harvested from rVSV-G1670A-prM-E-NS1 and rVSV-G1670A-prM-E. These results demonstrated that ZIKV E and NS1 proteins were highly expressed by MTase-defective rVSV.

Example 6 - mtdVSV-based vaccines are Highly Attenuate and Immunogenic

[151] In an illustrative method, the MTase-defective VSV (mtdVSV)-based vaccines were tested in BALB/c mice. Intranasal wild-type rVSV killed the mice within 10 days (Fig. 8A). Mice inoculated with rVSV-G1670A or rVSV-G1670A-E showed 13% and 7% weight loss respectively at day 7 post-infection but both recovered by day 10 (Fig. 8A). Mice inoculated with rVSV-G1670A-prM-E-NS1 or rVSV-G1670A-prM-E exhibited 1-2% loss of body weight but were not significantly different than the DMEM control (P>0.05) (Fig. 8A), and exhibited no VSV-associated clinical symptoms, indicating a high degree of attenuation.

[152] High levels of ZIKV E-specific antibody were detected by ELISA in rVSV-G1670A-prM-E and rVSV-G1670A-prM-E-NS1 mice at weeks 2 and 4 post-immunization, respectively (Fig. 8B). There was no significant difference in ELISA or neutralizing antibody titer (Fig. 8C) at week 5 between these two groups (P>0.05). No ZIKV specific antibody was detected in DMEM, rVSV-G1670A or rVSV-G1670A-E groups. Compared to the wild-type rVSV backbone, mtdVSV-based viruses had a delayed antibody response (*cf.* Fig. 6 and Fig. 8B), reflecting the significant attenuation of these recombinant viruses.

[153] In addition, all mice in the rVSV-G1670A-prM-E-NS1 group developed NS1-specific antibody as detected by ELISA at week 5 (Fig. 8D). These results demonstrated that mtdVSV-based ZIKV vaccine candidates are highly attenuated and immunogenic in mice.

Example 7 - Co-Expression of NS1 Has a Regulatory Effect on the Profile of T Cell Responses

[154] Induction of antigen-specific Ab and cytotoxic T cell responses capable of providing protection after immunization requires T helper cells (CD4+CD3+ cells). In an illustrative method, it was found that spleen cells from mice that had been intranasally immunized with rVSV-G1670A-E, rVSV-G1670A-prM-E, or rVSV-G1670A-prM-E-NS1 and restimulated *in vitro* with ZIKV E protein, increased the number of T helper cells (CD3+CD4+) (Fig. 9A). This finding indicates that immunization induced ZIKV E protein-specific T cells capable of proliferation after re-exposure to the E antigen.

[155] Th1 cells produce important cytokines (*i.e.*, IFN-γ and TNF-α) for the production of complement-

fixing Abs and cytotoxic T cells, which together are crucial for protection against intracellular pathogens such as viruses. Flow cytometry analysis of $CD3^+CD4^+$ cells producing Th1 cytokines revealed that only cells isolated from mice immunized with rVSV-G1670A-prM-E and rVSV-G1670A-prM-E-NS1 expressed ZIKV antigen-specific IFN-γ producing T helper cells ($CD4^+IFN\text{-}\gamma^+$) (Fig. 9B).

[156] TNF-α producing T helper cells ($CD4^+TNF\text{-}\alpha^+$) were detected in the spleens of mice immunized with rVSV-G1670A-prM-E, but not rVSV-G1670A-prM-E-NS1 (Fig. 9B). These results indicate that co-expression of NS1 enhances IFN-γ, but inhibits production of TNF-α by T helper cells.

[157] Th2 cells produce an array of cytokines which support the production of Abs more likely to protect against extracellular pathogens such as viruses. Interleukin 21, the signature product of follicular T helper cells (Tfh) and IL-17A, the product of Th17 cells, facilitate antibody production and affinity maturation. Both rVSV-G1670A-prM-E-NS1 and rVSV-G1670A-prM-E induced a similar level of $CD4^+IL\text{-}4^+$, a Th2 cytokine ($P>0.05$) in spleen cells after *in vitro* restimulation with ZIKV E protein (Fig. 9C). However, rVSV-G1670A-prM-E-NS1 induced significantly higher $CD4^+IL\text{-}5^+$ and $CD4^+IL\text{-}10^+$, the other two Th2 cytokines ($P<0.05$) (FIG. 9C).

[158] rVSV-G1670A-prM-E-NS1 also induced a significantly higher Th17 response ($CD4^+IL\text{-}17A^+$) than rVSV-G1670A-prM-E ($P<0.05$) (Fig. 9D). In addition, ZIKV E-specific Tfh cells ($CD4^+IL\text{-}21^+$) were produced at similar levels in rVSV-G1670A-prM-E-NS1 and rVSV-G1670A-prM-E inoculated mice ($P>0.05$) (Fig. 9E).

[159] These results demonstrated that mtdVSV-based vaccines triggered ZIKV-specific T cell responses and that co-expression of NS1 enhances Th2 and Th17 responses. The fact that co-expression of NS1 enhances IFN-γ indicates that NS1 modulated the Th1 response (Fig. 9B). Collectively, these results indicate that the presence of NS1 leads to a more balanced response including Th1, Th2, and Th17 cells.

Example 8 - A Single Dose of mtdVSV-Based Vaccines Protects BALB/c Mice from ZIKV Viremia

[160] In an illustrative method, the protective effect of mtdVSV-based ZIKV vaccines was determined in both female and male BALB/c mice. Mice were vaccinated intranasally with a single dose (10^6 PFU) of each recombinant virus, and were challenged with ZIKV Cambodian strain (FSS13025) at week 5 post-immunization. DNA vaccine (pCI-prM-E) was used as a control and was given intramuscularly twice (at week 0 and 2). Similar to the previous observation (Fig. 8A), prM-E-NS1 was the most attenuated virus, with mice experiencing no weight loss (Fig. 10). All other recombinant viruses resulted in 9-15% weight loss at early time points, but weights recovered by day 14 (Fig. 10).

[161] Previously, it was shown that the administration of anti-IFNAR1 antibody could render BALB/c mice more susceptible to ZIKV infection, resulting in significant weight loss and ZIKV-associated clinical signs upon challenge with a mouse-adapted African ZIKV strain (Dakar 41519). 1.8 mg of a

blocking antibody, anti-IFNAR1, was passively transferred to each mouse 24 h prior to challenge with the ZIKV. After ZIKV challenge, mice were monitored for 4 weeks. No significant weight loss or clinical symptoms were observed in any group including the unvaccinated but challenged controls (Fig. 11).

[162] The dynamics of viremia were monitored every 3-4 days until day 24 after ZIKV challenge (except the pCI-prM-E group, which was only monitored at days 3 and 7) and detected by real-time RT-PCR. For the unvaccinated challenged controls, the peak of viremia was observed at day 3, declined by days 7 and 10, and cleared by day 14 (Figs. 12A and 19A). This was consistent with previous observations that ZIKV only causes transient viremia in BALB/c mice. Similarly, mice in the rVSV-G1670A and rVSV-G1670A-E groups developed viremia, shedding an average of 3.7 logs of ZIKV PFU RNA/ml in blood samples collected at day 3 post-challenge (Fig. 12B). In contrast, mice that had been vaccinated with rVSV-G1670A-prM-E, rVSV-G1670A-prM-E-NS1, and pCI-prM-E were under the detection limit at day 3 (3 and 4 mice in rVSV-G1670A-prM-E and rVSV-G1670A-prM-E-NS1 had near detection limit level of viremia, respectively, and 1 mice in pCI-prM-E group had a high level of viremia) (Fig. 19A and Fig. 12B). In addition, viremia was under detection limit from days 7 to 24 in these groups (Fig. 19A)

[163] Collectively, these data indicate that a single dose vaccination of mtdVSV-based vaccines provides protection against ZIKV-induced viremia in BALB/c mice.

[164] To determine whether VSV was persistent in the vaccinated mice, brain tissues were collected at the termination of the study for detection of VSV. No infectious VSV was detected by plaque assay in any brain tissues in any group. However, 4-5 log VSV RNAs were detected in the brains of the rVSV-G1670A, rVSV-G1670A-E, and rVSV-G1670A-prM-E groups (Fig. 19B). In contrast, nearly no VSV RNA was detected in the rVSV-G1670A-prM-E-NS1 group (Fig. 19B). Therefore, rVSV-G1670A-prM-E-NS1 is the most attenuated of these viruses.

[165] The above animal experiment was repeated, where rVSV-G1670A-aE was included in the vaccination. Recombinant rVSV-G1670A-prM-E-NS1 and rVSV-G1670A-aE had no body weight loss whereas rVSV-G1670A-prM-E had approximately 4.2% body weight loss at day 7 (Fig. 22). High E-specific antibodies were observed in all animals vaccinated with rVSV-G1670A-prM-E or rVSV-G1670A-prM-E-NS1 at day 28 and further increased at day 35 post-vaccination (Fig. 19C). Only 1 out of 5 animals vaccinated with rVSV-G1670A-aE developed E-specific antibodies from day 7 to 28, and all animals developed E-specific antibodies at day 35 (Fig. 19C). NS1-specific antibodies were only detected in rVSV-G1670A-prM-E-NS1 group (Fig. 19D). Upon ZIKV challenge, mice did not exhibit body weight loss (Fig. 23). Mice vaccinated with rVSV-G1670A-prM-E and rVSV-G1670A-prM-E-NS1 were protected from viremia at days 3 (Fig. 19E) and 7 (Fig. 19F) post-challenge whereas mice received rVSV-G1670A-aE shed high titer of ZIKV RNA in blood in a level similar to the rVSV-G1670A and saline

control groups.

Example 9 - A Single Dose of mtdVSV-Based Vaccine Provides Complete Protection Against Lethal ZIKV Infection in INFAR-Lacking Mice

[166] In an illustrative method, the protective effect of mtdVSV-based ZIKV vaccines was assessed in A129 mice, which lack the interferon type I receptor (IFNAR) and, therefore, signaling responses to type I interferons. These mice have been demonstrated to be highly permissive for both ZIKV and VSV infection. A129 mice are so susceptible to wild-type VSV infection that a dose of 50 PFU is lethal.

[167] To reduce side effects, an intramuscular route was used for VSV vaccination. Since mtdVSV-based vaccines were significantly attenuated, a dose of 10^5 PFU was chosen for vaccination, which was 20,000 times higher than the wild-type VSV lethal dose. A129 mice were immunized intramuscularly with rVSV-G1670A-prM-E-NS1, rVSV-G1670A-prM-E, or rVSV-prM-E-NS1, and the safety and antibody response were monitored. It was observed thatVSV-G1670A-prM-E-NS1 was completely attenuated in A129 mice, exhibiting no body weight losses or any abnormal reactions (Fig. 13). However, rVSV-prM-E-NS1, which lacks the VSV attenuating mutation, was virulent in A129 mice, causing 2 deaths at day 7, and morbidity by day 10 that required termination of the others.

[168] Mice immunized with rVSV-G1670A-prM-E lost 20% of their weight but recovered and remained healthy. As illustrated by Fig. 14, all mice in rVSV-G1670A-prM-E-NS1 and rVSV-G1670A-prM-E groups developed high levels of antibody, detected by ELISA (Fig. 14A) and by neutralization (Fig. 14C) as early as week 1 post-vaccination. Ab titers remained high at week 3 (Figs. 14B and 14D). In addition, high levels of NS1-specific Ab were detected at weeks 1 and 3 in the rVSV-G1670A-prM-E-NS1 group (Figs. 14E and 14F). At week 4 post-immunization, each group was challenged with 10^5 PFU of the ZIKV Cambodian strain.

[169] Mice in the control, unvaccinated challenged group (immunized with the empty pCI plasmid) developed severe clinical signs (Fig. 15A) and had severe body weight loss (Fig. 15B). Because of the severity of disease in the pCI control group, these mice were terminated at day 7.

[170] In contrast, mice vaccinated with either rVSV-G1670A-prM-E-NS1 or rVSV-G1670A-prM-E did not exhibit any weight loss (Fig. 15B) or ZIKV associated clinical symptoms (Fig. 15A). ZIKV viremia was measured at days 3 and 7 post-challenge by real-time RT-PCR (Figs. 16A and 16B). An average of 5.8 log PFU equivalents of ZIKV was detected in the pCI control group at day 3. Low ZIKV PFU equivalents were detected at day 3 in the rVSV-G1670A-prM-E group but none in the rVSV-G1670A-prM-E-NS1 group. At day 7, high levels of ZIKV were detected in the blood of the pCI control group, whereas no or very low ZIKV was found in rVSV-G1670A-prM-E-NS1 and rVSV-G1670A-prM-E groups.

[171] Similarly, high levels of ZIKV were detected in the brain, uterus, lung, and spleen of the pCI

control group whereas under or near detection limit level ZIKV RNA was found in these organs in the rVSV-G1670A-prM-E and rVSV-G1670A-prM-E-NS1 groups (Figs. 16C-16F). In addition, histologic analysis of brain tissues showed that rVSV-G1670A-prM-E and rVSV-G1670A-prM-E-NS1 had completely protected the mice from ZIKV-induced encephalitis (Fig. 17). In contrast, severe encephalitis characterized by neuronal necrosis, gliosis, neuronal satellitosis and neuronophagia with lymphocytic perivascular cuffing was found in the control group (Fig. 17). The brain tissues were used for the detection of VSV RNA. It was found that VSV RNA was not detectable or near the detection limit in A129 mice vaccinated with rVSV-G1670A-prM-E-NS1 whereas approximately 6 log of VSV RNA were detected in rVSV-G1670A-prM-E group (Fig. 14G), indicating that rVSV-G1670A-prM-E-NS1 was significantly more attenuated than rVSV-G1670A-prM-E.

[172] These data demonstrate that a single low dose of mtdVSV-based vaccines provides complete protection against ZIKV challenge in A129 mice that are extremely sensitive to both VSV and ZIKV.

Example 10 - NS1 Provides Partial Protection Against ZIKV Challenge Without Inducing Neutralizing Antibody

[173] In an illustrative method, to determine whether prM-E or NS1 proteins, alone, can induce protection against a ZIKV challenge, DNA vaccination was used. DNA vaccine is safe to A129 mice. The NS1 gene with anchor C signal peptide was cloned into pCI vector. Both pCI-prM-E and pCI-NS1 expressed their intended proteins, E and NS1, in transfected 293T cells (*see* Figs. 18A & 18B). A129 mice were vaccinated intramuscularly with pCI-prM-E or pCI-NS1, and boosted with the same plasmid two weeks later. Only 1 out of 5 mice in the pCI-prM-E group had E-specific ELISA and neutralizing Ab at week 1 (Figs. 14A and 14C) but all of them had high levels of ZIKV E-specific Ab at week 3 (Figs. 14B and 14D). No ZIKV neutralizing Ab was detected in the pCI-NS1 group even after the boost (Figs. 14B and 14D), but Ab to NS1 was detected in 2 out of 5 mice at week 3 (Fig. 14F).

[174] Mice vaccinated with pCI-prM-E were protected from a ZIKV challenge at week 4 (Fig. 15). One of the five mice in the pCI-NS1 group only had 10% weight loss and quickly recovered (Fig. 15C). The other four mice in the pCI-NS1 group exhibited clinical signs, but less severe than the pCI group (Fig. 15A). Overall, weight loss in the pCI-NS1 group was less than the pCI control group (Fig. 15B). At day 3 post-challenge, the pCI-NS1 group had a level of viremia similar to the pCI control group ($P>0.05$) (Fig. 16A), but by day 7 the pCI-NS1 group had significantly less viremia ($P<0.05$) (Fig. 16B). Similarly, significantly less ZIKV was detected in spleen, uterus, lung, and brain in the pCI-NS1 group compared to the pCI control group ($P<0.05$ or $P<0.01$) (Fig. 16C-F). Histologic analysis showed that the pCI-NS1 group had less severe encephalitis compared to the pCI group (Fig. 17).

[175] Collectively, these data demonstrate that ZIKV NS1 was capable of conferring partial protection against ZIKV challenge in A129 mice in the absence of detectable ZIKV neutralizing Ab.

[176] To further improve the protection efficacy of NS1, mtdVSV expressing NS1 alone (rVSV-G1670A-NS1) was recovered, in which the ZIKV NS1 gene with anchor C was inserted at the gene junction between G and L genes. Western blot indicated that the NS1 expression in rVSV-G1670A-NS1-infected cells was significantly higher than pCI-NS1-transfected cells (Fig. 15D). A pilot experiment demonstrated that rVSV-G1670A-NS1 still caused considerable weight loss in A129 mice. The protection efficacy of rVSV-G1670A-NS1 was tested in BALB/c mice. BALB/c mice were immunized intramuscularly with two doses (50 μg each) of pCI-NS1 or intranasally with one dose (10^6 PFU) of rVSV-G1670A-NS1 or rVSV-G1670A-prM-E, and were challenged intravenously with 10^6 PFU of ZIKV at week 4 post-immunization. We found that rVSV-G1670A-NS1 triggered significantly higher NS1-specific antibody than pCI-NS1 in mice (Fig. 15E). As a positive control, recombinant rVSV-G1670A-prM-E triggered a high level of E-specific antibody (Fig. 15F). At days 3 post-challenge, mice in rVSV-G1670A-NS1 and pCI-NS1 groups had a similar level of viremia ($P>0.05$) but were significantly lower than pCI control ($P<0.05$) (Fig. 15G). As a positive control, the viremia level in the rVSV-G1670A-prM-E group was below or near detection limit (Fig. 156G). At day 7 post-challenge, mice in the rVSV-G1670A-NS1 and rVSV-G1670A-prM-E groups had no detectable viremia (except one in rVSV-G1670A-NS1 group which was near the detection limit) whereas mice in pCI and pCI-NS1 groups still had a significant level of viremia ($P<0.001$) (Fig. 15H). These results indicated that NS1 alone was capable of triggering significant protection against ZIKV-induced viremia and that rVSV-G1670A-NS1 had a higher protection efficacy than pCI-NS1.

Example 11 – Validation of the Safety and Efficacy of rVSV-G1670A-prM-E-NS1 in A129 Mice

[177] The protection efficacy of rVSV-G1670A-prM-E-NS1 was further validated in A129 mice by monitoring body weight and viremia for a prolonged time (until day 21 after challenge with ZIKV). As indicated in Fig. 20A, there were no significant differences in body weight gain among three groups ($P>0.05$), 10^5 PFU of rVSV-G1670A-prM-E-NS1, saline, and normal controls, demonstrating the high safety profile of rVSV-G1670A-prM-E-NS1 in A129 mice. rVSV-G1670A-prM-E-NS1 triggered a high level of E-specific (Fig. 20B) and NS1-specific (Fig. 20C) antibodies. Upon challenge with ZIKV Cambodian strain, mouse body weight and viremia were monitored every 1 or 3 days until day 21. Mice that received the saline control were all dead at day 6 post-challenge (Fig. 20D). The body weight in rVSV-G1670A-prM-E-NS1 group was indistinguishable from normal control ($P>0.05$) at all time points (Fig. 20D). Saline control group developed high levels of ZIKV induced viremia whereas rVSV-G1670A-prM-E-NS1 group had a baseline level of viremia at day 3 and no detectable viremia between days 3 and 21 (Fig. 20E). Collectively, rVSV-G1670A-prM-E-NS1 is of high safety and efficacy against ZIKV infection.

Example 12 – rVSV-D1762A Backbone

[178] In an illustrative method, a D1762A mutation was introduced into the plasmid encoding the VSV antigenome, pVSV-GxxL, resulting in pVSV-D1762A-GxxL. Using pVSV-D1762A-GxxL as the backbone, ZIKV NS1 gene was inserted into the gene junction between G and L. Five recombinant VSVs (rVSV) expressing ZIKV NS1 protein were recovered (Fig. 24). These recombinant viruses differed only in the signal peptide sequence which is fused to the N terminus of ZIKV NS1 protein. Recombinant rVSV-D1762A-a-NS1 also contains the anchor C sequence from the ZIKV genome, as the anchor C sequence has been shown to be essential for expression of ZIKV prM-E protein. Recombinant rVSV-D1762A-tPA-NS1 contains the signal sequence encoding human tissue plasminogen activator (t-PA) fused to NS1.

[179] Three recombinant viruses containing the transmembrane domain of the ZIKV E protein from the C-terminus of the E protein inserted at the C-terminus of the NS1 protein were also constructed. This domain can function as a signal peptide and potentially enhance NS1 protein expression. Since the exact length of this signal sequence is unclear, three recombinant viruses with different lengths of this signal sequence connected to the NS1 protein were constructed. These recombinant viruses were named rVSV-D1762A-456-NS1, rVSV-D1762A-484-NS1, and rVSV-D1762A-483-NS1 which contain amino acid residues from 456 to 504, 484 to 504, and 483 to 504 from C-terminal of ZIKV E protein, respectively as the signal peptide (Fig. 24).

[180] After recovery, all recombinant viruses were plaque purified. All recombinant viruses contained the desired insertions as indicated by sequencing. No mutations were found in the genome except for the D1762A substitution in the L gene. The parental rVSV formed large plaques at 36 h post-inoculation with an average diameter of 2.83 ± 0.57 mm (mean ± standard deviation). All other recombinant viruses formed significantly smaller plaques. The rVSV-D1762A-a-NS1 virus had a plaque size of 0.87 ± 0.18 mm, similar to the backbone virus rVSV-D1762A (0.85 ± 0.20 mm). The rVSV-D1762A-tPA-NS1, rVSV-D1762A-456-NS1, rVSV-D1762A-483-NS1, and rVSV-D1762A-484-NS1 viruses formed even smaller plaques with average size of 0.62 ± 0.12 mm, 0.57 ± 0.09 mm, 0.65 ± 0.10 mm, and 0.52 ± 0.15 mm, respectively. These results indicate that the D1762A methyltransferase-defective rVSVs (mtdVSVs) expressing NS1 had dramatically reduced replication and/or cell-to-cell spread.

[181] BALB/c mice were challenged intraperitoneally with 10^6 PFU of ZIKV Cambodian strain (FSSS13025) at week 4 post-immunization with mtdVSV-NS1 based vaccines or DNA vaccines. All mice were injected intraperitoneally with 2 mg of IFNAR1 antibody 24 h prior to ZIKV challenge. No significant body weight losses or ZIKV-associated clinical symptoms were observed in any group, including the unvaccinated but challenged controls, which was consistent with the observations of several other groups that ZIKV infection does not cause illness in immunocompetent mice.

[182] Blood samples were collected from each mouse at days 3 and 7 post-challenge to measure

viremia. The unvaccinated challenged groups (pCI and rVSV-D1762A) developed a high level of ZIKV viremia, reaching $10^{6.70}$ and $10^{7.00}$ RNA copies/ml post-challenge (Fig. 25A), which were equivalent to $10^{4.01}$ and $10^{4.33}$ PFU/ml of ZIKV, respectively (Fig.25C). The viremia in both control groups declined by day 7 (Figs. 25B and 25D). This result was consistent with previous observations that ZIKV only causes transient viremia in BALB/c mice. As the positive control, mice vaccinated with rVSV-G1670A-prM-E were completely protected from viremia at days 3 and 7, which was consistent with our previous study. The mice vaccinated with DNA vaccines or mtd-VSV-NS1 based vaccines had a significantly lower viremia at day 3 compared to the pCI and rVSV-D1762A controls (Figs. 25A and 25C). However, a certain level of viremia (3 log PFU/ml), approximately 1 log higher than the detection limit, was detected in the DNA vaccine or mtd-VSV-NS1 based vaccine groups (Fig. 25C). By day 7, viremia in the mtd-VSV-NS1 vaccine groups has been cleared whereas 2.5-3 log PFU were still detected in the DNA vaccine groups (Fig. 25D). Collectively, these results indicate that a single dose of mtd-VSV-NS1 vaccine or two doses of DNA vaccine expressing NS1 provided partial protection in BALB/c mice against ZIKV-induced viremia. The protective efficacy of mtd-VSV-based NS1 vaccine was higher than that of the DNA vaccines.

[183] Similar results were found when tested in A129 mice. rVSV-D1762A-483-NS1 was chosen for use in the A126 experiments because it had induced higher NS1 antibody than rVSV-D1762A-tPA-NS1 in BALB/c mice. A129 mice were intramuscularly immunized with 10^3 PFU of rVSV-D1762A-483-NS1. Two weeks later, A129 mice were boosted with 10^5 PFU of the same virus. At week 2 post-booster vaccination, mice were challenged with 10^5 PFU of ZIKV Cambodian strain. Body weight was monitored throughout the experiment. Mice inoculated with rVSV-D1762A-483-NS1 had no loss of body weight (Fig. 26A) or any abnormal reaction during the 4-week immunization time period, indicating that mtdVSV-based NS1 vaccine was completely attenuated in A129 mice. Two mice immunized with rVSV-D1762A-483-NS1 developed NS1-specific antibody at week 1 post-immunization and all the mice in this group developed high levels of NS1 antibody at week 2 post-immunization despite the fact that a relatively low dose (10^3 PFU) was used for vaccination (Fig. 26B). After booster vaccination, NS1 antibody did not significantly increase at weeks 3 and 4 (Fig. 26B). At week 4 post-immunization, mice from the unvaccinated group (saline) and rVSV-D1762A-483-NS1 group were challenged by ZIKV at a dose of 10^5 PFU per mouse. This dose was chosen because prM-E or prM-E-NS1 based vaccine candidates provided complete protection in A129 mice. After ZIKV challenge, both rVSV-D1762A-483-NS1 and saline groups developed ZIKV-associated clinical signs and had significant body weight loss (Fig. 26C). However, two mice immunized with rVSV-D1762A-483-NS1 showed less body weight loss (Fig. 26D) and three mice in this group survived at day 7 post-challenge (Fig. 26E). In contrast, all the mice in the saline group were dead at day 6 post-challenge. ZIKV-induced viremia in these mice was

measured at day 3 post-challenge. Results indicated that the mice immunized by rVSV-D1762A-483-NS1 had a significantly lower ZIKV viral load in blood compared with the saline group (Fig. 4F). Collectively, these data indicate that mtdVSV-NS1 vaccine provided partial protection against lethal ZIKV challenge.

Example 13 - Materials and Methods

[184] *Cell Line, Viruses, and Plasmid construction.* BHK-21 cells (ATCC no. CCL-10), Vero (ATCC no. CCL-81), and 293T cells (ATCC no. CRL-3216) were purchased from American Type Culture Collection (ATCC, Manassas, VA). BSRT7 cells, which stably express T7 RNA polymerase, are clones of BHK-21 cells. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% FBS. ZIKV Cambodian strain (FSS13025) was obtained and propagated in Vero cells, and titrated using a standard plaque assay.

[185] Plasmids encoding VSV N (pN), P (pP), and L (pL) genes, and an infectious cDNA clone of the viral genome, pVSV1(+), were obtained. Plasmid pVSV1(+) GxxL, which contains SmaI and XhoI at the G and L gene junction, was obtained. The full-length envelope (E) gene (from amino acids 1 to 504) and E truncation mutants (E404 (amino acids 1-404); E414 (amino acid 1-414), and E415 (amino acid 1-415)) lacking the predicted stem-transmembrane domain (TM) were amplified from an infectious cDNA clone of ZIKV Cambodian strain (GenBank accession no. MH158236) b by high fidelity PCR. These DNA fragments were digested with SmaI and XhoI and cloned into pVSV(+)GxxL at the same sites. The resulting plasmids were designated pVSV(+)-E, pVSV(+)-E404, pVSV(+)-E414, and pVSV(+)-E415.

[186] Using the same strategy, the anchor C (signal peptide) with E, E404, E414, and E415 were cloned into pVSV(+)GxxL at SmaI and XhoI sites resulted in construction of pVSV(+)-aE, pVSV(+)-aE404, pVSV(+)-aE414, and pVSV(+)-aE415 respectively. In addition, the anchor C -premembrane-envelope (prM-E), and anchor C-prM-E truncation mutants (prM-E404, prM-E414, and prM-E415), and anchor C-premembrane-envelope-nonstructural protein 1 (prM-E-NS1) genes were cloned into pVSV(+)GxxL at SmaI and XhoI sites. The resulting plasmids were designated pVSV(+)-prM-E, pVSV(+)-prM-E404, pVSV(+)-prM-E414, pVSV(+)-prM-E,415, and pVSV(+)-prM-E-NS1.

[187] Similarly, the anchor C-NS1 gene (amino acids 1-352) was cloned into pVSV(+)GxxL at the SmaI and XhoI sites, and the resultant plasmid was named pVSV(+)-NS1. To further attenuate the VSV vector, a point mutation, G1670A, in the large (L) polymerase protein was introduced, which rendered a recombinant virus that is specifically defective in mRNA cap G-N-7, but not 2'-O methylation. Using site-directed mutagenesis, G1670A mutation was introduced into pVSV(+)-E, pVSV(+)-aE, pVSV(+)-prM-E, pVSV(+)-prM-E-NS1, and pVSV(+)-NS1 which resulted in the construction of pVSV(+)-G1670A-E, pVSV(+)-G1670A-aE, pVSV(+)-G1670A-prM-E, pVSV(+)-G1670A-prM-E-NS1, and pVSV(+)-G1670A-NS1 respectively.

[188] To prepare DNA vaccine plasmids, the anchor C-prM-E and anchor C-NS1 genes were cloned

into pCI vector (Promega) which resulted in the construction of pCI-prM-E and pCI-NS1 respectively. All of the constructs were confirmed by sequencing.

[189] *Recovery of recombinant VSV expressing ZIKV antigens.* Recovery of recombinant VSV (rVSV) from the infectious clone was carried out. rVSV was recovered by cotransfection of plasmid encoding VSV genome, and support plasmids encoding VSV nucleocapsid complex (pN, pP, and pL) into BSRT7 cells infected with a recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase. At 96 h post-transfection, cell culture fluids were collected and filtered through a 0.2-μm filter, and the recombinant virus was further amplified in BSRT7 cells. Subsequently, the viruses were plaque purified as described previously. Individual plaques were isolated, and seed stocks were amplified in BSRT7 cells. The viral titer was determined by a plaque assay performed in Vero cells..

[190] *RT-PCR.* Viral RNA was extracted from recombinant VSVs by using an RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. ZIKV genes were amplified by a One Step RT-PCR kit (Qiagen) using primers annealing to VSV G gene at position 4524 (5' -CGAGTTGGTATTTATCTTTGC-3'; SEQ ID NO: 20) and L gene at position 4831 (5'-GTACGTCATGCGCTCATCG-3'; SEQ ID NO: 21) (numbering refers to the complete VSV Indiana genome sequence). The amplified products were analyzed on 1% agarose gel electrophoresis and sequenced.

[191] *Single-cycle growth curves.* Confluent BSRT7 cells were infected with individual viruses at a multiplicity of infection (MOI) of 3. After 1 h of absorption, the inoculum was removed, the cells were washed twice with Dulbecco's modified Eagle's medium (DMEM), fresh DMEM (supplemented with 2% fetal bovine serum) was added, and the infected cells were incubated at 37°C. Aliquots of the cell culture fluid were removed at the indicated intervals, and virus titers were determined by plaque assay in Vero cells.

[192] *Analysis of the expression of ZIKV antigens by VSV.* Confluent BSRT7 cells were infected with rVSV expressing ZIKV protein subunits, parental rVSV, or rVSV-G1670A at an MOI of 3.0. Three hours post-infection, cells were washed with methionine- and cysteine-free (M^-C^-) medium and incubated with fresh M^-C^- medium supplemented with actinomycin D (15 μg/ml). After 1 h of incubation, the medium was replaced with M^-C^- medium supplemented with EasyTag ^{35}S-Express (4 μCi/ml; Perkin-Elmer, Wellesley, MA). After 4 h of incubation, cytoplasmic extracts were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Labeled proteins were detected using a phosphorimager.

[193] *Detection of ZIKV antigen by Western blot.* BSRT7 cells were infected with each rVSV expressing ZIKV antigen as described above. For DNA vaccine plasmid, HEK293T cells were transfected with pCI, pCI-prM-E, or pCI-NS1 using lipofectimine 2000. At the indicated times post-infection, cell

culture medium was harvested and clarified at 3,000 rpm for 15 min and further concentrated at 30,000 rpm for 1.5 h. In the meantime, cells were lysed in lysis buffer containing 5% β-mercaptoethanol, 0.01% NP-40, and 2% SDS. Proteins were separated by 12% SDS-PAGE and transferred to a Hybond enhanced chemiluminescence nitrocellulose membrane (Amersham) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad). The blot was probed with rabbit anti-ZIKV E or NS1 antibody (Alpha diagnostic Intl Inc., San Antonio, TX) at a dilution of 1:2,000, followed by horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz) at a dilution of 1:5,000. The blot was developed with SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and exposed to Kodak BioMax MR film.

[194] *Production and purification of ZIKV VLPs by a VSV vector.* Recombinant rVSV-E, rVSV-prM-E, or rVSV-prM-E-NS1 was inoculated into 10 confluent T150 flasks of BSRT7 cells at an MOI of 0.01 in a volume of 2 ml of DMEM. At 1 h post absorption, 15 ml of DMEM (supplemented with 2% fetal bovine serum) was added to the cultures, and infected cells were incubated at 37°C for 24 to 48 h. Cell culture fluids were harvested when extensive cytopathic effect (CPE) was observed. Cell culture fluids were clarified by centrifugation at 3,000 g for 30 min. Virus was concentrated through a 40% (wt/vol) sucrose cushion by centrifugation at 30,000 g for 2 h at 4°C in a Ty 50.2 rotor (Beckman). The pellet was resuspended in NTE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA [pH 7.4]) and further purified through a CsCl isopycnic gradient by centrifugation at 35,000 g for 18 h at 4°C in an SW55 rotor (Beckman). The final pellet was resuspended in 0.3 ml of NTE buffer. Purified ZIKV VLPs were analyzed by SDS-PAGE, Western blotting, and electron microscopy. The protein concentrations of the VLPs were measured by using the Bradford reagent (Sigma Chemical Co., St. Louis, MO).

[195] *Purification of ZIKV.* Ten confluent T150 flasks of Vero cells were infected with ZIKV Cambodian strain at an MOI of 0.01 in a volume of 2 ml of DMEM. After 1 h of absorption, 20 ml of DMEM (supplemented with 5% fetal bovine serum) was added, and infected cells were incubated at 37°C for 72 h. When extensive cytopathic effect (CPE) was observed, cell culture fluids were harvested for ZIKV purification, which was the same procedure as VLP purification mentioned above.

[196] *Transmission electron microscopy.* Negative-staining electron microscopy of purified VLPs was performed. Briefly, 20 μl of VLP suspension was fixed in copper grids (Electron Microscopy Sciences, Inc.) and negatively stained with 1% ammonium molybdate. Virus particles were visualized by using a FEI Tecnai G2 Spirit transmission electron microscope (TEM) at 80 kV. Images were captured on a MegaView III side-mounted charge-coupled-device camera (Soft Imaging System, Lakewood, CO), and figures were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA).

[197] *Animal experiments.* Each inoculation group was separately housed in rodent cages under biosafety level 2 (BSL-2) conditions.

[198] *Experiment 1: determine whether VSV constructs are immunogenic in BALB/c mice.* Sixty 4 to 6-

week-old specific-pathogen-free female BALB/c mice were randomly divided into 12 groups (5 mice per group). Mice in group 1 were inoculated with parental rVSV (with no insertion). Mice in groups 2 to 10 were inoculated with nine different rVSVs expressing ZIKV antigens (rVSV-E404, rVSV-E414, rVSV-E415, rVSV-E, rVSV-prM-E404, rVSV-prM-E414, rVSV-prM-E415, rVSV-prM-E, and rVSV-prM-E-NS1). Mice in group 11 were inoculated with DMEM and served as uninfected controls (the normal control). Mice in group 12 were immunized with DNA vaccine pCI-prM-E. For VSV, each mouse was inoculated intranasally at a dose of 1×10^6 PFU in a volume of 50 μl. For DNA vaccine, mice were immunized intramuscularly with 50 μg of pCI-prM-E, and boosted with same dose two weeks later. After inoculation, the animals were evaluated twice every day for mortality and the presence of any symptoms of VSV infection. The severity of clinical signs associated with VSV infection was scored based on the following criteria: grade 3 (severe) was characterized by ruffled fur, hyperexcitability, tremors, circling, and paralysis; grade 2 (moderate) was characterized by ruffled fur with neurological symptoms such as circling; grade 1 (mild) was characterized by ruffled fur but no neurological symptoms; grade 0 was defined as no symptoms. The body weight of each mouse was monitored every three days. Blood samples were collected from each mouse weekly by bleeding facial vein, and serum was isolated for antibody detection. At week 5 post-inoculation, all mice were euthanized.

[199] *Experiment 2: determine antibody and T cell immune responses triggered by mtdVSV in BALB/c mice.* Thirty 6-week-old SPF female BALB/c mice were randomly divided into six groups (5 mice per group). Mice in group 1 were inoculated with DMEM and served as unimmunized controls (normal control). Mice in groups 2-6 were immunized with rVSV, rVSV-G1670A, rVSV-G1670A-E, rVSV-G1670A-prM-E, and rVSV-G1670A-prM-E-NS1. All mice were immunized intranasally at a dose of 1×10^6 PFU per mouse. After immunization, the animals were evaluated daily for body weight, mortality, and the presence of any symptoms of VSV infection. Blood samples were collected from each mouse weekly by bleeding facial vein, and serum was isolated for antibody detection. At week 5 post-inoculation, all mice were euthanized, and whole blood and spleens were isolated from each mouse for a T cell assay.

[200] *Experiment 3: determine whether mtdVSV vaccine can protect BALB/c mice against viremia until day 24 after ZIKV challenge.* Seventy 4-week-old SPF BALB/c mice were randomly divided into 7 groups (10 per group, 5 female and 5 male). Mice in groups 1-5 were immunized with DMEM, rVSV-G1670A, rVSV-G1670A-E, rVSV-G1670A-prM-E, or rVSV-G1670A-prM-E-NS1. Mice in group 6 were immunized with DNA vaccine. Mice in group 7 were served as normal control (immunized with DMEM and unchallenged). For VSV, mice were inoculated intranasally at a dose of 1×10^6 PFU per mouse. For DNA vaccine, mice were immunized intramuscularly with 50 μg of pCI-prM-E, and boosted with same dose two weeks later. After immunization, the presence of any VSV symptom induced by mtdVSV-based

ZIKV vaccine candidates was evaluated twice per day. At week 5 post-immunization, mice in groups 1-6 were challenged intravenously with ZIKV Cambodian strain at a dose of 1×10^6 PFU per mouse. At 24 h prior to ZIKV challenge, mice were intraperitoneally administered 1.8 mg of anti-IFNAR1 (Leinco Technologies, Fenton, MO) blocking antibody. After challenge, the animals were evaluated twice daily for mortality and the presence of any symptoms of ZIKV infection. The body weight for each mouse was monitored daily. At day 24 post-challenge, all mice from each group were euthanized. The blood, brain, lungs, liver, and spleen from each mouse were collected for virus quantification and histologic evaluation.

[201] *Experiment 4: determine whether mtdVSV vaccine can protect BALB/c mice against viremia until day 7 post-challenge*. Mice (6-week-old) in groups 1-5 were immunized with saline, rVSV-G1670A, rVSV-G1670A-aE, rVSV-G1670A-prM-E, or rVSV-G1670A-prM-E-NS1. The mice in group 6 served as a normal control (unimmunized unchallenged). The experimental procedure was identical to Experiment 3 except the mice were euthanized at day 7 after challenge with ZIKV Cambodian strain.

[202] *Experiment 5: determine whether mtdVSV vaccine and DNA vaccine can protect A129 mice against ZIKV challenge*. Thirty five 6-week-old female A129 mice were randomly divided into 7 groups (5 per group). Mice in groups 1-3 were immunized intramuscularly with pCI, pCI-prM-E, or pCI-NS1 at a dose of 50 μg DNA per mouse. Two weeks later, mice in groups 1-3 were boosted intramuscularly with the same plasmid at the same dose. Mice in groups 4-6 were administered intramuscularly using a single dose (1×10^5 PFU per mouse) of rVSV-G1670A-prM-E-NS1, rVSV-prM-E-NS1, or rVSV-G1670A-prM-E. Mice in group 7 were served as unvaccinated unchallenged control. After immunization, mice were evaluated every three days for body weight. The safety of mtdVSV-based ZIKV vaccine candidates was evaluated twice per day. Blood samples were collected at week 1 and 3 from each mouse for detection of antibody. At week 4 post-immunization, mice in groups 1-6 were intraperitoneally challenged with ZIKV Cambodian strain at a dose of 1×10^5 PFU per mouse. After challenge, the animals were evaluated twice every day for mortality and the presence of any symptoms of ZIKV infection. The severity of clinical disease was scored based the following criteria: 1 = heathy; 2 = mild; 3 = moderate; and 4 = severe, and early removal is required. The body weight for each mouse was monitored daily. Blood was collected at days 3 and 7 for the detection of viremia. At day 7 post-challenge, all mice from each group were euthanized, and brain, lungs, uterus/ovary, and spleen from each mouse were collected for virus quantification and histologic evaluation.

[203] *Experiment 6: determine whether NS1 alone can protect BALB/c mice against viremia*. Twenty-five 4-week-old female BALB/c mice were randomly divided into 5 groups (5 per group). Mice in groups 1-5 were immunized with DMEM, pCI, pCI-NS1, rVSV-G1670A-NS1, or rVSV-G1670A-prM-E. For VSV, mice were inoculated intranasally at a dose of 1×10^6 PFU per mouse. For DNA vaccine, mice were immunized intramuscularly with 50 μg of plasmid, and boosted with same dose two weeks later. At week

4 post-immunization, mice in groups 2-6 were intravenously challenged with ZIKV Cambodian strain at a dose of 1×10^6 PFU per mouse. At 24 h prior to ZIKV challenge, mice were intraperitoneally administered 1.8 mg of anti-IFNAR1 (Leinco Technologies) blocking antibody. At days 3 and 7 post-challenge, blood was collected from each mouse for detection of viremia by real-time RT-PCR.

[204] *Experiment 7: validate the safety and immunogenicity of rVSV-G1670A-prM-E-NS1 in A129 mice*. There were three groups in this study. Mice in group 1 were immunized intramuscularly with a single dose (1×10^5 PFU per mouse) of rVSV-G1670A-prM-E-NS1. Mice in groups 2 and 3 were served as unimmunized challenged control and normal control (unimmunized unchallenged). Blood was collected from each mouse weekly for antibody detection. At week 4, mice in groups 1 and 2 were intraperitoneally challenged with ZIKV Cambodian strain at a dose of 1×10^5 PFU per mouse. After challenge, mice were monitored for body weight changes and viremia every 1 or 3 days for 21 days.

[205] *Detection of ZIKV E or NS1-specific antibody by ELISA.* Ninety-six-well plates were first coated with 50 µl of highly purified ZIKV E or NS1 protein (MyBioSource, Inc., San Diego, CA) (4 µg/ml, in 50 mM Na2CO3 buffer, pH 9.6) per well at 4°C overnight, and then blocked with Bovine Serum Albumin (BSA, 1% W/V in PBS, 100 µl/well) at 37°C for 2 h. Subsequently, individual serum samples were tested for ZIKV-specific Ab on antigen-coated plates. Briefly, serum samples were 2-fold serially diluted and added to E or NS1 protein-coated wells. After incubation at room temperature for 2 h, the plates were washed five times with phosphate-buffered saline (PBS)-Tween (0.05%), followed by incubation with 50 µl of goat anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary Abs (Sigma) at a dilution of 1:2,000 for 1 h. Plates were washed, developed with 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB), stopped by 100 µl of H_2SO_4 (2 mol/L), and the optical density (OD) at 450 nm was determined by BioTek microplate reader. Endpoint titers were determined as the reciprocal of the highest dilution that had an absorbance value 2.1 folds greater than the background level (DMEM control). Ab titers were calculated by the geometric mean titers (GMT).

[206] *Detection of ZIKV neutralizing Ab.* ZIKV-specific neutralizing Ab was determined using a microneutralization (MN) assay. Serum samples were serially diluted twofold in 96-well micro-plates, and 100 µl of virus solution containing 50 PFU of ZIKV-Cambodian strain was added to 100 µl of each serum dilution and incubated at 37 °C for 1 h. The mixtures were then transferred to 24-well plates containing confluent Vero cell monolayers. After incubation for 60 h at 37 °C, cells were fixed with 4% (vol/vol) phosphate-buffered paraformaldehyde for 1 h and washed three times with PBS. After permeation by 0.4% (vol/vol) Triton-X 100 at room temperature for 20 min and washed for three times, a ZIKV E monoclonal antibody (MyBioSource, Inc.) was added to each well at a dilution of 1:1,000 and incubated at 37 °C for 2 h, followed by washing with PBS three times. A horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz) at a dilution of 1:2,000 was added to

each well and incubated at 37 °C for 1 h, followed by washing with PBS for three times. The plates were then developed with 3-Amino-9-ethylcarbazole (AEC) substrate for 1 h at room temperature and stained plaques in each well were counted under light microscope. The half maximal inhibitory concentration (IC_{50}) of neutralizing antibody in mice serum was calculated based on the number of plaques in each well compared with the average value of DMEM group.

[207] *Analysis of ZIKV-specific T cell responses.* To determine the nature of T cell responses that supported the development of ZIKV-specific Ab responses by rVSV expressing ZIKV antigens, we analyzed cytokine production by ZIKV E-specific spleen T cells. More specifically, spleen cells were aseptically removed from mice 35 days after immunization, and minced by pressing through a cell strainer. Red blood cells were removed by incubation in 0.84 % ammonium chloride and, following a series of washes in RPMI 1640, cells were resuspended in RPMI 1640 supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. The cell concentrations were adjusted to 3×10^6 cells/mL and 100 μl were added into each well of a 96-well microtiter plate and cultured either alone or in the presence of 20 μg/ml of ZIKV E protein for 5 days at 37°C in a 5% CO_2 atmosphere. Culture supernatants were collected from each well and frozen at -80°C until analysis of secreted cytokines using the Bio-Plex Pro Mouse Cytokine Standard 23-Plex, Group I (Bio-Rad Laboratories Inc, Hercules, CA) per manufacturer's instructions. The frequencies of ZIKV-specific Th1 ($IFN\text{-}\alpha^+CD4^+CD3^+$ and $TNF\text{-}\beta^+CD4^+CD3^+$), Th2 cells ($IL\text{-}4^+CD4^+CD3^+$, $IL\text{-}5^+CD4^+CD3^+$), Th17 ($IL\text{-}17A^+$ $CD4^+$ $CD3^+$), and Tfh ($IL\text{-}21^+$ $CD4^+$ $CD3^+$) cells were determined by intracellular staining with the corresponding anti-cytokine Abs (dilution of 1:5,000) after additional incubation in the presence of PMA and ionomycin. Cytokine-specific antibodies including Alexa Fluor 700 anti-CD3 (Cat. No. 100216), Alexa Fluor 750 anti-CD4 (Cat. No. 100460), Alexa Fluor 488 anti- IFNγ (Cat. No. 505813), PerCP Cy5.5 anti-TNFα (Cat. No. 506322), PE anti-IL-5 (Cat. No. 504307), Alexa Fluor 647 anti-IL-21 (Cat. No. 516803), PECy7 anti-IL-10 (Cat. No. 505026), Brilliant Violet 650 anti-IL-17 (Cat. No. 506929), Brilliant Violet 605 anti-IL-4 (Cat. No. 504125) were purchased from Biolegend (San Diego, CA). The cells were then analyzed with the aid of an Attune flow cytometer and data were expressed as mean % positive cells ± one SD and statistical differences are indicated and as * $p \leq 0.05$.

[208] *Measurement of viral burden.* At indicated time points after ZIKV challenge, blood was collected and organs were recovered. Organs were weighed and homogenized using a bead-beater apparatus (MagNA Lyser, Roche). The total RNA was extracted from tissue samples and blood by using TRIzol Reagent (Life technologies, Carlsbad, CA). Reverse transcription (RT) was conducted using a primer (5′-CTCGTCTCTTCTTCTCCTTCCTAGCATTGA-3′; SEQ ID NO: 22) targeting the E gene of ZIKV and the Superscript III transcriptase kit (Invitrogen, Carlsbad, CA). The RT products were then used to

perform real-time PCR using primers specifically targeting the E gene of ZIKV (forward, 5′-CATCAGGATGGTCTTGGCGATTCTAGC-3′ (SEQ ID NO: 23) reverse, 5′-CTCGTCTCTTCTTCTCCTTCCTAGCATTGA-3′ (SEQ ID NO: 24)) in a StepOne real-time PCR system (Applied Biosystems). A standard curve was generated using a ZIKV plasmid encoding E gene or a serial dilution of ZIKV RNA from known quantities of infectious virus. Amplification cycles used were 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. The threshold for detection of fluorescence above the background was set within the exponential phase of the amplification curves. For each assay, 10-fold dilutions of standard plasmid or viral RNA were generated, and negative-control samples and double-distilled water (ddH$_2$O) were included in each assay. Viral burden is expressed on a log10 scale as viral RNA equivalents per gram or per milliliter.

[209] *Histology.* Half of the tissues (brain, lung, uterus/ovary, and spleen) from each experiment were preserved in 4% (vol/vol) phosphate-buffered paraformaldehyde. Fixed tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin (HE) for the examination of histological changes by light microscopy.

[210] *Quantitative and statistical analyses.* Quantitative analysis was performed either by densitometric scanning of autoradiographs or by using a phosphorimager (Typhoon; GE Healthcare) and ImageQuant TL software (GE Healthcare, Piscataway, NJ). Statistical analysis was performed by one-way multiple comparisons using SPSS 8.0 statistical analysis software (SPSS Inc., Chicago, IL). A P value of ≤ 0.05 was considered statistically significant.

[211] Statement 1. A recombinant vector comprising a polynucleotide sequence encoding a Zika virus nonstructural protein 1 (NS1 protein).

[212] Statement 2. The recombinant vector of statement 1, wherein the Zika virus NS1 protein has at least 90% amino acid sequence identity with SEQ ID NO: 14.

[213] Statement 3. The recombinant vector of statement 1, wherein the Zika virus NS1 protein comprises an amino acid sequence according to SEQ ID NO: 14.

[214] Statement 4. The recombinant vector of any one of statements 1-3, wherein the recombinant vector further comprises one or more polynucleotide sequences encoding a Zika virus envelope (E) protein or truncation mutant thereof, and a Zika virus premembrane (prM) protein.

[215] Statement 5. The recombinant vector of statement 4, wherein: a) the Zika virus E protein has at least 90% amino acid sequence identity with SEQ ID NO: 4; b) the Zika virus E protein truncation mutant has at least 90% amino acid sequence identity with one of SEQ ID NO 10 (E404), SEQ ID NO: 8 (E414), or SEQ ID NO: 6 (E415); and c) the Zika virus prM protein has at least 90% amino acid sequence identity with SEQ ID NO: 12.

[216] Statement 6. The recombinant vector of any one of statements 1- 5, wherein the recombinant

vector encodes the Zika virus NS1 protein, the Zika virus E protein or truncation mutant thereof, and the Zika virus prM protein.

[217] Statement 7. The recombinant vector of any one of statements 1-6, wherein the recombinant vector comprises a DNA plasmid vector or an RNA viral vector.

[218] Statement 8. The recombinant vector of statement 7, wherein the viral vector is selected from the group comprising adenovirus, adeno-associated virus (AAV), retrovirus, lentivirus, vaccinia virus, cytomegalovirus, Sendai virus, modified vaccinia Ankara virus, and vesicular stomatitis virus (VSV).

[219] Statement 9. The recombinant vector of any one of statements 1-8, wherein the recombinant vector comprises a VSV vector.

[220] Statement 10. The recombinant vector of statement 9, wherein the VSV vector comprises at least one mutation in a methyltransferase-encoding region of an L protein of the VSV vector.

[221] Statement 11. The recombinant vector of statement 10, wherein the at least one mutation is a nucleic acid mutation that results in an amino acid mutation at a position in the VSV vector selected from the group of K1651, G1670, D1762, K1795, and E1833.

[222] Statement 12. The recombinant vector of statement 10 or statement 11, wherein the at least one mutation is a nucleic acid mutation that results in a G1670A mutation or a D1762A mutation in the VSV vector.

[223] Statement 13. The recombinant vector of statement 9, wherein the VSV vector comprises a nucleic acid sequence having at least 90% sequence identity to SEQ ID NO: 16.

[224] Statement 14. The recombinant vector of statement 9, wherein the VSV vector comprises a nucleic acid sequence according to SEQ ID NO: 16, or SEQ ID NO: 16 encoding a G→A mutation at amino acid position 1670 of VSV L protein, or SEQ ID NO: 16 encoding a D→A mutation at amino acid position 1762 of VSV L protein.

[225] Statement 15. An immunogenic composition comprising at least one recombinant vector according to any one of statements 1-14 and a pharmaceutically acceptable excipient.

[226] Statement 16. The immunogenic composition of statement 15, further comprising an adjuvant.

[227] Statement 17. A method for inducing an effective immune response against Zika virus in a subject, the method comprising administering to the subject an immunologically effective dose of the immunogenic composition of statement 15 or statement 16.

[228] Statement 18. The method of statement 17, wherein the subject is human.

[229] Statement 19. The method of statement 18, wherein the subject is pregnant, may be pregnant, or is trying to get pregnant.

[230] Statement 20. The method of any one of statements 17-19, wherein the immunogenic composition is administered to the subject via a route selected from intranasal administration,

subcutaneous administration, intramuscular administration, intradermal administration, and oral administration.

[231] Statement 21. The method of any one of statements 17-20, further comprising administering at least one subsequent immunologically effective dose of the immunogenic composition.

[232] Statement 22. A method for inducing an effective immune response against Zika virus in a subject, the method comprising expressing a Zika virus nonstructural protein 1 (NS1 protein) in cells of the subject.

[233] Statement 23. The method of statement 22, wherein the Zika virus NS1 protein has at least 90% amino acid sequence identity with SEQ ID NO: 14.

[234] Statement 24. The method of statement 22, wherein the Zika virus NS1 protein comprises an amino acid sequence according to SEQ ID NO: 14.

[235] Statement 25. The method of any one of statements 22-24, further comprising co-expressing a Zika virus envelope (E) protein or a truncation mutant thereof, and a Zika virus premembrane (prM) protein.

[236] Statement 26. The method of statement 25, wherein: a) the Zika virus E protein has at least 90% amino acid sequence identity with SEQ ID NO: 4; b) the Zika virus E protein truncation mutant has at least 90% sequence identity with one of SEQ ID NO 10 (E404), SEQ ID NO: 8 (E414), or SEQ ID NO: 6 (E415); and c) the Zika virus prM protein has at least 90% amino acid sequence identity with SEQ ID NO: 12.

[237] Statement 27. The method of any one of statements 22-26, wherein the Zika virus protein(s) are expressed from a recombinant vesicular stomatitis virus (VSV) vector.

[238] Statement 28. An expression cassette comprising a promoter operably linked to a polynucleotide encoding a Zika virus nonstructural protein 1 (NS1 protein).

[239] Statement 29. The expression cassette of statement 28, wherein the polynucleotide encoding the Zika virus NS1 protein further encodes a Zika virus envelope (E) protein or a truncation mutant thereof, and a Zika virus premembrane (prM) protein.

[240] Statement 30. The expression cassette of statement 29, wherein the Zika virus NS1 protein has at least 90% amino acid sequence identity with SEQ ID NO: 14, the Zika virus E protein has at least 90% amino acid sequence identity with SEQ ID NO: 4, the Zika virus E protein truncation mutant has at least 90% sequence identity with one of SEQ ID NO 10 (E404), SEQ ID NO: 8 (E414), or SEQ ID NO: 6 (E415), and the Zika virus prM protein has at least 90% amino acid sequence identity with SEQ ID NO: 12.

[241] Statement 31. An immunogenic composition comprising at least one recombinant vector according to any one of statements 1-14 and a pharmaceutically acceptable excipient for use in inducing

an effective immune response against Zika virus in a subject, the method comprising administering to the subject an immunologically effective dose of the immunogenic composition.

[242] Statement 32. The immunogenic composition of statement 31, wherein the immunogenic composition further comprises an adjuvant.

[243] Statement 33. The immunogenic composition of statement 31 or statement 32, wherein the subject is human, optionally wherein the human subject is pregnant, may be pregnant, or is trying to get pregnant.

[244] Statement 34. The immunogenic composition of and one of statements 31-33, wherein the immunogenic composition is administered to the subject via a route selected from intranasal administration, subcutaneous administration, intramuscular administration, intradermal administration, and oral administration, and optionally wherein at least one subsequent immunologically effective dose of the immunogenic composition is administered to the subject.

[245] Statement 35. An immunogenic composition comprising at least one recombinant vector according to any one of statements 1-14 and a pharmaceutically acceptable excipient for use in manufacturing a medicament.

CLAIMS

1. A recombinant vector comprising a polynucleotide sequence encoding a Zika virus nonstructural protein 1 (NS1 protein).

2. The recombinant vector of claim 1, wherein the Zika virus NS1 protein has at least 90% amino acid sequence identity with SEQ ID NO: 14.

3. The recombinant vector of claim 1, wherein the Zika virus NS1 protein comprises an amino acid sequence according to SEQ ID NO: 14.

4. The recombinant vector of any one of claims 1-3, wherein the recombinant vector further comprises one or more polynucleotide sequences encoding a Zika virus envelope (E) protein or truncation mutant thereof, and a Zika virus premembrane (prM) protein.

5. The recombinant vector of claim 4, wherein:
   the Zika virus E protein has at least 90% amino acid sequence identity with SEQ ID NO: 4;
   the Zika virus E protein truncation mutant has at least 90% amino acid sequence identity with one of SEQ ID NO 10 (E404), SEQ ID NO: 8 (E414), or SEQ ID NO: 6 (E415); and
   the Zika virus prM protein has at least 90% amino acid sequence identity with SEQ ID NO: 12.

6. The recombinant vector of claim 4 or claim 5, wherein the recombinant vector encodes the Zika virus NS1 protein, the Zika virus E protein or truncation mutant thereof, and the Zika virus prM protein.

7. The recombinant vector of any one of claims 1-6, wherein the recombinant vector comprises a DNA plasmid vector or an RNA viral vector.

8. The recombinant vector of claim 7, wherein the viral vector is selected from the group comprising adenovirus, adeno-associated virus (AAV), retrovirus, lentivirus, vaccinia virus, cytomegalovirus, Sendai virus, modified vaccinia Ankara virus, and vesicular stomatitis virus (VSV).

9. The recombinant vector of any one of claims 1-8, wherein the recombinant vector comprises a VSV vector.

10. The recombinant vector of claim 9, wherein the VSV vector comprises at least one mutation in a methyltransferase-encoding region of an L protein of the VSV vector.

11. The recombinant vector of claim 10, wherein the at least one mutation is a nucleic acid mutation that results in an amino acid mutation at a position in the VSV vector selected from the group of K1651, G1670, D1762, K1795, and E1833.

12. The recombinant vector of claim 10 or claim 11, wherein the at least one mutation is a nucleic acid mutation that results in a G1670A mutation or a D1762A mutation in the VSV vector.

13. The recombinant vector of claim 9, wherein the VSV vector comprises a nucleic acid sequence having at least 90% sequence identity to SEQ ID NO: 16.

14. The recombinant vector of claim 9, wherein the VSV vector comprises a nucleic acid sequence according to SEQ ID NO: 16, or SEQ ID NO: 16 encoding a G→A mutation at amino acid position 1670 of VSV L protein, or SEQ ID NO: 16 encoding a D→A mutation at amino acid position 1762 of VSV L protein.

15. An immunogenic composition comprising at least one recombinant vector according to any one of claims 1-14 and a pharmaceutically acceptable excipient.

16. The immunogenic composition of claim 15, further comprising an adjuvant.

17. A method for inducing an effective immune response against Zika virus in a subject, the method comprising administering to the subject an immunologically effective dose of the immunogenic composition of claim 15 or claim 16.

18. The method of claim 17, wherein the subject is human.

19. The method of claim 18, wherein the subject is pregnant, may be pregnant, or is trying to get pregnant.

20. The method of any one of claims 17-19, wherein the immunogenic composition is administered to the subject via a route selected from intranasal administration, subcutaneous administration,

intramuscular administration, intradermal administration, and oral administration.

21. The method of any one of claims 17-20, further comprising administering at least one subsequent immunologically effective dose of the immunogenic composition.

22. A method for inducing an effective immune response against Zika virus in a subject, the method comprising expressing a Zika virus nonstructural protein 1 (NS1 protein) in cells of the subject.

23. The method of claim 22, wherein the Zika virus NS1 protein has at least 90% amino acid sequence identity with SEQ ID NO: 14.

24. The method of claim 22, wherein the Zika virus NS1 protein comprises an amino acid sequence according to SEQ ID NO: 14.

25. The method of any one of claims 22-24, further comprising co-expressing a Zika virus envelope (E) protein or a truncation mutant thereof, and a Zika virus premembrane (prM) protein.

26. The method of claim 25, wherein:

    the Zika virus E protein has at least 90% amino acid sequence identity with SEQ ID NO: 4;
    the Zika virus E protein truncation mutant has at least 90% sequence identity with one of SEQ ID NO 10 (E404), SEQ ID NO: 8 (E414), or SEQ ID NO: 6 (E415); and
    the Zika virus prM protein has at least 90% amino acid sequence identity with SEQ ID NO: 12.

27. The method of any one of claims 22-26, wherein the Zika virus protein(s) are expressed from a recombinant vesicular stomatitis virus (VSV) vector.

28. An expression cassette comprising a promoter operably linked to a polynucleotide encoding a Zika virus nonstructural protein 1 (NS1 protein).

29. The expression cassette of claim 28, wherein the polynucleotide encoding the Zika virus NS1 protein further encodes a Zika virus envelope (E) protein or a truncation mutant thereof, and a Zika virus premembrane (prM) protein.

30. The expression cassette of claim 29, wherein the Zika virus NS1 protein has at least 90% amino acid

sequence identity with SEQ ID NO: 14, the Zika virus E protein has at least 90% amino acid sequence identity with SEQ ID NO: 4, the Zika virus E protein truncation mutant has at least 90% sequence identity with one of SEQ ID NO 10 (E404), SEQ ID NO: 8 (E414), or SEQ ID NO: 6 (E415), and the Zika virus prM protein has at least 90% amino acid sequence identity with SEQ ID NO: 12.

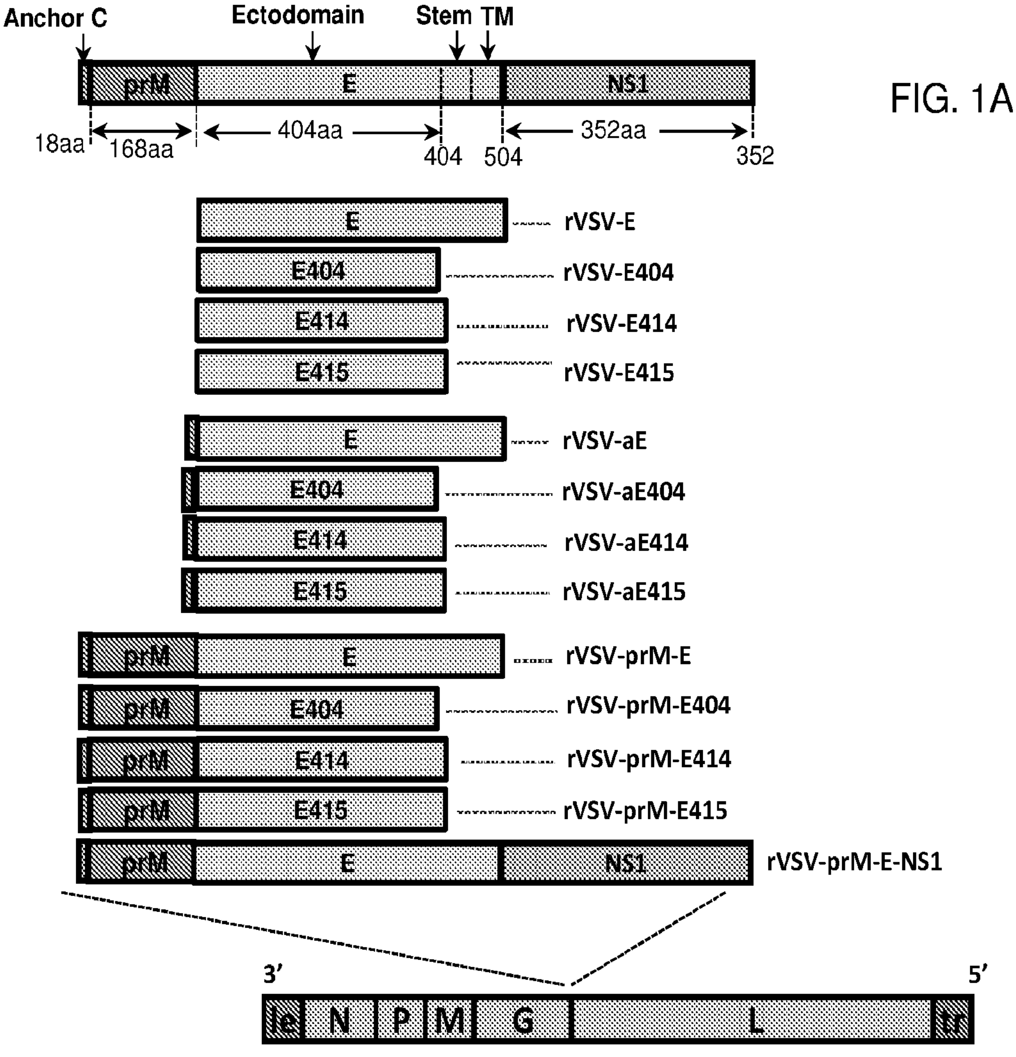
Anchor C
Ectodomain
Stem
TM
prM
E
NS1
18aa
168aa
404aa
404
504
352aa
352
E
rVSV-E
E404
rVSV-E404
E414
rVSV-E414
E415
rVSV-E415
E
rVSV-aE
E404
rVSV-aE404
E414
rVSV-aE414
E415
rVSV-aE415
prM
E
rVSV-prM-E
prM
E404
rVSV-prM-E404
prM
E414
rVSV-prM-E414
prM
E415
rVSV-prM-E415
prM
E
NS1
rVSV-prM-E-NS1
3'
5'
le
N
P
M
G
L
tr
VSV genome

FIG. 1A

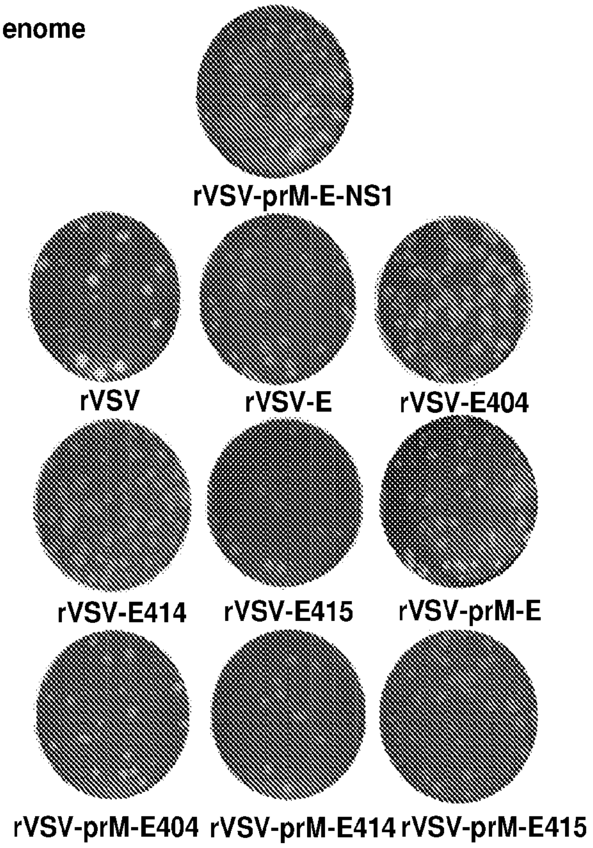
rVSV-prM-E-NS1
rVSV
rVSV-E
rVSV-E404
rVSV-E414
rVSV-E415
rVSV-prM-E
rVSV-prM-E404
rVSV-prM-E414
rVSV-prM-E415

FIG. 1B

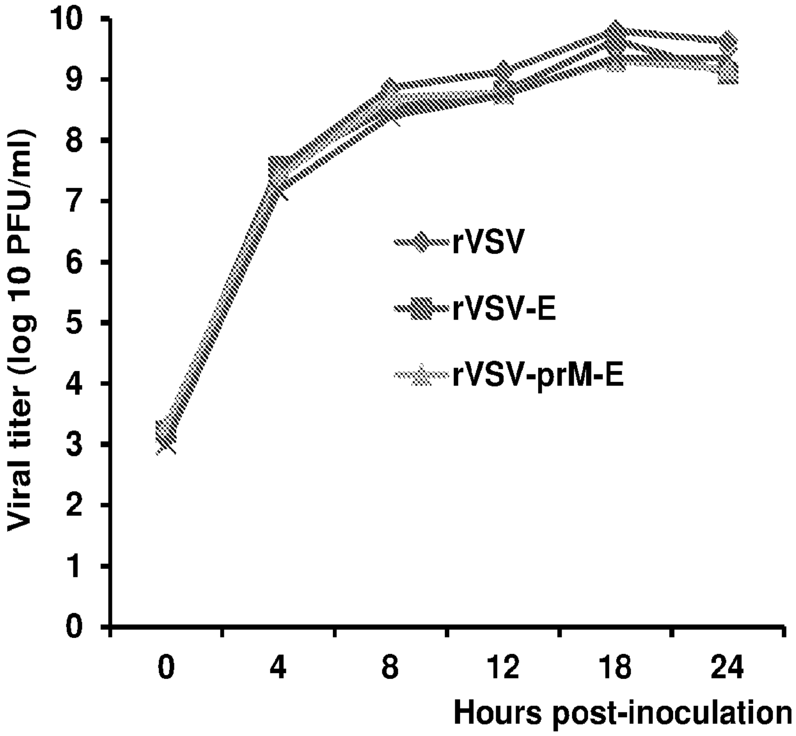
Viral titer (log 10 PFU/ml)
10
9
8
7
6
5
4
3
2
1
0
0
4
8
12
18
24
Hours post-inoculation
rVSV
rVSV-E
rVSV-prM-E

FIG. 2

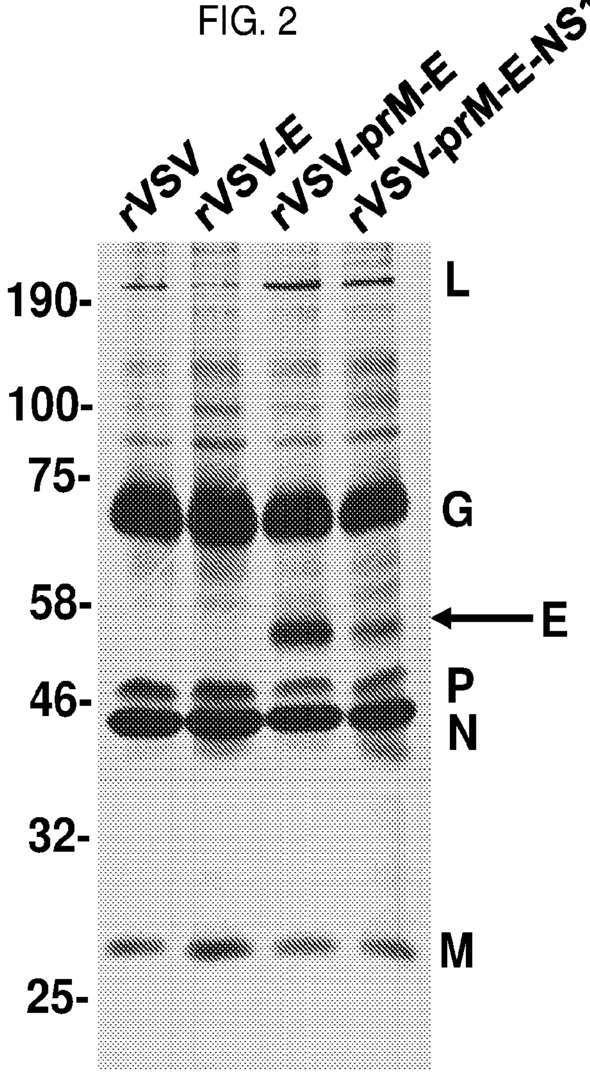
rVSV
rVSV-E
rVSV-prM-E
rVSV-prM-E-NS1
190-
100-
75-
58-
46-
32-
25-
L
G
E
P
N
M

FIG. 3

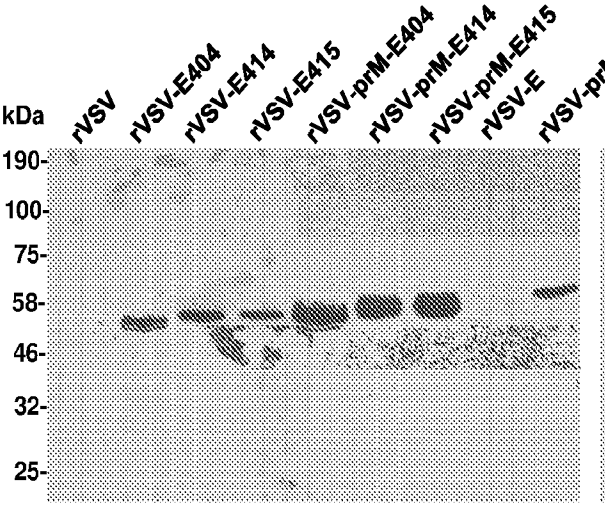

FIG. 4A

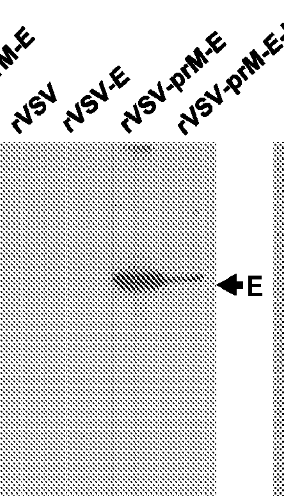

FIG. 4B

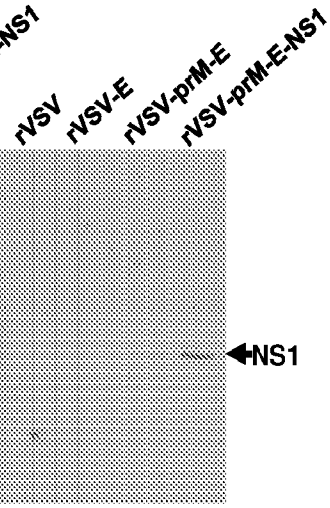

FIG. 4C

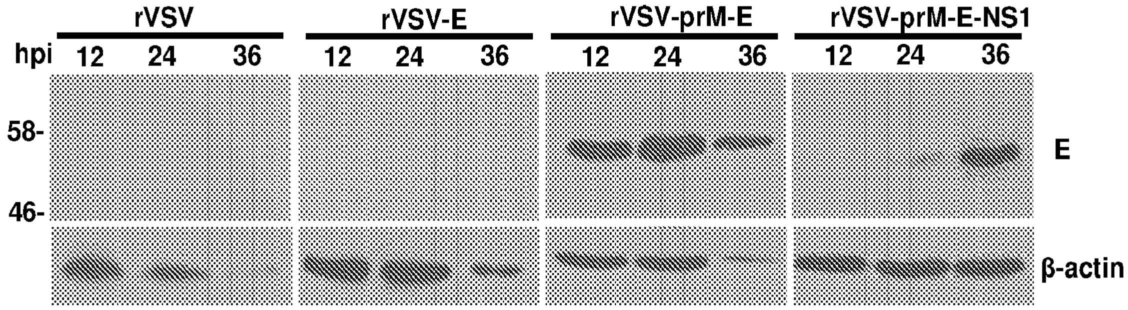

FIG. 4D

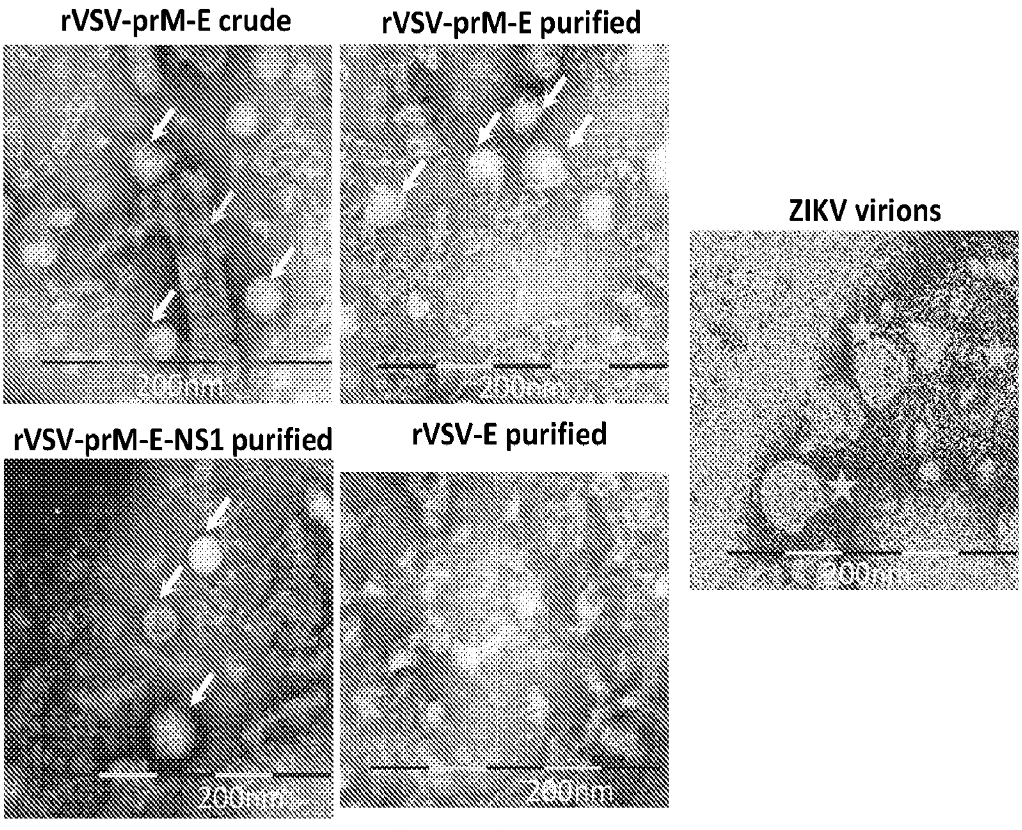

FIG. 4E

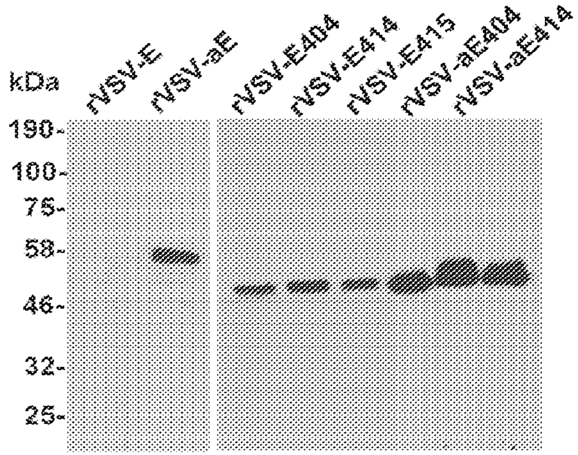

FIG. 4F

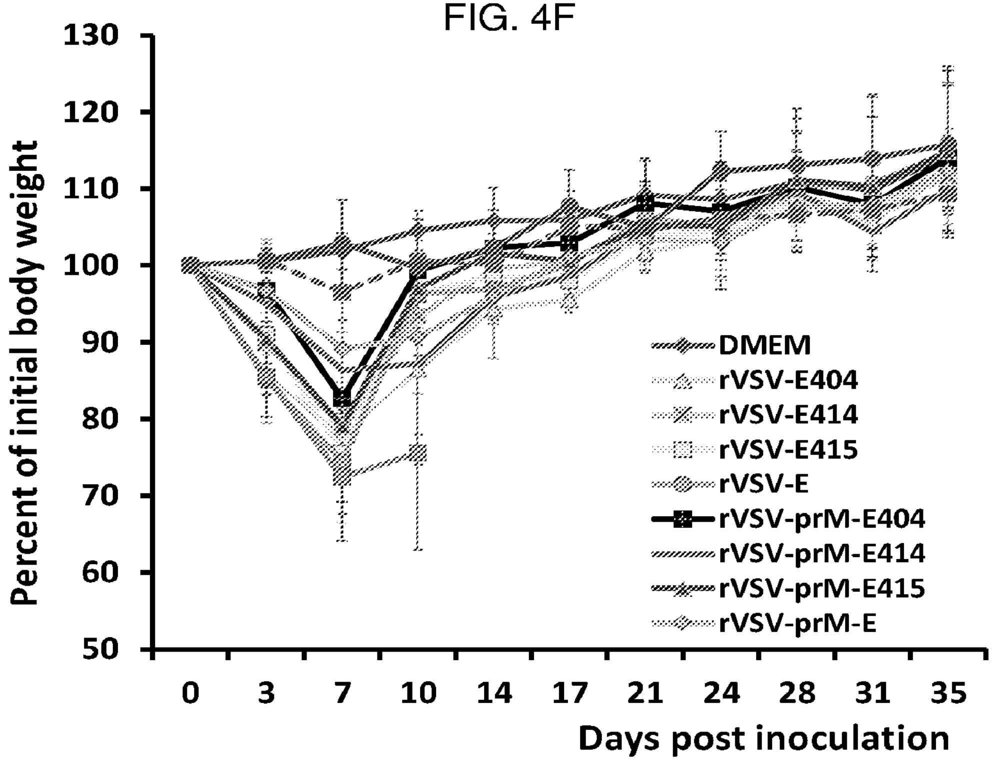

FIG. 5

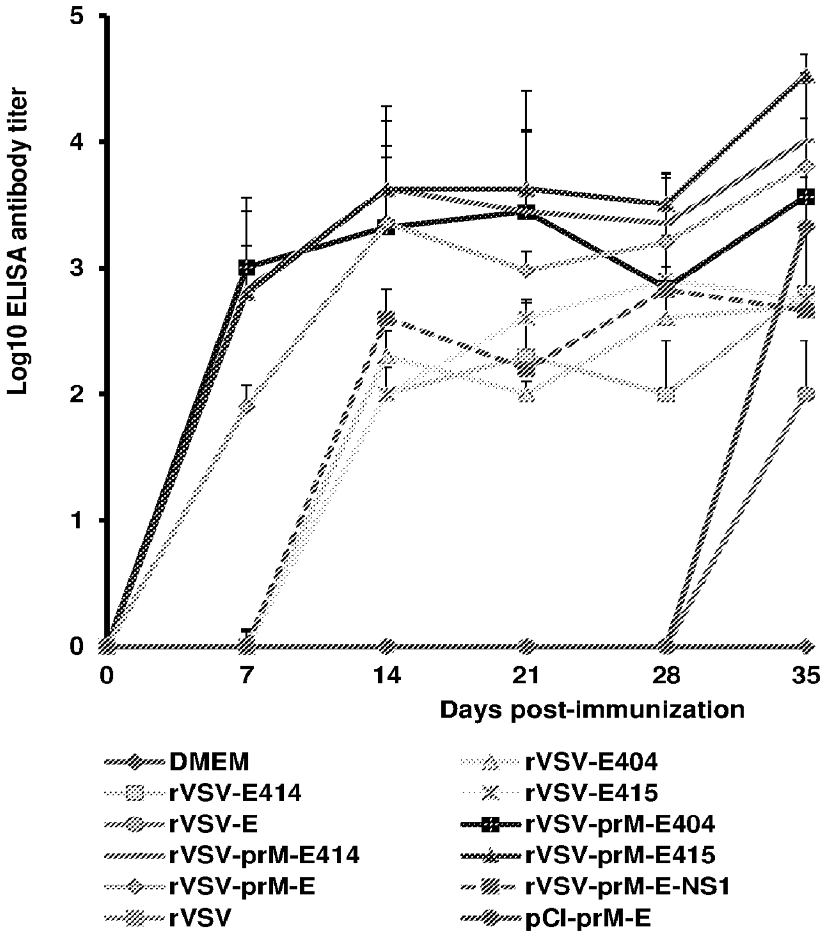

FIG. 6

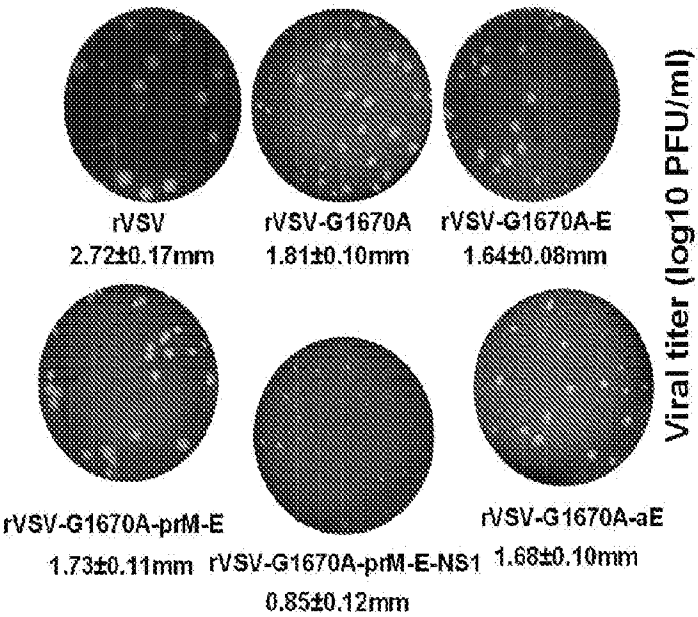

FIG. 7A

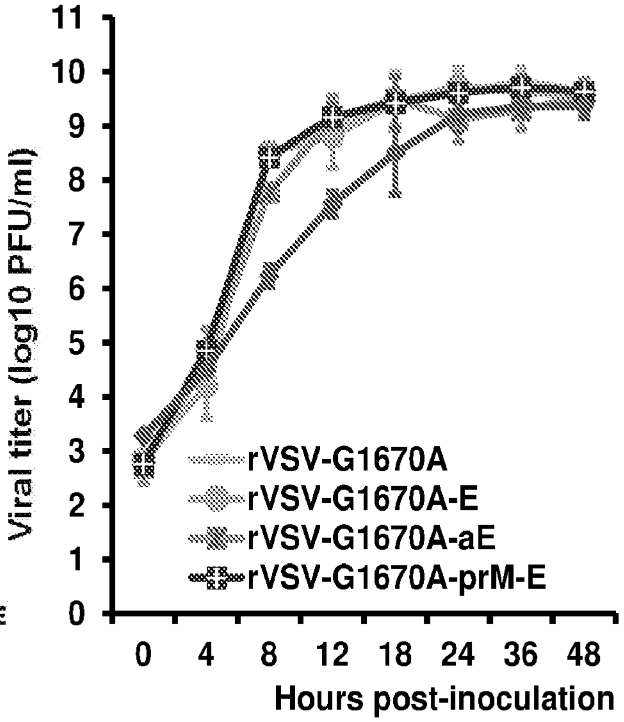

FIG. 7B

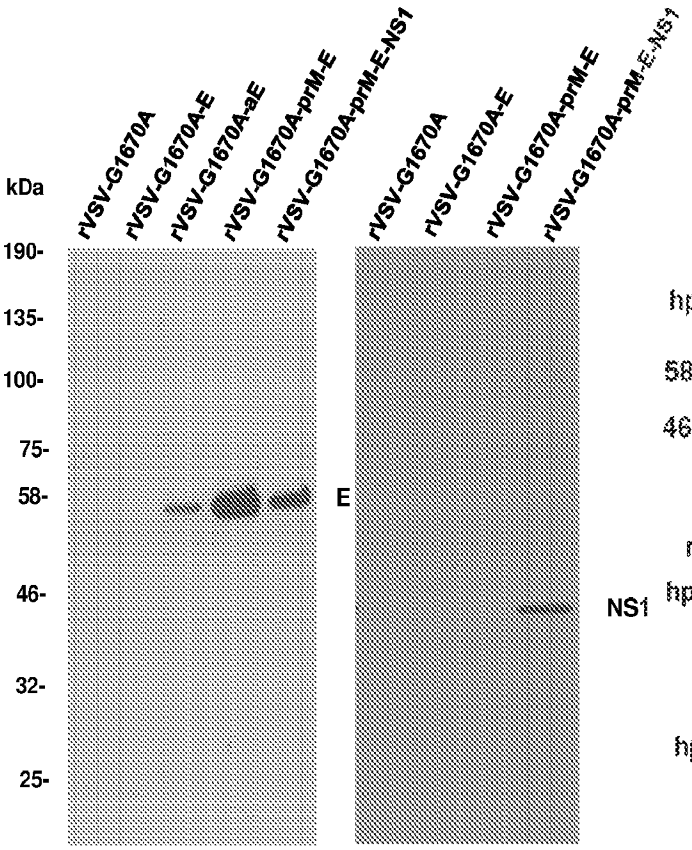

FIG. 7C

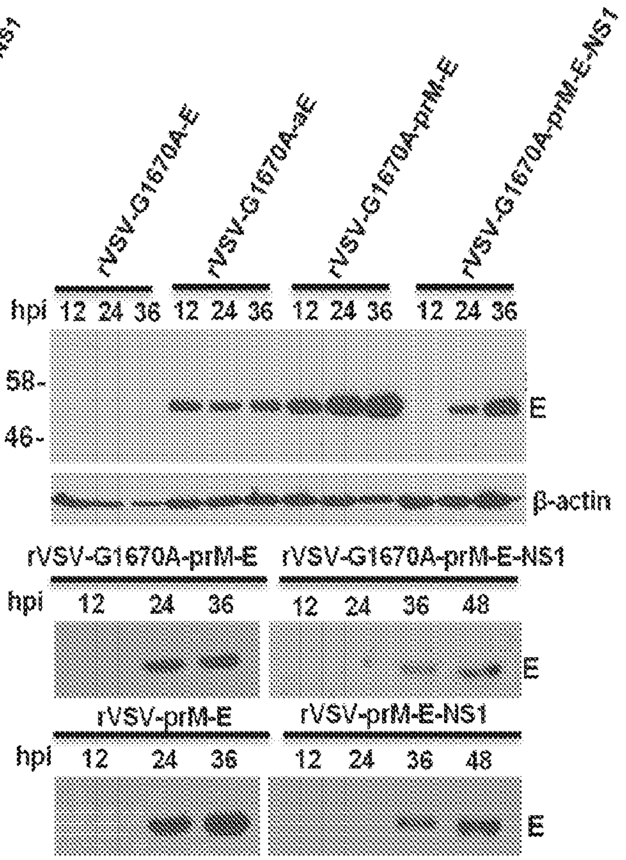

FIG. 7D

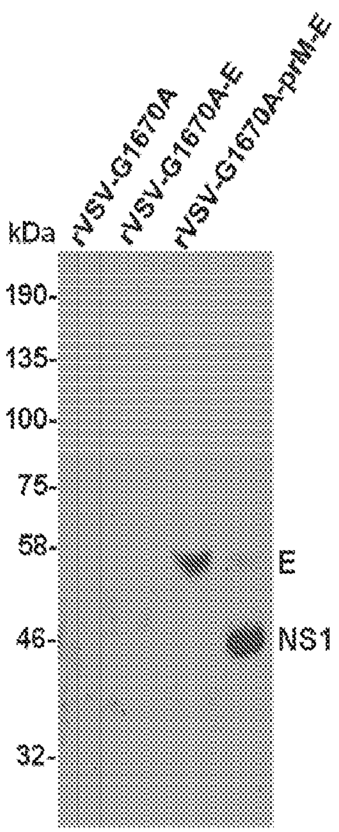

FIG. 7E

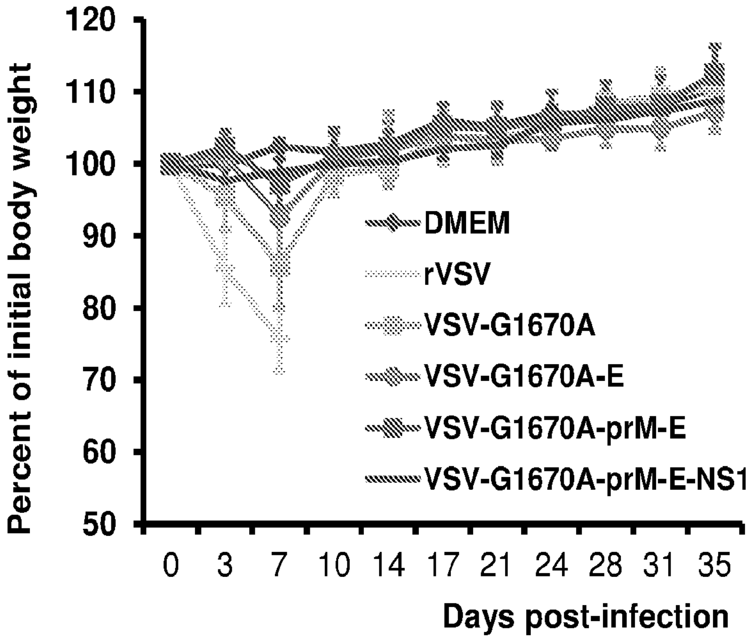

FIG. 8A

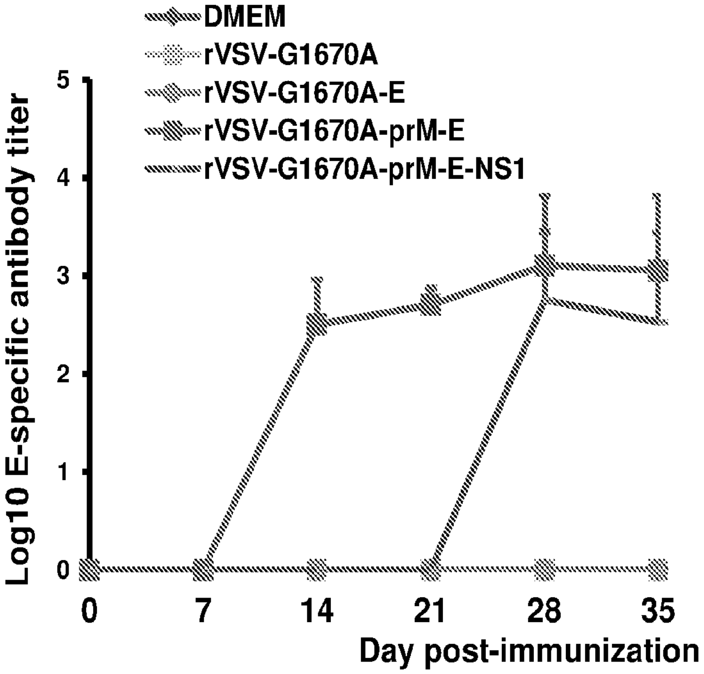

FIG. 8B

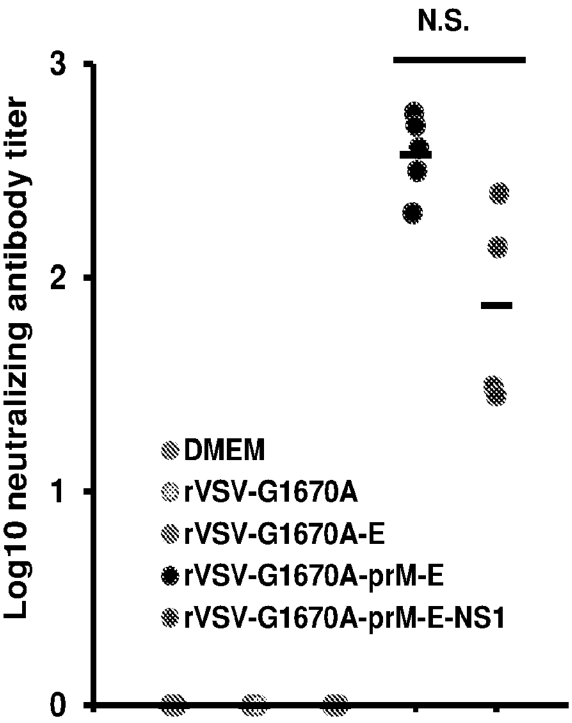
N.S.
Log10 neutralizing antibody titer
3
2
1
0
DMEM
rVSV-G1670A
rVSV-G1670A-E
rVSV-G1670A-prM-E
rVSV-G1670A-prM-E-NS1

FIG. 8C

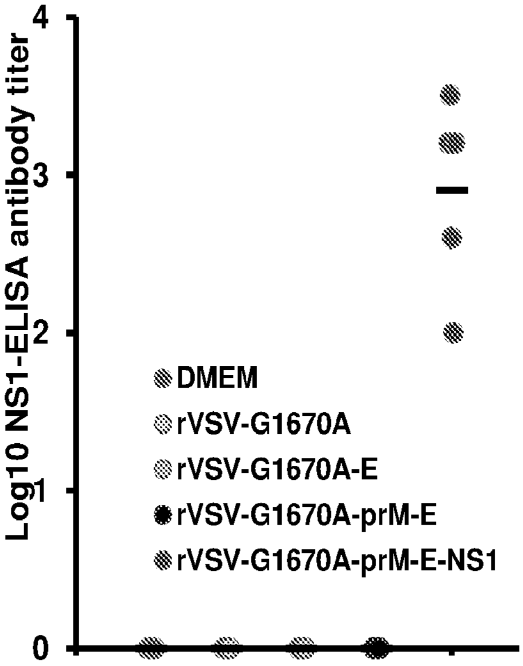
Log10 NS1-ELISA antibody titer
4
3
2
1
0
DMEM
rVSV-G1670A
rVSV-G1670A-E
rVSV-G1670A-prM-E
rVSV-G1670A-prM-E-NS1

FIG. 8D

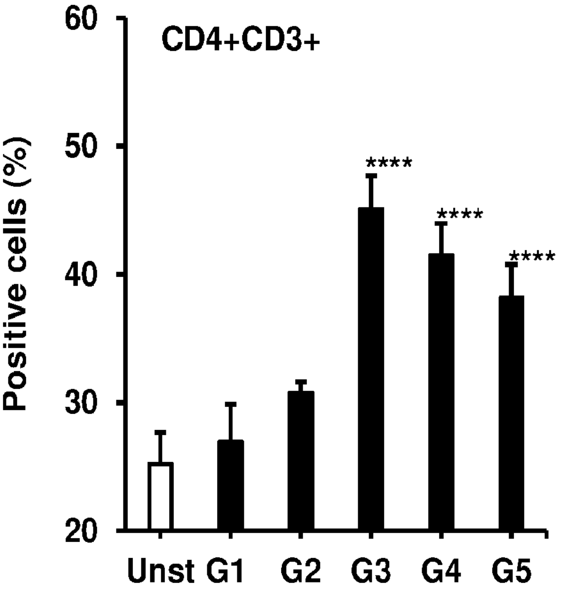

FIG. 9A

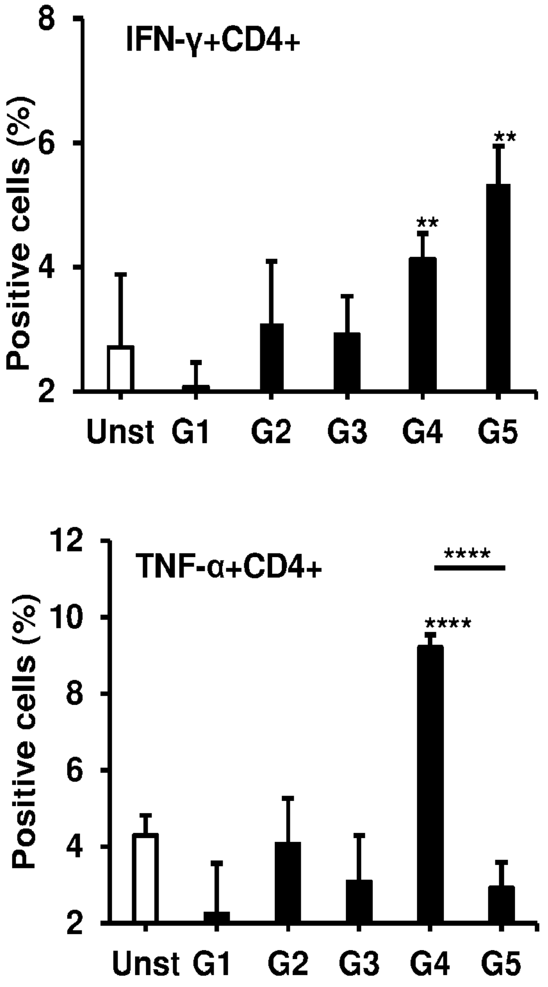

FIG. 9B

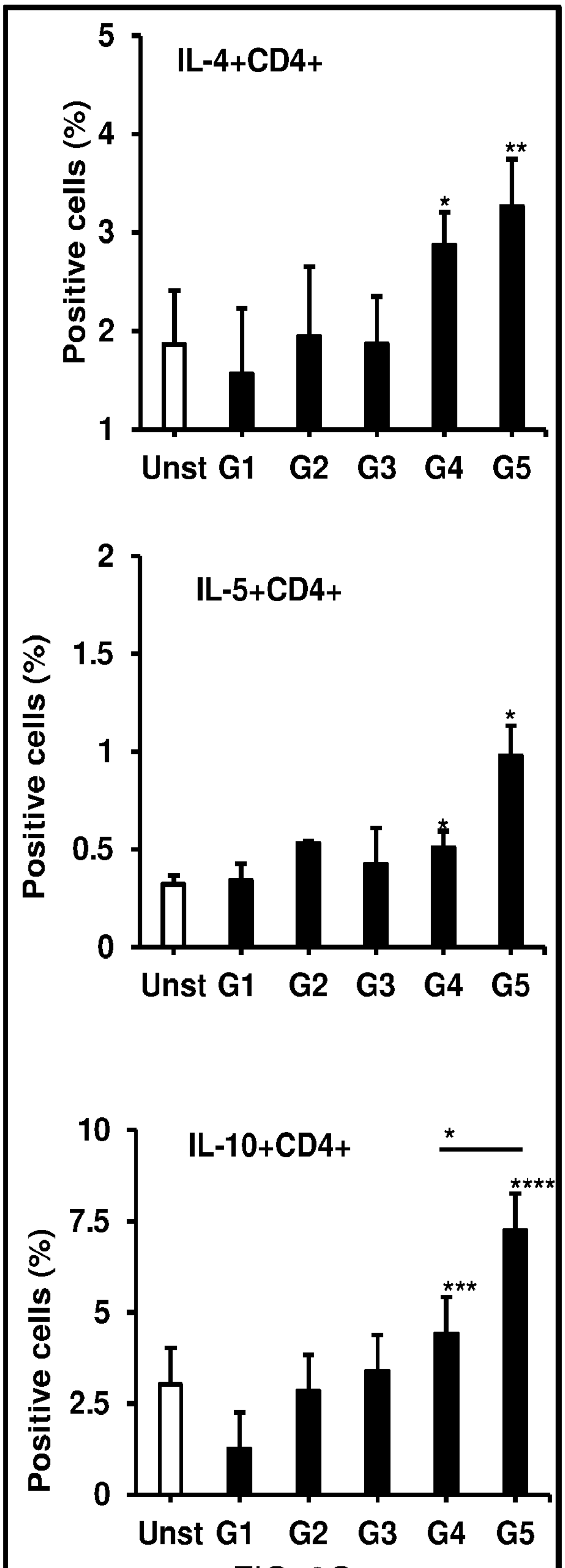

FIG. 9C

Unst: Unstimulated
G1: DMEM
G2: rVSV-G1670A
G3:rVSV-G1670A-E
G4:rVSV-G1670A-prM-E
G5:rVSV-G1670A-prM-E-NS1

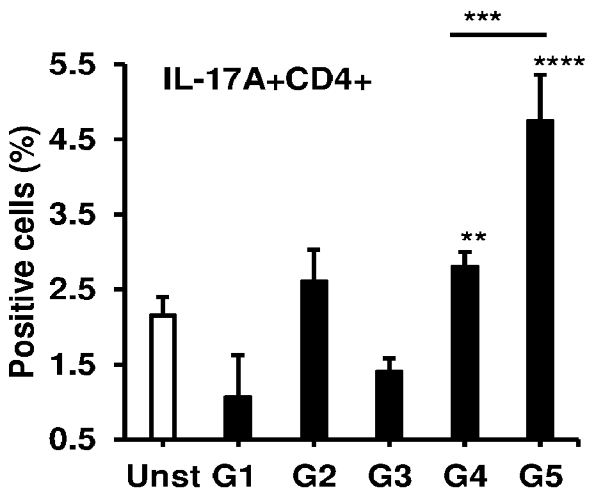

FIG. 9D

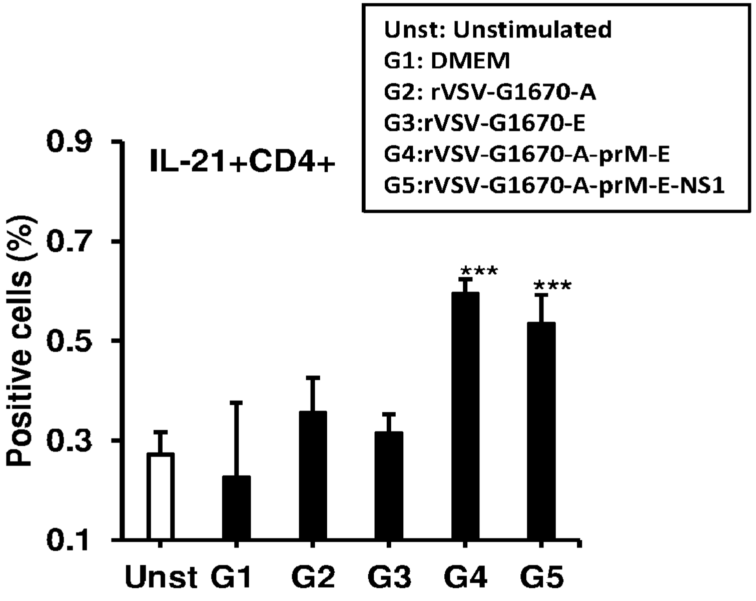

FIG. 9E

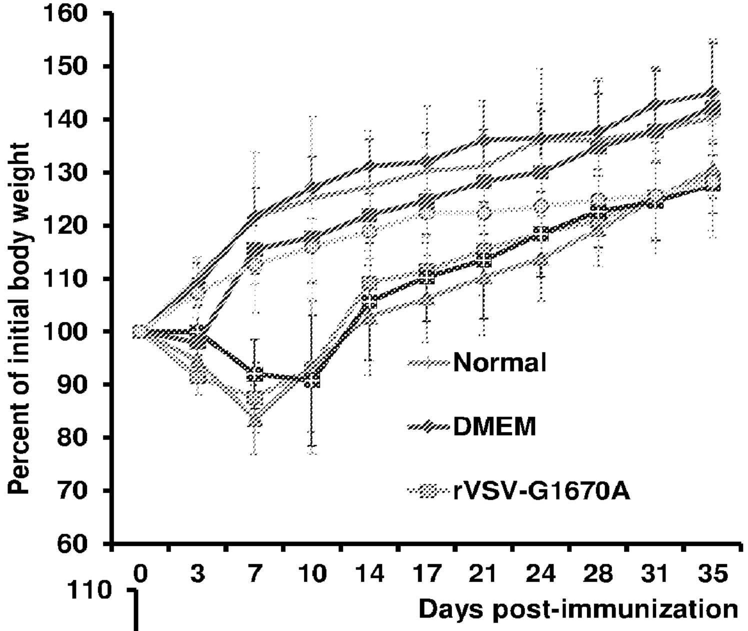

FIG. 10

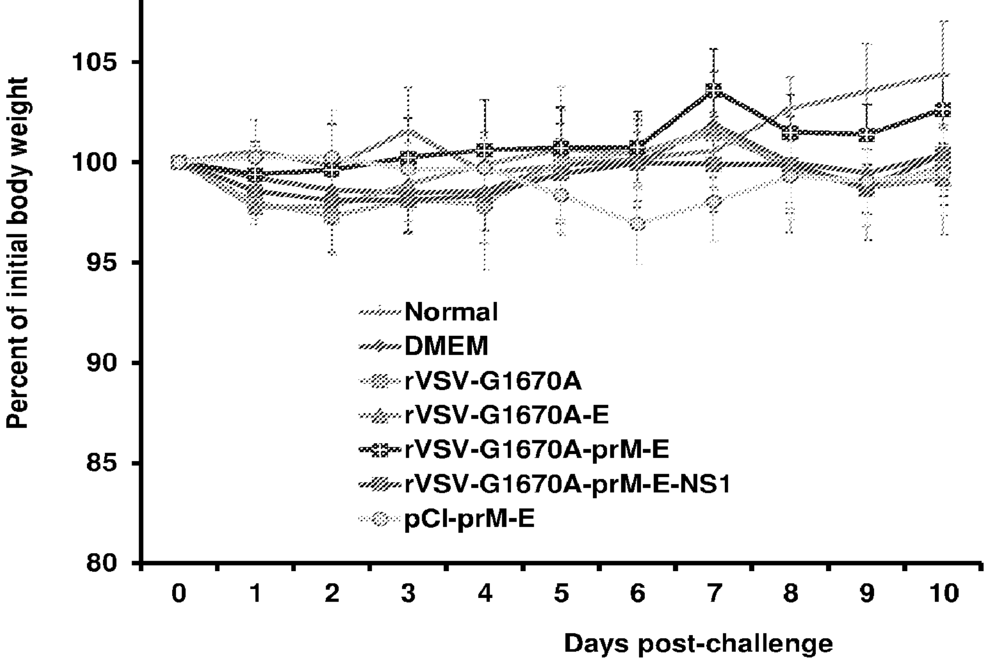

FIG. 11

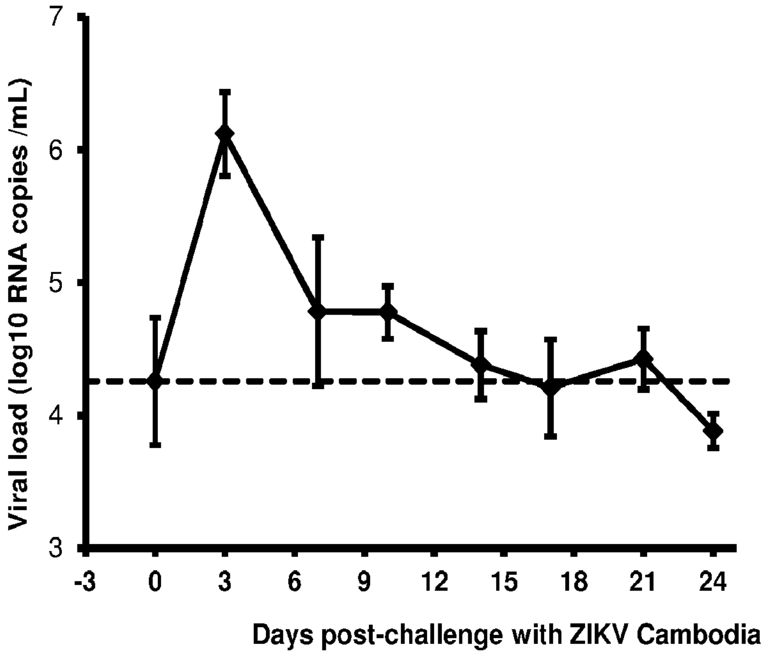

FIG. 12A

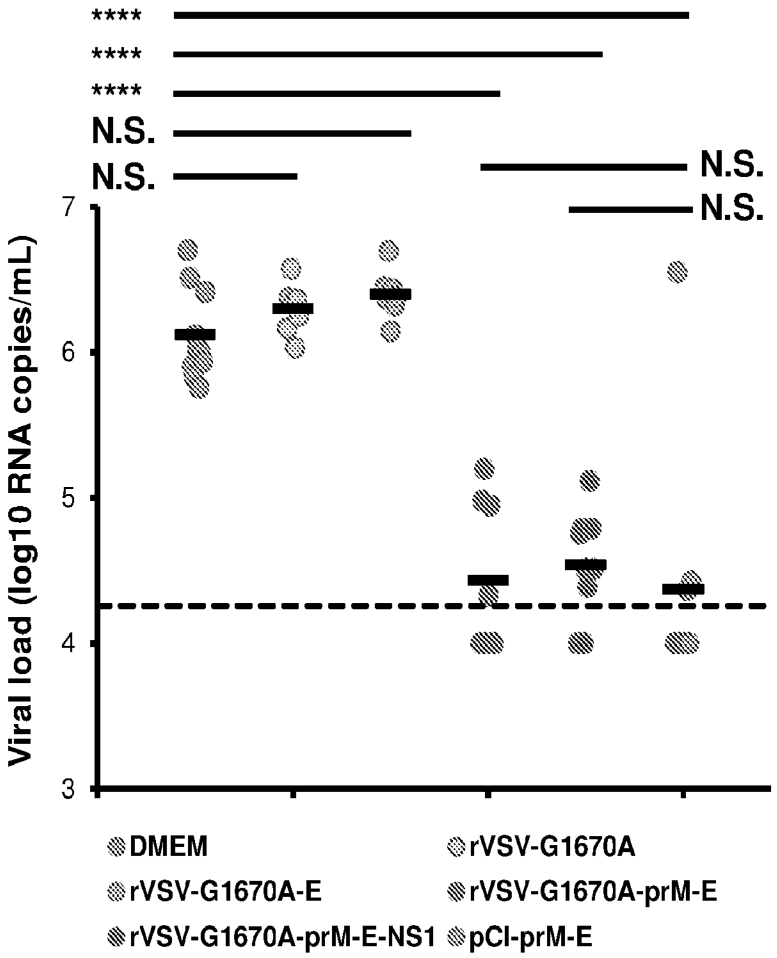

FIG. 12B

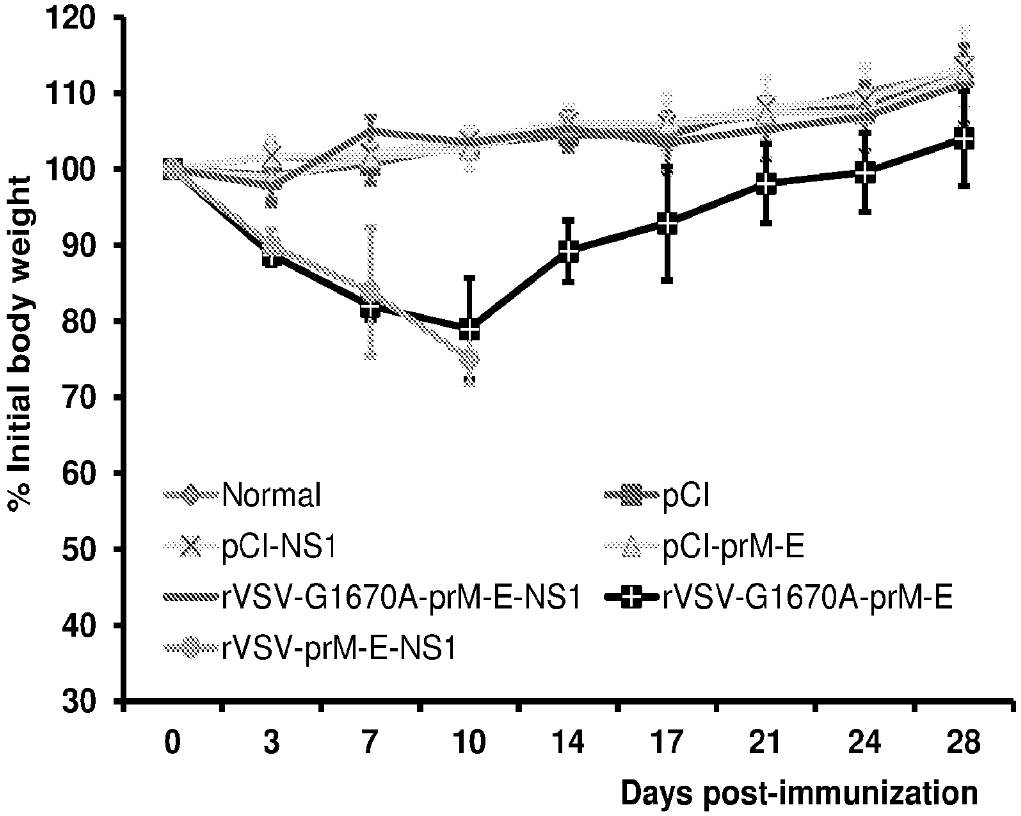

FIG. 13

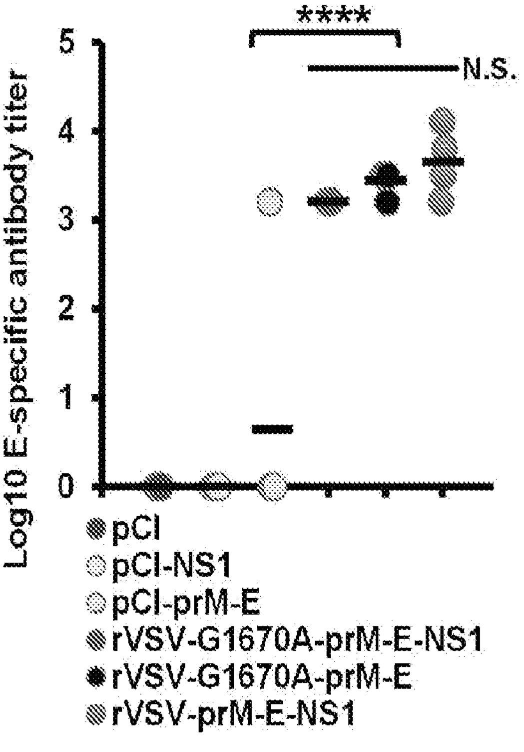

FIG. 14A

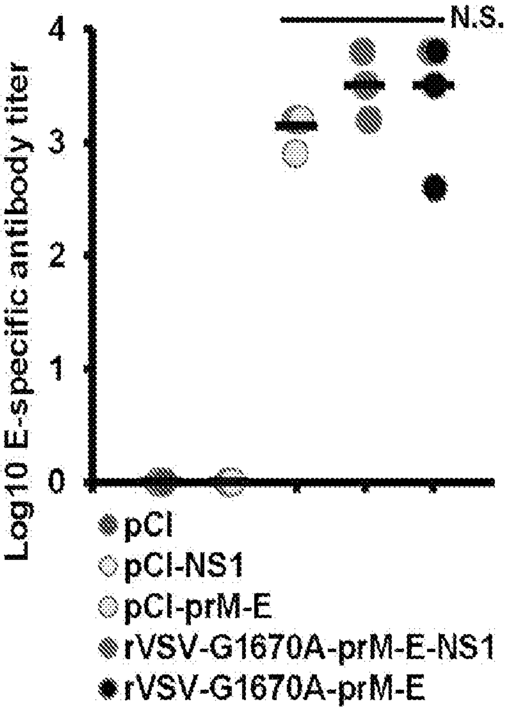

FIG. 14B

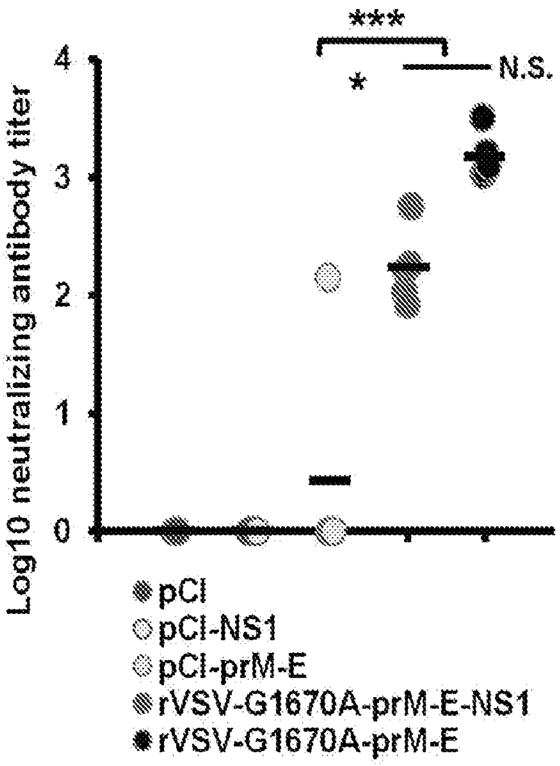

FIG. 14C

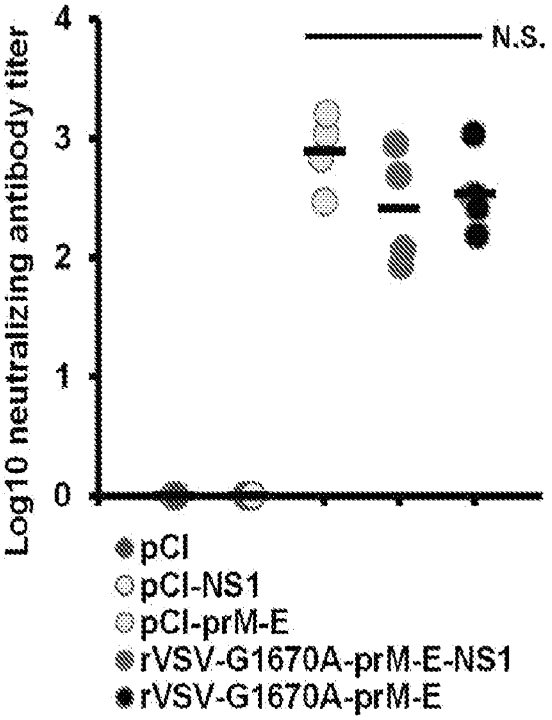

FIG. 14D

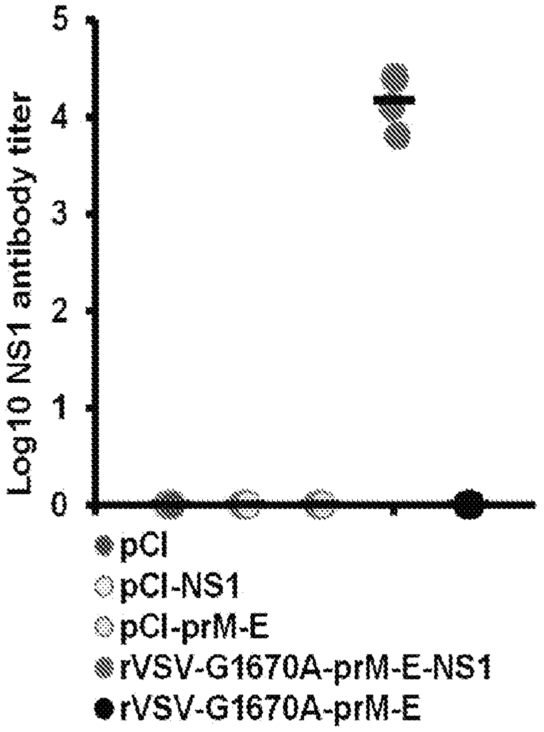

FIG. 14E

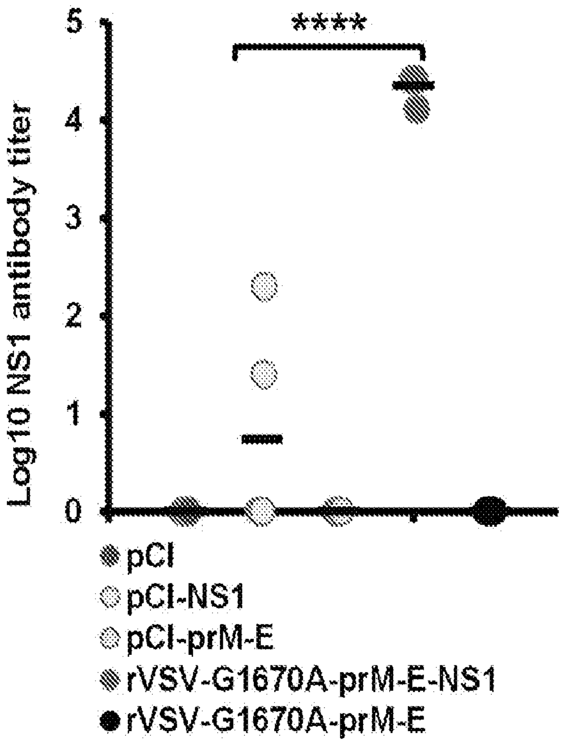

FIG. 14F

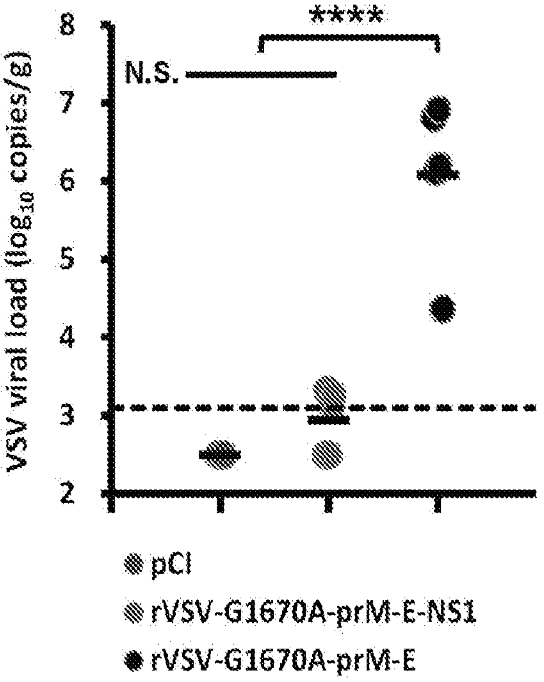

FIG. 14G

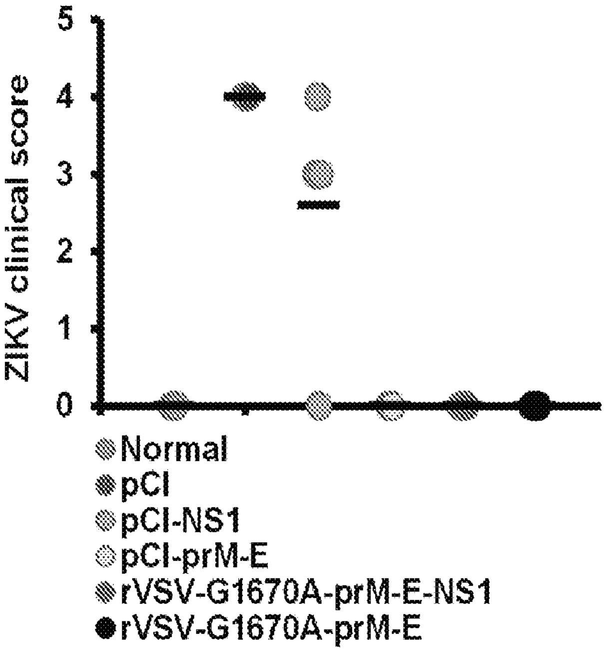

FIG. 15A

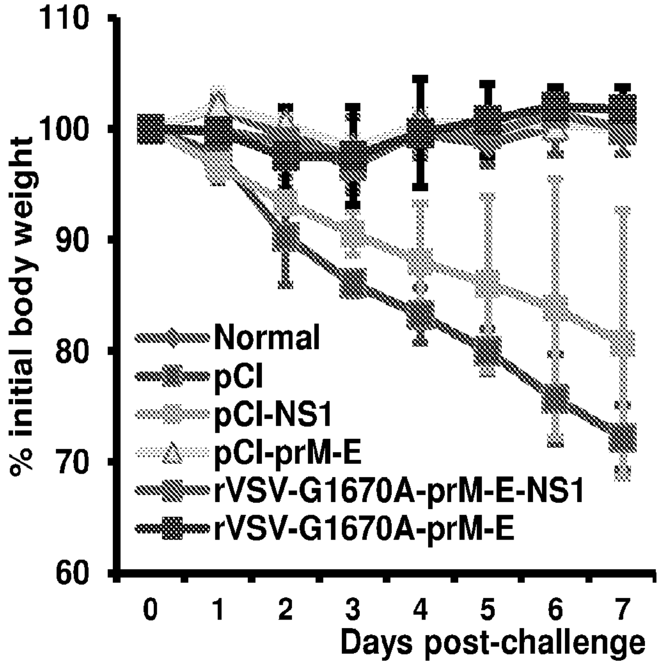

FIG. 15B

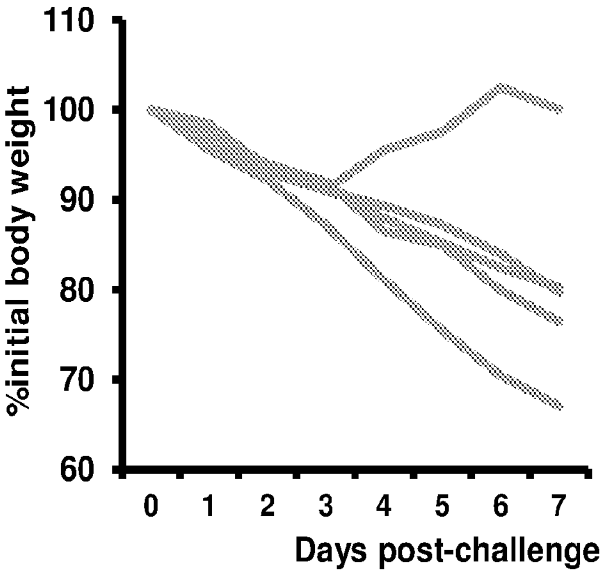

FIG. 15C

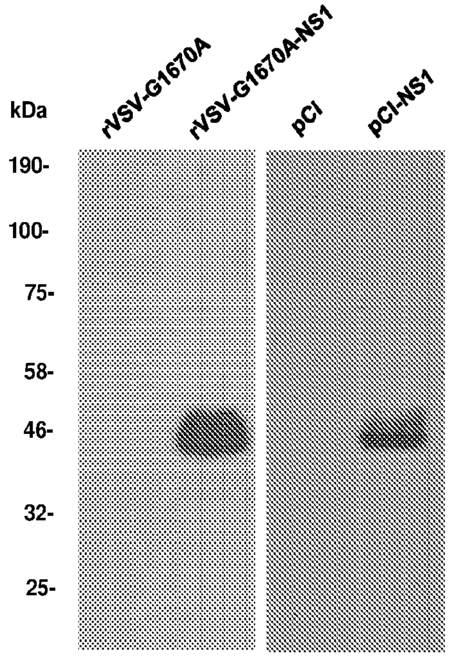

FIG. 15D

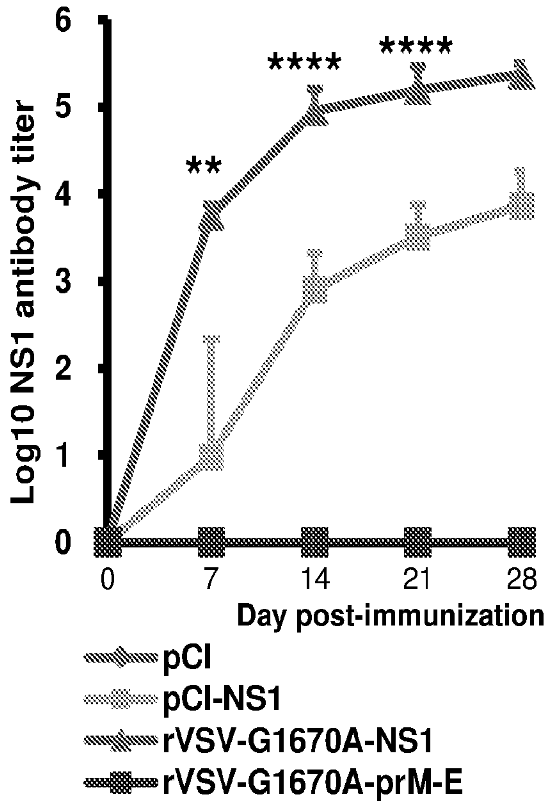

FIG. 15E

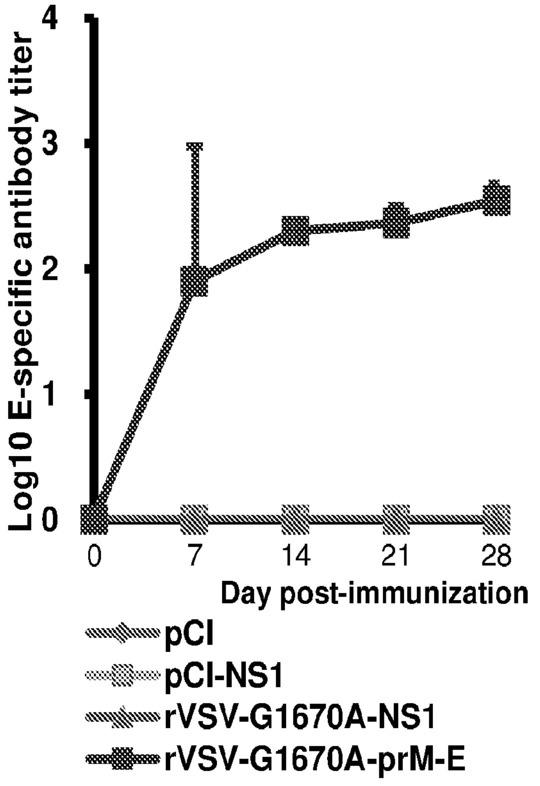

FIG. 15F

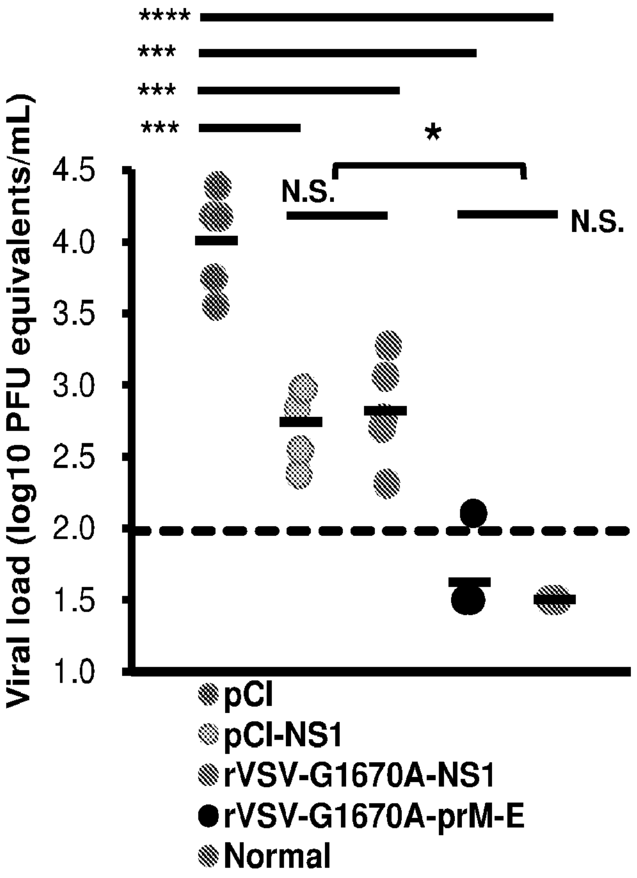

FIG. 15G

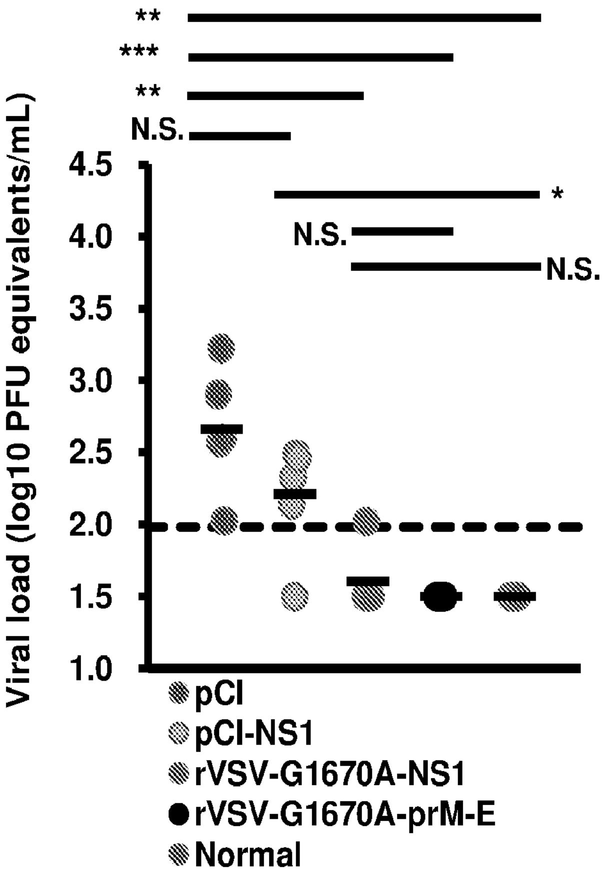
**
***
**
N.S.
*
N.S.
N.S.
Viral load (log10 PFU equivalents/mL)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
pCI
pCI-NS1
rVSV-G1670A-NS1
rVSV-G1670A-prM-E
Normal

FIG. 15H

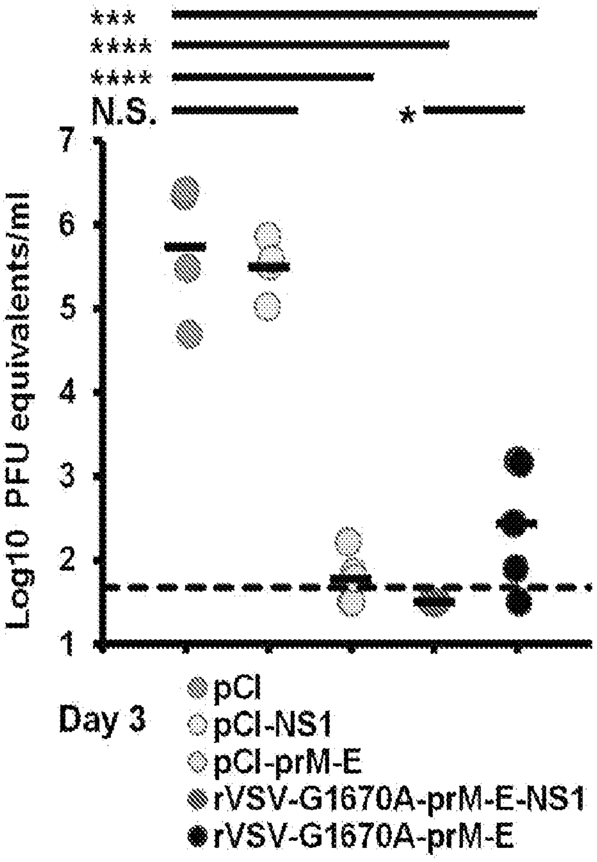

FIG. 16A

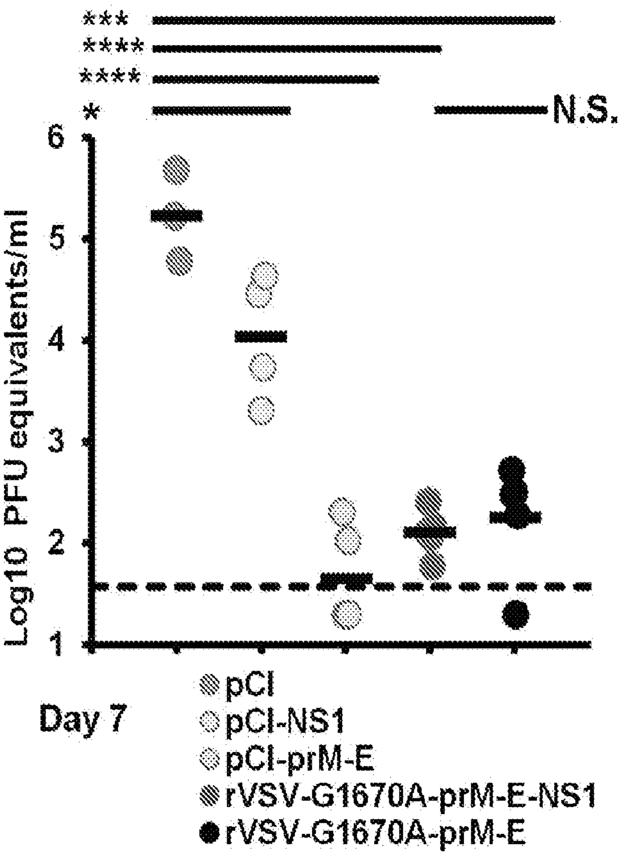

FIG. 16B

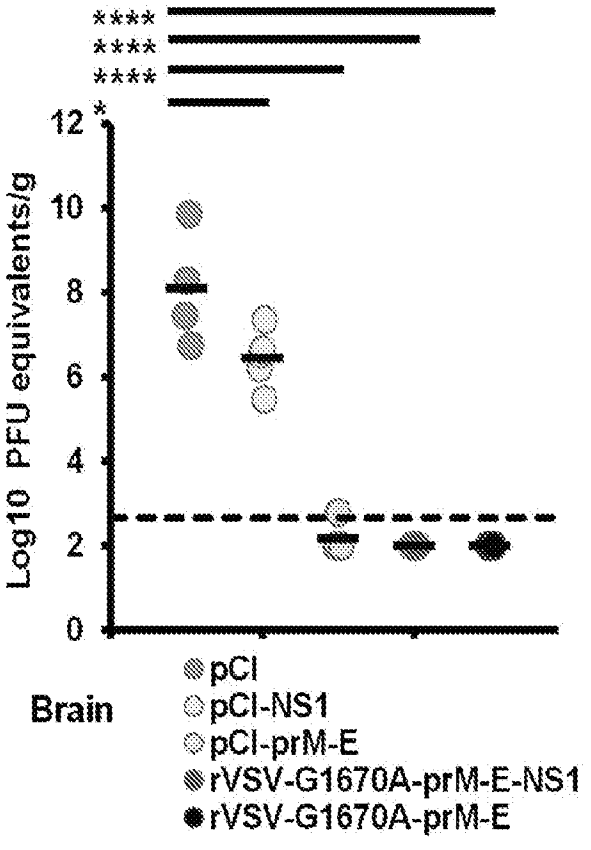

FIG. 16C

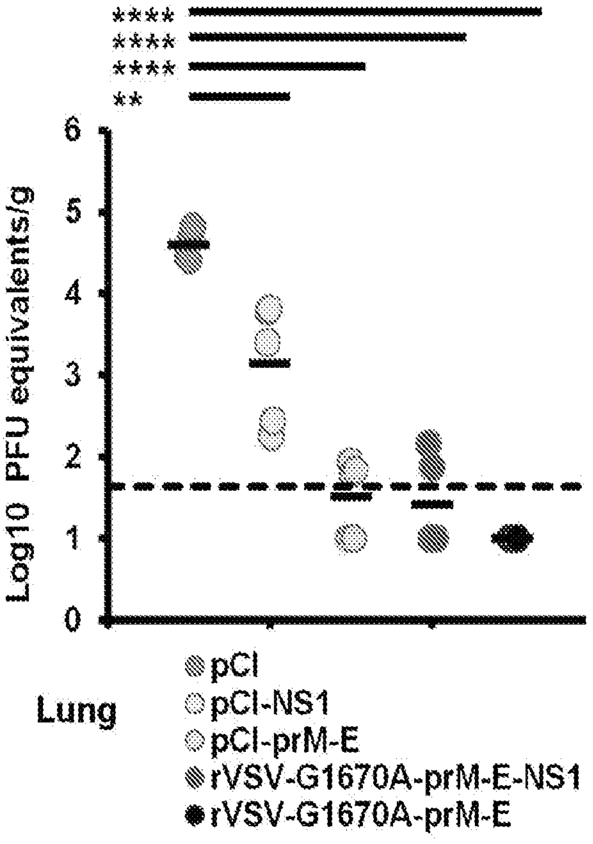

FIG. 16D

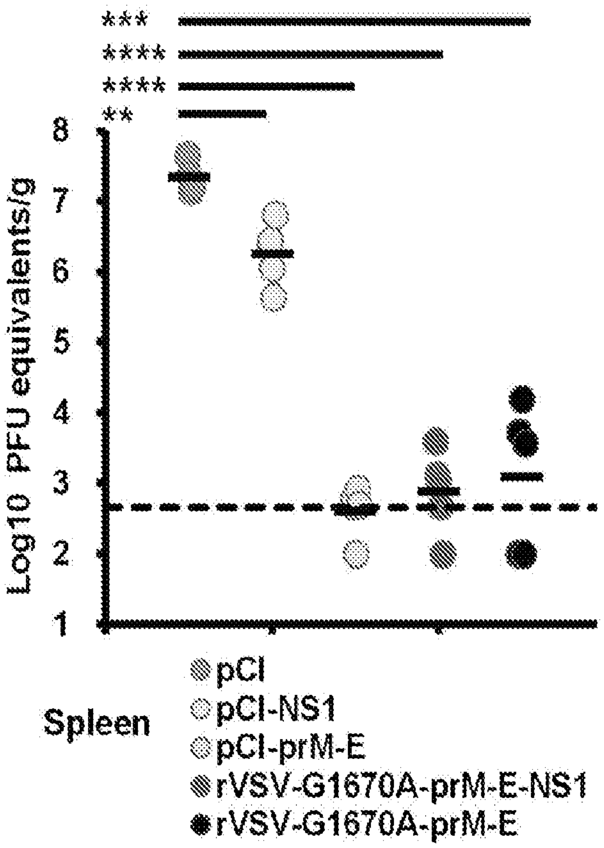

FIG. 16E

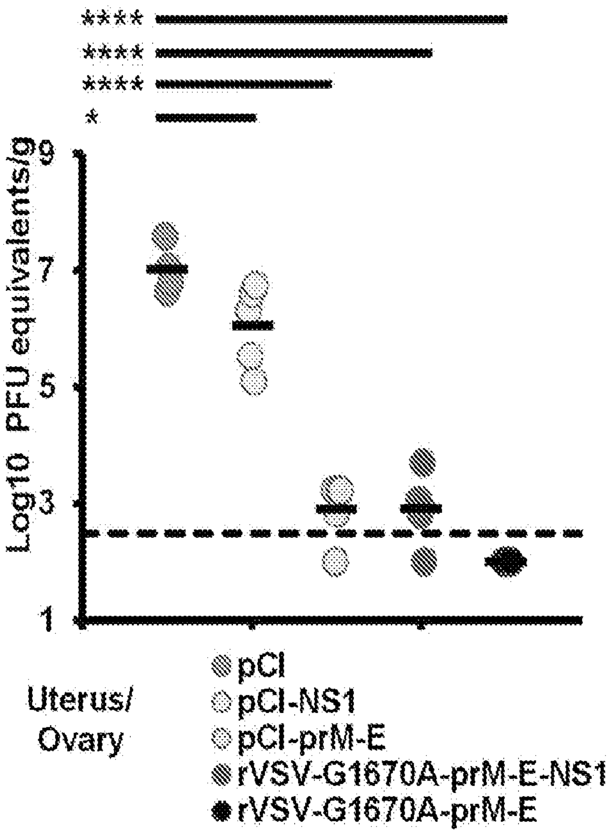

FIG. 16F

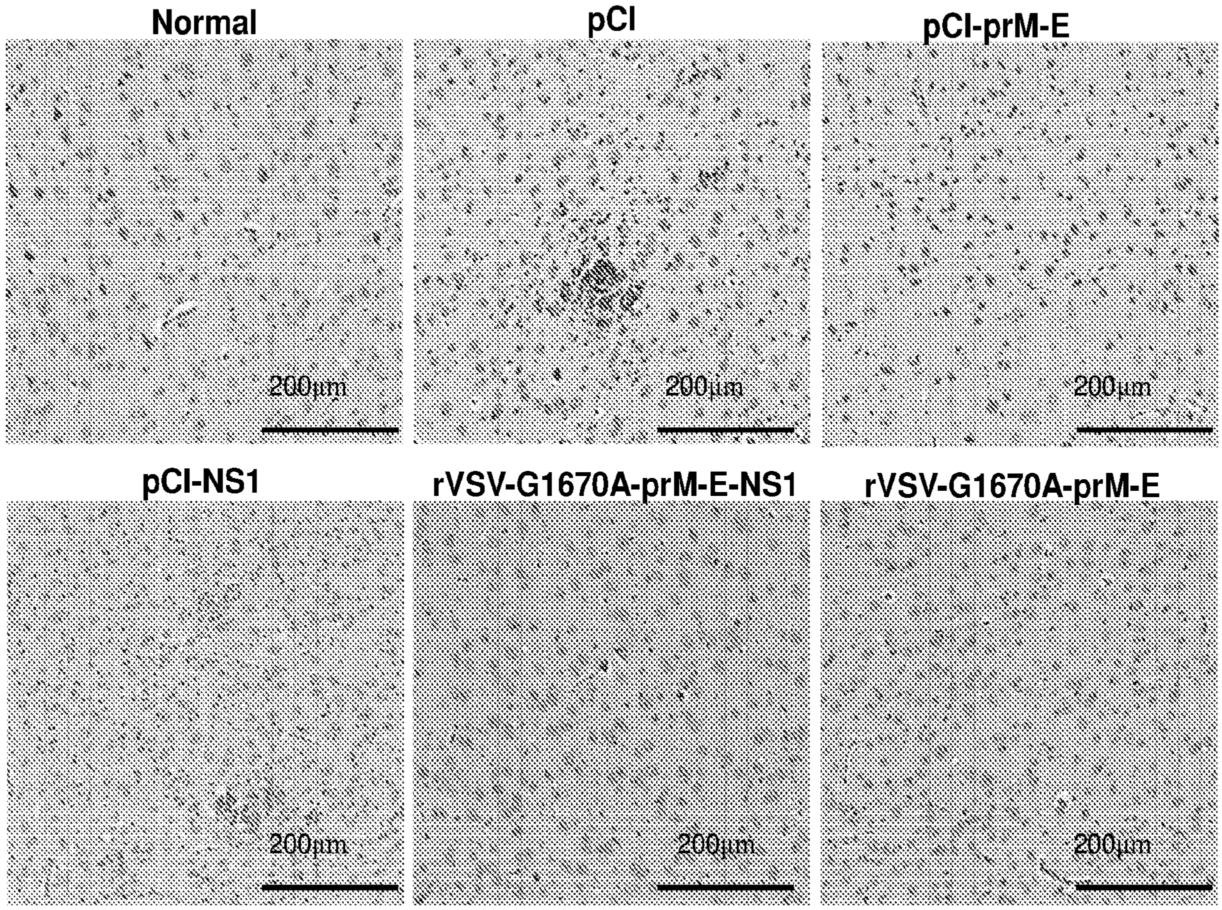

FIG. 17

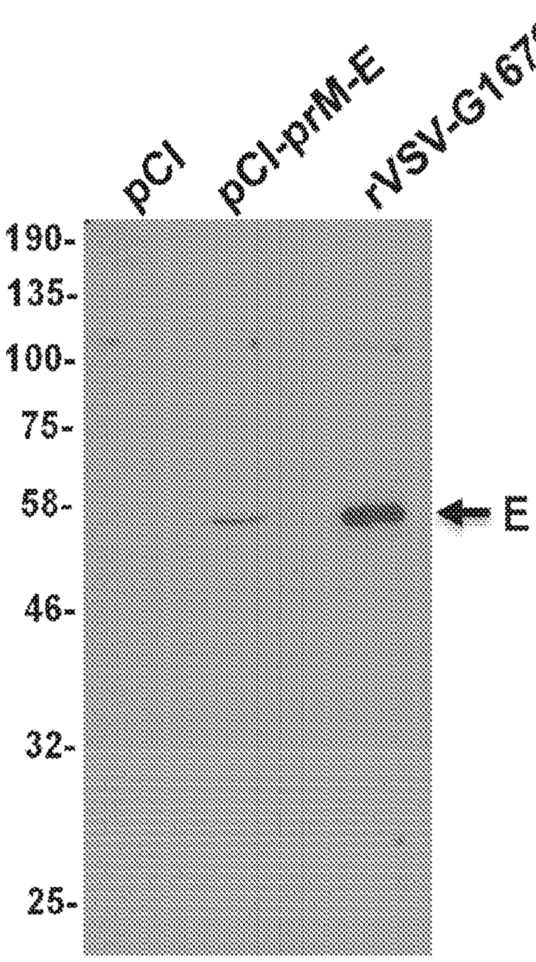

FIG. 18A

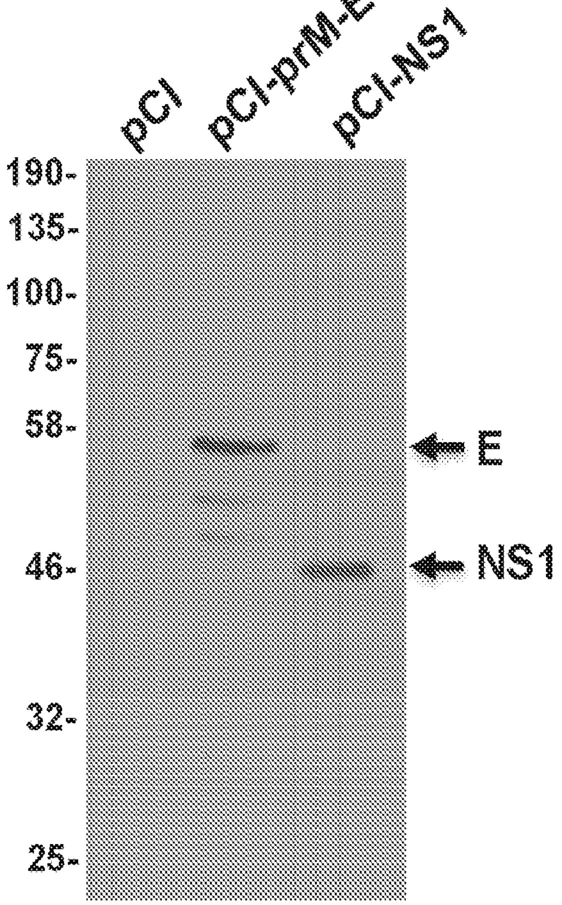

FIG. 18B

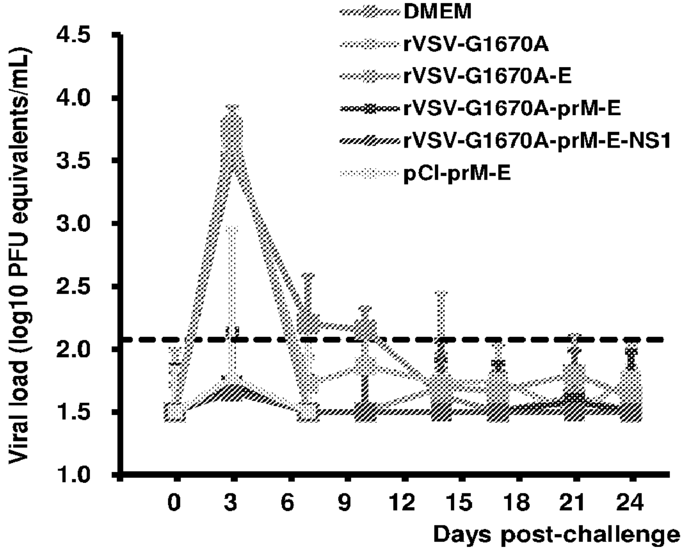

FIG. 19A

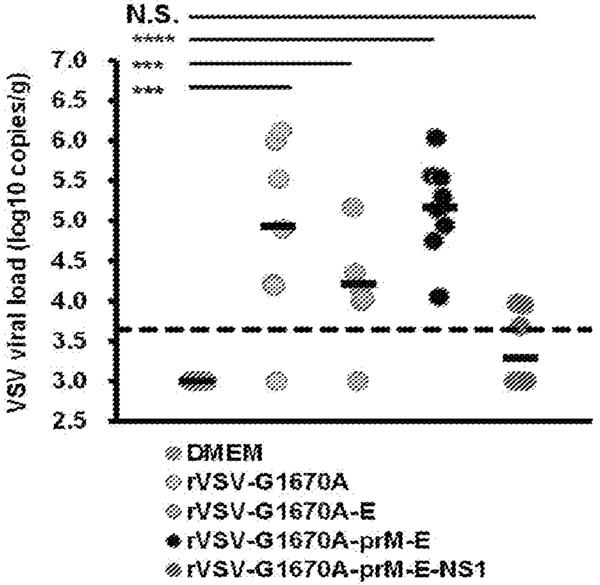

FIG. 19B

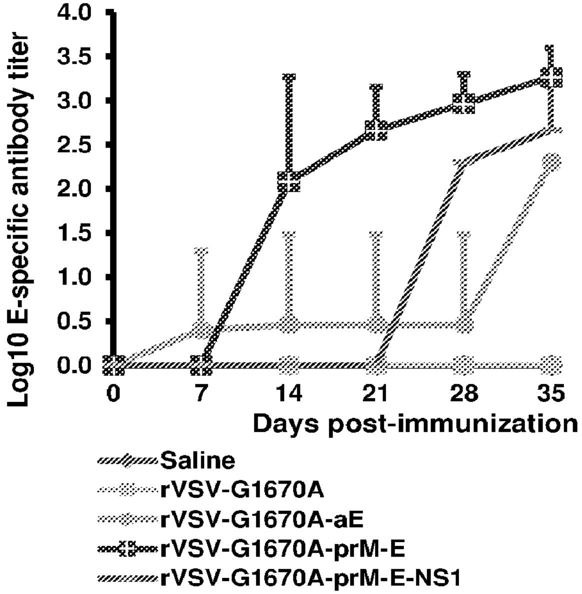

FIG. 19C

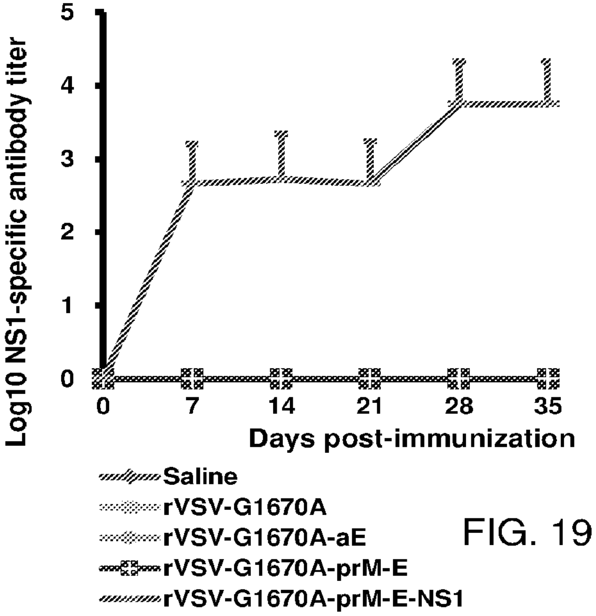

FIG. 19D

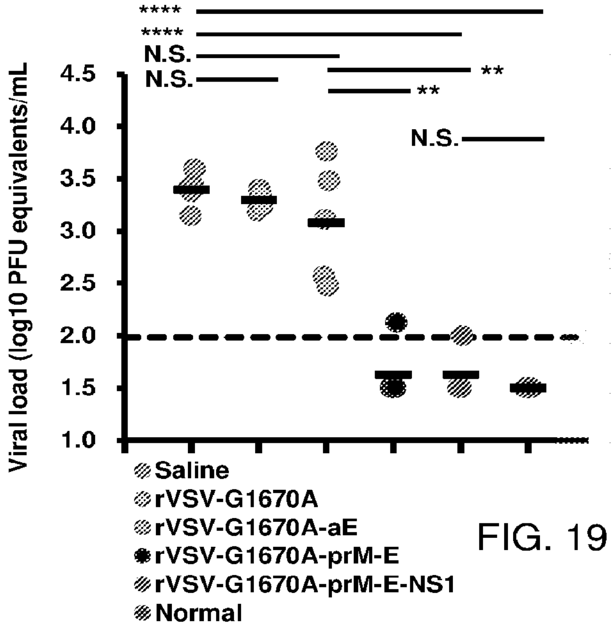

FIG. 19E

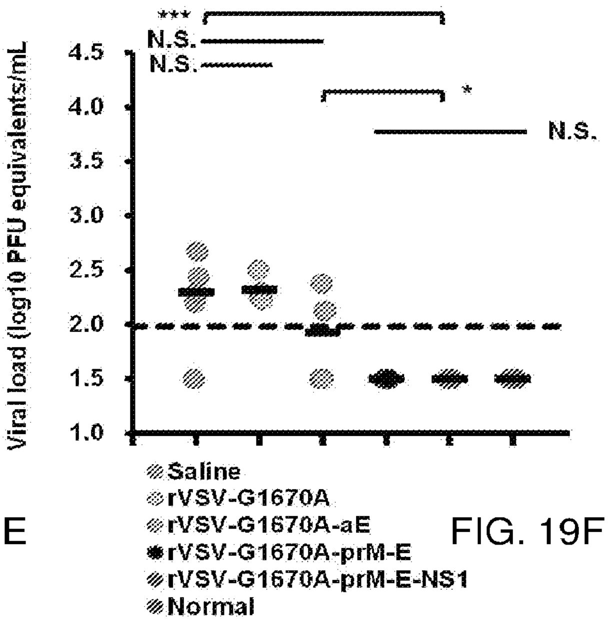

FIG. 19F

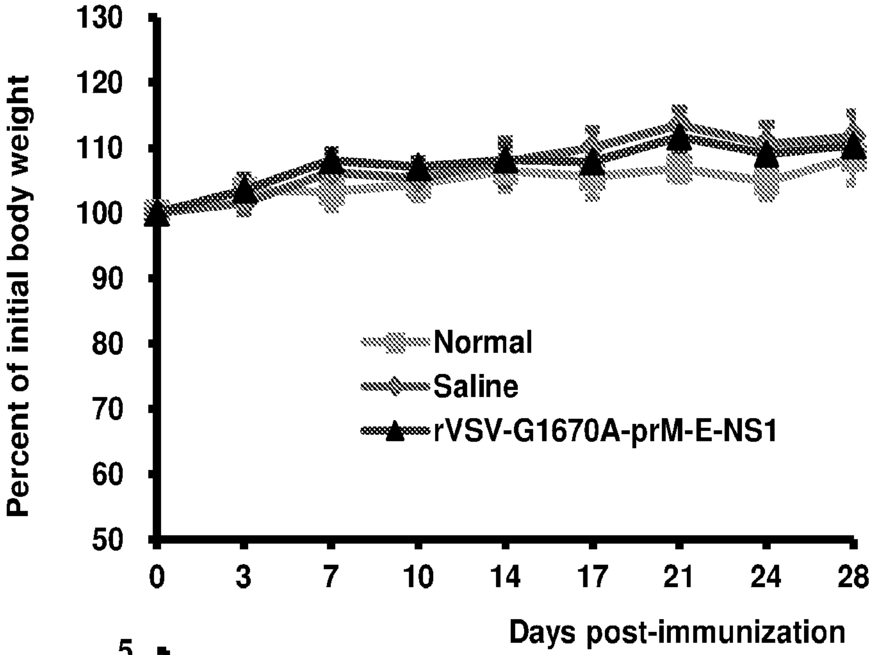

FIG. 20A

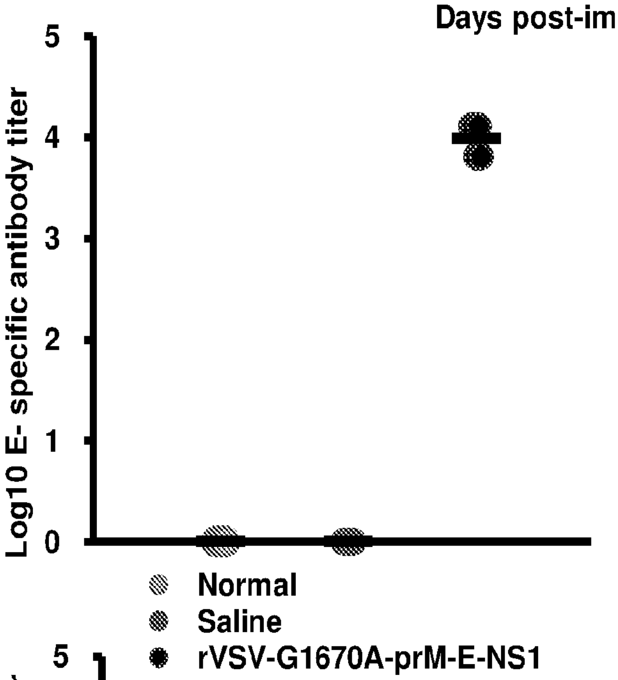

FIG. 20B

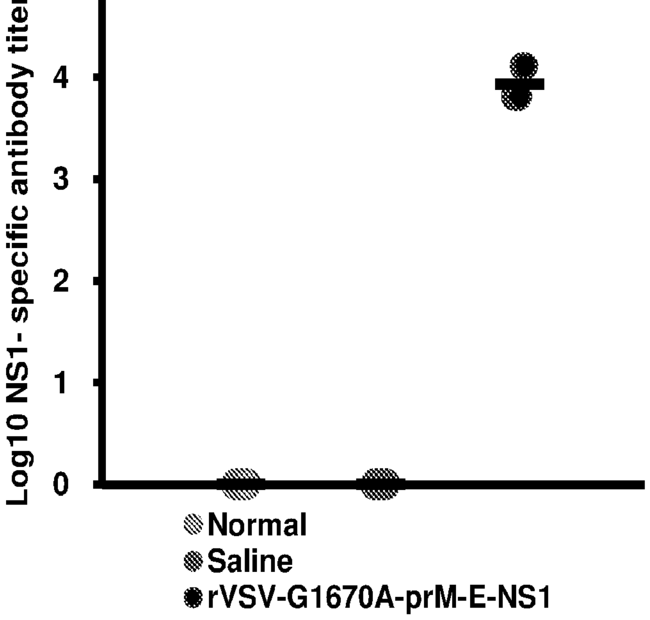

FIG. 20C

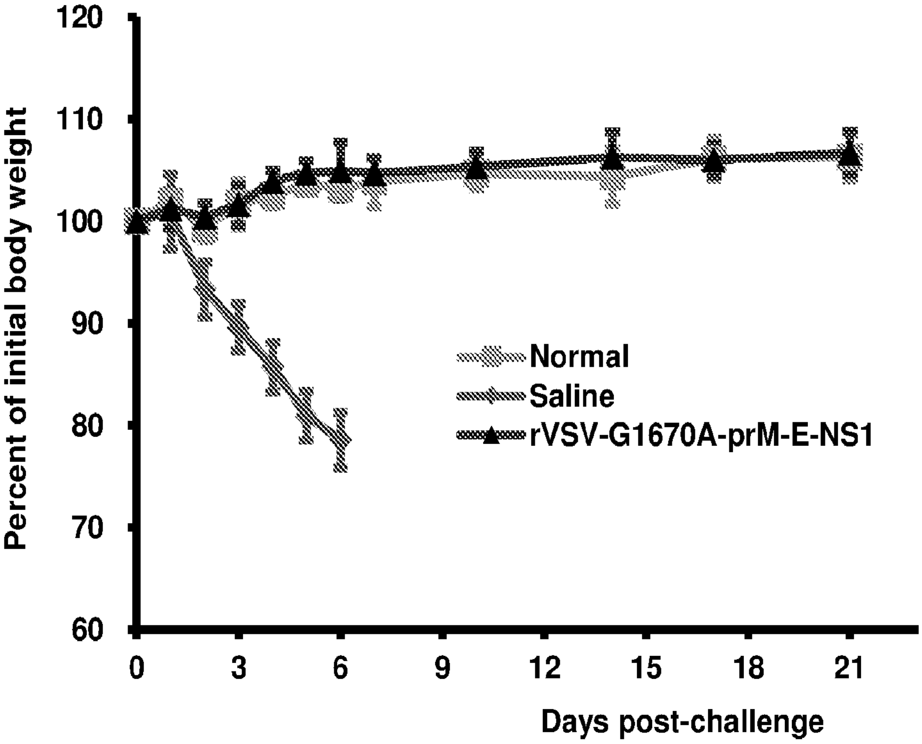

FIG. 20D

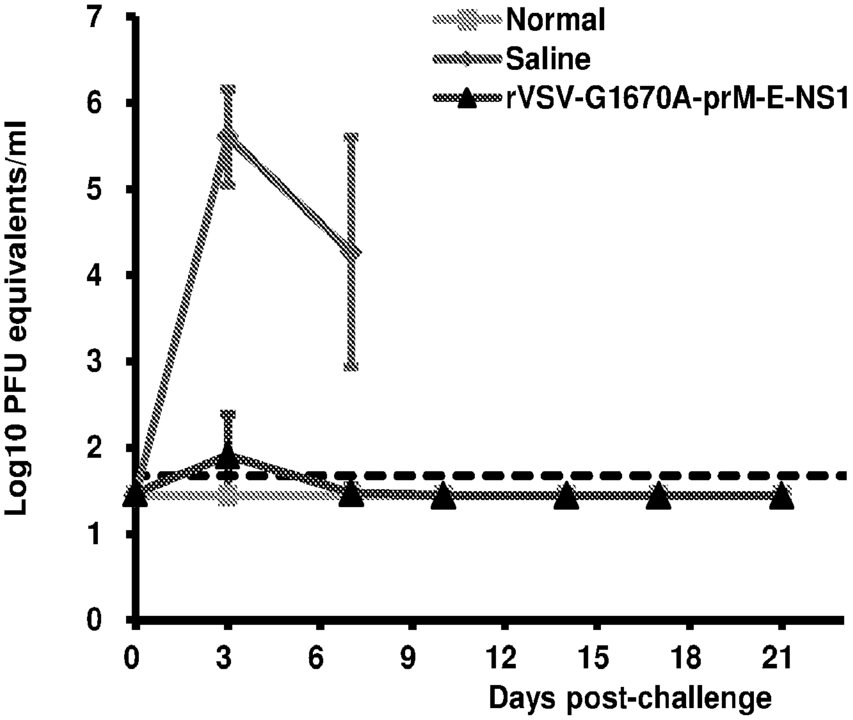

FIG. 20E

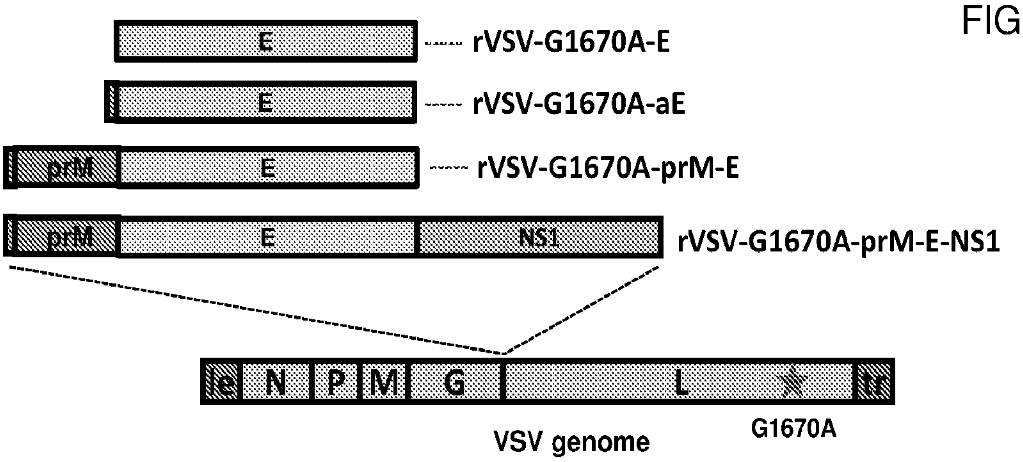

FIG. 21

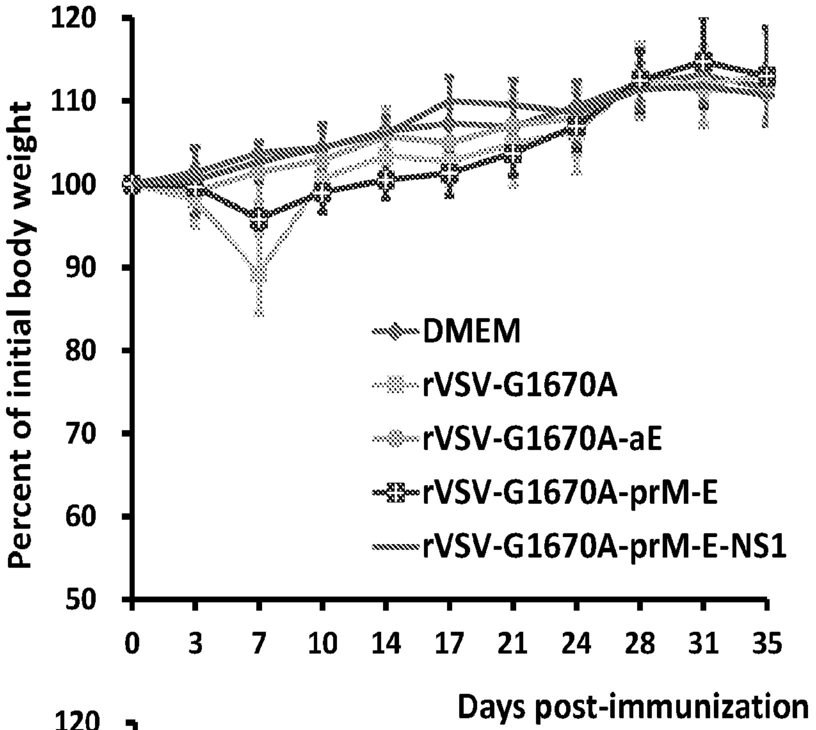

FIG. 22

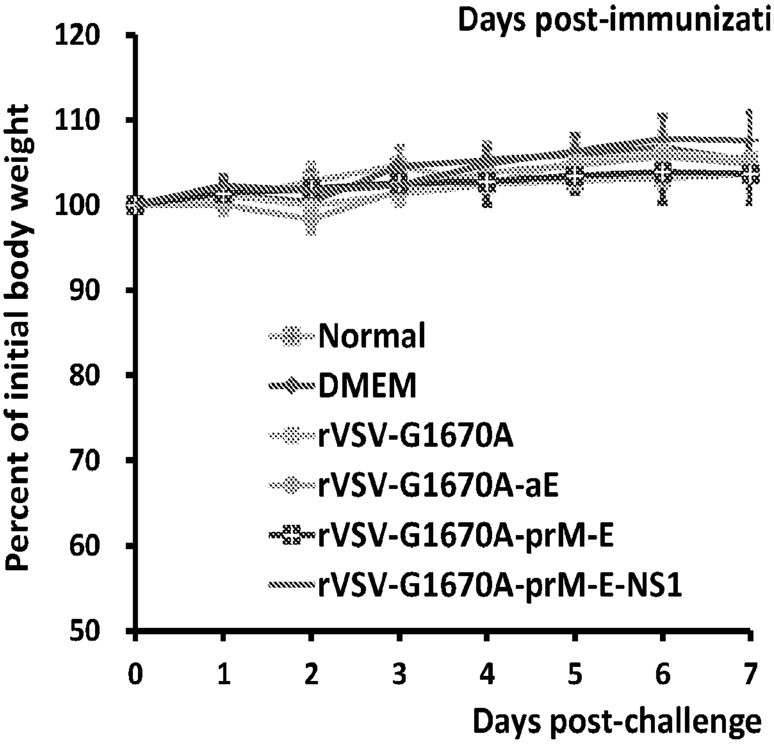

FIG. 23

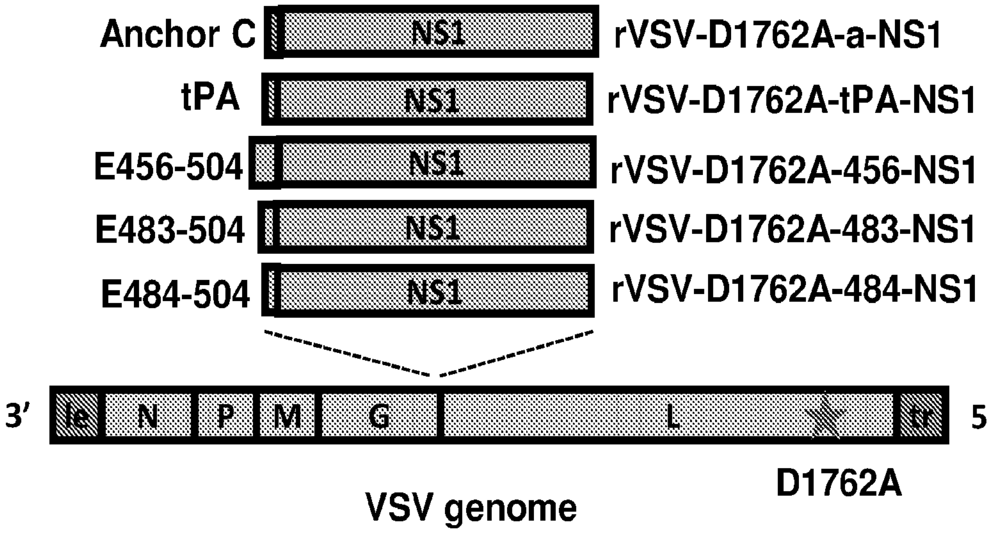

FIG. 24

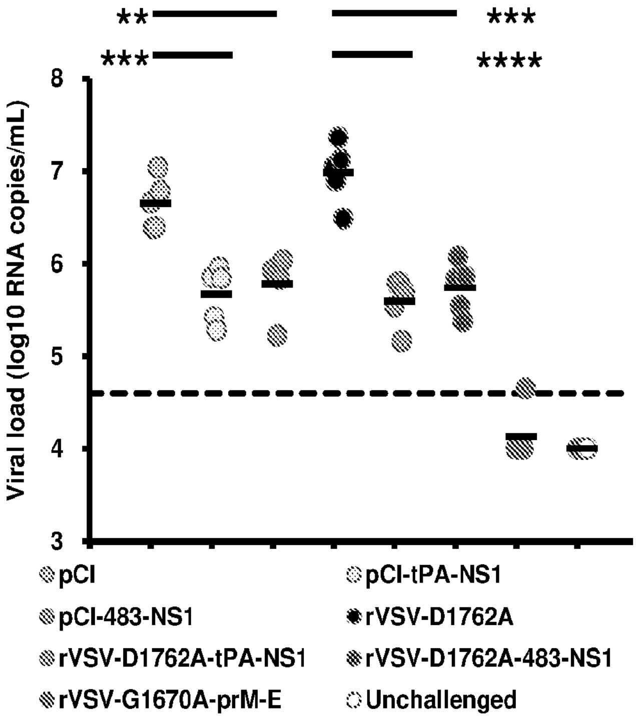

FIG. 25A

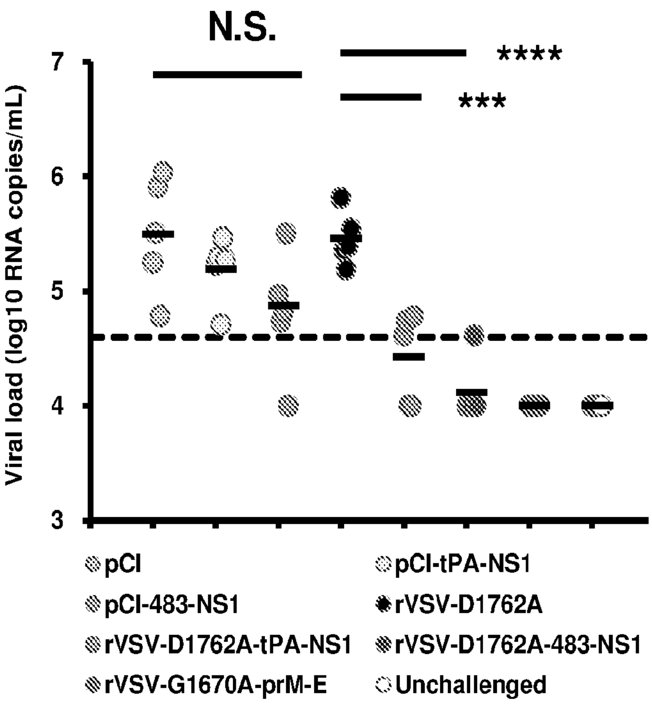

FIG. 25B

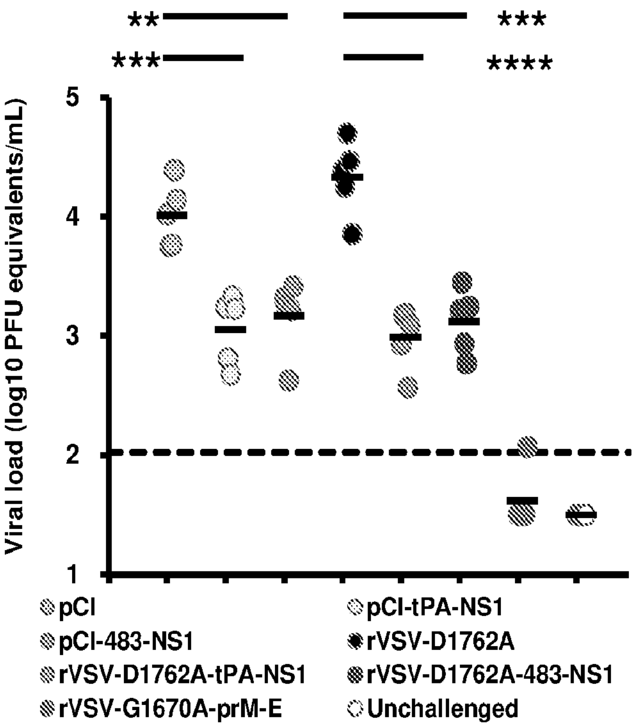

FIG. 25C

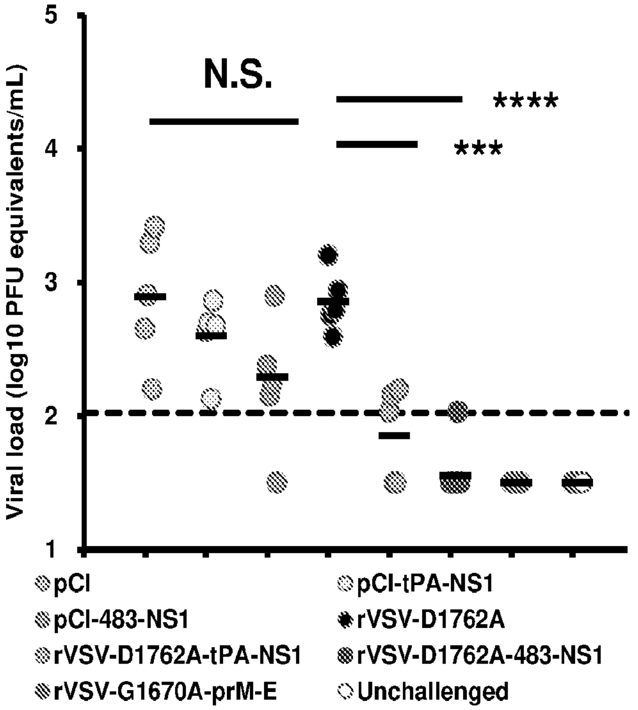

FIG. 25D

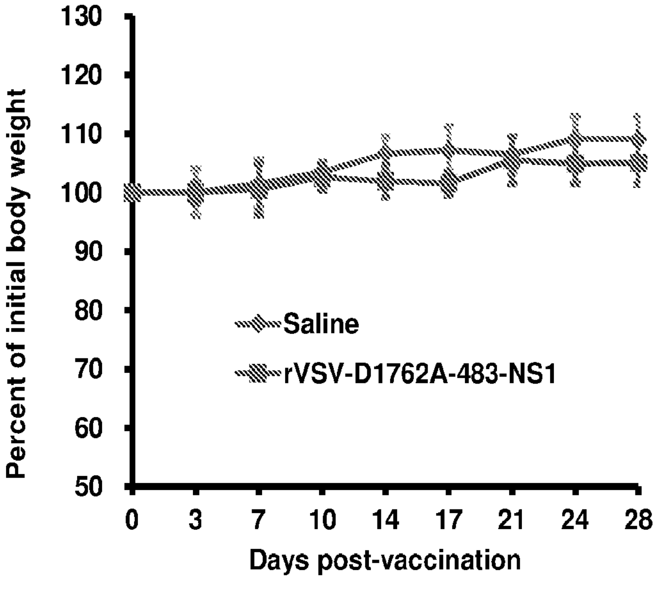

FIG. 26A

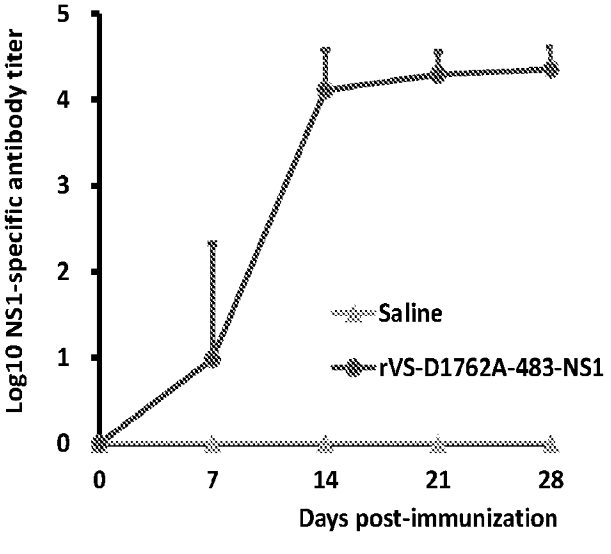

FIG. 26B

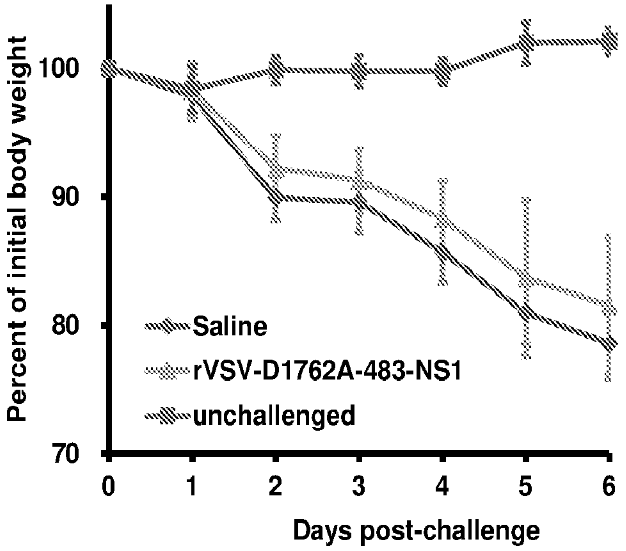

FIG. 26C

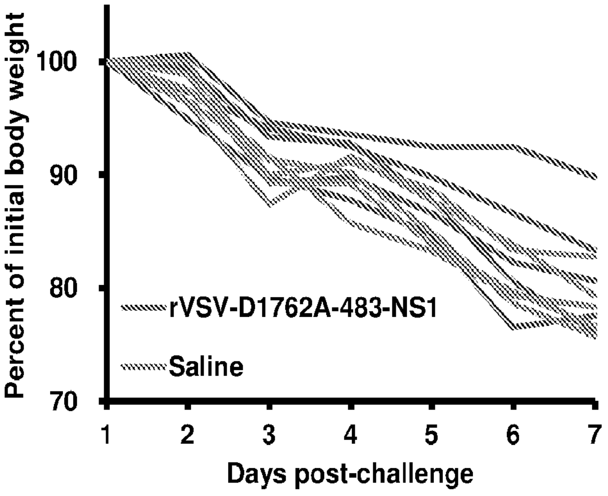

FIG. 26D

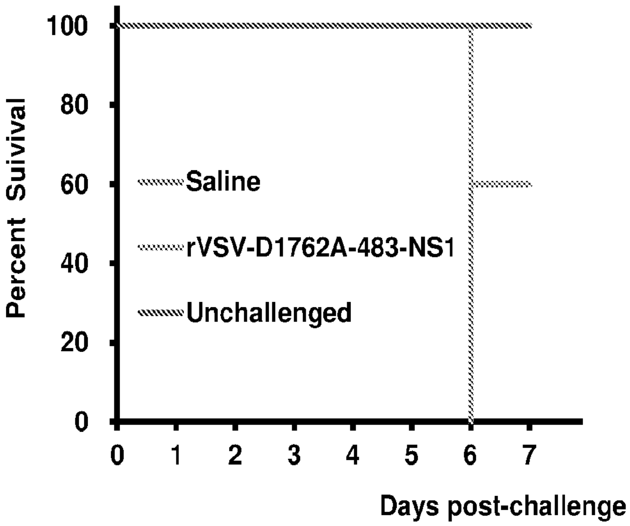

FIG. 26E

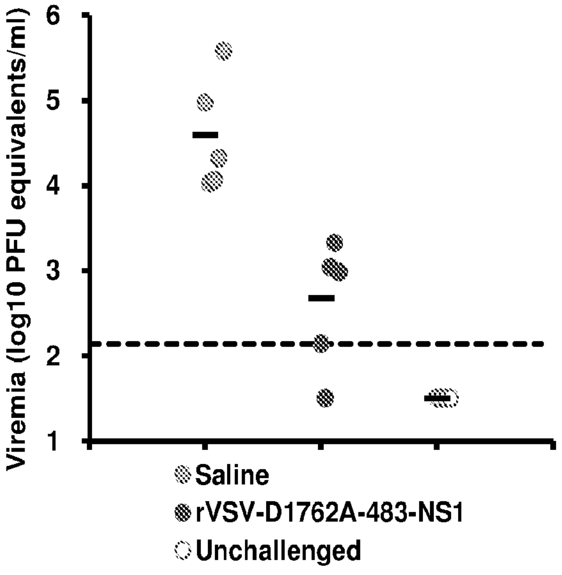

FIG. 26F

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/060137

Box No. I **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 4, 6, 8, 10, 12, and 14 were searched.

Box No. II **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 6-21, 27
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/060137

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/00; A61K 39/12; C07K 14/18; C12N 15/40; C12N 15/63; C12N 15/79 (2019.01)
CPC - A61K 39/12; C12N 2770/24134 (2019.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/204.1; 424/218.1; 435/320.1; 536/23.72; 536/23.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X – Y	WO 2017/136419 A1 (GEOVAX INC.) 10 August 2017 (10.08.2017) entire document	1, 4, 22, 25, 28, 29 ------------------------ 2, 5, 23, 26, 30
Y	WO 2017/140905 A1 (CUREVAC AG et al) 24 August 2017 (24.08.2017) entire document	2, 5, 23, 26, 30
P, X	LI et al. "A Zika virus vaccine expressing premembrane-envelope-NS1 polyprotein," Nat Commun, 03 August 2018 (03.08.2018), Vol. 9, No. 3067, Pgs. 1-17. entire document	1-5, 22-26, 28-30
A	US 2017/0014502 A1 (BHARAT BIOTECH INTERNATIONAL LIMITED) 19 January 2017 (19.01.2017) entire document	1-5, 22-26, 28-30
A	WO 2017/184696 A1 (INTEGRATED RESEARCH ASSOCIATES, LLC) 26 October 2017 (26.10.2017) entire document	1-5, 22-26, 28-30
A	BRAULT et al. "A Zika Vaccine Targeting NS1 Protein Protects Immunocompetent Adult Mice in a Lethal Challenge Model," Sci Rep, 07 November 2017 (07.11.2017), Vol. 7, No. 14769, Pgs. 1-11. entire document	1-5, 22-26, 28-30
A	WO 2017/132210 A1 (IOGENETICS, LLC) 03 August 2017 (03.08.2017) entire document	1-5, 22-26, 28-30
A	WO 2017/156511 A1 (THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH & HUMAN SERVICES et al) 14 September 2017 (14.09.2017) entire document	1-5, 22-26, 28-30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search 11 January 2019	Date of mailing of the international search report 28 JAN 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)